Resistance to *Oculimacula yallundae* and *Oculimacula acuformis* is conferred by *Pch2* in wheat

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The recent report of a differential response of wheat lines containing the *Pch2* gene to infection with the eyespot pathogens *Oculimacula yallundae* and *O. acuformis* has prompted this re-examination of the response to these fungi by the recombinant lines used to map *Pch2*. Homozygous recombinant substitution lines (RSL) derived from the hybridization of Chinese Spring (CS) and the CS chromosome substitution line Cappelle Desprez 7A (CS/CD7A), previously evaluated for response to glucuronidase (GUS)-transformed *O. yallundae*, were evaluated for response to infection with GUS-transformed *O. acuformis*. Based on visual scores and on GUS expression level, which reflects fungal colonization of seedling plants, evidence of a quantitative trait locus (QTL) conferring resistance to *O. acuformis* was found in two separate growth chamber experiments (logarithm of the odds, LOD, = 2·7 and 6·7 at 305 and 289 cM, respectively) that was equivalent in location to that for resistance to *O. yallundae* (LOD = 13·2 and 11·4 at 289 and 304 cM, respectively). These results confirm that *Pch2* confers some degree of resistance against both *O. yallundae* and *O. acuformis* under these conditions.

Keywords: eyespot of wheat, *Oculimacula acuformis*, *Oculimacula yallundae*, quantitative trait locus

Introduction

Since the identification of eyespot resistance gene *Pch2* on chromosome 7A in the French wheat cultivar Cappelle Desprez (CD) (Law *et al.*, 1976; Strausbaugh & Murray, 1989; de la Peña *et al.*, 1996), there has been much debate over the relative effectiveness of this gene for control of eyespot disease. Law *et al.* (1976) and Jahier *et al.* (1978) both reported a positive effect for chromosome 7A in reducing eyespot when the cultivar Chinese Spring (CS) was used as the susceptible parent. Both studies also found that chromosomes 2B and 5D contributed to resistance and found evidence that the effect of chromosome 7A was not entirely independent. More recently, Burt *et al.* (2011) identified a quantitative trait locus (QTL) on chromosome 5A of Cappelle Desprez that confers resistance in both the seedling and adult stages and confers a similar level of resistance as *Pch2*. Importantly, the QTL on chromosome 5A may enhance eyespot resistance when combined with *Pch2*, with the combination providing enhanced disease control.

Estimates of the effectiveness of *Pch2* in controlling eyespot vary greatly. Koebner & Martin (1990) reported that variation at the Ep-A1 isozyme locus on chromosome 7A accounted for 28% of the genetic variation in disease scores among CS:CD F_3 families. Chapman *et al.*

(2008) found that 35 to 40% of phenotypic variation among F_3 families from a cross between CS and the chromosome substitution line Chinese Spring–Cappelle Desprez 7A (CS/CD7A) was accounted for by nearest-marker variation, whereas Burt *et al.* (2010) found the effect in this region to be non-significant.

Compounding the uncertainty surrounding the effectiveness of *Pch2* is the difference among studies in the pathogen species used to evaluate plants for disease resistance. Eyespot disease of wheat and other cereals is caused by the related fungi *Oculimacula acuformis* (syn: *Tapesia acuformis*, anamorph *Pseudocercosporella herpotrichoides* var. *acuformis*; formerly rye (R)-type) and *O. yallundae* (syn: *Tapesia yallundae*, anamorph *Pseudocercosporella herpotrichoides* var. *herpotrichoides*; formerly wheat (W)-type) (Crous *et al.*, 2003). Populations of *O. acuformis* and *O. yallundae* co exist within fields of the US Pacific Northwest and other wheat growing regions (Murray, 2010); consequently, it is important that wheat varieties have effective resistance to both eyespot pathogens.

