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ORIGINAL PAPER

Identification of a candidate gene for the wheat endopeptidase *Ep-D1* locus and two other STS markers linked to the eyespot resistance gene *Pch1*

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Abstract Wheat is prone to strawbreaker foot rot (eyespot), a fungal disease caused by *Oculimacula yallundae* and *O. acuformis*. The most effective source of genetic resistance is *Pch1*, a gene derived from *Aegilops ventricosa*. The endopeptidase isozyme marker allele *Ep-D1b*, linked to *Pch1*, has been shown to be more effective for tracking resistance than DNA-based markers developed to date. Therefore, we sought to identify a candidate gene for *Ep-D1* as a basis for a DNA-based marker. Comparative mapping suggested that the endopeptidase loci *Ep-D1* (wheat), e*np1* (maize), and *Enp* (rice) were orthologous. Since the product of the maize endopeptidase locus *enp1* has been shown to exhibit biochemical properties similar to oligopeptidase B purified from *E. coli*, we reasoned that *Ep-D1* may also encode an oligopeptidase B. Consistent with this hypothesis, a sequence-tagged-site (STS) marker, *Xorw1*, derived from an oligopeptidase B-encoding wheat expressed-sequence-tag (EST) showed complete linkage with *Ep-D1* and *Pch1* in a population of 254 recombinant inbred lines (RILs) derived from a cross between wheat cultivars Coda and Brundage. Two other STS markers, *Xorw5* and *Xorw6*, and three microsatellite markers (*Xwmc14*, *Xbarc97*, and *Xcfd175*) were also completely linked to *Pch1*. On the other hand, *Xwmc14*, *Xbarc97*, and *Xcfd175* showed recombination in the W7984 × Opata85 RIL population suggesting that recombination near *Pch1* is reduced in the Coda/Brundage population. In a panel of 44 wheat varieties with known eyespot reactions, *Xorw1, Xorw5*, and *Xorw6* were 100% accurate in predicting the presence or absence of *Pch1* whereas *Xwmc14*, *Xbarc97*, and *Xcfd175* were less effective. Thus, linkage mapping and a germplasm survey suggest that the STS markers identified here should be useful for indirect selection of *Pch1*.

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Introduction

Eyespot, a fungal disease of wheat (*Triticum aestivum* L.) and other cereals (Wiese 1987), is caused by *Oculimacula yallundae* (Wallwork & Spooner) Crous & W. Gams (syn: *Tapesia yallundae*, *Pseudocercosporella herpotrichoides* var. *herpotrichoides*) and *O. acuformis* (Boerema, R. Pieters & Hamers) Crous & W. Gams (syn: *Tapesia acuformis*, *Pseudocercosporella herpotrichoides* var. *acuformis*) (Crous et al. 2003). Eye-shaped eliptical lesions on the lower portion of the stem give name to the disease also known as strawbreaker or footrot. Winter wheats grown in areas of high rainfall and moderate winters such as northwestern Europe and the Pacific Northwest (PNW) region of the United States are particularly vulnerable to infection. Severe infections can result in yield losses of up to 50% (Fitt et al. 1988). Fungicidal controls can be used but

require additional inputs and present some environmental concerns. In addition, the number of fungicide-resistant strains of the pathogen recovered from fields in the PNW has been increasing over time limiting the usefulness of this approach (Murray 1996). Thus, development of wheat cultivars with genetic resistance has been recognized as the most cost-effective and environment-friendly strategy.

Three sources of genetic resistance (*Pch1*, *Pch2*, and *Pch3*) are currently available for use in wheat. *Pch1* was introduced from *Aegilops ventricosa* Tausch into the hexaploid wheat breeding line VPM-1 (*Ae. ventricosa*/*T. persicum*//3*Marne) (Maia 1967; Doussinault et al. 1983) and mapped to the long arm of chromosome 7D (Worland et al. 1988). Since the 1950s, the French variety Cappelle-Desprez has been used as a source of the seedling-stage resistance provided by *Pch2*. This gene has been mapped to the long arm of chromosome 7A (de la Peña et al. 1997). A third potential source of resistance, $PchD^V$ (*Pch3*) that has not been tested in commercial wheat cultivars, has been identified and mapped to chromosome 4V of *Dasypyrum villosum* (L.) Candargy (Yildirim et al. 1998). Of these three genes, *Pch1* has been the most widely used in development of eyespot-resistant wheat cultivars.

Since bioassays or field screening for resistance can be difficult, there is both an interest and a need to identify a facile marker for indirect selection. Close association between *Pch1* and a codominant endopeptidase marker allele, *Ep-D1b*, was demonstrated by McMillin et al. (1986) and subsequently used to monitor introgression of eyespot-resistance (*Pch1*) from VPM-1 to elite lines. Improvements in the endopeptidase assay have recently been published and this assay was found to be completely accurate in predicting resistance (Santra et al. 2006). Since it is often difficult to distinguish the *Ep-D1b* allele from certain commonly occurring *Ep-A1* and *Ep-B1* homoeoalleles (Koebner et al. 1988), a more specific DNA-based marker would be desirable. Recently, an AFLP-derived microsatellite marker *XustSSR2001-7DL* closely linked to the *Ep-D1* locus has been reported (Groenewald et al. 2003). The authors observed two recombinants among 98 segregating progeny implying *XustSSR2001-7DL* lies approximately 2 cM distant from the *Ep-D1* locus. Therefore, it was not unexpected that Santra et al. (2006) found the microsatellite marker to be only 90% accurate in predicting the phenotype (presence or absence of *Pch1*) in 38 lines surveyed. Thus, the development of a DNA-based marker to replace the *Ep-D1b* isozyme assay has not been achieved.

