Identification and characterisation of eyespot resistance in wheat

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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 thesis was to provide further understanding of the genetic location, function and efficacy of resistances in the variety Cappelle Desprez (CD) and to fine-map the potent resistance gene *Pch1,* previously introduced into wheat from the relative *Aegilops ventricosa.*

In chapter 2, the genetic location of *Pch2* resistance from CD was confirmed on the distal portion of chromosome 7AL. By mapping sequences identified in a previous cDNA-AFLP expression study, two candidate genes for involvement in the *Pch2* resistance response were identified.

Further investigation into *Pch2* in chapter 3 revealed that it confers a significantly lower resistance against penetration by *O. yallundae* than against *O. acuformis*. Although a major QTL for *Pch2* resistance against *O. acuformis* was detected on chromosome 7A, no QTL was identified for resistance against *O. yallundae*. This was further demonstrated in wheat varieties and single chromosome substitution lines.

The adult plant resistance on chromosome 5A of CD was shown to also be effective in seedlings in chapter 4. Furthermore, it was shown to provide protection against both *O. yallundae* and *O. acuformis*. This resistance was mapped as a single major QTL on chromosome 5AL in both field trials and seedling bioassays, and the SSR marker *Xgwm639* was identified to be closely associated with the resistance.

In chapter 5, 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. Candidate gene regions of 364 Kb in *Brachypodium* and 178 Kb in rice were identified as a prelude to the map-based cloning of the resistance gene.

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Abbreviations

AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
BACs	bacterial artificial chromosomes
BARC	Beltsville Agricultural Research Station
Bez	Bezostaya
bp	base pair(s)
°C	degrees Celsius
CC	coiled-coil
CD	Cappelle Desprez
CER	controlled environment room
cDNA	complementary DNA
CFA/CFD	Clermont Ferrand (INRA) SSRs
cM	centimorgans
cm	centimetre(s)
COS	conserved orthologous sequence
CS	Chinese Spring
CTAB	hexadecyltrimethylammonium bromide
DMI	demethylation-inhibitor
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
ESTs	expressed sequence tags
GLM	general linear model
GS	growth stage
GWM	Gatersleben wheat microsatellite
HS	Hobbit 'sib'
JIC	John Innes Centre
Kb	kilobase(s) (1×10^3 bp)
LOD	logarithm of the odds (to the base 10)
MAS	marker-assisted selection
Mb	mega-base(s) (1×10^6 bp)
MBC	methyl-benzimadazole carbamates

M	mole(s)
mg	milligram(s)
mm	millimetre(s)
min	minute(s)
ml	millilitre(s)
MS	mean squares
MQM	multiple QTL mapping
NBCI	National Center for Biotechnology Information
NBS-LRR	nucleotide-binding site leucine-rich repeat
ng	nanogram(s)
OA	*Oculimacula acuformis*
OY	*Oculimacula yallundae*
P	probability
PCR	polymerase chain reaction
PDA	potato dextrose agar
pH	hydrogen ion concentration
%	percentage
QTL	quantitative trait loci
RFLP	restriction fragment length polymorphism
RILs	recombinant inbred lines
RT-PCR	reverse transcriptase polymerase chain reaction
s	seconds
SNPs	single nucleotide polymorphisms
SSCP	single-stranded conformation polymorphism
SSRs	simple sequence repeats
STS	sequence-tagged sites
µg	microgram(s)
µl	microlitre(s)
µM	micromole(s)
wESTs	wheat expressed sequence tags
WMC	wheat microsatellite club

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Appendices on CD

Papers published incorporating work presented in this thesis:

Appendix 1 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*. Theoretical and Applied Genetics 117: 425-433

Appendix 2 Chapman NH, Burt C, Nicholson P (2009) The identification of candidate genes associated with *Pch2* eyespot resistance in wheat using cDNA-AFLP. Theoretical and Applied Genetics 118: 1045-1057

Appendix 3 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 Pathology 59:819-828

Appendix 4 Burt C, Hollins TW, Nicholson P (2010) Identification of a QTL conferring seedling and adult plant resistance to eyespot on chromosome 5A of Cappelle Desprez. Theoretical and Applied Genetics DOI: 10.1007/s00122-010-1427-1

Chapter 1

General Introduction

1.1 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). The importance of eyespot increased considerably in Europe following the development of resistance to the previously effective methyl benzimadazole (MBC) fungicides (Brown et al. 1984; King and Griffin 1985). The economic impact of eyespot is difficult to quantify due to difficulties in accurately diagnosing the disease visually, particularly in the presence of other stem base pathogens such as brown foot rot and sharp eyespot (Polley and Turner 1995). However, UK national crop surveys (http://www.cropmonitor.csl.gov.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). Based on relationships identified by Scott and Hollins (1978), this amount of infection would result in an average yield loss in the UK of 130,000 tonnes per annum. This represents a 0.87% loss per annum using the five year average (2006-2009) UK wheat production of 14.9 million tonnes (http://www.nfuonline.com/News/NFU-Harvest-Survey--Results/). Based on an average UK wheat price from October 2008 to October 2010 of £110 / tonne (http://www.hgca.com/content.template/16/0/Markets/Markets/Markets%20Home%20Page.mspx), this equates to a financial cost of £14.3 million per annum.

Due to its economic impact, numerous studies have been carried out on eyespot disease and its causal organisms. Important discoveries have been made concerning pathogen taxonomy, epidemiology, fungicide resistance, population biology, pathogen genetics, and disease control.

1.2. Symptoms

Eyespot causes characteristic oval brown-bordered lesions, 15-30 mm in length, at the base of cereal stems (Figure 1.1). These often have black dots of fungal stroma in the centre, which cannot be removed by rubbing. Lesions usually occur on the first internode above the soil, but can be found as high as the base of the third internode, particularly in wet seasons (Goulds and Polley 1990). Initially eyespot lesions are diffuse and indistinct, but as the fungus penetrates the outer leaf sheath and subsequently the tissue below, lesions become more defined (Fitt 1992).

Figure 1.1: Eyespot lesions on seedlings of the susceptible wheat variety Chinese Spring.

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% (Scott and Hollins 1974). Less severe lesions may affect

water and nutrient uptake resulting in shrivelled ears, or 'whiteheads,' again resulting in yield loss (Scott and Hollins 1974). Furthermore, whiteheads may be colonised by secondary fungal pathogens.

1.3 Causal organisms

The two species of fungus that cause eyespot disease in cereals are *Oculimacula yallundae* and *Oculimacula acuformis.* These were initially classified as one species, *Cercosporella herpotrichoides* (Fron 1912), which was later renamed as *Pseudocercosporella herpotrichoides* (Deighton 1973). 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). A formal taxonomy for the pathotypes based on conidial and colony morphology subdivided the species into *P. herpotrichoides* var *herpotrichoides* (W-type) and *P. herpotrichoides* var *acuformis* (R-type) (Nirenberg 1981). Subsequently, both the W-type (Wallwork 1987) and R-type (Dyer et al. 1994) forms were found to produce ascospores and thus to be capable of sexual reproduction. The discovery that W- and R-types are sexually incompatible and hence form two separate breeding groups led to their re-classification as two distinct species, *Tapesia yallundae* and *Tapesia acuformis*. This was confirmed by genetic analysis of the two species using random amplified polymorphic DNA (RAPD) molecular markers (Dyer et al. 1996). The teleomorphs, describing the sexual stages, of the two species were further re-classified as *O. yallundae* and *O. acuformis*, and the genus of the anamorph, or asexual, stage was re-classified as *Helgardia*, by Crous et al. (2003) on the basis of sequence comparison from ribosomal internal transcribed spacer regions.

1.3.1 Identification and differentiation

Isolates of *O. yallundae* form fast-growing, even-edged colonies on potato dextrose agar, often with profuse aerial mycelium (Figure 1.2a), whilst *O. acuformis* forms slower growing, feathery-edged colonies (Figure 1.2b) (Hollins et al. 1985). *O. yallundae* tends to produce smaller conidia (Nirenberg 1981) and has a greater proportion of curved conidia than *O. acuformis* (Fitt et al. 1987). *O. yallundae* (W-

type) and *O. acuformis* (R-type) can also be differentiated on the basis of their differential pathogenicity to wheat and rye; *O. yallundae* isolates are more aggressive towards wheat than towards rye, whilst *O. acuformis* isolates are equally aggressive towards both wheat and rye (Hollins et al. 1985; Lange-de la Camp 1966).

(a) (b)

Figure 1.2: Isolates of (a) *Oculimacula yallundae* and (b) *Oculimacula acuformis* growing on potato dextrose agar.

Visual diagnosis of eyespot on field grown plants is complicated as stem base disease symptoms are often a complex of eyespot, sharp eyespot and brown foot rot (Turner et al. 1999), making it difficult to accurately assess the amount of disease visually in the field. Furthermore, it is impossible to differentiate *Oculimacula* species based on plant symptoms. However, accurate estimates of disease incidence and severity are important for disease management strategies and plant breeding programmes. There is also a need for accurate quantification of eyespot pathogens in seedling tests when identifying and characterising genetic sources of resistance to the disease in cereals or wild relatives (de la Peña and Murray 1994).

A number of biochemical and molecular techniques that can accurately identify, differentiate and quantify eyespot fungi in axenic culture and on plants have been developed. The first molecular method to be developed to differentiate *O. yallundae*

and *O. acuformis* species was isozyme polymorphisms (Julian and Lucas 1990). An enzyme-linked immunosorbent assay (ELISA) test was also developed by identifying and isolating *Oculimacula* specific proteins and directing antibodies against them (Lind 1990). ELISA values have been shown to correlate to visual disease scores and differences have been used to analyse resistance in different wheat cultivars at various growth stages (Lind 1992). However, the ELISA test requires the quantity of fungal antigen to exceed the limit of detection and hence is limited in its accuracy before anthesis in wheat (Lind 1992). Furthermore, it cannot distinguish between the two species of eyespot pathogen.

A number of DNA based methods have been developed for identifying and investigating eyespot. The two species can be differentiated using Restriction Fragment Length Polymophisms (RFLPs) of total DNA (Nicholson et al. 1991a), ribosomal DNA (Nicholson et al. 1991b, 1993) and mitochondrial DNA (Nicholson et al. 1993; Takeuchi and Kuninaga 1996). Polymorphisms in RFLP profiles provided some of the first molecular evidence that the W- and R- pathotypes are actually distinct species (Nicholson et al. 1993; Takeuchi and Kuninaga 1994, 1996).

However, RFLP methods are labour intensive and relatively expensive. Consequently polymerase chain reaction (PCR) based assays, which facilitate the rapid analysis of large numbers of isolates, have been developed for the detection and quantification of *O. yallundae* and *O. acuformis*. Furthermore, PCR assays are useful for the detection of pathogens before visual symptoms develop (Nicholson and Turner 2000). The first PCR-based assays for eyespot were developed from sequences from transcribed spacer (ITS) regions of ribosomal DNA genes from the different pathotypes (Gac et al. 1996; Poupard et al. 1993). This enabled the development of *O. yallundae* and *O. acuformis* specific primers to detect the two pathogen species. Random amplified polymorphic DNAs (RAPDs) have also been used to reliably detect and differentiate *O. yallundae* and *O. acuformis*. Interestingly, RAPDs have revealed a relatively high level of variability in *O. yallundae* isolates in comparison to *O. acuformis* isolates, thus suggesting that sexual reproduction is more prevalent in the life cycle of the former (Nicholson and Rezanoor 1994). A further diagnostic PCR assay to differentiate between *O. yallundae* and *O. acuformis* was developed based on polymorphisms in the CYP51 gene encoding cytochrome P450 sterol 14α-demethylase in both species. This is a key enzyme of the sterol

biosynthetic pathway and is inhibited by sterol 14α-demethylase inhibitor fungicides (DMIs). The primers were diagnostic of a mutation conferring resistance to DMIs in *O. acuformis* but not in *O. yallundae* and therefore PCR assay was able to differentiate field isolates with and without resistance to DMI fungicides and hence between the two species (Albertini et al. 2003).

None of the conventional PCR diagnostic methods are able to quantify the amount of pathogen present. Quantitative PCR assays to determine amounts of disease are of particular use when studying disease complexes in the field, such as stem base disease of cereals (Turner et al. 1999). As a result 'competitive' PCR methods have been developed (Nicholson et al. 1997), and more recently a real-time PCR method (Walsh et al. 2005) suitable for high-throughput quantitative assessment of a large number of samples.

Although molecular methods have been utilised extensively to provide quantification of eyespot in studies to identify novel sources of resistance (Cadle et al. 1997) and to map resistances (de la Peña et al. 1997), there are limitations to their use for this purpose. Techniques that assess the total amount of eyespot present, such as measuring GUS-transformed isolates (de la Peña and Murray, 1994) and quantification of fungal DNA (Nicholson et al, 1997) do not take into account the distribution of fungus within the plant tissues. In particular, they do not indicate whether the fungus has colonised leaf sheaths laterally or has penetrated through the sheaths. In contrast, the visual disease scoring method of Scott (1971) indicates the degree of penetration of the host. In this method, plants are harvested before tillering and successive leaf sheaths are peeled back and scored for eyespot disease on the following scale: 0 = seedling uninfected, 1 = coleoptile infected, 2 = coleoptile penetrated, 3 = first leaf sheath infected, 4 = first leaf sheath penetrated, 5 = second leaf sheath infected, 6 = second leaf sheath penetrated, 7 = third leaf sheath infected, 8 = third leaf sheath penetrated. This scale continues until the final infected leaf sheath is reached and the disease score for each wheat plant reflects the number of wheat leaf sheaths infected or penetrated. This method is intended to provide an indication of potential for yield loss, as this is dependent upon penetration of successive leaf sheaths leading to infection of the stem (Uslu et al. 1998). Consequently, visual disease scores may provide a better indication of the agronomic

effectiveness of an eyespot resistance than molecular methods that quantify the total amount of pathogen.

1.3.2 Population changes

Differences and shifts in populations of *O. yallundae* and *O. acuformis* are evident from a range of field data. Within the UK there is evidence to suggest that the greatest proportion of *O. acuformis* compared to *O. yallundae* is found in Scotland, whilst populations are more equal in Southern England (Hardwick et al. 2001; West et al. 1998). In some cases only one species may be present. For example, *O. yallundae* appears to be the only form of the disease present in South Africa, with *O. acuformis* absent (Campbell et al. 1996).

The relative proportion of each species on cereal crops has been shown to fluctuate each year (Leroux and Gredt 1997). However, trends have been observed. *O. yallundae* used to be the most common causal organism of eyespot, however in the 1980s and 1990s *O. acuformis* became more predominant in northern Europe, (King and Griffin 1985; Nicholson and Turner 2000; West et al. 1998) and the US Pacific Northwest (Douhan et al. 2002). This shift is thought to be due to differential sensitivity to carbendazim-generating (MBC) fungicides (Bateman et al. 1990; King and Griffin 1985). There is evidence that the predominance of *O. acuformis* was maintained during the 1990's by widespread use of sterol C-14 demethylation inhibitor (DMI) fungicides, particularly prochloraz, which may have favourably selected *O. acuformis*¸ both in the U.K (Bierman et al. 2002) and in the US Pacific Northwest (Douhan et al. 2002). Recent surveys suggested that *O. yallundae* and *O. acuformis* are present in the UK in approximately equal amounts (Burnett and Hughes 2004; Parnell et al. 2008). It has been suggested that this increase in *O. yallundae* could be due to a decreased use of prochloraz and an increase in the use of the anilinopyrimidine fungicide, cyprodinil, which is thought to select for *O. yallundae* (Parnell et al. 2008). It appears likely the relative proportion of each species will continue to change in the future as fungicide use and other agronomic practices change. These population shifts illustrate the necessity of controlling both species of the pathogen, as previous control of one species has led to an increase in

the other (Parnell et al. 2008) and has allowed host infection to continue (Bateman and Jenkyn 2001).

1.4 Epidemiology and pathogenicity

Eyespot is thought to be principally a monocyclic disease, as although secondary disease spread occurs, the progress of eyespot epidemics fits the model for a monocyclic disease (Fitt and White 1988). Epidemics of eyespot on cereals are composed of four overlapping phases: sporulation, conidial dispersal, infection of coleoptiles, and leaf sheath penetration and lesion development (Figure 1.3). Interestingly, a number of differences have been observed between *O. yallundae* and *O. acuformis* in some of these processes.

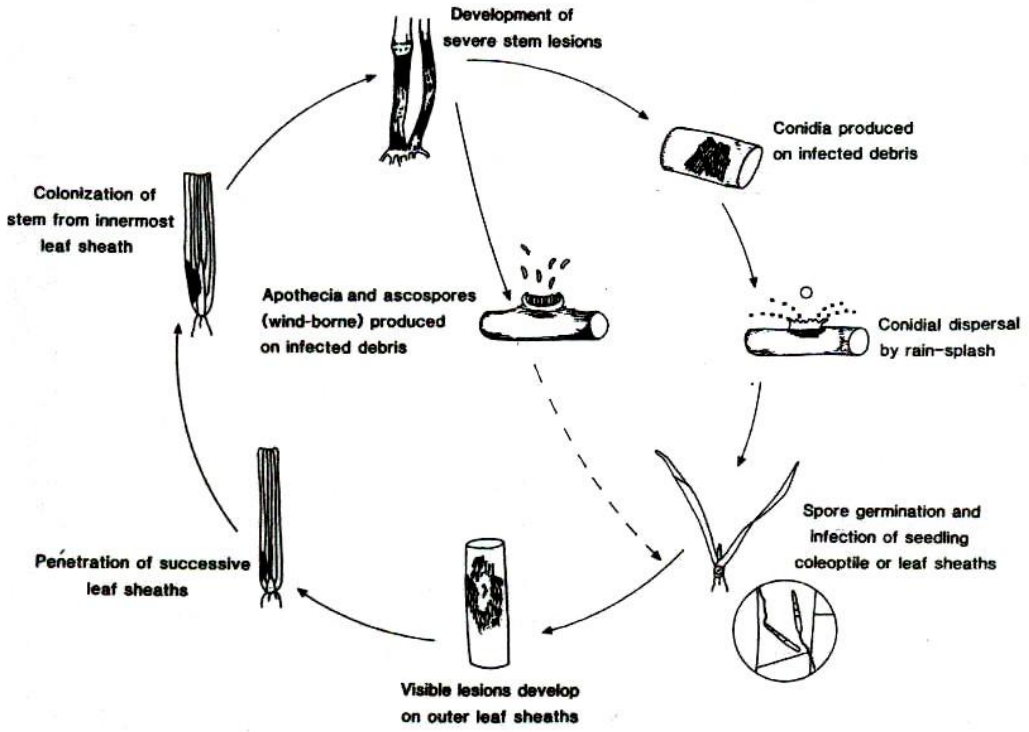

Figure 1.3. Life cycle of *Oculimacula* species showing the asexual cycle with conidia and the sexual cycle with apothecia and ascospores (From Fitt, 1992).

1.4.1 Sporulation, conidial dispersal and conidial adhesion

The predominant form of inoculum in the field is conidia, although ascospores have also been shown to be infective as part of the sexual cycle (see section 1.4.4). Conidia are produced on infected stem bases from a previous crop, particularly in mild wet winters. Sporulation occurs over a temperature range of 1-20°C, with an optimum on naturally infected debris of around 5°C (Fitt and Bainbridge 1983) and is dependent upon water absorption (Glynne 1953). In many areas where winter wheat is grown, conditions are favourable for sporulation for long periods. In northern Europe and North America viable conidia are produced on infected debris throughout the growing season, with a peak in March/April, followed by a decline as temperature increases (Higgins and Fitt 1984).

Conidia are dispersed over short distances to plants of the new crop by rain splash (Soleimani et al. 1996). Conidia are flexible so when they land on a host coleoptile they follow the topography of epidermal cells and even wrap around trichomes. The spores are surrounded in an extracellular matrix (ECM) of mucilage that aids adhesion on the hydrophobic surfaces of the host plant, protects against desiccation, and may facilitate the localisation of enzymes secreted during the early stages of development on the host (Daniels et al. 1991). Conidia only disperse over short distances; however ascospores are airborne and provide a method for long distance dispersal (see section 1.4.4).

1.4.2 Coleoptile invasion

The host coleoptile is important for the establishment of infection as it is a highly susceptible tissue and provides a base from which the pathogen can spread onto and penetrate leaf sheaths (Bateman and Taylor 1976). Following germination, germ tubes of *O. yallundae* extend along the anticlinal cell wall grooves between epidermal cells and form appressorium-like structures at intervals, suggesting a thigmotropic or chemical response to the host coleoptile surface. In contrast, *O. acuformis* follows a different, less orientated, growth strategy. Conidia tend to form multiple lateral germ tubes and produce appressoria at the tips of short hyphae randomly on the host coleoptile surface (Daniels et al. 1991). Penetration of the

coleoptile cuticle and epidermal cell wall occurs directly beneath appressoria (Daniels et al. 1991) and is thought to involve both pectolytic and cellulytic enzymes (Cooper et al. 1988).

Mycelial networks are established in *O. yallundae* through an intramural growth habit, predominantly in the middle lamellum. *O. acuformis* has a more prominent surface proliferation of mycelium. Rapidly growing runner hyphae are formed from the mycelial networks, which initiate colonisation of the underlying leaf sheath (Daniels et al. 1991). In conditions of high humidity the mycelial network on the coleoptile surface may produce further conidia, suggesting that a secondary infection cycle may take place (Soulie et al. 1985).

1.4.3 Leaf sheaf invasion and lesion development

After extension growth along vascular grooves on the leaf sheath, tip cells of runner hyphae differentiate to form discrete clusters of parenchyma-like cells termed infection plaques. The multicellular plaque formed on leaf sheaths is the most distinctive feature of the eyespot fungi. *O. yallundae* tends to form loose asymmetrical plaques extending along vascular grooves, whilst *O. acuformis* tends to form more compact, circular plaques of closely associated cells that are no larger in diameter than the width of the vascular groove they occupy (Daniels et al. 1991). The factors involved in triggering plaque induction are not clear. There is evidence that pressure signals are important in plaque induction, even in non-host plants, however there is no evidence that factors such as nutrients, oxygen status, leaf sheath topography and hydrophobicity of the plant surface are important (Lucas 2004).

The role of the infection plaque is host penetration, with closely attached central cells acting as a compound appressorium. Within the plaque, appressorial cells in contact with the host surface elongate and secrete an extracellular matrix to produce a seal. Fungal cell wall thickening and pigmentation takes place, probably due to the deposition of melanin. Penetration occurs simultaneously by the multiple appressorial cells, through formation of infection hyphae at the cell tips, which puncture the host cell wall (Lucas et al. 2000).

The precise method of penetration is unclear. The presence of melanin-pigmented appressoria suggests that *Oculimacula* species may use physical force to penetrate the plant cuticle and pigment mutants suggest that melanin is important in eyespot pathogenicity (Perry and Hocart 1998). In other plant pathogenic fungi, such as *Magnaporthe grisea*, appressorial melanin has been demonstrated to be important in cuticle penetration as it reduces cell wall permeability, and hence facilitates osmolyte accumulation and turgor pressure generation within the cell. This turgor pressure provides the force for penetration of the plant by the infection peg (Henson et al. 1999). However, localised erosion of the cuticle and alterations in the ultrastructure of the cell wall around the infection hyphae have been observed, suggesting that hydrolytic enzymes may also have a role in the penetration mechanism of eyespot (Lucas et al. 2000). Eyespot pathogens have been demonstrated to produce a series of extracellular enzymes, particularly hemicellulases suitable for digestion of the monocotyledon cell wall (Cooper et al. 1988; Mbwaga et al. 1997).

Following penetration, pathogens grow through leaf sheaths to emerge on the inner surface, where runner hyphae initiate infection of the underlying leaf sheath. In susceptible hosts this sequential colonisation and penetration of leaf sheaths eventually leads to infection of the stem and the appearance of characteristic eyespot lesions (Lucas et al. 2000). In resistant hosts, infection is slowed at an earlier stage and fewer leaf sheaths are colonised. Host resistance mechanisms include the deposition of osmiophilic material beneath infection plaques (Daniels et al. 1991), the formation of lignified cell wall appositions at penetration sites in epidermal cells (Murray and Bruehl 1983), and the hypersensitive response (Murray and Ye 1986).

There is evidence in *O. yallundae* that there may be a period of asymptomatic host colonisation prior to cell necrosis and symptom formation (Lucas et al. 2000). It has been suggested that the pathogen is biotrophic during this phase before switching to a necrotrophic phase once it reaches the first leaf sheath and that *O. yallundae* should be classified as a hemi-biotroph (Blein et al. 2009). There is microscopical evidence that *O. yallundae* is able to colonize the coleoptile and reach the first leaf sheath before the appearance of visual symptoms (Murray and Ye 1986) and without causing cell death (Blein et al. 2009). This is supported by PCR analyses that have detected and quantified pathogen presence before visible symptoms develop (Nicholson et al. 1997).

O. yallundae has been shown to colonise coleoptiles and leaf sheaths more rapidly than *O. acuformis* (Gac et al. 1999; Poupard et al. 1994). However, this may be temperature dependent. It has been demonstrated that *O. acuformis* is more pathogenic at low temperatures below 7 ºC, whilst *O. yallundae* is more pathogenic at higher temperatures of 10-15 ºC both in seedling tests and in field trials (Goulds and Fitt 1990). Furthermore, comparing infection rates of *O. acuformis* and *O. yallundae* using accumulated thermal time (degree-days) has demonstrated that *O. acuformis* penetrates leaf sheaths at a slower rate than *O. yallundae* at higher temperatures. *O. yallundae* was observed to penetrate plants at a rate of 0.0102 leaf sheaths/degree-day, whilst in comparison *O. acuformis* penetrated at a significantly slower rate ($P<0.001$) of 0.0067 leaf sheaths/degree-day (Wan et al. 2005). Current consensus predictions of climate change suggest that there will be a trend towards increased winter temperatures in the UK (Jenkins et al. 2009). This scenario could encourage the predominance of *O. yallundae* over *O. acuformis.*

1.4.4 The sexual stage

The sexual stage of *O. yallundae* was first identified on stems of various grass species within a wheat crop (Wallwork 1987) and has subsequently been found in New Zealand, Europe, the US Pacific Northwest and South Africa under natural field conditions (Lucas et al. 2000). The widespread occurrence of the sexual stage of *O. yallundae* in the field suggests that it is an intrinsic part of the fungal lifecyle. The fungus exhibits a two-allele heterothallic mating system with the complementary mating types *MAT-1* and *MAT-2* (Dyer et al. 1993). Both of these mating types are present throughout Europe (Dyer et al. 2001) and the Pacific Northwest USA (Douhan et al. 2002). A PCR-based method has been developed to identify the *MAT-1* and *MAT-2* mating types of the eyespot pathogens and has enabled investigations into the role of sexual reproduction in their life cycles (Dyer et al. 2001).

The sexual cycle occurs on straw stubble left after harvest, leading to the development of apothecia (Dyer et al. 1994). Ascospores are forcibly ejected from apothecia of *O. yallundae* (Sanderson and King 1988) and hence may become airborne and provide a method of long-range dispersal, once reaching a host following a similar pattern of infection to conidia (Daniels et al. 1995). In addition,

sexual reproduction involves genetic recombination and enables the production of new genotypes and a more variable pathogen population (Dyer et al. 1993).

Although apothecia of *O. acuformis* have been discovered in the field (King 1990), they appear to be rare and consequently the sexual stage is thought to be considerably less important in *O. acuformis* than *O. yallundae*. This may be because localised populations of *O. acuformis* consist predominantly of a single mating type (Dyer et al. 1996), although both *MAT-1* and *MAT-2* mating types have been discovered throughout Europe (Dyer et al. 2001). Alternatively, the low frequency of the sexual stage may be due to low fertility or specific environmental requirements (Dyer et al. 1996). The pathogen is thought to rely almost exclusively on asexual reproduction, thus explaining its low level of genetic variation (Nicholson and Rezanoor 1994). The processes of infection, penetration and colonisation from ascospores of *O. acuformis* on host plants have not yet been determined.

1.5 Chemical control of eyespot

No effective fungicides were available against eyespot until the introduction of inexpensive anti-microtubular methyl-benzimadazole carbamates (MBCs), such as carbendazim, in the mid-1970s (Fitt et al. 1990). These were extensively used until 1981, when widespread resistance to MBCs developed in *Oculimacula* populations in the UK (King and Griffin 1985) as a result of a mutation in a beta-tubulin gene (Albertini et al. 1999). Subsequently, MBCs were replaced by demethylation-inhibitor (DMI) fungicides, such as prochloraz. However, isolates resistant to prochloraz were initially reported in France (Leroux and Gredt 1997) and became widespread in the UK (Bateman et al. 1995) where the fungicide is thought to have contributed to selection for *O. acuformis* during the 1990's (Bierman et al. 2002). The reduced efficacy of prochloraz has led to extensive use of the anilinopyrimidine fungicide, cyprodinil. Anilinopyrimidines are thought to have a mode of action that involves inhibition of methionine biosynthesis (Fritz et al. 1997). However, field isolates of both *Oculimacula* species have been found with reduced sensitivity to cyprodinil, suggesting there is a risk that resistance could develop (Babij et al. 2000). Furthermore, as previously discussed (section 1.3.2) there is evidence that it has selected for *O. yallundae* isolates (Parnell et al. 2008) There have been attempts to

find a fungicide seed treatment that is effective against eyespot (Dawson and Bateman 2001), although as yet no commercial product has been released.

For economic and environmental reasons it is important that fungicide sprays are applied at optimum times and rates, and only when treatment against eyespot is needed. Sprays need to be applied before lesions become severe and hence it is generally recommended that MBC fungicides are applied at Zadoks' growth stage 30/31 (Zadoks et al. 1974) and prochloraz is applied at GS 30-37 to obtain control (Fitt et al. 1990). Several disease forecasting and risk assessment schemes, based on weather, cropping history, cultivars and environmental factors, were developed in the 1970s and 1980s to predict the occurrence of severe eyespot at a time when spray decisions need to be made (Fitt et al. 1988). A visual threshold of 20% infected shoots at stem extension has been used to predict where it would be cost-effective to apply an eyespot fungicide treatment (Burnett et al. 2000). These methods were reasonably effective whilst the UK eyespot population was predominantly *O. yallundae*. However, the increasing importance of *O. acuformis*, which develops more slowly at higher temperatures, and hence is more difficult to diagnose visually at stem extension, has made the use of forecasting and thresholds less effective (Burnett et al. 2000). The use of molecular diagnostics such as PCR assays may overcome this problem by detecting pathogens before visual symptoms develop (Nicholson and Turner 2000). Furthermore, the use of accumulated degree days rather than a chronological time scale could be used to produce a eyespot development scale, allowing for differences between the species, to more accurately predict disease severity and whether fungicide control is required (Wan et al. 2005).

1.6 Cultural control of eyespot

Cultural methods were essential for the control of eyespot before the introduction of cost-effective fungicides and are still useful in extensive agricultural systems where fungicides are prohibitively expensive. Economic and environmental pressures on agriculture have led to an increased consideration of cultural control methods. A number of cultural methods including sowing date, crop rotation and minimum tillage could be used to reduce eyespot levels.

Eyespot is less likely to be severe in late-sown crops, and hence the trend towards early sown crops may have increased the importance of eyespot in the UK (Yarham 1986). Furthermore, early sowing dates favour the slower developing *O. acuformis*, and may be partially responsible for its current predominance (West et al. 1998). Control of eyespot through late sowing can substantially reduce the risk of serious eyespot epidemics but is unlikely to be appropriate in the UK as early sowing is favourable for other agronomic reasons (Fitt et al. 1990). Excessive seed rates and high applications of nitrogen fertilisers are likely to produce very lush crops that favour eyespot, and hence should be avoided (Glynne and Slope 1959).

Crops most at risk from eyespot are second wheats, or wheat following a single years' break from cereals. Crop rotation using a single year break is not sufficient to ensure low disease levels. Using a two-year break, however, with non-susceptible crops and controlling volunteer cereals has been demonstrated to reduce eyespot to negligible levels as disease carry-over is prevented (Glynne and Slope 1959). There is a need for more alternative crops to cereals if eyespot is to be effectively and economically controlled by crop rotation.

Although it may be expected that burning stubble would reduce eyespot levels, the disease is less severe when straw from the previous crop is chopped and incorporated into the soil. This may due to competition from other micro-organisms (Jenkyn et al. 2001; Jenkyn et al. 2010). In addition, minimum cultivation may be a useful control technique as it has reduced eyespot levels in the Pacific north-west of the USA (Herman and Weise 1985). Cultivation needs to be used in conjunction with crop rotation to provide control. For example, if a wheat crop is grown in a yearly rotation with a non-host crop, then ploughing may bring infected wheat straw to the surface from two years previously (Colbach 1999).

1.7 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 (Fitt et al. 1990). However, there is evidence that farmers do not value genetic disease resistance but apply fungicides indiscriminately. A change in the paradigm of many farmers will be required if genetic sources of resistance to eyespot, and other plant pathogens, are to be utilised to their full potential (Stevens et al. 1997).

1.7.1 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, VPM1 (Maia 1967). This was achieved because *Ae. ventricosa* possesses the genome $M^vM^vD^vD^v$, and therefore the D^v chromosomes can pair with the D genome of wheat (Doussinault et al. 1983). The resistance gene *Pch1* was identified as a major dominant gene on the long arm of chromosome $7D^v$ (Worland et al. 1988). The gene does not confer immunity but does significantly reduce the rate of fungal colonisation and penetration from the outer leaf sheaths into the stem. In addition, there is evidence of variability in eyespot resistance amongst cultivars and lines carrying *Pch1*. Furthermore, the *Pch1* gene appears to have a strong effect on eyespot resistance in the early stages of plant growth but a lesser effect at later growth stages when quantitative factors gain increased importance (Lind 1999).

Although *Pch1* has been widely used as a source of resistance in attempts to produce eyespot resistant cultivars, the use of *Pch1* in commercial cultivars in the UK has been limited as the resistance gene is transferred to wheat from *Ae. ventricosa* as part of a segment of chromosome $7D^v$ along with agronomically undesirable genes from the wild grass. Hence a significant yield penalty may sometimes be observed in the absence of the disease (Koen et al. 2002). For example, an early *Pch1* variety, Rendezvous, reached the National Institute for Agricultural Botany (NIAB) provisionally recommended list in 1987 (NIAB 1987). Although it contained both *Pch1* and a second gene, believed to be *Pch2* (see below), and provided a higher level of resistance than any previous UK wheat cultivar (Hollins et al. 1988), the variety was not widely used as a result of its relatively low yield in the absence of the disease. This association between *Pch1* and yield limitations has proven difficult

to break because of limited recombination of the translocated *Ae. ventricosa* segment (Johnson 1992).

Recently, a number of *Pch1* carrying varieties have recently been released, such as Battalion and Marksman, 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 enhanced bread making ability through higher grain protein content and dough strength (Groos et al. 2004). However, an inverse correlation has been well established between yield and protein content in cereals (Simmonds 1995). Groos et al. (2004) did not control for differences in yield in their QTL analysis of wheat quality traits in 194 Renan (*Pch1* present) x Recital (*Pch1* absent) recombinant inbred lines (RILs) and it is possible that the observed enhanced grain protein observed in *Ae. ventricosa* may be associated with reduced yield.

1.7.2 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 partial 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). In addition, there is evidence to suggest that the resistance on chromosome 7A is less effective against *O. yallundae* than against *O. acuformis* (P. Nicholson, personal communication).

The resistance observed in Cappelle Desprez appears to be quite complex and may involve additional genes on chromosomes other than 7A. Law et al. (1976) identified potential resistances on chromosomes 2B and 5D in Cappelle Desprez, however these authors also identified potential susceptibility factors (relative to the variety Chinsese Spring) particularly on chromosomes 1A and 1B. More recently, a major gene for eyespot resistance, expressed in adult plants, has been identified on chromosome 5A of Cappelle Desprez (Muranty et al. 2002). This was shown to confer significant resistance at an adult plant stage in field trials over three years, but conferred relatively little resistance at the seedling stage. 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 resistance to eyespot that is effective at the adult plant stage.

1.7.4 Efficacy of resistances against the two species

There is a need to identify additional resistance genes against eyespot due to the paucity of genetic resistance available within wheat, the development of fungicide resistant isolates of the pathogen and the changes in proportion of the two pathogen species in field populations. The discovery of new resistance genes offers opportunities to improve eyespot control and to increase the genetic base in plant breeding programmes. However, identification of new eyespot resistance genes is difficult because resistance is often quantitative and variation occurs in disease development within pathogenicity tests (Lucas et al. 2000). Wild relatives of wheat have provided numerous sources of resistance towards a number of diseases, including eyespot. A number of potentially novel sources of resistance to eyespot, particularly towards *O. yallundae*, have been identified in wild relatives of wheat. These include *Triticum monococcum* (Burt 2002; Cadle et al. 1997), *Triticum tauschii* (Assefa and Fehrmann 1998; Yildirim et al. 1995), *Triticum durum*, *Triticum dicoccoides, Triticum turgidum* (Figliuolo et al. 1998), *Aegilops kotschyi* (Thiele et al. 2002), *Aegilops longissima* (Sheng and Murray 2009) and *Dasypyrum villosum* (Murray et al. 1994). Most of these sources of resistance have not been

characterised or mapped and little is known about any alien sources of resistance to *O. acuformis* specifically. An exception is the resistance identified on chromosome 4V of *D. villosum* by Murray et al. (1994) that has been mapped as a single dominant gene on the distal portion of the long arm of the chromosome and termed *Pch3* (Yildirim et al. 1998). Despite this, the *Pch3* resistance has not yet been introgressed into any commercial cultivars of wheat.

There is evidence that some resistance genes may be differentially effective against *O. yallundae* and *O. acuformis*. Notably, *Pch3* on chromosome 4V of *D. villosum* confers resistance to *O. yallundae*, but not to *O. acuformis* (Uslu et al. 1998). However, resistance to *O. acuformis* has been associated with factors on chromosomes 1V, 2V, 3V and 5V, but not 4V of *D. villosum* (Uslu et al. 1998). Evidence that the genetic basis of resistance against *O. yallundae* and *O. acuformis* may be different has also been obtained from studies of *T. monococcum* (Burt 2002). There is a need to screen against both species in any future chemical control research and plant breeding programmes.

1.8 Molecular markers for eyespot resistances

Phenotyping for eyespot resistance in breeding 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.

1.8.1 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). This linkage has been used for marker-assisted selection of eyespot resistance and has been applied by breeders in efforts to develop cultivars incorporating *Pch1*. Studies have reported a complete linkage between *Ep-D1b* and the resistance gene (Leonard et al. 2008; Santra et al. 2006) and it has been suggested that the endopeptidase protein is directly involved in providing *Pch1* resistance (Worland et al. 1988). However, recombinants have been detected between *Ep-D1b* and *Pch1* (Mena et al. 1992), suggesting that the endopeptidase protein does not itself confer the resistance and may not be entirely suitable as a diagnostic marker.

Isozyme methods can be technically challenging and time-consuming. Consequently there have been attempts to develop PCR markers that are closely linked to *Pch1*. A co-dominant microsatellite marker *XustSSR2001-7DL* was developed with apparent tight linkage to the isozyme locus *Ep-D1b* (Groenewald et al. 2003), however this was subsequently found to map around 3 cM from *Pch1* (Chapman 2005) and to have only a 90% success rate at predicting the correct phenotype in a collection of wheat cultivars compared to a 100% success rate for *Ep-D1b* (Santra et al. 2006). Dominant microsatellite marker loci *Xwmc14* and *Xbarc97* have both been found to co-segregate with *Pch1* resistance in the HS x RVPM7D recombinant chromosome 7D population (Chapman 2005). However, again these markers may not be suitable for marker assisted selection as Leonard et al. (2008) observed recombination between these markers and *Pch1* in a panel of USA wheat varieties.

The tight linkage observed between the endopeptidase isozyme *Ep-D1b* led to the development of a sequence-tagged site (STS) marker *Orw1*. This marker was developed by identifying the maize endopeptidase locus *enp1* as orthologous to *Ep-D1b*. The *enp1* locus in maize has been shown to exhibit biochemical properties

similar to an oligopeptidase B identified in *E. coli* and therefore it was reasoned that *Ep-D1b* may also encode an oligopeptidase B. A wheat expressed sequence tag (wEST) encoding an oligopeptidase B was identified and *Orw1* primers designed to it. This marker was subsequently found to co-segregate with both *Ep-D1b* and *Pch1* in a segregating population of 254 RILs derived from the *Pch1* carrying cultivar Coda and the *Pch1* lacking Brundage. Additional STS markers *Orw5* and *Orw6* were designed to wESTs encoding a callose synthase and a PSII assembly factor Hcf136, respectively. These were identified from predicted gene sequences on the rice BAC clone AP005750 containing the rice oligopeptidase B gene orthologue (Leonard et al. 2008).

Further markers to the distal end of long arm of chromosome 7D are required for the fine mapping of *Pch1* (Chapman 2005). Wheat SSR markers, such as *Wmc14* and *Barc97,* are often dominant, producing a specific product from wheat but failing to amplify one from the *Ae. ventricosa* introgression. Co-dominant markers that amplify from both wheat and introgressed DNA are favourable as they are suitable for differentiating heterozygotes from homozygotes in F_2 populations. The use of gene-based markers developed to wESTs may assist this process as PCR primers can be designed to exon sequences that tend to be more conserved between species than intron sequences (Varshney et al. 2005). However, EST-based markers are not always co-dominant when applied to alien introgressions (Leonard et al. 2008; Qi et al. 2008), depending on the level of relatedness (Zeid et al. 2010). Co-dominant markers for alien introgressions in wheat and other cereals could be generated through the development of cross-species transferable Conserved Orthologous Sequence (COS) markers. This involves designing primers to exon sequences that are highly conserved between the fully sequenced reference genomes of rice (International Rice Genome Sequencing Project 2005) or *Brachypodium distachyon* (Brachypodium) (International Brachypodium Initiative 2010) and available sequence from wheat or other cereals (Bertin et al. 2005). COS markers have been utilised for mapping studies in wheat (Quraishi et al. 2009) and Brachypodium (Garvin et al. 2010) and there is also evidence that they may be useful for mapping inter-specific hybrids between forage grass species (King et al. 2007).

1.8.2 Molecular markers for Pch2 resistance

There have also been attempts to find suitable biochemical or molecular markers for *Pch2* to facilitate marker-assisted selection. An endopeptidase marker, *Ep-A1b*, has previously been associated with *Pch2* (Koebner and Martin 1990). However, recombination frequencies of 15% have been observed and hence the linkage is not tight enough to be of use in marker-assisted selection (de la Peña et al. 1996). Flanking RFLP markers have been found for *Pch2* resistance to *O. yallundae*, positioning the gene on the long arm of 7A and it has been suggested that simultaneous selection of these RFLP markers could be used for marker assisted selection of the resistance gene (de la Peña et al. 1997). However, tightly linked PCR-based markers, such as microsatellites, are much more suitable for efficient marker-assisted selection in cultivar development. Recently, *Pch2* resistance to *O. acuformis* has been mapped as a QTL in a 7.5 cM region in between microsatellite 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* as suggested by Uslu et al. (1998). This would enable the development of closely linked PCR markers suitable for marker-assisted selection of eyespot resistance in breeding programmes.

1.8.3 Evidence for Pch1-Pch2 homoeology

There is currently uncertainty regarding the relationship between the resistance genes *Pch1* and *Pch2*, located on the distal portions of the long arms of chromosomes 7D and 7A respectively. It has been suggested that the genes are homoeoloci, based on their distal locations and proximity to the chromosome 7A and 7D homoeoloci of the endopeptidase isozyme markers *Ep-A1* and *Ep-D1* (De la Peña and Murray 1994; de la Peña et al. 1997). However, other studies have been unable to provide evidence for homoeology between the resistances (Leonard et al. 2008). Further work is required to determine whether the two genes are homoeoloci. The use of COS markers, which are likely to be transferable across wheat homoeologues as well as across cereal species, should enable the integration of 7A and 7D maps to provide

this evidence. If *Pch1* and *Pch2* do represent homoeoloci, then efforts to fine-map and clone one of these resistances would directly inform research on the other.

1.9 Cereal comparative genetics and exploitation of synteny

Comparative mapping in cereals has provided evidence for conservation of markers and gene order, termed colinearity, between genomes (Moore et al. 1995). The exploitation of comparative genetics can enable transfer of marker and sequence information from model species such as rice and Brachypodium to genetically complex species such as wheat (Salse and Feuillet 2007), or orphan species such as millet (Bertin et al. 2005).

The first comparative genetic studies utilised RFLP markers because these markers are generally transferable across species (Moore et al. 1995). These studies identified significant macrocolinearity between cereal genomes and led to the generation of a consensus grass map based on 25 rice linkage blocks (Devos and Gale 2000; Feuillet and Keller 2002). Although RFLP markers enabled the establishment of macrocolinearity between cereal species, their use is limited as they produce low-resolution maps that only allow the detection of large re-arrangements (Salse et al. 2008). More recently, genomic resources have enhanced the potential to exploit synteny between the cereal species. The increasing availability of DNA sequences has enabled *in-silico* comparisons and the development of dense molecular marker maps. For example, more than 1 million wEST sequences are now available (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) of which 7107 have been physically mapped using deletion bins (Qi et al. 2004). The publication of the rice genomic sequence has enabled a number of comparative mapping studies with wheat (Singh et al. 2007; Sorrells et al. 2003) to improve our understanding of colinearity between the genomes. Furthermore, in wheat it has facilitated fine-mapping (Alfares et al. 2009; Kuraparthy et al. 2009; Distelfeld et al. 2006), candidate gene identification (Handa et al. 2008) and gene cloning (Griffiths et al. 2006).

Rearrangements of gene content and order are often found in studies of micro-colinearity between rice and wheat (Bennetzen and Ramakrishna 2002; Feuillet and

Keller 2002), limiting the applicability of the rice sequence for studies in some regions of the wheat genome. Recently the full genomic sequence for Brachypodium was completed (International Brachypodium Initiative 2010). Brachypodium is more closely related to wheat and other members of the Pooideae sub-family of cool season cereals and grasses than is rice (Bossolini et al. 2007) Recent studies suggest that Brachypodium and wheat diverged from rice 40-54 million years (Myr) ago, whilst Brachypodium diverged from wheat 32-39 Myr ago (International Brachypodium Initiative). Therefore, it should thus provide a more suitable reference genome for wheat and provide higher levels of sequence conservation and co-linearity for marker development, fine-mapping and positional cloning (Kumar et al. 2009). Synteny between wheat and Brachypodium could be exploited to develop further markers and to advance the mapping of the eyespot resistance genes *Pch1*, *Pch2* and any novel resistance genes.

1.10 Overall objectives

Combining 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 specific objectives of this research 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 fine map *Pch1* by identifying recombinants in the *Ae. ventricosa* segment and by exploiting synteny between wheat and Brachypodium.

Chapter 2

Development of PCR-based markers associated with *Pch2* eyespot resistance and the mapping of candidate genes identified by cDNA-AFLP.

Data from this chapter has been included in the publications:

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*. Theoretical and Applied Genetics 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. Theoretical and Applied Genetics 118:1045-1057

Abstract:

Eyespot is a fungal disease of the stem base of cereal crops and causes lodging and the premature ripening of grain. Wheat cultivar Cappelle Desprez contains a durable eyespot partial resistance gene, *Pch*2 on the long arm of chromosome 7A. A single chromosome (7A) recombinant population segregating for *Pch2* was screened for eyespot resistance in two independent seedling tests and mapped using SSRs. QTL interval mapping closely associated *Pch2* with the SSR marker *Xwmc525*. A previous cDNA-AFLP analysis identified 29 sequences differentially expressed between the eyespot susceptible variety Chinese Spring (CS) and the Chinese Spring chromosome substitution line Cappelle Desprez 7A (CS/CD7A) which contains *Pch*2. Primers were designed to these sequences and 14 were located to chromosome 7A. Three of these mapped to the region of *Pch2* making them putative candidates for involvement in eyespot resistance. Of particular interest are two fragments; 4CD7A8 and 19CD7A4, which have homology to an *Oryza sativa* putative callose synthase protein and a putative cereal cyst nematode NBS-LRR disease resistance protein (RCCN) respectively.

2.1 Introduction:

Eyespot is caused by two species of fungus, *Oculimacula acuformis* (formerly *Tapesia acuformis*) and *O. yallundae* (*T. yallundae*) (Crous et al. 2003), which are considered to be necrotrophic pathogens. However, there is evidence of an early asymptomatic stage during coleoptile colonisation by *O. yallundae* and it could thus be considered to be a hemi-biotrophic pathogen (Daniels et al. 1991; Blein et al. 2008). Although both species cause similar symptoms, differences have been reported in their host pathogenicity, epidemiology, plant infection strategy and responses to fungicides (Hollins et al. 1985; Lange-de-la Camp, 1966; Scott and Hollins, 1980; Poupard et al. 1994, Wan et al. 2005, Daniels et al. 1991, Bateman et al. 1990, Bierman et al. 2002). The relative abundance of the two species on cereal crops has changed over time; during the 1980s, *O. yallundae* was the prevalent strain of eyespot fungus (Parnell et al. 2008). However by the 1990s, *O. acuformis* had surpassed *O. yallundae* to become predominant in northern Europe, and the US Pacific Northwest (Douhan et al. 2002). This shift is thought to be due to different sensitivities to the widely used fungicide, prochloraz (Parnell et al. 2008). Therefore, due to its dominance in field populations, *O. acuformis* was used in the present study to investigate *Pch2* resistance.

The first eyespot resistant wheat variety to be discovered was Cappelle Desprez (Vincent et al. 1952). Even though it has been widely used in Europe (Scott et al. 1989), Cappelle Desprez resistance has proven durable (Johnson 1984; Scott et al. 1989). It has widely been considered that most resistance to eyespot in Cappelle Desprez is conferred by a gene (*Pch2*) on chromosome 7AL (Law et al. 1976; de la Peña et al. 1997). However, there is evidence of eyespot resistance conferred by other chromosomes (Law et al., 1976; Muranty et al., 2002), most notably an adult plant resistance gene on chromosome 5A (Muranty et al., 2002).

De la Peña et al. (1996) observed a 1:1 segregation of recombinant substitution lines for eyespot resistance, suggesting that a single gene controls *Pch2* resistance in Cappelle-Desprez. Although the gene has been shown to be associated with the isozyme marker *Ep-A1b* (Koebner and Martin 1990), a 15% recombination rate has been observed, suggesting that the loci are not closely linked (de la Peña et al. 1996). Subsequently, *Pch*2 was shown to map to approximately 11 cM proximal to RFLP

marker *Xcdo347* and 18.8 cM distal to *Xwg380* on the distal end of the long arm of chromosome 7A (de la Peña et al., 1997). However, it was suggested that the two RFLP markers *Xcdo347* and *Xwg380* could be used to select for *Pch*2 as double recombination occurred only in 3% of the recombinant population. More recently, *Pch2* has been mapped to a 7 cM interval between SSR markers *Xwmc346* and *Xcfa2040* located on the distal end of the long arm of chromosome 7A and is closely associated with SSR *Xwmc525* (Chapman 2005).