Law *et al.* (1976) conducted a monosomic analysis of Cappelle Desprez using a pathogen isolate now known as *O. yallundae*. de la Peña *et al.* (1997) mapped *Pch2* to an interval on chromosome 7AL between the loci *Xcdo347* and *Xwg380* using a set of RSL they developed through hybridization of CS with CS/CD7A, and inoculated with a GUS-transformed isolate of *O. yallundae*. Koebner & Martin (1990) refined the location of *Pch2* using a mixture of isolates they referred to as *Pseudocercosporella herpotrichoides*, but did not indicate whether they were W- or R-type isolates, and found significant effects for genotype and genotype × environment

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interaction. Burt *et al.* (2010) used *O. yallundae* to evaluate the same lines for eyespot resistance that Chapman *et al.* (2008) evaluated using *O. acuformis*, but with different results. They concluded that *Pch2* provided seedling resistance against *O. acuformis*, but was much less effective against *O. yallundae*.

The goals of this project were to evaluate the RSL developed by de la Peña *et al.* (1997) for reaction to *O. acuformis*, to evaluate the effectiveness of *Pch2* in these lines against both eyespot pathogens, and to use QTL mapping to identify the presence (or lack) of a QTL conferring resistance to these pathogens.

Materials and methods

Plant materials

One hundred and twelve chromosome 7A homozygous RSL, segregating for the *Pch2* gene, from the cross CS × CS/CD7A (de la Peña *et al.*, 1996) were tested for resistance to *O. yallundae* and *O. acuformis*. In order to balance the data from all four experiments, 108 RSL were evaluated in the statistical analyses. For quality control purposes, wheat lines Cappelle Desprez (PI518787) and CS (CItr14108) were included in all eyespot resistance experiments. In addition, wheat lines Eltan (PI536994), Hyak (PI511674) and Madsen (PI511673) were included as checks in one *O. yallundae* resistance experiment, whereas Eltan, Hill-81(CItr17954) and Madsen were included as checks in both *O. acuformis* experiments. Cappelle Desprez contains *Pch2*, as well as loci on chromosomes 2B and 5D that contribute to eyespot resistance (Law *et al.*, 1976). Both Madsen and Hyak were derived from VPM-1 and contain *Pch1*. Eltan and Hill-81 are susceptible to eyespot disease.

Evaluation of eyespot resistance

Two independent growth chamber experiments of eyespot resistance experiments were performed for each pathogen species. Resistance was evaluated by visual score and β-glucuronidase (GUS) assay as described by de la Peña & Murray (1994). Briefly, two seeds of each entry were sown in 6·4 cm pots and grown at 12°C with 10 h per day illumination. At the two-leaf stage, seedlings were inoculated with GUS-transformed isolates of either *O. yallundae* or *O. acuformis* as a suspension of conidia in agar (1×10^5 conidia mL^{-1}) pipetted into a 1·5 cm section of plastic drinking straw placed around the seedling stem. Relative humidity in the growth chambers was maintained near 100% for 14 days after inoculation. Each experiment was arranged as a randomized complete block design with three to six replicates resulting in six to 12 plants per entry being evaluated.

Washed 4-cm-long basal stem sections were visually rated for eyespot lesion severity (de la Peña & Murray, 1994) 6–8 weeks post-inoculation and placed on ice or frozen until GUS assays were conducted. Visual ratings were performed on a 1–5 scale based on the area of stem with symptoms as described by de la Peña & Murray (1994), with larger numbers corresponding to increased lesion size. Stems were assayed for GUS activity using a procedure modified from Jefferson *et al.* (1987) and described by de la Peña & Murray (1994). Response to pathogen infection was represented as the fluorimetrically determined 4-methyl-umbelliferone (MU) content after conversion from 4-methylumbelliferyl glucuronide by GUS. Glucuronidase activity, as measured by this assay, is highly correlated with mycelial growth (de la Peña & Murray, 1994).

