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ORIGINAL PAPER

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

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Abstract Introgressions into wheat from related species have been widely used as a source of agronomically beneficial traits. One such 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 hindered by linkage drag with yield-limiting traits. Attempts to break this linkage have been frustrated by a lack of co-dominant PCR markers suitable for identifying heterozygotes in F_2 populations. We 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 a BC_6 F_2 population, segregating between wheat and *Ae. ventricosa* over the distal portion of 7DL, to identify recombinants in the region of *Pch1*. By exploiting co-linearity between wheat chromosome 7D, Brachypodium chromosome 1, rice chromosome 6 and sorghum chromosome 10, *Pch1* was located to an interval between the flanking markers *Xwg7S* and *Xcos7-9*. Furthermore candidate gene regions were identified in Brachypodium (364 Kb), rice (178 Kb) and sorghum (315 Kb) as a prelude to the map-based cloning of the gene. In addition, using homoeologue transferable markers, we obtained evidence that the eyespot resistances *Pch1* and *Pch2* on chromosomes 7D and 7A, respectively, are potentially homoeoloci. It is anticipated that the 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 greater use of these introgressions in commercial varieties.

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Introduction

Introgressions into wheat (*Triticum aestivum*) from species such as *Aegilops* (Schneider et al. 2008) and cultivated relatives such as rye (*Secale cereale*) (Schlegel and Korzun 1997) are potentially valuable sources 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 because of 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 for other traits 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 homoeologues (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 often limited.

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 there has been considerable interest in the development of COS markers for application in cereal (Bertin et al. 2005; Quraishi et al. 2009) and forage grass species (King et al. 2007). 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 model genomes to enable targeted marker development and candidate gene identification.

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 subfamily of cool season cereals and grasses, which includes wheat. However, there are limitations in the co-linearity between wheat and Brachypodium, and caution should be exercised if Brachypodium alone is to be used as a reference genome for map-based cloning (Bossolini et al. 2007). The combined use of the *Oryza sativa* (International Rice Genome Sequencing 2005), *Sorghum bicolor* (Paterson et al. 2009) and Brachypodium genome sequences, in conjunction with gene-based markers such as COS, will enable the triangulation of candidate gene regions in wheat and other cereals and grasses.

An 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). It provides a potent resistance that is effective against both eyespot pathogen species (Burt et al. 2010) at seedling and adult plant stages (Jahier et al. 1989). However, varieties carrying *Pch1* appear to achieve a lower yield in the absence of the disease compared to 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 has hindered the identification of useful recombinants across the introgressed segment and has limited the use *Pch1* in commercial varieties, particularly in Europe (Johnson 1992).

The exploitation of *Pch1* has been further 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 apparently tight linkage to *Pch1* resistance (Santra et al. 2006). Although this isozyme marker has been used widely, it is often difficult to distinguish the *Ep-D1b* allele from the *Ep-A1* and *Ep-D1a* homoeologues (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, 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, which resided on the same rice bacterial artificial chromosome (BAC), AP005750. However, all of these STS markers were dominant, providing either a wheat or *Ae. ventricosa* specific product. Similarly, the SSR markers *Xwmc14* and *Xbarc97*, identified by Chapman et al. (2008) as potential PCR-based markers for *Pch1*, are also dominant, failing to amplify *Ae. ventricosa* specific products.

Despite the limitations in utilising the *Ae. ventricosa* introgression, a significant interest remains in *Pch1* due to its potency and the paucity of alternative eyespot resistances. The only other sources of characterised resistance used in commercial wheat varieties derive from the French variety Cappelle Desprez (Vincent et al. 1952). These consist of 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* (Burt et al. 2011). The 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.

The specific objectives of this study were to (1) assess the applicability of COS markers to alien introgressions such as *Ae. ventricosa* chromosome $7D^v$ in wheat, (2) identify recombination events in the region of *Pch1* using a set of wheat varieties and a recombinant F_2 population; (3) identify co-linear regions in the sequenced Brachypodium, rice and sorghum genomes through gene-based markers to map *Pch1* and to identify candidate gene regions; and (4) examine the homoeologous relationship between *Pch1* on chromosome 7D and the *Pch2* eyespot resistance gene on chromosome 7A.

