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Expression profiling and mapping of defence response genes associated with the barley–*Pyrenophora teres* incompatible interaction

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SUMMARY

Barley net- and spot-form of net blotch disease are caused by two *formae* of the hemibiotrophic fungus *Pyrenophora teres* (*P. t.* f. *teres* and *P. t.* f. *maculata*). In the present study, suppression subtractive hybridization (SSH) was used in combination with quantitative real-time reverse transcriptase PCR to identify and profile the expression of defence response (*DR*) genes in the early stages of both barley–*P. teres* incompatible and compatible interactions. From a pool of 307 unique gene transcripts identified by SSH, 45 candidate *DR* genes were selected for temporal expression profiling in infected leaf epidermis. Differential expression profiles were observed for 28 of the selected candidates, which were grouped into clusters depending on their expression profiles within the first 48 h after inoculation. The expression profiles characteristic of each gene cluster were very similar in both barley–*P. t.* f. *teres* and barley–*P. t.* f. *maculata* interactions, indicating that resistance to both pathogens could be mediated by induction of the same group of *DR* genes. Chromosomal map locations for 21 *DR* genes were identified using four doubled-haploid mapping populations. The mapped *DR* genes were distributed across all seven barley chromosomes, with at least one gene mapping to within 15 cM of another on chromosomes 1H, 2H, 5H and 7H. Additionally, some *DR* genes appeared to co-localize with loci harbouring known resistance genes or quantitative trait loci for net blotch resistance on chromosomes 6H and 7H, as well as loci associated with resistance to other barley diseases. The *DR* genes are discussed with respect to their map locations and potential functional role in contributing to net blotch disease resistance.

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INTRODUCTION

Barley net- (NFNB) and spot-form (SFNB) of net blotch are two of the major diseases affecting barley crops around the world, with grain yield losses of up to 40% reported in severely affected regions (Khan, 1987). Relatively recent outbreaks of net blotch have been reported in all major barley-growing areas of the world (Mathre, 1997), yet despite having such a devastating impact on grain yield and quality, surprisingly very little is known about the molecular processes, in particular the genes, that regulate a successful defence response between barley and the two pathogens responsible for the disease. The pathogens in question are two *formae* of the hemibiotrophic fungus *Pyrenophora teres*, with *P. teres* f. *teres* (*Ptt*) responsible for NFNB and *P. teres* f. *maculata* (*Ptm*) responsible for SFNB (Smedegård-Petersen, 1971).

At present, the use of resistant barley cultivars is the most effective and economical method of controlling net blotch disease, and the identification of single dominant net blotch resistance (*R*) genes in barley using molecular markers (Graner *et al*., 1996; Manninen *et al*., 2000; Williams *et al*., 1999) has facilitated the development of resistant cultivars via marker-assisted selection. Nevertheless, the mixed (sexual and asexual) reproductive system of *P. teres* (Serenius *et al*., 2005) constitutes a high evolutionary risk for resistance breeding (McDonald and Linde, 2002). Instances where the pathogen has overcome the effectiveness of *R* genes in cultivars have been reported (Platz *et al*., 2000), and therefore as a long-term option for the control of this disease, alternative strategies need to be developed to account for the introduction or evolution of new virulent pathotypes.

Unlike *R* genes, which act as genes that encode for proteins involved in pathogen recognition (Dangl and Jones, 2001), defence response (*DR*) genes are defined as a group of genes that are induced downstream of the recognition event (Dixon and Harrison, 1990). *DR* genes often show altered expression in response to pathogen challenge and have previously been identified by differential screening methods such as differential display (Sánchez-Torres and González-Candelas, 2003), cDNA-AFLP

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(Zhang *et al*., 2003), suppression subtractive hybridization (SSH) (Bittner-Eddy *et al*., 2003) and microarray analysis (Zierold *et al*., 2005). The identified *DR* genes encode enzymes of secondary metabolism, proteins involved in cell-wall modification, hydrolases and pathogenesis-related (PR) proteins, and regulatory proteins controlling the expression of multiple downstream *DR* genes (Dixon and Harrison, 1990). In a number of pathosystems, the constitutive over-expression of *DR* genes in transgenic dicotyledonous and monocotyledonous plants has led to an enhanced resistance level, supporting the hypothesis that the products of these genes are involved in the biosynthesis of defence compounds and/or participate in one or more signal transduction pathways leading to effective host defence (Christensen *et al*., 2004; Leckband and Lörz, 1998; Oldach *et al*., 2001). For example, transgenic barley plants carrying a stilbene synthase gene derived from *Vitis vinifera* L. showed increased resistance to the fungal pathogen *Botrytis cinerea* (Leckband and Lörz, 1998) and transgenic wheat plants in which a barley chitinase was over-expressed showed increased resistance to two fungal pathogens, namely *Erysiphe graminis* f. sp. *tritici* and *Puccinia recondita* f. sp. *tritici* (Oldach *et al*., 2001).