It is not yet known if *Pch2* is constitutively expressed or induced by *Oculimacula spp*. Various physiological studies into the nature of *Pch2* resistance have proven inconclusive, providing evidence for both a constitutive and induced basis to this resistance. Non-inoculated mature wheat cultivars containing *Pch2* had a thicker hypodermis with more cell layers than equivalent susceptible cultivars suggesting that anatomy may play an important role in resistance (Murray and Bruehl 1983). This same study concluded that such a resistance mechanism may be less likely to be overcome by new pathogen strains which would in part explain the durability of *Pch2* resistance (Murray and Bruehl 1983). By contrast a second study showed that papillae formation in leaf sheaths, produced in response to eyespot infection, was higher in cultivars containing *Pch2* than in susceptible cultivars suggesting that resistance is induced (Murray and Ye 1986).

The cDNA-AFLP technique has been previously used to identify and isolate differentially expressed genes associated with plant-pathogen interactions e.g. the response of barley (*Hordeum vulgare*) to infection with powdery mildew (*Blumeria graminis* f.sp. *hordei*) (Eckey et al. 2004). It has also been used to identify markers that co-segregate with the powdery mildew resistance gene *Mlg* in barley and to generate markers closely linked to the tan spot (*Pyrenophora tritici-repentis*) resistance gene *Tsn1* in wheat (Korell et al. 2007; Haen et al. 2004).

Recently, a cDNA-AFLP approach was used to identify genes that were differentially expressed between the eyespot susceptible cultivar Chinese Spring (CS) and the (*Pch2*) resistant chromosome substitution line Chinese Spring / Cappelle Desprez 7A (CS/CD7A). Both constitutive differences in expression and differences induced by infection with *O. acuformis* were assessed. In addition, reverse transcriptase (RT)-PCR was used to determine whether the differential intensity of cDNA-AFLP fragments

was due to differential expression or allelic polymorphism between CS and CD on chromosome 7A (Chapman 2005). 34 fragments were found to be differentially expressed between CS and CS/CD7A and clones of 29 of these fragments were obtained. Sequences of these clones were compared to those in the NCBI database and it was determined that although these mostly had homology to genes involved in metabolism, cellular communication or development, four (14%) had homology to proteins involved in defence responses (Table 2.1). In comparison, 1% of predicted genes in rice have been identified as disease resistance (R) genes encoding nucleotide-binding-sites (NBS) (Zhou et al. 2004). This provides an indication that the cDNA-AFLP expression analysis identified a greater proportion of resistance genes than may be expected randomly. However, the proportion of the wheat genome consisting of defence related genes is currently unknown.

Table 2.1: Summary of differentially expressed fragments generated by cDNA-AFLP between Chinese Spring (CS) and Chinese Spring/Cappelle Desprez 7A (CS/CD7A) and homology of differentially expressed fragments with nucleotide or protein sequences in the NCBI (National Centre for Biotechnology Information) database (adapted from Chapman 2005).

Sequence name[a]	Isolated From	Homology with BLASTn or BLASTx[b]	BLAST E-value
Metabolism			
2CD7A1	CS/CD7A Constitutive	XM_482526.1 (*Oryza sativa*) putative dihydrolipoamide acetyltransferase (N)	2e-88
2CD7A20	CS/CD7A Constitutive	XM_482597.1 (*O. sativa*) putative diphosphate-fructose-6-phosphate (N)	6e-48
16CD7A9	CS/CD7A Constitutive	NP_910779.1 (*O. sativa*) putative NADH dehydrogenase (X)	8e-07
17CD7A13	CS/CD7A Constitutive	XM_482597.1 (*O. sativa*) putative diphosphate-fructose-6-phosphate 1-phosphotransferase (N)	4e-69
18CD7A12	CS/CD7A Constitutive	BAD67843.1 (*O. sativa*) putative prolyl aminopeptidase (X)	2e-30
20CD7A8	CS/CD7A Constitutive	XM_464982.1 (*O. sativa*) lipase class 3-like (N)	3e-52
25CD7A14	CS/CD7A Constitutive	AAA68209.1 (*Z. mays*) sus1 gene product (X)	1e-49

Table 2.1 Cont.

Sequence name[a]	Isolated From	Homology with BLASTn or BLASTx[b]	BLAST E-value
30CS8	CS Induced	XP_467559.1 (*O. sativa*) putative ribulose-1, 5 bisphoshate carboxylase/oxygenase small subunit N-methyltransferase (X)	2e-05
33CD7A8	CS/CD7A Constitutive	NP_196706 (*A. thaliana)* oxygen-evolving complex-related (X)	2e-13
33CD7A18	CS/CD7A Constitutive	NP_196706 (*A. thaliana*) oxygen-evolving complex-related (X)	3e-08
37CS17	CS Constitutive	CAA84022 (*H. vulgare*) beta-ketoacyl-ACP synthase (X)	8e-44
40CS7	CS Constitutive	AAM92706.1 (*T. aestivum*) putative cytochrome c oxidase subunit 6b (X)	5e-64
16CD7A18	CS/CD7A Constitutive	XM_479680.1 (*O. sativa*) phosphatidylinositol 3,5-kinase like (N)	2e-42
21CD7A14	CS/CD7A Induced	AP003832.3 (*O. sativa*) putative bZIP family transcription factor (N)	0.005
23CD7A8	CS/CD7A Constitutive	XM_464102.1 (*O. sativa*) GHMP kinaselike protein (X)	4e-42
28CD7A10	CS/CD7A Constitutive	XP_482875.1 (*O. sativa*) F-box protein family like (X)	0.13
31CD7A6	CS/ CD7A Constitutive	XP_464580.1 (*O. sativa*) ZIGA2 protein-like (X)	4e-17
36CS1	CS Induced	AAP53900.1 (*O. sativa*) putative DNA binding protein (X)	2e-42
36CS22	CS Induced	AAR82959.1 (*O. sativa*) transducin/WD-40 repeat protein (X)	3e-63
37CS3	CS Constitutive	BAD54671.1 (*O. sativa*) putative C2H2 zinc-finger protein SERRATE (X)	7e-40
Development			
4CD7A8	CS/CD7A Constitutive	NP_001058646 Callose synthase 1 catalytic subunit (X)	1e-80
22CD7A19	CS/CD7A Constitutive	XP_480766.1 (*O. sativa*)putative proteasome 26S non-ATPase subunit1 (X)	3e-19
25CD7A2	CS/CD7A Constitutive	AF542974.1 (*T. aestivum*) Emrl (N)	2e-50
38CS1	CS Constitutive	NP_186875.2 (*A. thaliana*) auxin transport protein (BIG) (X)	0.38
28CD7A3	CS/CD7A Constitutive	DQ334407 (*T. aestivum*) drought-responsive factor-like transcription factor DRFL1a (N)	9e-24
33CD7A2	CS/CD7A Constitutive	XM_470271 (*O. sativa*) putative glutathione reductase. (N)	4e-08

Table 2.1 cont.

Sequence name[a]	Isolated From	Homology with BLASTn or BLASTx[b]	BLAST E-value
Defence			
10CD7A7	CS/CD7A Constitutive	AY581258.1 (*Zea mays*) Rpl-D213 rust resistance protein (N)	2e-33
19CD7A4	CS/CD7A Induced	AF320848 (*Triticum aestivum*) NBS-LRR disease resistance protein RCCN3 (N)	4e-49
20CD7A12	CS/CD7A Induced	AAP54661.1 (*O .sativa*) putative plant disease resistance polyprotein (X)	3e-10
Transport			
14CD7A4	CS/CD7A Constitutive	AJ011921.1 (*Hordeum vulgare*) amino acid selective channel protein (N)	e-175
32CS20	CS Induced	NP_914242.1 (*O. sativa*) putative glucose inhibited division protein A (X)	4e-32
16CD7A3B	CS/CD7A Constitutive	AJ277799.1 (*H. vulgare*) putative elongation factor 1 beta (N)	5e-92
17CD7A12	CS/CD7A Constitutive	AJ277799.1 (*H. vulgare*) putative elongation factor 1 beta (N)	e-119
24CD7A20	CS/CD7A Constitutive	NM_125526 (*A. thaliana*) rRNA processing protein-related (N)	2e-23
28CD7A8	CS/CD7A Constitutive	AY049041.1 (*T. aestivum*) 28S ribosomal segment (N)	2e-07
31CD7A10	CS/CD7A Constitutive	NP_974263.1 (*A. thaliana*) putative mRNA capping enzyme (X)	8e-27
36CS8	CS Induced	BAD28853.1 (*O. sativa*) putative ribosomal protein L10a (X)	5e-27
6CD7A18	CS/CD7A Constitutive	AE017081.1 (*O. sativa*) putative retro-element (N)	0.063
21CD7A8	CS/CD7A Induced	AAP51781 (*O. sativa*) putative maize transposon MuDR-like (X)	2e-06
27CS17	CS Constitutive	AAP51893.1 (*O. sativa*) putative Tam3-like transposon protein (X)	1e-43
6CD7A12	CS/CD7A Constitutive	AAP44759.1 (*O. sativa*) unknown protein (X)	2e-39
14CD7A19	CS/CD7A Constitutive	XM_475185.1 (*O. sativa*) unknown protein (N)	5e-15
28CD7A4	CS/ CD7A Constitutive	AAO72604.1 (*O. sativa*) unknown protein (X)	2e-04
28CD7A21	CS/CD7A Constitutive	BAB09745.1 (*A. thaliana*) unnamed protein product (X)	1e-09
29CS22	CS Induced	CK207443.1 (*T. aestivum*) unknown function (N)	2e-55

Table 2.1 cont.

Sequence name[a]	Isolated From	Homology with BLASTn or BLASTx[b]	BLAST E-value
32CS18	CS Induced	XM_482549.1 (*O. sativa*) unknown protein (N)	4e-18
No matches			
8CD7A8	CS/CD7A Constitutive	No hits	-
12CD7A4	CS/CD7A Constitutive	No hits	-
26CD7A16	CS/CD7A Constitutive	No hits	-

[a] First number of the fragment name corresponds to the extracted cDNA-AFLP fragment e.g. 2CD7A1 and 2CD7A20 represent two sequences from the same fragment.

[b] N = BLASTn; X = BLASTx

In a previous study (Chapman 2005), Natalie Chapman conducted the cDNA-AFLP expression analysis, cloned and sequenced the fragments, conducted the initial BLASTn homology searches to determine putative functions of fragments, designed primers to directly amplify from 14 sequences originating from eight of the fragments (14 primer pairs in table 2.2), and conducted the controlled environment room phenotyping trial. The aim of the present study was to identify the genetic location of the sequences from the cDNA-AFLP fragments, and to determine whether they are in the region of *Pch2* and can therefore be considered as candidates for the resistance gene. PCR primers were designed directly to sequences from differentially expressed fragments or to wESTs identified from the fragment sequences. These primers were used to amplify DNA from CS, CS/CD7A and CS group 7 nullisomic-tetrasomic lines. PCR products were analysed by single strand conformational polymorphism (SSCP) assay to identify those originating from chromosome 7A. Furthermore, the physical map positions of these fragments were determined using chromosome 7A deletion bin stocks (Endo and Gill 1996) and, where possible, the genetic map positions were

determined using a CS x CS/CD7A F_2 population. In addition, the genetic location of *Pch2* relative to existing SSR markers and novel cDNA-AFLP derived markers was confirmed through a QTL analysis using phenotypic data produced from a glasshouse seedling bioassay inoculated with *O. acuformis* on F_3 families derived from CS x CS/CD7A.

2.2 Materials and Methods:

2.2.1 Plant material

Chinese Spring (CS), a susceptible spring wheat, and single chromosome substitution line Chinese Spring / Cappelle Desprez 7A (CS/CD7A) containing the *Pch2* resistance, were used. The substitution line was developed by A.J. Worland and is maintained at JIC. CS/CD7A was crossed to the eyespot susceptible variety CS. F_1 plants were selfed to generate an F_2 population of 192 plants and leaf material was removed for genotyping. F_2 plants were then grown on and selfed to produce F_3 families (Chapman 2005). In the current study, these F_3 families were used in a glasshouse seedling bioassay to determine their resistance to leaf sheath penetration by *O. acuformis*.

2.2.2 Primer development

Primers were designed either directly to cDNA-AFLP fragment sequences, or by comparison to wheat Expressed Sequence Tags (wESTs) homologous to the fragment sequences, with reference to the annotated rice genome, to identify regions flanking introns (Table 2.2). Primers were tested for secondary structures, hairpins, primer dimers and annealing temperatures (http://www.sigma-genosys.com/calc/DNACalc.asp). Primers were synthesised by Sigma-Genosys Ltd, U.K.

Table 2.2: PCR primers designed to cloned cDNA-AFLP fragments or homologous wEST sequence to amplify from genomic DNA template.

Specific primer	Sequence
2CD7A1F	CATCTTTATCAGCCGCCACT [b]
2CD7A1R	ACTCATCACACCCGTCCTG [b]
2CD7A20F	ATCCGGCCCTTATGGTTTAC [b]
2CD7A20R	ACATCGACCCGACGTACAAG [b]
4CD7A8F	TCTGGTTTCGAGTGGCAA [a]
4CD7A8R	GTCCAGTCATCAAAGTCCTCA [a]
6CD7A12F	GCTCGATGGTTGGTAGTTGC [b]
6CD7A12R	TCAAATGCAAGTTCAGACACG [b]
6CD7A18F	CCGTCAGTTTGTGGATGATG [b]
6CD7A18R	CATGGCAGGAAATCACAATG [b]
8CD7A8F	AGATCTCATGGTCCAGCG [a]
8CD7A8R	TGCCACAATGCCTTGAT [a]
10CD7A7F	GGATGGTATTTCTGCCACCT [b]
10CD7A7R	CAGAGGGCCTTATTGATTCG [b]
12CD7A4F	ACAACCAAGGGCTCATTCTG [b]
12CD7A4R	ACGATGCAAGCATTTTGTTC [b]
14CD7A4F	ATGTGCAAGGAGGGAGCATA [b]
14CD7A4R	CCGATAAAAACATCGCTTCG [b]
14CD7A19F	TTGCAAAGCTTTCTGTGCTG [b]
14CD7A19R	AGGGTGTTCAGGGTGAGTTG [b]
16CD7A9F	GGCCTGTGCAGGGTCTTAT [b]
16CD7A9R	CTCCATCAAAGCACACAAGG [b]
16CD7A18F	ATTTGGTGAACAGGGACTGC [b]
16CD7A18R	AAATGTGCAGAAGTTGGCTGT [b]
17CD7A12F	TGGATACCAAGCTTCCAAGG [b]
17CD7A12R	AGACAGCCAGAAGCAGAAGG [b]
17CD7A13F	GAGTGTTGCAGATGCAGGAA [b]
17CD7A13R	ATTTCAAGGGCATCGGTGT [b]
18CD7A12F	AAGTTGACGTACATGTCCTCG [a]
18CD7A12R	CGATGAGATCAATGCTCTACG [a]
19CD7A4F	GCCACCCACATTCTAACCAG [c]
19CD7A4R	CCACATTGCAGAAAAGCTGA [c]
20CD7A8F	CCAGGAGACCATGTATGTTCAGA [b]
20CD7A8R	AGGTGCAGCGGTTCTGATT [b]
20CD7A12F	TGCATAGCCCTTGTCATCAG [b]
20CD7A12R	CTGTCCCGTGTCGCTCAT [b]
21CD7A8F	TTTCATCAGCCGCCTCT [a]
21CD7A8R	GATAAACCTAGAAGGAAGCGG [a]

Table 2.2 cont.

Specific primer	Sequence
22CD7A19F	TATGATTCAGAGAGCAGGG [a]
22CD7A19R	GATGATGTTCGTAGGACTGC [a]
23CD7A8F	ACGATTCAGAGAGCAGGG [a]
23CD7A8R	GATGATGTTCGTAGGACTGC [a]
24CD7A20F	AACTCCTTGAGAAGTTGATG [a]
24CD7A20R	GAAATCTGATGTTGGTGTATT [a]
25CD7A19F	GCTCAGATGAACCGTGT [a]
25CD7A19R	ATGCCTCGATGACAGTAAG [a]
26CD7A16F	CAACCAAAAGGAGAGGTCC [a]
26CD7A16R	CACCCACCACTTCTTCAA [a]
27CD7A17F	AGTTGGTCAGCGCCTTG [a]
27CD7A17R	CAGTTCCTCTGCAGCTATATCAC [a]
28CD7A4F	TCTGCTACCACCACCTCGT [a]
28CD7A4R	GCTCCTCTGCCGCTCT [a]
29CS22F	ATTGGCACAGCTATCTTGAT [a]
29CS22R	GCGGATCCAGAATTCTG [a]
30CS8F	GATGAGGAGGCGCCTTT [a]
30CS8R	GGTATAGCCCCAGATGTG [a]
31CD7A10F	AGGCCTTCTGATCTTGAT [a]
31CD7A10R	CCCTAGGACATCATCATTAG [a]
32CS18F	GGTAGGCACACACACTTTGC [c]
32CS18R	TACGAGCCATACCCTGGATT [c]
33CD7A8F	CCTAATCTGTTTCGGCAC [a]
33CD7A8R	TGAAAGGATGCCACAGC [a]
36CS8F	GACTTCTTGGACCTGGTCTT [a]
36CS8R	TTGGACTCGAGGGATTCC [a]
37CS3F	CAGCGTTCATTCCTCCTTTC [a]
37CS3R	ATCACTACCCAGAAACGTGCT [a]
38CS1F	AACCACCTGCTTTCCGTATG [c]
38CS1R	TGCGTACATGCAGTTGTTGA [c]
40CS7F	GAGATCGGTAGTACTTGGCAA [a]
40CS7R	ACCTGCTGACTTCCGCTT [a]

[a] Primer designed to amplify genomic DNA based on wEST sequence homologous to fragment sequence.

[b] Primers previously designed by Chapman (2005) directly to fragment sequences

[c] Primers designed directly to fragment sequences in the current study.

2.2.3 Chromosomal origin of differentially expressed fragments

To determine whether cDNA-AFLP derived clones are located on chromosome 7A, and to detect any polymorphisms between CS and CS/CD7A, the primers (Table 2.2) were used in a PCR with DNA of parental lines CS and CS/CD7A, and Chinese Spring nullisomic-tetrasomic lines 7A, 7B and 7D. PCR products were analysed by SSCP as described by Martin-Lopes et al. (2001), using Sequa Gel® MD (National Diagnostics, U.K Ltd) and visualised by silver staining (Bassam et al. 1991).

2.2.4 Mapping of markers developed by cDNA-AFLP

To determine the physical map positions of the cDNA-AFLP clones originating from chromosome 7A, DNA from twenty-one homozygous deletion bin lines for chromosome 7A of Chinese Spring (Endo and Gill 1996) were used in a PCR with the genomic DNA primers (Table 2.2). A selection of the chromosome 7A SSR markers used by Chapman (2005), were included in the deletion bin analysis to provide a comparison between genetic and physical maps along the full length of the chromosome. Where clear polymorphisms were detected between CS and CS/CD7A, markers were genetically mapped using the F_2 population of 192 lines derived from CS x CS/CD7A. These results were combined with SSR marker data for the population previously generated by Chapman (2005) to generate a combined 7A genetic linkage map.

2.2.5 Phenotypic trials

Eyespot resistance was assessed for 183 F_3 families from CS x CS/CD7A by a seedling bioassay in an unheated unlit glasshouse at RAGT Seeds Ltd., Cambridge, between December 2006 and February 2007. This experiment was conducted in a randomised complete block design with six blocks. In each block, six seeds of each F_3 family and twelve seeds of both parental lines were sown onto peat and sand compost in 35 cm x 20 cm seed trays, with 20 lines per tray. *O. acuformis* isolates were kindly provided by Bill Hollins from the collection of RAGT Seeds Ltd.

Inoculum was prepared using the method of Bruehl and Nelson (1964). 225 ml threshed oat grains and 125 ml sterile distilled water was mixed in 2 litre conical flasks and autoclaved twice. These were inoculated with mycelial cultures of *O. acuformis* maintained on V8 agar and incubated at 20 °C for approximately 1 month, shaken vigorously every 2-3 days. After incubation the oat grain inoculum was mixed in a 1:1 ratio with peat and sand compost and applied to the seeds so that seedlings emerged through the inoculum mixture. Plants were harvested 10 weeks after sowing and scored to measure the degree of penetration into leaf sheaths as described by Scott (1971).

Data was obtained from a controlled environment room (CER) phenotypic trial, previously conducted by Chapman (2005). In the CER trial, nine plants from each of 162 F_3 families were grown in 5 x 5 cm pots arranged in a complete randomised block design with three blocks.

2.2.6 Statistical analysis

Analysis of variance (ANOVA) was calculated using a generalised linear model (GLM) in Genstat, ninth edition (Copyright 2006, Lawes Agricultural Trust (Rothamsted Experimental Station)) to assess variability attributed to block and genotype and to generate predicted mean disease scores for each line in the glasshouse and CER phenotypic trials independently. These means were used in a subsequent QTL analysis. Data from the glasshouse and CER trials were combined in a GLM to predict the mean disease score for each line combined across the two trials in a further QTL analysis.

2.2.7 Map construction and QTL analysis

Linkage maps were calculated from recombination frequencies (0.4) and a LOD of 3.0 was used in JoinMap® version 3.0 (van Ooijen et al. 2001). QTL analysis was carried out using MapQTL® version 4.0 (van Ooijen and Maliepaard, 1996). Phenotypic data from the two seedling trials for eyespot resistance in F_3 families from the

CSxCS/CD7A population were used in a QTL interval mapping analysis. A permutation test (1,000 cycles) was used to determine the LOD score at which the QTL was deemed to be present in the given genomic region with a confidence interval of 99 %.

2.3 Results:

2.3.1 Marker development

Wherever possible, intron spanning primers were developed in this chapter to increase the probability of finding polymorphisms between CS and CS/CD7A. For example, in pearl millet (*Pennisetum glaucum*) the frequency of SNPs or insertions and deletions (indels) has been estimated at 1 per 59 bp in introns and 1 per 714 bp in exons. Although the overall rate of polymorphism is lower in wheat, estimated at 1 SNP in 1,000 bp overall (Bryan et al. 1999), a higher rate of polymorphism has been observed in introns compared to exons (Bryan et al. 1999; Ishikawa et al. 2007).

Initially primer sets were designed directly to the clone sequences in order to amplify genomic DNA, with the aim of developing suitable markers for each cDNA-AFLP fragment. Clone sequences were aligned with rice orthologues and PCR primers were designed to flank introns. However, amplification was only successful for 7 of the 29 clones using this method (Chapman 2005). In the present study, primers were successfully designed directly to sequences for the clones 19CD7A4, 32CS18 and 38CS1. Sequences from the remaining 16 clones were compared to wESTs (GenBank, http://ncbi.nlm.nih.gov/entrez) using a BLASTn search. wESTs homologous to each clone were aligned using ClustalW (Chenna et al. 2003), and intron positions were identified by comparing these aligned EST sequences to genomic sequence from rice (GenBank, http://ncbi.nlm.nih.gov/entrez) and *Brachypodium* (www.modelcrop.org) through a further BLASTn search. PCR primers were again designed to produce amplicons that span introns with the aim of developing polymorphic markers on chromosome 7A. Of the fourteen clones for which two or more sequences were available, the sequence with the most suitable intron sizes (100-300 bp) and intron

spanning regions appropriate for primer design were selected for marker development. Primer sequence and origin of the design template are detailed in Table 2.2.

PCR assays to each of the 29 clones (Table 2.2) were used to amplify from DNA of CS, CS/CD7A and CS nullisomic-tetrasomic lines N7AT7D, N7BT7A and N7DT7B. Amplicons were subjected to gel-based single stranded conformational polymorphism (SSCP) analysis to determine whether they derived from chromosomes 7A, 7B and/or 7D and to detect any polymorphisms between CS and CS/CD7A. For example, there are no polymorphisms between lanes 1 and 2 (CS and CS/CD7A) for sequence 32CS18 (Figure 2.1). A CS and CS/CD7A mix was included in lane 3 to validate potential co-dominant polymorphic markers on an artificial heterozygote. The absence of the CS bands in the CS nullisomic-tetrasomic series (lanes 4, 5 and 6) suggests that 32CS18 has loci on chromosomes 7A, 7B and 7D (Figure 2.1).

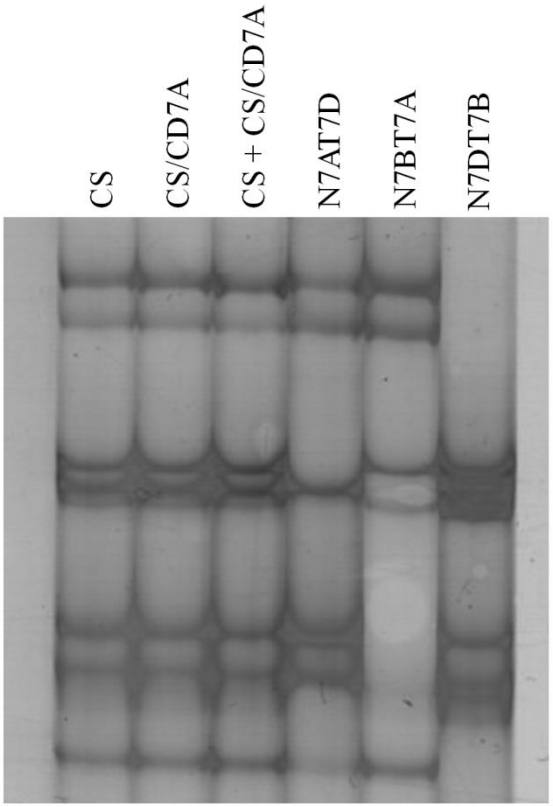

Figure 2.1 A SSCP gel of a screen between Chinese Spring (CS), the chromosome substitution line CS/CD7A, CS + CS/CD7A, and CS nullisomic-tetrasomic lines N7AT7D, N7BT7A and N7DT7B with sequence 32CS18.

SSCP analysis indicated that 16 sequences originated from group 7 chromosomes (Table 2.3). Fourteen of these could be shown to derive from genes on 7A of which eight specifically amplified from 7A. Two sequences were also present on 7B, one was also present on 7D, and three were also present on both 7B and 7D. Furthermore, one sequence originated from 7B, and one was from 7D. No bands relating to any of the sequences from the remaining 13 clones were absent on any of the CS group 7 nullisomic-tetrasomic lines, indicating that they probably derive from genes on other chromosomes.

Table 2.3: Chromosome location and physical map position of markers designed to cDNA-AFLP fragments.

cDNA-AFLP fragment	Primer template	Chromosome origin	Chromosome 7A deletion bin location
2CD7A20	Fragment	7A[a]	0.83 7AS12 - 0.73 7AS2
4CD7A8	wESTs	7A and 7B	0.99 7AL15
6CD7A18	Fragment	7A[a]	Centromere
8CD7A8	wESTs	7A and 7B	Centromere
10CD7A7	Fragment	7A[a]	Centromere
16CD7A18	Fragment	7A[a]	0.4 7AL11 - 0.31 7AL14
17CD7A13	Fragment	7A[a]	0.83 7AS12 - 0.73 7AS2
18CD7A12	wESTs	7A and 7D	0.83 7AS12 - 0.73 7AS2
19CD7A4	Fragment	7A	Not mapped
20CD7A12	Fragment	7A[a]	Centromere
22CD7A19	wESTs	7B	n/a
25CD7A14	wESTs	7A	0.89 7AS1
28CD7A4	wESTs	7A, 7B and 7D	0.49 7AL10 - 0.4 7AL11
30CS8	wESTs	7D	n/a
32CS18	Fragment	7A, 7B and 7D	0.74 7AL21 - 0.63 7AL5
33CD7A8	wESTs	7A, 7B and 7D	0.99 7AL15

[a] Data obtained from Chapman (2005).

2.3.2 Physical and genetic mapping

The physical map positions of the sequences associated with chromosome markers were determined using a set of CS7A deletion bin lines (Endo & Gill, 1996). These results are shown in Table 2.3 and in Figure 2.2. DNA markers were named according to the cDNA-AFLP fragment from which they derived (markers are shown with a 'X' prefix and in italic font in accordance with convention). Deletion bin locations were determined for 13 of the 14 cDNA-AFLP derived markers from chromosome 7A, and were found to be distributed evenly over the chromosome. Significantly, two of these markers, *X4CD7A8* and *X33CD7A8*, were located in the distal deletion bin of chromosome 7AL (0.997AL15) in the region of *Pch2* (Figure 2.2). In addition, the marker *X19CD7A4* was determined to derive from chromosome 7A as a clear polymorphism was observed between CS and CS/CD7A. However, it could not be bin-mapped because no bands were found to be absent in the nullisomic-tetrasomic or deletion bin lines. This is likely to be because products from the 7A, 7B and 7D homoeologues of CS are identical to each other and cannot be distinguished by SSCP, whereas there is a difference between the products from CS 7A and CD 7A.

Polymorphisms were detected between CS and CS/CD7A for the cDNA-AFLP markers *X6CD7A18*, *X20CD7A12* and *X19CD7A4*. These markers were genetically mapped using the F_2 population derived from CS x CS/CD7A, alongside a previously calculated SSR map (Chapman 2005), to identify markers associated with *Pch2* resistance (Figure 2.2). *X6CD7A8* and *X20CD7A12* both mapped genetically to the centromeric region, in confirmation of their deletion bin locations. *X19CD7A4* mapped distal to *Pch2* at the end of chromosome 7AL (Figure 2.2).

2.3.3 Pch2 resistance in the CS x CS/CD7A F_3 families

A total of 162 CS x CS/CD7A F_3 families were phenotyped for *Pch*2 resistance to *O. acuformis* in the CER trial (Chapman 2005) and 183 F_3 families were phenotyped in the glasshouse trial in the present study. Analysis of variance demonstrated that genotype was highly significant in both trials but block and residual variance were

both higher in the glasshouse trial, presumably due to a more heterogeneous environment in the unheated and unlit glasshouse (Table 2.4).

Table 2.4 Variance components of visual eyespot disease scores using generalised linear modelling for the two disease trials of the CS x CS/CD7A F_3 families.

Source of Variation	Glasshouse Trial		CER Trial[a]	
	MS	*F* value	MS	*F* value
Block	164.55	80.66***	8.32	8.76***
Genotype	16.94	8.3***	28.27	29.78***
Block x Genotype	4.16	2.04***	2.35	2.48***
Residual	2.04		0.95	

[a] Controlled environment room trial conducted by Chapman (2005).

MS mean squares, *** P<0.001

2.3.4 QTL interval mapping analysis

QTL interval mapping was used to determine the genomic location of *Pch2* on chromosome 7A and to identify SSR and cDNA-AFLP markers associated with the resistance. QTL analysis revealed that *Pch2* was most significantly associated with three SSRs; *Xwmc346*, *Xwmc525* and *Xcfa2040*, that mapped on the distal end of the long arm of chromosome 7A (Figure 2.2) and that the QTL position was consistent between both phenotypic trials.

Table 2.5. Summary of the QTL interval mapping analysis of *Pch2* on chromosome 7AL.

7AL Marker	Map Distance (Kosambi cM)	Glasshouse Test		CER[a]		Combined	
		LOD[b]	% Variance Explained	LOD[b]	% Variance Explained	LOD[b]	% Variance Explained
Xwmc83	0	0.7	2.1	0.4	1.3	0.7	1.8
X6CD7A18	18	0.15	0.4	0.1	0.3	0.3	0.8
Xbarc108	19	0.1	0.3	0.1	0.2	0.3	0.6
Xpsp3050	19	0.1	0.3	0.1	0.2	0.3	0.6
X20CD7A12	20	0.1	0.3	0.1	0.3	0.3	0.7
Xbarc29	32	0.3	0.7	0.3	0.8	0.3	0.7
Xgwm276	41	0.1	0.3	0.4	1.1	0.1	0.3
Xgwm332	70	3.1	8.3	0.2	0.7	1.9	5.0
Xcfa2019	76	4.5	15.1	0.8	3.4	3.3	10.2
Xwmc346	111	13.2	31.2	10.9	26.7	13.4	29.2
Xwmc525	115	18.2	40.1	15.1	35.1	17.6	36.3
Xcfa2040	119	18.1	40.0	11.0	27.1	14.1	30.7
X19CD7A4	132	12.8	32.4	8.7	23.7	10.0	24.4

[a] Controlled Environment Room trial conducted by Chapman (2005).

[b] Logarithm of the odds score

The marker most significantly associated with eyespot disease in all instances was SSR *Xwmc525*. This marker explained 35.1% of the phenotypic variance observed in the CER trial and 40.1% in the glasshouse trial (Table 2.5). The cDNA-AFLP markers *X6CD7A8* and *X20CD7A12* that mapped in the centromeric region accounted for little phenotypic variation. However, *X19CD7A4*, which mapped distal to *Pch2* at the end of chromosome 7AL accounted for 24.4% (Table 2.5).

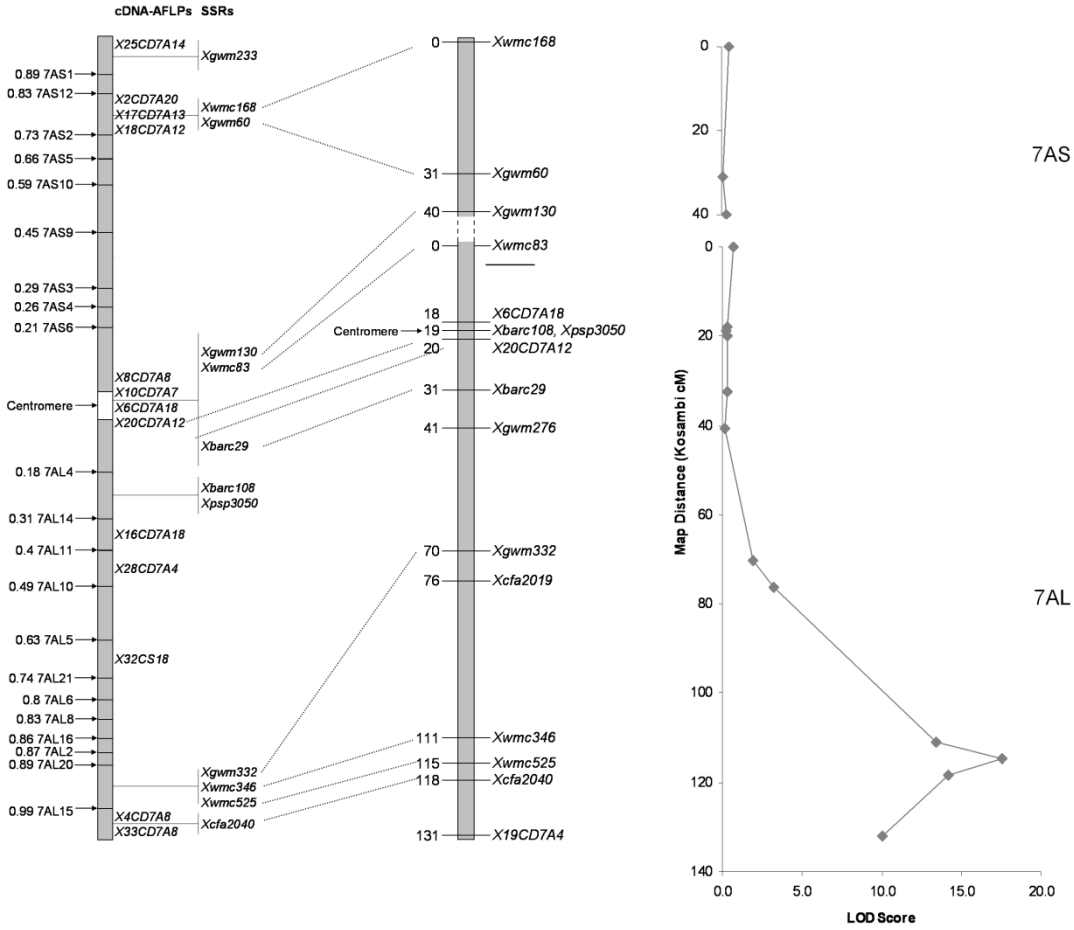

Figure 2.2 A comparison of integrated SSR and cDNA-AFLP physical and genetic maps of chromosome 7A. A LOD profile from the combined interval mapping analysis of *Pch2* on chromosome 7A is also shown aligned to the genetic map to demonstrate to position of *Pch2*.

2.4 Discussion

This study has confirmed the location of *Pch2* on the distal end of the long arm of chromosome 7A. Evidence from two independent phenotypic trials conducted in different environments, position *Pch2* at the distal end of the long arm of chromosome 7A closely linked to SSR marker *Xwmc525*, within the 7cM interval flanked by SSR markers *Xwmc346* and *Xcfa2040*. Although previous studies have mapped *Pch2* as a qualitative trait (de la Peña et al. 1997), it was not possible to clearly categorise F_3 families in the present study. This is presumably due to the lack of potency of *Pch2* compares to *Pch1* (Hollins et al. 1988) and because of the

variability associated with using F_3 families, particularly in the uncontrolled conditions of a glasshouse trial. Therefore, the resistance was analysed as a quantitative trait.

To identify further markers on chromosome 7A and to identify candidate genes for involvement in the *Pch2* resistance response, Chapman (2005) used a cDNA-AFLP approach. cDNA-AFLP is an open system allowing simultaneously comparison of both constitutive and induced differences in expression of host genes providing insight into the biological processes involved in plant-pathogen interactions. Wheat lines CS and CS/CD7A were used that differ only for the 7A chromosome that harbours *Pch2*, to increase the likelihood that differentially expressed sequences might be associated with this gene. A remarkably small proportion (0.7%), of the 4700 fragments observed was differentially expressed between CS and CS/CD7A. From these 34 differentially expressed fragments 29 were cloned and sequenced, putative function was determined by homology searches and six sequences were assigned to chromosome 7A (Chapman 2005). The present study has built upon this work, by determining which of the unassigned sequences originate from group 7 chromosomes, determining their genetic location either physically by CS deletion bins or where polymorphisms exist by genetic mapping using the CS x CS/CD7A F_2 population. Furthermore, these markers were related to *Pch2* phenotypic data using a QTL interval mapping analysis to determine whether they are likely to be candidates for involvement in the *Pch2* resistance response.

Using SSCP analysis, sequences from 16 of the cDNA-AFLP fragments were shown to be located on group 7 chromosomes, of which 14 mapped to chromosome 7A. Primers designed to six of the 7A sequences also amplified homoeologues on chromosomes 7B and/or 7D. This may be expected, as EST-based markers often amplify homoeologous genes in wheat (Parida et al. 2006, Ishikawa et al. 2007, Xue et al. 2008). Differences between CS and CS/CD7A detected by cDNA-AFLP might be expected to originate only from chromosome 7A. However, the sequences 22CD7A19 and 30CS8 appeared to originate from chromosome 7B and 7D respectively. This is probably a result of the SSCP assay for these sequences being inadvertently designed to homoeologues from these chromosomes, rather than 7A, based upon the wEST sequences available in the NCBI database. For fragments that appear to derive from non-group 7 chromosomes the observed differences in expression may indicate that

their expression is influenced by factors on 7A or that they are expressed differentially as a consequence of the differential resistance of the two lines. It should also be borne in mind that amplicons from other chromosomes may have been cloned as contaminants from the isolated cDNA-AFLP fragment (Chapman 2005).

Most of the fourteen sequences that could be assigned to chromosome 7A had homology to genes involved in metabolism, cellular communication, or development. Four had homology to proteins involved in defence responses and one had weak homology to a putative retroelement. Previous studies of differential gene expression following pathogen infection have identified genes with homology similar to those found herein. For example, Eckey et al. (2004), used cDNA-AFLP to examine gene expression in barley (*H. vulgare* L.) after inoculation with powdery mildew (*Blumeria graminis* f.sp. *hordei, Bgh*), and found that the majority of fragments showed homology with proteins involved in secondary and primary metabolism. Genes encoding proteins involved in signal transduction, metabolism, protein synthesis, stress and plant defence, were also isolated using the suppression subtractive hybridisation (SSH) technique from resistant potato cultivars that had been inoculated with *Phytophthora infestans* (potato late-blight) (Birch et al. 1999).

The present study showed that *Xwmc525* is located in the 7AL deletion bin (0.897AL20-0.997AL15), whilst *Xcfa2040* is located in the immediately distal, and terminal, deletion bin (0.99 7AL15). Therefore it appears likely that *Pch2* is positioned around the 0.99 bin breakpoint, possibly in the region within the terminal bin. Physical mapping using CS 7A deletion bin lines positioned two of the cDNA-AFLP derived markers (*X4CD7A8* and *X33CD7A8*) in the distal deletion bin of chromosome 7AL, in approximately the same region as *Pch2*. Therefore, the genes relating to these sequences can be considered as candidates 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 (Chapman 2005). Callose synthase is produced in response to wounding and as a defence response to pathogen attack (Østergaard et al. 2002; (Jacobs et al. 2003) as well as during cell wall development (Hong et al. 2001). Callose deposition (papillae) acts as a physical barrier preventing pathogens from penetrating the host cell (Holub and Cooper 2004). Furthermore, papillae formation

and lignification of cell walls in epidermal and pith cells of wheat stems infected with *Oculimacula spp*. has also been associated with resistance (Murray and Ye 1986). Callose synthase may be associated with papillae formation preventing *Oculimacula spp*. from penetrating and infecting CS/CD7A. Previous RT-PCR studies revealed that expression of 4CD7A8 increased significantly in CS/CD7A in response to infection by *O. acuformis*, suggesting an involvement of this gene in the resistance response of CS/CD7A. In contrast, although expression of this gene could be detected in CS, it was not significantly enhanced in response to infection (Chapman 2005). The identification of the fragment from the original cDNA-AFLP analysis suggests that a polymorphism exists between CS and CS/CD7A in the gene relating to the fragment 4CD7A8. Unfortunately, our assays for 4CD7A8 did not detect any polymorphism between CS and CS/CD7A. However, this assay was based only on the available sequence of the callose synthase gene that aligned to the wEST sequence data in the NCBI database. When additional sequence data becomes available for this gene it will be possible to determine whether polymorphisms exist between CS and CD.

The second marker that physically mapped into the terminal deletion bin in the region of *Pch2* (*X33CD7A8*) showed highest homology with the 23 kDa subunit of the oxygen evolving system of photosystem II (NP_196706 of *Arabidopsis thaliana*). The primers designed to this sequence did not produce a polymorphic product between CS and CS/CD7A so it was not possible to map it genetically in the CS x CS/CD7A population. While it is conceivable that this gene is involved in resistance to eyespot, it is, perhaps, more probable that the differential amplification of this cDNA-AFLP fragment reflects allelic diversity between CS and CD at this locus.

The three sequences (10CD7A7, 19CD7A4 and 20CD7A12), that demonstrated homology to proteins associated with plant disease resistance (Chapman 2005) were shown to be located to chromosome 7A and might therefore be involved in defence responses following recognition of *O. acuformis* by *Pch2* elsewhere on the chromosome. Sequence 10CD7A7 shows homology with a *Z. mays RP1*-D213 rust resistance protein that confers race-specific resistance to maize common rust (*Puccinia sorghi*) (Smith et al. 2004). *RP1-D* genes belong to the NBS-LRR class of R genes (Collins et al. 1999) that have been shown to recognise a number of fungal and bacterial pathogens (Ayliffe and Lagudah 2004). Sequence 19CD7A4 showed

homology with a putative cereal cyst nematode NBS-LRR disease resistance protein (RCCN), while 20CD7A12 showed homology to a putative plant disease polyprotein.

Sequences 19CD7A4 and 20CD7A12 were constitutively expressed only in CS/CD7A, whilst 10CD7A7 was constitutively expressed in both CS and CS/CD7A as previously shown by RT-PCR (Chapman 2005). Expression of 20CD7A12 showed no response to infection by *O. acuformis* while expression of both 10CD7A7 and 19CD7A4 increased significantly in CS/CD7A in response to *O. acuformis*. A few resistance genes have been shown to be constitutively expressed at low levels but, in response to infection, to be up-regulated. For example expression of a sunflower coiled coil (CC) domain NBS-LRR resistance gene (Radwan et al. 2005) and the tobacco mosaic virus resistance gene *N* (Levy et al. 2004) have both been shown to be up-regulated in response to infection.

Genetic mapping revealed that *X19CD7A4* is located 16 cM distal of the SSR marker *Xwmc525* and is therefore relatively close to the proposed location of *Pch2*. Moreover, the function, location and expression profile of 19CD7A4 suggests that it 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.

Although 19CD7A4 was located in the region of *Pch2*, both 10CD7A7 and 20CD7A12 are located at the centromere and, therefore, are not candidates for the eyespot resistance gene itself. A possible explanation for the increase in expression of 10CD7A7 following infection is that a subset of genes, including 10CD7A7, involved in resistance may be influenced by some form of feed-back mechanism from products derived from the *Pch2* resistance. This explanation is supported by recent transcriptome analyses of major stripe rust resistance genes in wheat. Wheat Gene Chip analyses of the *Yr5* (Coram et al. 2008a) and *Yr39* (Coram et al. 2008b) resistances identified numerous induced *R* gene homologues that may control increased signalling and expression of defence-related products. Several of these transcripts, however, did not originate from the major resistance gene locus (Coram et al. 2008c). It is possible that a similar mechanism may occur in *Pch2* conferred

eyespot resistance, leading to the expression of sequences such as 10CD7A7 that are not associated with the *Pch2* locus.

In addition to providing candidates for the *Pch2* gene(s), cDNA-AFLP has provided additional insight into the response of wheat to infection by *O. acuformis*. The sequence 16CD7A18 mapped to the deletion bin 0.31-7AL14 - 0.4-7AL11 and therefore cannot be considered as a direct candidate for *Pch2*. However, expression of this sequence was previously shown to increase in both CS and CS/CD7A in response to infection by *O. acuformis* (Chapman 2005). 16CD7A18 showed homology with phosphatidylinositol 3, 5-kinase like proteins that are thought to be involved in cell-signalling pathways (Contento et al. 2004). Increased synthesis and hydrolysis of polyphosphoinositide, a derivative of phosphatidylinositol, is observed in a number of different plants in response to drought, salinity, temperature stress and pathogen attack (van Leeuwen et al. 2004). Enhanced expression of the sequence in CS and CS/CD7A indicates that expression of phosphatidylinositol 3, 5-kinase like proteins may be associated with infection in general rather than specifically with resistance.

To summarise, this study, alongside data from Chapman (2005), suggests that the *Pch2* QTL spans 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. 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 29 sequences shown to be differentially expressed between the *Pch2* carrying CS/CD7A line and the susceptible CS line in a previous cDNA-AFLP study (Chapman 2005). 14 of these sequences were shown to be derived from chromosome 7A. Two particularly interesting sequences, 4CD7A8 and 19CD7A4, were identified among the fragments and, although it is not possible to demonstrate conclusively that these sequences are responsible for *Pch2* mediated eyespot resistance, their functions, expression patterns and genetic locations suggest that they are candidates worthwhile of further investigation.

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Chapter 3

Differential seedling resistance to the eyespot pathogens, *Oculimacula yallundae* and *Oculimacula acuformis,* conferred by *Pch2* in wheat and among accessions of *Triticum monococcum*.

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Abstract

Eyespot is an economically important stem-base disease of wheat caused by two fungal species; *Oculimacula yallundae* and *Oculimacula acuformis.* The genes *Pch1*, introgressed into hexaploid wheat from *Aegilops ventricosa*, and *Pch2*, identified in the variety Cappelle Desprez, have both been used as sources of resistance by plant breeders. We investigated the efficacy of both of these resistances to *O. yallundae* and *O. acuformis* separately. In a series of seedling bioassays we found *Pch1* to be highly effective against both species. Although we found *Pch2* to confer resistance against both pathogen species, it was significantly less effective against penetration from *O. yallundae* than *O. acuformis*. Furthermore, a QTL analysis was not able to locate any resistance to *O. yallundae* on chromosome 7A of Cappelle Desprez. 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. In addition, a set of twenty-two *T. monococcum* accessions was screened for resistance to both *O. yallundae* and *O. acuformis* to identify potentially novel resistances and to assess the accessions for evidence of differential resistance to the eyespot species. Significant differences in resistance to the two pathogens were identified in four of these lines, providing evidence for differential resistance in *T. monococcum.* This study demonstrates that resistance can be species specific and therefore future screening for novel sources of eyespot resistance should investigate both pathogen species.

3.1 Introduction

Eyespot is a fungal disease of the stem base of cereal crops including wheat, barley and rye, and is caused by two species of fungi, *Oculimacula acuformis* (formerly *Tapesia acuformis*) and *O. yallundae* (*T. yallundae*) (Crous et al. 2003). Severe infection results in lodging and premature ripening of grain leading to reduced crop yield and is considered economically important in temperate areas such as North West Europe, North West USA and New Zealand (Fitt, 1992).

Although *O. yallundae* and *O. acuformis* cause broadly similar symptoms (Ray et al. 2006), a number of differences have been reported between the pathogens. The species can be separated on the basis of their colony morphology and host pathogenicity. *O. yallundae* forms fast-growing, even-edged colonies on potato dextrose agar (PDA) and is more aggressive on wheat than on rye, whilst *O. acuformis* forms slow-growing uneven-edged colonies on PDA and is equally pathogenic on wheat and rye (Lange-de la Camp, 1966; Scott et al. 1975; Hollins et al. 1985). Further differences can be observed between the two species in their infection strategy on wheat seedlings. After spore germination, *O. acuformis* follows a disorientated growth pattern producing short hyphae terminating in appressoria at random positions on the host surface. From these appressoria *O. acuformis* penetrates directly into coleoptile epidermal cells with hyphae then crossing cell walls into adjacent cell wall layers. In contrast, *O. yallundae* follows an orientated hyphal growth pattern to form appressoria at intervals along the anticlinal cell wall grooves. *O. yallundae* then grows within these to the next cell layer where a network of branching hyphae subsequently colonises anticlinal and periclinal cell walls (Daniels et al. 1991). Furthermore, evidence suggests that *O. yallundae* may be hemi-biotrophic, as it exhibits an asymptomatic biotrophic phase during coleoptile colonisation before switching to a necrotrophic phase once the pathogen reaches the first leaf sheath (Blein et al. 2009).

O. yallundae and *O. acuformis* also demonstrate different levels of sensitivity to certain fungicides and this is thought to have caused shifts in the relative abundance of the two species in field populations. *O. yallundae* was the most common causal organism of eyespot in northern Europe until the early 1980s when *O. acuformis* became increasingly prevalent, possibly due to differential sensitivity to

carbendazim-generating (MBC) fungicides (King & Griffin, 1983; Bateman et al. 1990). The predominance of *O. acuformis* was maintained in the 1990s, probably due to widespread use of the demethylation inhibitor fungicide, prochloraz, which was more effective against *O. yallundae* and consequently favourably selected for *O. acuformis* (Bateman et al. 1990; Bierman et al. 2002). More recently, there is evidence that the prevalence of *O. acuformis* compared to *O. yallundae* has declined. This has been attributed to a reduction in the use of prochloraz and an increase in the use of the anilinopyrimidine fungicide, cyprodinil, which has been shown to select for *O. yallundae* (Parnell et al. 2008). These population shifts illustrate the necessity of controlling both species of the pathogen, as control of only one species will act to increase the proportion of the other in field populations and allows host infection to continue (Bateman & Jenkyn, 2001).