Materials and methods

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 within their *Ae. ventricosa* introgressed segments. 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*-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 1312 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.

COS marker analysis

Primer sets for a group of 13 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 (International Brachypodium Initiative 2010) using BLASTn 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 11 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 at locations with high levels of sequence conservation with Brachypodium and that 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 × 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 by Burt et al. (2011). PCR amplification was conducted using a touchdown programme consisting of a denaturing step of 95°C for 10 min; 16 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 30 cycles of 95°C for 15 s, 50°C for 15 s and 72°C for 1 min. 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. If no polymorphism was detected using 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).

SSR marker analysis

The variety set was screened with ten 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 *XustSSR2001-7DL* (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 × 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 by Burt et al. (2011) with the exception of the marker *XustSSR2001-7DL* for which PCR amplification conditions were those described by Groenewald et al. (2003).

Recombinant eyespot resistance phenotyping

The 3 recombinant varieties and 25 BC_6 F_3 families from HS × 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 6 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 (Burt et al. 2010). This partial eyespot resistance gene is present in HS and therefore in all the BC_6 F_3 families.

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).

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 × CS/CD7A F_2 population using an SSCP assay (Martins-Lopes et al. 2001). The marker data were added to previous data on this population for the SSR markers *Xgwm346*, *Xwmc525* and *Xcfa2040* (Chapman et al. 2008) and from the cDNA-AFLP derived marker *X19CD7A4* (Chapman et al. 2009). 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 × CS/CD7A (Chapman et al. 2008) 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.

Results

COS marker analysis

One of the 13 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 consequently these products are likely to derive from the same loci. The STS marker, *Xorw6*, developed in the same study, was also evaluated but 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 1).

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, 26 further COS markers were identified (Tr), or developed from aligned wESTs (Cos7) in this region. Of these, seven were polymorphic (27%) (Table 1) and all of these were determined to be located on the distal portion of chromosome 7DL. Six 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.

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 2). These markers confirmed that HS/VPM7D consisted of *Ae. ventricosa* DNA along the length of the chromosome, that RVPM25 consisted of *Ae. ventricosa* from marker *Xbarc53*, and that RVPM44 had 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 1) and were included in a seedling bioassay to determine the presence or absence of *Pch1*. This suggested that all three varieties contained *Pch1* with a recombination event proximal to the gene (Table 3).

Mapping Pch1 using SSR, STS and COS markers

Refining the position of *Pch1* was achieved by screening the HS × RVPM25 BC_6 F_2 population with nine polymorphic COS markers, two STS markers (Table 1) and four SSR markers (*Xbarc76*, *Xbarc97*, *Xwmc14* and *Xcfd175*). Twenty-five BC_6 F_2 plants from the 1,312 tested were identified with recombination events occurring between the SSR markers *Xbarc76* and *Xcfd175* (Table 3). These data were used to determine the marker order in the *Pch1* region (Table 3; Fig. 1), and a genetic map of chromosome 7D was constructed based on recombination in the HS × RVPM25 BC_6 F_2 population (Fig. 1). Where no recombination events had occurred between markers, the order in Table 3 was inferred based on the order of Brachypodium homologues.

The *Pch1* phenotype of the recombinant set was determined in a seedling bioassay using *O. yallundae* isolates. This demonstrated that the distal recombinant varieties, Hermann, Striker and RAGT 2, all carry *Pch1* and classified the HS × RVPM25 BC_6 F_2s as homozygous for either the presence or absence of *Pch1*, or as heterozygous (Table 3).

Phenotypic data were combined with marker data to determine the position of *Pch1* relative to the markers. It was possible to detect recombination events both proximal and distal to *Pch1*. The resistance gene was found to be located to a 0.26-cM region 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* (Table 3, Fig. 1). In addition, *Pch1* was found to co-segregate with the STS marker *Xorw1* and with the COS markers *Xcos7-11* and *Xtr40* (Table 3). The latter marker generates a dominant *Ae. ventricosa* product, but screening individual F_3 plants revealed no plants generating an HS haplotype at this locus indicating that line F2-195 was homozygous for *Ae. ventricosa* at this locus. 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 (Table 3), suggesting that they were not completely linked to the resistance gene.