Recent evidence suggests that *DR* genes may control the quantitative nature of disease resistance, and to date, several co-localizations between *DR* gene markers and quantitative trait loci (QTL) for disease resistance have been reported in rice (Wang *et al*., 2001; Wisser *et al*., 2005), potato (Trognitz *et al*., 2002), and pepper (Pflieger *et al*., 2001). For NFNB, QTL for disease resistance have been reported on all chromosomes, with several groups identifying what appears to be a common QTL on chromosome 6H (Cakir *et al*., 2003; Friesen *et al*., 2006; Manninen *et al*., 2000). In contrast, SFNB resistance loci have been mapped to different chromosomes in every study thus far reported (2H in Ho *et al*., 1996; 7H in Williams *et al*., 1999; 4H in Friesen *et al*., 2006; and 5H in Manninen *et al*., 2006). It is possible that some of the known net blotch resistance QTL may correspond to *DR* genes and that molecular polymorphisms within these genes result in allelic diversity that may be related to the observed quantitative resistance levels. At present, none of the numerous QTL controlling resistance to both forms of net blotch have been described at the molecular level.

Utilizing a common barley genotype that contains *R* loci effective against *Ptt* and *Ptm* isolates, the method of SSH (Diatchenko *et al*., 1996) was used in the present study to isolate *DR* gene transcripts that are differentially expressed in the epidermis of barley–*Ptt* and barley–*Ptm* incompatible interactions. The aims were to (1) use quantitative real-time reverse transcriptase (RT) PCR (Q-PCR) to profile the expression of *DR* genes during the first 48 h after inoculation (hai) in both incompatible and compatible interactions, and (2) determine if the differentially expressed genes map to known net blotch *R* loci or QTL. The epidermis was specifically targeted to look for *DR* genes because it represents the first barrier encountered by the pathogen and is directly penetrated by it (Keon and Hargreaves, 1983). Previously, transcriptional changes involving *DR* genes have been shown to occur in the epidermis of barley leaves infected with other pathogens that have a direct mode of penetration, such as *Blumeria graminis* f. sp. *hordei*, the fungus responsible for powdery mildew (Zierold *et al*., 2005), and *Rhynchosporium secalis*, the scald-causing fungus (Steiner-Lange *et al*., 2003).

RESULTS

Differential screening and sequencing of SSH clones

Two cDNA libraries containing candidate *DR* genes that are differentially expressed in the barley–*P. teres* incompatible interaction were generated following SSH. The NFNB- and SFNB-associated SSH libraries comprised 335 and 367 clones, respectively, with the average insert size being 222 bp. Macroarray analysis showed a low level of sensitivity resulting in the majority of arrayed clones, 242 NFNB (72%) and 302 SFNB (82%), showing weak or non-existent hybridization signals when probed with unsubtracted tester and driver cDNAs (data not shown). The sequencing of 54 clones for which a hybridization signal was detected, including all those that showed differential signal intensities, revealed a high proportion of clones with homology to genes encoding either ribulose-1,5-bisphosphate carboxylase (RUBISCO), the photosystem II (PSII) protein D1 or a subtilisin-chymotrypsin inhibitor 2 (CI2). These proteins are known to be abundant in nature and were not investigated further. The larger number of clones showing weak or no detectable hybridization signals were interpreted as being potentially of low abundance. The sequencing of these clones (241 NFNB and 199 SFNB) showed that 307 were representative of unique genes, 249 of which were represented by one copy in the library.

Functional annotation of SSH clones

The 307 unique expressed sequence tag (EST) sequences were used to annotate the function of their respective genes based on their alignment with known translated sequences using the BLASTx algorithm (Altschul *et al*., 1990) and the public nucleotide and amino acid sequence databases available at NCBI. According to putative function, each clone was distributed into one of ten functional categories (Fig. 1), including those related to secondary metabolism, cell maintenance and growth, stress and defence responses, gene expression, transport, signal transduction, protein degradation, and proteins of unknown function. From the 307 gene transcripts, 282 showed significant similarity to barley ESTs and therefore were assumed to be derived from the host and not the pathogens. The remaining 25 transcripts that did not show significant homology with barley ESTs were classified as

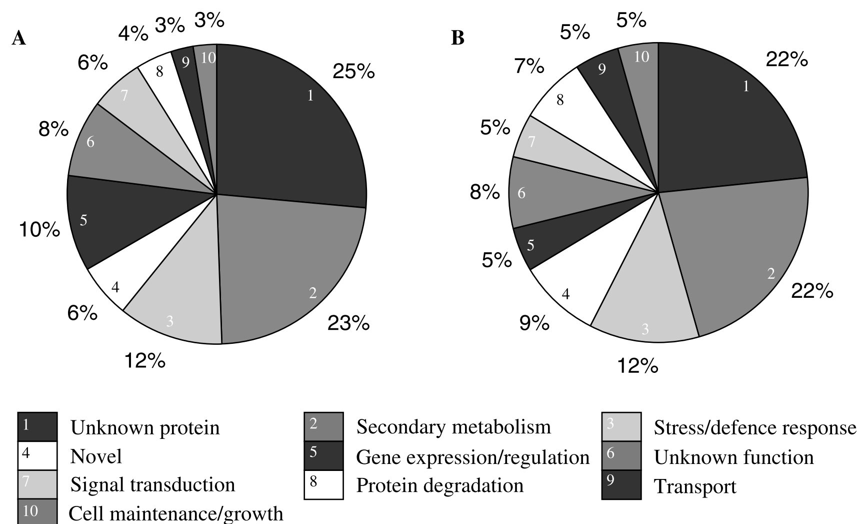


Fig. 1 Distribution of SSH clones from the NFNB (A: 335 clones) and SFNB (B: 367 clones) subtraction libraries into functional groups based on their sequence alignments using BLASTx.

