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ORIGINAL PAPER

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

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

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Introduction

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

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

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

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

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

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

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

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

Materials and methods

Plant material and inoculation

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

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

RNA isolation and cDNA synthesis

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

cDNA/amplified fragment length polymorphism analysis

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

Fragment isolation and sequencing

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

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

Primer development

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

Analysis of differentially expressed transcripts using reverse transcriptase PCR

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

Chromosomal origin of differentially expressed fragments

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

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

Mapping of markers developed by cDNA-AFLP

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

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

Results

cDNA-AFLP analysis of differentially expressed genes

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

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

Fragment characterisation

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

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

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

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

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

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

Table 1 continued

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

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

N blastN, *X* blastX

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

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

Marker development

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

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

Physical and genetic mapping

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

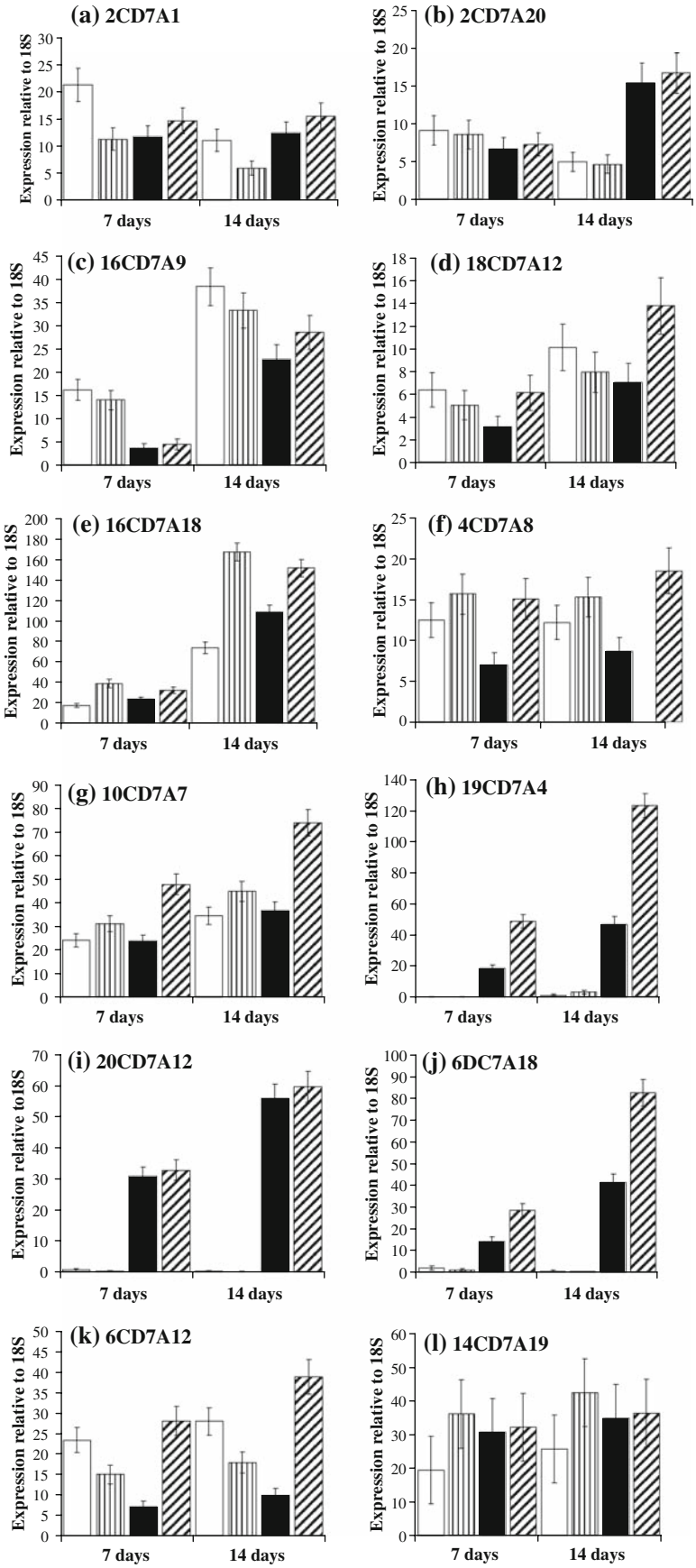

Fig. 1 Expression of cDNA-AFLP fragments using RT-PCR in Chinese Spring (*CS*) non-inoculated (*white boxes*) CS inoculated (*vertical lines*), CS chromosome substitution Cappelle Desprez 7A (CS/CD7A) non-inoculated (*black boxes*) and CS/CD7A inoculated (*diagonal lines*). Samples were taken at time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

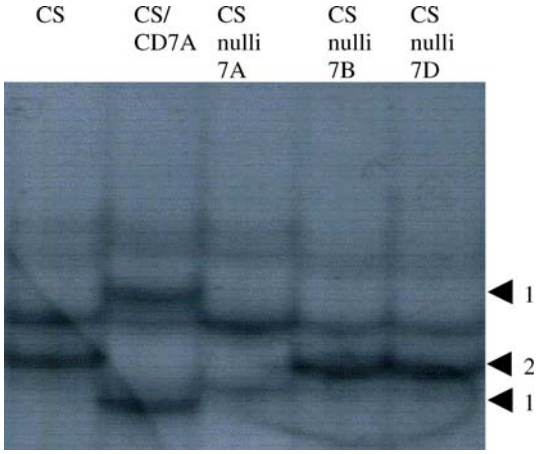

Fig. 2 An SSCP gel of a polymorphism screen between Chinese Spring (*CS*), CS chromosome substitution Cappelle Desprez 7A line (CS/CD7A) and CS nullisomic–tetrasomic lines 7A, 7B and 7D with sequence 6CD7A18. Sequence 6CD7A18 is derived from chromosome 7A, as a band is absent from CS nullisomic–tetrasomic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

