The eyespot resistance genes Pch1 and Pch2 of wheat are not homoeoloci

M. Pasquariello · J. Ham · C. Burt · J. Jahier · S. Paillard · C. Uauy · P. Nicholson

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Abstract

Phenotyping and mapping data reveal that chromosome intervals containing eyespot resistance genes Pch1 and Pch2 on 7D and 7A, respectively, do not overlap, and thus, these genes are not homoeoloci.

Introduction

Eyespot is an important fungal disease of the stem base of cereals growing in temperate regions, such as North West Europe, North West USA, and New Zealand. It is caused by two species of fungi, Oculimacula acuformis and O. yallundae (Crous et al. 2003), and affects a wide range of hosts, including wheat, barley, rye, and oats. In the cases of severe infection, this causes lodging and premature ripening of grain, leading to reduced crop yield (Fitt et al. 1988). Two main sources of resistance effective at the seedling stage are currently available for use in wheat (Pch1 and Pch2). The more potent of these is the single major gene Pch1 which was transferred to hexaploid wheat from an accession (termed Vent 10) of the wild relative Aegilops ventricosa and located on the distal end of chromosome 7D (Doussinault et al. 1983; Maia 1967). Pch1 has been mapped to a 0.26 cM region between five co-segregating proximal markers (Xcos7-6, X4CD7A8, Xorw5, Xtr383, and Xwg7S) and the distal COS (Conserved Orthologous Sequence) marker Xcos7-9 (Burt and Nicholson 2011). Until recently, marker-assisted selection for Pch1 commonly relied upon the presence of allele Ep-D1b of a co-segregating...
endopeptidase (McMillin et al. 1986). This assay has been replaced by the PCR-based STS (Sequence Tagged Site) marker Xorw1 developed by Leonard et al. (2008).

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

Pch1 has been widely used in attempts to increase eyespot resistance, but its presence in commercial wheat varieties in Europe has been limited because of linkage drag with undesirable genes derived from Ae. ventricosa. Although Pch1 is highly effective against both eyespot species, a significant reduction of yield and thousand-kernel weight was reported in a line that carried the Ep-D1b locus. Eyespot resistance was also transferred to durum wheat from a second Ae. ventricosa accession (Vent 11) (Huguet-Robert et al. 2001). This accession lacks a functional Ep-D1 endopeptidase allele further indicating that Pch1 is not a product of Ep-D1b.

Pch1 has been widely used in attempts to increase eyespot resistance, but its presence in commercial wheat varieties in Europe has been limited because of linkage drag with undesirable genes derived from Ae. ventricosa. Although Pch1 is highly effective against both eyespot species, a significant reduction of yield and thousand-kernel mass has been sometimes observed in the absence of resistance (Koen et al. 2002). Moreover, even though recombination does occur between the Ae. ventricosa 7D' and the wheat 7D chromosome segments, it does so at a much lower frequency than normal (Worland et al. 1988), and until now, it has been difficult to break the linkage between Pch1 and yield-limiting traits.

The second source of eyespot resistance derives from the French cultivar Cappelle Desprez (CD) (Vincent et al. 1952). Since the 1950s, this has proved to be a moderate but highly durable resistance, dominating European wheat markets for over two decades. Originally, most of the CD resistance effects were determined to be conferred by a seedling resistance locus termed Pch2, located on the distal end of chromosome 7AL (de la Peña et al. 1996), but more recently, an additional QTL of moderate effect has been located on chromosome 5A (Burt et al. 2011; Muranty et al. 2002). Pch2 has been mapped to a 7 cM interval between SSR markers Xwm346 and Xcfa2040, and it is closely associated with SSR Xwmc525 (Chapman et al. 2008).

The relative positions of Pch1 and Pch2 on the long arm of chromosomes 7D and 7A, respectively, suggest that they are homoeologous. A number of the previous studies support this hypothesis. For example, in the work of Chapman et al. (2008), the SSR marker Xcfa2040, which has homoeologous loci on 7A and 7D, was found to be close to Pch2 on 7A, and also to the Pch1-linked SSR marker Xwme14 on 7D. Unfortunately, due to the homoeologue-specific nature of most SSR markers, it was impossible to make other direct comparisons between the two regions. The potential homoeology is further supported by the study of de la Peña et al. (1997), where they mapped Ep-D1 and Ep-A1, respectively, on the distal portion of the long arms of the 7D and 7A chromosomes. However, Leonard et al. (2008), who identified a candidate gene for Ep-D1 and developed a DNA-based marker on it, reported a large discrepancy between the genetic distances of the two endopeptidase loci (Ep-D1 and Ep-A1) and the Pch loci on their respective chromosomes and could not provide clear evidence to support them being homoeologous based upon their genetic positions. More recently, Burt and Nicholson (2011) found that the region containing Pch1 (flanked by the markers Xcos7-6 and Xcos7-9) on chromosome 7D overlapped with the Pch2 QTL between markers Xcos7-6 and Xorw1. The Pch2 QTL region originally spanned a 7 cM region (Chapman et al. 2008), but the addition of COS and sequence tagged site (STS) markers extended both the map and the QTL interval (Burt and Nicholson 2011). The large size of the QTL interval was believed to be, largely, a result of inaccuracies in phenotyping F3 families for eyespot resistance conferred by Pch2, as this gene is only of moderate effect (Burt et al. 2010; Chapman et al. 2008).