Two main sources of genetic resistance to eyespot have been incorporated into cultivated wheat varieties, *Pch1* and *Pch2*. The more potent of these, *Pch1*, was introduced into wheat from the wild grass *Aegilops ventricosa* (Maia, 1967) and is located on the distal end of chromosome 7D (Doussinault et al. 1983; Worland et al. 1988), where it is linked to simple sequence repeat (SSR) markers *Xwmc14* and *Xbarc97* (Chapman et al. 2008). However, its use in commercial varieties has been limited as resistance is believed to be associated with a yield penalty in the absence of the disease (Johnson, 1992). The second resistance originates from the French variety Cappelle Desprez (Vincent et al. 1952). This partial resistance has been attributed to the gene *Pch2* on chromosome 7A (de la Peña et al. 1996; Law et al. 1976). *Pch2* resistance to *O. acuformis* has been mapped to the distal portion of chromosome 7AL (de la Peña et al. 1996). More recently, the SSR marker *Xwmc525* has been identified to be closely associated with the resistance gene (Chapman et al. 2008).

The paucity of genetic resistances available to plant breeders in hexaploid wheat and the limitations of the existing sources of resistance has led to a renewed interest in novel sources in wheat progenitors and relatives. These include *Triticum monococcum* (Cadle et al. 1997), *Triticum tauschii* (Yildirim et al. 1995), *Triticum durum* (Figliuolo et al. 1998), *Aegilops kotschyi* (Thiele et al. 2002) and *Dasypyrum villosum* (Murray et al. 1994). Although the majority of the work on novel resistances has focused solely on *O. yallundae*, there is evidence that some of these

resistance genes may confer different levels of resistance to *O. yallundae* and *O. acuformis*. A single dominant resistance gene, named *Pch3*, was identified in *D. villosum* and has been mapped to the distal portion of the long arm of chromosome 4V (Yildirim et al. 1995). Interestingly, this gene has been shown to confer resistance to *O. yallundae*, but not to *O. acuformis* (Uslu et al. 1998), suggesting that the genetic basis of resistance to the two eyespot pathogens can differ.

In order to provide effective eyespot control it is important that any genetic sources of resistance are effective against both forms of the pathogen. Therefore, a greater understanding of the effectiveness of *Pch1* and *Pch2* resistance genes towards both *O. yallundae* and *O. acuformis* is required. This study aims to determine the level of efficacy of *Pch1* and *Pch2* to *O. yallundae* and *O. acuformis* separately, through a series of seedling bioassays on wheat varieties and inter-varietal single chromosome substitution lines, and a QTL analysis of *Pch2* resistance. Furthermore, it is necessary to fully understand the efficacy of any novel resistances before they can be considered as useful candidates for introgression into hexaploid wheat. To this end, building on initial evidence from a previous study (Burt, 2002), I investigated potentially novel resistances in accessions of *T. monococcum* for evidence of differential resistance to the eyespot pathogens.

3.2 Materials and Methods

3.2.1 Plant and fungal material

The inter-varietal single chromosome substitution lines; Hobbit 'sib'-VPM7D (HS/VPM7D) containing the resistance gene *Pch1* (Worland et al. 1988) and Chinese Spring-Cappelle Desprez 7A (CS/CD7A) containing *Pch2* (Law et al. 1976), were obtained from the John Innes Centre (JIC) wheat collection. Hobbit 'sib' (HS), which does not contain *Pch1* but is believed to carry *Pch2* (Worland et al. 1988), and Chinese Spring (CS) containing no eyespot resistance genes, were used as controls.

186 F_3 families developed from the CS x CS/CD7A population, as previously described by Chapman et al. (2008), were phenotyped for resistance to *O. yallundae* in controlled environment room (CER) and glasshouse seedling bioassays. This data

was used in a QTL analysis to examine the chromosomal location of resistance to *O. yallundae* from CD7A and to compare this to the previously identified location of *Pch2* resistance to *O. acuformis.*

Wheat cultivars known to have different susceptibilities to eyespot were obtained from the John Innes Centre collection. Andante (Nicholson et al. 1997), Lynx (Nicholson et al. 2002) and Rendezvous (Hollins et al. 1988) are highly resistant; Cappelle Desprez (Law et al. 1976), Riband (Nicholson et al. 1997) and HS (Worland et al. 1988) are moderately resistant; whilst Holdfast (Scott & Hollins, 1980) and Talon (Nicholson et al. 1997) are susceptible. However, little is known about the relative levels of resistance in these lines to *O. yallundae* and *O. acuformis*. HS/VPM7D was included as a highly resistant control, and CS was included as a susceptible control.

To identify novel eyespot resistances, and to assess for differential responses to *O. yallundae* and *O. acuformis*; twenty-two accessions of *Triticum monococcum* were obtained from Professor John Snape, John Innes Centre and Professor Giles Waines, University of California, Riverside, and were screened for resistance to both pathogen species in seedling bioassays. In these seedling bioassays Cappelle Desprez (moderately resistant) and Chinese Spring (susceptible) were included as wheat controls.

All *O. yallundae* and *O. acuformis* isolates used in the JIC trials were from the JIC culture collection. For each species, a homogenised mixture of six different isolates, as detailed by Chapman et al. 2008, was used for inoculations. For *O. yallundae* these were: 22.20, AG98/372, AG98/244, P149/631/1, AG98/241, 432 536/1; and for *O. acuformis* these were: (P37, P38, AG98/167, AG98/174, AG98/119, C93/786) A mixture of different isolates was used to ensure that a successful infection was achieved in case of lack of virulence of one or more of the isolates. In the trials conducted at RAGT, single vigorous isolates for each species were selected from the company's isolate collection and used for inoculations.

3.2.2 SSR genotyping analysis

The wheat lines Andante, Cappelle Desprez, CS, CS/CD7A, Holdfast, HS, HS/VPM7D, Lynx, Rendezvous, Riband, and Talon were genotyped with the *Pch1*-linked SSR markers *Xwmc14* and *Xbarc97*, and the *Pch2*-linked SSR markers *Xwmc346*, *Xwmc525* and *Xcfa2040*, as identified by Chapman et al. (2008), to determine the presence or absence of the known eyespot resistance genes *Pch1* and *Pch2*. Primer sets used were from Beltsville Agricultural Research Station (*Xbarc97*) (Song et al. 2002), Wheat Microsatellite Consortium (*Xwmc14*, *Xwmc346* and *Xwmc525*) (http://wheat.pw.usda.gov/ggpages/SSR/WMC/), and INRA (*Xcfa2040*) (http://wheat.pw.usda.gov/ggpages/SSRclub/).

DNA was extracted from each wheat line using the CTAB method (Nicholson et al. 1996), quantified using a PicoDrop spectrophotometer (Picodrop Ltd.), and diluted to 6ng/ul in sterile distilled water for use in PCRs. A 6.25 μl reaction volume consisted of 2.5 μl of DNA, 3.125 μl of Taq mastermix (Qiagen) and 0.625 μl of the relevant primer pair (2 μM). The forward primer for each marker was labelled with 6-FAM, NED, PET or VIC fluorescent dyes (Applied Biosystems). PCR conditions were as described by Bryan et al. (1997). Samples were prepared by adding 1 μl of a 1:40 dilution of the PCR product to 10 μl formamide and 0.2 μl of LIZ 500 size standard (Applied Biosystems). Samples were run on an ABI 3700 capillary sequencer (Applied Biosystems) and the output data were analysed using Peak Scanner v1.0 (Applied Biosystems) to determine the product size of the amplicons. Lines were deduced to carry *Pch1* if they contained the VPM7D allele at both *Xwmc14* and *Xbarc97*, and were deduced to carry *Pch2* if they contained the Cappelle Desprez allele at *Xwmc346*, *Xwmc525* and *Xcfa2040*.

3.2.3 Seedling bioassays

In all CER seedling bioassays, plants were grown in a 10 ºC growth chamber with 12 h daylength. 3 cm long transparent PVC cylinders (5 mm internal diameter), were placed over emerging shoot tips and after 21 days plants were inoculated by pipetting approximately 400 μl of agar slurry of six mixed isolates of either *O. yallundae* or *O. acuformis*. Plants were incubated for 6-8 weeks, harvested and

scored for disease on a scale reflecting the number of wheat leaf sheaths infected or penetrated where, 0 = seedling uninfected, 1 = coleoptile infected, 2 = coleoptile penetrated, 3 = first leaf sheath infected, 4 = first leaf sheath penetrated, 5 = second leaf sheath infected, 6 = second leaf sheath penetrated, etc (Scott, 1971). In all glasshouse trials, plants were grown over-winter in unheated unlit glasshouses. In the JIC glasshouse trials plants were inoculated and scored as above. In the RAGT glasshouse trials plants were inoculated using the oat grain method of Bruehl and Nelson (1964) as described in Chapter 2, harvested 10 weeks after sowing, and scored as above.

3.2.4 Seedling bioassays of inter-varietal single chromosome substitution lines

In the CER experiment, seeds of each line were grown in peat and sand compost in ten 7 x 7 cm diameter pots, five seeds per pot. Pots were arranged as five complete randomised blocks, each block containing two pots of each line. In each block, one pot of each line was inoculated with *O. yallundae* and the other was inoculated with *O. acuformis.* This experiment was subsequently repeated using identical methods to confirm the findings.

A total of three glasshouse trials were conducted; two at the JIC, Norwich and one at RAGT Seeds Ltd., Cambridge. In each of the JIC glasshouse trials, seeds of each line were grown in John Innes No.2 compost in eight 2 litre pots, seven seeds per pot. Pots were arranged in a complete randomised block design consisting of four blocks, each containing two pots of each line. Again, in each block, one pot of each line was inoculated with *O. yallundae* and the other with *O. acuformis.* In the glasshouse trial at RAGT Seeds, a complete randomised split-plot design was conducted with six blocks. In each block, twelve seeds of each line were sown onto peat and sand compost in 35 cm x 20 cm seed trays, with six seeds in one tray and six in another. One tray in each block was inoculated with *O. acuformis* and the other with *O. yallundae*.

3.2.5 Phenotyping F_3 population CSxCS/CD7A for resistance to O. yallundae

186 F_3 families and the parents CS and CS/CD7A were phenotyped for resistance to *O. yallundae* by seedling bioassays in a CER at JIC and in a glasshouse at RAGT. In the CER experiment nine plants from each of the F_3 families from the population CS x CS/CD7A were grown in 9 x 9 cm pots containing peat and sand compost. Three replicates of each F_3 family were arranged in a randomised complete block design. Parental lines, CS and CS/CD7A, were included as references in each block. The glasshouse trial at RAGT was conducted using a complete randomised block design with seven blocks. In each block, six seeds of each F_3 family and twelve seeds of both parental lines were sown onto peat and sand compost in seed trays and inoculated with *O. yallundae.* This trial was conducted alongside a similar phenotyping trial of the same population for resistance to *O. acuformis*, consisting of six blocks, as reported in chapter 2, to provide a direct comparison of the genetic basis of resistance to the two species.

3.2.6 Seedling bioassays of wheat varieties

Seeds of each variety were grown in John Innes No.2 compost in sixteen 9 x 9 cm pots, eight seeds per pot. Pots were arranged in a complete randomised block design with eight blocks and with two pots of each genotype per block. In each block, one pot of each line was inoculated with *O. yallundae* and the other was inoculated with *O. acuformis*.

3.2.7 Seedling bioassays of Triticum monococcum accessions

In a previous study (Burt, 2002) seeds of each *T. monoccoccum* accession were grown in peat and sand compost in ten 7 x 7cm pots and seeds of each wheat control variety, CS and Cappelle Desprez, were grown in twenty pots, with five seeds per pot. Pots were arranged as ten randomised blocks, each block containing one pot of each of the twenty-two *T. monococcum* accessions and two pots of each wheat control variety. Five blocks were inoculated with *Oculimacula yallundae* and the other five were inoculated with *Oculimacula acuformis*.

To confirm resistance and susceptibility a repeat screening was carried out in the present study using ten selected accessions and the two control lines, using an experimental design and procedure as above. Insufficient seed was available to re-test all the potentially resistant lines.

3.2.8 Statistical analysis

For all experiments, analysis of variance was performed on visual disease scores to assess the variation attributable to block, line, pathogen species and line x pathogen species interactions, using a general linear model (GLM) in Genstat v.11 (Lawes Agricultural Trust, 2008). In all experiments predicted mean disease scores were calculated for each line for *O. yallundae* and *O. acuformis* inoculations using the GLM. In the substitution line seedling bioassays, predicted mean disease scores were calculated using individual GLMs for CER and glasshouse experiments. A GLM incorporating both CER and glasshouse data, with blocks included in the model as sub-plots of experiments, was used to calculate overall means for each line. Similarly in the *T. monococcum* experiment, predicted mean disease scores were calculated for each line using individual GLMs for experiment 1 and 2 and overall means were calculated through a GLM incorporating data from both experiments.

To directly compare the level of resistance of each line to *O. acuformis* and *O. yallundae*, mean disease scores were compared using t-probabilities calculated within each of the GLMs outlined above. Analysis of variance incorporating a Tukey's test was used to separate mean disease scores for each line for *O. yallundae* and *O. acuformis* inoculations independently.

The mean disease score for each F_3 family was predicted in a GLM for the CER trial and glasshouse trial separately, adjusting for the block effect in each case, and was used in the subsequent QTL analysis.

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

Predicted mean disease scores for *O. yallundae* inoculations of each F_3 family from the CER and glasshouse trials were used alongside an existing map of chromosome

7A generated from SSR and cDNA-AFLP marker data (Chapman et al. 2008; 2009) for interval mapping of *O. yallundae* resistance in Map-QTL® version 4.0 (van Ooijen & Maliepaard, 1996). Existing datasets of predicted mean disease scores for *O. acuformis* inoculations of each F_3 family from CER and glasshouse trials, previously published by Chapman et al. (2008), were used for multiple QTL mapping (MQM) to finalize the location of the previously identified *Pch2* QTL for *O. acuformis* resistance. A permutation test (1,000 cycles) was used to determine the LOD score at which the QTL was deemed to be present in the given genomic region with a confidence interval of 99%.

3.3 Results

3.3.1 SSR genotyping analysis

The SSR marker analysis confirmed the presence of *Pch1* in HS/VPM7D and *Pch2* in CS/CD7A (Table 1). In addition, it demonstrated that HS and HS/VPM7D carry the same haplotype as Cappelle Desprez at the *Pch2* linked loci *Xwmc346, Xwmc525* and *Xcfa2040*, indicating that HS is likely to carry the resistance gene *Pch2* and that HS/VPM7D is likely to contain both *Pch1* and *Pch2* resistance genes. CS was confirmed to have a susceptible haplotype possessing neither *Pch1* nor *Pch2*. Haplotypes of the seven wheat cultivars showed that two contain both *Pch1* and *Pch2,* two contain *Pch2* alone, one contains *Pch1* alone, and two have neither of these resistance genes (Table 3.1).

Table 3.1: Allele sizes (bp) for SSR markers linked to *Pch1* (7D) and *Pch2* (7A) and the resistance genes inferred.

Line	*Pch1*		*Pch2*			Inferred Resistance Genes
	Barc97	Wmc14	Wmc346	Wmc525	Cfa2040	
CS	254	252	210	210	297	None
CS/CD7A	254	252	204	208	317	*Pch2*
HS	257	252	204	208	317	*Pch2*
HS/VPM7D	Null	Null	204	208	317	*Pch1* and *Pch2*
Lynx	Null	Null	204	208	317	*Pch1* and *Pch2*
Rendezvous	Null	Null	204	208	317	*Pch1* and *Pch2*
Cappelle Desprez	254	252	204	208	317	*Pch2*
Riband	259	252	204	208	317	*Pch2*
Holdfast	259	252	212	210	295	None
Andante	Null	Null	218	202	295	*Pch1*
Talon	259	252	212	210	295	None

3.3.2 Eyespot resistance in inter-varietal single chromosome substitution lines

Significant effects of line ($P<0.001$), pathogen species ($P<0.001$) and a significant interaction ($P<0.001$) between pathogen species and line was detected in both CER and glasshouse experiments, providing evidence for differential levels of resistance to the two pathogens amongst the host lines (Table 3.2). The effects of block within each experiment were accounted for within the GLMs, but are not presented in table 3.2.

Table 3.2: Summary of variance components for eyespot disease scores using general linear modelling for seedling bioassays of single chromosome substitution lines, wheat varieties and *Triticum monococcum* accessions.

Source of Variation	Single Chromosome Substitution Lines				Wheat Varieties		*Triticum monococcum* Accessions			
	CER		Glasshouse				Experiment 1		Experiment 2	
	MS	*F* value	MS	*F* value	MS	*F* value	MS	*F* value	MS	*F* value
Line	301.4	190.4***	361.6	104.8***	150.0	214.7***	27.8	11.8***	35.2	16.3***
Pathogen	46.8	29.6***	146.8	42.5***	7.7	11.0***	10.6	4.5*	0.1	0.9
Line x Pathogen	23.2	14.7***	21.9	6.4***	7.9	11.4***	3.9	1.7*	3.5	1.6
Residual	1.6		3.5		1.8		2.4		2.1	

MS = mean squares

* =P<0.05, **=P<0.01, ***=P<0.001

HS/VPM7D was highly resistant to both *O. yallundae* and *O. acuformis* in both the CER and glasshouse (Table 3.3). The disease scores for HS/VPM7D were significantly lower than the *Pch2* carrying HS (P<0.05) when inoculated with either species, and although there was some evidence of increased susceptibility to *O. yallundae* in the glasshouse bioassay (P=0.03) there was no overall evidence of differential resistance to the two pathogens (P=0.117). Both of the *Pch2*-containing lines, HS and CS/CD7A had significantly lower disease scores than CS when inoculated with either pathogen (P>0.05). However, both of these lines exhibited significantly less resistance to *O. yallundae* than to *O. acuformis* overall (P<0.001), and furthermore this differential was highly significant in both CER (P<0.001) and glasshouse bioassays (P<0.001) (Table 3.3).

The level of penetration by the two pathogens on the susceptible line CS was very similar in both environments, suggesting that the two species develop at a similar rate in these conditions on a fully susceptible host. Therefore, disease levels of the two pathogens on each line have been compared directly (Table 3.3).

Table 3.3: Predicted mean disease scores from general linear modelling (GLM) 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[a]	2.7[a]	0.433	4.4[a]	5.0[a]	0.030	3.6[a]	3.9[a]	0.117
Hobbit-Sib	*Pch2*	5.3[b]	5.8[b]	<0.001	6.5[b]	7.6[b]	<0.001	5.8[b]	6.7[b]	<0.001
CS/CD7A	*Pch2*	5.2[b]	6.4[b]	<0.001	6.4[b]	7.6[b]	<0.001	5.8[b]	7.0[b]	<0.001
Chinese Spring	None	7.3[c]	6.9[c]	0.163	8.3[c]	8.3[c]	0.889	7.8[c]	7.7[c]	0.3

[*] The statistical significance of the differences between OA and OY disease scores for each line in CER experiments, glasshouse experiments, and across both experiments, are shown by t-probabilities calculated within the GLMs.

[†]Different letters within columns represent significant differences (P<0.05) between disease scores for each line for OA and OY inoculations separately, using a Tukey's test.

3.3.3 QTL analysis of Pch2 resistance to O. yallundae

Previously a significant QTL on chromosome 7A of Cappelle Desprez was detected for *Pch2* resistance to *O. acuformis* (Chapter 2) using QTL interval mapping. The location of this QTL was confirmed to be centred on SSR marker *Xwmc525* using MQM (Figure 3.1) and accounted for 35 and 40% of phenotypic variation at this locus from CER and glasshouse experiments respectively. Because MQM was used to locate *Pch2* resistance to *O. acuformis* in Figure 3.1, the SSR marker *Xwmc525* has absorbed the effects of other linked markers and was identified as the most significant. 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. Consequently, it was not possible to conduct an MQM analysis and the results of a

QTL interval mapping analysis of *O. yallundae* resistance are presented in Figure 3.1.

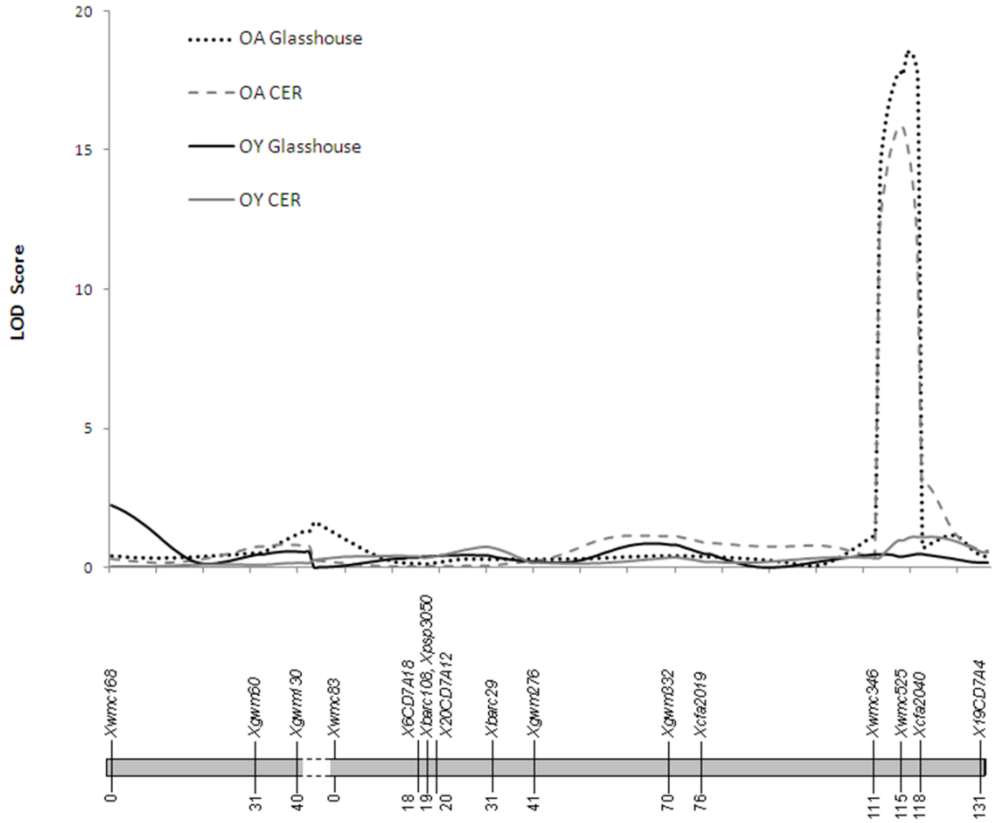

Figure 3.1: A comparison of QTL mapping analyses of *Pch2* resistance to *Oculimacula acuformis* using multiple QTL mapping (MQM), and *Oculimacula yallundae* using interval mapping, in CER and glasshouse trials of the wheat Chinese Spring x Chinese Spring-Cappelle Desprez 7A F_3 families. Genetic map data and phenotypic data for *Pch2* resistance to *O. acuformis* were utilised from Chapter 2.

3.3.4 Eyespot resistance in wheat varieties

Andante, which contains *Pch1*, provided a high level of resistance to both *O. acuformis* and *O. yallundae*. Varieties containing both *Pch1* and *Pch2*, HS/VPM7D, Lynx and Rendezvous, had similarly low disease scores for both pathogen species (Table 3.4). However, there was evidence for differential resistance to the two

pathogens in the experiment as a significant interaction between variety and pathogen species (P<0.001) was detected (Table 3.2). This was mostly due to the differential efficacy of *Pch2*. Cappelle Desprez and Riband both contain *Pch2*, and although both cultivars demonstrated greater resistance than Chinese Spring to both species, they also demonstrated a significantly lower level of resistance to *O. yallundae* compared to *O. acuformis* (Table 3.4), confirming the findings from the inter-varietal chromosome substitution line experiments and QTL analysis. In comparison varieties containing *Pch1* demonstrated no differential levels of resistance to the two pathogen species (Table 3.4).

Table 3.4: Predicted mean disease scores from general linear modeling (GLM) for wheat cultivars when inoculated with *Oculimacula acuformis* (OA) and with *Oculimacula yallundae* (OY).

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

[†]Different letters within columns represent significant differences (P<0.05) between varietal mean disease scores for OA and OY inoculations separately, using a Tukey's test.

Again, there were similar amounts of penetration by the two pathogen species on the susceptible control Chinese Spring, suggesting that it is possible to make direct comparisons between the two pathogen species for each line. The other susceptible lines, Holdfast and Talon, demonstrated high disease scores when inoculated with either pathogen, however, a greater level of penetration by *O. yallundae* was detected in Holdfast and a greater level of penetration by *O. acuformis* was detected in Talon.

3.3.5 Eyespot resistance in Triticum monococcum

In the initial test of twenty-two *T. monococcum* accessions conducted as part of a previous study (Burt, 2002), sixteen demonstrated resistance to *O. yallundae*, to *O. acuformis*, or to both forms of the pathogen, with disease scores significantly lower ($P<0.05$) than the susceptible control Chinese Spring (Table 3.5). Three of these sixteen resistant lines demonstrated a high level of resistance, that was significantly lower than that observed in the moderately resistant Cappelle Desprez ($P<0.05$) to either *O. yallundae* or *O. acuformis*. In addition, there was some evidence of a significant interaction ($P<0.05$) between line and pathogen species (Table 3.2), suggesting that there is differential resistance to the two pathogens amongst the accessions.

In the present study, repeat testing was conducted to both pathogens where seed quantity permitted, including eight accessions that demonstrated resistance, and two accessions that were as susceptible as CS. Generally the results of experiment 2 confirmed the results of experiment 1, with resistance confirmed in seven of the eight accessions (Table 3.5). However, it was not possible to detect any significant interaction between line and pathogen species in this repeat experiment (Table 3.2).

Table 3.5: Predicted mean disease scores from general linear modeling (GLM) for *Triticum monococcum* accessions and control lines when inoculated with *Oculimacula acuformis* (OA) and with *Oculimacula yallundae* (OY).

Genotype	Experiment 1		Experiment 2		Overall		
	OY†	OA†	OY†	OA†	OY†	OA†	t-prob*
Chinese Spring	4.7	4.4	4.2	3.9	4.6	4.3	NS
Cappelle Desprez	2.8	2.7	2.9	1.8	2.8	2.4	0.05
G1261	1.2[ab]	2.7[a]	0.3[a]	2.3	0.9[ab]	2.6[a]	0.04
G1391	2.0[a]	3.1					
G2901	3.6	3.2					
G3371	1.4[a]	2.0[a]	1.9[a]	1.3[a]	1.5[a]	1.8[a]	NS
G4325	3	3.4					
G4483	2.4[a]	2.4[a]					
G4502	2.3[a]	2.7[a]					
G4507	3.2	1.3[ab]	1.4[a]	0.8[a]	2.3[a]	1.1[a]	<0.001
G4509	3.8	3.5					
G4511	2.8[a]	2.5[a]					
G4519	3.7	3.8	3.2	4	3.4	3.9	NS
G4535	2.6[a]	2.3[a]					
G5163	3.5	3.2					
G5191	2.3[a]	2.5[a]					
G5212	3.5	2.5[a]					
G5214	3	2.4[a]	3.1	3.1	3.0	2.7[a]	NS
G5215	4.2	3.3	3.8	3.3	4.1	3.3	0.02
G806	1.8[a]	2.1[a]					
V97020	2.5[a]	1.9[a]	1.2[a]	1.7	1.8[a]	1.8[a]	NS
V97031	2[a]	2.4[a]	1.4[a]	1.6[a]	1.7[a]	2.0[a]	NS
V97052	2.3[a]	1.0[a]	0.7[a]	1.2[a]	1.5[ab]	1.1[a]	NS
V97151	0.5[ab]	1.3[a]	1.4[a]	2.3	1.0[ab]	1.9[a]	0.05

*The statistical significance of the difference between OA and OY disease scores for each line present in both experiments is shown by t-probabilities calculated within a GLM incorporating data from experiments 1 and 2.

† Statistically significant difference ($P<0.05$) between *T. monococcum* accession and control line mean disease scores for OY and OA inoculations separately using a Tukey's test whereby, a = significantly different from Chinese Spring, b= significantly different from Cappelle Desprez.

Predicted overall mean disease scores for *O. yallundae* and *O. acuformis* inoculations, calculated across both experiments, were compared to determine whether any line demonstrated differential resistance to the two eyespot species. There was evidence of a higher level of resistance to *O. acuformis* in two lines, G4507 ($P<0.001$) and G5215 ($P=0.02$), and also some evidence of a higher level of resistance to *O. yallundae* in two lines, G1261 ($P=0.04$) and V97151 ($P=0.05$). Although the extent of the differential between *O. yallundae* and *O. acuformis* resistance in these lines varied between the two experiments, the trend for a higher level of resistance to one species was consistent within each of these lines (Table 3.5).

In confirmation of other results presented herein, overall disease scores for CS were highly similar for *O. yallundae* and *O. acuformis* enabling a direct comparison of disease scores between accessions. In addition, the *Pch2* containing Cappelle Desprez again demonstrated a higher level of resistance ($P=0.04$) to *O. acuformis*.

3.4 Discussion

The eyespot resistance gene *Pch2* was found to confer enhanced resistance to penetration by *O. acuformis* relative to CS, but to provide a lower level of resistance against penetration by *O. yallundae* (Table 3.3). This was demonstrated by the chromosome substitution line CS/CD7A, which had significantly greater disease scores when inoculated with *O. yallundae* than when inoculated with *O. acuformis* across a range of environments and experiments.

Although *Pch2* has previously identified as a major QTL for *O. acuformis* resistance located on the distal portion of chromosome 7A (Chapman et al. 2008), it was not possible to detect any QTL for resistance to *O. yallundae* in the same CS x CS/CD7A population under identical conditions in the present study, providing further evidence that *Pch2* confers relatively little resistance to *O. yallundae* at the seedling stage. However, it was possible to detect a low level of resistance to *O. yallundae* in the substitution line CS/CD7A and it may be expected that the genetic location of this weak resistance would have been identified in the QTL analysis

conducted in the present study. However, the high level of environmental variation often associated with large-scale seedling bioassays of eyespot resistance (de la Peña et al. 1996), particularly in an uncontrolled glasshouse environment (Chapman et al. 2008), and additional variation arising from the use of F_3 families, of which approximately 50% will contain heterozygotes at the *Pch2* locus, may have prevented detection of a QTL for *Pch2* resistance to *O. yallundae*. To overcome this limitation, it would be advisable for future studies to use fixed populations with low heterozygosity, such as recombinant inbred lines or double haploids. This would reduce the variation due to heterozygotes and would allow additional replication (Burr & Burr, 1991), to assist the detection of any QTL for *Pch2* resistance to *O. yallundae*.

The differential resistance conferred by *Pch2* was confirmed in seedling bioassays of three varieties that, on the basis of SSR haplotypes, appear to carry *Pch2* without *Pch1*; Cappelle Desprez, Riband and Hobbit 'sib' (Table 3.4). Although these varieties did demonstrate resistance to *O. yallundae*, they consistently demonstrated a significantly higher level of resistance to *O. acuformis* than to *O. yallundae*. This lower level of *O. yallundae* resistance could be conferred by *Pch2* itself. 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 the variety experiments, the susceptible line CS exhibited very similar susceptibility to the two pathogens, suggesting a similar rate of penetration in the absence of any resistances. However, the susceptible variety Talon, without *Pch1* or *Pch2*, demonstrated significantly lower disease scores when inoculated with *O. yallundae* than when inoculated with *O. acuformis*. In addition, Holdfast demonstrated weak resistance to *O. acuformis* suggesting that these varieties may carry pathogen species specific resistances, albeit of minor effect.

Although it was not possible to identify a genetic location for *Pch2* resistance to *O. yallundae* in the present study, *Pch2* has previously been located to a 28.9 cM interval between the RFLP markers *Xcdo347* and *Xwg380* using a GUS-transformed *O. yallundae* isolate (de la Peña et al. 1997). It has been suggested that measuring disease levels using a GUS technique provides a more sensitive measurement of

resistance in wheat genotypes than visual disease scores (de la Peña et al. 1996). However, techniques that assess the total amount of eyespot present, such as measuring GUS-transformed isolates and quantification of fungal DNA, do not take into account the distribution of fungus within the plant tissues. The disease scoring method of Scott (1971), as used in the present study, indicates the degree of penetration of the host and is intended to provide an indication of potential for yield loss, as this is dependent upon penetration of successive leaf sheaths leading to infection of the stem (Uslu et al. 1998). Consequently, visual disease scores may provide a better indication of the agronomic effectiveness of an eyespot resistance. The previous identification of *Pch2* resistance to *O. yallundae* using a GUS-transformed isolate, combined with the inability to detect a QTL through visual disease assays, suggests that *Pch2* may prevent lateral colonisation of leaf sheaths of *O. yallundae* but has a lesser effect against penetration of leaf sheaths by the pathogen.

It is not possible to infer the mechanism responsible for the differential resistance conferred by *Pch2* on the basis of current evidence. However, it may be related to the differences in the modes of infection, particularly coleoptile penetration, of the two *Oculimacula* species, as described by Daniels et al. (1991). It is possible that *Pch2* provides a resistance that is effective against the apparently random penetration strategy of *O. acuformis* but is ineffective against the more ordered, possibly hemi-biotrophic (Blein et al. 2009), *O. yallundae* infection mechanism. Further investigation into the infection processes of both species on wheat genotypes with and without *Pch2* would be of interest to determine the mechanisms underlying the resistance differential and to provide insight into the function of this resistance.

Pch2 was previously assigned to chromosome 7A in seedling tests by Law et al. (1976) using inoculum that would now be classified as *O. yallundae*. However, a number of experiments were required by Law et al. (1976) to assign the effect and this is consistent with our ability to detect a small effect of resistance to *O. yallundae* in *Pch2* carrying lines. *Pch2* has also previously been associated with the endopeptidase marker *Ep-A1b* (Koebner & Martin, 1990), using F_3 families derived from the cross Chinese Spring x Cappelle Desprez and a mixture of *Pseudocercosporella herpotrichoides* isolates. No clear explanation can be offered for the apparent contradiction between this and my results. However, both *O.*

yallundae and *O. acuformis* isolates were used in this study in separate replicates and there is evidence that at temperatures less than 7 °C *O. acuformis* develops more rapidly than *O. yallundae* (Wan et al. 2005), and furthermore cool glasshouse conditions as used by Koebner and Martin (1990) have been observed to provide conditions that favour development of *O. acuformis* over *O. yallundae* (Nicholson et al. 1997). This may have resulted in a predominance of *O. acuformis* when averaging across replicates in the final data analysis, resulting in the detection of *Pch2* resistance in their study.

Pch1 conferred a high level of resistance to eyespot both in the inter-varietal substitution line HS/VPM7D and in wheat varieties (Tables 3.3 and 3.4). Furthermore, in contrast to *Pch2*, no difference was detected in the level of resistance conferred by *Pch1* towards the two pathogens. Previous studies have provided evidence that varieties combining both *Pch1* and *Pch2* genes have a greater level of adult plant resistance than varieties containing *Pch1* alone in mixed inoculum field trials (Hollins et al. 1988). However, it was not possible to detect a greater level of seedling resistance in the varieties combining both genes (e.g. Lynx) in the present study than those containing *Pch1* alone (e.g. Andante). It is possible that an additional effect of *Pch2* can be observed at the adult plant stage (Hollins et al. 1988), presumably as a result of enhanced control of *O. acuformis*, but this effect cannot be detected over the relatively short time-scale of a seedling bioassay.

No reduction in resistance to *O. yallundae* was observed in lines combining *Pch1* and *Pch2*, suggesting that the potent effect of *Pch1* is sufficient to mask the differential resistance conferred by *Pch2*. The potency of *Pch1* to both eyespot species in a range of genetic backgrounds as demonstrated in the present study, combined with its widespread use in areas of high eyespot pressure such as the pacific north-west USA in varieties such as Hyak (Allan et al. 1990) and Finch (Campbell et al. 2005), clearly demonstrates the agricultural value of the gene. However, the use of *Pch1* in NW Europe has been limited due to the apparent linkage between the resistance gene and yield limiting traits (Johnson, 1992). Although it is likely that the *Ae. ventricosa* segment has recombined in some varieties (Leonard et al. 2008), further work is required to develop more markers in this region to facilitate the identification of more recombinants and accurate mapping of this potent resistance gene.

Sixteen *T. monococcum* accessions out of twenty-two tested were identified to have eyespot resistance. Previous studies, such as Cadle et al. (1997), have also identified a high proportion of resistant accessions in collections of *T. monococcum*, suggesting that the species may be a useful source of novel eyespot resistances. Furthermore, some of these resistances identified in the present study were potent, providing a level of resistance greater than that of *Pch2* (Table 3.5). Those accessions that provided a high level of resistance to both species could potentially be useful sources of novel eyespot resistance. However, it is not possible to infer the genetic basis of these resistances from our data.

The resistances identified could potentially be allelic to *Pch2* as *T. monococcum* is closely related to *T. uratu*, the diploid A genome donor of wheat (Dvorak et al. 1993). In the relatively small group of twenty-two *T. monococcum* accessions examined, it was possible to identify two accessions with evidence of greater resistance to *O. yallundae* and two accessions with evidence of greater resistance to *O. acuformis* over the two experiments. This suggests that different levels of resistance to *O. yallundae* and *O. acuformis* may occur within *T. monococcum*. In conjunction with our findings for *Pch2* and previous findings of differential resistance in accessions of *D. villosum* (Uslu et al. 1998), this suggests that any future screening of germplasm collections should examine resistance to the two *Oculimacula* species separately.

If any of the *T. monococcum* resistances are to be considered as candidates for introgression into wheat, genetic mapping studies are required to determine the number of genes conferring resistance and their genetic location. This could involve mapping in bi-parental crosses between suitable *T. monococcum* accessions or a wider association genetics approach examining genotype and phenotype of a large collection of accessions. These approaches would also determine whether any of the resistances present in *T. monococcum* are allelic to *Pch2*. If genes of interest are identified in *T. monococcum* then these can be transferred to hexaploid wheat by bridging crosses with tetraploid wheats such as *T. durum* e.g. Chhuneja et al. (2008).

In conclusion, my results demonstrate that *Pch2* provides effective resistance against leaf sheath penetration by *O. acuformis* but provides a significantly lower level of resistance against *O. yallundae*. Furthermore, differential resistance was observed in

accessions of *T. monococcum*, suggesting that a different genetic basis of resistance to the two pathogens may be present in relatives of wheat. These findings may be related to the differences in the infection processes of the two pathogens (Daniels et al. 1991) and requires further investigation. In conjunction with previous evidence that *Pch2* is ineffective towards *O. yallundae* at the adult plant stage (Muranty et al. 2002), these results have important implications for the use of eyespot resistance genes in commercial varieties, as my results indicate that the use of *Pch2* as a sole resistance would not provide effective protection against eyespot where the predominant species is *O. yallundae*.

Acknowledgements

The CER phenotyping trial of CS x CS/CD7A with *O. yallundae* was conducted by Nicola Powell (JIC). Data from experiment 1 of the assessment for eyespot resistance in *Triticum monococcum* (Section 3.3.5) was previously presented in my MSc thesis (Burt 2002), but was re-analysed in the present study incorporating data from repeat experiments. I would like to thank Navideh Rezanoor for technical support on the wheat variety trials. I would also like to thank Bill Hollins, Bob Evans and Hilary Butler for their assistance with the glasshouse trials at RAGT seeds.

Chapter 4

Identification of a QTL conferring seedling and adult plant resistance to eyespot on chromosome 5A of Cappelle Desprez.

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Abstract

Eyespot is an economically important fungal disease of wheat and other cereals caused by two fungal species; *Oculimacula yallundae* and *Oculimacula acuformis*. However, only two eyespot resistance genes have been characterised and molecular markers made available to plant breeders. These resistances are *Pch1*, introduced into wheat from the relative *Aegilops ventricosa*, and *Pch2*, originally identified in the variety Cappelle Desprez. 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. An additional resistance has been reported on chromosome 5A of Cappelle Desprez that confers resistance to eyespot in adult plants. In the present study we demonstrate that resistance on this chromosome is effective against both *O. yallundae* and *O. acuformis* eyespot pathogens and confers resistance at both seedling and adult plant stages. This resistance was mapped in both seedling bioassays and field trials in a 5A recombinant population derived from a cross between Cappelle Desprez (CD) and a CD single chromosome substitution line carrying 5A from the susceptible line Bezostaya. The resistance was also mapped using seedling bioassays in a 5A recombinant population derived from a cross between the susceptible line Chinese Spring (CS) and a single chromosome substitution line carrying 5A from CD. A single major QTL on the long arm of chromosome 5A was detected in all experiments. Furthermore, the SSR marker *Xgwm639* was found to be closely associated with the resistance and could be used for marker assisted selection of the eyespot resistance by plant breeders.

4.1 Introduction

Eyespot is an economically important fungal disease of cereal crops such as wheat, barley and rye. It is caused by two species of fungi, *Oculimacula yallundae* (formally *Tapesia yallundae*) and *O. acuformis* (*T. acuformis*). It is widespread in areas where mild and damp autumns promote the growth and spread of the pathogen, particularly northwest Europe and northwest USA. High levels of the disease can lead to significant economic losses (Hardwick et al. 2001; Murray 1996). Unfortunately, control with fungicides may not be cost effective (Nicholson and Turner 2000) and resistance has arisen in numerous eyespot pathogen populations to a number of different chemicals (Parnell et al. 2008). Therefore, the use of disease resistant varieties is thought to be the most effective strategy to control the disease.

There are only two sources of resistance that are known to be widely used in commercial wheat varieties. The more potent of these is the dominant resistance gene *Pch1*, which was derived from the wheat relative *Aegilops ventricosa*. This gene was introduced into hexaploid wheat (Maia 1967) and has been located to the long arm of chromosome 7D (Worland et al. 1988), where it is associated with the SSR markers *Xwmc14* and *Xbarc97* (Chapman et al. 2008) and the STS markers *Xorw1*, *Xorw5* and *Xorw6* (Leonard et al. 2008). Although *Pch1* significantly reduces the rate of penetration from the outer leaf sheaths into the stem (Mauler and Fehrmann 1987), there is evidence that the gene is most effective at the seedling stage and that additional quantitative resistances are required to confer a high level of resistance at the adult plant stage (Lind 1999). A further limitation is that it appears to be difficult to break a linkage between *Pch1* and yield limiting traits also introgressed from *Ae. ventricosa* on the same segment (Koen et al. 2002). Consequently there is interest in alternative sources of resistance to use either in combination with *Pch1* to increase the level of resistance, or in place of *Pch1* to avoid the problem of linkage drag with deleterious traits.

The second source of resistance is from the variety Cappelle Desprez, which was widely grown in Europe for over 20 years from 1953 (Silvey 1978). The resistance observed in Cappelle Desprez has been shown to be partly due to a seedling resistance termed *Pch2* on chromosome 7A (Law et al. 1976). This gene has been

mapped to the long arm of chromosome 7AL (de la Peña et al. 1996) and is delimited by the SSR markers *Xgwm346*, *Xwmc525* and *Xcfa2040* (Chapman et al. 2008). Additional quantitative resistances are believed to be present in Cappelle Desprez, notably an adult plant resistance identified on chromosome 5A (Muranty et al. 2002). Chromosome 5A appears to be an important component of the eyespot resistance observed in Cappelle Desprez at the adult plant stage, particularly as *Pch2* appears to confer little adult plant resistance as assessed by field trials (Muranty et al. 2002). Previous studies (Hollins et al. 1988; Lind 1999) have demonstrated that varieties carrying *Pch1* and with Cappelle Desprez in their pedigree, such as Rendezvous, have enhanced adult plant resistance. However, it has not been determined whether this is due to the effect of *Pch2*, as known to be carried by Rendezvous (Chapter 3), or other Cappelle Desprez quantitative resistances that are effective at the adult plant stage such as the 5A resistance.

There is evidence from a number of sources that the genetic basis of resistance to *O. yallundae* and *O. acuformis* can differ. Notably, *Pch2* was shown to confer a lower level of resistance to *O. yallundae* than to *O. acuformis* in Chapter 3. Furthermore, *PchD*^{v} (*Pch3*), a third eyespot resistance mapped to chromosome 4V of the wheat relative *Dasypyrum villosum* (Yildirim et al. 1998), but not used in wheat varieties, appears to be less effective against *O. acuformis* than *O. yallundae* (Uslu et al. 1998). There is also evidence that potentially novel resistances identified in the wheat relative *Triticum monococcum* (Chapter 3) may confer differential resistance to the two pathogen species. The species coexist in field populations and control of one species tends to increase the proportion of the other and fails to prevent disease (Parnell et al. 2008). To provide effective disease control in commercial wheat varieties any novel sources of resistance that are used, such as the Cappelle Desprez chromosome 5A resistance, should be effective against both forms of the pathogen.

There are other genes of agronomic importance on chromosome 5A, such as QTL controlling time to ear emergence (Griffiths et al. 2009) and resistance to Fusarium head blight (Buerstmayr et al. 2003), and therefore it is highly desirable to identify molecular markers linked to the eyespot resistance in order to introduce this alongside genes for other beneficial traits carried on this chromosome in other varieties. No previous studies have determined the genetic basis of this resistance or sought to identify molecular markers for its selection by plant breeders.

The aim of the present study was to characterise the adult plant eyespot resistance previously identified on chromosome 5A of Cappelle Desprez and determine whether its presence could be detected at the seedling stage and whether it confers resistance towards both *O. yallundae* and *O. acuformis*. I 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.

4.2 Materials and Methods

4.2.1 Plant and fungal material

Cappelle Desprez (CD) carries both *Pch2* and 5A resistances, while Chinese Spring (CS) and Bezostaya (Bez) contain no known eyespot resistance genes. The inter-varietal single chromosome substitution lines Chinese Spring-Cappelle Desprez 7A (CS/CD7A) containing *Pch2,* Chinese Spring-Cappelle Desprez 5A (CS/CD5A) containing the chromosome 5A resistance, Cappelle Desprez-Bezostaya 7A (CD/Bez7A) lacking *Pch2* but containing the 5A resistance, and Cappelle Desprez-Bezostaya 5A (CD/Bez5A) lacking the 5A resistance but carrying *Pch2,* were all obtained from the John Innes Centre (JIC) wheat collection. The parental lines CD, CS and Bez were used as controls.

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.

Eyespot isolates were grown on V8 agar (9 g of bacto agar, 50 ml of V8 vegetable juice in 450 ml of de-ionized water) at 15°C for 21 days. In the seedling bioassays conducted at the JIC mixtures of six isolates were used for both the *O. yallundae* and *O. acuformis* inoculations. Inoculum for the field trials was prepared using colonised oat grain as described by Bruehl and Nelson (1964). All twelve isolates were used in equal proportions for the field trials at JIC to provide a 1:1 inoculum mix of the two pathogen species. In the field trials conducted at RAGT Seeds, three *O. yallundae*

and two *O. acuformis* isolates were selected from the company's collection and used in a 3:2 inoculum mix. A mixture of different isolates was used to ensure that a successful infection was achieved in case of lack of virulence of one or more of the isolates.

4.2.2 Inter-varietal single chromosome substitution lines experiments

Seedling bioassays were conducted to determine the relative effectiveness of *Pch2* and 5A resistances at the seedling stage using wheat lines CS, CS/CD7A, CS/CD5A, CD, CD/Bez 5A, CD/Bez5A and Bez. In all seedling bioassays, plants were grown in 7 x 7 cm pots in peat and sand compost, with five plants per pot. All plants were grown under 12 h daylength in controlled environment rooms (CERs). Twenty pots per line were arranged in a complete randomised block design consisting of ten blocks at 5 °C and ten at 10 °C, with one pot of each line per block. At each temperature five blocks were inoculated with *O. yallundae* and five blocks were inoculated with *O. acuformis.* Plants were inoculated at growth stage (GS) 12 (Zadoks et al. 1974) using a PVC cylinder and inoculum slurry method as described in Chapter 3, harvested 6-8 weeks after inoculation, and scored for disease on the basis of leaf sheath penetration (Scott 1971). This experiment was subsequently replicated as an independent experiment using identical methods to confirm the findings.

4.2.3 Phenotyping populations

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. These were conducted as independent randomised complete block experiments, each consisting of six blocks, and each block containing one pot (five plants) per line and three pots (fifteen plants) per parent line. Seedlings were inoculated as described above.

The seedling bioassays of single chromosome substitution lines suggested that the differential in disease between lines with or without the 5A resistance was greater between CD and CD/Bez5A at 5 °C than at 10 °C, whereas the differential between CS and CS/CD5A was greater at 10 °C, although neither of these differences were significant. For this reason the CD x CD/Bez5A experiment was conducted in a CER at 5 °C and the CS x CS/CD5A experiment was conducted at 10 °C in an effort to ensure maximal discrimination between lines with and without the chromosome 5A resistance.

To map 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. Both trials were drilled in autumn 2007 and plants were harvested and scored in summer 2008. Each trial was arranged in a randomized block design, each block containing two plots of each line and the two parents. Each plot consisted of three 1 m rows of plants. Using inoculum mixes and colonised oat grain as described above, both trials were inoculated when seedlings had reached GS 12 at an application rate of 20 g/m^2. To prevent unwanted foliar fungal pathogens, plants in the JIC trial were sprayed once at approximately GS 45 with 'Amistar Opti' (Azoxystrobin and Chlorothalonil) at a rate of 1.5 litre ha^{-1}. Plants in the RAGT trial were untreated with fungicides. Plants were assessed for penetration of eyespot into the main stem at GS 70, using the method of Scott and Hollins (1974). 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.

4.2.4 Statistical analysis

For the chromosome substitution line seedling bioassays, *O. yallundae* and *O. acuformis* inoculations were analysed as separate experiments. General linear modelling (GLM) was used to calculate predicted mean disease scores for each substitution line and control line across the two replicated seedling bioassay experiments and temperatures within each experiment, also accounting for the environmental effects of blocks. Mean disease scores for lines were compared using t-probabilities calculated within the GLM.

Data from the population phenotyping experiments were also analysed using GLM to assess variability due to block and genotype. Interactions between block and genotype were also assessed as each field trial block contained two plots per genotype and each seedling bioassay block contained five plants per genotype. GLMs were used to predict mean disease scores for RILs from the CD x CD/Bez5A population seedling bioassays and field trials, and for RILs from the CS x CS/CD5A population seedling bioassay. The predicted means were subsequently used for the QTL analysis detailed below. All analyses were conducted using Genstat v.12 (Payne et al. 2009).

4.2.5 SSR analysis

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. Primer sets used were from IPK Gatersleben (*Gwm*) (Roder et al. 1998), Beltsville Agricultural Research Station (*Barc*) (Song et al. 2002), Wheat Microsatellite Consortium (*Wmc*) (http://wheat.pw.usda.gov/ggpages/SSR/WMC/), and INRA (*Cfa/Cfd*/*Gpw*) http://wheat.pw.usda.gov/ggpages/SSRclub/). Markers were identified to provide an even coverage of chromosome 5A on the basis of deletion bin locations (Goyal et al. 2005) and published consensus maps (Somers et al. 2004). Wherever possible, markers polymorphic in both populations were used for mapping, in order to make direct comparisons.