The main objective of this study was to identify a wheat EST candidate for *Ep-D1* to be used as a basis for designing a DNA-based marker tightly linked to *Pch1*. Observations regarding both the biochemical properties and chromosomal locations of putative homoeologous endopeptidases from wheat, rice (*Oryza sativa* L), and maize (*Zea mays* L) suggested that *Ep-D1* may encode a serine protease, oligopeptidase B. The report of a purified wheat oligopeptidase B linked to a wheat EST provided a starting point for marker development. Here, we present the identification of this candidate gene and two other DNA-based markers more closely linked to *Pch1* than *XustSSR2001-7DL*. We also report an apparent reduction in recombination surrounding *Pch1* in the population used in this study.

Materials and methods

Plant materials

A collection of 44 wheat accessions (related by pedigree) was used (Table 1). Twenty-two of these lines were eyespot-resistant and carried *Pch1* whereas, 22 were eyespot-susceptible and did not carry *Pch1*. This panel of genotypes was used to test candidate markers for association with *Pch1*. Markers that showed an association with *Pch1* were genetically mapped using a population of 254 F_6-derived recombinant inbred lines (RILs) from a cross between the eyespot-resistant club wheat cultivar Coda (Allan et al. 2000) and the eyespot-susceptible common wheat cultivar Brundage (Zemetra et al. 1998). Genetic mapping of some microsatellite markers was also carried out using 94 RILs of the International Triticeae Mapping Initiative (ITMI) W7984 × Opata85 population (Nelson et al. 1995).

DNA isolation, molecular markers, and mapping

DNA was extracted from approximately 50 mg of leaf tissue using the protocol of Riera-Lizarazu et al. (2000). Sequences for previously mapped microsatellites were obtained from the GrainGenes website (http://wheat.pw.usda.gov/GG2/index.shtml). BLAST searches were conducted on databases at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), The J. Craig Venter Institute (http://www.tigr.org/), and the Gene Index Project at the Dana-Farber Cancer Institute (http://compbio.dfci.harvard.edu/tgi/). Primer sequences for new STS markers and the DNA sequence accessions used for their design are listed in Table 2. Polymerase chain reactions (PCR) were carried out in 10-µl volumes comprising 0.3 U *Taq* polymerase with 1X PCR buffer containing 1.5 mM $MgCl_2$ (Qiagen, Valencia, CA, USA), 2% sucrose in 0.04% cresol red, 0.2 mM of each dNTP, and 0.5 µM of each primer. The PCR consisted of denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 50–60°C (depending on primers) for 30 s, and 72° for 1 min, with final extension at 72°C for 5 min. Microsatellite-specific primer pairs that produced PCR products with size polymorphisms greater than 4 bp were resolved on 3%

Table 1 Germplasm used for association mapping of *Pch1*-related markers

Line	Accession[a]	Pedigree	*Pch1* present
Chukar	PI 628641	WA 7665/Rulo	Yes
Coda	PI 594372	Tres//Madsen/Tres	Yes
Finch	PI 628640	Dusty//Wa7164/Dusty	Yes
FR-50	PI 494183	VPM-1/McCall	Yes
Hyak	PI 511674	VPM-1/Moisson 421 //2*Tyee	Yes
Madsen	PI 511673	VPM-1/Moisson 951//2* Hill 81	Yes
Rendezvous[b]		VPM-1/Hobbit//Virtue	Yes
Roazon	PI 422330	VPM-1/Moisson	Yes
Rulo	PI 578137	Tyee//Roazon/Tres	Yes
Simon	PI 636132	Haven/Lambert//Madsen'	Yes
Temple	PI 599665	Tres/VPM-1	Yes
Tubbs	PI 629114	Madsen/Malcolm	Yes
VPM-1	PI 519303	*Ae ventricosa*/*T. persicum*/3*Marne	Yes
VPM-1/Moisson 421[c]		VPM-1/Moisson	Yes
VPM-1/Moisson 951[c]		VPM-1/Moisson	Yes
WA 7217	PI 561035	VPM/Moisson-951//2*Barbee	Yes
WA 7621	PI 566815	VPM/Moisson 421/2/VH 66354/WA 5827/WA 6241/3/Tres	Yes
WA 7625	PI 561031	VPM-1/Moisson 951//2*Hill 81	Yes
WA 7666	PI 561030	VPM/Moisson 951//CI 13438	Yes
WA 7671	PI 566816	VPM-1/Moisson 421//VH-66354/WA 5827/WA 6241/3/2*Hill 81	Yes
WA 7690	PI 597665	VPM-1/Moisson 951//Yamhill / Hyslop/Hill 81/3/WA6910	Yes
Weatherford	PI 602861	Malcolm/3/VPM/Moisson 951//Hill/4/VPM/Moisson 951//2*Hill	Yes
Barbee	CItr 17417	WA 3969//PI 178383/Vogel-1	No
Brundage	PI 599193	Stephens/Geneva	No
Capelle Desprez	PI 262223	Vilmorin 27/Hybride du Joncquois	No
Daws	CItr 17419	WA 4877/VH 66336	No
Dusty	PI 486429	Brevor/CI 15923//Nugaines	No
Haven	PI 592150	Hedgehog/Norman//Moulin	No
Hill 81	CItr 17954	Yamhill/Hyslop	No
Hobbit	PI 428521	Professeur Marchal//Marne Desprez/VG-9144/3/TJB-16	No
Hyslop	CItr 14564	Nord Desprez/2*Pullman101	No
Lambert	PI 583372	Stephens/Sprague	No
Malcolm	PI 497672	Cappelle Desprez/Pullman 101//Bezostaya/3/Stephens	No
Moisson	PI 315998	Cappelle Desprez//Hybride 80–3/Etoile de Choisy	No
Peck	CItr 17298	Gaines/CI 17250	No
Pullman Selection 101	CItr 13438	Norin 10/Brevor//Orfed/Hybrid 50/3/Burt	No
Stephens	CItr 17596	Nord Desprez/Pullman Selection 101	No
Tres	CItr 17917	Suwon 92 /6*Omar/3/Tr .Sp/Coastal//3*Omar	No
Tyee	CItr 17773	Vogel-1/CI 78051//CI 13447 /3/3*Omar	No
Vilmorin 27	PI 125093	Dattel//Japhet/Parsel/3/Hatif Inversable/Bon Fermier	No
Virtue	PI 447429	Maris Huntsman/Maris Durin	No
WA 5827	PI 518952	PI 178383/2*Burt//Omar/WA 1834	No
WA 7665	PI 561029	Tyee//Capelle Desprez/Tres	No
Yamhill	CItr 14563	Heines VII/Alba	No