A high level of genome-synteny between wheat and model species, such as rice (Oryza sativa) sorghum (Sorghum bicolor) and Brachypodium (Brachypodium distachyon), has been widely reported (Vogel et al. 2010), with Brachypodium providing a greater level of co-linearity with wheat than the other two due to its closer evolutionary relationship. Notably, the panel of co-dominant COS markers developed by Burt and Nicholson (2011) was obtained by exploiting the syntenic relationship between mainly Brachypodium (but also rice and sorghum) and wheat. The availability of complete genomic sequence of these species is a great advantage for the development of molecular markers, for the identification of candidate genes for traits of interest and for predicting biological gene functions. Such comparative and functional genomic approaches have already helped to uncover the molecular basis of important wheat characters, including disease resistance (Krattinger et al. 2009), vernalisation requirement (Yan et al. 2006), and flowering time (Lv et al. 2014). To aid the genetic dissection of eyespot resistance, this co-linear relationship could be further explored for proceeding to the map-based cloning of Pch1 and at the same time, it could be helpful for developing homoeologue (7A/7D) transferable markers by which to resolve the potential homoeology between Pch1 and Pch2.

Although there are limitations in utilising the Ae. ventricosa introgression, it is being actively used both in Europe and in the USA by wheat breeders. However, considering the fact that Pch1-carrying varieties can be affected by significant eyespot-induced grain yield losses in situations of severe attack, there is both an interest and a need to further enhance the resistance of wheat. A first strategy to achieve this goal is through pyramiding of Pch1 and the Pch2 and
5A QTL of Cappelle Desprez. Pyramiding entails stacking multiple resistance genes or QTLs in a variety to develop stronger and durable resistance. A first attempt in this direction for eyespot was reported by Hollins et al. (1988) who stated that the variety Rendezvous, derived from crosses involving VPM ([Ae. ventricosa × T. persicum] × Marne) and CD derived lines, displays higher resistance than VPM. Consequently, they suggested that Rendezvous possesses resistance from both Ae. ventricosa and CD, but at that time, it was not possible to confirm this, as suitable molecular markers were not available for Pch2. Later, Burt et al. (2010), using SSR markers, identified some additional wheat lines containing both Pch1 and Pch2, such as the intergenotypic single chromosome substitution lines Hobbit ‘sib’-VPM7D (HS/VPM7D) and the variety Lynx.

A second strategy to improve eyespot resistance in wheat is the generation of genotypes carrying additional copies of Pch1 on either 7A and/or 7B chromosomes as well as 7D. Stacking multiple Pch1 copies in the genome to improve the level of resistance compared to a single copy is based on the observation that plants homozygous for Pch1 are more resistant to eyespot than those that are heterozygous (Huguet-Robert et al. 2001). As a first step towards this objective, Huguet-Robert et al. (2001) produced meiotically stable Pch1-carrying tetraploid wheat lines derived from backcross progenies of a cross between Ae. ventricosa and T. durum cv. Creso ph1c. The Pch1 transfer, resulting from recombination between homeologous chromosomes, was located on the long arm of chromosome 7A. This conclusion was based on the inability to detect the 7A specific SSR marker Xgwm698 or the Ep-A1 protein in the homozygous recombinant lines, suggesting that a recombination event had occurred between the 7A and 7D chromosomes.

The four goals of the present study were to: (1) develop new gene-based molecular markers closely linked to Pch1 and Pch2 by exploiting synteny between wheat and Brachypodium distachyon; (2) refine the genetic position of Pch1 and Pch2, respectively, on the 7D and 7A chromosomes; (3) clarify the potential homeologous relationship between Pch1 and Pch2; (4) characterize the T. durum wheat lines carrying Pch1 on the 7A chromosome created by Huguet-Robert et al. (2001) using SSRs and new developed KASP markers.

Materials and methods

Plant material

Nine hundred and forty-four BC6 F2 plants from the cross between the recombinant substitution line RVPM25 and Hobbit-Sib (HS) produced by Burt and Nicholson (2011) were genotyped to identify additional recombinants in the Pch1 region. The 25 BC6 F2 plants reported by Burt and Nicholson (2011) to be recombinant between the SSR markers Xbarc76 and Xcfd175 were re-analysed with additional markers to refine the recombination break points.

A total of 92 F7 lines obtained by single seed decent (SSD) and deriving from a cross between Chinese Spring (CS) and the substitution line Chinese Spring/Cappelle Desprez 7A (CS/CD7A) produced by Chapman et al. (2008) were used to define the genetic position of Pch2.

The T. durum line 301 produced by Huguet-Robert et al. (2001) and containing the Pch1 locus from Ae. ventricosa (Vent11) on chromosome 7A was crossed to Creso ph1c as described previously Huguet-Robert et al. (2001) to reduce further the size of the Ae. ventricosa introgression. Two lines, Red1 and Red5, were selected for further analysis based upon the presence of the 7A-specific SSR marker Xgwm698 in these lines (data not shown). Red1 and Red5 were characterized using the sets of markers for Pch1 and Pch2 developed within the present study along with a panel of 7A and 7D specific SSRs to better define the size of the Ae. ventricosa introgression in each line.

Seedling bioassays

The 92 CS × CS/CD7A F7 lines were tested for eyespot resistance against O. acuformis at the seedling stage. Five plants from each of the F7 lines were grown in 7 × 7 cm square pots containing peat and sand compost. Three replicate pots of each, with five plants per pot, were arranged in a complete randomized block design. The parental lines, CS and CS/CD7A, were included in each block as a control. The plants were grown for 2–3 weeks in a controlled-environment room (CER) at 8 °C and 8/16 h light/dark condition, then inoculated using inoculum slurry constrained within PVC cylinders, and incubated as described by Chapman et al. (2008). Plants were harvested 8 weeks after inoculation and scored for disease using the method described by Scott (1971). This experiment was subsequently repeated using identical methods to confirm the findings.