Fresh leaf tissue (50 mg) from 5-week-old plants of the two populations was harvested into 96-well plates on dry ice. DNA was extracted from samples, quantified using a PicoDrop spectrophotometer (Picodrop Ltd.), and diluted to 6ng/ul in sterile distilled water for use in PCRs. A 6.25 µl reaction volume consisted of 2.5 µl of DNA, 3.125 µl of Taq mastermix (Qiagen) and 0.625 µl of the relevant primer pair (2 µM). The forward primer for each marker was labelled with 6-FAM, NED, PET or VIC fluorescent dyes (Applied Biosystems). PCR conditions were as described by Bryan et al. (1997) with annealing temperatures as indicated by the GrainGenes website (http://wheat.pw.usda.gov/GG2/index.shtml). Samples were

prepared by adding 1 μl of a 1:40 dilution of the PCR product to 10 μl formamide and 0.2 μl of LIZ 500 size standard (Applied Biosystems). Samples were run on an ABI 3700 capillary sequencer (Applied Biosystems) and the output data were analysed using Peak Scanner v1.0 (Applied Biosystems) to determine the product size of the amplicons.

4.2.6 Map construction and QTL analysis

Linkage maps were generated for both populations in JoinMap® (version 3.0) (van Ooijen and Voorips 2001) using 0.4 as the maximum recombination fraction and 3.0 as the logarithm of the odds ratio (LOD). Linkage map data was combined with phenotypic data from the seedling bioassays and two field trials of the populations independently for a QTL analysis using Map-QTL® version 4.0 (van Ooijen and Maliepaard 1996). QTLs were initially identified using the non-parametric single marker-based Kruskal-Wallis test to test the significance of markers when examined individually. Secondly, approximate locations, LOD scores and phenotypic effects of the potential QTLs were determined using interval mapping for each experiment independently. Interval mapping provides a systematic way to scan the genetic map for evidence of QTL by using flanking markers to create a region in which to search for the presence of QTL. Finally, multiple QTL mapping (MQM) was carried out to finalize the locations, using the QTLs detected as co-factors. The selected cofactors absorb the effects of nearby QTLs, thereby increasing the power for mapping other segregating QTLs and enabling the separation and mapping of linked QTLs (van Ooijen and Maliepaard, 1996). The minimum significant logarithm of the odds (LOD) scores were calculated by permutation tests (1,000 permutations) to identify the appropriate significance thresholds ($P<0.05$) to declare the presence of a QTL for eyespot resistance.

4.2.7 Verification experiments

To confirm the QTL location of resistance to both pathogen species at the seedling stage, 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 consisted of five blocks, each block consisting of two pots per line, one of which was inoculated with *O. yallundae* and the other was inoculated with *O. acuformis*. CS, CS/CD5A, CD and CD/Bez5A were included as controls in each block. Mean disease scores from the seedling bioassays of recombinant lines were used alongside marker data in a single marker regression analysis to confirm QTL location for *O. yallundae* and *O. acuformis* resistance separately.

4.3 Results

4.3.1 Seedling bioassays of inter-varietal single chromosome substitution lines

The *Pch2* and the 5A resistances were similarly expressed in the experiments conducted at 5 °C and 10 ºC and additionally in the two independent experiments. Therefore combined data across temperatures and experiments are presented in Figure 4.1. However, the differential between CD and CD/Bez5A was greater, but not significantly so, at 5 ºC than at 10^{o}C whereas the reverse was the case between CS and CS/CD5A. Although there is no clear explanation for this, it could partly be influenced by differences in growth habit between CD and CS.

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 4.1a), demonstrating that chromosome 5A of CD confers resistance at the seedling stage in a susceptible CS background. CS/CD7A contains *Pch2* resistance in the same susceptible background and the level of resistance observed in CS/CD7A was similar to that observed in CS/CD5A (Figure 4.1a) to *O. yallundae* ($P = 0.297$) and to *O. acuformis* ($P = 0.107$).

The levels of penetration by *O. yallundae* and *O. acuformis* on CS/CD7A are similar ($P = 0.972$). Although this is inconsistent with the findings of chapter 3, which indicated that *Pch2* is less effective against *O. yallundae* than *O. acuformis*, a significantly higher level of penetration was demonstrated by *O. acuformis* than by *O. yallundae* on the susceptible control CS ($P<0.001$). Therefore it is not possible to make direct comparisons between penetration levels of the two pathogen species on substitution lines in a CS background in this experiment.

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 4.1b). 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. CD/Bez7A, which contains the CD5A resistance but lacks *Pch2*, had significantly higher disease scores than CD (P<0.01) when inoculated with *O. acuformis* but not when inoculated with *O. yallundae* (Figure 4.1b), suggesting that the loss of any effect of *Pch2* in the CD resistance reduces the level of resistance to *O. acuformis* but not to *O. yallundae*. This is consistent with the findings of chapter 3 which provided evidence that *Pch2* is less effective against *O. yallundae* than *O. acuformis*.

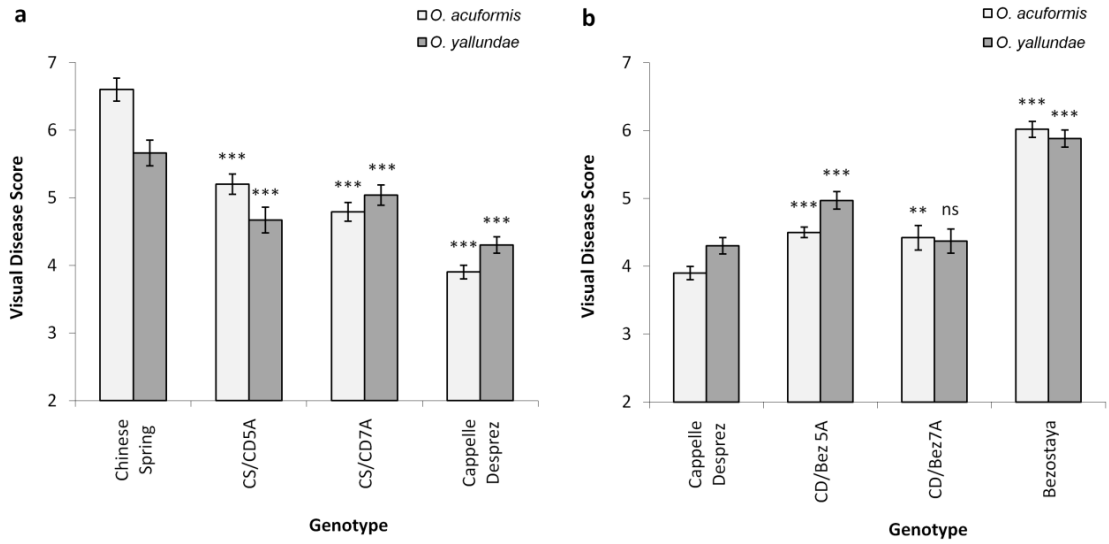

Figure 4.1: Predicted mean disease scores for (a) Chinese Spring – Cappelle Desprez substitution lines and (b) Cappelle Desprez – Bezostaya substitution lines when inoculated with *Oculimacula yallundae* and *Oculimacula acuformis* in seedling bioassays. Error bars are all ± standard error of the mean. Mean disease scores are compared to Chinese Spring in 1a, and to Cappelle Desprez in 1b, using t-probabilities calculated within general linear models: ns = non-significant, * P<0.05, ** P<0.01, ***P<0.001.

4.3.2 Seedling and adult plant resistance in CS x CS/CD5A and CD x CD/Bez5A populations

Analysis of variance demonstrated that the effect of genotype was highly significant (P<0.001) in all trials apart from the field trial of CD x CD/Bez5A conducted at RAGT, in which it was also significant, albeit at a lower level (P<0.05). A highly significant block effect (P<0.001) was observed in all experiments. This was higher in the field trials than the CER seedling bioassays and was particularly high in the RAGT field trial. The large block effect in both field trials may in part have been due to fungal development within stems during the harvesting and scoring process. Trials were harvested and scored in blocks, each block taking approximately two days to complete, during which time the fungus continued to grow within the stems. This would increase block variation but would limit the residual component of the analysis of variance. No significant block x genotype interaction could be detected in either field trial although a significant interaction was observed in the seedling bioassays of the populations (Table 4.1).

Table 4.1: Variance components of visual disease scores from phenotyping experiments, calculated using general linear modelling.

Variance Component	CS x CS/CD5A		CD x CD/Bez5A					
	Seedling OA		Seedling OY		JIC Field		RAGT Field	
	MS	F-Value	MS	F-Value	MS	F-Value	MS	F-Value
Block	28.6	25.0***	44.3	31.8***	445.4	19.6***	5174.5	111.0***
Genotype	4.9	4.3***	8.5	6.1***	41.2	1.8***	62.7	1.3*
Block x Genotype	2.4	2.1***	3.7	2.7***	28.0	1.2^{ns}	41.9	0.9^{ns}
Residual	1.1		1.4		22.7		46.6	

OA, *Oculimacula acuformis*; OY, *Oculimacula yallundae*; MS, mean squares.

^{ns} not significant, *P<0.05, **P<0.01, ***P<0.001

4.3.3 SSR analysis, map construction and QTL analysis

Of the 47 SSR markers tested 40% (19) were polymorphic between CS and CS/CD5A and 36% (17) were polymorphic between CD and CD/Bez5A. All of the 17 that were polymorphic between CD and CD/Bez5A were also polymorphic between CS and CS/CD5A. These markers were applied to the populations and linkage maps were calculated using JoinMap (version 3.0). The markers were resolved into identical orders in both populations and it was possible to compare QTL locations in the two populations directly (Figure 4.2). However, the linkage map of chromosome 5A was shorter in CD x CD/Bez5A (76 cM) than in CS x CS/CD5A (131 cM). This is probably due to the combined effect of a smaller population size and a lower level of recombination in this population.

QTLs were detected in the same location in both populations conferring both seedling and adult plant resistance (Figure 4.2). In the CD x CD/Bez 5A population a QTL for eyespot resistance at the adult plant stage was detected on the long arm of chromosome 5A, centred on the SSR marker *Xgwm639* (Table 4.2), in both the JIC (LOD 4.7, R^2 24%) and RAGT field trials (LOD 4.8, R^2 23%). The seedling bioassay of CD x CD/Bez5A, identified a QTL for seedling resistance to *O. yallundae* (LOD 4.6, R^2 24%), which was most significantly associated with the SSR marker *Xbarc197* (Table 2). 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 4.2). 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 4.2), again centred on marker *Xgwm639* (LOD 10.6, R^2 34%).

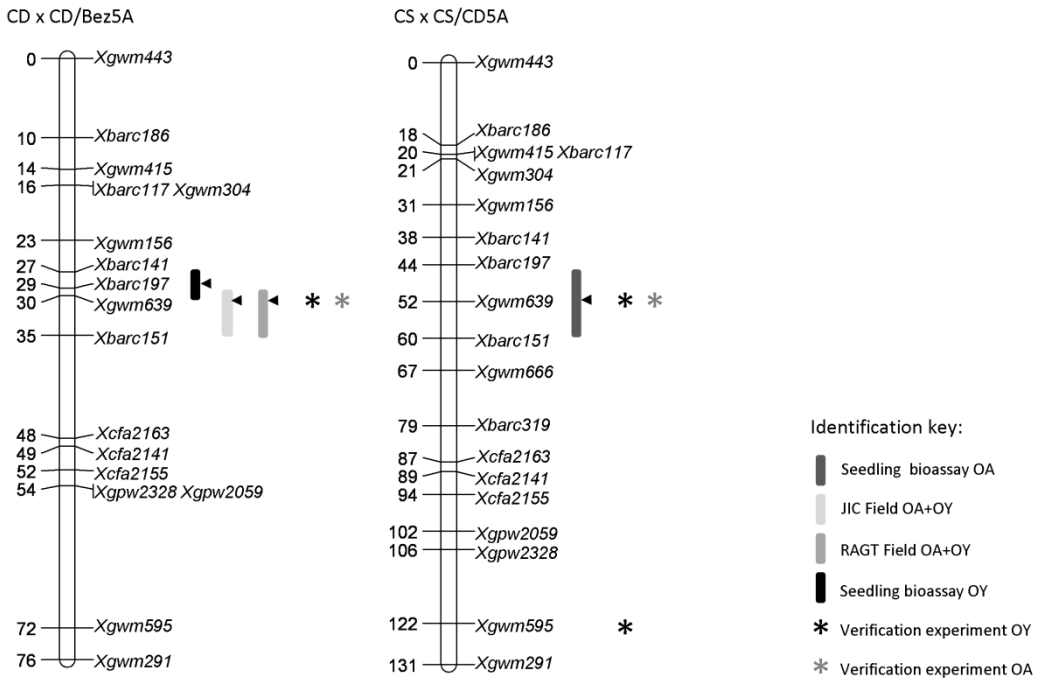

Figure 4.2: Genetic maps of chromosome 5A in CD x CD/Bez5A and CS x CS x CS/CD5A populations. Distances are measured in Kosambi cM units. QTL positions for resistance to eyespot are shown to the right of the genetic maps by bars that indicate areas on the map with a LOD score greater than the significance threshold (P<0.05) and arrows indicate the location of the peak LOD score. Asterisks indicate markers with significant associations (P<0.05) with resistance to *Oculimacula yallundae* and *Oculimacula acuformis* in the verification experiments using a single marker regression analysis. OA refers to inoculation with *Oculimacula acuformis* and OY refers to inoculation with *Oculimacula yallundae*.

Table 4.2: 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.

4.3.4 Verification experiment

The single marker regression in the verification experiment confirmed *Xgwm639* as the marker most significantly associated with seedling resistance to *O. yallundae* and *O. acuformis* in both populations (Figure 4.2). The presence of a CD allele at the *Xgwm639* locus was associated with reduction in disease scores of 0.82 for *O. acuformis* and of 0.85 to *O. yallundae* in the recombinant lines from CD x CD/Bez5A ($P<0.05$). explaining 29.1 % of phenotypic variance for resistance to *O. acuformis* and 36.2% of phenotypic variance for resistance to *O. yallundae* (Table 4.3). Similarly, the presence of a CD allele at *Xgwm639* was associated with a reduction in disease scores of 0.51 for *O. acuformis* and 0.60 for *O. yallundae* in the recombinant lines from CS x CS/CD5A ($P<0.001$), explaining 43.9% of phenotypic variance for resistance to *O. acuformis* and 43.3% of variance for resistance to *O. yallundae* (Table 4.3).

Table 4.3: Single marker regression of predicted mean disease scores, calculated in a general linear model, for recombinant lines from CD x CD/Bez 5A and CS x CS/CD5A.

Marker	CD x CD/Bez5A				CS x CS/CD5A			
	O. acuformis		*O. yallundae*		*O. acuformis*		*O. yallundae*	
	R^2	P-Value	R^2	P-Value	R^2	P-Value	R^2	P-Value
Xgwm443	0	0.789	0	0.864	1.1	0.269	0	0.940
Xbarc186	2.2	0.283	0	0.41	0	0.479	0.1	0.322
Xbarc117	6.3	0.206	7.9	0.182	2.9	0.123	5.9	0.123
Xgwm415	2.5	0.278	0	0.437	6.1	0.109	7.1	0.092
Xgwm304	8	0.205	8.7	0.179	6.1	0.109	7.1	0.092
Xgwm156	6.6	0.202	8.9	0.168	0.8	0.281	0.8	0.278
Xbarc141	1.2	0.311	6.1	0.21	0	0.635	0	0.846
Xbarc197	9.1	0.167	3	0.267	9.1	0.064	4.8	0.135
Xgwm639	29.1	0.031	36.2	0.017	43.9	<0.001	43.3	<0.001
Xbarc151	0	0.698	1.5	0.296	5.4	0.123	0	0.328
Xcfa2163	0	0.721	0	0.356	0	0.456	0	0.635
Xcfa2141	0	0.721	0	0.356	0	0.958	0	0.699
Xcfa2155	0	0.622	0	0.758	0	0.858	0	0.991
Xgpw2059	0	0.674	0	0.578	0	0.595	0	0.777
Xgpw2328	0	0.674	0	0.578	0	0.641	0	0.915
Xgwm595	0	0.582	0	0.987	8.8	0.077	12.6	0.042
Xgwm291	0	0.977	0	0.53	4.1	0.154	6.8	0.097

R^2 , percentage phenotypic variance explained

In addition, it was possible to detect a minor resistance to both pathogens, at the *Xgwm595* locus on the distal portion of 5AL from CD in the CS x CS/CD5A recombinant lines, but not in the CD x CD/Bez5A recombinant lines. The presence of a CD allele at *Xgwm595* in CS x CS/CD5A explained 8.8% of phenotypic variance for resistance to *O. acuformis* and 12.6% of variance for resistance to *O. yallundae.* Only the *O. yallundae* resistance was below the P=0.05 significance threshold (P<0.042) at this locus (Figure 4.2, Table 4.3). This probably represents a minor resistance, too small to be detected in the original QTL analysis, which can only be detected in a fully susceptible background. Alternatively, it is possible that this represents a minor effect that is carried by both Cappelle Desprez and Bezostaya but is absent from Chinese Spring.

4.4 Discussion

Resistance to eyespot in the variety Cappelle Desprez has previously been attributed to the seedling resistance gene *Pch2* on chromosome 7A (de la Peña et al. 1996; Law et al. 1976) and to an unmapped adult plant resistance located on chromosome 5A (Muranty et al. 2002). Through seedling bioassays it was determined that the Cappelle Desprez chromosome 5A resistance is also effective at the seedling stage, and that it is an important component of the resistance observed in Cappelle Desprez, conferring a similar level of resistance to *Pch2*. The resistance was demonstrated in the absence of *Pch2* in a fully susceptible background in the substitution line CS/CD5A (Figure 4.1), suggesting it could be used alone to provide eyespot resistance in varieties. Furthermore, the resistance was also observed in the presence of *Pch2* in lines from the CD x CD/Bez5A population that contained both resistances, suggesting that the 5A resistance confers an additive effect when combined with *Pch2*, and therefore could be introduced into varieties alongside *Pch2* to provide a higher level of protection against the disease.

In contrast to my findings, neither Law et al. (1976) nor Muranty et al. (2002) were able to detect any significant resistance conferred by Cappelle Desprez chromosome 5A at the seedling stage. The apparent contradiction between these studies and my data may be due to differences between the methods of inoculation, particularly considering the environmental variability often associated with a necrotrophic fungus such as eyespot (Chapman et al. 2008; de la Peña et al. 1996). In my seedling bioassays, inoculum slurry was pipetted into a PVC cylinder around each seedling stem base as described by Chapman et al. (2008). In contrast, both Law et al. (1976) and Muranty et al. (2002) used the Macer technique whereby seedlings are infected from inoculated straw (Macer, 1966). The PVC cylinder and slurry method is likely to provide a more uniform infection than the Macer technique, as inoculum is maintained in contact with the entire surface of the stem base and this may prevent disease escape or delays in infection and so enable the detection of moderate seedling resistances, such as that conferred by Cappelle Desprez chromosome 5A. However, it is possible that other differences in experimental conditions such as the isolates used, temperature, humidity and plant growth stage at inoculation or harvest could also contribute to the disparity between our findings and those of previous studies.

Although adult plant resistances have been identified that are only activated once a plant reaches a particular developmental stage (Hugot et al. 1999), other adult plant resistances have been identified in wheat that can also be detected at the seedling stage, particularly towards rust diseases (Ma and Singh 1996; Singh and Huerta-Espino 2003). For example, the broad-spectrum adult plant resistance gene *Lr34*, which was first determined to confer resistance to leaf rust at the adult plant stage (Dyck 1987), was shown in later studies to be expressed at the seedling stage. Near-isogenic lines of *Lr34* have demonstrated some evidence of resistance as early as the first leaf stage and a highly significant level of resistance by the 4-leaf stage when inoculated with the causal agent of leaf rust, *Puccinia triticina* (Singh and Huerta-Espino 2003). This suggests that resistance genes once thought to be specific to adult growth stages, such as *Lr34* resistance to leaf rust and resistance to eyespot on Cappelle Desprez chromosome 5A, may be active at earlier stages and that this can be detected with accurate phenotyping methods. It is also possible that resistances such as these may have a cumulative effect becoming more evident in older plants and although not conferring complete resistance may inhibit pathogen development sufficiently to prevent the disease becoming a significant problem.

Importantly, I determined that the Cappelle Desprez 5A resistance functioning at both the seedling and adult plant stages against *O. yallundae* and *O. acuformis* is conferred by the same genetic location. A major QTL, closely associated with SSR marker *Xgwm639*, was identified for adult plant resistance in field trials of the 5A recombinant population CD x CD/Bez5A at both RAGT and JIC sites. As discussed above, I was also able to detect a significant effect of CD5A on eyespot resistance at the seedling stage and therefore we conducted seedling bioassays of both 5A recombinant populations to map the resistance at the seedling stage. A major QTL was detected in CS x CS/CD5A when inoculated with *O. acuformis*, again centred on *Xgwm639*, in the same position as detected in the field trials for adult plant resistance. Although the QTL identified from the seedling test on CD x CD/Bez5A using *O. yallundae* was centred on a different SSR marker, *Xbarc197*, it is probable that they represent the same genetic location as the QTL areas ($P<0.05$) overlapped and *Xbarc197* was only 1 cM proximal from the QTL peak position detected in the other three experiments.

A verification experiment was conducted to confirm that resistance to both pathogen species is conferred by the same genetic location. This consisted of seedling bioassays on a sub-set of recombinant lines from CD x CD/Bez5A and CS x CS/CD5A with separate inoculations with *O. yallundae* and *O. acuformis*. A single marker regression analysis on the mean disease scores from this experiment demonstrated that *Xwmc639* was the marker most closely associated with both *O. yallundae* and *O. acuformis* resistance in both populations. Alongside data from the field trials and seedling bioassays of the complete populations, this provides supporting evidence that a single major QTL on 5A, associated with *Xgwm639,* confers resistance to both *O. yallundae* and *O. acuformis*. Although the eyespot resistances; *Pch1* (Chapman et al. 2009), *Pch2* (de la Peña et al. 1996) and *Pch3* (Yildirim et al. 1998), have previously been mapped and annotated as single genes, they provide quantitative phenotypes. Due to the variability associated with screening populations for resistance to eyespot, particularly in field trials, it was not possible to map the Cappelle Desprez 5A resistance as a qualitative trait and therefore it was characterised as a quantitative trait. To my knowledge this is the first eyespot resistance QTL to be characterised and I propose that is designated as *QPch.jic-5A.*

The QTL detected in the CS x CS/CD5A seedling trial accounted for a higher percentage of phenotypic variation than those detected in field trials. This may be due to the lower level of environmental variation observed in CER experiments compared to field trials (Table 4.1). However, the QTL detected in the seedling bioassay of CD x CD/Bez5A was also of lower significance and accounted for less phenotypic variation than the QTL detected in the seedling test of CS x CS/CD5A. This difference was also observed in the seedling bioassays for the verification experiment. This may be partly because the CD x CD/Bez5A population has *QPch.jic-5A* segregating always in the presence of *Pch2*. This could reduce the differential between lines with and without the QTL and hence reduce its significance. In comparison, *QPch.jic-5A* in CS x CS/CD5A is segregating in a susceptible Chinese Spring background and provides more accurate phenotypic data for locating the QTL, and this is reflected in the greater effect of the detected QTL. Any further work to refine the QTL position should focus on the CS x CS/CD5A population as this provides a clearer phenotypic difference. In addition, CS x

CS/CD5A is a larger population and was found to have a higher recombination rate than CD x CD/Bez5A, providing a more accurate resource for mapping *QPch.jic-5A* at a higher resolution.

Although an SSR marker that appears to be closely linked to *QPch.jic-5A* has been identified in this study, it should be possible to improve the predicted genetic location of the gene and to develop more tightly linked PCR based markers. The linked SSR marker, *Xgwm639*, has been physically mapped to the deletion bin location 5AL-6 0.68 – 5AL-17 0.78 (Goyal et al. 2005). Further PCR markers could be developed in this region using wESTs that have been positioned in this deletion bin. In addition, co-linearity between this region in wheat and potentially syntenous regions in the sequenced genomes of *Brachypodium distachyon* (International Brachypodium Initiative 2010) and *Oryza sativa* (International Rice Genome Sequencing Project 2005) could be used to target the region of interest on chromosome 5A more accurately.

Q.Pch.jic-5A could be of greater use than *Pch2* as it appears to provide resistance to both eyespot species at all growth stages, whilst *Pch2* provides a lower level of resistance to *O. yallundae* (Chapter 3) and confers little effect at adult plant stages (Muranty et al. 2002). Although not in the scope of this study it would be beneficial to validate the 5A resistance QTL to determine whether it is sufficiently potent for use in a range of genetic backgrounds in commercial varieties. Varieties could be screened for the presence and absence of Cappelle Desprez haplotypes at the SSR loci associated with the resistance, and then phenotyped through seedling tests or field trials, to determine whether the markers identified herein provide a suitable predictor of eyespot resistance.

In conclusion, we identified a single major QTL, *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. This could provide breeders with the ability to select for a previously uncharacterised source of resistance that is effective against both forms of the pathogen and is effective at both seedling and adult plant stages.

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Chapter 5

Exploiting co-linearity among grass-species to fine-map the *Aegilops ventricosa* derived *Pch1* eyespot resistance in wheat and establish its relationship to *Pch2*.

Abstract

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. 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. This has been exacerbated by a lack of co-dominant PCR markers suitable for identifying heterozygotes in F_2 populations. I developed conserved orthologous sequence (COS) markers, utilising the *Brachypodium distachyon* (Brachypodium) genome sequence, to provide co-dominant markers in the *Pch1* region. These were supplemented with previously developed sequence-tagged site (STS) markers and simple sequence repeat (SSR) markers. Markers were applied to a panel of varieties and to an F_2 population, segregating between wheat and *Ae. ventricosa* over the distal portion of 7DL, to identify recombinants in the approximate region of *Pch1*. By exploiting co-linearity between wheat chromosome 7D, Brachypodium chromosome 1 and rice chromosome 6, *Pch1* was located to an interval between the flanking markers *Xwg7S* and *Xcos7-9*, and furthermore a 364 Kb candidate gene region in Brachypodium and an 178 Kb region in rice were identified as a prelude to the map-based cloning of the gene. In addition, using homoeologue transferable markers, I obtained evidence that the eyespot resistances *Pch1* and *Pch2* on chromosomes 7D and 7A respectively are homoeoloci. It is anticipated that COS marker methodology could be used for the identification of recombinants in other introgressions into wheat from wild relatives. This would assist the mapping of genes of interest and the breaking of deleterious linkages to enable the use of these introgressions in commercial varieties.

5.1 Introduction

Introgressions into wheat (*Triticum aestivum*) from cultivated relatives such as rye (*Secale cereale*) (Schlegel and Korzun 1997) and species such as *Aegilops* (Schneider et al. 2008) provide a valuable source of genetic diversity for plant breeders. Such introductions provide novel alleles for numerous traits including disease resistance, abiotic stress tolerance and enhanced yield potential. However, use of this germplasm in wheat breeding is often limited by linkage on the introgressed segments between the traits of interest and deleterious traits (Feuillet et al. 2008). Furthermore, the presence of a large non-recombining introgressed segment may prevent beneficial alleles from other sources being introduced into these regions. However, reducing the size of alien introgressions and breaking adverse linkages is often difficult due to suppressed recombination between wheat chromosomes and wild relative homeologues (Riley et al. 1959).

Identifying recombination within alien introgressions in wheat is further complicated by a lack of suitable co-dominant PCR markers that produce specific products from both the wheat and introgressed DNA. Although dominant PCR markers that generate a specific product from only one species can be used for screening homozygous lines, they are not suitable for differentiating heterozygotes from homozygotes in F_2 populations (Chapman et al. 2008). Hence the potential for rapidly identifying recombinants in large populations and reducing the size of alien segments is limited. In particular, SSR markers are often genome specific, with low transferability across related species (Mullan et al. 2005). EST based markers tend to be more transferable because they are designed to relatively conserved exon sequences within genes (Varshney et al. 2005). However, transferability depends upon the level of relatedness between wheat and the introgression species (Zeid et al. 2010), and co-dominant markers are not always successfully developed (Qi et al. 2008).

Comparative mapping in grass species has provided evidence for conservation of markers and gene order, termed colinearity, between genomes (Devos and Gale 2000; Moore et al. 1995). The release of the rice genome sequence and the development of large EST collections from other cereals have greatly enhanced the resolving power of comparative mapping and has enabled co-linearity based gene

cloning (Kilian et al. 1997). However, small rearrangements of gene content and order are often found in studies of micro-colinearity between rice and wheat (Bennetzen and Ramakrishna 2002; Feuillet and Keller 2002). It is anticipated that the annotated *Brachypodium distachyon* (Brachypodium) genome (International Brachypodium Initiative 2010) will further enable the exploitation of co-linearity in the economically important Pooideae sub-family of cool season cereals and grasses, which includes wheat. It is estimated that Brachypodium and wheat diverged from rice 40-54 million years (Myr) ago, whilst Brachypodium diverged from wheat 32-39 Myr ago (International Brachypodium Initiative 2010). Therefore, the Brachypodium resource should provide higher levels of sequence conservation and co-linearity for marker development, fine-mapping and positional cloning in wheat. However, there are limitations in the co-linearity between wheat and Brachypodium, and caution should be exercised if Brachypodium is to be used as a reference genome for map-based cloning. For example, only two thirds of the genes on five wheat bacterial artificial chromosomes (BACs) in the *Lr34* region were found to be co-linear with the *Lr34* region of Brachypodium, whilst the remaining one third were not co-linear, with orthologues elsewhere in the Brachypodium genome (Bossolini et al. 2007).

Conserved Orthologous Sequence (COS) markers are gene-based markers that are designed to exon sequences that are highly conserved between the fully sequenced reference genome of a model species and available sequence from the target species (Fulton et al. 2002). As a result they are highly transferable, and consequently there has been considerable interest in the development of COS markers for application in cereal species (Bertin et al. 2005; Quraishi et al. 2009). There are two other major advantages of COS markers. Firstly, as they are designed to span introns, using gene structure information from the reference genome, they exploit a greater frequency of single nucleotide polymorphisms (SNPs) than found in exons and are potentially highly polymorphic. Secondly, they define orthologous regions in the model genome to enable targeted marker development and candidate gene identification.

The utility of COS markers was demonstrated by a recent study in which a set of 695 genome-wide COS markers was designed for wheat, using rice as a reference genome and successfully used to fine-map a pentosan viscosity quantitative trait locus on chromosome 7A and to identify candidate genes in rice (Quraishi et al.

2009). In addition, a high proportion of primers designed to rice sequences have provided successful amplification when applied to hybrids between the forage grasses *Lolium perenne* and *Festuca pratensis* (King et al. 2007). As a direct result of this cross-species transferability, an important application for COS markers in wheat and other cereals could be the identification of recombinants in introgressions from related species, and the subsequent mapping of these segments. The Brachypodium sequence should provide an additional resource for the development of further COS markers applicable to wheat and its relatives.

The introgression from the wild grass *Aegilops ventricosa* into hexaploid wheat (Doussinault et al. 1983; Maia 1967) provides a relevant example of a beneficial gene that is restricted in its commercial use by the problems associated with alien introgressions. A large segment of chromosome $7D^V$ of *Ae. ventricosa* was introgressed into chromosome 7D of wheat as a source of resistance to the economically important cereal disease, eyespot. The *Ae. ventricosa* eyespot resistance is 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 eyespot pathogen species (Chapter 3) 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 lower frequency than normally observed in crosses between traditional hexaploid varieties (Worland et al. 1988). This, together with the lack of co-dominant markers (Chapman et al. 2008), has hindered the identification of useful recombinants across the introgressed segment. This has resulted in a limited use of the *Pch1* gene in commercial varieties, particularly in Europe (Johnson 1992).

Despite linkage-drag on the *Ae. ventricosa* introgression, a significant interest remains in *Pch1* due to its potency and also to the paucity of alternative eyespot resistances. The only other source of resistance known to be used in commercial wheat varieties derives from the French variety Cappelle Desprez (Vincent et al. 1952). This has been shown to be controlled by a seedling resistance gene, termed *Pch2* on chromosome 7A (de la Peña et al. 1996), and a recently identified QTL of moderate effect on chromosome 5A, *QPch.jic-5A* (Chapter 4). Neither of these

resistances is as potent as *Pch1* as demonstrated by a significantly lower resistance to both *O. yallundae* and *O. acuformis* in Cappelle Desprez in comparison to the *Pch1* containing variety Andante in Chapter 3 of this study (Table 3.4). Although there is evidence that the Cappelle Desprez resistances may provide an additional level of protection against eyespot when used in combination with the *Pch1* introgression (Hollins et al. 1988; Lind 1999), data from Chapter 3 of this study suggests that the resistance conferred by *Pch1* alone is similar to the effect of *Pch1* and *Pch2* combined (Table 3.4).

The exploitation of *Pch1* has been significantly hampered by a lack of co-dominant PCR markers that generate specific polymorphic products from both wheat and *Ae. ventricosa* DNA (Chapman et al. 2008). 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, it is often difficult to distinguish the *Ep-D1b* allele from the *Ep-A1* and *Ep-D1a* homeologues (Koebner et al. 1988) and consequently 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 *Xorw1*, 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 *Xorw5* and *Xorw6* 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). However, all of these STS markers were dominant, providing either a wheat or *Ae. ventricosa* specific product. Similarly, Chapman et al. (2008) identified the SSR markers *Xwmc14* and *Xbarc97* as potential PCR based markers for *Pch1*. However, these were also dominant, failing to amplify *Ae. ventricosa* specific products and they have additionally been shown not to be diagnostic of *Pch1* resistance in a panel of wheat varieties (Leonard et al. 2008).

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 assess the applicability of COS markers to alien introgression

by applying them to identify recombinants within the *Ae. ventricosa* chromosome 7D introgression into wheat. To identify recombination events in the region of *Pch1*; a combination of SSR, STS and COS markers were applied to a set of wheat varieties and a recombinant F_2 population. I utilised co-linear regions in the sequenced Brachypodium and rice genomes identified through gene-based markers, to fine-map the gene and to identify candidate gene regions. Furthermore, using COS markers, I investigated the homoeologous relationship between *Pch1* on chromosome 7D and the *Pch2* eyespot resistance gene on chromosome 7A.

5.2 Materials and Methods

5.2.1 Plant material

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 SSR markers to identify recombination in their *Ae. ventricosa* introgressions. Hobbit 'sib' (HS) was used as a control variety lacking *Pch1*, and the inter-varietal chromosome substitution line Hobbit-sib-VPM7D (HS/VPM7D) was used as an *Ae. ventricosa* (*Pch1*-containing) control. The recombinant substitution lines RVPM25 and RVPM44, previously developed from a BC_5 recombinant population between HS and HS/VPM7D (Worland et al. 1988) were used as *Pch1* resistance carrying controls in a susceptible HS background but with known differences in their *Ae. ventricosa* introgressions (Chapman et al. 2008).

The recombinant substitution line RVPM25 was further backcrossed to HS and a recombinant population of 376 BC_6 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. Recombinant lines were selfed to produce BC_6 F_3 families for phenotypic evaluation of eyespot resistance.

5.2.2 COS marker analysis

Primer sets for a group of thirteen COS markers (designated *Wg*) previously developed by colleagues at the John Innes Centre and known to be located on group 7 chromosomes (http://www.wgin.org.uk/resources/Markers/TAmarkers.php) were obtained. A further COS marker was developed to the wheat EST (wEST) CJ641756, previously identified from a *Pch2* candidate cDNA-AFLP fragment 4CD7A8 (Chapman et al. 2009). Two further informative gene-based markers were identified as the STS markers, *Xorw1* and *Xorw5*, previously developed as *Pch1*-linked markers by Leonard et al. (2008). The wEST sequences on which these markers were based were aligned to the Brachypodium sequence using BLASTn (International Brachypodium Initiative 2010) to identify the region of Brachypodium that corresponds to the Pch1 region on wheat 7DL.

The Brachypodium sequence corresponding to the *Pch1* region was visually examined using the Brachypodium (Bd21) Genome Browser (http://www.modelcrop.org/cgi-bin/gbrowse/brachyv1/) to identify further markers. Fifteen COS markers (designated Tr) were identified in the region of *Pch1*, aligning to the Brachypodium sequence using BLASTn. For fine-mapping *Pch1* these were supplemented by a set of ten additional COS markers (designated Cos7) that were developed utilising wESTs aligned to the targeted Brachypodium region by BLASTn. To generate novel COS markers, PCR primers were designed to wEST sequences to locations with high levels of sequence conservation with Brachypodium and to flank introns as predicted by alignment with the annotated Brachypodium genomic sequence.

All of the COS and the two STS markers were tested on HS, HS/VPM7D, RVPM25 and RVPM44 to identify polymorphisms and to determine whether the markers were located on the distal portion of chromosome 7D. Polymorphic markers were applied to the HS x RVPM25 $BC_6 F_2$ population and to three recombinant wheat varieties; Hermann, Striker and RAGT 2. DNA extractions and PCR reactions were prepared as described previously (Chapter 3). PCR amplification was conducted using a touchdown programme consisting of a denaturing step of 95 °C for ten min; sixteen touchdown cycles of 95 °C for 15 s, 58 °C for 1 min decreasing 0.5 °C per cycle,

72°C for 1 min; then thirty cycles of 95 °C for 15 s, 50 °C for 15 s and 72°C for 1 min. Samples were initially run on an ABI 3700 capillary sequencer (Applied Biosystems) and the output data were analysed using Peak Scanner v1.0 (Applied Biosystems) to determine the product size of the amplicons. If no suitable polymorphism was detected through this method, then products were examined by single strand conformation polymorphism (SSCP) assay (Martins-Lopes et al. 2001) using Sequa Gel® MD (National Diagnostics, UK Ltd.) and visualised by silver staining (Bassam et al. 1991).

5.2.3 SSR marker analysis

The variety set was screened with 10 publicly available SSR markers that were polymorphic between HS and HS/VPM7D. Markers were identified to provide a uniform coverage of chromosome 7D. Primer sets used were from IPK Gatersleben (*Xgwm428*) (Roder et al. 1998), Beltsville Agricultural Research Station (*Xbarc53, Xbarc76, Xbarc97, Xbarc111* and *Xbarc121*) (Song et al. 2002), Wheat Microsatellite Consortium (*Xwmc14* and *Xwmc221*) (http://wheat.pw.usda.gov/ggpages/SSR/WMC/), and INRA (*Xcfd175*) (http://wheat.pw.usda.gov/ggpages/SSRclub). The SSR marker X*ustSSR2001-7DL* (Groenewald et al. 2003) was also included. Four of these SSR markers in the distal portion of chromosome 7D; *Xbarc76*, *Xbarc97*, *Xwmc14* and *Xcfd175*, were applied to the HS x RVPM25 BC_6 F_2 population to identify recombinants in the region of *Pch1*. DNA extractions, PCRs, and amplicon analyses were all conducted as described in Chapter 3, with the exception of the marker *XustSSR2001-7DL* for which PCR amplification conditions were those described by Groenewald et al. (2003).

5.2.4 Recombinant eyespot resistance phenotyping

The three recombinant varieties and seven BC_6 F_3 families from HS x RVPM25 recombinant BC_6 F_2 plants were phenotyped for resistance to *O. yallundae* in seedling bioassays alongside the susceptible control HS and *Pch1*-carrying controls HS/VPM7D and RVPM25. Thirty plants per line were arranged in a randomised

complete block design consisting of six blocks, inoculated with *O. yallundae*, incubated for six weeks and scored for disease; as described by Chapman et al. (2008). Isolates of *O. yallundae* were used because this pathogen species is known to successfully penetrate stem bases of plants carrying the resistance gene *Pch2* on chromosome 7A. This partial eyespot resistance gene is present in HS (Chapter 3) and therefore is present in all the BC_6F_3 families.

5.2.5 Statistical analysis

Analysis of variance was performed on visual disease scores from the recombinant lines to assess the variation attributable to block and genotype using a general linear model (GLM) in Genstat v.12 (Payne et al. 2009). Predicted mean disease scores were calculated for each line using the GLM and these were compared to the mean disease scores for HS and RVPM25 using t-probabilities calculated within the GLM. Lines with disease scores significantly different ($P<0.001$) from HS but not from RVPM25 were determined to be homozygous for *Pch1*, lines significantly different ($P<0.001$) from both HS and RVPM25 were considered heterozygous for *Pch1*, and lines significantly different ($P<0.001$) from RVPM25 but not from HS were determined to be homozygous for the absence of *Pch1*. A high significance threshold ($P<0.001$) was used to limit the possibility of Type-I errors generated by multiple comparisons of genotypes as described by Olmos et al. (2003).

5.2.6 7A and 7D homoeology

The 7D polymorphic COS and STS markers were applied to Chinese Spring (CS) nullisomic–tetrasomic lines N7AT7B, N7BT7D and N7DT7A, and to the three CS 7AL terminal deletion bin lines (Endo and Gill 1996) to determine if they have homoeoloci in the *Pch2* region on the distal portion of chromosome 7AL. These markers were also applied to the single chromosome substitution line Chinese Spring-Cappelle Desprez 7A (CS/CD7A) to determine if they were polymorphic between CS and CS/CD7A, the parents of a 7A (*Pch2*) recombinant F_2 population previously used to locate the QTL position of *Pch2* (Chapman et al. 2008).

Those markers that were polymorphic between CS and CS/CD7A were applied to 186 lines from the CS x CS/CD7A F_2 population using an SSCP assay (Martins-Lopes et al. 2001). This marker data was added to previous data on this population for the SSR markers *Xgwm346, Xwmc525* and *Xcfa2040* (Chapman 2005) and from the cDNA-AFLP derived marker *X19CD7A4* (Chapter 2). A new map of the distal portion of chromosome 7A was calculated using Joinmap version 3.0 (van Ooijen and Voorips 2001). To assess the relationship between the novel markers and *Pch2* resistance the new 7A map was combined with previously obtained phenotypic data for the *Pch2* resistance to *O. acuformis* in CS x CS/CD7A (Chapman 2005; Chapter 2) in a new QTL interval mapping analysis using MapQTL version 4.0 (van Ooijen and Maliepaard 1996). The minimum significant logarithm of the odds (LOD) score was calculated by a permutation test (1,000 permutations) to identify the appropriate significance threshold ($P<0.01$) to declare the presence of a QTL for eyespot resistance.

5.3 Results

5.3.1 COS marker analysis

One of the thirteen WGIN COS markers, *Xwg7S*, was found to be polymorphic between HS and HS/VPM7D. Furthermore, this marker was also found to be distally located on 7D in the *Pch1* region producing an *Ae. ventricosa* haplotype from RVPM25 and RVPM44. As expected, the STS markers *Xorw1* and *Xorw5* were also found to be polymorphic and distally located on 7DL. However, in contrast to the findings of Leonard et al. (2008), co-dominant products were detected for both markers using the touchdown PCR programme described above. Although it is conceivable that the polymorphic products detected from *Xorw1* and *Xorw5* were from different loci, polymorphisms were assessed between HS and the recombinant substitution lines RVPM25 and RVPM44 with reduced *Ae. ventricosa* segments on the distal portion of chromosome 7DL, and it is highly probable that the products derive from the same loci. The STS marker, *Xorw6*, developed in the same study, was also evaluated but was found to generate a complicated profile with weak amplification under our conditions, and was therefore not utilised further in the present study. The cDNA-AFLP derived COS marker *X4CD7A8* was also found to

be polymorphic between HS and HS/VPM7D, and to be distally located on 7DL in the region of *Pch1*. The wEST sequences to which these four informative markers were developed were found to have homologous locations on Brachypodium chromosome 1 (Bd1) (Table 5.1).

Table 5.1: Summary of conserved orthologous set (COS) markers, STS markers and cDNA-AFLP derived markers used in the study to identify recombinants in the region of *Pch1*.

Locus Name	Wheat EST	Primers	Resolution Method	Brachypodium Bd1 Physical Location	Brachypodium Predicted Transcript
Xwg7S	BF484041	CTTGATCAGACGGAAGACGAGC TGGTTTATGGCTTCTCATGGGTT	ABI 3700	25,174,226.. 25,174,579	Bd1g29620
Xtr40	CJ579531	TAAAGGACCTCCATGCACAG CCAGGCAGCACGAGAAAA	ABI 3700	24,824,436.. 24,826,323	Bd1g29320
Xtr331	BQ743827	GGAACCCCCTACTTGGTGAT CAGAGTGGGGATGCTTGAAT	ABI 3700	25,563,782.. 25,565,973	Bd1g30180
Xtr370	CD490497	CAATGGCAAAGCTTGACAAA TGAAAGGGATGGAACTGAGG	ABI 3700	25,904,597.. 25,906,532	Bd1g30580
Xtr383	CJ726064	TGACATCTTCGTCTGCATCC GACCATCTCAACGACAGGCT	ABI 3700	25,174,811.. 25,176,125	Bd1g29620
Xcos7-6	BF473539	CTGTTTTTAAGTTGGAGGTTGC AGATGAAGGAGGCATTCCAG	SSCP Gel	25,232,127.. 25,232,366	Bd1g29690
Xcos7-9	CD885492	CTGCCGAGATCTTGAGGAAG ACAGTGCTTCCCGTACCATC	SSCP Gel	24,811,725.. 24,811,256	Bd1g29290
Xorw1	AB246917	CTATTACATGAAATCTTATTCTC CCAGCAGTAACGAGAATGTGG	ABI 3700	24,990,182.. 24,990,550	Bd1g 29400
Xorw5	TC252872	GCATCCTCGCCTTCATGC CGACCATCTCGACCACAGG	ABI 3700	25,175,591.. 25,175,504	Bd1g29620
X4CD7A8	CJ641756	CAGGCTTGATCGCTTGG TCTGCATCCTCGCCTTC	ABI 3700	25,175,591.. 25,175,877	Bd1g29620

Examining the Brachypodium region surrounding these locations revealed that a number of 7DL terminal deletion-bin mapped wESTs also have homologous locations in this region of Bd1. Therefore, an approximately 1.1 Mb region (24,811,700-25,906,600) was targeted on Bd1 to identify further markers and to define the extent of the co-linear relationship with the *Pch1* region on wheat chromosome 7DL. Using this region of the Brachypodium sequence twenty-five

further COS markers were identified (Tr), or were developed from aligned wESTs (Cos7) in this region. Of these, six were polymorphic (24%) (Table 5.1) and all of these were determined to be located on the distal portion of chromosome 7DL. Five of these markers were co-dominant, yielding specific products from both wheat and *Ae. ventricosa*. One COS marker, *Xtr40*, was polymorphic but dominant, yielding only an *Ae. ventricosa* specific product of 435 bp.

5.3.2 Identification of recombination in wheat varieties

Ten polymorphic SSR markers and the COS markers *Xwg7S* and *Xtr40* were applied to the panel of varieties and control lines (Table 5.2). These markers confirmed that HS/VPM7D consists of *Ae. ventricosa* DNA along the length of the chromosome, that RVPM25 consists of *Ae. ventricosa* from marker *Xbarc53*, and that RVPM44 has a double recombination along the chromosome resulting in a short *Ae. ventricosa* segment in the distal portion. 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, with an *Ae. ventricosa* haplotype at the loci *Xtr40* and *Xcfd175*. These distal recombinant varieties were included in further analysis using the full set of polymorphic COS and STS markers (Table 5.1) and were included in a seedling bioassay (Figure 5.1) 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 (Table 5.3).

Table 5.2: 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
RVPM44	B	B	A	A	A	A	A	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*

5.3.3 Mapping Pch1 using SSR, STS and COS markers

The eight polymorphic COS markers, two STS markers (Table 5.1) and four SSR markers (*Xbarc76*, *Xbarc97*, *Xwmc14* and *Xcfd175*) were all applied to the HS x RVPM25 BC_6F_2 population. Eight BC_6F_2 plants from the 376 tested were identified with recombination events occurring between the SSR markers *Xbarc76* and *Xcfd175* (Table 5.3). 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 (Table 5.3; Figure 5.2). Where no recombination events have occurred between markers, the order was inferred based on the order of Brachypodium homologues. An approximate map of chromosome 7D was constructed to demonstrate marker order (Figure 5.2). However, it was not possible to indicate genetic distance as it was based upon recombination from an unknown number of recombination events in the varietal panel.

The *Pch1* phenotype of the recombinant set was determined in a seedling bioassay using *O. yallundae* isolates demonstrating that the recombinant varieties all carry *Pch1* and classifying the HS x RVPM25 BC_6F_2s as homozygous for either the presence or absence of *Pch1*, or as heterozygous (Figure 5.1).

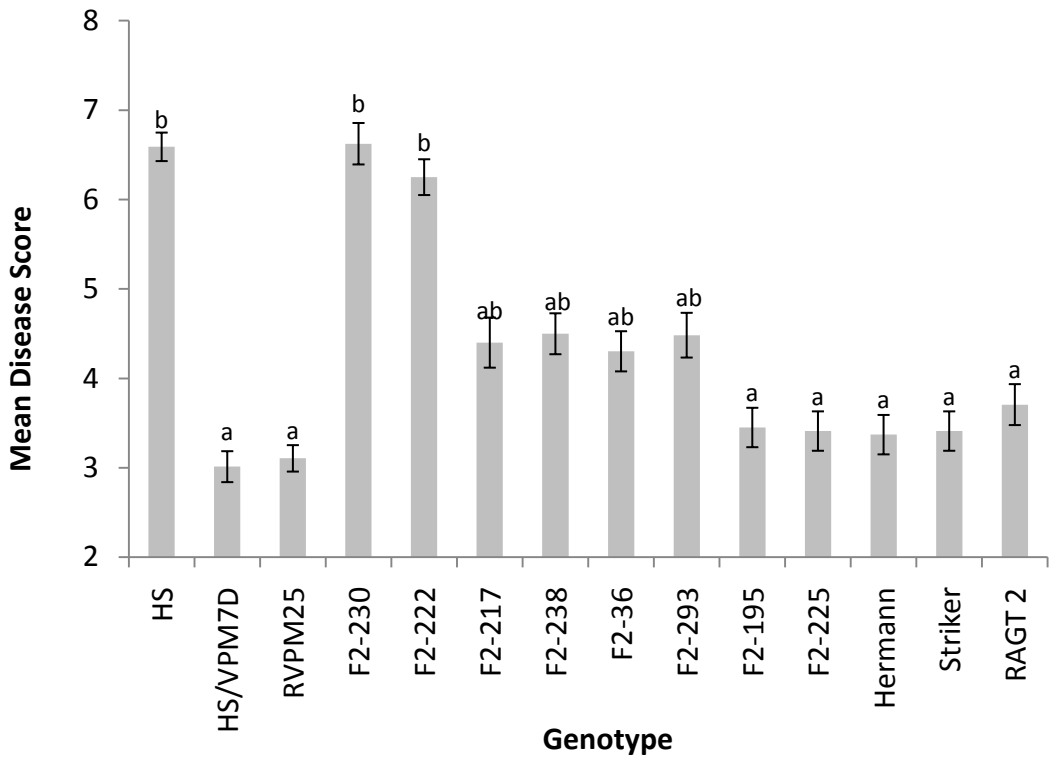

Figure 5.1: Predicted mean disease scores for recombinant BC_6 F_3 families derived from HS x RVPM25 and for *Pch1* carrying varieties calculated within a general linear model (GLM). Error bars are +/- standard errors. Lines were compared to controls using t-probabilites calculated within the GLM ($P<0.001$); where a = significantly different from the susceptible HS, and b = significantly different from the *Pch1* carrying RVPM25.