'novel' genes. They also did not share any similarities to sequences from 25 fungal genomes available from the Broad Institute, including that of *Pyrenophora tritici repentis*. The grouping of SSH clones into functional categories shows that a similar distribution pattern exists in both NFNB- and SFNB-associated subtraction libraries (Fig. 1A,B).

Expression profiling of SSH clones by Q-PCR

One of the technical limitations of the present study was the difficulty associated with further analysing all of the clones identified by SSH. Therefore, a subset consisting of 45 candidate *DR* gene transcripts was selected for detailed expression profiling. Some genes were selected at random while others were chosen on the basis of their association with proteins putatively involved in general defence responses, signal transduction, carbohydrate metabolism and detoxification. Candidate *DR* gene expression profiles were monitored in both barley–*Ptm* and barley–*Ptt* incompatible and compatible interactions at six time points within 48 hai by Q-PCR, using a 1.5-fold cut-off value to assign genes as being differentially expressed. The prefix *HvPtr* (for *Hordeum vulgare P. teres* resistance) was used to name the differentially expressed *DR* genes. The candidate genes were grouped into one of eight clusters depending on the kinetics of their expression in infected tissue relative to water-inoculated (mock) controls (Fig. 2). The non-replication of both *Ptt* and *Ptm* inoculation experiments necessitated a form of statistical analysis which treated these two inoculations as replicates. By including all six time points within a gene cluster in the same analysis, 12 degrees of freedom were generated within the residual variance estimate, which yielded secure *t*-values in almost all cases. The statistical analysis of both incompatible and both compatible interactions within a cluster revealed that their respective expression profiles were very similar (broken lines, Fig. 2). The only point pairs that showed a relatively high level of disagreement were those of very low expression before 12 hai in gene cluster 6 (compatible and incompatible interactions), as well as at points 6 and 12 hai in gene clusters 3 and 5, respectively (compatible interaction only) (data not shown). Because of the overall high level of similarity, the means of each different interaction were used to generate consensus profiles (solid lines, Fig. 2). When individual expression profiles from each of the seven differential clusters were compared with those of gene cluster 8 (non-differential), significant differences ($P < 0.05$) between either the incompatible or compatible interactions were identified at certain time points, which enabled these clusters (especially gene clusters 1–4 in which the fold change in expression was low) to be clearly distinguished from that of gene cluster 8.

The first cluster (cluster 1) contained three genes (*HvPtr1* to *HvPtr3*) that were up-regulated by a factor of 2 in the incompatible interaction very early after infection (1 and 3 hai). In the compatible interaction, the same genes were found to be down-regulated by a factor of 2 at the corresponding time points. The combined expression profiles of seven genes (*HvPtr4* to *HvPtr10*) made up cluster 2, which was characterized by a four-fold induction in gene expression at 12 hai in the resistant response compared with both susceptible and control responses. A possible delayed induction of these genes was observed in the susceptible genotype at 48 hai. The expression profile of clones included in cluster 3 shows that this group of genes (*HvPtr11* to *HvPtr17*) was induced later and stronger in the incompatible interaction. The genes appear to be initially down-regulated from 1 to 6 hai, and then up-regulated from 6 to 24 hai, where there is a 2.5-fold increase in transcript levels relative to controls. In contrast, the compatible interaction shows an inverse mode of expression for