For the eyespot inoculation, a homogenized mixture of 25 isolates of O. acuformis was selected from the JIC culture collection. A mixture of different isolates was used to ensure that a successful infection was achieved in the case of lack of virulence of one or more isolates. Each isolate was grown on V8 agar (9 g of bacto-agar and 50 ml of V8 vegetable juice in 450 ml of deionised water) at 15 °C for 21 days prior to preparation of inoculum as described previously (Chapman et al. 2008).

Statistical analysis

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

**Map construction and QTL analysis**

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

The search for QTLs was done using the Single Trait Linkage Analysis of Genstat v.16 (Copyright 2009 Lawes Agricultural Trust, Rothamsted Experimental Station, UK) in three different steps: (1) initial genome-wide scan by simple interval mapping (SIM) to obtain candidate QTL positions; (2) one or more rounds of composite interval mapping (CIM), in the presence of cofactors, which are potential QTL positions detected at the previous step; and (3) fit the final QTL model. Default threshold based on the estimation of the effective number of tests (Li and Ji 2005) has been chosen for the QTL analysis.

**Molecular markers**

The mapping reported by Burt and Nicholson (2011) located *Pch1* to the region between the markers *Xcos7-6* (corresponding to *Bradi1g29690*) and *Xcos7-9* (corresponding to *Bradi1g29287*). To refine the position of *Pch1* on the chromosome 7D, markers were developed taking advantage of the syntenic relationship between *Brachypodium distachyon* (Bd) chromosome 1 and wheat Group 7 chromosomes. The *Pch1* homologous location on Bd chromosome 1 was identified in EnsemblPlants (http://plants.ensembl.org/Brachypodium_distachyon/Info/Index) and found to cover approximately a region of 424 Kb (Bd1:24810968-25235129) containing 43 genes from *Bradi1g29690* to *Bradi1g29287*.

Wheat genes on the group 7 chromosomes corresponding to each of these Bd genes were identified, and PCR primer pairs were designed on 23 of them at locations with high levels of sequence conservation between Bd and wheat to maximise the probability of obtaining successful amplification of the *Ae. ventricosa* introgressed DNA. These primers were tested on genomic DNA from HS and RVPM25, and PCR products were used in two different strategies for producing either Single-Strand Conformation Polymorphism (SSCP) or Kompetitive Allele Specific PCR (KASP) markers. In the first case, PCR products were examined by SSCP assay (Martins-Lopes et al. 2001) using Sequa Gel® MD (National Diagnostics, UK Ltd.) and visualised by silver staining (Bassam et al. 1991). For KASPs, instead, PCR products were sequenced and aligned to identify SNPs between *Ae. ventricosa* and wheat 7D. KASP marker PCR assays were manually designed on the basis of the identified SNPs.

A similar procedure was followed for identifying new markers to define the *Pch2* locus and to allow the genetic maps for 7A and 7D to be related to one another. *Pch2* was originally located on the chromosome 7A to a position between the markers *Xwm346* (SSR) and *Xorw1* (corresponding to *Bradi1g29400*) (Burt and Nicholson 2011). Due to the non-gene-based nature of SSR markers, it was impossible to identify a syntenic position for *Xwm346* on Bd1. Therefore, to ensure the entire coverage of the potential homologous region of *Pch2* on Brachypodium, a large Bd1 region containing about 100 genes was identified. As one of the aims of this work is to determine the homoeologous relationship between *Pch1* and *Pch2*, most attention was focused on developing markers to the overlapping segment of the two loci. Eight Bd genes were selected at equal intervals to span the region from *Bradi1g29480* at the proximal end of *Pch1* to *Bradi1g29960* at the distal end of *Pch2*.

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

A second set of KASP markers specific for the *Pch2* map was developed using the high-density single nucleotide polymorphism (SNP) genotyping iSelect array of 81,587 SNPs published by Wang et al. (2014) and available for 400 wheat lines on the CerealDB website (http://www.cerealsdb.uk.net/cerealgensomics/CerealsDB/iseselect_search.php). Both Chinese Spring (CS) and Cappelle Desprez were included among this panel. After analysing the complete set of SNPs mapping to 7A and polymorphic between the two parental lines CS and Cappelle Desprez, a set of SNPs located across the *Pch2* region interval was selected. KASP primers were designed using Polymarker, an automated bioinformatics pipeline for SNP assay development which is designed to increase the probability of generating homoeologue-specific assays for polyploid wheat [http://polymarker.tgac.ac.uk/ (Ramirez-Gonzalez et al. 2015)].

Thermodynamic properties of designed primers were verified after adding the standard FAM or HEX compatible tails (LGC ltd).
7A and 7D specific KASP markers were initially tested against the parental lines (CS and CS/CD7A, HS, and RVPM25, respectively), and the ones which were polymorphic between the parental line pairs were then applied to the F2 CsxCS/CD7A and BC6 F2 HSxRVPM25 populations, respectively.