The two *O. yallundae* experiments were performed in 1997 and 2000, while the *O. acuformis* experiments were performed in 2011 and 2012. There was some variation in GUS assay methodology among experiments as a result of the time span between the first and last experiments. First, treatment of stems from the *O. yallundae* resistance experiments were as described by de la Peña *et al.* (1997) and consisted of grinding the stems in liquid nitrogen, then soaking in 600 μL GUS extraction buffer for 15 min at 4°C. Stems from the *O. acuformis* resistance experiments were mechanically crushed in a leaf squeezer (Ravenel Specialties Company) and the sap washed into a collection tube with 2·5 mL GUS extraction buffer. Secondly, the MU content of samples inoculated with *O. yallundae* was determined using a Molecular Devices Emax microplate reader (Molecular Devices Co.) whereas that of samples inoculated with *O. acuformis* was determined using a SpectraMax M2 microplate reader (Molecular Devices Co.). Thirdly, the first *O. yallundae* experiment used in these analyses was performed with four replicates, the second with six replicates, and both *O. acuformis* experiments with three replicates.

Statistical analyses

For these analyses, MU content was treated as a quantitative variable and log-transformed prior to analysis because of the non-independence of mean and variance. A mixed model analysis was implemented using the SAS statistical software package (SAS v. 9.2, SAS Institute Inc.) to evaluate line, pathogen and interaction effects using the combined data from all experiments in a split-plot design. In this analysis, pathogen was the whole plot factor with experiments nested within pathogen as the error term. Because replicates were nested within both pathogen and experiment, that term was included at the whole plot level. Recombinant substitution lines were the subplot factor. Line × pathogen and line × experiment within pathogen interaction effects were included in the model. Pathogens and lines were considered as fixed effects. A *P*-value < 0·05 was considered statistically significant. Quantitative trait locus analysis was performed within experiments on line least squares mean values.

Twenty-eight markers, all on wheat chromosome 7A, previously described by de la Peña *et al.* (1996, 1997) were used in the analysis. Marker map order was as determined by de la Peña *et al.* (1996). One hundred and eight RSL with both phenotype and genotype measures were used in the QTL analysis. Interval linkage mapping, as implemented in QTL CARTOGRAPHER (Basten *et al.*, 1994), was used to determine the logarithm of the odds (LOD) score for the presence of a QTL at 1 cM intervals on chromosome 7A. The threshold for considering a LOD score as statistically significant evidence of a QTL was determined using a permutation test of 1000 cycles.

Results

Response to *O. acuformis* and *O. yallundae* infection varied among lines within experiments (Table 1). The MU values of the previous *O. yallundae* experiments by de la Peña *et al.* (1997) were analysed as a categorical variable based on non-significant differences from the resistant parent. Visual scores from that experiment were no longer available. Visual scores and log-transformed MU values from all experiments (where available) were

Table 1 Response of 102 recombinant substitution lines (RSL) and six wheat cultivars to *Oculimacula acuformis* and *O. yallundae* infection measured as either a visual rating or GUS score

Species	Year	Visual score			GUS score		
		n	Mean (SD)[a]	Range	*n*	Mean (SD)[b]	Range
O. acuformis	2011	672	2·7 (0·85)	0–4	672	3·79 (0·33)	2·67–4·96
	2012	690	1·9 (0·91)	0–4	690	4·00 (0·30)	2·76–4·65
O. yallundae	1997	NA	NA	NA	452	3·07 (0·35)	1·85–4·02
	2000	1241	3·1 (0·62)	1–4	1237	4·50 (0·46)	2·80–5·73