Co-linearity between wheat chromosome 7D, Brachypodium chromosome 1 (Bd1), sorghum chromosome 10 (Sb10) and rice chromosome 6 (Os6)

The physical location of orthologues of COS and STS markers could be identified over an approximately 1.1-Mb region on Bd1, a 4.9-Mb region on Os6, and a 6.3-Mb region on Sb10. The order of the markers on wheat chromosome 7D was fully conserved with the physical location of markers on Bd1 and Sb10 (Fig. 1). It was not possible to

Table 1 Summary of conserved orthologous sequence (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	CTTGATCAGACGGAAGACGAGCT GGTTTATGGCTTCTCATGGGTT	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 TGAAAGGATGGAACTGAGG	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
Xcos7-11	DQ205351	TTGCTTGGGTGACTGTATCTCAAA GGGTTGGCATTTGAATATGG	SSCP Gel	25,032,560.. 25,033,704	Bd1g29450
Xorw1	AB246917	CTATTACATGAAATCTTATTCTCCCAGCAGTAACGAGAATGTGG	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

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 Sb10 and the genetic order on wheat 7D were 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 and Sorghum, but distally in rice (Fig. 1). Therefore, Brachypodium and Sorghum may provide a greater level of co-linearity to wheat than rice at these loci, as *Xtr40* maps proximally to *Xcos7-9* in the HS × RVPM25 BC_6 F_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 (Fig. 1). This region contains 34 predicted transcripts (Fig. 2), according to the Brachypodium genome annotation v.1.0 (International Brachypodium Initiative 2010). It was possible to assign putative functions for 27 of these through significant similarity to genes encoding known proteins from other cereal species (Table S1) using BLASTn. 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 26 genes (Fig. 2), for which function could be assigned to 19 (Table S1). Similarly, the *Pch1* region in sorghum (Paterson et al. 2009) was defined as a 315-Kb region on Sb10 containing 32 genes (Fig. 2), of which 22 could be assigned function (Table S1). BLASTn revealed that 15 of the genes found in the *Pch1* regions of rice, sorghum and Brachypodium were shared (Fig. 2). These genes were in a mostly co-linear order in all three species, with the exception of the inversion between rice and Brachypodium/sorghum in the order of genes delimited by the markers *Xcos7-9* and *Xtr40* (Fig. 1; Table S1). In addition, within this inverted region it appears that there has been either a gene duplication event in Brachypodium or possibly inaccurate gene annotation, as two Brachypodium genes (Bradi1g29310/Bradi1g29320) are represented by a single rice gene (Os06g51520) or a single sorghum gene (Sb10g31220) (Table S1).

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

Table 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

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 (Fig. 3). Four of the markers located to the 7AL terminal deletion bin, *Xcos7-6, Xorw1*, *Xcos7-11* and *Xtr40*, were found to be polymorphic between CS and CS/CD7A and were therefore mapped in the CS × 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 (Fig. 3). 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 LOD > 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. In comparison, on chromosome 7D, *Pch1* was located at an interval between the loci of *Xwg7S* and *Xcos7-9*. Consequently, there is an overlap between the *Pch1* and *Pch2* regions on chromosomes 7D and 7A, respectively (Fig. 3).

Discussion

The exploitation of beneficial alleles from introgressions of related species into wheat has often been limited by linkage

Table 3 Haplotypes of the distal recombinant varieties Hermann, Striker and RAGT 2 and BC6 F2 lines from HS × RVPM25