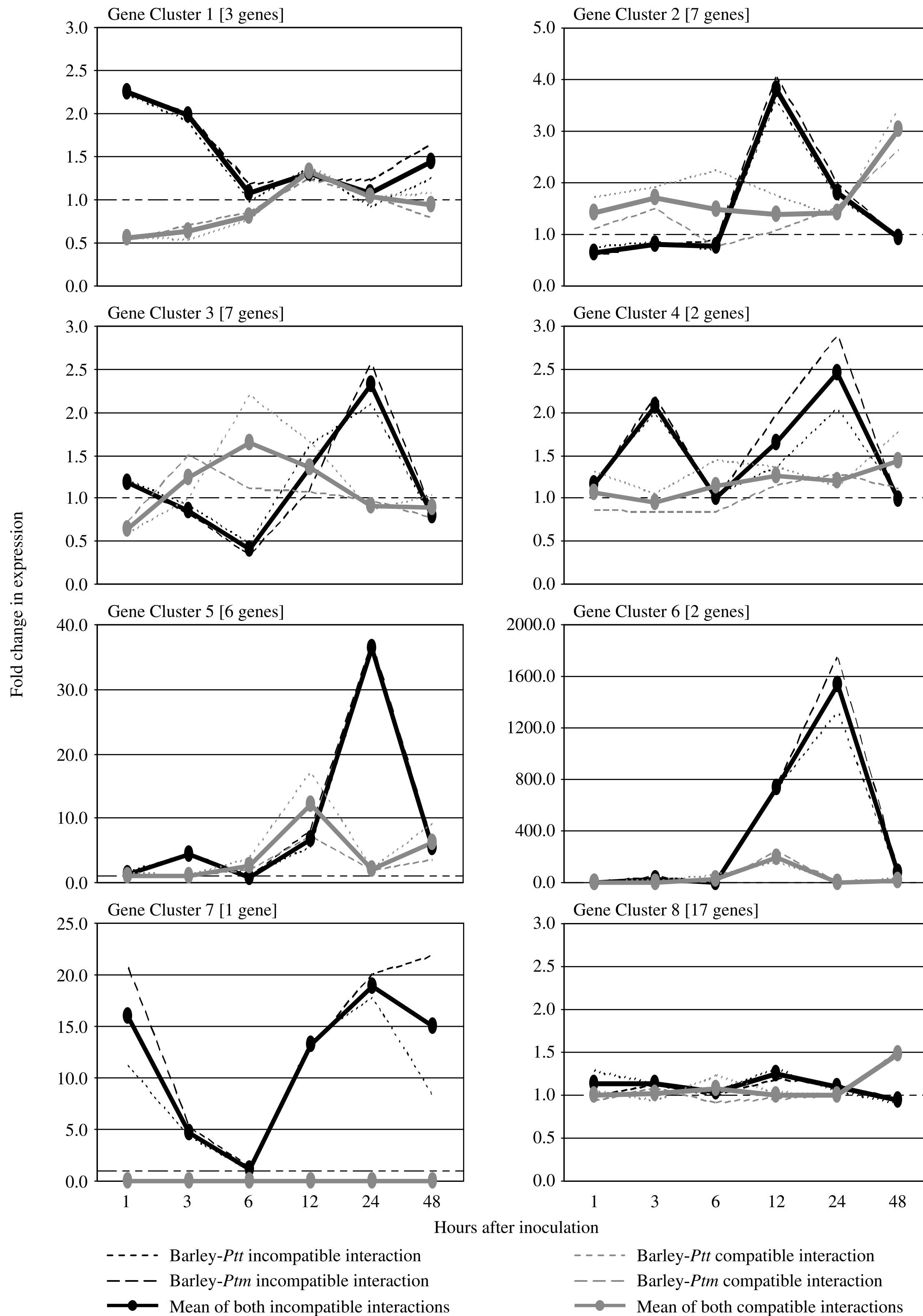


Fig. 2 Grouping of 45 candidate *DR* genes into gene expression clusters based on their temporal expression profiles in the barley epidermis following infection with *Ptm* and *Ptt*. Broken lines represent mean expression profiles for genes in both barley–*Ptm* and barley–*Ptt* incompatible and compatible interactions. Solid lines represent consensus expression profiles generated from the means of both sets of incompatible and compatible interactions combined. The *y*-axis indicates the fold change in expression for all genes in a given cluster relative to mock-inoculated control plants.

this cluster. The mean expression profiles of two genes (*HvPtr18* and *HvPtr19*) form cluster 4, which is highlighted by an early and late up-regulation of these genes at 3 and 24 hai in the resistant genotype only. The same genes do not appear to be differentially expressed in the susceptible genotype. Clusters 5 and 6 have a similar expression profile with their representative genes (*HvPtr20* to *HvPtr27*) being strongly up-regulated at 24 hai in the incompatible interaction. The difference between the two clusters is in the level of up-regulation observed at this time point, with cluster 5 genes being up-regulated 40-fold and cluster 6 genes 1500-fold. The genes grouped into clusters 5 and 6 are also up-regulated at 12 hai in both resistant and susceptible genotypes; however, in the resistant response the gene transcripts are further induced at 24 hai whereas in the susceptible response their expression returns to control levels. Cluster 7 was unique to one gene (*HvPtr28*), which appeared to be differentially expressed in the incompatible interaction only. The gene was up-regulated 15-fold in the resistant genotype within 1 hai before returning to control levels at 6 hai. It was then induced again and continued to be up-regulated by approximately 15-fold from 12 to 48 hai. Cluster 8 represented 17 genes that showed no differential expression and thus were considered as false positives. In total, 28 of 45 candidate *DR* genes were differentially expressed, indicating that a subtraction efficiency of 62% was achieved from the SSH. These 28 *DR* genes formed the group of genes to be analysed further by genetic mapping. Their putative functions based on homologies to genes encoding known proteins are summarized in Table 1. The 17 genes from cluster 8 that were not differentially expressed and their putative functions are listed in Supporting Information Table S1.

Mapping of *DR* genes and their association with known net blotch resistance loci

Of the 28 *DR* genes identified by expression profiling, 14 could be mapped by direct PCR or by converting them into cleaved amplified polymorphic sequence (CAPS) markers. Those not able to be mapped in this way, i.e. no simple sequence repeats (SSRs) or single nucleotide polymorphisms (SNPs) identified between mapping parents, were subsequently examined for their utility as restriction fragment length polymorphism (RFLP) probes. RFLPs were detected in at least one set of mapping parents for seven candidate genes. All of the *DR* gene probes used for RFLP analysis detected single loci with variable copy number (data not shown). In total, 21 of the 28 candidates were able to be mapped. Corresponding chromosomal locations for each clone are illustrated in Fig. 3 and marker types are summarized in Table 1.