[a]Visual disease ratings were performed on a 0–4 scale, where 0 = no symptoms (healthy), 1 = a lesion only on the first leaf sheath, 2 = a lesion on the first leaf sheath and a small lesion on the second leaf sheath, 3 = a lesion covering the first leaf sheath and up to half of the second sheath, and 4 = a lesion covering the first and second sheaths (nearly dead).
[b]GUS scores represent the transformed value of 4-methylumbelliferone produced from the β-glucuronidase (GUS) assay (log(MU)+1).
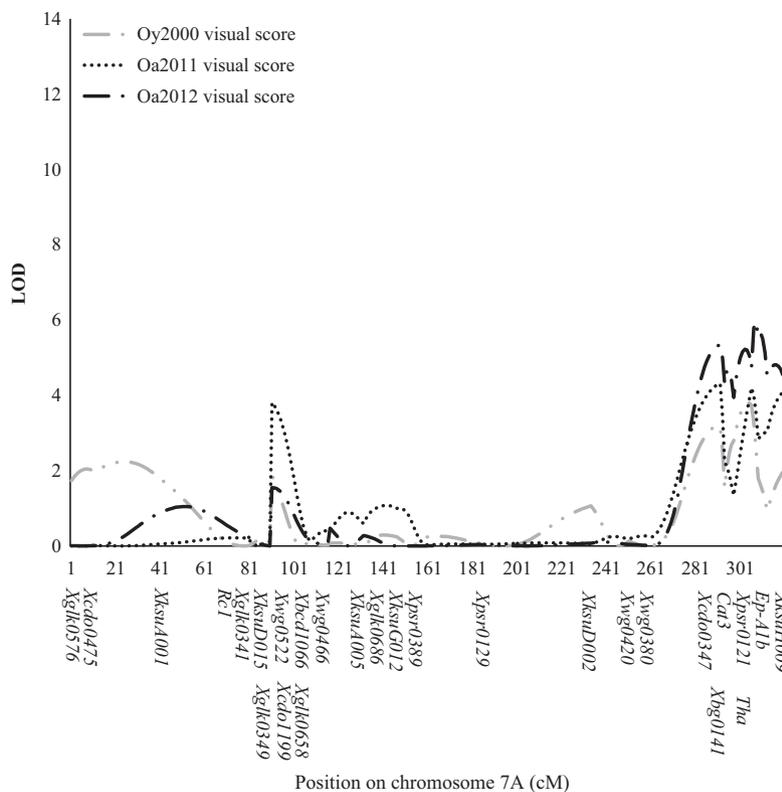NA: eyepot visual ratings were not available for the 1997 experiment.

Figure 1 Logarithm of the odds (LOD) scores on wheat chromosome 7A from interval linkage mapping of resistance to *Oculimacula acuformis* and *O. yallundae* as indicated by visual assessment of disease severity. Oy2000 indicates the results of an *O. yallundae* trial performed in 2000; Oa2011 indicates the results of an *O. acuformis* trial performed in 2011; Oa2012 indicates the results of an *O. acuformis* trial performed in 2012. A LOD score of 2·5 is the threshold for statistical significance.

normally distributed (skew and kurtosis <|1·0|). Combining experiments, the visual disease scores and MU values from *O. acuformis* experiments did not differ from those of the *O. yallundae* experiments ($P > 0.05$). Recombinant substitution line and line × pathogen interaction effects were statistically significant ($P < 0.0001$ and $P < 0.001$, respectively for both log MU and visual scores). Within pathogen species, the 1997 experiment resulted in lower MU values than the 2000 experiment ($P < 0.0001$). The 2011 *O. acuformis* experiment had greater visual scores on average, but lower MU values, than did the 2012 experiment ($P < 0.0001$).

For both *O. yallundae* and *O. acuformis*, visual ratings and GUS scores yielded two peaks <18 cM apart, but not overlapping in their 1-LOD confidence intervals. Peak

LOD scores for the visual disease scoring system were 3·8 at 304 cM for the *O. yallundae* experiment in 2000, and 4·3 at 292 cM and 6·0 at 307 cM for the *O. acuformis* 2011 and 2012 experiments, respectively (Fig. 1). Peak LOD scores for GUS activity were 13·2 at 289 cM and 11·4 at 304 cM for the *O. yallundae* 1997 and 2000 experiments; and 2·7 at 305 cM and 6·7 at 289 cM for the *O. acuformis* 2011 and 2012 experiments, respectively (Fig. 2). The threshold for statistical significance was LOD = 2·5 as determined by permutation test.