	Xbarc76 [1]	*Xtr370* [1]	*Xtr331* [1]	*Xbarc97*	*Xwmc14*	*Xcos7-6* [2]	*X4CD7A8* [2]	*Xorw5* [2]	*Xtr383* [2]	*Xwg7s* [2]	*Xcos7-11* [2]	*Pch1* [3]	*Xorw1* [2]	*Xtr40* [2]	*Xcos7-9* [1]	*Xcfd175* [1]	Mean Disease Score	SEM
HS	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	6.6	0.16
R25	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	3.1	0.15
Hermann	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	3.4	0.22
Striker	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	3.4	0.22
RAGT 2	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	3.7	0.23
F2-293	A	H	H	C	C	H	H	H	H	H	H	H	H	D	H	H	4.5	0.25
F2-1144	A	A	A	C	C	A	A	A	A	A	H	H	H	D	H	H	4.8	0.22
F2-929	A	A	A	C	C	A	A	A	A	A	H	H	H	D	H	H	5.1	0.27
F2-1293	A	A	A	C	C	A	A	A	A	A	A	A	A	A	A	H	6.4	0.21
F2-1051	A	A	A	C	C	A	A	A	A	A	A	A	A	A	A	H	6.3	0.20
F2-1037	H	A	A	C	C	A	A	A	A	A	A	A	A	A	A	A	5.9	0.34
F2-222	H	A	A	C	C	A	A	A	A	A	A	A	A	A	A	A	6.3	0.20
F2-230	H	H	H	C	C	A	A	A	A	A	A	A	A	A	A	A	6.6	0.23
F2-883	H	H	H	C	C	H	H	H	H	H	A	A	A	A	A	A	5.7	0.30
F2-217	H	H	H	C	C	H	H	H	H	H	H	H	H	D	A	A	4.5	0.31
F2-238	H	H	H	C	C	H	H	H	H	H	H	H	H	D	A	A	4.5	0.22
F2-36	H	H	H	C	C	H	H	H	H	H	H	H	H	D	H	A	4.3	0.22
F2-1170	H	H	B	B	B	B	B	B	B	B	B	B	B	D	B	B	3.7	0.11
F2-1194	H	H	H	C	C	H	H	H	H	H	B	B	B	D	B	B	3.4	0.22
F2-868	H	H	H	C	C	H	H	H	H	H	H	H	H	D	H	B	4.3	0.35
F2-912	B	A	A	C	C	A	A	A	A	A	A	A	A	A	A	A	5.7	0.26
F2-1303	B	A	A	C	C	A	A	A	A	A	A	A	A	A	A	A	5.8	0.36
F2-902	B	B	B	B	B	B	B	B	B	B	B	B	B	D	B	A	3.4	0.11
F2-952	B	B	B	B	B	B	B	B	B	B	B	B	B	D	B	A	3.6	0.19
F2-960	B	B	B	B	B	B	B	B	B	B	B	B	B	D	B	A	3.5	0.21
F2-195	B	B	B	B	B	B	B	B	B	B	B	B	B	B*	H	H	3.5	0.22
F2-225	B	B	B	B	B	B	B	B	B	B	B	B	B	D	B	H	3.4	0.22
F2-422	B	B	B	B	B	B	B	B	B	B	B	B	B	D	B	H	3.5	0.11
F2-383	B	B	B	B	B	B	B	B	B	B	B	B	B	D	B	H	4	0.12
F2-1013	B	B	B	B	B	B	B	B	B	B	B	B	B	D	B	H	3.4	0.20

Haplotypes are shaded for ease of interpretation with dominant markers shaded according to the most likely haplotype, based on results for surrounding loci. Predicted mean disease scores and associated standard errors (SEMs) for recombinant BC_6 F_3 families and varieties inoculated with *O. yallundae* were calculated within a general linear model (GLM)

A Wheat/Hobbit 'sib'

B Aegilops ventricosa

H Heterozygote

C A or H (Hobbit 'sib' dominant marker)

D B or H (*Ae. ventricosa* dominant marker)

Marker order was determined by [1]recombination events in varieties and BC_6 F_2 lines, [2]recombination events followed by homologous Brachypodium marker positions, [3]eyespot resistance phenotype

* Due to its importance in determining the *Pch1* interval, the haplotype of F2-195 for *Xtr40* was determined using DNA from six individuals from the BC_6 F_3 family

to deleterious traits, a low level of recombination and a paucity of PCR-based co-dominant markers. Our results demonstrated that cereal COS markers provide a means of identifying 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 aid the selection of recombinant lines carrying desirable traits. However, the applicability of COS markers for this purpose is still likely to be limited by an absence of recombination within introgressions from more distantly related species such as rye.