Two *HvPtr DR* genes, *HvPtr12* and *HvPtr18*, were mapped onto chromosome 1 in the G*HN and C*S populations, respectively. The resulting loci displayed comparable positions between maps as indicated by common markers linked via the C*H map. On chromosome 2H, four genes, *HvPtr5*, *HvPtr16*, *HvPtr20* and *HvPtr21*, were mapped in the C*S population. *HvPtr5* and *HvPtr16*, *HvPtr16* and *HvPtr21*, and *HvPtr21* and *HvPtr20* are separated by distances of 53.7, 25.1 and 10.7 cM, respectively. Three genes, *HvPtr4*, *HvPtr8* and *HvPtr19*, were mapped onto chromosome 3H in the G*HN and C*S populations. *HvPtr19* and *HvPtr4* are 38.3 cM apart, while the distance between *HvPtr4* and *HvPtr8* is probably even greater as shown by their positions in relation to the common marker WG940. Interestingly, *HvPtr19*, which encodes a putative casein kinase, appears to be located in the same region as the *Pt. a* gene, which confers resistance to NFNB (Graner *et al*., 1996). Only one *DR* gene, *HvPtr3*, was mapped onto chromosome 4H. The chromosome to which the highest number of *HvPtr* genes mapped was 5H. It comprised five genes, *HvPtr2*, *HvPtr9*, *HvPtr10*, *HvPtr14* and *HvPtr15*. *HvPtr9*, *HvPtr14* and *HvPtr15* were mapped in the C*S population, whereas *HvPtr2* and *HvPtr10* were mapped in the A*S and G*HN populations, respectively. Due to their close proximity to common markers, *HvPtr10* and *HvPtr15* appear to have comparable map positions. Likewise, *HvPtr9* and *HvPtr14*, separated by 14.4 cM, are also located close to one another. It seems that the corresponding sets of genes are not tightly linked as the distance between *HvPtr9* and *HvPtr15* on the C*S map is 41.2 cM. It is possible that *HvPtr2* also shares a similar map position to *HvPtr14*. On chromosome 6H, two genes, *HvPtr11* and *HvPtr28*, were mapped in the C*S population, along with SSR markers HVM14, EBmac0874 and Bmag0173, which in previous studies have been shown to be linked to QTL for NFNB resistance (Cakir *et al*., 2003; Friesen *et al*., 2006; Manninen *et al*., 2000, 2006). In the C*S population, *HvPtr11*, which encodes a putative invertase, mapped to the same position as HVM14 and Ebmac0874, with Bmag0173 situated 2.7 cM away. *HvPtr28* mapped to a location 51.6 cM away from *HvPtr11*. Four genes, *HvPtr7*, *HvPtr17*, *HvPtr24* and *HvPtr27*, were mapped onto chromosome 7H in the C*H, G*HN and C*S mapping populations. *HvPtr7* and *HvPtr17*, which share homology with genes encoding a major facilitator superfamily (MFS) protein and a cytochrome P450 monooxygenase, respectively, appear to flank the *Rpt4* gene, which confers resistance to SFNB (Williams *et al*., 1999). *HvPtr7* is 18.6 cM away from *Rpt4*, whereas *HvPtr17* is likely to be much closer as indicated by its close proximity to the marker AWBMA11, which is only 4.7 cM away from *Rpt4* on the G*HN map. *HvPtr24*, which shares similarity to a copine-encoding gene, may also be situated near *Rpt4*. In contrast, *HvPtr27* does not map near this locus. It is located 53.1 cM away from *Rpt4* and 34.5 cM away from *HvPtr7*.

Co-localization of *DR* genes with loci conferring resistance to other barley diseases

In addition to the co-localization of *HvPtr* genes with known net blotch resistance loci, some of them also mapped closely to

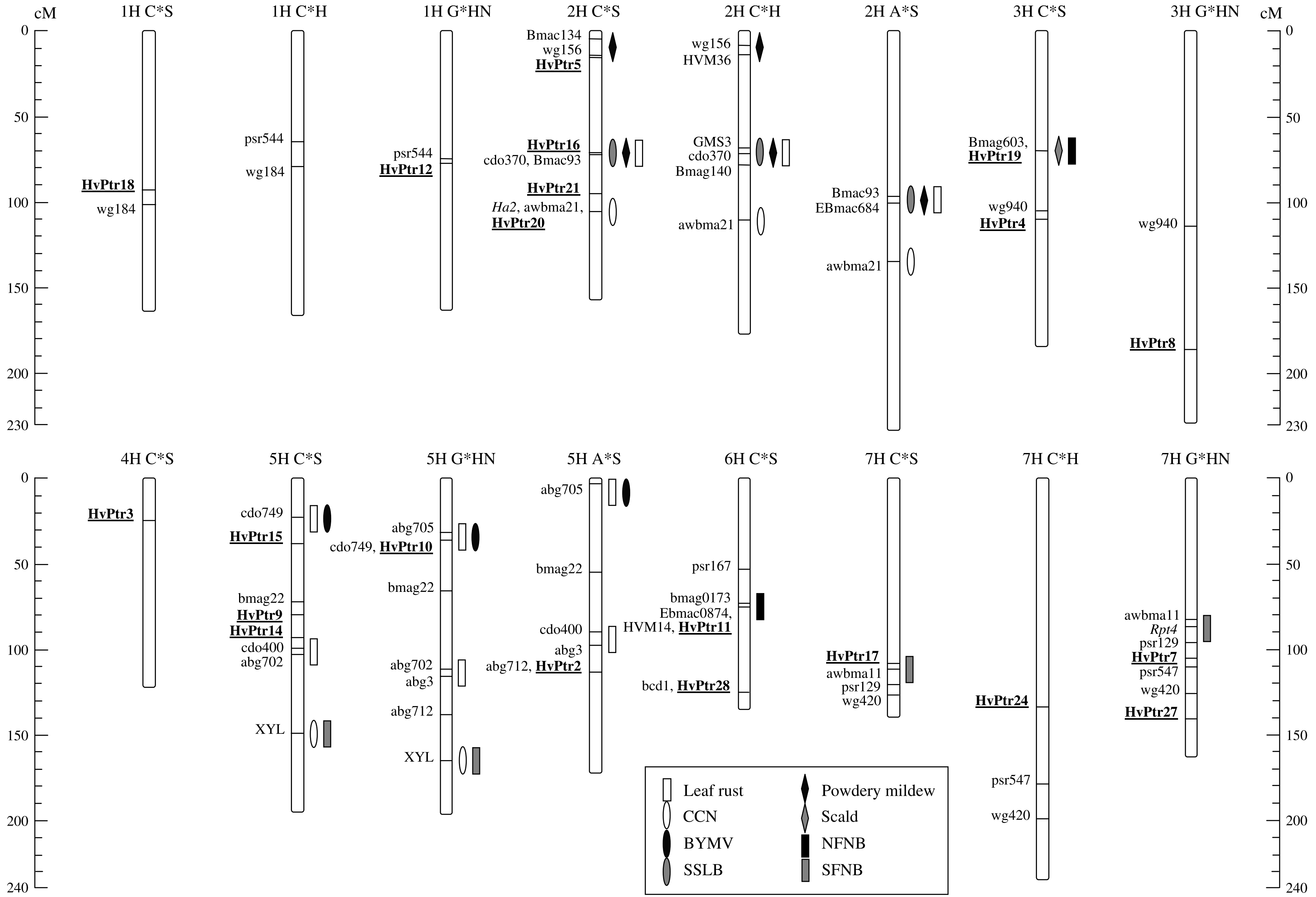
Table 1 Summary of 28 *DR* gene clones differentially expressed in both barley–*Ptt* and barley–*Ptm* incompatible interactions.