Statistically significant evidence for linkage with a QTL for visual disease severity was also observed at 92 cM near *Xwg522* (Fig. 1) in the 1997 and 2000 *O. yallundae* experiments (LOD = 2·80 and 4·31, respectively) and in the 2011 *O. acuformis* experiment

(LOD = 3·81). This peak is on the short arm of chromosome 7A and was not investigated further in this study.

Evidence of linkage from all experiments was strongest between *Xcdo347* and *Xpsr121* (Figs 1 & 2). Variation at the *Xcdo347* locus accounted for 38·9 and 25·8% of phenotypic variation in GUS activity in the 1997 and 2012 experiments, respectively, and 20·1% of visual score variation in the 2011 experiment. Variation at the RFLP locus *Tha* accounted for 37·9 and 10·8% of phenotypic variation in GUS activity in the 2000 and 2011 experiments, respectively, and 14·5% of variation in visual score in the 2000 experiment. Variation at *Xpsr121* accounted for 21·1% of 2012 visual disease score phenotypic variation.

Discussion

Re-analysis of the response of these homozygous RSL to infection by GUS-transformed *O. yallundae*, including data from a previously unpublished trial, confirmed the presence of an eyespot disease resistance locus at the terminal end of the long arm of chromosome 7A observed by de la Peña *et al.* (1997). Two new experiments evaluating these lines for their response to *O. acuformis* also provided evidence of an eyespot resistance QTL in the same region. In the previous two-point linkage analysis, in which *Pch2* was considered as a qualitative trait, *Xcdo347* was the most strongly linked locus (de la Peña *et al.*, 1997). In this study, resistance was considered a quantitative trait and evaluated using the original mapping data. The strongest evidence for a QTL was at or

somewhat distal to *Xcdo347*. The length of the map as published by de la Peña *et al.* (1997) may indicate the presence of genotyping errors when regarded in the context of more contemporary studies. In addition, the existence of two nearby peaks and the variation in peak LOD score location among experiments may indicate inconsistencies between the estimated and true map order of markers in this region, or be a result of stochastic variation. Regardless, this QTL appears to be at, or very near, the location reported by Chapman *et al.* (2008). Although the marker set in the current study and theirs did not overlap, the publically available linkage map for Courtot × Chinese Spring (Courtot/CS; http://wheat.pw.usda.gov) contains flanking markers for this region used in both studies. Marker loci *Xcfa2019* and *Xcfa2040* at 224 and 256·2 cM, respectively, on the Courtot/CS map flanked *Pch2* on the Chapman map; whereas, *XksuD002* and *XksuH009* at 232·8 and 267·5 cM, respectively, on the Courtot/CS map flanked the QTL on the map in the current study. Together, this places *Pch2* in the interval between the *Xcfa2019* and *XksuH009* loci and provides strong evidence that the QTL identified in these two studies is the same.