Using the Brachypodium genome and by exploiting synteny with wheat, new COS markers were developed and used alongside existing COS and STS markers 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 × RVPM25 BC_6 F_2 population to identify novel *Pch1* recombinants. Marker data from BC_6 F_2 recombinants were used to calculate a genetic map of chromosome 7D from the population and combined with phenotypic data to locate *Pch1*to a 0.26-cM region between five co-segregating proximal markers; *Xcos7-6*, *X4CD7A8*, *Xorw5*, *Xtr383* and *Xwg7S* and the distal COS marker

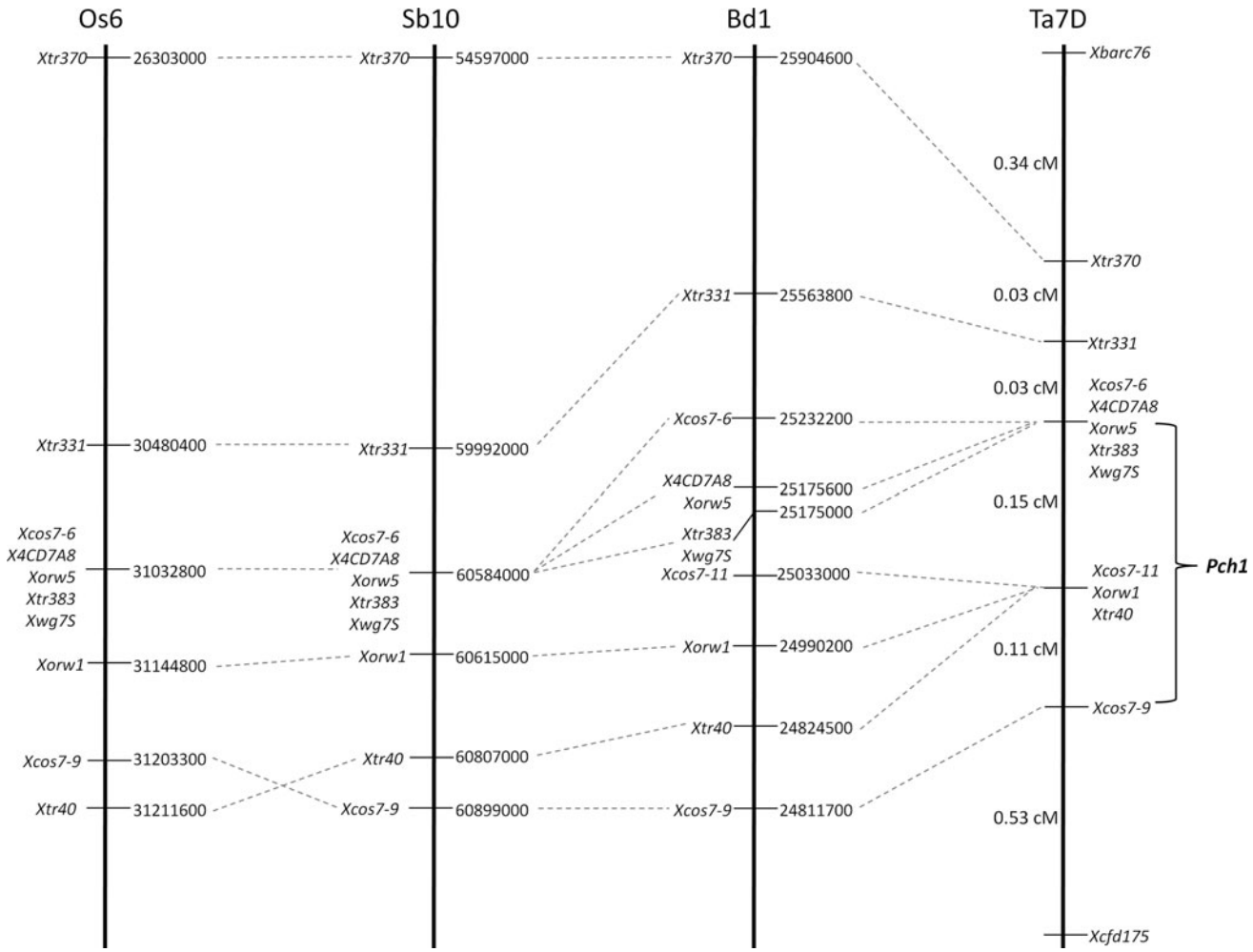

Fig. 1 Comparison of the HS × RVPM25 wheat chromosome 7D (Ta7D) approximate genetic map including *Pch1* with physical marker locations on Brachypodium chromosome 1 (Bd1), sorghum chromosome (Sb10) and rice chromosome 6 (Os6)

Xcos7-9. 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_6 F_2 recombinants using seedling bioassays with relatively few plants per line (approximately, 30). The process of screening a larger population for further mapping and potentially map-based cloning of the gene will be greatly facilitated by the ability to use F_3 families to phenotype this material without the need to generate homozygous lines.