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

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

A set of publicly available SSR markers was used to test the two T. durum lines Red1 and Red5 produced by Huguet-Robert et al. (2001). Markers were chosen to cover the 7A and 7D regions, where KASPs were not predictive. Primer sets were selected from the Wheat Microsatellite Consortium, as described within ‘GrainGenes’ (http://wheat.pw.usda.gov/GG2/index.shtml). PCR conditions for SSR amplification were as described previously with annealing temperatures as indicated by the ‘GrainGenes’ website. Samples were prepared by adding 1 µl of a 1:5 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.

Results

Refining the position of Pch1 on chromosome 7D

To refine the position of Pch1 on chromosome 7D, the available set of six COS and SSR markers (Xtr331, Xtr370, Xwg7s, Xorw5, Xbarc76, and Xcfld175) previously produced by Burt and Nicholson (2011) was supplemented by a set of 11 new markers, three SSCPs (X29550, X29570, and X29577) and eight KASPs (Xorw1_D, Xcos7-11_D, Xcos7-9_D, Xtr40_D, X29457, X29500, X29515, and X29560) developed utilizing the syntenic relationship between wheat and Bd. They were named using an “X” followed by a five digit number indicating the syntenic Bd gene and are listed in Table 1 along with primer sequences, the relevant syntenic Bd gene number, and the corresponding 7D wheat gene. Different names were used for Xorw1_D, Xcos7-11_D, Xcos7-9_D, and Xtr40_D to clearly indicate that new KASP markers were developed on the same genes previously described and mapped by Burt and Nicholson (2011). A set of 23 7D located wheat genes covering the Pch1 homologous location on Brachypodium chromosome 1 was used to design molecular markers, but only 11 of them were useful for development of 7D/ventricosa specific KASPs or SSCPs.

Two main reasons account for this low successful ratio (48 %): (1) PCR primers designed at locations with high levels of sequence conservation between Bd and wheat failed to amplify a specific PCR product on the Ae. ventricosa introgressed DNA (seven primer pairs; 30 %) and (2) after sequencing of PCR products, no useful SNPs between Ae. ventricosa and wheat 7D genes were identified (five primer pairs; 22 %).

These markers were applied to 2256 lines of the HS x RVPM25 BC6 F2 population [the original 1312 lines reported by Burt and Nicholson (2011) plus an additional 944 lines]. Six BC6 F2 plants from the 2256 tested (0.26 %) were identified with recombination events occurring between the KASP markers, two groups of co-segregating markers, Xtr40_D, X29500, X29550, X29570, and X29577 (corresponding to Brad1g29287 and Xcos7-9_D) were identified within the six plants. This probably reflects the very low recombination rate in the region, and suggests that they sit very close to or within the Ae. ventricosa introgressed segment, where the recombination is inhibited or hindered. The available recombination events were used to determine the marker order in the Pch1 region, and a new genetic map was constructed, as shown in Fig. 1. In the new version of the map, Pch1 spans a genetic region of 0.07 cM from the distal marker Xcos7-9_D (Brad1g29287) to the proximal group of co-segregating markers X29500-X29457-Xcos7-11_D-Xorw1_D-Xtr40_D.
The refined position of \( \text{Pch}1 \) on the chromosome 7D eliminates 19 genes of the syntenic region on Bd1 from the list of candidate genes in comparison with the previous version of the map published by Burt and Nicholson (2011). As a result, the \( \text{Pch}1 \) syntenic region of Bd1 is reduced from 424 to 250 Kb long and includes 24 genes (Fig. 1).

### Pch2 mapping and QTL analysis

A new set of 21 KASP markers was used to refine the position of \( \text{Pch}2 \) on chromosome 7A. 11 of these (X29577, X29570, X29560, X29550, X29457, Xorw1_D, Xcos7-11_D, Xtr40_D, and Xcos7-9_D) are gene-based markers and were developed taking advantage of the syntenic relationship between Bd and wheat. In particular, X29577, X29570, X29560, X29550, and X29457 were designed on 7A wheat genes, which, based on the synteny with Brachypodium, were determined to be located in the overlapping segment of \( \text{Pch}1 \) and \( \text{Pch}2 \). Moreover, Xcos7-11_A, Xorw1_A, and Xtr40_A are new gene-based KASP markers developed on the same genes previously described and mapped on the 7A map by Burt and Nicholson (2011).

The other ten markers (XBS1_30210, XBS2, XBS3_29990, XBS4_29980, XBS5, XBS6, XBS7, XBS8_29590, XBS9_29370, and XBS10) were developed from the high-density single nucleotide polymorphism (SNP) genotyping iSelect array (Wang et al. 2014) and are based on 7A specific SNPs able to distinguish between CS and CD. Only five of them could be related to wheat genes and their corresponding Bd gene code is indicated in their nomenclature (XBS1_30210, XBS3_29990, XBS4_29980, XBS8_29590, and XBS9_29370).
These two new sets of markers, the Bd-wheat based set (X) and the iSelect set (XBS), are listed in Table 2 along with primer sequences, corresponding Bd gene code (where available) and 7A wheat ESTs’ gene names where known.

These two sets of markers, together with the ones previously described by Burt and Nicholson (2011) (Xwmc346, Xwmc525, and Xcfa2040) were used to genotype 92 lines of the CS × CS/CD7A SSD F7 population, and a new genetic map of the region, spanning a total of 43 cM, was produced (Fig. 2).