In contrast to Burt *et al.* (2010), statistically significant evidence of a QTL for resistance to *O. yallundae* on chromosome 7A was found. Both studies used mapping populations developed from Chinese Spring and the chromosome substitution line Chinese Spring–Cappelle Desprez 7A. One reason for this disparity may be the use, in this study, of completely homozygous RSL as opposed to the F₃ families used by Burt *et al.* (2010),
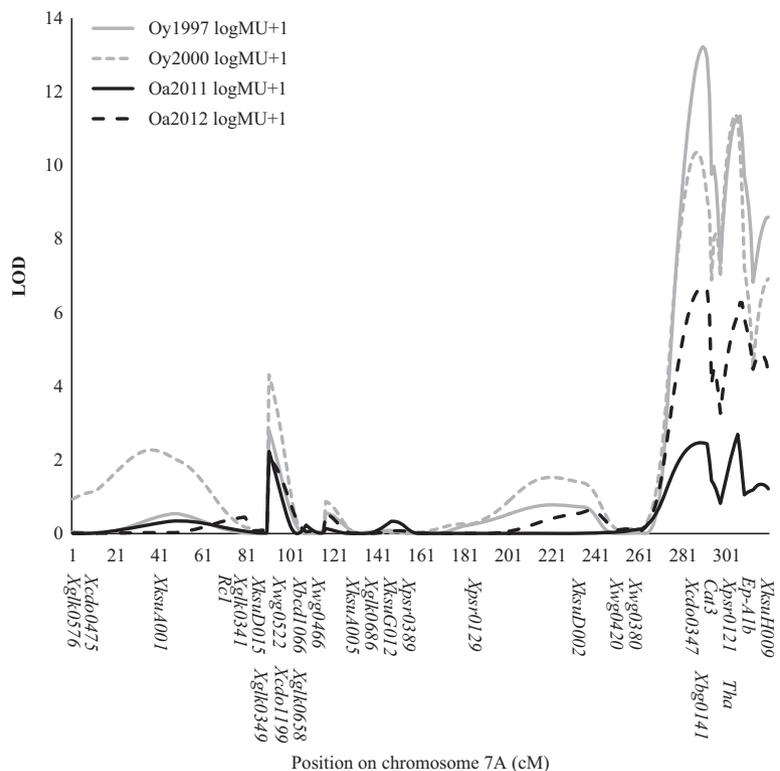

Figure 2 Logarithm of the odds (LOD) scores on wheat chromosome 7A from interval linkage mapping of resistance to *β*-glucuronidase (GUS)-transformed *Oculimacula acuformis* and *O. yallundae* as indicated by GUS activity, which is highly correlated with pathogen growth. Oy1997 are the results of an *O. yallundae* trial performed in 1997; Oy2000 are the results of an *O. yallundae* trial performed in 2000; Oa2011 are the results of an *O. acuformis* trial performed in 2011; Oa2012 are the results of an *O. acuformis* trial performed in 2012; MU is the 4-methylumbelliferone level. A LOD score of 2·5 is the threshold for statistical significance.

which may have suffered from reduced power to detect linkage as a result of heterozygosity at the *Pch2* locus. Alternatively, the extreme variation in estimates of the size of the *Pch2* effect among studies and experiments within studies (0–40% of phenotypic variation) may be because of differing contributions to phenotypic variance × environment or gene × environment interaction. Indeed, in this current investigation at least, pathogen species and experiment-specific environment effects were confounded because of the necessity of using separate growth chamber trials. Other potential sources of disparity between this study and Burt *et al.* (2010) include methodological differences in the controlled environment tests and the use of GUS-transformed isolates in this study. In addition, differences in the visual scoring systems include the use in this study of a 5-point rather than a 7-point scale and assessment based more on lesion spread than on penetration into the leaf sheaths. These differences may have had consequences for the ability to detect a QTL under *O. yallundae* infection.

Burt *et al.* (2010) found significantly greater visual disease severity for *O. yallundae* than *O. acuformis*, which may be interpreted as less effective resistance against *O. yallundae*. In the current study, neither the visual disease scores nor the MU values differed between pathogen species, but it is unreliable to draw conclusions from this about disease severity given the confounding between experiment and pathogen effects that are a consequence of the experimental design. It would be of interest to investigate potential differences in disease severity measures between the eyespot pathogen species, given indications that they differ in their distribution within plant tissues and in how they trigger the plant hypersensitive response, but not in their colonization rate (Murray & Ye, 1986).

Given the non-uniform experimental conditions in published works to date, it is premature to draw conclusions concerning the relative effectiveness of *Pch2* against *O. acuformis* and *O. yallundae*. Indeed, caution must be used when drawing conclusions about pathogen species difference in disease severity from these data, given the methodological changes between the *O. yallundae* and the more contemporary *O. acuformis* experiments. Nevertheless, evidence was found of a QTL on chromosome 7AL that influences eyespot disease resistance, regardless of pathogen species or disease rating method, suggesting that *Pch2* confers some level of resistance against both species under these conditions.

Acknowledgements

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