The STS marker *Xorw1* co-segregated with *Pch1* in the HS × 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 (Santra et al. 2006; McMillin et al. 1986) or the derived PCR-based marker (Leonard et al. 2008; Meyer et al. 2011). It has been speculated that *Pch1* resistance may be conferred by the *Ep-D1b* protein (Worland et al. 1988). Similar to others, our data did not identify any recombination between *Xorw1* and *Pch1*, and on this evidence the oligopeptidase B gene may 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 was not a product of the *Ep-D* locus. Furthermore, the null form of the *Ep-D1* endopeptidase allele was identified in resistant introgression lines when *Pch1* was introduced from *Ae. ventricosa* into durum wheat, also suggesting that *Pch1* resistance was not conferred by the *Ep-D1b* protein (Huguet-Robert et al. 2001). If *Orw1* and *Ep-D1b* are derived from the same gene, then

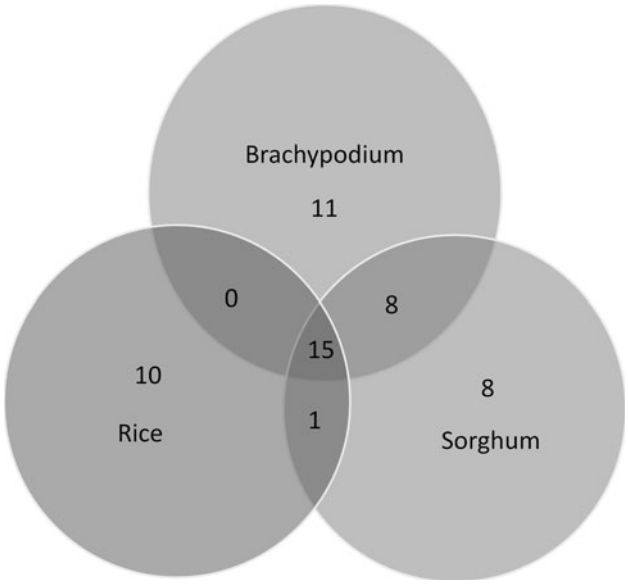

Fig. 2 Venn diagram showing the distribution of shared genes in the *Pch1* candidate gene regions of *Oryza sativa* (Rice), *Brachypodium distachyon* (Brachypodium) and *Sorghum bicolor* (Sorghum)

the findings of Mena et al. (1992) and Huguet-Robert et al. (2001) would discount the oligopeptidase B gene as a candidate for *Pch1*.

The SSR analysis of a panel of wheat varieties and breeding lines identified a number of independent recombination events over the length of the *Ae. ventricosa* segment as a result of crossing in breeding programmes. A relatively small *Ae. ventricosa* segment was detected in Hermann, Striker and RAGT 2, and these lines were included in the COS marker analysis. Taken in conjunction with evidence for recombination both in the BC_6 F_2 population, and 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. However, only a 1.2% recombination rate was observed between the SSR markers *Xbarc76* and *Xcfd175* in the HS × RVPM25 population in comparison to a rate of approximately 10% between these markers in the consensus wheat genetic maps generated by Somers et al. (2004). Thus the number of HS × RVPM25 BC_6 F_2s that will need to be screened to identify additional recombination events in the *Pch1* region to reduce the interval and the number of candidate genes will be greater than for a conventional wheat × wheat cross.