Clone	Size (bp)	GenBank accession no.	Protein similarity	Sequence origin	BLASTx: Accession no. of similar sequence	BLASTx: Similarity	Gene cluster	Chromosomal position and marker type
Carbohydrate metabolism								
HvPtr6	203	EY965255	Sugar transporter	*O. sativa*	ABF94465	1e-40 (73%, 115aa)	2	not mapped
HvPtr11	424	EY965260	Putative neutral/alkaline invertase	*O. sativa*	BAD33266	6e-48 (94%, 97aa)	3	6H, SSR
Detoxification								
HvPtr7	121	EY965256	Major facilitator superfamily protein	*O. sativa*	EAZ19564	8e-08 (80%, 45aa)	2	7H, RFLP
HvPtr8	393	EY965257	Putative sterol delta-7 reductase	*A. thaliana*	BAD19845	8e-60 (90%, 130aa)	2	3H, CAPS
HvPtr12	290	EY965261	Mitochondrial aldehyde dehydrogenase	*H. vulgare*	BAB62757	4e-10 (72%, 50aa)	3	1H, RFLP
HvPtr15	249	EY965264	Aldehyde dehydrogenase	*O. sativa*	AAG43027	3e-29 (95%, 82aa)	3	5H, PCR
HvPtr17	278	EY965266	Putative cytochrome P450	*L. rigidum*	AAK38091	8e-45 (91%, 92aa)	3	7H, PCR
HvPtr23	275	EY965272	MATE efflux protein family	*O. sativa*	BAC83974	6e-07 (50%, 63aa)	5	not mapped
HvPtr27	176	EY965276	Putative flavin mono-oxygenase	*O. sativa*	EAY93143	2e-22 (82%, 58aa)	6	7H, RFLP
Signal transduction								
HvPtr2	366	EY965251	Putative receptor-like protein kinase	*O. sativa*	AAP68881	4e-06 (69%, 42aa)	1	5H, RFLP
HvPtr3	392	EY965252	14-3-3-like protein A	*H. vulgare*	P29305	2e-52 (100%, 103aa)	1	4H, CAPS
HvPtr5	452	EY965254	Putative receptor-like protein kinase	*O. sativa*	CAH67715	1e-41 (87%, 93aa)	2	2H, RFLP
HvPtr9	319	EY965258	Putative B-box-type zinc finger protein	*O. sativa*	BAD46368	1e-60 (77%, 148aa)	2	5H, CAPS
HvPtr14	159	EY965263	Putative ser/thr protein kinase	*O. sativa*	BAC79157	4e-17 (83%, 48aa)	3	5H, CAPS
HvPtr18	156	EY965267	Leucine-rich repeat protein family	*O. sativa*	AAV31268	4e-36 (73%, 109aa)	4	1H, CAPS
HvPtr19	152	EY965268	Casein kinase I-like	*O. sativa*	BAB92346	2e-20 (96%, 50aa)	4	3H, SSR
Calcium signal perception								
HvPtr1	111	EY965250	Ser/thr protein kinase, NPH1-1	*A. sativa*	AAC05083	5e-48 (89%, 104aa)	1	not mapped
HvPtr10	499	EY965259	Putative calcineurin B-like protein	*O. sativa*	AAL31965	5e-22 (90%, 53aa)	2	5H, CAPS
HvPtr24	260	EY965273	Putative copine I	*O. sativa*	BAD35622	5e-22 (93%, 49aa)	5	7H, PCR
Stress or defence response								
HvPtr4	154	EY965253	Chitinase	*O. sativa*	BAA23809	4e-64 (75%, 172aa)	2	3H, RFLP
HvPtr25	106	EY965274	Putative proteinase inhibitor	*T. aestivum*	AAS49905	5e-43 (100%, 90aa)	5	not mapped
HvPtr26	217	EY965275	Pathogenesis-related protein 1	*H. vulgare*	CAA79703	9e-17 (100%, 39aa)	6	not mapped
Miscellaneous								
HvPtr13	237	EY965262	Alpha-galactosidase	*H. vulgare*	CAA74160	1e-36 (97%, 78aa)	3	not mapped
HvPtr16	166	EY965265	CBS domain containing protein	*O. sativa*	EAY93850	3e-22 (90%, 55aa)	3	2H, SSR
HvPtr20	176	EY965269	Putative threonine aldolase	*O. sativa*	EAY94851	1e-19 (82%, 58aa)	5	2H, CAPS
HvPtr21	333	EY965270	Glutamate dehydrogenase	*O. sativa*	BAE48298	5e-28 (90%, 63aa)	5	2H, RFLP
HvPtr22	180	EY965271	Putative ABA-responsive protein	*O. sativa*	BAD25079	8e-59 (76%, 139aa)	5	not mapped
HvPtr28	413	EY965277	Putative iron/ascorbate-dependent oxidoreductase	*O. sativa*	EAZ36021	4e-27 (59%, 99aa)	7	6H, CAPS