[a] Accession starting with CItr and PI were obtained from the US Department of Agriculture, National Small Grains Collection, Aberdeen, ID, USA

[b] Seed for the variety Rendezvous was obtained from Dr. Xianming Chen, US Department of Agriculture, Agricultural Research Service, Pullman, WA, USA

[c] Seed for VPM-1/Moisson 421 and VPM-1/Moisson 951 were obtained from Dr. Robert E. Allan, US Department of Agriculture, Agricultural Research Service, Pullman WA, USA

Table 2 Target gene, primer sequence, approximate product size, and annealing temperatures for three *Pch1*-linked STS markers

Putative gene	Primer	Primer sequence	Accession number	*T*m (°C)	Product size (bp)[b]
Oligopeptidase B	orw1F	5′-CTATTACATGAAATCTTATTCTCC-3′	AB246917[a]	55	180
	orw1R	5′-CAGCAGTAACGAGAATGTGG-3′			
Callose synthase	orw5F	5′-GCATCCTCGCCTTCATGC-3′	TC252872[c]	56	Null
	orw5R	5′-CGACCATCTCGACCACAGG-3′			
PSII assembly factor Hcf136	orw6F	5′-AGGGCCGCAGATAACATCC-3′	TC268469[c]	56	166
	orw6R	5′-CACAAACCCTTGGTTGTCG-3′			

[a] Genbank accession number, NCBI (http://www.ncbi.nlm.nih.gov/)

[b] Amplification product fragment size produced from DNA from *Pch1*-carrying genotypes

[c] Tentative contig from the Wheat Gene Index; Gene Index Project, Dana-Farber Cancer Institute (http://compbio.dfci.harvard.edu/tgi/tgipage.html)

agarose gels. Otherwise, one primer per pair was labeled with a fluorescent dye and fragment analysis was carried out using an ABI Prism 3100 Genetic Analyzer. ABI GeneScan, version 2.1, and Genotyper, version 2.0, software (Applied Biosystems, Foster City, CA, USA) were used to size fragments based on an internal lane standard. Maps were constructed using Joinmap 3.0 (Van Ooijen and Voorrips 2001) utilizing the Kosambi mapping function.

Endopeptidase assay

The allelic constitution at the *Ep-D1* locus for Coda (*Ep-D1b*), Brundage (*Ep-D1a*), and the Coda/Brundage RIL population was determined using the endopeptidase assay described by Santra et al. (2006). Wheat seeds 50% submerged in tap water were grown 5–6 days at ambient temperatures (20–25°C) with 16 h days under the light of a standard 60 W tungsten electric bulb. Proteins extracted in 10 µL of ice-cold extraction buffer from ~4 cm root samples were absorbed onto 4 × 10 mm Whatman No.3 filter paper strips and electrophoresed on an 18% starch gel (Starch Art Corp., Smithville, TX, USA) for 3–3.5 h at 4°C under a constant current of 40 mA. Gel slices were incubated for 1-2 h in staining solution [25 ml 0.1 M Tris–Maleate-NaOH, pH 6.4, containing 6.4 mg Black K Salt and 2.8 mg N-α-Benzoyl-DL-Arginine β-Naphthylamide (BANA)] at ambient temperature in the dark.

Disease screening

The allelic constitution with respect to the *Pch1* locus in Coda (*Pch1* present), Brundage (*Pch1* absent), and the Coda/Brundage RIL population was determined using bioassays for strawbreaker foot rot resistance according to Macer (1966) with some modifications. Four replicates of five plants per RIL were grown in 73 well-trays (Growing Systems Inc., Milwaukee, WI) along with the two parents, Brundage and Coda, in a Conviron GR48 growth chamber (Controlled Environments Limited, Winnipeg, MB, Canada) set at a 16 h/24°C days and 15°C nights. Upon emergence, a 3-cm plastic straw cylinder was placed over the coleoptiles to hold inoculum and to improve uniformity of contact with the stem base. After 2 weeks, a plate of actively growing *O. yallundae* was blended in 250 mL sterile water to produce an inoculum slurry. Plants were individually inoculated with 500 µL of inoculum (~1×10^5 conidia mL^{-1}). Two weeks after inoculation the photoperiod was reduced to 12 h at 20°C. After another week, day length was reduced to 10 h, and day/night temperature was reduced to 4/4°C over a 5-day period then steadily maintained for 6 weeks.