Moreover, the same set of lines was phenotyped for eyespot resistance in two replicated and independent seedling disease bioassays inoculated with O. acuformis. It has been reported that Pch2 is more effective against O. acuformis than against O. yallundae (Burt et al. 2010). Earlier attempts to localise Pch2 were hindered because of the use of F3 families (still segregating for Pch2) rather than more advanced fixed lines for assessing disease resistance (Chapman et al. 2008, 2009). For these reasons, in the current work, disease assessment was carried out using an SSD F7 population challenged with isolates of O. acuformis.
Table 2  Summary of markers used to identify recombinants in the Pch2 region

| Marker name | Brachypodium gene | Wheat 7A homolog | BS SNP code | Marker type | Primer |
|-------------|-------------------|------------------|-------------|-------------|--------|
| X29390      | Bradi1g29390      | Traes_7AL_ACE7F3AC3 | –           | KASP        | gtttgtgtagaaagaggaagcagT  
gtttgtgtagaaagaggaagcagG  
acacataactttgactcgcg   |
| X29480      | Bradi1g29480      | Traes_7AL_1522CB55B | –           | KASP        | gttgtctggttctactcttGC  
gttgtctggttctactcttTG  
gcgcgtgtgtgtgtctactttat  |
| X29500      | Bradi1g29500      | Traes_7AL_AFA2C29C9 | –           | KASP        | ggtgtgtgtagaaagaggaagcagA  
ggtgtgtgtagaaagaggaagcagC  
agagagagagagagagagagatgag   |
| X29560      | Bradi1g29560      | Traes_7AL_3F2C16688 | –           | KASP        | tctctctcttataagtagttT  
tctctctcttataagtagttG  
gacgactgctgttgcctagctag   |
| X29776      | Bradi1g29776      | Traes_7AL_13AE7EEC0 | –           | KASP        | atttaggtgagaaagaggaatacA  
atttaggtgagaaagaggaatacG  
tagctgtgtagaaagaggaagcag   |
| X29792      | Bradi1g29792      | Traes_7AL_DA8343C6B  | BS00072731  | KASP        | cctctctcttataagtagttatc  
cctctctcttataagtagttatcA  
cctctctcttataagtagttatcG  
cctctctcttataagtagttatcT  
cctctctcttataagtagttatcC  
cctctctcttataagtagttatcG  
cctctctcttataagtagttatcA  
cctctctcttataagtagttatcC  
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cctctctcttataagtagttatcC  
cctctctcttataagtagttatcG  
cctctctcttataagtagttatcA  
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cctctctcttataagtagttatcC  
cctctctcttataagtagttatcG  
cctctctcttataagtagttatcA  
cctctctcttataagtagttatcC  
cctctctcttataagtagttatcG  
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cctctctcttataagtagttatcC  
cctctctcttataagtagttatcG  
cctctctcttataagtagttatcA  
cctctctcttataagtagttatcC  
cctctctcttataagtagttatcG  
cctctctcttataagtagttatcA  
cctctctcttataagtagttatcC  
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An interval mapping QTL analysis was carried out combining marker data with phenotypic results, and it revealed that the \( Pch2 \) QTL peaks at \( Xcfa2040 \) in the two bioassays explaining 26.1 and 33.5% of the phenotypic variance observed in the first and second tests, respectively (Table 3). The combined data from the two trials also revealed that \( Xcfa2040 \) (LOD = 10.41) was the marker most significantly associated with resistance (\( Pch2 \)) explaining 42.4% of the phenotypic variance observed. Overall, the analysis determined that the \( Pch2 \) QTL region spans 15.6 cM in the interval between \( XBS1_30210 \) and the co-segregating group of markers \( XBS3_29990 \)-\( XBS4_29980 \)-\( X29960 \)-\( XBS5 \) (Fig. 3; Table 3).

**Pch1–Pch2 homoeology**

The new genetic maps of 7D and 7A with the respective positions of \( Pch1 \) and \( Pch2 \) were then used to examine the potential homoeology between the two loci. The two maps were aligned to the relevant region of chromosome 1 of Bd on the basis of the overall syntenic relationship with Bd. Where possible, marker positions on both 7A and 7D chromosomes were anchored to the Bd gene-spaced map of the region (Fig. 4), and co-linearity was checked for both chromosomes against Bd1. Overall co-linearity was conserved across the entire length of the \( Pch1 \) map. However, a breakdown of the micro synteny was identified in the \( Pch2 \) map, where \( X29447 \), \( X29776 \), \( X29370 \), and \( Xcos7-11\_A \) seem to be located in a different position in respect to the physical order in Bd1 (Fig. 4). Using Bd1 as reference, the homoeologous region to \( Pch1 \) on 7D was identified on chromosome 7A. The equivalent locus was located in a genetic region of 0.5 cM between the two co-segregating groups of markers \( X29500 \)-\( X29480 \)-\( Xorw1\_A \) and \( X29390 \)-\( Xcos7\_11\_A \)-\( Xtr40\_A \). The genetic distance between the most proximal of these groups (\( X29500 \)-\( X29480 \)-\( Xorw1\_A \)) and the most distal group of markers (\( XBS3_29990 \)-\( XBS4_29980 \)-\( X29960 \)-\( XBS5 \)) for the \( Pch2 \) QTL was 23.6 cM. On the basis of these new findings, it is clear that the \( Pch1 \) and \( Pch2 \) respective regions on chromosomes 7D and 7A do not overlap, and hence, there is no evidence that they represent homoeoloci.