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

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

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

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

Discussion

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

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

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

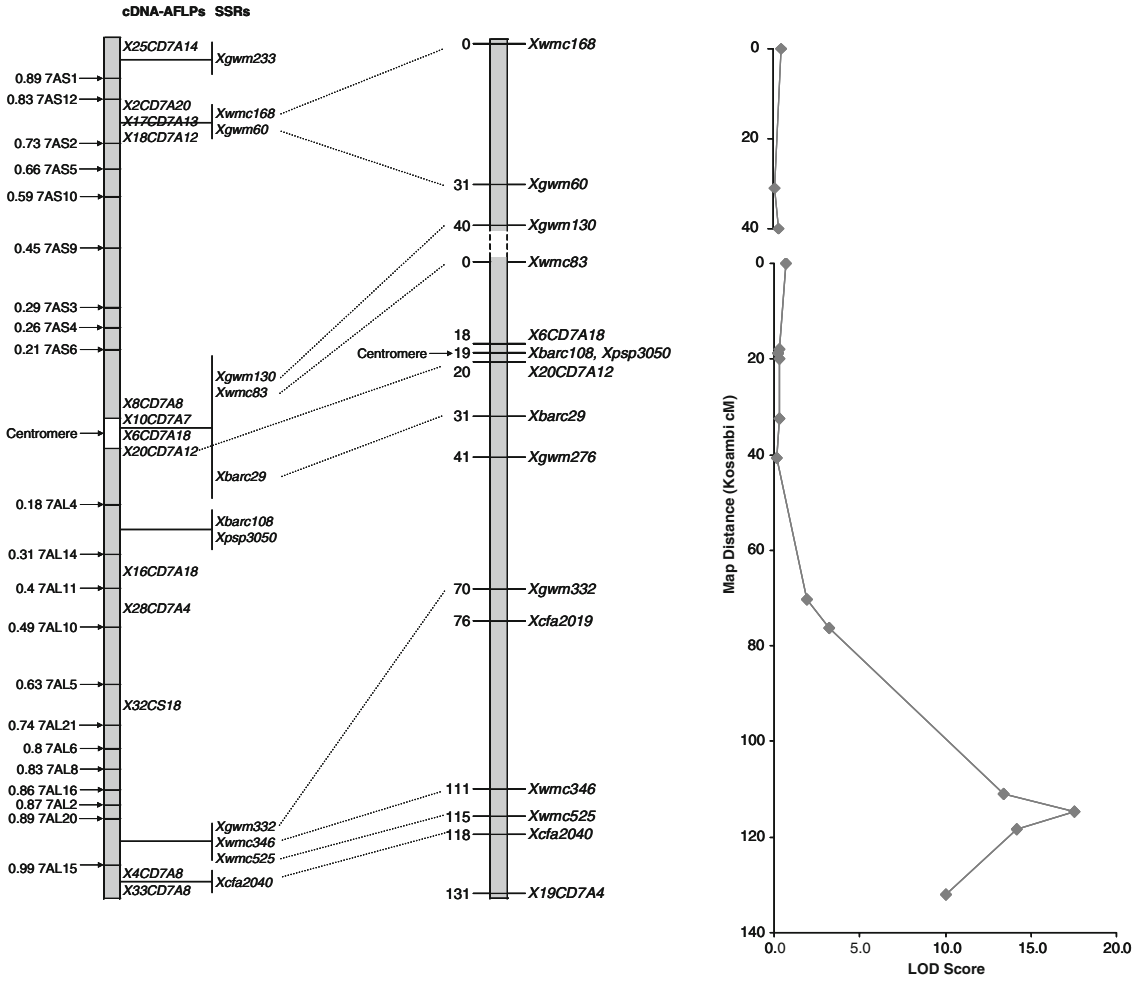

Fig. 3 A comparison of integrated SSR and cDNA-AFLP physical and genetic maps of chromosome 7A. A LOD profile from the QTL interval mapping analysis of *Pch2* on chromosome 7A is also shown aligned to the genetic map to demonstrate the position of *Pch2*

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

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

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

breakpoint, possibly in the region within the terminal bin. Physical mapping using CS 7A deletion bin lines positioned two of the cDNA-AFLP derived markers (*X4CD7A8* and *X33CD7A8*) in the distal deletion bin of chromosome 7AL, in approximately the same region as *Pch2*. Therefore, the genes relating to these sequences can be considered as candidates for *Pch2*.

Sequence 4CD7A8 is particularly interesting as a candidate for *Pch2* as in addition to its chromosomal location, it showed homology to an *Oryza sativa* putative callose synthase protein. Callose synthase is produced in response to wounding and as a defence response to pathogen attack (Østergaard et al. 2002; Jacobs et al. 2003) as well as during cell wall development (Hong et al. 2001). Callose deposition (papillae) acts as a physical barrier preventing pathogens from penetrating the host cell (Holub and Cooper 2004). Furthermore, papillae formation and lignification of cell walls in epidermal and pith cells of wheat stems infected with *Oculimacula* spp. has also been associated with resistance (Murray and Ye 1986). Callose synthase may be associated with papillae formation preventing *Oculimacula* spp. from penetrating and infecting CS/CD7A. RT-PCR revealed that expression of 4CD7A8 increased significantly in CS/CD7A, in response to infection by *O. acuformis* at both 7 and 14 dpi suggesting an involvement of this gene in the resistance response of CS/CD7A. In contrast, although we could detect expression of this gene in CS it was not significantly enhanced in response to infection at either time point. The identification of the fragment from the original cDNA-AFLP analysis suggests that a polymorphism exists between CS and CS/CD7A in the gene relating to the fragment 4CD7A8. Unfortunately, our assay for 4CD7A8 did not detect any polymorphism between CS and CS/CD7A. However, this assay was based only on the available sequence of the callose synthase gene that aligned to the wheat EST sequence data in the NCBI database. When additional sequence data becomes available for this gene it will be possible to determine whether polymorphisms exist between CS and CD.

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

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

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

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

genetic map along with the development, identification and disease testing of more recombinant lines.

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

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

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

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