Table 5.3: Haplotypes of a recombinant set of varieties and BC_6F_2 lines from HS x RVPM25.

Line	*Xbarc76* [1]	*Xtr370* [2]	*Xtr331* [2]	*Xbarc97*	*Xwmc14*	*Xcos7-6* [2]	*X4CD7A8* [2]	*Xorw5* [2]	*Xtr383* [2]	*Xwg7s* [2]	*Pch1* [3]	*Xorw1* [2]	*Xtr40* [2]	*Xcos7-9* [1]	*Xcfd175* [1]
HS	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
R25	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Hermann	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B
Striker	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B
RAGT 2	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B
F2-222	H	A	A	A/H	A/H	A	A	A	A	A	A	A	A	A	A
F2-230	H	H	H	A/H	A/H	A	A	A	A	A	A	A	A	A	A
F2-217	H	H	H	A/H	A/H	H	H	H	H	H	H	H	B/H	A	A
F2-238	H	H	H	A/H	A/H	H	H	H	H	H	H	H	B/H	A	A
F2-36	H	H	H	A/H	A/H	H	H	H	H	H	H	H	B/H	H	A
F2-293	A	H	H	A/H	A/H	H	H	H	H	H	H	H	B/H	H	H
F2-195	B	B	B	B	B	B	B	B	B	B	B	B	B/H	H	H
F2-225	B	B	B	B	B	B	B	B	B	B	B	B	B/H	B	H

A = wheat

B = *Aegilops ventricosa*

H = heterozygous

Marker order was determined by;

[1] recombination events in varieties and BC_6F_2 lines,

[2] recombination events followed by homologous Brachypodium marker positions,

[3] eyespot resistance phenotyping.

Phenotypic data (Figure 5.1) was combined with marker data to determine the position of *Pch1* relative to the markers, using data from both the recombinant set of varieties and the BC_6F_2 lines. It was possible to detect recombination events both proximal and distal to *Pch1*. 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* due to a recombination detected in the varieties Hermann, Striker and RAGT 2 (Table 5.3). In addition, *Pch1* was found to co-segregate with the STS marker *Xorw1*. It is also possible that

the dominant COS marker *Xtr40* co-segregates with *Pch1* (Table 5.3) and is indicated as such in Figure 5.2. However, as this marker generates a dominant *Ae. ventricosa* product, it is not possible to determine if line F2-195 is heterozygous or homozygous *Ae. ventricosa* at this locus in order to orientate *Xtr40* relative to *Xorw1* and *Pch1*.

Neither the STS marker *Xorw5*, nor the SSR markers *Xbarc97* and *Xwmc14*, previously identified for marker-assisted selection of *Pch1* co-segregated with the resistance, based on data from the variety panel (Table 5.2) (Table 5.3), suggesting that they are not completely linked to the resistance gene.

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

The order of the markers on wheat chromosome 7D was fully conserved with the physical location of markers on Bd1 (Figure 5.2). It was not possible to determine the order of a set of five co-segregating loci in wheat (*Xcos7-6*, *X4CD7A8*, *Xorw5*, *Xtr383* and *Xwg7S*). However, this may be expected for *X4CD7A8*, *Xorw5*, *Xtr383* and *Xwg7S* as these four markers were found to be extremely close on Bd1, aligning to two locations only approximately 600 bp apart within the same predicted transcript (Bradi1g29620). The other co-segregating marker at this locus, *Xcos7-6*, is also relatively close, locating approximately 57 Kb away to the predicted transcript Bradi1g29690. The physical marker order on Bd1 and the genetic order on wheat 7D was largely conserved on Os6, with the exception of an inversion event between the loci *Xtr40* and *Xcos7-9*. *Xtr40* locates proximally to *Xcos7-9* in Brachypodium but distally in rice (Figure 5.2). Therefore, Brachypodium may provide a greater level of co-linearity to wheat than rice at these loci as *Xtr40* appears to map proximally to *Xcos7-9* in the HS x RVPM25 BC_6F_2 population.

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 5.2). This region contains thirty-four predicted transcripts according to the Brachypodium genome annotation (International Brachypodium Initiative 2010). It was possible to assign putative functions for twenty-five of these through significant similarity to

known proteins from other cereal species (Table 5.4). Of the remaining nine, seven had similarity with expressed proteins of unknown function and two had no significant similarity to any known genes. It is possible that the two genes with no homology may represent pseudogenes, predicted by the automated pipeline employed in the Brachypodium genome annotation (International Brachypodium Initiative 2010).

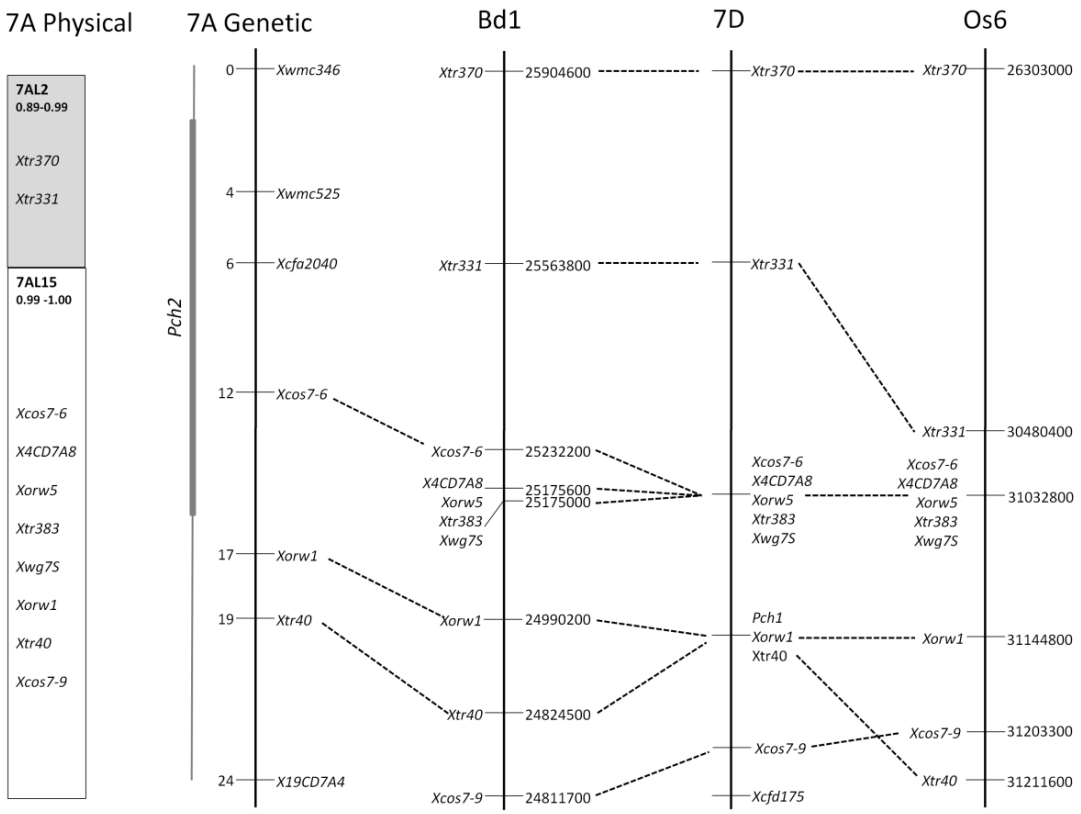

Figure 5.2: Comparison of the HS x RVPM25 wheat chromosome 7D approximate genetic map including *Pch1* with physical marker locations on Brachypodium chromosome 1 (Bd1) and rice chromosome 6 (Os6), and also with the CS x CS/CD7A chromosome 7A genetic and CS deletion bin maps. The location of the *Pch2* QTL region determined on the basis of interval mapping is also indicated as a thin bar to represent intervals above the P<0.01 significance threshold of LOD = 3.0 and as a thick bar to represent intervals with LOD>15.2.

The candidate gene region in Brachypodium contains four genes with homology to proteins associated with plant disease resistance (Table 5.4). Located between *Xwg7s* and *Xorw1*, Bradi1g29450 was found to have homology to a wheat complete coding sequence (CDS) DQ205351 that encodes a nucleotide-binding site–leucine-rich repeat (NBS-LRR) disease resistance protein (Wang et al. 2006). Between *Xtr40* and *Xorw1*, the genes Bradi1g29360 and Bradi1g29370 both had homology to the same rice gene (Os11g39550), which encodes a LRR family protein. This could represent a gene duplication event in Brachypodium. In addition, the gene Bradi1g29390, also located between *Xtr40* and *Xorw1*, was found to have homology to a rice gene (Os06g51420) that encodes a coiled-coil domain containing protein.

By aligning the *Pch1* flanking markers to the TIGR rice genome annotation (Ouyang et al. 2007); the *Pch1* region in rice can be defined as an approximately 178 Kb region on Os6 containing twenty-six genes. BLASTn revealed that sixteen of the twenty-six genes in the *Pch1* region of rice were shared with the Brachypodium *Pch1* region in a mostly co-linear order (Table 5.4). Of the genes potentially involved in disease resistance in the Brachypodium *Pch1* region, only Bradi1g29390 (coiled-coil domain containing protein) was found to have an orthologue (Os06g51420) in the rice *Pch1* region. No other genes with functions directly associated with plant disease resistance were detected in the rice candidate gene region.

Interestingly, the genes Bradi1g29310 and Bradi1g29320 have been annotated separetly in Brachypodium but both have homology to one gene (Os06g51520) in the rice candidate gene region (Table 5.4). These two pairs of predicted transcripts are inverted between rice and Brachypodium as revealed by the COS markers *Xcos7-9* and *Xtr40* (Figure 5.2) that locate to Bradi1g29290 and Bradi1g29320 respectively (Table 5.4).

Table 5.4: Comparison of *Pch1* candidate gene regions in Brachypodium and rice. In cases where corresponding genes were not identified in both regions this is indicated as a dash (-).

COS Marker	Brachypodium Gene	Gene Function	Rice Gene	Gene Function
Xcos7-9	Bradi1g29290	Expressed protein	Os06g51500	Expressed protein
	Bradi1g29300	Expressed protein	Os06g51510	Expressed protein
	Bradi1g29310	Lysine ketoglutarate reductase trans-splicing related 1	Os06g51520	Lysine ketoglutarate reductase trans-splicing related 1
Xtr40	Bradi1g29320			
	Bradi1g29330	No homology	-	-
	Bradi1g29340	Expressed protein	-	-
	Bradi1g29350	Protein kinase like domain	-	-
	Bradi1g29360	Leucine rich repeat family protein	-	-
	Bradi1g29370		-	-
	Bradi1g29380	Retrotransposon protein	-	-
	-	-	Os06g51490	PHD-finger domain containing protein
	-	-	Os06g51480	Expressed protein
	-	-	Os06g51470	Expressed protein
	-	-	Os06g51460	White-brown complex homolog protein
	-	-	Os06g51450	PHD-finger domain containing protein
	-	-	Os06g51440	Transposon protein
	-	-	Os06g51430	Signal peptide peptidase-like
	Bradi1g29390	Coiled-coil domain-containing protein 12	Os06g51420	Coiled-coil domain-containing protein
Xorw1	Bradi1g29400	Putative prolyl oligopeptidase [a]	Os06g51410	Putative prolyl oligopeptidase [a]

Table 5.4 cont.

COS Marker	Brachypodium Gene	Gene Function	Rice Gene	Gene Function
	Bradi1g29410	Expressed protein	-	-
	Bradi1g29420	Expressed protein	Os06g51400	Expressed protein
	Bradi1g29430	Retrotransposon protein	-	-
	Bradi1g29440	No homology	-	-
	Bradi1g29450	NBS-LRR disease resistance protein	-	-
	Bradi1g29460	Expressed protein	Os06g51390	Expressed protein
	Bradi1g29470	Root hairless 1	Os06g51380	Root hairless 1
	-	-	Os06g51370	Putative serine carboxypeptidase homologue
	Bradi1g29480	LysM domain containing protein	Os06g51360	LysM domain containing protein
	Bradi1g29490	Isopentenyl transferase family protein	Os06g51350	Isopentenyl transferase family protein
	-	-	Os06g51340	Retrotransposon protein
	Bradi1g29500	Z. mays plastid high chlorophyll fluorescence 136 precursor	-	-
	Bradi1g29510	Expressed protein	Os06g51330	Expressed protein
	-	-	Os06g51320	GASR8 - Gibberellin-regulated GASA/GAST/Snakin family protein precursor
	Bradi1g29520	Z. mays brassinosteroid biosynthesis-like protein (DWF-1)	-	-
	Bradi1g29530	Nucleic acid binding protein	-	-
	Bradi1g29540	Cyclin-L1-1, putative, expressed	-	-
	Bradi1g29550	Regulator of ribonuclease activity A	-	-
	Bradi1g29560	Pollen signalling protein with adenyl cyclase activity	-	-

Table 5.4 cont.

COS Marker	Brachypodium Gene	Gene Function	Rice Gene	Gene Function
	Bradi1g29570	Sucrose synthase	-	-
	Bradi1g29580	PAZ domain containing protein	Os06g51310	PAZ domain containing protein,
	-	-	Os06g51300	Conserved hypothetical protein
	Bradi1g29590	Phytoene synthase, chloroplast precursor	Os06g51290	Phytoene synthase, chloroplast precursor
	Bradi1g29600	Acetolactate synthase 1, chloroplast precursor	Os06g51280	Acetolactate synthase 1, chloroplast precursor
	Bradi1g29610	RING-H2 finger protein ATL1R	-	-
Xwg7S	Bradi1g29620	1,3-beta-glucan synthase [b]	Os06g51270	1,3-beta-glucan synthase [b]

[a] Homologous to wheat oligopeptidase B encoding expressed sequence tag (AB246917) (Leonard et al. 2008).

[b] Homologous to wheat 1,3-beta-glucan expressed sequence tag (DQ086484) involved in callose synthesis (Voigt et al. 2006).

5.3.5 7A-7D Homoeology

All of the gene-based COS and STS markers were also determined to have homoeoloci on chromosome 7AL. Testing these markers on the 7AL deletion bin series demonstrated that the two most proximal 7D COS markers *Xtr370* and *Xtr331* located to the bin adjacent to the terminal 7AL deletion bin, whilst the remaining COS markers all located to the terminal 7AL bin (Figure 5.2). Three of the markers located to the 7AL terminal deletion bin, *Xcos7-6, Xorw1* and *Xtr40*, were found to be polymorphic between CS and CS/CD7A and were therefore mapped in the CS x CS/CD7A F_2 population. These mapped to a region between the SSR marker *Xcfa2040* and the cDNA-AFLP derived marker *X19CD7A4* and resolved into the same order as found on wheat chromosome 7D and on Bd1 (Figure 5.2). Incorporating the new 7A COS markers into the QTL interval mapping analysis

suggested that the *Pch2* QTL peaks at a locus between *Xwmc525* and *Xcfa2040* (LOD = 17.2) and covers a region based on the peak LOD -2 (LOD threshold > 15.2) from a locus between the SSR markers *Xwmc346* and *Xwmc525* to a locus between the COS marker *Xcos7-6* and the STS marker *Xorw1*. The addition of COS markers distal to *Xcfa2040* extended the *Pch2* QTL (LOD>15.2) to a 14 cM region. This compares to a narrower 7 cM region identified by interval mapping in Chapter 2. In comparison, on chromosome 7D, *Pch1* was located to an interval between the loci of *Xwg7S* and *Xcos7-9*. Therefore, it is possible that there is an overlap between the *Pch1* and *Pch2* containing regions on chromosomes 7D and 7A respectively (Figure 5.2).

5.4 Discussion

The exploitation of beneficial alleles from introgressions of related species into wheat has often been limited by linkage to deleterious traits, a low level of recombination and insufficient PCR-based co-dominant markers. Our results demonstrated that cereal COS markers provide a means to identify recombinants in the *Ae. ventricosa* segment on chromosome 7D that has previously been recalcitrant to genetic analysis. Furthermore, we utilised COS markers to fine-map the potent eyespot resistance gene *Pch1* present on the introgression. The cross-species transferability of COS markers across cereals (Bertin et al. 2005; Garvin et al. 2010), combined with evidence from the present study suggests that they could be applied to other introgressions from relatives into wheat to assist selection of desirable traits. However, the applicability of COS markers for this purpose is likely to be limited by an absence of recombination in introgressions from more distantly related species such as rye.

Using the sequenced and annotated Brachypodium genome and by exploiting synteny with wheat, new COS markers were developed and existing COS and STS markers were identified to produce a panel of co-dominant markers, targeted to the *Pch1* region on chromosome 7D of hexaploid wheat. These markers were applied to the HS x RVPM25 BC_6 F_2 population to identify novel *Pch1* recombinants. In addition, an SSR analysis of a panel of wheat varieties and breeding lines identified further recombination events, notably in Hermann, Striker and RAGT 2. Marker data

from BC_6F_2 and varietal recombinants were combined to determine the marker order over the *Pch1* region of chromosome 7D. However, it was not possible to calculate genetic map distances because the marker order was determined partly based on recombination observed in the varietal panel.

Importantly, due to the potency of the *Pch1* resistance it was possible to characterise the gene as a qualitative trait. A clear phenotype could be assigned to F_3 families generated from the BC_6F_2 recombinants using seedling bioassays with relatively few plants per line (approximately 30). Because it is possible to use F_3 families for phenotyping in this material without the need to generate homozygous lines, the process of screening larger populations for further mapping and potentially map-based cloning of the gene will be greatly speeded up.

The physical location of orthologues of COS and STS markers could be identified over an approximately 1.1 Mb region on Bd1 and a 4.9 Mb region on Os6. 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.* This suggests that both rice and Brachypodium provide a useful resource for further marker development and fine-mapping of *Pch1*.

Using the annotated sequence of Brachypodium it was possible to generate and orientate a panel of markers and to locate *Pch1* to an interval between a proximal group of five co-segregating flanking markers; *Xcos7-6*, *X4CD7A8*, *Xorw5*, *Xtr383* and *Xwg7S*; and the distal flanking marker *Xcos7-9*. Four of the co-segregating proximal flanking markers aligned to the same Brachypodium (Bd1g29620) and rice genes (Os06g51270) in the syntenous regions of both species. Furthermore, wEST sequences for these four markers all had significant similarity to a wheat putative 1,3-beta-glucan synthase 3 gene (DQ086484) thought to be involved in callose synthesis (Voigt et al. 2006).

Callose synthesis and its deposition at wound sites have been widely implicated in disease resistance (Chen and Kim 2009) and furthermore have been implicated in *Pch2* resistance to *O. acuformis* through the identification of up-regulation of expression of callose synthase in cDNA-AFLP studies (Chapman et al. 2009). As such, callose synthase genes might be considered as candidates for involvement in

Pch1 resistance. The four callose synthase gene markers identified did not co-segregate with *Pch1* in the wheat lines Hermann, Striker and RAGT 2 and were determined to flank the resistance proximally. However, it is possible that this callose synthase gene could still be a candidate for *Pch1* depending on the relative locations of recombination events and any crucial polymorphisms between the wheat and *Ae. ventricosa* versions of the gene that cause a functional change in the protein. Nonetheless, this is unlikely as the four markers were all designed to wESTs that align to exons at the 3' end of the gene, and therefore any functional polymorphism would need to occur in a small (approximately 500 bp) region.

The marker *Xorw1* was determined to co-segregate with *Pch1* in the HS x RVPM25 BC_6 F_2 population and confirms the findings of Leonard et al. (2008) that it is suitable for marker assisted selection of the resistance gene by plant breeders. This marker was designed to an oligopeptidase B gene as a PCR replacement for the endopeptidase protein isozyme marker *Ep-D1b* (Leonard et al. 2008). A number of previous studies have failed to detect recombination between *Pch1* and either the isozyme marker (McMillin et al. 1986; Santra et al. 2006) or the PCR-based marker (Leonard et al. 2008). It has been speculated that *Pch1* resistance may be conferred by the *Ep-D1b* protein (Worland et al. 1988). Our data did not identify any recombination between *Xorw1* and *Pch1*, and on this evidence the oligopeptidase B gene can be considered a candidate for the resistance. However, Mena et al. (1992) identified a recombination between *Ep-D1b* and *Pch1* in a line that carried the endopeptidase allele *Ep-D1b* but was susceptible to eyespot, suggesting that the resistance is not a product of the *Ep-D* locus. If *Orw1* and *Ep-D1b* are derived from the same gene, then the findings of Mena et al. (1992) would discount the oligopeptidase B gene as a candidate for *Pch1*.

Our data demonstrates that the COS marker *Xtr40* is tightly linked to *Pch1*. However, this marker was designed to a wEST with homology to a gene in rice encoding an enzyme involved in lysine metabolism (Anderson et al. 2010), which appears unlikely to be directly involved in plant defence responses. It was not possible to accurately determine the halpotype of all the HS x RVPM25 BC_6 F_2 population at *Xtr40* because it is a dominant marker generating an *Ae. ventricosa* specific product. Consequently, there could be undetected recombination between *Pch1* and *Xtr40*. BC_6 F_3 plants were retained from all recombinant HS x RVPM25

BC_6 F_2 lines, selfed and BC_6 F_4 seed produced so that homozygous material will be available to future studies to validate the recombinants, and to fully resolve haplotypes at loci based on dominant markers such as *Xtr40*.

It was possible to identify candidate gene regions for *Pch1* in rice and in Brachypodium on the basis of orthologous locations of the flanking markers. An approximately 364 Kb region containing thirty-four predicted transcripts was identified on Bd1 and an approximately 178 Kb region containing twenty-two putative genes was identified on Os6. Sixteen of the genes in the Brachypodium *Pch1* region are also present in the rice *Pch1* region and gene order is largely conserved. However, there is evidence that micro co-linearity between rice and Brachypodium has broken down in this region as there are eighteen additional genes present in Brachypodium but absent in rice, and eight additional genes in rice that are not present in Brachypodium. In addition, there is an inversion in the order of genes between the markers *Xcos7-9* and *Xtr40* between rice and Brachypodium. Interestingly, within this inverted region it appears that there has been either a gene duplication event in Brachypodium or possibly inaccurate gene annotation, as two pairs of Brachypodium genes (Bradi1g29290/Bradi1g29300 and Bradi1g29310/Bradi1g29320) are represented by two single rice genes (Os06g51510 and Os06g51520 respectively). At this stage it is not possible to infer whether rice or Brachypodium provides a more suitable reference sequence for further dissection of this region in wheat. However, the utilisation of both of these resources together should enable the development of further markers and identification of candidate genes to facilitate positional cloning of *Pch1*. For example, further markers could be directly developed in the candidate gene region using 7DL terminal deletion bin mapped wESTs aligning to the rice genes Os06g51310, Os06g51330, and Os06g51480.

A number of independent recombination events were detected in the panel of varieties, demonstrating that recombination has occurred over the length of the *Ae. ventricosa* segment in breeding programmes. Taken in conjunction with evidence for recombination in the BC_6 F_2 population, and evidence for recombination in previous studies (Leonard et al. 2008; Mena et al. 1992), this demonstrates that recombination does occur within the introgressed segment and that further mapping should be possible. In order to map *Pch1* with increased precision, more HS x RVPM25 BC_6

F_2s could be tested to identify additional recombination events in the *Pch1* region to reduce the interval and the number of candidate genes. This would also enable the calculation of genetic distances between markers as recombination events in the varietal panel would not need to be considered. However, due to the reduced recombination observed between wheat and *Ae. ventricosa*, a greater number of F_2 individuals would need to be screened than in a conventional wheat cross. A 1 % recombination rate was observed between the SSR markers *Xbarc76* and *Xcfd175* in the HS x RVPM25 population. In comparison, the consensus wheat genetic maps generated by Somers et al. (2004) indicates an approximately 10% recombination rate between these markers. However, recombination rates may not be equal over the region. A recombination rate of 0.2 % was recorded between the SSR markers *Xbarc76* and *Xwmc14* compared to an approximately 8 % recombination rate in the Somers et al. (2004) consensus map, whereas a recombination rate of 0.8 % was identified between *Xwmc14* and *Xcfd175* compared to a 1% recombination rate in the consensus map.

To my knowledge, no resistance genes of quantitative effect against a necrotrophic fungal pathogen have been cloned and characterised in wheat and therefore I have no prior expectations as to the likely function of *Pch1*. Many of the cloned single dominant resistance (R) genes in plants that are involved in pathogen recognition and signal transduction encode a protein with a nucleotide binding site followed by a leucine rich repeat domain (NBS-LRR). These domains are commonly preceded at the N-terminal by coiled-coil (CC) or Toll and mammalian interleukin (IL)-1 receptors (TIR) domains (Dangl and Jones 2001). Although it has been suggested that such R-genes are not generally effective against necrotrophic pathogens such as eyespot (Glazebrook 2005), resistance genes have been cloned in melon (Joobeur et al. 2004) and tomato (Ori et al. 1997) that are effective against the necrotrophic fungus *Fusarium oxysporum*, and have been found to encode NBS-LRR type proteins. Most R-genes confer complete immunity and furthermore rarely provide durable resistance (McDonald 2010). In contrast, *Pch1* provides a high level of partial resistance and has, proven durable to date. Although *Pch1* may not be anticipated to be an R gene, from the putative gene functions identified within the rice and Brachypodium *Pch1* regions, there are candidate genes with R-gene motifs. Three genes (Bradi1g29360, Bradi1g29370 and Bradi1g29450) were identified in the

Brachypodium *Pch1* region with homology to LRR domain containing proteins in rice and wheat. In addition, a gene encoding a coiled-coil domain containing protein was found in both Brachypodium (Bradi1g29390) and rice (Os06g51420) in a co-linear position.

It is possible that indications of function for a resistance such as *Pch1* could be identified from partial resistances to biotrophic pathogens. For example, the partial stripe rust resistance gene *Yr36* has been determined to contain a kinase and a putative START lipid-binding domain (Fu et al. 2009), and the broad-spectrum leaf-rust, stripe rust and powdery mildew resistance *Lr34* has been shown to be an adenosine triphosphate–binding cassette (ABC) transporter (Krattinger et al. 2009). Interestingly, a gene identified in the *Pch1* region of rice annotated as encoding a White-brown complex homolog protein (Os06g51460) has significant homology with a gene encoding a putative ABC transporter in *Arabidopsis thaliana* (At1g53390).

Previous studies have suggested that *Pch1* on chromosome 7D and *Pch2* on chromosome 7A may be homoeoloci as they are both located on the distal portion of the long arms of the homoeologous chromosomes. De la Peña et al. (1997) suggested that *Pch1* and *Pch2* could be homoeoloci based on their positions relative to *Ep-D1* and *Ep-A1* respectively. This was supported by evidence from the SSR marker *Xcfa2040*, which has homoeoloci on 7A and 7D. *Xcfa2040* was found to be close to *Pch2* on 7A, and also to the *Pch1* linked SSR marker *Xwmc14* on 7D (Chapman et al. 2008). However, the frequent use of homoeologue-specific SSR markers has made it difficult to make direct comparisons between the two regions and Leonard et al. (2008) were unable to provide evidence to support homoeology between the two resistance genes. The direct transferability of gene-based COS and STS markers across chromosomes 7A and 7D enabled the partial integration of the maps of the two chromosomes, in this study.

The data from the present study suggests that there is a potential overlap in the region of *Pch1* flanked by the markers *Xcos7-6* and *Xcos7-9* on chromosome 7D and the region of the *Pch2* QTL between markers *Xcos7-6* and *Xorw1*. However, the addition of COS markers to the 7A map enlarged the region (LOD<15.2) of the *Pch2* QTL, as identified by interval mapping, from a 7 cM region identified in chapter 2 to

a 14 cM region. Although there is no clear explanation for this, it is possibly due to inaccuracies when phenotyping F_3 families from CS x CS/CD7A for eyespot resistance particularly considering the high level of environmental variation often associated with large-scale seedling bioassays of eyespot resistance (de la Peña et al. 1996). This may be overcome by the future development and phenotyping of RILs developed from F_3 families of the CS x CS/CD7A population to provide a more precise QTL location. If the two genes are indeed homoeoloci then it is anticipated that the higher level of recombination on chromosome 7A in *Pch2* populations may assist the orientation of homoeologue transferable COS markers on chromosome 7D and directly inform mapping of the more potent *Pch1* resistance.

In conclusion, I have demonstrated that COS markers developed using the annotated Brachypodium genome 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 provide valuable insight into the function of a necrotrophic disease resistance gene in wheat and 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.

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Chapter 6

General Discussion

Eyespot is an economically important fungal disease of wheat that attacks the stem base and causes significant yield losses in northern Europe (Hardwick et al. 2001) and the USA Pacific Northwest (Murray 1996). The disease is caused by two species of fungi *Oculimacula yallundae* and *O. acuformis*. 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 in the pathogen species to many of the widely used chemicals (Parnell et al. 2008). There are only two genetic sources of eyespot resistances in wheat for which molecular markers have been identified; *Pch1* and *Pch2*. There is a requirement for further characterisation of these resistances and for the identification of novel resistances that can be used by plant breeders to increase the level of resistance to eyespot in commercial varieties of wheat.

The first source of varietal resistance against eyespot to be identified was the variety Cappelle Desprez (Vincent et al. 1952). This resistance was determined to be partly due to *Pch2* on chromosome 7A (Koebner and Martin 1990; Law et al. 1976). *Pch2* was more recently mapped on 7AL to a 7.5cM interval between the SSR loci *Xwmc346* and *Xcfa2040* (Chapman 2005). In chapter 2, the location of *Pch2* resistance against *O. acuformis* was also located to this interval based on QTL interval mapping using a glasshouse seedling bioassay of the 7A recombinant population, CS x CS/CD7A. In an attempt to identify candidate genes for *Pch2*, a cDNA-AFLP approach was used by Chapman (2005) to identify genes differentially expressed between the susceptible variety Chinese Spring (CS) and the *Pch2* carrying single chromosome substitution line Chinese Spring – Cappelle Desprez 7A (CS/CD7A) when inoculated with *O. acuformis*. In chapter 2, the genetic locations of the cDNA-AFLP fragment sequences were determined. From the 29 fragment sequences generated by Chapman (2005), fourteen were mapped onto chromosome 7A, and three of these were found to locate to the *Pch2* region. Of these three, two fragments (4CD7A8 and 19CD7A4) had predicted functions that may indicate an

involvement in *Pch2* resistance. 4CD7A8 was homologous to a rice callose synthase gene and 19CD7A4 showed homology with a putative cereal cyst nematode NBS-LRR disease resistance protein (RCCN). Furthermore, the genes corresponding to both of these fragments were previously identified to have up-regulated expression patterns in CS/CD7A plants inoculated with *O. acuformis* (Chapman 2005). Although it has not yet been demonstrated conclusively that these sequences are responsible for *Pch2* mediated eyespot resistance, their functions, expression patterns and genetic locations suggest that they are candidates worthwhile of further investigation.

Evidence presented in chapter 3 demonstrates that *Pch2* is differentially effective against the two eyespot pathogen species, conferring a significantly lower level of resistance against penetration by *O. yallundae* compared to *O. acuformis.* This was demonstrated across a range of environments in both varieties and single chromosome substitution lines containing *Pch2*. Furthermore, although it was possible to detect a major QTL representing *Pch2* resistance against *O. acuformis* it was not possible to detect any resistance to *O. yallundae* in the population CS x CS/CD7A, in both glasshouse and controlled environment room experiments. Although it is not possible to infer the mechanism responsible for the differential resistance conferred by *Pch2* on the basis the evidence presented in this thesis, it may be related to the differences in the modes of infection of the two pathogen species, as described by Daniels et al. (1991).

The lack of effectiveness of *Pch2* against *O. yallundae* suggests that it is of limited use when deployed as a stand-alone resistance in commercial varieties, particularly as evidence from studies on the effects of fungicides suggest that controlling one species of the pathogen acts to increase the predominance of the other, with little reduction on the overall disease occurrence (Parnell et al. 2008). In addition, *Pch2* has previously been shown to be ineffective at the adult plant stage (Muranty et al. 2002), further limiting its efficacy in commercial varieties. However, *Pch2* may be useful in conjunction with other resistances, because it has been shown to provide an enhanced resistance when combined with *Pch1* (Hollins et al. 1988; Lind 1999), although it was not possible to detect an enhanced effect in the varieties tested in chapter 3.

There is a requirement for further resistances to eyespot because of the deleterious linkages associated with *Pch1* and the limitations in efficacy associated with *Pch2*. A number of additional genetic factors have been detected on other chromosomes of Cappelle Desprez (Law et al. 1976), notably an adult plant resistance on chromosome 5A (Muranty et al. 2002). Eyespot resistances should be effective at the adult plant stage to limit stem infection and prevent yield losses. However, it is preferable if resistances can also be identified at the seedling stage to facilitate mapping and selection in breeding programmes. Importantly the resistance on chromosome 5A of CD was determined to also be effective at the seedling stage using CS/CD5A and CD/Bez5A substitution lines in chapter 4.

A recombinant inbred line (RIL) population derived from CD x CD/Bez5A was used to identify the genetic location of the resistance on chromosome 5A in chapter 4. A QTL of moderate effect was detected close to the SSR marker *Gwm639* in the same location in a seedling bioassay using *O. yallundae* and in two independent field trials. A RIL population derived from CS x CS/CD5A was also used in a seedling bioassay inoculated with *O. acuformis* and located a significant QTL in the same genetic location. This was confirmed by a validation experiment on recombinant lines from both CD x CD/Bez5A and CS x CS/CD5A in seedling bioassays inoculated with both *O. yallundae* and *O. acuformis* independently. This suggests that the QTL for eyespot resistance on chromosome 5A, designated as *QPch.jic-5A*, provides a moderate level of all-round eyespot resistance, being effective against both pathogen species at seedling and adult plant stages. 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. As CD is within the pedigrees of many UK wheat varieties, *Pch2* and/or *QPch.jic-5A* may have been retained in these varieties. It would be interesting to establish whether varieties with moderate non-*Pch1* eyespot resistance, as determined by HGCA Recommended List trials, have either or both *Pch2* and *QPch.jic-5A*, or whether their resistance is potentially due to as yet uncharacterised genes.

Pch1 was introduced from the relative *Aegilops ventricosa* onto chromosome 7D (Doussinault et al. 1983; Worland et al. 1988) and is the most potent of the known eyespot resistances. However, as *Pch1* was introgressed into wheat as part of a large chromosome segment from *Ae. ventricosa* it is linked to yield limiting traits (Koen et

al. 2002), and it has proven difficult to break this linkage due to low rates of recombination between wheat and the wild relative (Worland et al. 1988). This association with lower yields limited the use of early *Pch1* carrying varieties, such as Rendezvous.

The isozyme marker *Ep-D1b* (McMillin et al. 1986), the Simple-Sequence-Repeat (SSR) markers *Xbarc97* and *Xwmc14* (Chapman 2005), and the Sequence-Tagged-Site (STS) markers *Xorw1, Xorw5* and *Xorw6* (Leonard et al. 2008), have all been shown to be linked to *Pch1*. Furthermore, it is possible that the markers previously identified to be linked to the resistance may not be physically close, but appear to co-segregate due to the low recombination rate. In this study recombination was detected between *Pch1* and the STS markers *Xorw5* (Leonard et al. 2008) and SSR markers *Xbarc97* and *Xwmc14* (Chapman et al. 2008) previously identified for selection of the resistance gene. This suggests that none of these markers used alone would provide completely accurate marker-assisted selection. However, the STS marker *Xorw1* previously identified as a *Pch1*-linked marker (Leonard et al. 2008) was found to co-segregate with the resistance, suggesting that it is suitable for the marker-assisted selection of the resistance.

The previously developed *Pch1* markers are all dominant, and do not provide specific polymorphic PCR amplicons from both wheat and *Ae. ventricosa* DNA. There is a need for co-dominant markers to assist in the identification of recombinants in F_2 populations that contain heterozygote plants. In chapter 5, co-dominant markers were developed in the region of *Pch1* by identifying conserved orthologous sequence (COS) markers using co-linearity between wheat and Brachypodium. In addition, the STS markers *Xorw1* and *Xorw5* were also converted into co-dominant markers by altering the PCR conditions to reduce the specificity of the primers. COS markers could be applied more widely to other introgressions from related species into wheat to identify recombination events and to select for beneficial traits such as disease resistance (Qi et al. 2008), abiotic stress tolerance (Mullan et al. 2009), and enhanced grain quality (Garg et al. 2009).

In chapter 5, a number of recombination events were detected in the panel of *Pch1* carrying varieties suggesting that although recombination may be impaired between wheat and *Ae. ventricosa*, it has occurred in a number of independent occasions in

breeding programmes. Furthermore, it was possible to generate novel recombination in the HS x RVPM25 F_2 population. *Pch1* is thought to be linked to yield limiting traits on the introgressed *Ae. ventricosa* segment (Koen et al. 2002), but also to high protein content conferring enhanced bread making ability (Groos et al. 2004). The F_2 recombinants identified in chapter 5 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.

Using gene-based COS and STS markers it was possible to identify a candidate gene region of 364 Kb in the annotated Brachypodium genome containing thirty four genes and a 178 Kb region in rice containing twenty-two genes. Although there are currently no clear expectations as to the likely function of a necrotrophic resistance gene in wheat, it was possible to identify three genes in the Brachypodium *Pch1* region with plant disease resistance functions, of which one was also present in rice.

Furthermore, evidence from chapter 5 suggests that *Pch1* on the distal portion of 7DL and *Pch2* on the distal portion of 7AL may be homoeoloci. The application of COS markers from chromosome 7D to chromosome 7A, in order to anchor the maps of the homoeologous chromosomes together, resulted in an extension of the *Pch2* QTL region. This extension suggests that the location of *Pch2* on chromosome 7A could overlap with the location of *Pch1* on chromosome 7D. However, more precise eyespot resistance phenotyping using RILs developed from the CS x CS/CD7A population and the application of further chromosome 7A and 7D transferable COS markers in the region would refine the *Pch2* QTL and provide evidence on whether the genes are homoeologous. If the resistance genes are homoeoloci then further study into one of these resistances should provide insight into the other.

The cloning of *Pch1* would be of significant interest in terms of functionally characterising a necrotrophic disease resistance gene in wheat, and also for the development of perfect markers for accurate selection of the resistance in plant breeding programmes. The development of targeted co-dominant markers, the identification of recombinants in the region, the ability to accurately and rapidly determine phenotypes on BC_6 F_3 families, and the delimitation of candidate gene

regions in rice and Brachypodium, will facilitate any future fine mapping and positional cloning of *Pch1*.

DNA is available from 1,200 further BC_6 F_2s for screening for recombinants in future studies. In addition, BC_6 F_3 seed was generated from these plants for phenotyping to position *Pch1* relative to the molecular markers. However, a large F_2 population will be required to clone *Pch1*, particularly due to the reduced level of recombination in the population. To this end, seed has been produced for 5,000 further BC_6 F_2s that could be screened for further recombinants to reduce the candidate gene interval.

Additional markers could be rapidly generated using further bin-mapped wESTs that align to candidate genes in rice, as discussed in chapter 5. A further important source of wheat sequence is the recently released Chinese Spring sequence, currently at five-fold coverage (http://www.cerealsdb.uk.net/search_reads.htm). This was generated in a collaborative project between the Universities of Bristol and Liverpool and the John Innes Centre using Roche 454 pyrosequencing technology (http://454.com/about-454/index.asp) to produce a database of non-annotated, unassembled sequence reads at an average length of 400 bp. Chinese Spring sequence in the region of *Pch1* can now be identified through a BLASTn search using sequence from genes in the candidate regions of rice and Brachypodium. A further resource that could be utilised for cloning *Pch1* is the BAC library of the *Pch1* containing variety Renan (Chalhoub et al. 2004). This could be screened using probes for the Brachypodium and rice genes in the candidate region to establish gene content in the *Pch1* region of wheat.

The identification and characterisation of eyespot resistances in the present study was greatly facilitated by the use of inter-varietal chromosome substitution lines. These have been widely used for studying inheritance of quantitative traits in wheat, particularly highly environmentally influenced traits such as Fusarium head blight resistance (Garvin et al. 2009) and yield traits (Dilbirligi et al. 2006). Furthermore chromosome substitution lines of wild tetraploid *Triticum turgidum* var. *dicoccoides* into the tetraploid durum variety Langdon, and populations subsequently developed from recombinant chromosome substitution lines, have been used for fine mapping, candidate gene identification and cloning (Distelfield et al. 2004; Distelfield et al.

2006; Olmos et al. 2003; Uauy et al. 2006) of the high grain protein content gene *Gpc-B1*, and of the linked high-temperature stripe rust resistance gene *Yr36* (Fu et al. 2009; Uauy et al. 2005).

The single chromosome substitution lines used in the present study were developed at the Plant Breeding Institute, Cambridge, in the 1970's and 1980's through a time consuming procedure involving repeated backcrossing to a recurrent monosomic or monotelocentric parent followed by cytological selection of monosomic segregants of each backcross (Law and Worland, 1973). Previous studies utilised the substitution lines CS/CD7A and CD/Bez5A to identify the chromosomal origins of *Pch2* (Law et al. 1976) and the 5A resistance (Muranty, 2002). In the present study, the use of populations developed from these single chromosome substitution lines CS/CD7A, CS/CD5A and CD/Bez5A were used for the characterisation of *Pch2* and *QPch.jic-5A* as quantitative traits. The segregation of a single chromosome in these precise genetic resources enabled the elimination of background variation for eyespot resistance to provide accurate phenotypic data. This is particularly important when phenotyping populations for a highly environmentally influenced trait such as eyespot resistance. Furthermore, the use of a backcross population developed from a recombinant single chromosome substitution line (RVPM25) enabled the quantitative *Pch1* resistance to be mapped as a qualitative trait in BC_6 F_3 families.

To summarise, this thesis provides important tools to 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*.

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Appendix 1

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*. Theoretical and Applied Genetics 117: 425-433

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ORIGINAL PAPER

The development of PCR-based markers for the selection of eyespot resistance genes *Pch1* and *Pch2*

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Abstract Two eyespot resistance genes (*Pch1* and *Pch2*) have been characterised in wheat. The potent resistance gene *Pch1*, transferred from *Aegilops ventricosa*, is located on the distal end of the long arm of chromosome 7D (7DL). *Pch2* derives from the variety Cappelle Desprez and is located at the distal end of chromosome 7AL. The RFLP marker *Xpsr121* and the endopeptidase isozyme allele *Ep-D1b*, are very closely linked to *Pch1,* probably due to reduced recombination in the region of the introgressed *A. ventricosa* segment. *Pch2* is less closely linked to these markers but is thought to be closer to *Xpsr121* than to *Ep-A1b*. In the present study simple sequence repeat (SSR) markers were integrated into the genetic map of a single chromosome (7D) recombinant (RVPM) population segregating for *Pch1*. Sequence-tagged-site (STS)-based assays were developed for *Xpsp121* and a 7DL wheat EST containing a SSR. SSR markers *Xwmc14* and *Xbarc97* and the *Xpsr121*-derived marker co-segregated with *Pch1* in the RVPM population. A single chromosome (7A) recombinant population segregating for *Pch2* was screened for eyespot resistance and mapped using SSRs. QTL interval mapping closely associated *Pch2* with the SSR marker *Xwmc525*.

N. H. Chapman and C. Burt contributed equally to this work.

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Introduction

Eyespot, caused by *Oculimacula acuformis* (formerly *Tapesia acuformis*) and *O. yallundae* (*T. yallundae*) (Crous et al. 2003) is one of the most important diseases of the stem base of cereals in temperate regions (Muranty et al. 2002). A wide range of cereals including wheat, barley, oats and rye are susceptible. Symptoms include leaf sheath browning followed by the development of stem base lesions (Fitt 1992). Severe infection results in lodging and premature ripening of grain, reducing crop yield.

Two main sources of resistance effective at the seedling stage have been incorporated into cultivated wheat varieties. The most potent resistance derives from the wild grass species *Aegilops ventricosa*. The single dominant gene *Pch1* was introduced into the wheat line VPM1 by Maia (1967), and is located on the distal end of the long arm of chromosome 7D (7DL) (Doussinault et al. 1983; Worland et al. 1988).

The second resistance originates from the variety Cappelle Desprez which dominated European wheat markets for two decades from 1953 (Hollins et al. 1988). Despite being widely used across Europe (Scott et al. 1989), the Cappelle Desprez resistance has proved highly durable (Johnson 1984; Scott et al. 1989). Resistance is transferable (Scott et al. 1989) and several new varieties have been found to

contain levels of resistance similar to Cappelle Desprez (Johnson 1992). Most of the resistance of Cappelle Desprez was found to be conferred by resistance gene(s) *Pch2*, located on chromosome 7A with additional effects associated with chromosomes 2B and 5D suggesting the involvement of genes of minor effect (Law et al. 1976). De la Peña et al. (1996), however, observed a 1:1 segregation of recombinant substitution lines for eyespot resistance, suggesting that a single gene controls *Pch2* resistance in Cappelle-Desprez.

De la Peña et al. (1997) used RFLP markers to map *Pch2*. A partial map of the long arm of chromosome 7A was constructed but no RFLP markers showed close linkage to resistance gene *Pch2*. However, two markers *Xcdo347* and *Xwg380* could be used to select for *Pch2* as double recombination occurred only in 3% of the recombinant population. *Pch2* mapped to approximately 11 cM proximal to RFLP marker *Xcdo347* and 18.8 cM distal to *Xwg380* on the distal end of the long arm of chromosome 7A (de la Peña et al. 1997).

Isozyme loci for endopeptidase are located on chromosomes 7AL, 7BL and 7DL (Hart and Langston 1977) and it was shown that *Pch1* co-segregated with the *Ep-D1b* allele (McMillin et al. 1986) with no recombination observed between *Ep-D1* and *Pch1* (Worland et al. 1988). Koebner et al. (1988) refined the isozyme assay for the group 7 endopeptidase-1 (*Ep-1*) homoeoallelic series and this is used by several plant breeders for selection of *Pch1* in breeding material. Recently, a STS marker was developed to an oligopeptidase B gene suggested to encode *Ep-D1* (Leonard et al. 2007). Leonard and colleagues found this marker to be completely linked to *Ep-D1* and *Pch1*. Although *Pch1* and *Ep-D1* are genetically linked these loci may not be in close physical proximity because the presence of the translocated *Ae. ventricosa* chromosome segment may prevent recombination between *Ep-D1* and *Pch1*. This is supported by the report of Chao and colleagues who found *Pch1* to co-localise with both the *Ep-D1* and *Xpsr121-7D* loci in a VPM1 7D single chromosome recombinant population of Hobbit 'S' Chao et al. (1989). In contrast, the *Ep-D1* and *Xpsr121* homoeo-loci were determined to be 9 and 10 cM apart on chromosomes 7A and 7B respectively, with the latter marker being distal. A later study to map *Pch2* found *Ep-A1* to be 3.9 cM proximal to *Xpsp121* (de la Peña et al. 1996). They also reported that *Pch2* was probably closer to *Xpsr121* than to *Ep-A1*, mapping 27.9 cM proximal to *Xpsr121* on the long arm of 7A. The relative positions of *Pch1* and *Pch2* on the long arm of chromosomes 7D and 7A respectively, strongly suggest that they are homoeo-loci (de la Peña et al. 1996).

A sequence tagged site (STS) marker has been developed that is closely linked to *Pch1* (Groenewald et al. 2003) but a significant amount of recombination between *Pch1* and this marker has been found (Santra et al. 2006). To date no PCR-based marker has been reported for the selection of *Pch2*. We sought to identify SSR markers closely linked to *Pch1* and *Pch2* and to use genomics resources to develop additional PCR-based assays to facilitate selection of these two eyespot resistance genes.

Materials and methods

Plant material

The inter-varietal chromosome 7D substitution line, Hobbit 'sib'-VPM7D (HS/VPM7D) carries *Pch1* (Worland et al. 1988). A BC_5 recombinant population (RVPM7D) of 90 lines, between HS/VPM7D and the eyespot susceptible line Hobbit 'sib' (HS), (Worland et al. 1988) was used to map *Pch1*. Similarly the inter-varietal chromosome substitution line Chinese Spring-Cappelle Desprez 7A (CS/CD7A) carries *Pch2*. CS/CD7A was crossed to the eyespot susceptible variety Chinese spring (CS). F_1 plants were selfed to generate an F_2 population of 192 plants and leaf material was removed for genotyping. F_2 plants were grown on and selfed to produce F_3 families that were phenotyped for eyespot resistance in two trials.

SSR analysis

The parental lines in the two populations were screened with SSRs reported to be located on chromosomes 7A and 7D (Table 1). Six sources of SSR markers were used: John Innes Centre hexaploid wheat SSRs (Bryan et al. 1997), Gatersleben D genome bread wheat microsatellite markers (Pestsova et al. 2000), Gatersleben wheat microsatellites (Röder et al. 1998), wheat microsatellite consortium (http://wheat.pw.usda.gov), US Wheat and Barley Scab Initiative (BARC) primers (http://www.scabusa.org/pdfs/BARC_maps_011106.pdf) and the *XustSSR2001-7DL* marker (Groenewald et al. 2003).

Leaf tissue (50 mg) from 5-week-old plants of the recombinant population RVPM and the F_2 CS:CS/CD7A population was harvested into 96-well plates on dry ice. Samples were stored at −70°C prior to DNA extraction using the DNeasy 96 Plant Kit (Qiagen Ltd.) according to manufacturer's instructions.

Development of SSR marker to7D using wheat EST deletion bins

Wheat EST sequences mapping to deletion bin 7DL3-0.821-1.00, located at the distal end of chromosome 7D were obtained from the *GrainGenes* database (http://wheat.pw.usda.gov). ESTs were analysed for SSR sequences

Table 1 SSR markers used to map *Pch1* and *Pch2* on chromosomes 7D and 7A respectively showing allele sizes for the parental lines used to generate the mapping populations in each case

7D marker	Allele size (bp)		7A marker	Allele size (bp)	
	HS	HS/VPM7D		CS	CS/CD7A
gdm86	154	150	barc108	159	155
wmc221	298	260	barc29	199	191
gdm150	104	–	gwm130	128	126
gdm46	150	140	gwm276	93	130
gdm67	140	134	gwm332	230	211
psp3045	115	–	gwm60	213	217
gwm428	152	–	psp3050	159	125
wmc273	260	175	wmc168	328	312
gwm37	201	240	wmc346	210	204
ustSSR2001-7DL	220	240	wmc83	163	155
wmc14	252	–	wmc525	210	208
barc97	257	–	cfa2019	–	221
W7DESTSSR	350	–	cfa2040	297	317

using Tandom Repeats Finder version 3.21 (Benson 1999). EST BE446395 was the only sequence found to contain SSR elements. A primer set (W7Dest-F: CACTAATCTTC TTGCTCTCTCT and W7Dest-R: CAATCTCTTTGTCAG TATCT) was designed for BE446395 using Primer3 (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and tested for secondary structures, hairpins, primer dimers and annealing temperatures using the Genosys website (http://www.sigma-genosys.co.uk/oligos/frameset.html). W7Dest primers were tested on the parental varieties HS and HS/VPM7D and were mapped using the *Pch1* RVPM7D mapping population. PCR conditions for SSRs were as described by Bryan et al. (1997) with W7DestF/R having an annealing temperature of 50°C. PCR products were separated on 6% polyacrylamide gel and visualised by silver staining (Bassam et al. 1991).