The first set of 94 RILs plus parents (Coda and Brundage) were visually rated for disease severity on a scale of 0–2, where 0 = no lesion, 1 = lesions present on first and second leaf sheath, and 2 = large lesions on leaf sheaths and stem or plant senescence. The distribution of disease scores for these 94 RILs was bimodal (Fig. 1a) with some overlap between groups. Thus, we used analysis of variance (completely randomized design with four replications) and Fisher's least significant difference (LSD) to separate resistant from susceptible genotypes. A susceptible line (*Pch1* absent) was defined as having a mean trait value that was at least one LSD (0.5) lower than Brundage (1.78), the susceptible parent. A resistant line (*Pch1* present) was defined as having a mean trait value that was up to one LSD greater than Coda (0.70), the resistant parent. Using this classification, individuals with mean visual ratings ranging from 0 to 1.2 were considered resistant (*Pch1* present) whereas plants with mean ratings ranging from 1.28 to 2 were considered susceptible (*Pch1* absent). Two RILs had a mean disease score between 1.2 and 1.28 and could not be classified. These two RILs were retested in addition to the remaining 160 RILs of the population as well as the parental lines. Because the 0–2 rating scale was narrow, all subsequent screenings relied on disease severity ratings on a 0–4 scale, where 0 = no lesion, 1 = a lesion under the first leaf sheath, 2 = several lesions on the second leaf sheath, 3 = large lesion around the tiller involving both the first and second

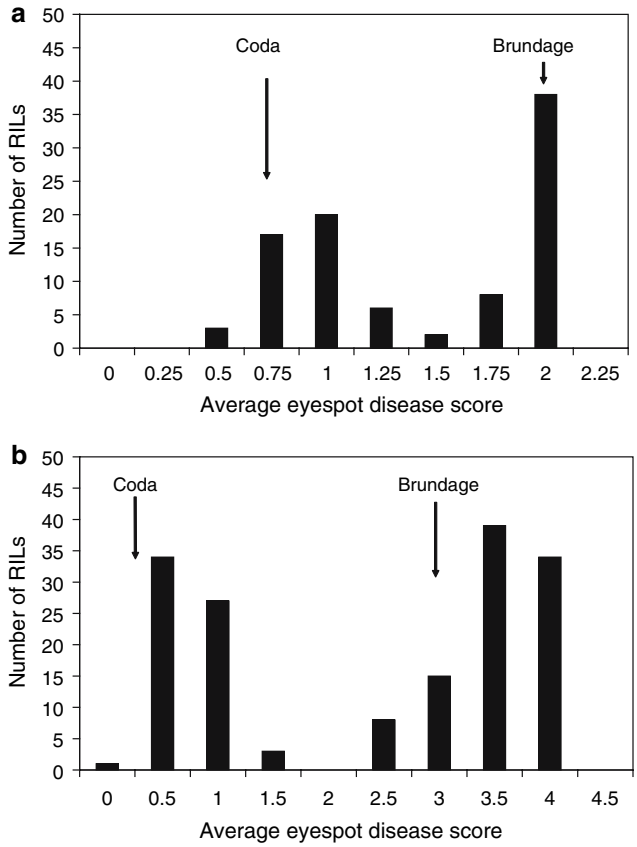

Fig. 1 Frequency distributions of average eyespot disease scores for recombinant inbred lines (RILs) from the Coda × Brundage cross. **a** Frequency distribution for the first 94 Coda\Brundage RILs using a 0–2 scoring scale. The average disease scores for Coda and Brundage were 0.70 and 1.78, respectively. The least significant difference (LSD) for the disease score was 0.50. **b** Frequency distribution for an additional 160 RILs using the 0–4 scoring scale. The average disease scores for Coda and Brundage were 0.1 and 2.7, respectively. The least significant difference (LSD) for disease score was 0.62

leaf sheath, 4 = prominent lesion visible on the tiller or plant death. Disease scores had a bimodal distribution with two distinct groups (Fig. 1b). Thus, individuals with mean visual ratings ranging from 0 to 1.5 were considered resistant (*Pch1* present) whereas plants with mean ratings ranging from 2.5 to 4 were considered susceptible (*Pch1* absent).

Results

Identification of a candidate gene

A series of three orthologous wheat genes *Ep-A1*, *Ep-B1*, and *Ep-D1* encode enzymes that cleave the substrate N_{α}-Benzoyl-DL-Arginine-β Naphthylamide (BANA). These enzymes are assumed to be endopeptidases as BANA does not possess a free carboxyl or an amino terminus for exopeptidase activity. *Ep-A1*, *Ep-B1*, and *Ep-D1* have been localized to the long arms of wheat homeologous group 7 chromosomes (Hart and Langston 1977; Koebner et al. 1988). Furthermore, *Ep-D1* has been mapped to the distal end of the long arm of chromosome 7D (Gale et al. 1995). Rice and maize also possess endopeptidase loci that cleave the substrate BANA (Vodkin and Scandalios 1980; de Kochko 1987; Cardy et al. 1982; Pham et al. 1989). Both the maize endopeptidase locus, *enp1* (Edwards et al. 1992), and the rice endopeptidase locus *Enp* (Pham et al. 1989) are located on chromosome 6 of their respective species, which are homoeologous to the long arm of wheat 7D (Hossain et al. 2004). These observations suggested that wheat, maize, and rice endopeptidase loci might be orthologous.