**Molecular characterization of \( T. \) durum wheat lines carrying \( Pch1 \) on 7A**

Huguet-Robert et al. (2001) produced meiotically stable lines carrying \( Pch1 \) on chromosome 7A in a background of the tetraploid \( T. \) durum variety Creso. Initially, a hybrid was obtained by crossing \( Ae. \) ventricosa accession 11 (Vent 11) containing \( Pch1 \) on the 7D\( ^{v} \) chromosome and \( T. \) durum cv. Creso \( ph1c \) mutant. The \( Ph1 \) gene suppresses homoeologous pairing in wheat (Sears and Oka- moto 1958), and mutations of this gene (\( ph1b \) and \( ph1c \)) were used to increase the chance of transferring \( Pch1 \) containing 7D\( ^{v} \) segment to wheat 7A or 7B chromosomes by homoeologous recombination. The hybrid was colchicine doubled and then put through a series of backcrossing to Creso \( ph1c \) and the hexaploid wheat variety Courtot \( ph1b \).
with the aim of reducing and stabilising the chromosome number. Five homozygous eyespot resistant lines containing either 28 or 29 chromosomes were selected from this cross. The \( Pch1 \) transfer in these lines was deduced to be located to the long arm of chromosome 7A, based on the apparent loss of the 7A specific SSR marker \( Xgwm698 \) and the \( Ep-A1 \) protein. This suggested that a recombination event had occurred between 7A and 7D\( ^v \) and so eliminating the region of 7A harbouring these loci. Some of these lines were used to produce F\(^3\) families in a backcross to \( Creso \) \( ph1c \) to further reduce the size of the \( Pch1 \) introgression, and two homozygous eyespot resistant lines derived from line 301 (Huguet-Robert et al. 2001), namely, Red1 and Red5, were selected as potentially containing relatively small introgressions.

To better characterize the nature and size of the \( Pch1 \) introgression on chromosome 7A of these two lines, they were assayed for the presence of three 7D specific KASPs located within the region of the \( Pch1 \) locus (\( X29500 \), \( Xtr40_D \), and \( Xcos7-11_D \)). The three KASP assays revealed that Red1 and Red5 contained \( Ae. \) \( ventricosa \) alleles, so confirming that the \( Pch1 \) locus had been transferred to the end of 7A chromosome. In an attempt to establish the extent of the \( Ae. \) \( ventricosa \) introgression and

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**Fig. 2** Comparison of the wheat chromosome 7A genetic map, including \( Pch2 \), published by Burt and Nicholson (2011) and the new version of the same map using physical marker locations on Brachypodium chromosome 1 as a reference. a \( Pch2 \) genetic map published by Burt and Nicholson (2011). b Gene-spaced map of Brachypodium chromosome 1. c New genetic map of \( Pch2 \)
determine whether it extended into the region of Pch2, two 7A specific SSRs (Xwmc525 and Xcfa2040) were run on the parental lines (Creso ph1c, Courtot ph1b, and Ae. ventricosa 11) and the two recombinant lines Red1 and Red5. Capillary electrophoresis of the Xwmc525 and Xcfa2040 PCR products revealed that they were identical to those of Creso, the T. durum recurrent parent (Fig. 5). This evidence indicates that the 7Dv introgression on 7A does not extend into the region, where Pch2 is located. In the original report, evidence was provided that a portion of the Courtot hexaploid wheat parent was also transferred to the 7A chromosome of the T. durum (Huguet-Robert et al. 2001). The analysis of the lines using three 7D specific SSRs (Xorw5, Xwgs7S, and Xtr383) SSRs revealed that the product size detected in Red1 and Red5 was identical to that of Courtot (Fig. 5). These findings confirm the chimaeric nature of the arrangement of the 7A chromosome previously found by Huguet-Robert et al. (2001), and it is schematically represented in Fig. 5.

Discussion

The main objective of this work was to refine the position of Pch1 and Pch2 on wheat chromosome 7D and 7A, respectively, to determine whether they are homoeoloci. The evidence generated by the present study clearly indicates that the two genes are not homoeoloci, and are genetically independent.

The Pch1 resistance gene was introgressed to wheat from Ae. ventricosa. The cloning of this gene has been hampered by two main factors: marker availability and recombination rate. To deal with these issues, we exploited the syntenic relationship between Brachypodium and wheat for identifying a list of Conserved Orthologous Sequence (COS) markers located inside the Pch1 region on chromosome 7D. These genes were used to tag interspecific (ventricosa-wheat) SNPs on which KASP markers were developed. These markers were then applied to the HS × RVPM25 BC6F2 population to discover new recombinants, and consequently, Pch1 was located to a very small genetic region spanning only 0.07 cM between Xcos7-9_D and the group of co-segregating markers X29947-X29940-X29457-Xcos7-11_D-Xorw1_D-Xtr40_D (Fig. 1). Despite the larger number of lines screened (4512 gametes), a total of only 36 recombination events were identified between the two most external markers Xbarc76 and Xcdf175, corresponding to recombination rate of 0.8 %. Considering that the rate of recombination between homoeologous chromosomes of wheat and wild grass relatives is estimated at around 3 % (Niu et al. 2011), our results suggest that the actual Pch1 physical region is very small and that a recombination event within the introgressed segment is very unlikely to be observed. Further confirmation of this hypothesis is that the Pch1 corresponding genetic region mapped on the chromosome 7A in the cross CS × CS/CD7A (wheat × wheat), in which the recombination rate should be normal, spans only 0.5 cM (Fig. 4). Finally, in respect to the previous version of the map published by Burt and Nicholson (2011), the refined position of Pch1 on the 7D eliminates 19 genes of the orthologous region on Bd1 (Fig. 1), which now physically spans only 250 Kb and contains 24 annotated genes. Taken together, these results are encouraging with respect to efforts towards cloning the Pch1 gene, but indicate that