We were able to identify candidate gene regions for *Pch1* in rice, Brachypodium and sorghum on the basis of orthologous locations of the flanking markers. Within these regions, 15 genes were conserved across the three species. However, there is evidence that micro co-linearity between rice, sorghum and Brachypodium has broken down in this region. For example, there are 11 Brachypodium specific genes, 10 rice specific genes and 8 sorghum specific genes (Fig. 2). It is anticipated that Brachypodium should provide a greater level of co-linearity with wheat than rice and sorghum due to its closer evolutionary relationship. Eight genes were common between sorghum and Brachypodium, whilst none was common between rice and Brachypodium in the *Pch1* region. This suggests that Sorghum may provide a greater level of co-linearity than rice to the *Pch1* region in wheat. This is supported by the inversion observed in rice between the markers *Xcos7-9* and *Xtr40* compared to the marker order in wheat, Brachypodium and rice. However, the use of these three reference genomes together should enable the development of further markers, the triangulation of a smaller set of candidate genes, and facilitate positional cloning of *Pch1*.

Currently, we have no prior expectations as to the likely function of *Pch1* and, consequently, from the putative gene functions identified in rice, Sorghum and Brachypodium, there are no clear candidates for *Pch1*. It is possible that indications of function for a resistance such as *Pch1* can be identified from partial resistances to biotrophic pathogens. For example, 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 (Os06g51460) has significant homology with a gene encoding a putative ABC transporter in *Arabidopsis thaliana* (At1g53390).

wEST sequences for four of the five *Pch1* proximal flanking markers (*Xcos7-6*, *X4CD7A8*, *Xorw5*, *Xtr383* and *Xwg7S*) 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). The *Pch1* eyespot resistance has also been associated with papillae formation at attempted infection points (Murray and Ye 1986). As such, callose synthase genes might be considered as candidates for involvement in *Pch1* resistance. However, recombination was detected between these callose synthase markers and *Pch1*, in both the wheat varieties and the HS × RVPM25 population, suggesting that this callose synthase gene is unlikely to be candidate for *Pch2*. The recombination detected between the STS markers *Xorw5* and *Xorw1* indicates that recombination has occurred within the wheat region syntenous to the rice BAC AP005750, to which these markers were designed by Leonard et al. (2008).