Since the maize *enp1* locus encodes an enzyme with substrate specificities and inhibitor characteristics (Vodkin and Scandalios 1980; Doi et al. 1986) similar to oligopeptidase B purified from *E. coli* (Table 3), we hypothesized that the wheat *Ep-D1* locus might also encode an oligopeptidase B. The existence of a wheat oligopeptidase B homologue has been shown by Tsuji et al. (2004), who purified a serine protease from developing wheat embryos with peptide sequence homology to *E. coli* oligopeptidase B. One of seven peptides sequenced from the purified wheat protein was found to be encoded by the wheat expressed sequence tag (EST) BU100257. This presented a likely candidate for one of the three wheat endopeptidase homoeologues.

Tsuji et al. (2004) also identified a rice cDNA (AK070316) that fully encoded three of seven peptides sequenced from the putative wheat oligopeptidase B. The identity between three other of the sequenced wheat peptides and the predicted peptide sequence of AK070316 ranged from 80 to 94%. The wheat EST BU100257 and rice cDNA AK070316 are 85% identical over their 741-nt overlap. A search of the TIGR Rice Genome Annotation Database and Resource (http://www.tigr.org/tdb/e2k1/osa1/) identified a predicted coding region LOC_Os06g51410 as the source of rice cDNA AK070316. LOC_Os06g51410 is located in a region of rice chromosome 6 with homoeology to the most terminal deletion bin of chromosome 7D (7DL3) (Hossain et al. 2004; Fig. 2), a likely chromosome bin location of *Ep-D1* (Fig. 2). These observations suggested that cDNA AK070316 might the product of the rice *Enp* locus and that a wheat homologue of the rice cDNA AK070316 (BU100257) may also be present in the 7DL3 wheat chromosome bin.

To determine if a wheat oligopeptidase B homologue was associated with *Pch1*, we developed a PCR-based marker and tested it on a panel of 22 lines known to carry *Pch1* and 22 lines known to be lacking this gene (Table 1). For marker development, we identified a near full-length wheat transcript (AB246917) in the NCBI database which includes and extends the previously identified EST

Table 3 Characteristics of genetically defined endopeptidases from wheat, maize, and rice (*Ep-D1*, *enp1*, *Enp*, respectively) compared to purified oligopeptidase B proteins from *E. coli* and wheat

Genetic locus or gene	Chromosomal location	Enzyme substrate[f]	Inhibitors[g]
Ep-D1[a] (wheat)	Long arm of 7D	BANA	ND[h]
enp1[b] (maize)	Short arm of 6	BANA BAPNA	TLCK, leupeptin, antipain, *p*-mercuribenzoate
Enp[c] (rice)	Short arm of 6	BANA	ND[h]
E. coli oligopeptidase B[d]	N/A[i]	BAPNA Bz-Arg-NHMec	TLCK, leupeptin, antipain DFP
Wheat oligopeptidase B[e]	Long arm of 7D	Z-Arg-NHMec	TLCK, leupeptine, antipain, DFP, benzamidine

[a] Based on Hart and Langston (1977) and Koebner et al. (1988)

[b] Based on Vodkin and Scandalios (1980), Cardy et al. (1982), Doi et al. (1986), and Edwards et al. (1992)

[c] Based on de Kochko (1987) and Pham et al. (1989)

[d] Based on Tsuru and Yoshimoto (1994) and MEROPS the Peptidase Database (http://merops.sanger.ac.uk)

[e] Based on Tsuji et al. (2004) and the present study

[f] BANA, Bz-Arg-NHNap; BAPNA, Bz-Arg-NHPhNO2; Z, benzoyl; Bz, carbobenzoxy; Mec, 4-methylcoumaryl 7-amide