Note: protein similarities for clones in bold type were derived from the best barley EST match. aa, amino acids.

genetic loci associated with resistance to a variety of other diseases affecting barley (Fig. 3). *HvPtr5*, which shares homology with a gene encoding a receptor-like protein kinase, mapped to within a few centi-Morgans of chromosome 2H markers linked to QTL for resistance to powdery mildew (HVM36: von Korff *et al.*, 2005; Bmac134: Řepková *et al.*, 2006). *HvPtr20*, which shares sequence homology with a threonine aldolase-encoding gene, appears to be closely linked to the cereal cyst nematode (CCN) resistance gene, *Ha2* (Kretschmer *et al.*, 1997). Furthermore, additional markers associated with QTL for resistance to septoria speckled leaf blotch (SSLB) (GMS3, Bmag140: Yun *et al.*, 2005) as well as powdery mildew and leaf rust (Ebmac684, GMS3: von Korff *et al.*, 2005) resistance are located on chromosome 2H near *HvPtr16*, whose gene transcript encodes a putative

Fig. 3 Chromosomal locations of 21 *DR* genes in relation to known *R* gene loci and QTL contributing to resistance against NFNB, SFNB and other barley diseases. The *DR* genes were mapped in either C*S, G*HN, C*H or A*S doubled haploid mapping populations (Willsmore *et al.*, 2006) and are shown in bold and underlined. Reference markers for comparative analysis are also shown. Map positions of known *R* gene loci and QTL for disease resistance (bars) were inferred from published data.


cM
1H C*S
1H C*H
1H G*HN
2H C*S
2H C*H
2H A*S
3H C*S
3H G*HN
HvPtr18
wg184
psr544
wg184
psr544
HvPtr12
Bmac134
wg156
HvPtr5
HvPtr16
cdo370, Bmac93
HvPtr21
Ha2, awbma21,
HvPtr20
wg156
HVM36
GMS3
cdo370
Bmag140
awbma21
Bmac93
EBmac684
awbma21
Bmag603,
HvPtr19
wg940
HvPtr4
wg940
HvPtr8
4H C*S
5H C*S
5H G*HN
5H A*S
6H C*S
7H C*S
7H C*H
7H G*HN
HvPtr3
cdo749
HvPtr15
bmag22
HvPtr9
HvPtr14
cdo400
abg702
XYL
abg705
cdo749, HvPtr10
bmag22
abg702
abg3
abg712
XYL
abg705
bmag22
cdo400
abg3
abg712, HvPtr2
psr167
bmag0173
Ebmac0874,
HVM14, HvPtr11
bcd1, HvPtr28
HvPtr17
awbma11
psr129
wg420
HvPtr24
psr547
wg420
awbma11
Rpt4
psr129
HvPtr7
psr547
wg420
HvPtr27
Leaf rust
CCN
BYMV
SSLB
Powdery mildew
Scald
NFNB
SFNB
0
50
100
150
200
230
240

cystathionine-β-synthase (CBS) domain-containing protein. On chromosome 3H, *HvPtr19* is also linked closely to the *Rh* gene, which confers resistance to scald (Graner and Tekauz, 1996). The *Pt. a* and *Rh* genes were shown to be genetically linked in repulsion phase at less than 0.2 cM in the cross Igri*Franka (Graner *et al.*, 1996). *HvPtr10* and *HvPtr15*, which share homology with genes encoding a calcineurin b-like (CBL) protein and aldehyde dehydrogenase, respectively, have comparable map positions on chromosome 5H with markers for the barley yellow mosaic virus (BYMV) *R* gene, *ym3* (abg705: Saeki *et al.*, 1999), and the leaf rust *R* gene, *Rph2* (cdo749: Borovkova *et al.*, 1997). *HvPtr14* appeared to map near a marker linked closely to another gene conferring resistance to leaf rust, *Rph9* (abg3: Borovkova *et al.*, 1998).