| Locus     | Genetic position | Trial 1 LOD* score | Trial 2 LOD* score | Combined data LOD* score |
|-----------|------------------|--------------------|--------------------|--------------------------|
| XBS1_30210 | 0                | 2.35               | 3.54               | 3.54                     |
| XBS2      | 0.7              | 2.63               | 3.79               | 3.99                     |
| Xwmc346   | 2.7              | 4.02               | 3.9                | 4.7                      |
| Xwmc525   | 9.6              | 6.18               | 5.54               | 7.46                     |
| Xcfa2040  | 10.9             | 7.42               | 7.32               | 10.41                    |
| X29994    | 15.2             | 5.92               | 4.4                | 5.03                     |
| X29960    | 15.6             | 5.76               | 4.39               | 5.01                     |
| XBS4_29980| 15.6             | 5.76               | 4.39               | 5.01                     |
| XBS3_29990| 15.6             | 5.76               | 4.39               | 5.01                     |
| XBS5      | 15.6             | 5.76               | 4.39               | 5.01                     |
| XBS7      | 20.3             | 3.08               | –                  | –                        |
| XBS6      | 20.3             | 3.08               | –                  | –                        |
| X29792    | 26.9             | 2.79               | –                  | –                        |

QTL effect: Xcfa2040 Xcfa2040 Xcfa2040

% Expl. Var: 26.1 33.5 42.4

*a* LOD score = −Log10(P). Significance threshold is 2.079
different approaches have to be considered to overcome the biological (lack of recombination) and technical (marker saturation) limitations. The conventional approach would involve obtaining the physical sequence spanning the genetic interval through the screening of *ad hoc* created BAC libraries of the resistant genotype to identify a short list of candidates and test their nature by complementation. However, with the advent of the next-generation sequencing technologies, powerful alternatives to this conventional approach include mutagenesis, successfully used for cloning single-copy disease resistance genes in wheat (Feuillet et al. 2003; Periyannan et al. 2013), or exome capture methodologies and sequencing (Gardiner et al. 2014; Henry et al. 2014), which facilitate the cloning of genes from wheat and its relatives.

To refine the position of *Pch2*, we exploited the syntenic relationship between wheat and Brachypodium together with the high-density SNP genotyping 90 K array iSelect (Wang et al. 2014) to develop a new set of 20 KASP markers. The QTL analysis performed combining marker and phenotypic data resulted in positioning *Pch2* in a genetic region of 15.6 cM between *XBS1_30210* and the co-segregating group of markers *XBS3_29990-XBS4_29980-X29960-XBS5* (Fig. 3). Although this work used an increased number of markers combined with phenotyping of *F*$_7$ lines, the genetic size of the interval containing *Pch2* remains unchanged in comparison with the previous version published by Burt and Nicholson (2011). Possible explanations for this could be the moderate phenotypic effect of *Pch2* (Burt et al. 2010; Chapman et al. 2008), which makes it difficult to monitor small differences in susceptibility using a visual disease scoring method based on leaf sheath penetration (Scott 1971) as well as the small size of the population (97 lines) used to perform the QTL analysis. However, the distal edge of the *Pch2* locus moved from the *Xcos7-11_A* marker to the group of co-segregating

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**Fig. 3** QTL interval mapping analysis of *Pch2* resistance to *Oculimacula acuformis* of the 92 wheat CS×CS/CD7A 7A *F*$_7$ lines. This LOD profile derives from combined data of two CER trials. Genetic map data are aligned to the LOD profile.
markers XBS3_29990-XBS4_29980-X29960-XBS5, which sits 24.1 cM away from Xcos7-11_A (Fig. 2). Moreover, using Brachypodium as a syntenic reference, this distance (from Xcos7-11_A corresponding to Bradi1g29441 to X29960 corresponding to Bradi1g29960) corresponds to a region containing 56 genes on Bd1 (Fig. 2).

In this work, two KASP markers, Xorw1_A and Xorw1_D, were designed to the 7A (Traes_7AL_D1DB-1B9EE) and 7D (Traes_7DL_DBBA5FD6C) orthologous genes of Bradi1g29400. Xorw1_D is the KASP replacement of the STS marker (Xorw1) developed by (Leonard et al. 2008) towards the wheat EST AB246917. AB246917 is 96 and 91 % similar to Traes_7DL_DBBA5FD6C and Bradi29400, respectively, and encodes the endopeptidase protein Ep-D1b. A number of the previous studies detected a complete linkage between Pch1 and either the isozyme marker (McMillin et al. 1986; Santra et al. 2006) or the STS marker (Burt and Nicholson 2011; Leonard et al. 2008; Meyer et al. 2011). In accordance with these studies, our data also did not identify any recombination event between Xorw1_D and Pch1, not making it possible to refute the hypothesis that Ep-D1b could be a candidate for Pch1. However, Mena et al. (1992) reported recombination between Pch1 and allele Ep-D1b leading to the production

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Fig. 4 Comparison of the location of Pch1 on the HSxRVPM25 genetic map of chromosome 7D (Ta7D) with the CSxCS/CD7A chromosome 7A genetic of Pch2 using physical marker locations on Brachypodium chromosome 1 (Bd1) as a reference.
of a line susceptible to eyespot but containing the Ep-D1b allele. Moreover, Huguet-Robert et al. (2001) obtained durum wheat lines using a second Ae. ventricosa accession (Vent 11), which are resistant to eyespot but lack a functional version of the Ep-D1 endopeptidase. Taken together, these results suggest that, while Ep-D1b is very closely linked to Pch1, it is not, itself, responsible for the enhanced eyespot resistance.