Marker development from *Xpsr121*

The RFLP probe *Xpsr121* encodes a partial sequence for a beta-glucanase. Wheat EST BG604756 is annotated as encoding for beta-glucanase and maps to the distal 7DL wheat deletion bin (7DL3-0.821-1.00), located at the end of chromosome 7D and was used to develop a 7D specific marker. PCR primers to the BG604756 sequence (B-glu7D-F: TGGAGGTTCTGTACCCGTTC and B-glu7D-R: GAAGTCCGGTGGGTTGTCTA) were designed and mapped as above using a PCR programme with 60°C annealing temperature. PCR products were examined by single strand conformational polymorphism (SSCP) assay using Sequa Gel® MD (National Diagnostics, U.K Ltd) and visualised by silver staining (Bassam et al. 1991).

Phenotyping F_3 population CS:CS/CD7A families for *Pch2* resistance

Trial 1 (2005)

Nine plants from each of 162 F_3 families were grown in 5 × 5 cm pots (Plantpak™ Cookson Plantpak Ltd), containing peat and sand compost. Three replicates of each F_3 family were arranged in a randomised complete block design in propagation trays (20 pots/tray). Parental lines, CS and CS/CD7A, were included as references in each tray. Plants were grown in a 10°C growth chamber with 12 h day length and 3 cm long transparent PVC cylinders (5 mm I.D), were placed over emerging shoot tips. After 21 days plants were inoculated by pipetting agar slurry (400 µl) into each cylinder. Isolates of *O. acuformis* (P37, P38, AG98/167, AG98/174, AG98/119, C93/786) from the JIC culture collection were grown on V8 agar (9 g of bacto agar, 50 ml of V8 in 450 ml of de-ionized water) at 15°C for 21 days. Inoculum was prepared by homogenising the agar and associated fungal colonies with water (2:1). Trays were well watered and propagator lids were used to increase humidity to aid fungal infection. Plants were returned to incubate at 10°C for 8 weeks when plants were harvested and disease was scored according to Scott (1971).

Trial 2 (2006/7)

Eyespot resistance was assessed for 183 F_3 families by a seedling bioassay in an unheated unlit glasshouse at RAGT Seeds Ltd., Cambridge, between December 2006 and February 2007. This experiment was conducted in complete randomised blocks with six replicates. In each replicate, six seeds of

each F_3 family and twelve seeds of both parental lines were sown onto peat and sand compost in 35 cm × 20 cm seed trays, with 20 lines per tray. Seeds were inoculated by applying a mixture of oat grains previously colonised by *O. acuformis* using the method of Bruehl and Nelson (1964), and peat and sand compost, through which the seedlings emerged. *O. acuformis* isolates were kindly provided by Bill Hollins from the collection of RAGT Seeds Ltd. Plants were harvested 10 weeks after sowing and scored as above.

Statistical analysis

Regression analysis and analysis of variance (ANOVA) were calculated in Genstat, ninth edition (Copyright 2006, Lawes Agricultural Trust (Rothamsted Experimental Station)) to assess variability attributed to replicate and genotype. The mean disease score for each line was estimated using generalized linear modelling and was used in the subsequent QTL analysis.

Map construction and QTL analysis

Linkage maps were calculated from recombination frequencies (0.4) and a LOD of 3.0 was used in JoinMap® (version 3.0). QTL analysis was carried out using MapQTL® version 4.0 (van Ooijen and Maliepaard 1996). Interval mapping was carried out on the two linkage groups created following the map construction of chromosome 7A using JoinMap® (version 3.0). A permutation test (1,000 cycles) was used to determine the LOD score at which the QTL was deemed to be present in the given genomic region with a confidence interval of 99%.

Results

SSR and PCR marker analysis and map construction for chromosome 7DL

The HS and HS/VPM7D parents of the 7D recombinant (RVPM) population were screened using 46 SSR markers on the long arm of chromosome 7D. Twelve (26%) of the 46 SSRs and the *XustSSR2001-7DL* marker were polymorphic between HS and HS/VPM7D and co-dominant producing allele sizes shown in Table 1.

Single sequence repeats (SSRs) development using wheat EST deletion bins

Wheat ESTs mapping to the distal portion of the long arm of chromosome 7D were used to develop additional SSR markers for mapping of *Pch1*. Only one EST (BE446395) in the distal deletion bin 7DL3-0.82-1.00 was found to contain SSR elements (CT and CA repeats) (data not shown). The W7Dest primer set amplified a product in HS but not in HS/VPM7D, providing a dominant (presence / absence) marker.

Marker development using 7D Beta-glucanase wheat EST

Initial efforts to produce a PCR primer set based upon the RFLP probe *Xpsr121* that encodes a beta-glucanase were not successful. Following screening of nullisomic lines of Chinese Spring, it was determined that this probe derives from wheat Group 1 chromosomes. The wheat EST BG604756 maps to wheat deletion bin 7DL3-0.821-1.00, located at the distal end of chromosome 7D. BG604756 is annotated as encoding for beta-glucanase and this sequence was used to develop a marker specific for beta-glucanase on chromosome 7D. The B-glu7D primer set amplified a product in HS but not in HS/VPM7D, providing a dominant marker.

The RVPM population was screened with 14 SSR markers (12 conventional SSRs, and the *XustSSR2001-7DL* and *XW7Dest* SSR markers targeted to *Pch1*) and the B-glu7D primer set.

A genetic map of chromosome 7D was constructed by combining the data obtained in the present study with the RFLP and phenotype data, including location of *Pch1* from Chao et al. (1989). The RFLP based map of chromosome 7D produced by Chao et al. (1989) was constructed from 68 RVPM lines and an additional two lines had to be excluded from the analysis because of missing data. JoinMap 3.0 resolved three linkage groups and the map was orientated and the position of the centromere was determined on the basis of the Somers et al. (2004) SSR consensus map for chromosome 7D (Fig. 1). Two SSR markers (*Xwmc14* and *Xbarc97*) mapped to the same position as *Pch1* and the markers *Ep-D1* and *Xpsr121* that were shown to co-segregate with *Pch1* previously (Chao et al. 1989). The B-glu7D marker designed to EST BG604756 (beta-glucanase) also co-segregated with *Pch1* (Fig. 1). The full set of RVPM lines were screened with *Xwmc14*, *Xbarc97* and *XB-glu7D* and all three markers were found to co-segregate with *Pch1* (results not shown). The W7Dest SSR marker derived from the SSR-containing EST (BE446395) within deletion bin 7DL3-0.82-1.00 was found to map to the distal end of chromosome 7D but did not co-segregate with *Pch1,* being separated by 6 cM (Fig. 1).

SSR analysis and map construction of chromosome 7A

The two parents CS and CS/CD7A were screened using 16 7A SSR markers. Thirteen (81%) of the 7A SSRs were found to be polymorphic between CS and CS/CD7A (Table 1). The 13 polymorphic SSRs were used screen 192 CS:CS/CD7A F_2 lines and the data used to construct a map of chromosome 7A. The software package JoinMap®

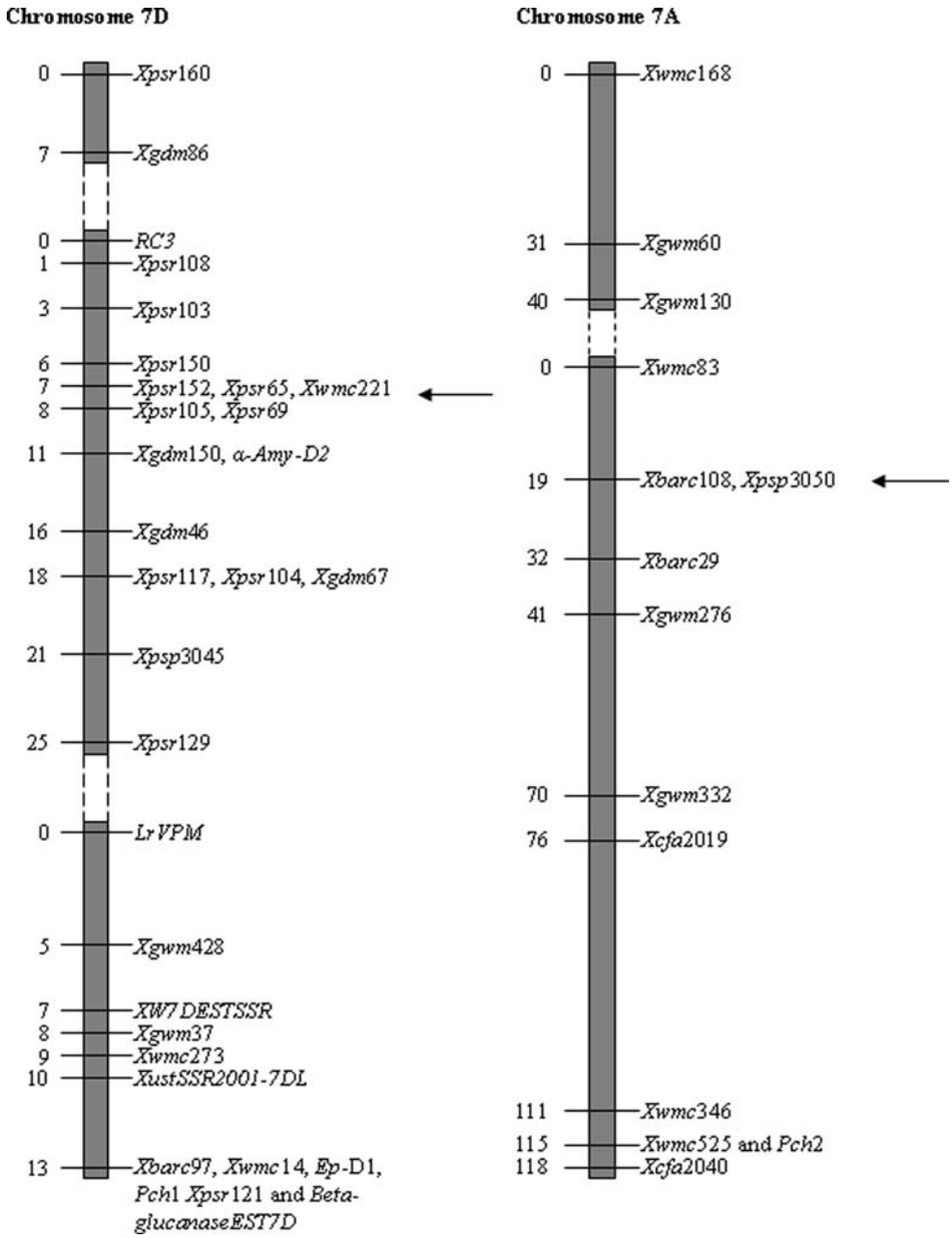

Fig. 1 Genetic map of chromosome 7D in the Hobbit-sib (HS): Hobbit-sib/VPM7D (HS/VPM7D) recombinant population (RVPM lines) and a genetic map of chromosome 7A in Chinese Spring (CS): CS chromosome substitution line Cappelle Desprez (CS/CD7A) recombinant lines. Distances are measured in Kosambi cM units. The *broken vertical line* represents no linkage. The centromere is indicated by the *arrow*

(version 3.0) resolved two linkage groups (Fig. 1) and the linkage groups were orientated according to the Somers et al. (2004) SSR consensus map for chromosome 7A.

Pch2 resistance in the CS:CS/CD7A F_3 families

A total of 162 CS:CS/CD7A F_3 families were phenotyped for *Pch2* resistance to *O. acuformis* in the first trial and 183 F_3 families were phenotyped in the second trial. Analysis of variance demonstrated that genotype was highly significant in both trials but that variance due to replicate was also high in Trial 2 carried out in an unheated glasshouse (Table 2).

QTL interval mapping analysis

QTL interval mapping was also used to determine the genomic location of *Pch2* on chromosome 7A and identify SSR markers associated with the resistance. The QTL analysis of results from the individual trials revealed that *Pch2* was associated with three SSRs; (*Xwmc346*, *Xwmc525* and *Xcfa2040*) that mapped together on the distal end of the long arm of chromosome 7A (Fig. 1). The marker most significantly associated with eyespot disease in all instances was SSR *Xwmc525*. This marker explained 35.1% of the phenotypic variance observed in Trial 1 and 40.1% in Trial 2 (Table 3). The combined data from the two trials also

Table 2 Variance components of visual eyespot disease scores using generalised linear modelling for the two disease trials of the CS × CS/CD7A F_3 families

Source of variation	Trial 1		Trial 2	
	MS	*F* value	MS	*F* value
Replicate	8.32	8.76***	164.55	80.66***
Genotype	28.27	29.78***	16.94	8.3***
Replicate x Genotype	2.35	2.48***	4.16	2.04***
Residual	0.95		2.04	

MS mean squares

*** $P < 0.001$

showed that *Xwmc525* was the marker most significantly associated with resistance (*Pch2*) explaining 36.3% of the phenotypic variance observed (Table 3).

Discussion

Pch1 on chromosome 7D.

We have integrated SSR marker data into the RFLP-based map of 7D from RVPM lines generated by Chao et al. (1989) to provide PCR-based markers for *Pch1*. Two SSR markers, *Xbarc97* and *Xwmc14*, co-segregated with the *Pch1* locus that confers eyespot resistance. These two SSR markers were also found to be completely linked to *Pch1* in a large mapping population (Leonard et al. 2007). However, evidence was presented to suggest that linkage between *Pch1* and these SSRs can be broken. Unfortunately, both SSR markers are dominant, failing to amplify the *Ae. ventricosa* allele, making them suitable only for screening homozygous material in breeding programmes. Furthermore, exclusive use of dominant markers makes it impossible to detect failed reactions (Varshney et al. 2005). This problem can be easily overcome by including a second co-dominant marker in the PCR reaction. Overall, six of the thirteen SSRs used herein were found to be dominant markers (absent in VPM), in the RVPM population. Other studies have also found SSR markers to be less transferable between closely related genera than within a genus (Röder et al. 1995; Peakall et al. 1998). For example, Gupta et al. (2003) found 66% of EST-based SSRs to be polymorphic between 18 alien species and *Triticum aestivum* L. due to null alleles in the alien. We found the *XustSSR2001-7DL* marker to be the closest co-dominant SSR marker to *Pch1* mapping 3 cM from the resistance locus. The SSR *XustSSR2001-7DL* was recently reported as a highly polymorphic marker linked to *Pch1* and *Ep-D1*, with a calculated recombination frequency of 2% (Groenewald et al. 2003). However, use of this marker to predict eyespot resistance (*Pch1*) is reported to be only about 90% efficient and it has been concluded that the *Ep-D1* isozyme marker was more efficient in the MAS selection of *Pch1* than *XustSSR2001-7DL* (Santra et al. 2006). A single EST in the 7DL distal deletion bin (7DL3-0.821-1.00) was found to contain SSR motifs. The SSR marker (*XW7Dest*) designed to this EST mapped to the distal end of chromosome 7D but 6 cM away from *Pch1*, and therefore, like *XustSSR2001-7DL*, cannot be used for reliable MAS selection of *Pch1*, as there is a high probability that recombination would occur between the marker and *Pch1*.

Ep-D1 is widely used for selecting *Pch1* resistance (Koebner and Summers 2002). It was originally suggested that the eyespot resistance conferred by *Pch1* might be a product of *Ep-D1b* (Worland et al. 1988). Later study showed this not to be the case because wheat line H-93-51 was found to carry the *Ep-D1b* allele but is susceptible to eyespot (Mena et al. 1992). The authors concluded that *Ep-D1b* and *Pch1* can be separated and eyespot resistance is not a product of the *Ep-D1* locus (Mena et al. 1992). However, a recent study reported that a STS marker to *Ep-D1* showed complete linkage to *Pch1* in a cross between the

Table 3 Summary of the QTL interval mapping analysis of *Pch2* on chromosome 7AL

Marker	Map distance (cM)	Trial 1		Trial 2		Combined	
		LOD[a] score	% variance explained	LOD score	% variance explained	LOD score	% variance explained
wmc83	0	0.4	1.3	0.7	2.1	0.7	1.8
barc108	19	0.1	0.2	0.1	0.3	0.3	0.6
psp3050	19	0.1	0.2	0.1	0.3	0.3	0.6
barc29	32	0.3	0.8	0.3	0.7	0.3	0.7
gwm276	41	0.4	1.1	0.1	0.3	0.1	0.3
gwm332	70	0.2	0.7	3.1	8.3	1.9	5.0
cfa2019	76	0.8	3.4	4.5	15.1	3.3	10.2
wmc346	111	10.9	26.7	13.2	31.2	13.4	29.2
wmc525	115	15.1	35.1	18.2	40.1	17.6	36.3
cfa2040	119	11.0	27.1	18.1	40.0	14.1	30.7

[a] Logarithm of the odds score

eyespot resistant cultivar Coda and the susceptible cultivar Brundage (Leonard et al. 2007).

The RFLP marker *Xpsr121* co-segregates with *Pch1* in the RVPM population (Chao et al. 1989). This probe includes sequence for a beta-glucanase gene. Initial attempts to design a PCR-based marker to this probe failed to produce a marker to 7D, amplifying instead products from Group 1 chromosomes (results not shown). The beta-glucanase sequence hybridising to *Xpsr121* on 7D appears to be a paralogue to the original probe. Fortunately, the EST BG604756 encodes beta-glucanase and has been mapped to the distal deletion bin on chromosome 7DL. PCR primers designed to the 7D beta-glucanase EST produced a dominant marker, amplifying a product from HS but not HS/VPM7D. The *XB-glu7D* marker co-segregated with the RFLP marker *Xpsr121* and, hence *Pch1*.

On the basis of RFLP data, recombination within chromosome 7D in RVPM lines carrying the introgressed segment from *Ae. ventricosa* was shown to be significantly reduced relative to that in chromosomes 7A and 7B. Chao et al. (1989) reported that the *Ep-B1* and *Xpsr121* loci on 7B were 9.9 cM apart and, similarly, the *Ep-A1* and *Xpsr121* loci on 7A are separated by 3.9 cM (de la Pena et al. 1997). In contrast, these markers were found to be completely linked for the 7D chromosome of RVPM lines (Chao et al. 1989). This finding is confirmed by the SSR markers added in the present study. For example, loci *Xwmc273* and *Xwmc14* were separated by 4 cM on the 7D chromosome carrying *Pch1* from *Ae. ventricosa* whereas these loci are reported to be separated by 10 cM on the native 7D chromosome of wheat (Somers et al. 2004). This difference in recombination frequency may result from the alien 7D chromosome *Ae. ventricosa*. The alien segment may prevent chiasmas from occurring between the 7D chromosome of wheat and the 7D chromosome of *Ae. ventricosa* reducing recombination (de la Peña et al. 1996). It has also been suggested that an inversion of the VPM segment involving the region between *Ep-D1* and *Xpsr121* would also give an appearance of complete linkage (Chao et al. 1989).

Pch2 on chromosome 7A

Eighty one percent of the SSRs were polymorphic between CS and CS/CD7A. This figure is high compared to the level of polymorphism identified for Group 7 chromosomal RFLP probes in diverse wheat recombinant populations, where polymorphism levels averaged 9% (Chao et al. 1989). Even though non-genic SSRs tend to have higher levels of polymorphism compared to cDNA derived RFLP markers (Chalmers et al. 2001) the level of SSR polymorphism is generally 20–40% in single-wheat mapping populations (Somers et al. 2004), which is lower than that observed here between CS and CS/CD7A. The genetic map developed herein agrees well with that of Somers et al. (2004) with the exception of the relationship between *Xcfa2019* and *Xwmc346*. While the distance between these loci was estimated as 6 cM (Somers et al. 2004) we found the distance to be much greater, being approximately 49 cM. The reasons for this disparity are not known.

Despite the difficulties of carrying out screening for resistance to a necrotrophic pathogen using an F_3 population, the phenotype data from the first trial indicated that *Pch2* functions as a single gene, which corresponds with previous studies (Strausbaugh and Murray 1989; de la Pena et al. 1997). However, *Pch2* was analysed as a quantitative trait due to its lack of potency compared to *Pch1* (Hollins et al. 1988) and because of the variability associated with using an F_3 population, particularly in the uncontrolled conditions of the second trial. QTL interval mapping associated *Pch2* resistance with three SSRs, *Xwmc346*, *Xwmc525* and *Xcfa2040*. The most significant association was with SSR *Xwmc525* which accounted for 35.1 and 40.1% of variance explained in the first and second trial, respectively (Table 2). Thus, results from both trials indicated that *Pch2* is located at the distal end of the long arm of chromosome 7A and is closely linked to SSR *Xwmc525*.

Pch2 has previously been mapped to the long arm of chromosome 7A, being located between RFLP markers *Xcdo*347 and *Xwg380* (de la Peña et al. 1997) which are separated by 29.8 cM (de la Peña et al. 1997). The authors concluded that the RFLP markers *Xcdo*347 and *Xwg380* could be used in combination to select for *Pch2* resistance because double recombination between these markers occurred in only 3% of the population. In the current study we have identified three SSRs (*Xwmc346*, *Xwmc525* and *Xcfa2040*) that are closely linked to *Pch2*. Our results indicate that *Pch2* maps close to *Xwmc525* within the 7 cM interval flanked by *Xwmc346* and *Xcfa2040*. These provide the first reported PCR-based markers to this eyespot resistance gene.

Taken together with the results from previous reports, the results from the present study strongly suggest that *Pch1* and *Pch2* are homeoloci. The 7D locus of *Xcfa2040* is close to that for *Xwmc14* which we have shown to co-localise with *Pch1* in the RVPM population, suggesting that this locus is close to *Pch1,* whilst we have also shown that the 7A locus of *Xcfa2040* is close to *Pch2*. Probably as a result of reduced recombination, the endopeptidase marker *Ep-D1b* and the RFLP marker *Xpsr121* are closely linked to *Pch1* and may be used for the selection of *Pch1* resistance (Chao et al. 1989). However, on chromosome 7A, the homeologous endopeptidase marker *Ep-A1b* is only weakly linked to *Pch2* (Koebner and Martin 1990), and cannot be used to follow this resistance in breeding programmes (de la Peña et al. 1996). Both *Xpsr121* and *Ep-A1b* appear

distal to *Pch2* on chromosome 7A (de la Peña et al. 1997), although the former is closer to *Pch2*. Assuming that *Pch1* and *Pch2* are homeologous it would be anticipated that the *XB-glu7D* marker developed in the present study, would be closer to *Pch1* than the endopeptidase marker *Ep-D1b*. In the current study we have developed a series of PCR-based markers to facilitate selection of both the major eyespot resistance genes, *Pch1* and *Pch2,* in wheat breeding programmes.

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Appendix 2

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ORIGINAL PAPER

The identification of candidate genes associated with *Pch2* eyespot resistance in wheat using cDNA-AFLP

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Abstract Eyespot is a fungal disease of the stem base of cereal crops and causes lodging and the premature ripening of grain. Wheat cultivar Cappelle Desprez contains a highly durable eyespot resistance gene, *Pch2* on the long arm of chromosome 7A. A cDNA-amplified fragment length polymorphism (AFLP) platform was used to identify genes differentially expressed between the eyespot susceptible variety Chinese Spring (CS) and the CS chromosome substitution line Cappelle Desprez 7A (CS/CD7A) which contains *Pch2*. Induced and constitutive gene expression was examined to compare differences between non-infected and plants infected with *Oculimacula acuformis*. Only 34 of approximately 4,700 cDNA-AFLP fragments were differentially expressed between CS and CS/CD7A. Clones were obtained for 29 fragments, of which four had homology to proteins involved with plant defence responses. Fourteen clones mapped to chromosome 7A and three of these mapped in the region of *Pch2* making them putative candidates for involvement in eyespot resistance. Of particular importance are two fragments; 4CD7A8 and 19CD7A4, which have homology to an *Oryza sativa* putative callose synthase protein and a putative cereal cyst nematode NBS-LRR disease resistance protein (RCCN) respectively. Differential expression associated with *Pch2* was examined by semi-quantitative RT-PCR. Of those genes tested, only four were differentially expressed at 14 days post inoculation. We therefore suggest that a majority of the differences in the cDNA-AFLP profiles are due to allelic polymorphisms between CS and CD alleles rather than differences in expression.

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Introduction

Eyespot is a fungal disease of the stem base of cereal crops. Infection results in lodging and premature ripening of grain leading to reduced crop yield. This disease affects a wide range of hosts, including wheat, barley, rye, oats and wild and cultivated grasses and is considered economically important in temperate areas such as North West Europe, North West USA and New Zealand.

Eyespot is caused by two species of fungus, *Oculimacula acuformis* (formerly *Tapesia acuformis*) and *O. yallundae* (*T. yallundae*) (Crous et al. 2003), which are considered to be necrotrophic pathogens. However, there is evidence of an early asymptomatic stage during coleoptile colonisation and they could thus be considered to be hemi-biotrophic pathogens (Daniels et al. 1991; Blein et al. 2008). Although both species cause similar symptoms, differences have been reported in their host pathogenicity, epidemiology, plant infection strategy and responses to fungicide (Hollins et al. 1985; Lange-de la Camp 1966; Scott and Hollins 1980;

Poupard et al. 1994; Wan et al. 2005; Daniels et al. 1991; Bateman et al. 1990; Bierman et al. 2002).

The relative abundance of the two species on cereal crops has changed over time; during the 1980s, *O. yallundae* was the prevalent strain of eyespot fungus (Parnell et al. 2008). However by the 1990s, *O. acuformis* had surpassed *O. yallundae* to become predominant in northern Europe, and the US Pacific Northwest (Douhan et al. 2002). This shift is thought to be due to different sensitivities to the widely used fungicide, prochloraz (Parnell et al. 2008). Therefore, due to its dominance in field populations, *O. acuformis* was used in the present study to investigate *Pch2* resistance.

The first eyespot resistant wheat variety to be discovered was Cappelle Desprez (Vincent et al. 1952). Even though it has been widely used in Europe (Scott et al. 1989), Cappelle Desprez resistance has proven durable (Johnson 1984; Scott et al. 1989). Most resistance to eyespot in Cappelle Desprez is seemingly conferred by a gene (*Pch2*) on chromosome 7AL (Law et al. 1976; de la Peña et al. 1997). However, there is evidence of eyespot resistance conferred by other chromosomes (Law et al. 1976; Muranty et al. 2002), most notably an adult plant resistance gene on chromosome 5A (Muranty et al. 2002). *Pch2* has been mapped to a 7-cM interval between SSR markers *Xwmc346* and *Xcfa2040* located on the distal end of the long arm of chromosome 7A and is closely associated with SSR *Xwmc525*. Another eyespot resistance gene *Pch1*, derived from *Aegilops ventricosa*, maps to the distal end of the long arm of chromosome 7D and it is thought that *Pch1* and *Pch2* are homeoloci (Chapman et al. 2008).

It is not yet known if *Pch2* is constitutively expressed or induced by *Oculimacula* spp. Various physiological studies into the nature of *Pch2* resistance have proven inconclusive, providing evidence for both a constitutive and induced basis to this resistance. Non-inoculated mature wheat cultivars containing *Pch2* had thicker hypodermis with more cell layers than equivalent susceptible cultivars suggesting that anatomy may play an important role in resistance (Murray and Bruehl 1983). This same study concluded that such a resistance mechanism may be less likely to be overcome by new pathogen strains which would in part explain the durability of *Pch2* resistance (Murray and Bruehl 1983). By contrast a second study showed that papillae formation in leaf sheaths, produced in response to eyespot infection, was higher in cultivars containing *Pch2* than in susceptible cultivars suggesting resistance is induced (Murray and Ye 1986).We know of no studies to date which have examined the expression of the eyespot resistance gene *Pch2* at a molecular level.

The cDNA-amplified fragment length polymorphism (AFLP) technique has been previously used to identify and isolate differentially expressed genes associated with plant–pathogen interactions, e.g. the response of barley (*Hordeum vulgare*) to infection with powdery mildew (*Blumeria graminis* f. sp. *hordei*) (Eckey et al. 2004). It has also been used to identify markers that co-segregate with the powdery mildew resistance gene *Mlg* in barley and to generate markers closely linked to the tan spot (*Pyrenophora tritici-repentis*) resistance gene *Tsn1* in wheat (Korell et al. 2007; Haen et al. 2004).

In this study we sought to identify candidate genes for *Pch2*. We used a cDNA-AFLP approach to identify genes which are differentially expressed between the eyespot susceptible cultivar Chinese Spring (CS) and the (*Pch2*) resistant chromosome substitution line CS/Cappelle Desprez 7A (CS/CD7A). Differences in resistance responses between susceptible and resistant wheat cultivars have previously been observed from 8 to 18 days after inoculation in physiological studies (Murray and Ye 1986). Studies of *O. acuformis* development during infection of wheat coleoptile tissue have shown that surface proliferation of mycelium occurred 5 days post inoculation (dpi) and the formation of infection plaques 7 dpi (Daniels et al. 1991). Therefore RNA samples were taken at 7 and 14 days after inoculation, in order to maximise the chances of observing infection-associated differences in gene expression. Both constitutive differences in expression and differences induced by infection with *O. acuformis* were assessed. PCR primers were designed to clones from differentially expressed fragments and used to amplify DNA from CS, CS/CD7A and CS group 7 nullisomic–tetrasomic lines. PCR products were analysed by single strand conformational polymorphism (SSCP) assay to identify those originating from chromosome 7A. Furthermore, the physical map positions of these fragments were determined using chromosome 7A deletion bin stocks (Endo and Gill 1996) and, where possible, the genetic map positions were determined using a CS × CS/CD7A F_2 population. In addition, RT-PCR was used to determine whether the differential intensity of cDNA-AFLP fragments was due to differential expression or allelic polymorphism between CS and CD on chromosome 7A.

Materials and methods

Plant material and inoculation

Chinese Spring, a susceptible spring wheat and CS substitution line Cappelle Desprez 7A (CS/CD7A) containing the *Pch2* resistance were used throughout. The substitution line was developed by A.J. Worland and is maintained at JIC. Isolates of *O. acuformis* (P37, P38, AG98/167, AG98/174, AG98/119, C93/786) from the JIC culture collection were grown on V8 agar (9 g of bacto agar, 50 ml of V8 in 450 ml of de-ionised water) in a 15°C growth cabinet (16-h day

length). Three week-old associated colonies and agar were homogenised (2:1) with water. Plants were grown in 60 cell trays (Plantpak™ Cookson Plantpak Ltd) of peat and sand mix in a 10°C growth cabinet (12 h day length) for 3 weeks. Transparent PVC cylinders (5 mm I.D), 3 cm were placed over emerging shoot tips. Inoculum slurry (400 µl) was pipetted into each cylinder. Equivalent numbers of plants were inoculated with agar alone acting as a control. Water was added to each tray and propagator lids were used to increase humidity to aid fungal infection. Samples consisting of 3 cm of stem base from 20 plants were taken at 7 and 14 dpi.

RNA isolation and cDNA synthesis

The stem base sections were washed clear of agar, snap frozen in liquid nitrogen and ground to a fine powder. Total RNA was extracted using Tri-Reagent™ Sigma (UK), DNase treated using DNA-*free*™ kit (Ambion Ltd, UK) and cDNA synthesised using Superscript III (Invitrogen) all according to manufacturer's instructions.

cDNA/amplified fragment length polymorphism analysis

Samples taken 14 days post infection, consisting of two independent biological replicates per line and treatment (inoculated and non-inoculated) were used to detect differentially expressed sequences as described by Bachem et al. (1996). The cDNA was digested with *Pst*I and *Mse*I restriction enzymes. Adapters *Pst*I (5′-CTCGTAGACTGCGTACATGCA-3′ and bottom strand 5′-TGTACGCAGTCTAC-3′) and *Mse*I (5′-GACGATGAGTCCTGAG-3′ and bottom strand 5′-TACTCAGGACTCAT-3′) were ligated to restricted fragments. PCR was carried out as described by Vos et al. (1995). Selective PCR products were run on SequaGelXR Extended Range acrylamide gel (National Diagnostics, UK Ltd), buffered in 1 × TBE. Gels were run for 2 h at 60 W. Samples were then visualised by silver staining (Bassam et al. 1991).

Fragment isolation and sequencing

Fragments were excised from gels into 100 µl TE buffer, diluted a 100-fold in water and used as template in the re-amplification PCR, using the same primers as in the selective PCR step. PCR products were purified using QIAquick® Spin Kit (Qiagen), cloned using P-Gem Easy Kit-Plasmid vectors (Promega), and transformed into electro-competent *E. coli* (strain DH10β). Clones were sequenced using Big dye version 3.1 system (Applied Biosystems) and run on an ABI 3700 capillary sequencer by the John Innes Centre Genome Laboratory (JGL).

Sequences were analysed using the Wisconsin software package version 10.1 [Genetics Computer Group (GCG), Madison, WI, USA]. Sequences were subjected to blastN and blastX similarity search analysis against the National Centre for Biotechnology Information (NCBI) database and characterised according to their homology with known nucleotide and protein sequences.

Primer development

PCR primers were designed from the sequences of cloned fragments for cDNA amplification. For amplification of genomic DNA, primers were designed either directly to fragment sequences, or by comparison to wheat ESTs homologous to the fragment sequences, with reference to the annotated rice genome to identify regions flanking introns (Supplementary Table 1). Primers were tested for secondary structures, hairpins, primer dimers and annealing temperatures (http://www.sigma-genosys.com/calc/DNACalc.asp). Primers were synthesised by Sigma-Genosys Ltd, UK.

Analysis of differentially expressed transcripts using reverse transcriptase PCR

RNA samples from both time points consisting of two independent biological replicates per line and treatment were subjected to semi-quantitative RT-PCR using 18S ribosomal RNA as a reference. PCR was carried out using a touchdown programme consisting of; a denaturing step of 94°C for two min, five cycles of 94°C for 20 s, 64°C for 20 s, 72°C for 30 s, five cycles of 94°C for 20 s, 62°C for 20 s and 72°C for 30 s and 30 cycles of 94°C for 20 s, 60°C for 20 s, 72°C for 30 s, a final extension step of 5 min at 72°C. PCR products were quantified, using Quantity One® version 4.5 software for Windows and Macintosh (Bio-Rad). Correlation and means of concentration data from two 18S replicate reactions were calculated in Microsoft® Excel 2002 (Microsoft Corp., USA). The data for each fragment were logarithmically transformed prior to analysis due to non-independence of mean and variance. Regression analysis and analysis of variance were performed in Genstat ninth edition [Copyright 2006, Lawes Agricultural Trust (Rothamsted Experimental Station)], and *t*-probabilities were calculated to compare expression levels of each fragment between CS and CS/CD7A, and inoculated and un-inoculated samples, at 7 and 14 dpi.

Chromosomal origin of differentially expressed fragments

To determine whether cDNA-AFLP derived clones are located on chromosome 7A, and to detect any polymorphisms between CS and CS/CD7A, the genomic DNA primers (Supplementary Table 1) were used in a PCR with

DNA of parental lines CS and CS/CD7A, and CS nullisomic–tetrasomic lines 7A, 7B and 7D. PCR products were analysed by SSCP using Sequa Gel® MD (National Diagnostics, UK Ltd) and visualised by silver staining.

Mapping of markers developed by cDNA-AFLP

To determine the physical map positions of the cDNA-AFLP clones originating from chromosome 7A, DNA from 21 homozygous deletion bin lines for chromosome 7A of CS (Endo and Gill 1996) were used in a PCR with the genomic DNA primers (Supplementary Table 1). A selection of the chromosome 7A SSR markers used by Chapman et al. (2008) were included in the deletion bin analysis to provide a comparison between genetic and physical maps along the full length of the chromosome.

Where clear polymorphisms were detected between CS and CS/CD7A, markers were genetically mapped using an F2 population of 192 lines derived from CS × CS/CD7A. These results were combined with SSR marker data for the population previously generated by Chapman et al. (2008). The combined 7A genetic linkage map was calculated for the 7A SSRs and the cDNA-AFLP derived markers from recombination frequencies (0.4) and a LOD of 3.0 in JoinMap®3.0 (van Ooijen and Voorips 2001). Combined phenotypic data from two seedling trials for eyespot resistance in F_3 families from the CS × CS/CD7A population previously generated by (Chapman et al. 2008) were used in a QTL interval mapping analysis using Map-QTL® 4.0 (van Ooijen and Maliepaard 1996).

Results

cDNA-AFLP analysis of differentially expressed genes

Approximately 4,700 fragments ranging from 50 to 1,000 base pairs (bp) were generated from 168 *Mse*I and *Pst*I AFLP primer combinations of cDNA samples from CS and CS/CD7A seedlings 14 days after inoculation with agar colonised by *Oculimacula acuformis* (induced samples) or with agar alone (constitutive samples). Only 34 fragments were differentially expressed between CS and CS/CD7A. Sixteen fragments were expressed in both CS/CD7A inoculated (induced samples) and non-inoculated samples (constitutive samples) but not in CS and two fragments were expressed only in CS/CD7A inoculated samples. Four fragments were expressed only in CS/CD7A non-inoculated samples, six fragments were expressed in both inoculated and non-inoculated CS samples but not in CS/CD7A and six fragments were expressed in only CS inoculated samples.

Twenty-nine of the 34 differentially expressed fragments were successfully cloned. Among these, 15 originated from CS/CD7A inoculated and non-inoculated samples, four from CS/CD7A non-inoculated samples two from CS/CD7A inoculated samples, four from CS inoculated and non-inoculated samples and four from CS inoculated samples (Supplementary Table 2). Attempts to clone the remaining five fragments were unsuccessful.

Fragment characterisation

Isolated fragments may not consist of a single product and, for this reason, at least eight clones were sequenced for each fragment. The sequences of each clone were compared and the predominant sequence present was assumed to be that responsible for the observed cDNA-AFLP product. A total of 49 different sequences were obtained from clones of the 29 fragments and these are summarised in Table 1. A single sequence predominated in clones derived from 15 of the 29 cDNA-AFLP fragments. However, two commonly occurring sequences were present among clones of ten fragments and three sequences were present among clones of each of three fragments. Five sequences were present among clones of one fragment (28CD/M47P14). The sequences were compared with those in the NCBI database and broadly characterised according to their homology with known nucleotide and protein sequences (Table 1). The majority of sequences showed homology to proteins of known function: plant metabolism, cellular communication and signal transduction, plant development, stress response, disease resistance, transport, cell division, protein synthesis and retro-elements. Six sequences showed homology to unknown proteins and three had no matches in the database.

The wheat cDNA-AFLP derived sequences were used to develop PCR primer sets for amplifying cDNA for RT-PCR. In addition, sequences were also compared to orthologues from rice using a blastN search to infer the position of introns (NCBI, http://ncbi.nlm.nih.gov/entrez). PCR primers were designed, where possible, to flank putative introns to take advantage of the relatively higher levels of polymorphism in these regions, so enhancing the potential to produce assays to facilitate mapping.

Expression of sequences derived from cDNA-AFLP fragments at 7 and 14 dpi

RT-PCR was used to examine expression of the cDNA-AFLP derived sequences in inoculated and non-inoculated CS and CS/CD7A samples. Assays to 12 sequences (2CD7A1, 2CD7A20, 4CD7A8, 6CD7A12, 6CD7A18, 10CD7A7, 14CD7A19, 16CD7A9, 16CD7A18, 18CD7A12, 19CD7A4 and 20CD7A12) were successfully developed to amplify from cDNA template and RT-PCR was used to examine expression at an earlier time point

Table 1 Summary of differentially expressed fragments generated by cDNA-AFLP between Chinese Spring (CS) and Chinese Spring/Cappelle Desprez 7A (CS/CD7A) and homology of differentially expressed fragments with nucleotide or protein sequences in the National Centre for Biotechnology Information (NCBI) database

AFLP primer/ sequence name[a]	Length (bp)	Isolated from	Homology with blastN or blastX	Blast *E*-value
Metabolism				
M48P19/2CD7A1	400	CS/CD7A Constitutive	XM_482526.1 (*Oryza sativa*) putative dihydrolipoamide acetyltransferase (N)	2e-88
M48P19/2CD7A20	400	CS/CD7A constitutive	XM_482597.1 (*O. sativa*) putative diphosphate-fructose-6-phosphate (N)	6e-48
M55P15/16CD7A9	340	CS/CD7A constitutive	NP_910779.1 (*O. sativa*) putative NADH dehydrogenase (X)	8e-07
M56P19/17CD7A13	380	CS/CD7A constitutive	XM_482597.1 (*O. sativa*) putative diphosphate-fructose-6-phosphate 1-phosphotransferase (N)	4e-69
M49P17/18CD7A12	300	CS/CD7A constitutive	BAD67843.1 (*O. sativa*) putative prolyl aminopeptidase (X)	2e-30
M52P14/20CD7A8	220	CS/CD7A constitutive	XM_464982.1 (*O. sativa*) lipase class 3-like (N)	3e-52
M54P19 Upper band/25CD7A14	350	CS/CD7A Constitutive	AAA68209.1 (*Z. mays*) sus1 gene product (X)	1e-49
M53P19/30CS8	250	CS induced	XP_467559.1 (*O. sativa*) putative ribulose-1, 5 bisphosphate carboxylase/oxygenase small subunit N-methyltransferase (X)	2e-05
M55915/33CD7A8	250	CS/CD7A constitutive	NP_196706 (*A. thaliana)* oxygen-evolving complex-related (X)	2e-13
M55P14/33CD7A18	250	CS/CD7A constitutive	NP_196706 (*A. thaliana*) oxygen-evolving complex-related (X)	3e-08
M55P20/37CS17	300	CS constitutive	CAA84022 (*H. vulgare*) beta-ketoacyl-ACP synthase (X)	8e-44
M48P17/40CS7	330	CS constitutive	AAM92706.1 (*T. aestivum*) putative cytochrome c oxidase subunit 6b (X)	5e-64
Cellular communication and signal transduction				
M55P15/16CD7A18	340	CS/CD7A constitutive	XM_479680.1 (*O. sativa*) phosphatidylinositol 3,5-kinase like (N)	2e-42
M52P14/21CD7A14	130	CS/CD7A induced	AP003832.3 (*O. sativa*) putative bZIP family transcription factor (N)	0.005
M53P24/23CD7A8	250	CS/CD7A constitutive	XM_464102.1 (*O. sativa*) GHMP kinase like protein (X)	4e-42
M47P14/28CD7A10	230	CS/CD7A constitutive	XP_482875.1 (*O. sativa*) F-box protein family like (X)	0.13
M56P14/31CD7A6	380	CS/CD7A constitutive	XP_464580.1 (*O. sativa*) ZIGA2 protein-like (X)	4e-17
M55P20/36CS1	375	CS induced	AAP53900.1 (*O. sativa*) putative DNA binding protein (X)	2e-42
M55P20/36CS22	375	CS induced	AAR82959.1 (*O. sativa*) transducin/WD-40 repeat protein (X)	3e-63
M55P20/37CS3	300	CS constitutive	BAD54671.1 (*O. sativa*) putative C2H2 zinc-finger protein SERRATE (X)	7e-40
Development				
M49P26/4CD7A8	290	CS/CD7A constitutive	NP_001058646 (*O. sativa*) callose synthase 1 catalytic subunit (X)	1e-80
M52P26/22CD7A19	130	CS/CD7A constitutive	XP_480766.1 (*O. sativa*)putative proteasome 26S non-ATPase subunit1 (X)	3e-19

Table 1 continued

AFLP primer/ sequence name[a]	Length (bp)	Isolated from	Homology with blastN or blastX	Blast *E*-value
M54P19 Upper band/25CD7A2	350	CS/CD7A constitutive	AF542974.1 (*T. aestivum*) Emrl (N)	2e-50
M35P26 Upper band/38CS1	280	CS constitutive	NP_186875.2 (*A. thaliana*) auxin transport protein (BIG) (X)	0.38
Stress related				
M47P14/28CD7A3	230	CS/CD7A constitutive	DQ334407 (*T. aestivum*) drought-responsive factor-like transcription factor DRFL1a (N)	9e-24
M55P14/33CD7A2	250	CS/CD7A constitutive	XM_470271 (*O. sativa*) putative glutathione reductase (N)	4e-08
Defence				
M31P13/10CD7A7	380	CS/CD7A constitutive	AY581258.1 (*Zea mays*) Rpl-D213 rust resistance protein (N)	2e-33
M52P14/19CD7A4	320	CS/CD7A induced	AF320848 (*Triticum aestivum*) NBS-LRR disease resistance protein RCCN3 (N)	4e-49
M52P14/20CD7A12	220	CS/CD7A induced	AAP54661.1 (*O. sativa*) putative plant disease resistance polyprotein (X)	3e-10
Transport				
M48P21/14CD7A4	400	CS/CD7A constitutive	AJ011921.1 (*Hordeum vulgare*) amino acid selective channel protein (N)	e-175
Cell division				
M56P14/32CS20	290	CS induced	NP_914242.1 (*O. sativa*) putative glucose inhibited division protein A (X)	4e-32
Protein synthesis				
M55P15/16CD7A3B	340	CS/CD7A constitutive	AJ277799.1 (*H. vulgare*) putative elongation factor 1 beta (N)	5e-92
M56P19/17CD7A12	380	CS/CD7A constitutive	AJ277799.1 (*H. vulgare*) putative elongation factor 1 beta (N)	e-119
M47P14/24CD7A20	350	CS/CD7A constitutive	NM_125526 (*A. thaliana*) rRNA processing protein-related (N)	2e-23
M47P14/28CD7A8	230	CS/CD7A constitutive	AY049041.1 (*T. aestivum*) 28S ribosomal segment (N)	2e-07
M56P14/31CD7A10	380	CS/CD7A constitutive	NP_974263.1 (*A. thaliana*) putative mRNA capping enzyme (X)	8e-27
M55P20/36CS8	375	CS induced	BAD28853.1 (*O. sativa*) putative ribosomal protein L10a (X)	5e-27
Retro-elements				
M52P16/6CD7A18	340	CS/CD7A constitutive	AE017081.1 (*O. sativa*) putative retro-element (N)	0.063
M52P14/21CD7A8	130	CS/CD7A induced	AAP51781 (*O. sativa*) putative maize transposon MuDR-like (X)	2e-06
M34P16/27CS17	215	CS constitutive	AAP51893.1 (*O. sativa*) putative Tam3-like transposon protein (X)	1e-43
Unknown proteins				
M52P16/6CD7A12	340	CS/CD7A constitutive	AAP44759.1 (*O. sativa*) unknown protein (X)	2e-39
M48P21/14CD7A19	400	CS/CD7A constitutive	XM_475185.1 (*O. sativa*) unknown protein (N)	5e-15
M47P14/28CD7A4/	230	CS/CD7A constitutive	AAO72604.1 (*O. sativa*) unknown protein (X)	2e-04
M47P14/28CD7A21	230	CS/CD7A constitutive	BAB09745.1 (*A. thaliana*) unnamed protein product (X)	1e-09
M31P13/29CS22	210	CS induced	CK207443.1 (*T. aestivum*) unknown function (N)	2e-55
M56P14/32CS18/	290	CS induced	XM_482549.1 (*O. sativa*) unknown protein (N)	4e-18
No matches				
M32P19/8CD7A8	280	CS/CD7A constitutive	No hits	–
M45P17/12CD7A4	400	CS/CD7A constitutive	No hits	–
M54P19 Lower band/26CD7A16	350	CS/CD7A constitutive	No hits	–

[a] First number of the fragment name corresponds to the extracted cDNA-AFLP fragment, e.g. 2CD7A1 and 2CD7A20 represent two sequences from the same fragment

N blastN, *X* blastX

(7 dpi). At 7 dpi, the formation of *O. acuformis* infection plaques occurs in infected wheat coleoptile tissue (Daniels et al. 1991). The later time point of 14 dpi was also examined to confirm whether the genes relating to these sequences were differentially expressed in the manner indicated by the cDNA-AFLP profiles at 14 dpi (Table 1). In susceptible and moderately resistant cultivars penetration of the first leaf sheath occurs within an approximate 2-week period after infection (Murray and Ye 1986). Expression levels were normalised by reference to 18S (ribosomal RNA) for each sample. Four sequences, 2CD7A20, 6CD7A18, 19CD7A4 and 20CD7A12 (Fig. 1b, h, i, j) were constitutively expressed at 14 dpi only in CS/CD7A. This pattern of expression mirrored that observed by cDNA-AFLP for three of the fragments. However, one (19CD7A4) had appeared to be induced by infection in cDNA-AFLP profiles. Interestingly, two of these sequences (19CD7A4, 20CD7A12) are homologous to disease resistance proteins and no corresponding transcript was obtained from the susceptible cultivar CS at either time point. Furthermore, expression of the disease resistance like sequence (19CD7A4) increased significantly in inoculated CS/CD7A samples ($P < 0.001$), as did the retro-element (6CD7A18) ($P < 0.001$).

The pattern of expression of the other eight sequences did not reflect that indicated by the cDNA-AFLP profiles. Expression of four sequences (18CD7A12, 4CD7A8, 10CD7A7 and 6CD7A12) increased significantly in CS/CD7A ($P < 0.05$) in response to infection by *O. acuformis* at both time points, while no significant increase in expression was observed for the susceptible cultivar CS indicating that they may be involved in the resistance response (Fig. 1d, f, g, k). Expression of one sequence (16CD7A18) was significantly increased in both genotypes ($P < 0.001$) in response to infection at 14 dpi (Fig. 1e) indicating that it may be involved in a general response to infection. For the three remaining sequences, two (2CD7A1, 14CD7A19) showed no difference in expression between genotypes and did not appear to respond to infection by *O. acuformis* (Fig. 1a, l). The remaining sequence (16CD7A9) appeared to be expressed to a greater extent in CS than in CS/CD7A, particularly at 7 dpi ($P < 0.001$) but, again, did not respond to infection.

Marker development

Initially primer sets were designed directly to the clone sequences in order to amplify genomic DNA, with the aim of developing suitable markers for each cDNA-AFLP fragment. Clone sequences were aligned with rice orthologues and PCR primers were designed to flank introns to increase the probability of finding polymorphisms between CS and CS/CD7A. However, amplification was only successful for 10 of the 29 clones using this method. For the remaining 19 clones, sequences were compared to wheat ESTs (GenBank, http://ncbi.nlm.nih.gov/entrez) using a blastN search. ESTs homologous to each clone were aligned using ClustalW (Chenna et al. 2003), and intron positions were identified by comparing these aligned EST sequences to genomic sequence from rice (GenBank, http://ncbi.nlm.nih.gov/entrez) and *Brachypodium* (http://www.modelcrop.org) through a further blastN search. PCR primers were again designed to produce amplicons that span introns with the aim of developing polymorphic markers on chromosome 7A. Of the 14 clones for which two or more sequences were available, the sequence with the most suitable intron sizes (100-300 bp) and intron spanning regions appropriate for primer design were selected for marker development. Primer sequence and the design method are detailed in Supplementary Table 1.