[g] TLCK, N-tosyl-L-lysine chloromethyl ketone; DFP, diisopropyl fluorophosphate

[h] ND, Not determined

[i] N/A, Not applicable

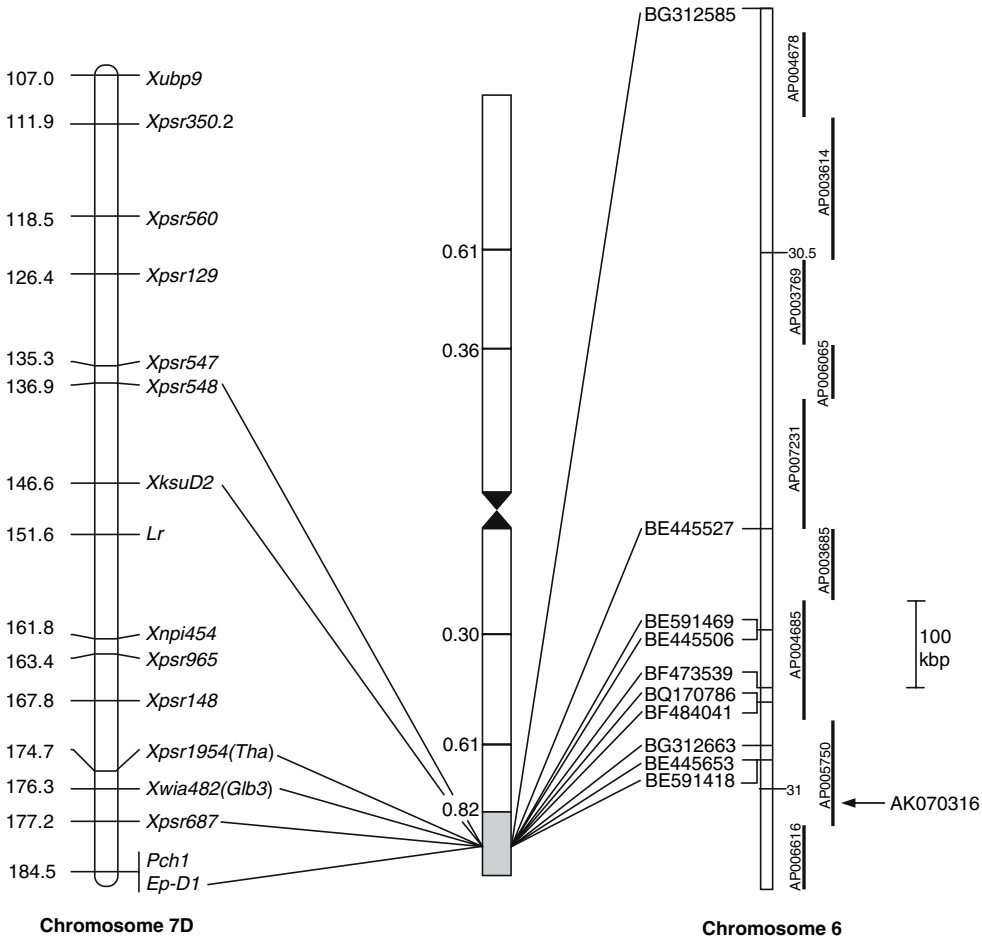

Fig. 2 Deletion bin location of markers on the long arm of chromosome 7D and bin-mapped wheat expressed sequence tags (ESTs) that have homologues on the terminus of rice chromosome 6. *Left*, genetic map of the long arm of chromosome 7D (Gale et al. 1995). *Center*, ideogram of chromosome 7D showing seven deletion-defined chromosome bins (Hossain et al. 2004). Deletion bin 7DL3 (0.82–1.0) is shown in *grey*. *Right*, bin-mapped wheat expressed sequence tags (ESTs) that have homologues on the terminus of rice chromosome 6. Lines connect markers and ESTs that have been mapped to the 7DL3 deletion bin of chromosome 7D (Hossain et al. 2004; Boyco et al. 2002). BAC tiling path of a terminal segment of rice chromosome 6 is indicated by bars and the location of oligopeptidase B-encoding transcript AK070316 is indicated by an *arrow*. *Numbers* on the wheat genetic map represent cM while *numbers* on rice chromosome 6 indicate megabasepairs

BU100257, and aligned it with genomic sequence of the putative rice oligopeptidase B LOC_Os06g51410 using the computer program Spidey (NCBI Database) to predict intron/exon boundaries for intron-scanning PCR. This analysis showed a number of small, closely spaced predicted introns near the carboxy terminus. The orw1F/orw1R primer pair (Table 2), designed to be complementary to the putative wheat oligopeptidase B transcript that spanned predicted intron 9, revealed an informative polymorphism. PCR reactions with the orw1 primer pair produced 167- and/or 178-bp fragments among both eyespot-resistant and -sensitive lines. But all *Pch1*-carrying lines, including the donor line VPM-1, produced a 180-bp fragment while none of the eyespot-sensitive lines yielded this product. This panel of wheat genotypes was also tested with the *Pch1*-associated marker *XustSSR2001-7DL*. Similar to the report by Santra et al. (2006), we found that the PCR reactions utilizing DNA from eyespot-resistant lines produced the *Pch1*-associated 237-bp product whereas DNA from the eyespot-resistant variety Coda yielded a 222-bp fragment associated with the absence of *Pch1* (Groenewald et al. 2003). This suggested that the putative oligopeptidase B locus templating the 180-bp fragment was more tightly associated with *Pch1* than *XustSSR2001-7DL*.

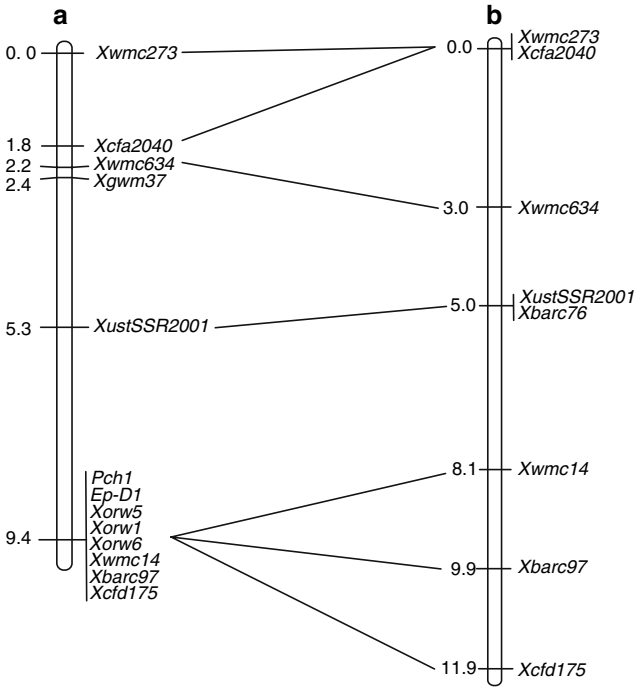

Fig. 3 Genetic maps of the 7DL terminus. **a** Map based on 254 RILs derived from the cross between eyespot-resistant Coda and eyespot-sensitive Brundage. **b** Map based on 94 RILs from the ITMI W7984 × Opata85 RIL population. *Numbers* represent distances in cM