Koebner and Martin (1990) reported the eyespot resistance on the chromosome 7A of Cappelle Desprez to be associated to the endopeptidase allele Ep-A1b, and the isozyme marker of Ep-A1b was located at the end of chromosome 7A by de la Peña et al. (1997). In this work, we developed a KASP marker (Xorw1_A) based on Traes_7AL_D1DB1B99E. Several lines of evidence lead us to consider this gene to be a good candidate for the Ep-A1b gene: Traes_7AL_D1DB1B9EE encodes an oligopeptidase B domain; it is located at the end of chromosome 7A (181.240 genetic bin); and it is the orthologous gene of Bradi29400, positioned in the Pch1/Pch2 syntenic region of Bd1. Thus, Xorw1_A can be considered the first DNA-based replacement of the isozyme marker for Ep-A1b. Our data revealed that the recombination between Pch2 and Xorw1_A was extensive with Xorw1_A being 28.1 cM from the Pch2 peak marker (Xcfa2040) (Fig. 2). These results confirm the findings of de la Peña et al. (1997) who mapped the isozyme marker for Ep-A1b 32.8 cM distal to Pch2. However, the genetic distance of Xorw1_A from the QTL peak suggests that this marker is not suitable for the selection of Pch2 resistance in wheat breeding.

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

The data from the present study do not support the view that Pch1 and Pch2 are homoeologous. In fact, we found that the homoeologous region to Pch1 on chromosome 7A is located 23.6 cM away from the group of co-segregating markers (XBS3_29990-XBS4_29980-X29960-XBS5) that define the distal end of the Pch2 QTL (Fig. 4). The most probable explanation for these discordant results is the difference in the resolution of genotyping and phenotyping of the plant material. We performed the eyespot resistance test on F7 families from the CS × CS/CD7A cross, minimising the influence of heterozygosity on phenotyping the moderately potent effect of Pch2. Moreover, adding a total of 11 and 21 new markers, respectively, to the Pch1 and Pch2 genetic maps allowed us to refine their positions on the respective chromosomes making a clear separation between them. In conclusion, our results reveal that Pch1 and Pch2 do not overlap and that they do not appear to be homoeologous.

Finally, in this study, we have characterized Red1 and Red5 lines carrying Pch1 on chromosome 7A in a background of the tetraploid T. durum variety Cresco produced by Huguet-Robert et al. (2001).

Our aim was to define the nature and size of the Pch1 introgression on chromosome 7A of these two lines and determine whether it extended into the region of Pch2. Marker analysis performed using three 7D specific KASPs located within the Pch1 locus (X29500, Xtr40_D, and Xcos7-11_D) and two 7A specific SSRs (Xwmc525 and Xcfa2040) located within the Pch2 locus confirmed that Red1 and Red5 both contained the Pch1 locus which had been transferred to the end of 7A chromosome. In addition, both the lines were identical to Cresco, the T. durum recurrent parent in the Pch2 region. Thus, the Ae. ventricosa introgression in these lines does not extend into the
region of the 7A chromosome, where Pch2 is located, making Red1 and Red5 potentially very interesting materials for future breeding programs to improve eyespot resistance. For example, it would be possible to cross these lines to a hexaploid wheat line containing Pch2 and take advantage of recombination between 7A chromosomes from tetraploid and hexaploid wheat, to pyramid Pch1 and Pch2 in a tetraploid background. In addition, based on the finding that plants homozygous for Pch1 are significantly more resistant than those that are heterozygous (Huguet-Robert et al. 2001), Red1 and Red5 can be used to create hexaploid genotypes with an increased copy number of Pch1, with one copy on 7D and one on 7A. Furthermore, it would be possible to cross them to Pch1 containing lines, such as Rendezvous or Lynx, which also contain Pch2 to produce lines containing both Pch2 and one copy of Pch1 on the chromosome 7A and one copy of Pch1 on the 7D.

In conclusion, we provide evidence that does not support the view that Pch1 and Pch2 are homoeoloci. Consequently, this result has implications for the cloning of Pch1, because it prevents Pch2 being used as a proxy target in which the higher level of recombination in wheat × wheat crosses could be exploited to fine map and identify the causal gene. On the other hand, the high level of wheat-Brachypodium syntenic has been proven to be extremely helpful for guiding the development of new molecular markers and the identification of candidate genes, as a means to towards the cloning of Pch1. At the same time, the refined positions of Pch1 and Pch2 allowed us to characterize some potentially interesting material for pyramiding and/or increasing the copy number of different sources of eyespot resistance.

Author contribution statement MP, PN, CB, and CU made substantial contributions to the conception and design of the work. MP and JH carried out the acquisition and analysis of data and together with PN, CB, and JJ performed interpretation of data. JJ, SP, and CB contributed to the germplasm development used in the experiments. MP and PN wrote the paper. All the authors participated in revising it critically and gave approval of the final version to be submitted.

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Compliance with ethical standards

Ethical statement This manuscript is original, has not been published before, and is not currently being considered for publication elsewhere. Moreover, the manuscript has been read and approved by all named authors, and there are no other persons who satisfied the criteria for authorship but are not listed. The order of authors listed in the manuscript has been approved by all co-authors. Informed consent was obtained from all individual participants included in the study. Finally, this manuscript does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

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