PCR assays to each of the 29 clones (Supplementary Table 1) were used to amplify from DNA of CS, CS/CD7A and CS nullisomic–tetrasomic lines 7A, 7B and 7D. Amplicons were subjected to gel-based SSCP analysis to determine whether they derived from chromosomes 7A, 7B and/or 7D and to detect any polymorphisms between CS and CS/CD7A. Sequence 6CD7A18 originates from chromosome 7A (Fig. 2). A polymorphism between lanes 1 and 2 (CS and CS/CD7A) allowed mapping of the marker. The loss of fragment from CS/CD7A in lanes 3, 4 and 5 determined the location of 6CD7A18 to group 7 chromosomes and the absence of the CS band in CS nullisomic–tetrasomic 7A suggests that the sequence originates from chromosome 7A. SSCP analysis indicated that 16 sequences originated from group 7 chromosomes (Table 2). Fourteen of these could be shown to derive from genes on 7A of which eight specifically amplified from 7A. Two sequences were also present on 7B, one was also present on 7D and three were also present on both 7B and 7D. Furthermore, one sequence originated from 7B, and one was from 7D. No bands relating to any of the sequences from the remaining 13 clones were absent on any of the CS group 7 nullisomic–tetrasomic lines, indicating that they probably derive from genes on other chromosomes. Although no bands were absent from 19CD7A4, this marker was determined to be 7A specific, as a clear polymorphism was observed between CS and CS/CD7A (data not shown).

Physical and genetic mapping

The physical map positions of the sequences associated with chromosome markers were determined using a set of CS7A deletion bin lines (Endo and Gill 1996). These results are shown in Table 2 and in Fig. 3. DNA markers were named according to the cDNA-AFLP fragment from which they derived (markers are shown with a 'X' prefix

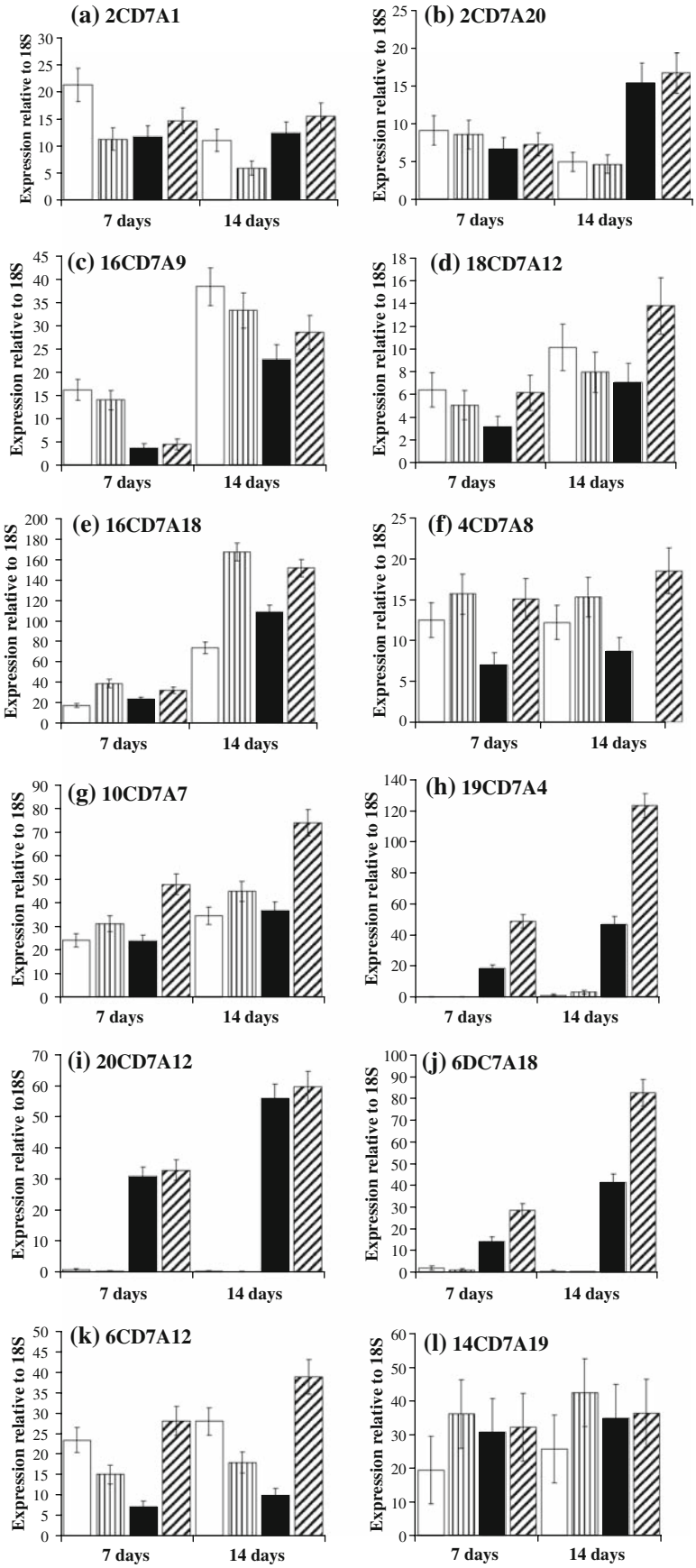

Fig. 1 Expression of cDNA-AFLP fragments using RT-PCR in Chinese Spring (*CS*) non-inoculated (*white boxes*) CS inoculated (*vertical lines*), CS chromosome substitution Cappelle Desprez 7A (CS/CD7A) non-inoculated (*black boxes*) and CS/CD7A inoculated (*diagonal lines*). Samples were taken at time points 7 and 14 days post inoculation (*dpi*). Expression was normalised according to 18S. **a** sequence 2CD7A1, **b** sequence 2CD7A20, **c** sequence 16CD7A9, **d** sequence 18CD7A12, **e** sequence 16CD7A18, **f** sequence 4CD7A8, **g** sequence 10CD7A7, **h** sequence 19CD7A4, **i** sequence 20CD7A12, **j** sequence 6CD7A18, **k** sequence 6CD7A12, **l** sequence 14CD7A19

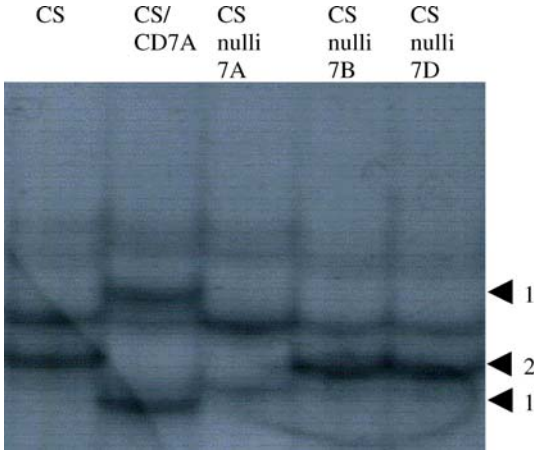

Fig. 2 An SSCP gel of a polymorphism screen between Chinese Spring (*CS*), CS chromosome substitution Cappelle Desprez 7A line (CS/CD7A) and CS nullisomic–tetrasomic lines 7A, 7B and 7D with sequence 6CD7A18. Sequence 6CD7A18 is derived from chromosome 7A, as a band is absent from CS nullisomic–tetrasomic line 7A. *1* Band present in CS/CD7A but absent from CS, *2* band present in CS, CS nullisomic–tetrasomic lines 7B and 7D but absent in CS/CD7A and CS nullisomic–tetrasomic line 7A

Table 2 Chromosome location and physical map position of markers designed to cDNA-AFLP fragments

cDNA-AFLP fragment	Primer template	Chromosome origin	Chromosome 7A deletion bin location
2CD7A20	Fragment	7A	0.83 7AS12-0.73 7AS2
4CD7A8	wESTs	7A and 7B	0.99 7AL15
6CD7A18	Fragment	7A	Centromere
8CD7A8	wESTs	7A, 7B	Centromere
10CD7A7	Fragment	7A	Centromere
16CD7A18	Fragment	7A	0.4 7AL11–0.31 7AL14
17CD7A13	Fragment	7A	0.83 7AS12–0.73 7AS2
18CD7A12	wESTs	7A and 7D	0.83 7AS12–0.73 7AS2
19CD7A4	Fragment	7A	Not mapped
20CD7A12	Fragment	7A	Centromere
22CD7A19	wESTs	7B	n/a
25CD7A14	wESTs	7A	0.89 7AS1
28CD7A4	wESTs	7A, 7B and 7D	0.49 7AL10–0.4 7AL11
30CS8	wESTs	7D	n/a
32CS18	Fragment	7A, 7B and 7D	0.74 7AL21–0.63 7AL5
33CD7A8	wESTs	7A, 7B and 7D	0.99 7AL15

and in *italic* font in accordance with convention). cDNA-AFLP derived markers were found to be distributed evenly over chromosome 7A. Significantly, two of these markers, *X4CD7A8* and *X33CD7A8*, were located in the distal deletion bin of chromosome 7AL (0.997AL15) in the region of *Pch2*.

Polymorphisms were detected between CS and CS/CD7A for the cDNA-AFLP markers *X6CD7A18*, *X20CD7A12* and *X19CD7A4*. These markers were genetically mapped using an F_2 population derived from CS × CS/CD7A and were included in a QTL interval mapping analysis (Fig. 3) alongside the published SSR map (Chapman et al. 2008), to identify markers associated with *Pch2* resistance. *X6CD7A8* and *X20CD7A12* both mapped to a centromeric region and accounted for little of the phenotypic variance in eyespot resistance, but *X19CD7A4* mapped distal to *Pch2* at the end of chromosome 7AL (Fig. 3).

Discussion

cDNA-AFLP is an open system allowing simultaneous comparison of both constitutive and induced differences in expression of host genes providing insight into the biological processes involved in plant–pathogen interactions. Wheat lines CS and CS/CD7A that differ only for the 7A chromosome that harbours *Pch2* were used to increase the likelihood that differentially expressed sequences might be associated with this gene. A remarkably small proportion (0.7%), of the 4,700 fragments observed was differentially expressed between CS and CS/CD7A.

Most of the 14 sequences that could be assigned to chromosome 7A had homology to genes involved in metabolism, cellular communication or development, four had homology to proteins involved in defence responses and one had weak homology to a putative retro-element. Previous studies of differential gene expression following pathogen infection have identified genes with homology similar to those found herein. For example, Eckey et al. (2004), used cDNA-AFLP to examine gene expression in barley (*H. vulgare* L.) after inoculation with powdery mildew (*Blumeria graminis* f. sp. *hordei*, *Bgh*), and found that the majority of fragments showed homology with proteins involved in secondary and primary metabolism. Genes encoding proteins involved in signal transduction, metabolism, protein synthesis, stress and plant defence, were also isolated using the suppression subtractive hybridisation technique from resistant potato cultivars that had been inoculated with *Phytophthora infestans* (potato late-blight) (Birch et al. 1999).

Using SSCP analysis, sequences from 16 of the cDNA-AFLP fragments were shown to be located on group 7 chromosomes of which 14 mapped to chromosome 7A. Primers designed to six 7A sequences also amplified homoeologues on chromosomes 7B and/or 7D. This may be expected, as EST-based markers often amplify homoeologous genes in wheat (Parida et al. 2006; Ishikawa et al. 2007; Xue et al. 2008). Differences between CS and CS/CD7A detected by cDNA-AFLP might be expected to originate only from chromosome 7A. However, the sequences 22CD7A17 and

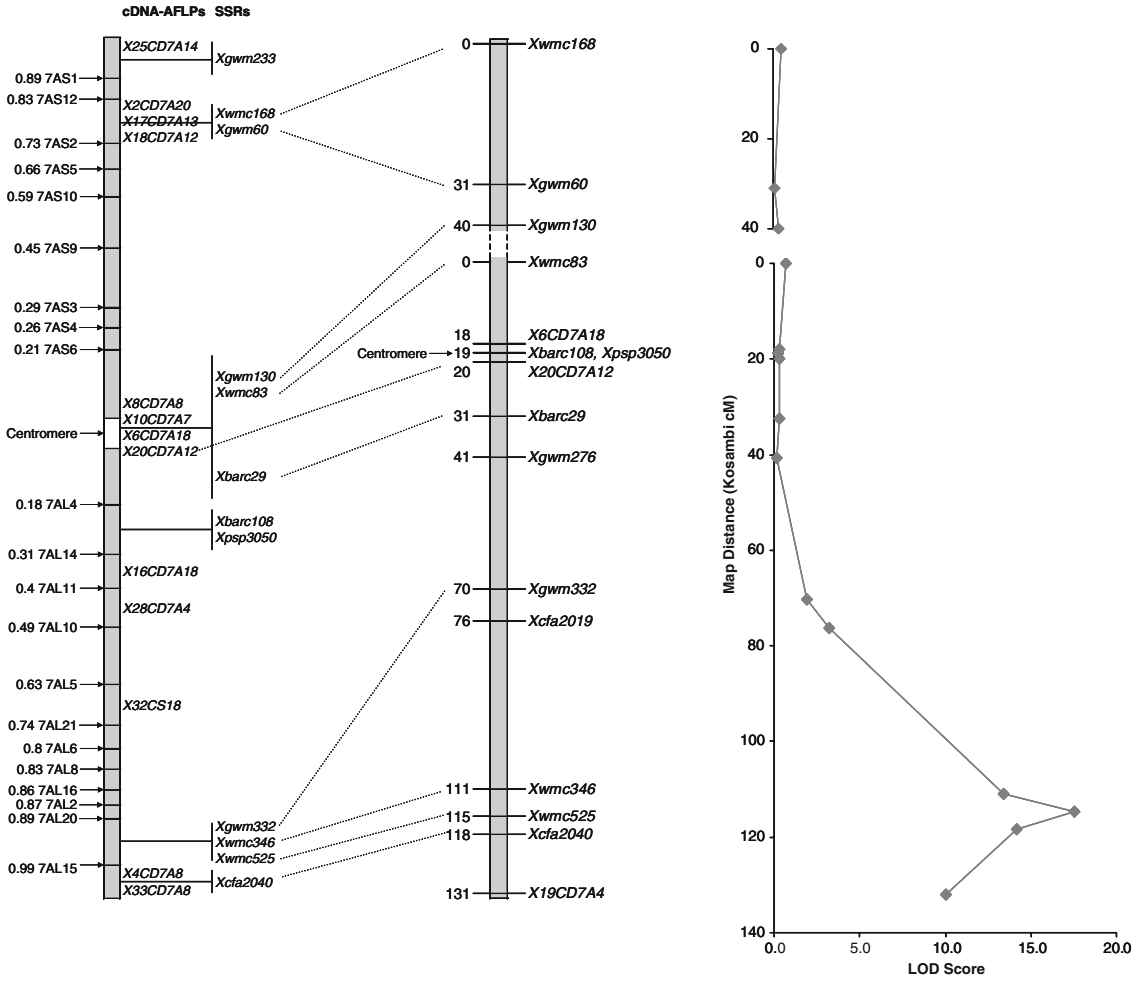

Fig. 3 A comparison of integrated SSR and cDNA-AFLP physical and genetic maps of chromosome 7A. A LOD profile from the QTL interval mapping analysis of *Pch2* on chromosome 7A is also shown aligned to the genetic map to demonstrate the position of *Pch2*

30CS18 appeared to originate from chromosome 7B and 7D respectively. This is probably a result of the SSCP assay for these sequences being inadvertently designed to homoeologues from these chromosomes, rather than 7A, based upon the sequences available in the NCBI database. For fragments that appear to derive from non-group 7 chromosomes the observed differences in expression may indicate that their expression is influenced by factors on 7A or that they are expressed differentially as a consequence of the differential resistance of the two lines. It should also be borne in mind that amplicons from other chromosomes may be cloned as contaminants from the isolated cDNA-AFLP fragment.

RT-PCR of sequences derived from cDNA-AFLP fragments that were differentially expressed between CS and CS/CD7A showed that only 25% of sequences (2CD7A20, 20CD7A12 and 6CD7A18) mirrored the pattern from cDNA-AFLP at 14 dpi. The majority of sequences were not differentially expressed as determined by RT-PCR and may have been identified as a result of allelic differences between the genes located on chromosome 7A of CS and CD producing a novel cDNA-AFLP product. RT-PCR analysis of hexaploid wheat is complicated by the co-amplification of homoeologous and paralogous transcripts (Poole et al. 2007). This may in part explain the reduced number of genes found to be differentially expressed between CS and CS/CD7A using RT-PCR when compared to cDNA-AFLP. Interestingly, RT-PCR indicated that three (2CD7A1, 14CD7A19 and 16CD7A9) of the four sequences that did not appear to derive from 7A showed no difference in expression between samples at 14 dpi. Thus they probably represent clones originating from contaminating amplicons within the cDNA-AFLP fragment.

Previous work has mapped *Pch2* on the long arm of 7A close to the SSR marker *Xwmc525* and within the 7cM interval flanked by SSR markers *Xwmc346* and *Xcfa2040* (Chapman et al. 2008). The present study showed that *Xwmc525* is located in the 7AL deletion bin (0.897AL20-0.997AL15), whilst *Xcfa2040* is located in the immediately distal, and terminal, deletion bin (0.99 7AL15). Therefore it appears likely that *Pch2* is positioned around the 0.99 bin

breakpoint, possibly in the region within the terminal bin. Physical mapping using CS 7A deletion bin lines positioned two of the cDNA-AFLP derived markers (*X4CD7A8* and *X33CD7A8*) in the distal deletion bin of chromosome 7AL, in approximately the same region as *Pch2*. Therefore, the genes relating to these sequences can be considered as candidates 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; Jacobs et al. 2003) as well as during cell wall development (Hong et al. 2001). Callose deposition (papillae) acts as a physical barrier preventing pathogens from penetrating the host cell (Holub and Cooper 2004). Furthermore, papillae formation and lignification of cell walls in epidermal and pith cells of wheat stems infected with *Oculimacula* spp. has also been associated with resistance (Murray and Ye 1986). Callose synthase may be associated with papillae formation preventing *Oculimacula* spp. from penetrating and infecting CS/CD7A. RT-PCR revealed that expression of 4CD7A8 increased significantly in CS/CD7A, in response to infection by *O. acuformis* at both 7 and 14 dpi suggesting an involvement of this gene in the resistance response of CS/CD7A. In contrast, although we could detect expression of this gene in CS it was not significantly enhanced in response to infection at either time point. The identification of the fragment from the original cDNA-AFLP analysis suggests that a polymorphism exists between CS and CS/CD7A in the gene relating to the fragment 4CD7A8. Unfortunately, our assay for 4CD7A8 did not detect any polymorphism between CS and CS/CD7A. However, this assay was based only on the available sequence of the callose synthase gene that aligned to the wheat EST sequence data in the NCBI database. When additional sequence data becomes available for this gene it will be possible to determine whether polymorphisms exist between CS and CD.

The second marker that mapped in the region of *Pch2* (*X33CD7A8*) was notable in that a second cDNA-AFLP product (33CD7A18) also appeared to derive from the same gene, showing highest homology with the 23 kDa subunit of the oxygen evolving system of photosystem II (NP_196706 of *Arabidopsis thaliana*). While it is conceivable that this gene is involved in resistance to eyespot, it is, perhaps, more probable that the differential amplification of this cDNA-AFLP fragment reflects allelic diversity between CS and CD at this locus.

The three sequences (10CD7A7, 19CD7A4 and 20CD7A12), that demonstrated homology to proteins associated with plant disease resistance were shown to be located to chromosome 7A and might therefore be involved in defence responses following recognition of *O. acuformis* by *Pch2* elsewhere on the chromosome. Sequence 10CD7A7 shows homology with a *Z. mays RP1*-D213 rust resistance protein that confers race-specific resistance to maize common rust (*Puccinia sorghi*) (Smith et al. 2004). *RP1*-D genes belong to the NBS-LRR class of R genes (Collins et al. 1999) that have been shown to recognise a number of fungal and bacterial pathogens (Ayliffe and Lagudah 2004). Sequence 19CD7A4 showed homology with a putative cereal cyst nematode NBS-LRR disease resistance protein (RCCN), while 20CD7A12 showed homology to a putative plant disease polyprotein.

Sequences 19CD7A4 and 20CD7A12 were constitutively expressed only in CS/CD7A, whilst 10CD7A7 was constitutively expressed in both CS and CS/CD7A as shown by RT-PCR (Fig. 1g, h). Expression of 20CD7A12 showed no response to infection by *O. acuformis* while expression of both 10CD7A7 and 19CD7A4 increased significantly in CS/CD7A in response to *O. acuformis*. A few resistance genes have been shown to be constitutively expressed at low levels but, in response to infection, to be up-regulated. For example expression of a sunflower coiled coil domain NBS-LRR resistance gene (Radwan et al. 2005) and the tobacco mosaic virus resistance gene *N* (Levy et al. 2004) have both been shown to be up-regulated in response to infection. Genetic mapping revealed that, whereas 19CD7A4 was located in the region of *Pch2*, both 10CD7A7 and 20CD7A12 are located at the centromere and, therefore, are not candidates for the eyespot resistance gene itself. A possible explanation for the increase in expression of 10CD7A7 following infection is that a subset of genes, including 10CD7A7, involved in resistance may be influenced by some form of feed-back mechanism from products derived from the *Pch2* resistance. This explanation is supported by recent transcriptome analyses of major stripe rust resistance genes in wheat. Wheat Gene Chip analyses of the *Yr5* (Coram et al. 2008a) and *Yr39* (Coram et al. 2008b) resistances identified numerous induced *R* gene homologues that may control increased signalling and expression of defence-related products. Several of these transcripts, however, did not originate from the major resistance gene locus (Coram et al. 2008c). It is possible that a similar mechanism may occur in *Pch2* conferred eyespot resistance, leading to the expression of sequences such as 10CD7A7 that are not associated with the *Pch2* locus.

X19CD7A4 was genetically mapped 16 cM distal of the SSR marker *Xwmc525* and is therefore relatively close to the proposed location of *Pch2*. Moreover, the function, location and expression profile of 19CD7A4 suggests that it may contribute towards *Pch2* resistance potentially by pathogen recognition. This will 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.

In addition to providing candidates for the *Pch2* gene(s), cDNA-AFLP also provided insight into the response to infection by *O. acuformis* of wheat lines differing in resistance to eyespot. For example, expression of sequence 16CD7A18 increased in both CS and CS/CD7A in response to infection. 16CD7A18 showed homology with phosphatidylinositol 3, 5-kinase like proteins that are thought to be involved in cell-signalling pathways (Contento et al. 2004). Increased synthesis and hydrolysis of polyphosphoinositide, a derivative of phosphatidylinositol, is observed in a number of different plants in response to drought, salinity, temperature stress and pathogen attack (van Leeuwen et al. 2004). Enhanced expression in CS and CS/CD7A indicates that expression of phosphatidylinositol 3, 5-kinase like proteins may be associated with infection in general rather than specifically with resistance.

Sequence 6CD7A18, that showed weak homology with a retrotransposon, was detected only in CS/CD7A and expression was significantly enhanced upon infection by *O. acuformis* (Fig. 1j). Retrotransposons are generally quiescent during normal plant development but become active in response to stress. For example treatment of wheat lines with the fungal mycotoxin deoxynivalenol leads to accumulation of transcripts in resistant and susceptible lines (Ansari et al. 2007). Cellular stress responses result in rapid re-amplification of retrotransposons (Alix and Heslop-Harrison 2004) and activation of retrotransposons may lead to altered expression of adjacent genes in wheat (Kashkush et al. 2003).

To summarise, cDNA-AFLP proved a successful approach in identifying gene-based polymorphisms between, and differences in the response of, the eyespot resistant wheat line CS/CD7A carrying *Pch2* and the susceptible wheat line CS. Of the 29 fragments that were successfully cloned and sequenced, sequences from 14 were shown to be derived from chromosome 7A and several of these were shown to be differentially expressed between the *Pch2* carrying CS/CD7A line and the susceptible CS line. Two particularly interesting sequences, 4CD7A8 and 19CD7A4, were identified among the fragments and, although it is not possible to demonstrate conclusively from the present study that these sequences are responsible for *Pch2* mediated eyespot resistance, their functions, expression patterns and genetic locations suggest that they are candidates worthwhile of further investigation.

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Appendix 3

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 Pathology 59:819-828

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Differential seedling resistance to the eyespot pathogens, *Oculimacula yallundae* and *Oculimacula acuformis*, conferred by *Pch2* in wheat and among accessions of *Triticum monococcum*

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Eyespot is an economically important stem-base disease of wheat caused by two fungal species: *Oculimacula yallundae* and *Oculimacula acuformis*. This study investigated the efficacy of two sources of resistance, viz. the genes *Pch1*, introgressed into hexaploid wheat from *Aegilops ventricosa*, and *Pch2*, identified in wheat cv. Cappelle Desprez, against *O. yallundae* and *O. acuformis* separately. In a series of seedling bioassays *Pch1* was found to be highly effective against both species. Although *Pch2* was found to confer resistance against both pathogen species, it was significantly less effective against penetration from *O. yallundae* than *O. acuformis*. Furthermore, a quantitative trait locus (QTL) analysis was not able to locate any resistance to *O. yallundae* on chromosome 7A of Cappelle Desprez. This has important implications for the use of *Pch2* in commercial cultivars as it is necessary to have genes that confer resistance to both pathogens for effective eyespot control. In addition, a set of 22 *T. monococcum* accessions was screened for resistance to both *O. yallundae* and *O. acuformis* to identify potentially novel resistances and to assess the accessions for evidence of differential resistance to the eyespot species. Significant differences in resistance to the two pathogens were identified in four of these lines, providing evidence for differential resistance in *T. monococcum*. This study demonstrates that future screening for novel sources of eyespot resistance should investigate both pathogen species.

Keywords: eyespot of wheat, *Oculimacula acuformis*, *Oculimacula yallundae*, quantitative trait loci analysis, *Triticum aestivum*, *Triticum monococcum*

Introduction

Eyespot is a fungal disease of the stem base of cereal crops including wheat, barley and rye, and is caused by two species of fungi, *Oculimacula acuformis* (formerly *Tapesia acuformis*) and *O. yallundae* (*T. yallundae*) (Crous *et al.*, 2003). Severe infection results in lodging and premature ripening of grain, leading to reduced crop yield, and is considered economically important in temperate areas such as North West Europe, North West USA and New Zealand (Fitt, 1992).

Although *O. yallundae* and *O. acuformis* cause broadly similar symptoms (Ray *et al.*, 2006), a number of differences have been reported between the pathogens. The species can be separated on the basis of their colony morphology and host pathogenicity. *Oculimacula yallundae* forms fast-growing, even-edged colonies on potato dextrose agar (PDA) and is more aggressive on wheat than on rye, whilst *O. acuformis* forms slow-growing uneven-edged colonies on PDA and is equally pathogenic on wheat and rye (Lange-de la Camp, 1966; Scott *et al.*, 1975; Hollins *et al.*, 1985). Further differences can be observed between the two species in their infection strategy on wheat seedlings. After spore germination, *O. acuformis* follows a disorientated growth pattern, producing short hyphae terminating in appressoria at random positions on the host surface. From these appressoria *O. acuformis* penetrates directly into coleoptile epidermal cells with hyphae then crossing cell walls into adjacent cell walls. In contrast, *Oculimacula yallundae* follows an orientated hyphal growth pattern to form appressoria at intervals along the anticlinal cell wall grooves. *O. yallundae* then grows within these to the next cell layer where a network of branching hyphae subsequently colonizes anticlinal and periclinal cell walls (Daniels *et al.*, 1991). Furthermore, evidence suggests that *O. yallundae* may be hemibiotrophic, as it exhibits an asymptomatic biotrophic phase during coleoptile

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colonization before switching to a necrotrophic phase once the pathogen reaches the first leaf sheath (Blein *et al.*, 2009).

Oculimacula yallundae and *O. acuformis* also demonstrate different levels of sensitivity to certain fungicides and this is thought to have caused shifts in the relative abundance of the two species in field populations. *Oculimacula yallundae* was the most common causal organism of eyespot in northern Europe until the early 1980s, when *O. acuformis* became increasingly prevalent, possibly because of differential sensitivity to carbendazim-generating (MBC) fungicides (King & Griffin, 1985; Bateman *et al.*, 1990). The predominance of *O. acuformis* was maintained in the 1990s, probably as a result of widespread use of the demethylation inhibitor fungicide prochloraz, which was more effective against *O. yallundae* and consequently favourably selected for *O. acuformis* (Bateman *et al.*, 1990; Bierman *et al.*, 2002). More recently, there has been evidence that the prevalence of *O. acuformis* over *O. yallundae* has declined. This has been attributed to a reduction in the use of prochloraz and an increase in the use of the anilinopyrimidine fungicide cyprodinil, which was shown to select for *O. yallundae* (Parnell *et al.*, 2008). These population shifts illustrate the necessity of controlling both species of the pathogen, as control of only one species will act to increase the proportion of the other in field populations and allow host infection to continue (Bateman & Jenkyn, 2001).

Two main sources of genetic resistance to eyespot, *Pch1* and *Pch2,* have been incorporated into cultivated wheat genotypes. The more potent of these, *Pch1*, was introduced into wheat from the wild grass *Aegilops ventricosa* (Maia, 1967) and is located on the distal end of chromosome 7D (Doussinault *et al.*, 1983; Worland *et al.*, 1988), where it is linked to simple sequence repeat (SSR) markers *Xwmc14* and *Xbarc97* (Chapman *et al.*, 2008). However, its use in commercial cultivars has been limited as resistance is believed to be associated with a yield penalty in the absence of the disease (Johnson, 1992). The second resistance originates from the French cultivar Cappelle Desprez (Vincent *et al.*, 1952). This moderate resistance was attributed to the gene *Pch2* on chromosome 7A (Law *et al.*, 1976; de la Peña *et al.*, 1996). *Pch2* resistance to *O. acuformis* was mapped to the distal portion of chromosome 7AL (de la Peña *et al.*, 1996). More recently, the SSR marker *Xwmc525* was found to be closely associated with the resistance gene (Chapman *et al.*, 2008).

The paucity of genetic resistances available to plant breeders in hexaploid wheat and the limitations of the existing sources of resistance has led to a renewed interest in novel sources in wheat progenitors and relatives. These include *Triticum monococcum* (Cadle *et al.*, 1997), *T. tauschii* (Yildirim *et al.*, 1995), *T. durum* (Figliuolo *et al.*, 1998), *A. kotschyi* (Thiele *et al.*, 2002) and *Dasypyrum villosum* (Murray *et al.*, 1994). Although the majority of the work on novel resistances has focused solely on *O. yallundae*, there is evidence that some of these resistance genes may confer different levels of resistance to *O. yallundae* and *O. acuformis*. A single dominant resistance gene, named *Pch3*, was identified in *D. villosum* and mapped to the distal portion of the long arm of chromosome 4V (Yildirim *et al.*, 1995). Interestingly, this gene was shown to confer resistance to *O. yallundae*, but not to *O. acuformis* (Uslu *et al.*, 1998), suggesting that the genetic basis of resistance to the two eyespot pathogens can differ.

In order to provide effective eyespot control it is important that any genetic sources of resistance are effective against both forms of the pathogen. Therefore, a greater understanding of the effectiveness of *Pch1* and *Pch2* resistance genes towards both *O. yallundae* and *O. acuformis* is required. This study aimed to determine the level of efficacy of *Pch1* and *Pch2* to *O. yallundae* and *O. acuformis* separately, through a series of seedling bioassays on wheat cultivars and intergenotypic single chromosome substitution lines, and a quantitative trait locus (QTL) analysis of *Pch2* resistance. Furthermore, it is necessary to fully understand the efficacy of any novel resistances before they can be considered as useful candidates for introgression into hexaploid wheat. To this end, potentially novel resistances in accessions of *T. monococcum* were investigated for evidence of differential resistance to the eyespot pathogens.

Materials and methods

Plant and fungal material

The intergenotypic single chromosome substitution lines Hobbit 'sib'-VPM7D (HS/VPM7D) containing the resistance gene *Pch1* (Worland *et al.*, 1988) and Chinese Spring-Cappelle Desprez 7A (CS/CD7A) containing *Pch2* (Law *et al.*, 1976) were obtained from the John Innes Centre (JIC) wheat collection. Hobbit 'sib' (HS), which does not contain *Pch1* but is believed to carry *Pch2* (Worland *et al.*, 1988), and Chinese Spring (CS) containing no eyespot resistance genes, were used as controls.

A total of 186 F_3 families developed from the CS × CS/CD7A population, as previously described by Chapman *et al.* (2008), were phenotyped for resistance to *O. yallundae* in controlled-environment-room (CER) and glasshouse seedling bioassays. This data was used in a QTL analysis to examine the chromosomal location of resistance to *O. yallundae* from CD7A and to compare this with the previously identified location of *Pch2* resistance to *O. acuformis*.

Wheat cultivars known to have different susceptibilities to eyespot were obtained from the JIC collection. Andante (Nicholson *et al.*, 1997), Lynx (Nicholson *et al.*, 2002) and Rendezvous (Hollins *et al.*, 1988) are highly resistant; Cappelle Desprez (Law *et al.*, 1976), Riband (Nicholson *et al.*, 1997) and HS (Worland *et al.*, 1988) are moderately resistant; whilst Holdfast (Scott & Hollins, 1980) and Talon (Nicholson *et al.*, 1997) are susceptible. However, little is known about the relative levels of resistance in these lines to *O. yallundae* and

O. acuformis. HS/VPM7D was included as a highly resistant control and CS was included as a susceptible control.

To identify novel eyespot resistances and to assess for differential responses to *O. yallundae* and *O. acuformis*, 22 accessions of *Triticum monococcum* were obtained from Professor John Snape, JIC and Professor Giles Waines, University of California, Riverside, and were screened for resistance to both pathogen species in seedling bioassays. In these seedling bioassays Cappelle Desprez (moderately resistant) and Chinese Spring (susceptible) were included as wheat controls.

All *O. yallundae* and *O. acuformis* isolates used in the JIC trials were from the JIC culture collection. For each species, a homogenized mixture of six different isolates, as detailed by Chapman *et al.* (2008), was used for inoculations. A mixture of different isolates was used to ensure that a successful infection was achieved in case of lack of virulence of one or more of the isolates. In the trials conducted at RAGT Seeds, single vigorous isolates for each species were selected from the company's isolate collection and used for inoculations.

SSR genotyping analysis

The wheat lines Andante, Cappelle Desprez, CS, CS/CD7A, Holdfast, HS, HS/VPM7D, Lynx, Rendezvous, Riband and Talon were genotyped with the *Pch1*-linked SSR markers *Xwmc14* and *Xbarc97* and the *Pch2*-linked SSR markers *Xwmc346*, *Xwmc525* and *Xcfa2040*, as identified by Chapman *et al.* (2008), to determine the presence or absence of the known eyespot-resistance genes *Pch1* and *Pch2*.

DNA was extracted from each wheat line using the CTAB method (Nicholson *et al.*, 1996), quantified using a PicoDrop spectrophotometer and diluted to 6 ng μL^{-1} in sterile distilled water for use in PCRs. A 6·25-μL reaction volume consisted of 2·5 μL DNA, 3·125 μL Taq mastermix (Qiagen) and 0·625 μL of the relevant primer pair (2 μM). The forward primer for each marker was labelled with 6-FAM, NED, PET or VIC fluorescent dye (Applied Biosystems). PCR conditions were as described by Bryan *et al.* (1997). Samples were prepared by adding 1 μL of a 1:40 dilution of the PCR product to 10 μL formamide and 0·2 μL LIZ 500 size standard (Applied Biosystems). Samples were run on an ABI 3700 capillary sequencer (Applied Biosystems) and the output data were analysed using PEAK SCANNER v1.0 (Applied Biosystems) to determine the product size of the amplicons. Lines were deduced to carry *Pch1* if they contained the VPM7D allele at both *Xwmc14* and *Xbarc97*, and were deduced to carry *Pch2* if they contained the Cappelle Desprez allele at *Xwmc346*, *Xwmc525* and *Xcfa2040*.

Seedling bioassays

In all CER seedling bioassays, plants were grown, inoculated and incubated as described by Chapman *et al.* (2008). Plants were harvested 6–8 weeks after inoculation and scored for disease on a scale reflecting the number of wheat leaf sheaths infected or penetrated: 0 = seedling uninfected, 1 = coleoptile infected; 2 = coleoptile penetrated; 3 = first leaf sheath infected; 4 = first leaf sheath penetrated; 5 = second leaf sheath infected; and 6 = second leaf sheath penetrated, etc. (Scott, 1971). In all glasshouse trials, plants were grown over winter in unheated, unlit glasshouses. In the JIC glasshouse trials plants were inoculated and scored as above. In the RAGT Seeds glasshouse trials plants were inoculated using the method of Bruehl & Nelson (1964), harvested 10 weeks after sowing and scored as above.

Seedling bioassays of intergenotypic single chromosome substitution lines

In the CER experiment, seeds of each line were grown in peat and sand compost in 10 pots (7 × 7 cm square) with five seeds per pot. Pots were arranged as five complete randomized blocks, each block containing two pots of each line. In each block, one pot of each line was inoculated with *O. yallundae* and the other with *O. acuformis*. This experiment was subsequently repeated using identical methods to confirm the findings.

A total of three glasshouse trials were conducted; two at the JIC, Norwich and one at RAGT Seeds Ltd, Cambridge. In each of the JIC glasshouse trials, seeds of each line were grown in John Innes No.2 compost in eight 2-L pots, seven seeds per pot. Pots were arranged in a complete randomized block design consisting of four blocks, each containing two pots of each line. Again, in each block, one pot of each line was inoculated with *O. yallundae* and the other with *O. acuformis*. In the glasshouse trial at RAGT Seeds, a complete randomized split-plot design was conducted with six blocks. In each block, 12 seeds of each line were sown onto peat and sand compost in 35- × 20-cm seed trays, with six seeds in one tray and six in another. One tray in each block was inoculated with *O. acuformis* and the other with *O. yallundae*.

Phenotyping F_3 population CS × CS/CD7A families for resistance to *O. yallundae*

A total of 186 F_3 families and the parents CS and CS/CD7A were phenotyped for resistance to *O. yallundae* by seedling bioassays in a CER at JIC and in a glasshouse at RAGT Seeds. In the CER experiment nine plants from each of the F_3 families from the population CS × CS/CD7A were grown in 9- × 9-cm square pots containing peat and sand compost. Three replicates of each F_3 family were arranged in a randomized complete block design. Parental lines, CS and CS/CD7A, were included as references in each block. The glasshouse trial at RAGT Seeds was conducted using a complete randomized block design with seven blocks. In each block, six seeds of each F_3 family and 12 seeds of both parental lines were sown onto peat and sand compost in seed trays and inoculated with *O. yallundae*. This trial was conducted alongside a similar phenotyping trial of the same population for resistance to *O. acuformis*, as reported by

Chapman *et al.* (2008), to provide a direct comparison of the genetic basis of resistance to the two species.

Seedling bioassays of wheat cultivars

Seeds of each cultivar were grown in John Innes No. 2 compost in 16 pots (9 × 9 cm square), eight seeds per pot. Pots were arranged in a complete randomized block design with eight blocks and with two pots of each genotype per block. In each block, one pot of each line was inoculated with *O. yallundae* and the other with *O. acuformis*.

Seedling bioassays of *T. monococcum* accessions

In experiment 1, seeds of each *T. monococcum* accession were grown in peat and sand compost in 10 pots (7 × 7 cm square) and seeds of each wheat control cultivar, CS and Cappelle Desprez, were grown in 20 pots, with five seeds per pot. Pots were arranged as 10 randomized blocks, each block containing one pot of each of the 22 *T. monococcum* accessions and two pots of each wheat control cultivar. Five blocks were inoculated with *O. yallundae* and the other five with *O. acuformis*. To confirm resistance and susceptibility a repeat screening (experiment 2) was carried out using 10 selected accessions and the two control lines, using an experimental design and procedure as in experiment 1. Insufficient seed was available to retest all the potentially resistant lines.

Statistical analysis

For all experiments, analysis of variance was performed on visual disease scores to assess the variation attributable to line, pathogen species and line × pathogen species interactions, using a general linear model (GLM) in GENSTAT v.11 (Lawes Agricultural Trust, Rothamsted Research, Harpenden, AL5 2JQ, UK). In all experiments predicted mean disease scores were calculated for each line for *O. yallundae* and *O. acuformis* inoculations using the GLM. In the substitution line seedling bioassays, predicted mean disease scores were calculated using individual GLMs for CER and glasshouse experiments. A GLM incorporating both CER and glasshouse data with experimental replicates as subplots was used to calculate overall means for each line. Similarly, in the *T. monococcum* experiment, predicted mean disease scores were calculated for each line using individual GLMs for experiments 1 and 2 and overall means were calculated through a GLM incorporating data from both experiments.

To compare the level of resistance of each line to *O. acuformis* and *O. yallundae*, mean disease scores were compared using *t*-probabilities calculated within each of the GLMs outlined above. Analysis of variance incorporating a Tukey's test was used to separate mean disease scores for each line for *O. yallundae* and *O. acuformis* inoculations independently.

The mean disease score for each F_3 family was predicted in a GLM for the CER trial and glasshouse trial separately, adjusting for the block effect in each case, and was used in the subsequent QTL analysis.

QTL analysis of CS × CS/CD7A resistance to *O. yallundae* and *O. acuformis*

Predicted mean disease scores for *O. yallundae* inoculations of each F_3 family from the CER and glasshouse trials were used alongside an existing map of chromosome 7A generated from SSR and cDNA-AFLP marker data (Chapman *et al.*, 2008, 2009) for interval mapping of *O. yallundae* resistance in MAP-QTL® version 4.0 (van Ooijen & Maliepaard, 1996). Existing datasets of predicted mean disease scores for *O. acuformis* inoculations of each F_3 family from CER and glasshouse trials, previously published by Chapman *et al.* (2008), were used for multiple QTL mapping (MQM) to finalize the location of the previously identified *Pch2* QTL for *O. acuformis* resistance. A permutation test (1000 cycles) was used to determine the logarithm of the odds (LOD) score at which the QTL was deemed to be present in the given genomic region with a confidence interval of 99%.

Results

SSR genotyping analysis

The SSR marker analysis confirmed the presence of *Pch1* in HS/VPM7D and *Pch2* in CS/CD7A (Table 1). In addition, it demonstrated that HS and HS/VPM7D carry the same haplotype as Cappelle Desprez at the *Pch2*-linked loci *Xwmc346*, *Xwmc525* and *Xcfa2040*, indicating that HS carries the resistance gene *Pch2* and HS/VPM7D has both *Pch1* and *Pch2* resistance genes. CS was confirmed to have a susceptible haplotype possessing neither *Pch1* nor *Pch2*. Haplotypes of the seven wheat cultivars showed that two contain both *Pch1* and *Pch2*, two contain *Pch2* alone, one contains *Pch1* alone, and two have neither of these resistance genes (Table 1).

Eyespot resistance in intergenotypic single chromosome substitution lines

Significant effects of line ($P < 0{\cdot}001$), pathogen species ($P < 0{\cdot}001$) and a significant interaction ($P < 0{\cdot}001$) between pathogen species and line was detected in both CER and glasshouse experiments, providing evidence for differential levels of resistance to the two pathogens amongst the host lines (Table 2). HS/VPM7D was highly resistant to both *O. yallundae* and *O. acuformis* in both the CER and glasshouse (Table 3). The disease scores for HS/VPM7D were significantly lower than for the *Pch2*-carrying HS ($P < 0{\cdot}05$) when inoculated with either pathogen species, and although there was some evidence of increased susceptibility to *O. yallundae* in the glasshouse bioassay ($P = 0{\cdot}03$), there was no overall evidence of differential resistance to the two pathogens ($P = 0{\cdot}117$). Both of the *Pch2*-containing lines, HS and CS/CD7A, had significantly lower disease scores than CS when

Table 1 Allele sizes (bp) for SSR markers linked to *Pch1* (7D) and *Pch2* (7A) and the resistance genes inferred in lines of *Triticum* spp

	Pch1		*Pch2*			
Line	*Xbarc97*	*Xwmc14*	*Xwmc346*	*Xwmc525*	*Xcfa2040*	Inferred resistance gene(s)
CS	254	252	210	210	297	None
CS/CD7A	254	252	204	208	317	*Pch2*
HS	257	252	204	208	317	*Pch2*
HS/VPM7D	Null	Null	204	208	317	*Pch1* and *Pch2*
Lynx	Null	Null	204	208	317	*Pch1* and *Pch2*
Rendezvous	Null	Null	204	208	317	*Pch1* and *Pch2*
Cappelle Desprez	254	252	204	208	317	*Pch2*
Riband	259	252	204	208	317	*Pch2*
Holdfast	259	252	212	210	295	None
Andante	Null	Null	218	202	295	*Pch1*
Talon	259	252	212	210	295	None

Table 2 Summary of variance components for eyespot disease scores using general linear modelling for seedling bioassays of single chromosome substitution lines, wheat cultivars and *Triticum monococcum* accessions

	Single chromosome substitution lines						*Triticum monococcum* accessions			
	CER		Glasshouse		Wheat cultivars		Experiment 1		Experiment 2	
Source of variation	MS	*F*-value	MS	*F*-value	MS	*F*-value	MS	*F*-value	MS	*F*-value
Line	301·4	190·4***	361·6	104·8***	150·0	214·7***	27·8	11·8***	35·2	16·3***
Pathogen	46·8	29·6***	146·8	42·5***	7·7	11·0***	10·6	4·5*	0·1	0·9
Line × Pathogen	23·2	14·7***	21·9	6·4***	7·9	11·4***	3·9	1·7*	3·5	1·6
Residual	1·6		3·5		1·8		2·4		2·1	

MS = mean squares.
* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

Table 3 Predicted mean disease scores from general linear modelling (GLM) for the intergenotypic substitution lines when inoculated with *Oculimacula acuformis* (OA) or *O. yallundae* (OY)

		CER			Glasshouse			Overall		
Line	Resistance gene(s)	OA[a]	OY[a]	*t*-prob[b]	OA[a]	OY[a]	*t*-prob[b]	OA[a]	OY[a]	*t*-prob[b]
HS/VPM7D	*Pch1* and *Pch2*	2·9a	2·7a	0·433	4·4a	5·0a	0·030	3·6a	3·9a	0·117
Hobbit-Sib	*Pch2*	5·3b	5·8b	<0·001	6·5b	7·6b	<0·001	5·8b	6·7b	<0·001
CS/CD7A	*Pch2*	5·2b	6·4b	<0·001	6·4b	7·6b	<0·001	5·8b	7·0b	<0·001
Chinese Spring	None	7·3c	6·9c	0·163	8·3c	8·3c	0·889	7·8c	7·7c	0·3

[a]Different letters within columns represent significant differences ($P < 0{\cdot}05$) between disease scores for each line for OA and OY inoculations separately, using Tukey's test.
[b]Statistical significance of difference between OA and OY disease scores for each line in CER experiments, glasshouse experiments, and across both experiments, shown as *t*-probability calculated within GLM.

inoculated with either pathogen ($P > 0{\cdot}05$). However, both of these lines exhibited significantly less resistance to *O. yallundae* than to *O. acuformis* overall ($P < 0{\cdot}001$); furthermore this differential was highly significant in both CER ($P < 0{\cdot}001$) and glasshouse bioassays ($P < 0{\cdot}001$) (Table 3).

The level of penetration by the two pathogens on the susceptible line CS was very similar in both environments, suggesting that the two species develop at a similar rate in these conditions on a fully susceptible host. Therefore, disease levels of the two pathogens on each line were directly compared (Table 3).

QTL analysis of *Pch2* resistance to *O. yallundae*

A significant QTL on chromosome 7A of Cappelle Desprez for *Pch2* resistance to *O. acuformis* was previously detected using QTL interval mapping (Chapman *et al.*, 2008). The location of this QTL was confirmed to be centred on SSR marker *Xwmc525* using MQM (Fig. 1) and accounted for 35 and 40% of phenotypic variation at this locus from CER and glasshouse experiments, respectively. 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. Consequently,

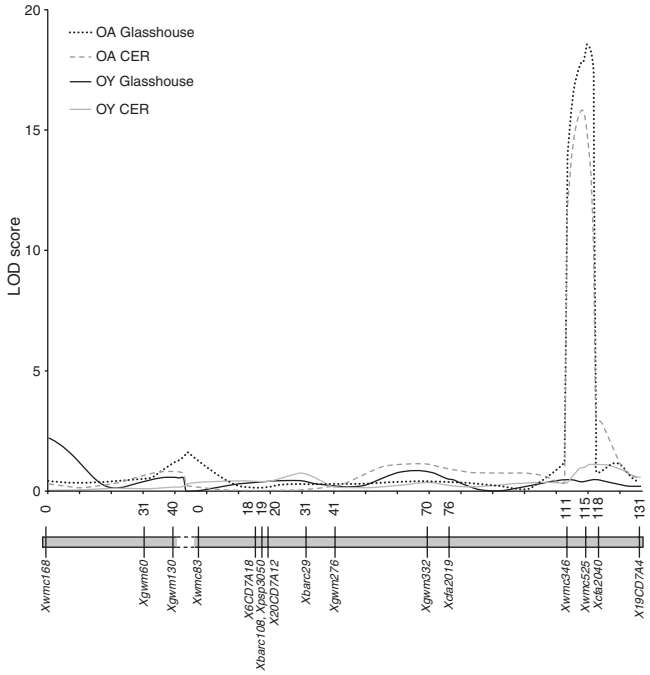

Figure 1 Comparison of QTL mapping analyses of *Pch2* resistance to *Oculimacula acuformis* using multiple QTL mapping (MQM) and to *O. yallundae* using interval mapping, in CER and glasshouse trials of the wheat Chinese Spring × Chinese Spring-Cappelle Desprez 7A F_3 families. Genetic map data and phenotypic data for *Pch2* resistance to *O. acuformis* were adapted from Chapman *et al.* (2009).

it was not possible to conduct an MQM analysis and the results of a QTL interval mapping analysis of *O. yallundae* resistance are presented in Fig. 1.

Eyespot resistance in wheat cultivars

Andante, which contains *Pch1*, provided a high level of resistance to both *O. acuformis* and *O. yallundae*. Cultivars containing both *Pch1* and *Pch2*, i.e. HS/VPM7D, Lynx and Rendezvous, had similarly low disease scores for both pathogen species (Table 4). However, there was evidence for differential resistance to the two pathogens in the experiment as a significant interaction between cultivar and pathogen species ($P < 0{\cdot}001$) was detected (Table 2). Cappelle Desprez and Riband both contain *Pch2*, and although both cultivars demonstrated greater resistance than Chinese Spring to both species, they also demonstrated a significantly lower level of resistance to *O. yallundae* than to *O. acuformis* (Table 4), confirming the findings from the intergenotypic chromosome substitution line experiments and QTL analysis.

Again, there were similar amounts of penetration by the two pathogen species on the susceptible control Chinese Spring, suggesting that it is possible to make direct comparisons between the two pathogen species for each line. The other susceptible lines, Holdfast and Talon, demonstrated high disease scores when inoculated with either pathogen, although a greater level of penetration by *O. yallundae* was detected in Holdfast and a greater level of penetration by *O. acuformis* was detected in Talon.