Mapping the *Pch1* region

We used microsatellite markers, the *Pch1*-associated marker *XustSSR2001-7DL*, and the orw1 primers to define a genetic locus (*Xorw1*) using 254 Coda/Brundage-derived RILs. *Xorw1* mapped to the distal end of wheat chromosome 7DL (Fig. 3a). The endopeptidase isozyme assay (Santra et al. 2006) was used on the mapping population to genetically locate *Ep-D1*. The population was also screened for disease reaction against *O. yallundae* to map *Pch1*. We detected no recombination between *Xorw1*, *Ep-D1* and *Pch1*.

Additional EST-derived markers for the *Pch1* region were designed from predicted gene sequences located on the BAC clone OSJNBa0069C14 (AP005750) encoding the putative rice oligopeptidase B. BLASTn searches of The Gene Index Project (presently housed at the Dana-Farber Cancer Institute) were used to identify homologous wheat putative EST contigs. The sequences returned were then used for an intron-scanning screen for polymorphic PCR products, tested on the association panel, and subsequently mapped. This exercise yielded two additional mapped loci. *Xorw5* derives from a putative rice callose synthase (LOC_Os06g51270). Orw5 primer pairs produced a 170-bp amplicon in all 44 members of the association panel but a 156-bp fragment only in lines that lacked *Pch1*. *Xorw6* derives from rice transcript assembly TA3304_4530, a putative photosystem II assembly factor HCF136. The orw6 primers detected two apparent loci not associated with *Pch1* in the panel. One locus produced either a null allele or a 158-bp product while the second locus yielded products of 191, 194 bp, or a null allele. A third locus, however, produced a unique 166-bp product only in *Pch1* carrying lines but no similarly sized product in lines lacking *Pch1*. When tested on the Coda/Brundage mapping population, *Xorw5* and *Xorw6* were also completely linked to *Pch1* together with a group of three microsatellite loci *Xcfd175*, *Xbarc97*, and *Xwmc14* (Fig. 3a).

As the three rice gene models corresponding to *Xorw1*, *Xorw5*, and *Xorw6* span less than 112 kbp on rice chromosome 6, we considered that tight linkage might reflect close physical position in wheat. Alternatively, reduced recombination between the introduced *Ae. ventricosa* $7D^{V}L$ fragment and common wheat 7DL might be the source of lack of segregation of these loci. To test this, we mapped markers using 94 RILs of the ITMI mapping population. The three EST-derived markers, *Xorw1*, *Xorw5*, and *Xorw6* were not polymorphic and could not be mapped. However, seven common microsatellite markers were mapped (Fig. 3b). Four identically ordered loci proximal to *Pch1* showed comparable genetic distances in both maps. The three loci completely linked to *Pch1* on the Coda/Brundage map, *Xwmc14*, *Xbarc97*, and *Xcfd175*, were each approximately 2 cM distant on the ITMI map.

We looked for evidence of recombination in lines carrying *Ae. ventricosa* segments by analyzing 22 lines carrying *Pch1* with 11 markers (Fig. 4). All accessions carrying *Pch1* were identical to VPM-1 at *Xorw1*, *Xorw5*, and *Xorw6*. Eleven of the 22 lines were identical to VPM-1 (carrying only *Ae. ventricosa*-derived alleles) at all 11 loci. Two accessions, Temple and Coda, carried alternate alleles at loci proximal to the three linked STS loci. Eight lines showed alternate alleles at some or all of the three distal SSR markers *Xwmc14*, *Xbarc97*, and *Xcfd175* indicating historical recombinations in the regions flanking *Pch1*. In the panel of 44 lines we analyzed, *Xorw1*, *Xorw5*, and *Xorw6* were 100% accurate in predicting the presence or absence of *Pch1* whereas the accuracy of prediction for markers *Xgwm37*, *Xbarc76*, *XustSSR2001-7DL*, *Xwmc14*, *Xbarc97*, and *Xcfd175* was 95, 95, 98, 98, 93, and 77%, respectively.

Discussion

In this study, we sought to identify a candidate gene for the endopeptidase locus *Ep-D1* that could be used to develop a DNA-based marker to track the economically important eyespot-resistance gene *Pch1* in breeding programs. The biochemical isolation and characterization of a wheat oligopeptidase B enzyme and observations concerning homoeology between rice and maize chromosome 6 and the terminus of wheat 7DL were key to identifying wheat ESTs that could be used as the basis for PCR marker development.

The complete association observed between the unique PCR products amplified from 22 wheat cultivars carrying *Pch1* with primers designed to wheat oligopeptidase B-encoding wheat EST BU100257 strengthened the connection between the isozyme marker (*Ep-D1*) and the putative oligopeptidase B transcript. Complete linkage observed between *Pch1* and the STS marker *Xorw1* in our genetic map constructed with a population of 254 RILs, lead us to propose that they represent the same locus. However, the lack of recombination in this region in our study leaves open the possibility that *Xorw1* represents a different, albeit tightly linked, locus.