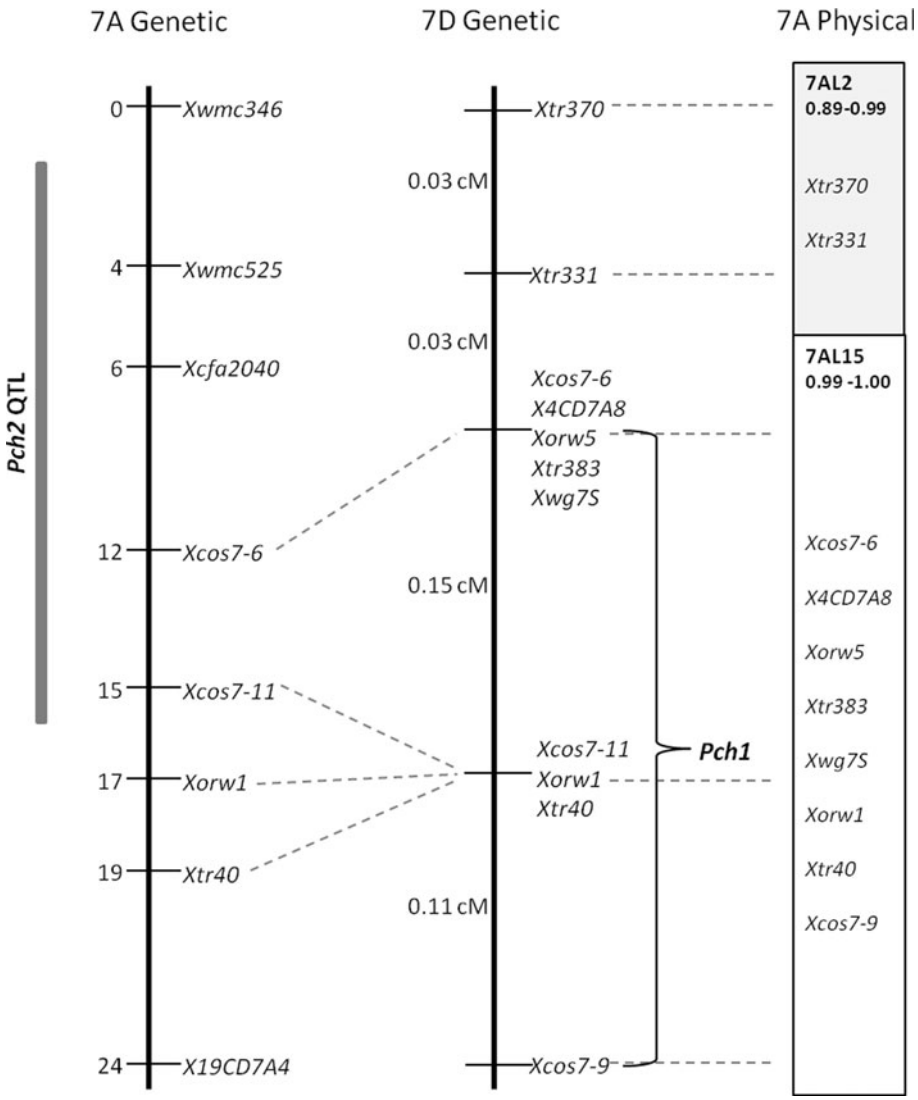

Fig. 3 Comparison of the location of *Pch1* on the genetic map of chromosome 7D with the CS × CS/CD7A chromosome 7A genetic and CS deletion bin maps. The location of the *Pch2* QTL region, determined on the basis of interval mapping, is indicated as a *bar* to represent intervals above the $P < 0.01$ significance threshold of LOD > 15.2

MLO related proteins were first discovered in barley and have been shown to have crucial roles as negative regulators of basal defence responses (Büschges et al. 1997). The recessive *mlo* allele confers durable broad-range resistance against the biotrophic fungus *Blumeria graminis* (Jørgensen 1992). Interestingly, a gene encoding a protein with homology to a transmembrane MLO protein was identified in the Sorghum *Pch1* candidate gene region (Sb10g31010).

Single dominant resistance genes (R-genes) that confer complete immunity rarely provide durable resistance (McDonald 2010). In contrast, *Pch1* provides a high level of partial resistance and has proven durable to date, and it may not be anticipated to be an R gene. To our knowledge, no resistance genes of quantitative effect against a necrotrophic fungal pathogen such as eyespot have been cloned and characterised in wheat. However, genes encoding NBS-LRR type proteins have been cloned in melon (Joobeur et al. 2004) and tomato (Ori et al. 1997) that are effective against the necrotrophic fungus *Fusarium oxysporum*. There are four candidate genes (Bradi1g29360, Bradi1g29370, Bradi1g29390 and Bradi1g29450) with R-gene motifs (NBS-LRR domains) in the Brachypodium *Pch1* region. Of these, Bradi1g29450 has homology to the wheat complete coding sequence DQ205351, identified to encode an NBS-LRR resistance (Wang et al. 2006), and to which the *Pch1* co-segregating COS marker *Xcos7-11* was designed. In addition, Bradi1g29390 shares homology to genes in the *Pch1* regions of rice (Os06g51420) and sorghum (Sb10g031190).

De la Peña et al. (1997) suggested that *Pch1* on chromosome 7D and *Pch2* on chromosome 7A could be homoeoloci based on their positions relative to *Ep-D1* and *Ep-A1,* respectively, on the distal portion of the long arms of the respective homoeologous chromosomes. 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 homoeologue-specific nature of most 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 indicate that 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* do, indeed, overlap suggesting that the genes could potentially be homoeoloci. 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 by Chapman et al. (2008) 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 × CS/CD7A for eyespot resistance conferred by the *Pch2* gene, which is only of moderate effect (Chapman et al. 2008; Burt et al. 2010). In addition, a considerable degree of variability due to the influence of environmental factors has often been noted in 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 × CS/CD7A population to enable the position of *Pch2* to be refined. 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, COS markers developed using the Brachypodium genome were successfully used to identify recombinants in a previously recalcitrant *Ae. ventricosa* chromosome segment introgressed into wheat. Using COS markers and previously developed STS markers, we 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, sorghum and Brachypodium. Consequently, 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 allow the development of perfect markers for accurate selection of the resistance by plant breeders. Application of the same techniques to identify recombinant lines for other introgressions from relatives of wheat may assist in the exploitation of the beneficial alleles that they contain and overcome linkage drag to agronomically deleterious characteristics.

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