Eyespot resistance in *Triticum monococcum*

In the initial test of 22 *T. monococcum* accessions, 16 demonstrated resistance to *O. yallundae*, to *O. acuformis*, or to both forms of the pathogen, with disease scores significantly lower ($P < 0{\cdot}05$) than the susceptible control Chinese Spring (Table 5). Three of these 16 resistant lines demonstrated a high level of resistance with disease scores significantly lower than that observed in the moderately resistant Cappelle Desprez ($P < 0{\cdot}05$) when inoculated with either *O. yallundae* or *O. acuformis*. In

Table 4 Predicted mean disease scores from general linear modeling (GLM) for wheat cultivars when inoculated with *Oculimacula acuformis* (OA) or *O. yallundae* (OY)

Wheat cultivar	Resistance gene(s)	OA[a]	OY[a]	*t*-prob[b]
Andante	*Pch1*	3·0a	2·9a	0·473
HS/VPM7D	*Pch1* and *Pch2*	3·1a	3·2a	0·246
Lynx	*Pch1* and *Pch2*	3·4a	3·2a	0·185
Rendezvous	*Pch1* and *Pch2*	3·2a	3·1a	0·568
Cappelle Desprez	*Pch2*	3·9b	4·6b	<0·001
Riband	*Pch2*	4·0b	4·9b	<0·001
Holdfast	None	5·3c	6·0d	<0·001
Talon	None	5·1c	4·7b	0·002
Chinese Spring	None	5·8d	5·5c	0·104

[a]Different letters within columns represent significant differences ($P < 0{\cdot}05$) between mean disease scores for each line for OA and OY inoculations separately, using Tukey's test.
[b]Statistical significance of difference between OA and OY disease scores for each line, shown as *t*-probability calculated within GLM.

Table 5 Predicted mean disease scores from general linear modeling (GLM) for *Triticum monococcum* accessions and control lines when inoculated with *Oculimacula acuformis* (OA) or *O. yallundae* (OY)

	Experiment 1		Experiment 2		Overall		
Genotype	OY[a]	OA[a]	OY[a]	OA[a]	OY[a]	OA[a]	*t*-prob[b]
Chinese Spring	4·7	4·4	4·2	3·9	4·6	4·3	NS
Cappelle Desprez	2·8	2·7	2·9	1·8	2·8	2·4	0·05
G1261	1·2ab	2·7a	0·3a	2·3	0·9ab	2·6a	0·04
G1391	2·0a	3·1					
G2901	3·6	3·2					
G3371	1·4a	2·0a	1·9a	1·3a	1·5a	1·8a	NS
G4325	3	3·4					
G4483	2·4a	2·4a					
G4502	2·3a	2·7a					
G4507	3·2	1·3ab	1·4a	0·8a	2·3a	1·1a	<0·001
G4509	3·8	3·5					
G4511	2·8a	2·5a					
G4519	3·7	3·8	3·2	4	3·4	3·9	NS
G4535	2·6a	2·3a					
G5163	3·5	3·2					
G5191	2·3a	2·5a					
G5212	3·5	2·5a					
G5214	3	2·4a	3·1	3·1	3·0	2·7a	NS
G5215	4·2	3·3	3·8	3·3	4·1	3·3	0·02
G806	1·8a	2·1a					
V97020	2·5a	1·9a	1·2a	1·7	1·8a	1·8a	NS
V97031	2a	2·4a	1·4a	1·6a	1·7a	2·0a	NS
V97052	2·3a	1·0a	0·7a	1·2a	1·5ab	1·1a	NS
V97151	0·5ab	1·3a	1·4a	2·3	1·0ab	1·9a	0·05

[a]Statistically significant differences ($P < 0·05$) between *T. monococcum* accession and control line mean disease scores for OY and OA inoculations separately using Tukey's test: a = significantly different from Chinese Spring, b = significantly different from Cappelle Desprez.

[b]Statistical significance of difference between OA and OY disease scores for each line, shown as *t*-probability calculated within GLM, incorporating data from experiments 1 and 2.

addition, there was some evidence of a significant interaction ($P < 0·05$) between line and pathogen species (Table 2), suggesting differential resistance to the two pathogens amongst the accessions.

Repeat testing was conducted against both pathogens, where seed quantity permitted. This included eight accessions that demonstrated resistance and two accessions that were as susceptible as CS. Generally, the results of experiment 2 confirmed the results of experiment 1, with resistance confirmed in seven of the eight accessions (Table 5). However, it was not possible to detect any significant interaction between line and pathogen species in this repeat experiment (Table 2).

Predicted overall mean disease scores for *O. yallundae* and *O. acuformis* inoculations, calculated across both experiments, were compared to determine whether any line demonstrated differential resistance to the two eyespot species. There was evidence of a higher level of resistance to *O. acuformis* in two lines, G4507 ($P < 0·001$) and G5215 ($P = 0·02$), and also some evidence of a higher level of resistance to *O. yallundae* in two lines, G1261 ($P = 0·04$) and V97151 ($P = 0·05$). Although the extent of the difference between *O. yallundae* and *O. acuformis* resistance in these lines varied between the two experiments, the trend for a higher level of resistance to one species was consistent within each of these lines (Table 5).

In confirmation of other results presented herein, overall disease scores for CS were highly similar for *O. yallundae* and *O. acuformis*, enabling disease scores to be compared directly between accessions. In addition, cv. Cappelle Desprez, containing *Pch2*, again demonstrated a higher level of resistance ($P = 0·04$) to *O. acuformis*.

Discussion

The eyespot resistance gene *Pch2* was found to confer enhanced resistance to penetration by *O. acuformis* relative to Chinese Spring (CS), but to provide a lower level of resistance against penetration by *O. yallundae* (Table 3). This was demonstrated by the chromosome substitution line CS/CD7A, which had significantly greater disease scores when inoculated with *O. yallundae* than when inoculated with *O. acuformis* across a range of environments and experiments.

Although *Pch2* was previously identified as a major QTL for *O. acuformis* resistance located on the distal portion of chromosome 7A (Chapman *et al.*, 2008), it was not possible to detect any QTL for resistance to *O. yallundae* in the same CS × CS/CD7A population under identical conditions in the present study, providing further evidence that *Pch2* confers relatively little resistance to *O. yallundae* at the seedling stage. However, it was possible to detect a low level of resistance to *O. yallundae* in the substitution line CS/CD7A and it might have been expected that the genetic location of this weak resistance would have been identified in the QTL analysis conducted in the present study. However, the high level of environmental variation often associated with large-scale seedling bioassays of eyespot resistance (de la Peña *et al.*, 1996), particularly in an uncontrolled glasshouse environment (Chapman *et al.*, 2008), and additional variation arising from the use of F_3 families, of which approximately 50% will contain heterozygotes at the *Pch2* locus, may have prevented detection of a QTL for *Pch2* resistance to *O. yallundae*. To overcome this limitation, it would be advisable for future studies to use fixed populations with low heterozygosity, such as recombinant inbred lines or double haploids. This would reduce the variation resulting from heterozygotes and would allow additional replication (Burr & Burr, 1991), to assist the detection of any QTL for *Pch2* resistance to *O. yallundae*.

The differential resistance conferred by *Pch2* was confirmed in seedling bioassays of three cultivars that, on the basis of SSR haplotypes, appear to carry *Pch2* without *Pch1*, i.e. Cappelle Desprez, Riband and Hobbit-sib (Table 4). Although these cultivars did demonstrate resistance to *O. yallundae*, they consistently demonstrated a significantly higher level of resistance to *O. acuformis*

than to *O. yallundae*. This lower level of *O. yallundae* resistance could be conferred by *Pch2* itself. However, it is also possible that other as yet uncharacterized genes may be influencing resistance. An additional resistance to eyespot was previously 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 cultivar than in Riband. In the cultivar experiments, the susceptible line CS exhibited very similar susceptibility to the two pathogens, suggesting a similar rate of penetration in the absence of any resistances. However, the susceptible cv. Talon, without *Pch1* or *Pch2*, demonstrated significantly lower disease scores when inoculated with *O. yallundae* than when inoculated with *O. acuformis*. In addition, Holdfast demonstrated weak resistance to *O. acuformis*, suggesting that these cultivars may carry pathogen species-specific resistances, albeit of minor effect.

Although this study was unable to identify a genetic location for *Pch2* resistance to *O. yallundae*, *Pch2* was previously located to a 28·9-cM interval between the RFLP markers *Xcdo347* and *Xwg380* using a GUS-transformed *O. yallundae* isolate (de la Peña *et al.*, 1997). It was suggested that measuring disease levels using a GUS technique provides a more sensitive measurement of resistance in wheat genotypes than visual disease scores (de la Peña *et al.*, 1996). However, techniques that assess the total amount of eyespot present, such as measuring GUS-transformed isolates and quantification of fungal DNA, do not take into account the distribution of fungus within the plant tissues. The disease scoring method of Scott (1971), as used in the present study, indicates the degree of penetration of the host and is intended to provide an indication of potential for yield loss, as this is dependent upon penetration of successive leaf sheaths, leading to infection of the stem (Uslu *et al.*, 1998). Consequently, visual disease scores may provide a better indication of the agronomic effectiveness of an eyespot resistance. The previous identification of *Pch2* resistance to *O. yallundae* using a GUS-transformed isolate, combined with the inability to detect a QTL through visual disease assays, suggests that *Pch2* may prevent lateral colonization of leaf sheaths of *O. yallundae*, but has a lesser effect against penetration of leaf sheaths by the pathogen.

It is not possible to infer the mechanism responsible for the differential resistance conferred by *Pch2* on the basis of current evidence. However, it may be related to the differences in the modes of infection, particularly coleoptile penetration, of the two *Oculimacula* species, as described by Daniels *et al.* (1991). It is possible that *Pch2* provides a resistance that is effective against the apparently random penetration strategy of *O. acuformis* but ineffective against the more ordered, possibly hemibiotrophic (Blein *et al.*, 2009), *O. yallundae* infection mechanism. Further investigation into the infection processes of both species on wheat genotypes with and without *Pch2* would be of interest to determine the mechanisms underlying the resistance differential and to provide insight into the function of this resistance.

Pch2 was previously assigned to chromosome 7A in seedling tests by Law *et al.* (1976) using inoculum that would now be classified as *O. yallundae*. However, a number of experiments were required by Law *et al.* (1976) to assign the effect and this is consistent with the detection of a small effect of resistance to *O. yallundae* in *Pch2*-carrying lines in the present study. *Pch2* was also previously associated with the endopeptidase marker *Ep-A1b* (Koebner & Martin, 1990), using F_3 families derived from the cross Chinese Spring × Cappelle Desprez and a mixture of *Pseudocercosporella herpotrichoides* isolates. No clear explanation can be offered for the apparent contradiction between that work and the present results. However, both *O. yallundae* and *O. acuformis* isolates were used in separate replicates in cool glasshouse conditions by Koebner & Martin (1990) and there is evidence that at temperatures less than 7°C *O. acuformis* develops more rapidly than *O. yallundae* (Wan *et al.*, 2005). Furthermore, cool glasshouse conditions, were found to provide conditions favouring development of *O. acuformis* over *O. yallundae* (Nicholson *et al.*, 1997). This may have resulted in a predominance of *O. acuformis* when averaging across replicates in the final data analysis, resulting in the detection of *Pch2* resistance in their study.

Pch1 conferred a high level of resistance to eyespot both in the intergenotypic substitution line HS/VPM7D and in wheat cultivars (Tables 3 and 4). Furthermore, in contrast to *Pch2*, no difference was detected in the level of resistance conferred by *Pch1* towards the two pathogens. Previous studies provided evidence that cultivars combining both *Pch1* and *Pch2* genes have a greater level of adult plant resistance than those containing *Pch1* alone in mixed-inoculum field trials (Hollins *et al.*, 1988). However, it was not possible to detect a greater level of seedling resistance in the cultivars combining both genes (e.g. Lynx) in the present study than in those containing *Pch1* alone (e.g. Andante). It is possible that an additional effect of *Pch2* may be observed at the adult plant stage (Hollins *et al.*, 1988), presumably as a result of enhanced control of *O. acuformis*, but this effect cannot be detected over the relatively short time scale of a seedling bioassay.

No reduction in resistance to *O. yallundae* was observed in lines combining *Pch1* and *Pch2*, suggesting that the potent effect of *Pch1* is sufficient to mask the differential resistance conferred by *Pch2*. The potency of *Pch1* to both eyespot species in a range of genetic backgrounds, as demonstrated in the present study, combined with its widespread use in areas of high eyespot pressure, such as the Pacific northwest USA in cultivars such as Hyak (Allan *et al.*, 1990) and Finch (Campbell *et al.*, 2005), clearly demonstrates the agricultural value of the gene. However, the use of *Pch1* in NW Europe has been limited because of the apparent linkage between the resistance gene and yield-limiting traits (Johnson, 1992). Although it is likely that the *A. ventricosa* segment has recombined in some cultivars (Leonard *et al.*, 2008), further work is required to develop more markers in this region to facilitate the identification of more

recombinants and accurate mapping of this potent resistance gene.

Sixteen *T. monococcum* accessions out of 22 tested were identified as having eyespot resistance. Previous studies, such as that of Cadle *et al.* (1997), have also identified a high proportion of resistant accessions in collections of *T. monococcum*, suggesting that the species may be a useful source of novel eyespot resistances. Furthermore, some of these resistances identified in the present study were potent, providing a level of resistance greater than that of *Pch2* (Table 5). Those accessions that provided a high level of resistance to both species could potentially be useful sources of novel eyespot resistance. However, it is not possible to infer the genetic basis of these resistances from the data of the present study. If any of the *T. monococcum* resistances are to be considered as candidates for introgression into wheat, genetic mapping studies are required to determine the number of genes conferring resistance and their genetic locations. The resistances identified could potentially be allelic to *Pch2*, as *T. monococcum* is closely related to *T. uratu*, the diploid A-genome donor of wheat (Dvořák *et al.*, 1993). In the relatively small group of 22 *T. monococcum* accessions examined, it was possible to identify two accessions with evidence of greater resistance to *O. yallundae* and two accessions with evidence of greater resistance to *O. acuformis* over the two experiments. This suggests that different levels of resistance to *O. yallundae* and *O. acuformis* may occur within *T. monococcum*. In conjunction with the present findings for *Pch2* and previous findings of differential resistance in accessions of *D. villosum* (Uslu *et al.*, 1998), this suggests that any future screening of germplasm collections should examine resistance to the two *Oculimacula* species separately.

In conclusion, the results of this study demonstrate that *Pch2* provides effective resistance against leaf sheath penetration by *O. acuformis*, but provides a significantly lower level of resistance against *O. yallundae*. Furthermore, differential resistance was observed in accessions of *T. monococcum*, suggesting that a different genetic basis of resistance to the two pathogens may be present in relatives of wheat. These findings may be related to the differences in the infection processes of the two pathogens (Daniels *et al.*, 1991) and requires further investigation. In conjunction with previous evidence that *Pch2* is ineffective towards *O. yallundae* at the adult plant stage (Muranty *et al.*, 2002), these results have important implications for the use of eyespot resistance genes in commercial cultivars, as they indicate that the use of *Pch2* as a sole source of resistance would not provide effective protection against eyespot where the predominant species is *O. yallundae*.

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Appendix 4

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ORIGINAL PAPER

Identification of a QTL conferring seedling and adult plant resistance to eyespot on chromosome 5A of Cappelle Desprez

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Abstract Eyespot is an economically important fungal disease of wheat and other cereals caused by two fungal species: *Oculimacula yallundae* and *Oculimacula acuformis*. However, only two eyespot resistance genes have been characterised and molecular markers made available to plant breeders. These resistances are *Pch1*, introduced into wheat from the relative *Aegilops ventricosa*, and *Pch2*, originally identified in the cultivar Cappelle Desprez (CD). 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. An additional resistance has been reported on chromosome 5A of CD that confers resistance to eyespot in adult plants. In the present study, we demonstrate that resistance on this chromosome is effective against both *O. yallundae* and *O. acuformis* eyespot pathogens and confers resistance at both seedling and adult plant stages. This resistance was mapped in both seedling bioassays and field trials in a 5A recombinant population derived from a cross between CD and a CD single chromosome substitution line carrying 5A from the susceptible line Bezostaya. The resistance was also mapped using seedling bioassays in a 5A recombinant population derived from a cross between the susceptible line Chinese Spring (CS) and a single chromosome substitution line carrying 5A from CD. A single major QTL on the long arm of chromosome 5A was detected in all experiments. Furthermore, the SSR marker *Xgwm639* was found to be closely associated with the resistance and could be used for marker-assisted selection of the eyespot resistance by plant breeders.

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Introduction

Eyespot is an economically important fungal disease of cereal crops such as wheat, barley and rye. It is caused by two species of fungi, *Oculimacula yallundae* (formally *Tapesia yallundae*) and *O. acuformis* (*T. acuformis*). It is widespread in areas where mild and damp autumns promote the growth and spread of the pathogen, particularly northwest Europe and northwest USA. High levels of the disease can lead to significant economic losses (Hardwick et al. 2001; Murray 1996). Unfortunately, control with fungicides may not be cost effective (Nicholson and Turner 2000) and resistance has arisen in numerous eyespot pathogen populations to a number of different chemicals (Parnell et al. 2008). Therefore, the use of disease-resistant cultivars is thought to be the most effective strategy to control the disease.

There are only two sources of resistance that are known to be widely used in commercial wheat cultivars. The more potent of these is the dominant resistance gene *Pch1*, which was derived from the wheat relative *Aegilops ventricosa.* This gene was introduced into hexaploid wheat (Maia 1967) and has been located to the long arm of chromosome 7D (Worland et al. 1988), where it is associated with the SSR markers *Wmc14* and *Barc97* (Chapman et al. 2008) and the STS markers *Orw1, Orw5* and *Orw6* (Leonard et al. 2008). Although *Pch1* significantly reduces the rate of penetration from the outer leaf sheaths into the stem

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(Mauler and Fehrmann 1987), there is evidence that the gene is most effective at the seedling stage and that additional quantitative resistances are required to confer a high level of resistance at the adult plant stage (Lind 1999). A further limitation is that it appears to be difficult to break a linkage between *Pch1* and yield-limiting traits also introgressed from *Ae. ventricosa* on the same segment (Koen et al. 2002). Consequently, there is interest in alternative sources of resistance to use either in combination with *Pch1* to increase the level of resistance, or in place of *Pch1* to avoid the problem of linkage drag with deleterious traits.

The second source of resistance is from the cultivar Cappelle Desprez (CD), which was widely grown in Europe for over 20 years from 1953 (Silvey 1978). The resistance observed in CD has been shown to be partly due to a seedling resistance termed *Pch2* on chromosome 7A (Law et al. 1976). This gene has been mapped to the long arm of chromosome 7AL (de la Peña et al. 1996) and is delimited by the SSR markers *Xgwm346*, *Xwmc525* and *Xcfa2040* (Chapman et al. 2008). Additional quantitative resistances are believed to be present in CD, notably an adult plant resistance identified on chromosome 5A (Muranty et al. 2002). Chromosome 5A appears to be an important component of the eyespot resistance observed in CD at the adult plant stage, particularly as *Pch2* appears to confer little adult plant resistance as assessed by field trials (Muranty et al. 2002). Previous studies (Hollins et al. 1988; Lind 1999) have demonstrated that cultivars carrying *Pch1* and with CD in their pedigree, such as Rendezvous, have enhanced adult plant resistance. However, it has not been determined whether this is due to the effect of *Pch2*, as known to be carried by Rendezvous (Burt et al. 2010), or other CD quantitative resistances that are effective at the adult plant stage such as the 5A resistance.

There is evidence from a number of sources that the genetic basis of resistance to *O. yallundae* and *O. acuformis* can differ. *Pch2* has recently been shown to confer a lower level of resistance to *O. yallundae* than to *O. acuformis* (Burt et al. 2010). Furthermore, $PchD^{v}$ (*Pch3*), a third eyespot resistance mapped to chromosome 4 V of the wheat relative *Dasypyrum villosum* (Yildirim et al. 1998), but not used in wheat cultivars, appears to be less effective against *O. acuformis* than *O. yallundae* (Uslu et al. 1998). There is also evidence that potentially novel resistances identified in the wheat relative *Triticum monococcum* (Burt et al. 2010) may confer differential resistance to the two pathogen species. The species coexist in field populations, and control of one species tends to increase the proportion of the other and fails to prevent disease (Parnell et al. 2008). To provide effective disease control in commercial wheat cultivars any novel sources of resistance that are used, such as the CD chromosome 5A resistance, should be effective against both forms of the pathogen.

There are other genes of agronomic importance on chromosome 5A, such as QTL controlling time to ear emergence (Griffiths et al. 2009) and resistance to Fusarium head blight (Buerstmayr et al. 2003), and therefore it is highly desirable to identify molecular markers linked to the eyespot resistance in order to introduce this alongside genes for other beneficial traits carried on this chromosome in other cultivars. To our knowledge, no previous studies have determined the genetic basis of this resistance or sought to identify molecular markers for its selection by plant breeders.

The aim of the present study was to characterise the adult plant eyespot resistance previously identified on chromosome 5A of CD and determine whether its presence could be detected at the seedling stage and whether it confers resistance towards both *O. yallundae* and *O. acuformis*. We 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.

Materials and methods

Plant and fungal material

Cappelle Desprez carries both *Pch2* and 5A resistances, while Chinese Spring (CS) and Bezostaya (Bez) contain no known eyespot resistance genes. The inter-varietal single chromosome substitution lines Chinese Spring-Cappelle Desprez 7A (CS/CD7A) containing *Pch2,* Chinese Spring-Cappelle Desprez 5A (CS/CD5A) containing the chromosome 5A resistance, Cappelle Desprez-Bezostaya 7A (CD/Bez7A) lacking *Pch2* but containing the 5A resistance, and Cappelle Desprez-Bezostaya 5A (CD/Bez5A) lacking the 5A resistance but carrying *Pch2*, were all obtained from the John Innes Centre (JIC) wheat collection. The parental lines CD, CS and Bez were used as controls.

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 × CD/Bez5A by Tony Worland at the Plant Breeding Institute, Cambridge, and a population of 147 RILs generated from the cross CS × CS/CD5A.

Eyespot isolates were grown on V8 agar (9 g of bacto agar, 50 ml of V8 vegetable juice in 450 ml of de-ionised water) at 15°C for 21 days. In the seedling bioassays conducted at the JIC mixtures of six isolates were used for both the *O. yallundae* and *O. acuformis* inoculations. Inoculum for the field trials was prepared using colonised oat grain as described by Bruehl and Nelson (1964). All 12

isolates were used in equal proportions for the field trials at JIC to provide a 1:1 inoculum mix of the two pathogen species. In the field trials conducted at RAGT Seeds, three *O. yallundae* and two *O. acuformis* isolates were selected from the company's collection and used in a 3:2 inoculum mix. A mixture of different isolates was used to ensure that a successful infection was achieved in case of lack of virulence of one or more of the isolates.

Inter-varietal single chromosome substitution lines experiments

Seedling bioassays were conducted to determine the relative effectiveness of *Pch2* and 5A resistances at the seedling stage using wheat lines CS, CS/CD7A, CS/CD5A, CD, CD/Bez5A, CD/Bez7A, and Bez. In all seedling bioassays, plants were grown in 7 × 7 cm pots in peat and sand compost, with five plants per pot. All plants were grown under 12 h day length in controlled environment rooms (CERs). Twenty pots per line were arranged in a complete randomised block design consisting of ten blocks at 5°C and ten at 10°C, with one pot of each line per block. At each temperature five blocks were inoculated with *O. yallundae* and five blocks were inoculated with *O. acuformis.* Plants were inoculated at growth stage (GS) 12 (Zadoks et al. 1974) using a PVC cylinder and inoculum slurry method as described by Chapman et al. (2008), harvested 6–8 weeks after inoculation, and scored for disease on the basis of leaf sheath penetration (Scott 1971). This experiment was subsequently repeated using identical methods to confirm the findings.

Phenotyping populations

To identify the genetic location of the resistance at the seedling stage, 88 RILs from the CD × CD/Bez5A population were phenotyped for resistance to *O. yallundae* and 147 RILs from the CS × CS/CD5A population were phenotyped for seedling resistance to *O. acuformis* in seedling bioassays. These were conducted as independent randomised complete block experiments, each consisting of six blocks, and each block containing one pot (five plants) per line and three pots (15 plants) per parent line. Seedlings were inoculated as described above. The seedling bioassays of single chromosome substitution lines suggested that the differential in disease between lines with or without the 5A resistance was greater between CD and CD/Bez5A at 5 than at 10°C, whereas the reverse was the case for the differential between CS and CS/CD5A. For this reason the CD × CD/Bez5A experiment was conducted in a CER at 5°C, and the CS × CS/CD5A experiment was conducted at 10°C in an effort to ensure maximal discrimination between lines with and without the chromosome 5A resistance.

To map the 5A resistance at the adult plant stage, 88 RILs from the population CD × CD/Bez5A were grown in two independent field trials at RAGT Seeds, Cambridge, UK, and at JIC, Norwich, UK. Both trials were drilled in autumn 2007 and plants were harvested and scored in summer 2008. Each trial was arranged in a randomised block design, each block containing two plots of each line and the two parents. Each plot consisted of a 1-m strip with three rows of plants. Using inoculum mixes as described above, both trials were inoculated when seedlings had reached GS 12 at an application rate of 20 g/m^2. To prevent unwanted foliar fungal pathogens, plants in the JIC trial were sprayed once at approximately GS 45 with 'Amistar Opti' (Azoxystrobin and Chlorothalonil) at a rate of 1.5 l ha^{-1}. Plants in the RAGT trial were untreated with fungicides. Plants were assessed for penetration of eyespot into the main stem at GS 70, using the method of Scott and Hollins (1974). It was not possible to conduct a field trial to map adult plant resistance in the CS × CS/CD5A population because of the very poor agronomic performance and growth habit of CS-based materials in the field.

Statistical analysis

For the chromosome substitution line seedling bioassays, *O. yallundae* and *O. acuformis* inoculations were analysed as separate experiments. General linear modelling (GLM) was used to calculate predicted mean disease scores for each substitution line and control line across the two replicated seedling bioassay experiments and temperatures within each experiment, also accounting for the environmental effects of blocks. Mean disease scores for lines were compared using *t* probabilities calculated within the GLM.

Data from the population phenotyping experiments were also analysed using GLM to assess variability due to block and genotype. Interactions between block and genotype were also assessed as each field trial block contained two plots per genotype and each seedling bioassay block contained five plants per genotype. GLMs were used to predict mean disease scores for RILs from the CD × CD/Bez5A population seedling bioassays and field trials, and for RILs from the CS × CS/CD5A population seedling bioassay. The predicted means were subsequently used for the QTL analysis detailed below. All analyses were conducted using Genstat v.12 (Copyright 2009 Lawes Agricultural Trust, Rothamsted Experimental Station, UK).

SSR analysis

The parent lines of the two populations, CS, CS/CD5A, CD and CD/Bez5A, were screened with 47 publicly available SSR markers, reported to be located on chromosome 5A, to identify markers which were polymorphic in either or both

populations. Primer sets used were from IPK Gatersleben (*Gwm*), Wheat Microsatellite Consortium (*Wmc*), Beltsville Agricultural Research Station (*Barc*) and INRA (*Cfa/Cfd/Gpw*), and are described on the GrainGenes website (http://wheat.pw.usda.gov/cgi-bin/graingenes/). Markers were identified to provide an even coverage of chromosome 5A on the basis of deletion bin locations (Goyal et al. 2005) and published consensus maps (Somers et al. 2004). Wherever possible, markers polymorphic in both populations were used for mapping, in order to make direct comparisons.

Fresh leaf tissue (50 mg) from 5-week-old plants of the two populations was harvested into 96-well plates on dry ice. DNA was extracted from samples, quantified using a PicoDrop spectrophotometer (Picodrop Ltd.), and diluted to 6 ng/µl in sterile distilled water for use in PCRs. A 6.25 µl reaction volume consisted of 2.5 µl of DNA, 3.125 µl of Taq mastermix (Qiagen) and 0.625 µl of the relevant primer pair (2 µM). The forward primer for each marker was labelled with 6-FAM, NED, PET or VIC fluorescent dyes (Applied Biosystems). PCR conditions were as described by Bryan et al. (1997) with annealing temperatures as indicated by the GrainGenes website. Samples were prepared by adding 1 µl of a 1:40 dilution of the PCR product to 10 µl formamide and 0.2 µl of LIZ 500 size standard (Applied Biosystems). Samples were run on an ABI 3700 capillary sequencer (Applied Biosystems), and the output data were analysed using Peak Scanner v1.0 (Applied Biosystems) to determine the product size of the amplicons.

Map construction and QTL analysis

Linkage maps were generated for both populations in JoinMap® (version 3.0) using 0.4 as the maximum recombination fraction and 3.0 as the logarithm of the odds ratio (LOD). Linkage map data were combined with phenotypic data from the seedling bioassays and two field trials of the populations independently for a QTL analysis using Map-QTL® version 4.0 (van Ooijen and Maliepaard 1996). QTLs were initially identified using the Kruskal–Wallis test. Second, approximate locations of the QTLs were determined using interval mapping for each experiment independently. Finally, multiple QTL mapping (MQM) was carried out to finalise the locations, using the QTLs detected as co-factors. The minimum significant logarithm of the odds (LOD) scores was calculated by permutation tests (1,000 permutations) to identify the appropriate significance thresholds ($P < 0.05$) to declare the presence of a QTL for eyespot resistance.

Verification experiments

To confirm the QTL location of resistance to both pathogen species at the seedling stage, seedling bioassays were conducted on sub-sets of 13 lines from the CD × CD/Bez5A population and 28 lines from the CS × CS/CD5A population. These lines were selected on the basis of recombination around the detected QTL. This experiment consisted of five blocks, each block consisting of two pots per line, one of which was inoculated with *O. yallundae* and the other was inoculated with *O. acuformis*. CS, CS/CD5A, CD and CD/Bez5A were included as controls in each block. Mean disease scores from the seedling bioassays of recombinant lines were used alongside marker data in a single marker regression analysis to confirm QTL location for *O. yallundae* and *O. acuformis* resistance separately.

Results

Seedling bioassays of inter-varietal single chromosome substitution lines

The *Pch2* and the 5A resistances were similarly expressed in the experiments conducted at 5 and 10°C and therefore combined data across temperatures are presented in Fig. 1. However, the differential between CD and CD/Bez5A was greater, but not significantly so, at 5°C than at 10°C whereas the reverse was the case between CS and CS/CD5A. We have no clear explanation for this, although it could partly be influenced by differences in growth habit between CD and CS.

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* (Fig. 1a), demonstrating that chromosome 5A of CD confers resistance at the seedling stage in a susceptible CS background. The level of resistance observed in CS/CD5A was similar to that observed in CS/CD7A, which contains *Pch2* in the same susceptible background (Fig. 1a).

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* (Fig. 1b). 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. CD/Bez7A, which contains the CD5A resistance but lacks *Pch2*, had significantly higher disease scores than CD ($P < 0.05$) when inoculated with *O. acuformis,* but not when inoculated with *O. yallundae* (Fig. 1b), suggesting that the loss of any effect of *Pch2* in the CD resistance reduces the level of resistance to *O. acuformis,* but not to *O. yallundae*.

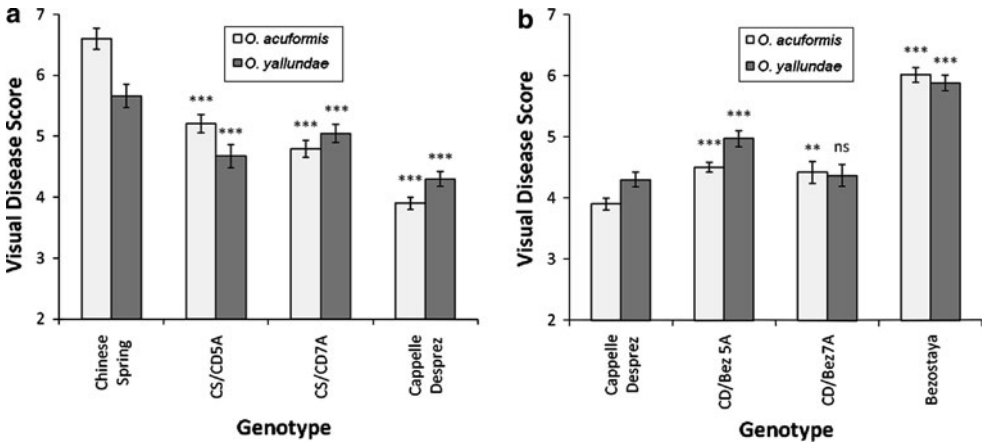

Fig. 1 Predicted mean disease scores for **a** Chinese Spring–Cappelle Desprez substitution lines and **b** Cappelle Desprez–Bezostaya substitution lines when inoculated with *Oculimacula yallundae* and *Oculimacula acuformis* in seedling bioassays. *Error bars* are all ±standard error of the mean. Mean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

Seedling and adult plant resistance in CS × CS/CD5A and CD × CD/Bez5A populations

Analysis of variance demonstrated that the effect of genotype was highly significant ($P < 0.001$) in all trials apart from the field trial of CD × CD/Bez5A conducted at RAGT, in which it was also significant, albeit at a lower level ($P < 0.05$). A highly significant block effect ($P < 0.001$) was observed in all experiments. This was higher in the field trials than the CER seedling bioassays and was particularly high in the RAGT field trial. The large block effect in both field trials may in part have been due to fungal development within stems during the harvesting and scoring process. Trials were harvested and scored in blocks, each block taking approximately 2 days to complete, during which time the fungus continued to grow within the stems. This would increase block variation but would limit the residual component of the analysis of variance. No significant block × genotype interaction could be detected in either field trial although a significant interaction was observed in the seedling bioassays of the populations (Table 1).

SSR analysis, map construction and QTL analysis

Of the 47 SSR markers tested 40% (19) were polymorphic between CS and CS/CD5A and 36% (17) were polymorphic between CD and CD/Bez5A. All of the 17 that were polymorphic between CD and CD/Bez5A were also polymorphic between CS and CS/CD5A. These markers were applied to the populations and linkage maps were calculated using JoinMap (version 3.0). The markers were resolved into identical orders in both populations and it was possible to compare QTL locations in the two populations directly (Fig. 2). However, the linkage map of chromosome 5A was shorter in CD × CD/Bez5A (76 cM) than in CS × CS/CD5A (131 cM). This is probably due to the combined effect of a smaller population size and a lower level of recombination in this population.

QTLs were detected in the same location in both populations conferring both seedling and adult plant resistance (Fig. 2). In the CD × CD/Bez5A population, a QTL for eyespot resistance at the adult plant stage was detected on the long arm of chromosome 5A, centred on the SSR marker

Table 1 Variance components of visual disease scores from phenotyping experiments, calculated using general linear modelling

Variance Component	CS × CS/CD5A		CD × CD/Bez5A					
	Seedling OA		Seedling OY		JIC field		RAGT field	
	MS	*F* value	MS	*F* value	MS	*F* value	MS	*F* value
Block	28.6	25.0***	44.3	31.8***	445.4	19.6***	5174.5	111.0***
Genotype	4.9	4.3***	8.5	6.1***	41.2	1.8***	62.7	1.3*
Block × Genotype	2.4	2.1***	3.7	2.7***	28.0	1.2^{ns}	41.9	0.9^{ns}
Residual	1.1		1.4		22.7		46.6	

OA, *Oculimacula acuformis*; OY, *Oculimacula yallundae*; MS, mean squares; ns, not significant

* $P < 0.05$, $**P < 0.01$, $***P < 0.001$

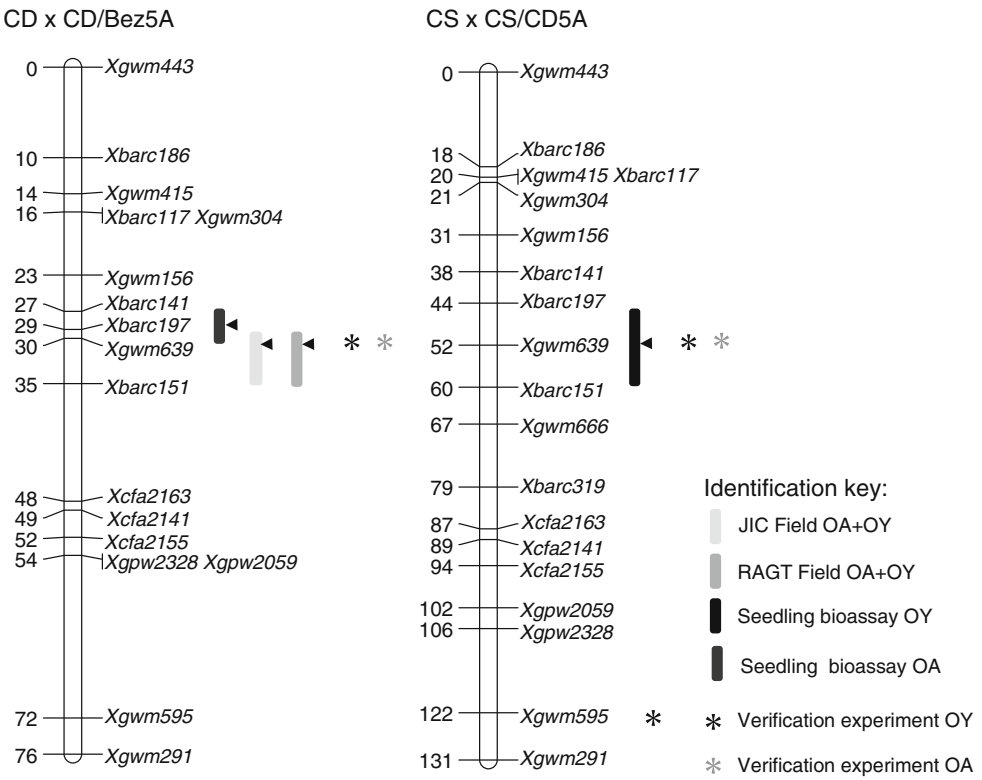

Fig. 2 Genetic maps of chromosome 5A in CD × CD/Bez5A and CS × CS × CS/CD5A populations. Distances are measured in Kosambi cM units. QTL positions for resistance to eyespot are shown to the *right* of the *genetic maps* by *bars* that indicate areas on the map with a LOD score greater than the significance threshold ($P < 0.05$) and *arrowheads* indicate the location of the peak LOD score. *Asterisks* indicate markers with significant associations ($P < 0.05$) with resistance to *Oculimacula yallundae* and *Oculimacula acuformis* in the verification experiments using a single marker regression analysis. *OA* refers to inoculation with *Oculimacula acuformis* and *OY* refers to inoculation with *Oculimacula yallundae*

Table 2 QTL identified in CS × CS/CD5A and CD × CD/Bez5A populations

Population	Test	Pathogen	Closest marker	Map position	LOD threshold	LOD	R^2
CS × CS/CD5A	Seedling	OA	*Xgwm639*	52	2.0	10.62	33.9
CD × CD/Bez5A	Seedling	OY	*Xbarc197*	29	1.9	4.62	23.9
CD × CD/Bez5A	Field JIC	OA + OY	*Xgwm639*	30	1.8	4.72	23.2
CD × 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

Xgwm639 (Table 2), in both the JIC (LOD 4.7, R^2 24%) and RAGT field trials (LOD 4.8, R^2 23%). The seedling bioassay of CD × CD/Bez5A identified a QTL for seedling resistance to *O. yallundae* (LOD 4.6, R^2 24%), which was most significantly associated with the SSR marker *Xbarc197* (Table 2). 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 (Fig. 2). The seedling bioassay of the CS × CS/CD5A population also identified a single major QTL for resistance to *O. acuformis* in the same location (Table 2), again centred on marker *Xgwm639* (LOD 10.6, R^2 34%).

Verification experiment

The single marker regression in the verification experiment confirmed *Xgwm639* as the marker most significantly associated with seedling resistance to *O. yallundae* and *O. acuformis* in both populations (Fig. 2). The presence of a CD allele at the *Xgwm639* locus was associated with a significant reduction in disease scores for both pathogen species in the recombinant lines from CD × CD/Bez5A ($P < 0.05$), explaining 29.1% of phenotypic variance for resistance to *O. acuformis* and 36.2% of phenotypic variance for resistance to *O. yallundae* (Table 3). Similarly, the presence of a CD allele at *Xgwm639* was associated with a highly significant reduction in disease scores for both pathogen species in recombinant lines from CS × CS/CD5A ($P < 0.001$), explaining 43.9% of phenotypic variance for resistance to *O. acuformis* and 43.3% of variance for resistance to *O. yallundae* (Table 3). In addition, it was possible to detect a minor resistance to both pathogens at the *Xgwm595* locus on the distal portion of 5AL from CD in the CS × CS/CD5A recombinant lines, but not in the CD × CD/Bez5A recombinant lines. The presence of a CD

Table 3 Single marker regression of predicted mean disease scores, calculated in a general linear model, for recombinant lines from CD × CD/Bez5A and CS × CS/CD5A

Marker	CD × CD/Bez5A				CS × CS/CD5A			
	O. acuformis		*O. yallundae*		*O. acuformis*		*O. yallundae*	
	R^2	*P* value	R^2	*P* value	R^2	*P* value	R^2	*P* value
Xgwm443	0	0.789	0	0.864	1.1	0.269	0	0.940
Xbarc186	2.2	0.283	0	0.41	0	0.479	0.1	0.322
Xbarc117	6.3	0.206	7.9	0.182	2.9	0.123	5.9	0.123
Xgwm415	2.5	0.278	0	0.437	6.1	0.109	7.1	0.092
Xgwm304	8	0.205	8.7	0.179	6.1	0.109	7.1	0.092
Xgwm156	6.6	0.202	8.9	0.168	0.8	0.281	0.8	0.278
Xbarc141	1.2	0.311	6.1	0.21	0	0.635	0	0.846
Xbarc197	9.1	0.167	3	0.267	9.1	0.064	4.8	0.135
Xgwm639	29.1	0.031	36.2	0.017	43.9	<0.001	43.3	<0.001
Xbarc151	0	0.698	1.5	0.296	5.4	0.123	0	0.328
Xcfa2163	0	0.721	0	0.356	0	0.456	0	0.635
Xcfa2141	0	0.721	0	0.356	0	0.958	0	0.699
Xcfa2155	0	0.622	0	0.758	0	0.858	0	0.991
Xgpw2059	0	0.674	0	0.578	0	0.595	0	0.777
Xgpw2328	0	0.674	0	0.578	0	0.641	0	0.915
Xgwm595	0	0.582	0	0.987	8.8	0.077	12.6	0.042
Xgwm291	0	0.977	0	0.53	4.1	0.154	6.8	0.097

R^2, percentage phenotypic variance explained

allele at *Xgwm595* in CS × CS/CD5A explained 8.8% of phenotypic variance for resistance to *O. acuformis* and 12.6% of variance for resistance to *O. yallundae*. Only the *O. yallundae* resistance was below the $P = 0.05$ significance threshold ($P < 0.042$) (Fig. 2). This probably represents a minor resistance, too small to be detected in the original QTL analysis, which can only be detected in a fully susceptible background. Alternatively, it is possible that this represents a minor effect that is carried by both CD and Bezostaya, but is absent from CS.

Discussion

Resistance to eyespot in the cultivar CD has previously been attributed to the seedling resistance gene *Pch2* on chromosome 7A (de la Peña et al. 1996; Law et al. 1976) and to an unmapped adult plant resistance located on chromosome 5A (Muranty et al. 2002). Through seedling bioassays we determined that the CD chromosome 5A resistance is also effective at the seedling stage, and that it is an important component of the resistance observed in CD, conferring a similar level of resistance to *Pch2*. The resistance was demonstrated in the absence of *Pch2* in a fully susceptible background in the substitution line CS/CD5A (Fig. 1), suggesting it could be used alone to provide eyespot resistance in cultivars. Furthermore, the resistance was also observed in the presence of *Pch2* in lines from the CD × CD/Bez5A population that contained both resistances, suggesting that the 5A resistance confers an enhanced effect when combined with *Pch2*, and therefore could be introduced into cultivars alongside *Pch2* to provide a higher level of protection against the disease.

In contrast to our findings, neither Law et al. (1976) nor Muranty et al. (2002) were able to detect any significant resistance conferred by CD chromosome 5A at the seedling stage. The apparent contradiction between these studies and our data may be due to differences between the methods of inoculation, particularly considering the environmental variability often associated with a necrotrophic fungus such as eyespot (Chapman et al. 2008; de la Peña et al. 1996). In our seedling bioassays, inoculum slurry was pipetted into a PVC cylinder around each seedling stem base as described by Chapman et al. (2008). In contrast, both Law et al. (1976) and Muranty et al. (2002) used the Macer technique whereby seedlings are infected from inoculated straw (Macer 1966). The PVC cylinder and slurry method is likely to provide a more uniform infection than the Macer technique, as inoculum is maintained in contact with the entire surface of the stem base, and this may prevent disease escape or delays in infection and so enable the detection of moderate seedling resistances, such as that conferred by CD chromosome 5A. However, it is possible that other differences in experimental conditions such as temperature, humidity and plant growth stage at inoculation or harvest could also contribute to the disparity between our findings and those of previous studies.

Although adult plant resistances have been identified that are only activated once a plant reaches a particular

developmental stage (Hugot et al. 1999), other adult plant resistances have been identified in wheat that can also be detected at the seedling stage, particularly towards rust diseases (Ma and Singh 1996; Singh and Huerta-Espino 2003). For example, the broad-spectrum adult plant resistance gene *Lr34*, which was first determined to confer resistance to leaf rust at the adult plant stage (Dyck 1987), was shown in later studies to be expressed at the seedling stage. Near-isogenic lines of *Lr34* have demonstrated some evidence of resistance as early as the first leaf stage and a highly significant level of resistance by the 4-leaf stage when inoculated with the causal agent of leaf rust, *Puccinia triticina* (Singh and Huerta-Espino 2003). This suggests that resistance genes once thought to be specific to adult growth stages, such as *Lr34* resistance to leaf rust and resistance to eyespot on CD chromosome 5A, may be active at earlier stages and that this can be detected with accurate phenotyping methods. It is also possible that resistances such as these may have a cumulative effect becoming more evident in older plants and although not conferring complete resistance may inhibit pathogen development sufficiently to prevent the disease becoming a significant problem.

Importantly, we determined that the CD 5A resistance functioning at both the seedling and adult plant stages against *O. yallundae* and *O. acuformis* is conferred by the same genetic location. A major QTL, closely associated with SSR marker *Xgwm639*, was identified for adult plant resistance in field trials of the 5A recombinant population CD × CD/Bez5A at both RAGT and JIC sites. As discussed above, we were also able to detect a significant effect of CD5A on eyespot resistance at the seedling stage and therefore we conducted seedling bioassays of both 5A recombinant populations to map the resistance at the seedling stage. A major QTL was detected in CS × CS/CD5A when inoculated with *O. acuformis*, again centred on *Xgwm639*, in the same position as detected in the field trials for adult plant resistance. Although the QTL identified from the seedling test on CD × CD/Bez5A using *O. yallundae* was centred on a different SSR marker, *Xbarc197*, it is probable that they represent the same genetic location as the QTL areas ($P < 0.05$) overlapped, and *Xbarc197* was only 1 cM proximal from the QTL peak position detected in the other three experiments.

A verification experiment was conducted to confirm that resistance to both pathogen species is conferred by the same genetic location. This consisted of seedling bioassays on a sub-set of recombinant lines from CD × CD/Bez5A and CS × CS/CD5A with separate inoculations with *O. yallundae* and *O. acuformis*. A single marker regression analysis on the mean disease scores from this experiment demonstrated that *Xwmc639* was the marker most closely associated with both *O. yallundae* and *O. acuformis* resistance in both populations. Alongside data from the field trials and seedling bioassays of the complete populations, this provides supporting evidence that a single major QTL on 5A, associated with *Xgwm639,* confers resistance to both *O. yallundae* and *O. acuformis*. Although the eyespot resistances; *Pch1* (Chapman et al. 2008), *Pch2* (de la Peña et al. 1996) and *Pch3* (Yildirim et al. 1998), have previously been mapped and annotated as single genes, they provide quantitative phenotypes. Due to the variability associated with screening populations for resistance to eyespot, particularly in field trials, we characterised the CD 5A resistance as a quantitative trait. To our knowledge, this is the first eyespot resistance QTL to be characterised and we propose that is designated as *QPch.jic-5A*.

The QTL detected in the CS × CS/CD5A seedling trial accounted for a higher percentage of phenotypic variation than those detected in field trials. This may be due to the lower level of environmental variation observed in CER experiments compared to field trials (Table 1). However, the QTL detected in the seedling bioassay of CD × CD/Bez5A was also of lower significance and accounted for less phenotypic variation than the QTL detected in the seedling test of CS × CS/CD5A. This difference was also observed in the seedling bioassays for the verification experiment. This may be partly because the CD × CD/Bez5A population has *QPch.jic-5A* segregating always in the presence of *Pch2*. This could reduce the differential between lines with and without the QTL and hence reduce its significance. In comparison, *QPch.jic-5A* in CS × CS/CD5A is segregating in a susceptible CS background and provides more accurate phenotypic data for locating the QTL, and this is reflected in the greater effect of the detected QTL. Any further work to refine the QTL position should focus on the CS × CS/CD5A population as this provides a clearer phenotypic difference. In addition, CS × CS/CD5A is a larger population and was found to have a higher recombination rate than CD × CD/Bez5A, providing a more appropriate resource for mapping *QPch.jic-5A* at a higher resolution.

Although we have identified an SSR marker that appears to be closely linked to *QPch.jic-5A*, it should be possible to improve the predicted genetic location of the gene and to develop more tightly linked PCR based markers. The linked SSR marker, *Xgwm639*, has been physically mapped to the deletion bin location 5AL-6 0.68–5AL-17 0.78 (Goyal et al. 2005). Further PCR markers could be developed in this region using wESTs that have been positioned in this deletion bin. In addition, synteny between wheat and the sequenced genomes of *Brachypodium distachyon* (International Brachypodium Initiative 2010) and *Oryza sativa* (International Rice Genome Sequencing Project 2005) could be used to target the region of interest on chromosome 5A more accurately.

Q.Pch.jic-5A could be of greater use than *Pch2* as it appears to provide resistance to both eyespot species at all growth stages, whilst *Pch2* provides a lower level of resistance to *O. yallundae* (Burt et al. 2010) and confers little effect at adult plant stages (Muranty et al. 2002). Although not in the scope of this study it would be beneficial to validate the 5A resistance QTL to determine whether it is sufficiently potent for use in a range of genetic backgrounds in commercial cultivars. Cultivars could be screened for the presence and absence of CD haplotypes at the SSR loci associated with the resistance, and then phenotyped through seedling tests or field trials, to determine whether the markers identified herein provide a suitable predictor of eyespot resistance.

In conclusion, we identified a single major QTL, *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. This could provide breeders with the ability to select for a previously uncharacterised source of resistance that is effective against both forms of the pathogen and is effective at both seedling and adult plant stages.

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