A goal of this study was to derive DNA-based markers more closely linked to *Pch1* than *XustSSR2001-7DL* for marker assisted selection (MAS). A recombination frequency between *XustSSR2001-7DL* and *Ep-D1* of 2% in a population of 98 plants was reported by the developers of the marker (Groenewald et al. 2003). Similarly, we mapped a distance of 3.9 cM between the same loci in our larger population. We also identified one cultivar, Coda, of the 22 *Pch1* carrying cultivars that does not carry the 237-bp *Ae. ventricosa*-derived *XustSSR2001-7DL* allele, confirming the need for a more tightly linked marker. The three EST-derived markers, *Xorw1*, *Xorw5*, and *Xorw6* reported here are all completely linked to *Pch1* in the genetic map (Fig. 3a) and reveal unique alleles in all 22 of the *Pch1*-carrying cultivars not found in the 22 eyespot-susceptible lines we assayed. All of the STS markers that we have developed are dominant, but the 156-bp *Triticum*-derived allele of *Xorw5* could be paired with the unique *Aegilops*-derived alleles of *Xorw1* or *Xorw6* to assess zygosity in segregating lines. The 180-bp *Xorw1* allele can easily be distinguished from a ubiquitous 178-bp product by fragment analysis in a capillary electrophoresis system, and the 166-bp *Xorw6*

variety	Xbarc53	Xcfa2040	Xgwm37	Xbarc76	XustSSR2001	Xorw5	Xorw1	Xorw6	Xwmc14	Xbarc97	Xcfd175
WA7690	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	T
Chukar	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	T
WA7671	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	T
Rendezvous	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	T
FR50	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	?	T
VPM/Moisson 951	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	T	T
Simon	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	T	T
Weatherford	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	T	T	T
VPM-1	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae	Ae
Coda	T	T	T	T	T	Ae	Ae	Ae	Ae	Ae	Ae
Temple	T	T	T	Ae	Ae	Ae	Ae	Ae	?	Ae	Ae

Fig. 4 Haplotypes of eyespot-resistant lines that differ from VPM-1 in the region between *Xbarc53* and *Xcfd175*. *Ae* indicates an allele from VPM-1 (originally derived from *Ae. ventricosa*) and *T* indicates an allele from *T. aestivum*. Markers are arranged in map order except for *Xorw5*, *Xorw1* and *Xor6*, which are completely linked. Their order is suggested by the orientation of homologues on rice chromosome 6. An additional 11 eyespot-resistant lines were identical to VPM-1 for all loci in this region and are not included in this figure

allele can be resolved from an unlinked 156-bp product making them useful markers for introgression of *Pch1* in a breeding program.

Attempts to develop a more densely populated genetic map of the *Pch1* region were frustrated by the complete linkage between *Xorw1*, *Xorw5*, and *Xorw6*, as well as three microsatellite markers (*Xwmc14*, *Xbarc97*, and *Xcfd175*). Recombination observed between the *Xwmc14*, *Xbarc97*, and *Xcfd175* in the smaller ITMI population suggest that recombination is locally inhibited in the Coda/Brundage population. Comparison of maps (Fig. 3) suggests that this region lies distal to *XustSSR2001-7DL*. However, historical recombinations involving *Xwmc14* and the two distal markers *Xbarc97* and *Xcfd175* (Fig. 4), imply that recombination is not eliminated in the 7DL terminus, but perhaps only reduced. Although the hexaploid wheat 7DL and the *Ae. ventricosa* $7D^{V}L$ chromosome segments carry homologous loci for the markers we used, it is possible there are some structural differences that locally inhibit recombination. Determining the extent of this inhibition will require further genetic analyses in other populations. If this is a general phenomenon, it seriously impacts the feasibility of cloning *Pch1* through a map-based approach.

It was proposed that similar positions of *Pch1* and *Pch2* on 7DL and 7AL, respectively, suggest they are homoeologues (de la Peña and Murray 1994). However, the 32.8 cM distance between *Pch2* and the *Ep-D1* homoeologue *Ep-A1* (de la Peña et al. 1997), contrasts sharply with the tight linkage observed between *Pch1* and *Ep-D1*. The physical distance between *Pch1* and *Ep-D1* may not be as be tight as implied by the genetic map because of the lack of recombination we observed in this region. Yet, the 4.1 cM distance calculated between *XustSSR2001-7DL* and *Xcfd175* on the Coda/Brundage map is only 2.8 cM shorter than that measured between the same markers on the ITMI map. Therefore, we also report a large discrepancy between the genetic distances of the endopeptidase loci, *Ep-D1* and *Ep-A1*, and the *Pch* loci on their respective chromosomes and offer no evidence to support their homoeology based upon their genetic positions.

The three rice genes used to develop markers in this report span 112 kbp in the rice genome. The complete linkage observed in both our genetic map and association panel suggest they might be similarly located in wheat. Ordering and sequencing of BACs hybridized, in turn, with these three EST-based markers might be used to establish their relative positions in the wheat genome. This would also allow a determination of microcolinearity between the wheat and rice genome in this region. Although it is interesting to speculate that the *Pch1*-derived disease resistance might actually be the product of *Ep-D1b* (Worland et al. 1988), our work cannot suggest any candidates for *Pch1* until a population segregating in this region can be established.

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