DISCUSSION

The SSH method was used to identify genes that are differentially expressed in the incompatible interaction between barley and two *P. teres* isolates that induce NFNB or SFNB disease symptoms. The cloning of SSH transcripts yielded two EST libraries corresponding to inoculations of barley epidermis with either the *Ptm* or the *Ptt* isolate. It was anticipated that differentially expressed transcripts would be identified by looking at differences in the signal intensity of arrayed clones when probed with unsubtracted tester or driver cDNAs; however, limitations in sensitivity made this differential screening procedure inefficient. Grenier *et al.* (2002) also encountered a similar lack of sensitivity when using macroarrays to analyse the differential expression of SSH-derived transcripts. Thus, although it was not possible to determine if all SSH clones were differentially expressed by macroarray analysis, the method was still useful in selecting clones for further analysis because abundant transcripts encoding RUBISCO, PSII protein D1 and CI2 could be discarded.

Although the SSH procedure enabled the isolation of 307 individual candidate *DR* gene transcripts, one technical limitation was that it was not possible to generate expression profiles for them all. From the 45 candidates selected for detailed expression profiling, 28 were found to be differentially expressed in both barley–*Ptt* and barley–*Ptm* incompatible interactions. Although this equates to a subtraction efficiency of 62%, the true figure is likely to be lower considering that results from the macroarray analysis, which revealed 28 and 18% of clones from the NFNB and SFNB libraries, respectively, to be non-differential or of little importance, were not factored into the calculation. Due to the highly abundant nature of their corresponding proteins (e.g. RUBISCO), these clones are likely to have escaped subtraction. It would appear then that SSH was not entirely comprehensive in this study—a finding commonly encountered with this method (e.g. Birch *et al.*, 1999; Grenier *et al.*, 2002).

Expression profiles of barley defence responses to *Ptm* and *Ptt* overlap

It was notable that the transcript levels of the 28 differentially expressed genes analysed by Q-PCR followed the same profiles over the 48-h time-course, irrespective of whether *Ptt* or *Ptm* was used for inoculation. In addition, it is evident that the entire repertoire of differentially expressed genes in the barley–*P. teres* incompatible interaction was not isolated from one subtraction alone as clones isolated from one of the net blotch incompatible interactions were also differentially expressed but not identified in the other. Although the expression data presented for both barley–*Ptt* and barley–*Ptm* interactions were based on one inoculation experiment, the fact that their individual expression profiles are so similar indicates that the observed expression patterns accurately reflect the way *DR* genes are differentially regulated following infection by either pathogen. Maximum induction of gene clusters ranged from approximately two- to 1532-fold over the mock-inoculated controls. Although a change of 1532-fold may seem extreme, a similar high level of *DR* gene induction was recently reported (Adhikari *et al.*, 2007).

The similarities that exist between the two sets of expression profiles imply that the barley–*Ptt* and barley–*Ptm* incompatible interaction involves coordinated expression of the same group of *DR* genes, even though the *R* genes thought to be responsible for specifically recognizing each pathogen are likely to be different. Resistance to NFNB and SFNB has been shown to be inherited independently (Ho *et al.*, 1996) with *R* loci effective against both causal pathogens being different (Graner *et al.*, 1996; Williams *et al.*, 1999), even in the same genetic background (Manninen *et al.*, 2006). The Ethiopian two-rowed barley line CI 9819, for example, has been shown to carry at least two independent genes for net blotch resistance: *Rpt5* effective against NFNB isolates, and *Rpt6* effective against SFNB isolates, on chromosomes 6H and 5H, respectively (Manninen *et al.*, 2006). Based on expression profile similarities of the *DR* genes investigated, we propose that although different *R* genes appear to be involved in *Ptm* and *Ptt* recognition, resistance to both pathogens is controlled by an overlapping or even identical defence response that is triggered downstream of the recognition event.

Unlike other studies in which expression profiles of pathogen-induced genes during incompatible and compatible interactions have been shown to be qualitatively similar but quantitatively different (Tao *et al.*, 2003; Zierold *et al.*, 2005), this was not the case here. Although a quantitative difference was observed in all clusters, expression profiles between barley–*P. teres* incompatible and compatible interactions were not qualitatively similar. The incompatible interaction was generally characterized by genes that were up-regulated while induction of the same genes was suppressed or they were down-regulated in the compatible interaction. Also, the compatible interaction did not appear to be

associated with a quantitative increase in *DR* gene induction at later time points as has been reported by other groups (Boyd *et al*., 1994; Tao *et al*., 2003). The observed differences could reflect variations that exist in the way defence responses are activated against pathogens that have necrotrophic versus biotrophic stages of infection. Indeed, quantitative but not qualitative differences in *DR* gene expression were also observed in wheat cultivars that were resistant and susceptible to septoria tritici blotch caused by another hemibiotrophic fungal pathogen, *Mycosphaerella graminicola* (Adhikari *et al*., 2007).

DR genes involved in carbohydrate metabolism

Two *DR* genes, *HvPtr6* and *HvPtr11*, encode proteins putatively associated with carbohydrate metabolism. *HvPtr6* encodes a sugar transporter, and several studies have shown that sugar transporter genes are induced in plants following pathogen attack (Williams *et al*., 2000; Zierold *et al*., 2005). Correlations have also been made between increased sugar levels and *DR* gene induction (Herbers *et al*., 2000). *HvPtr11* encodes a putative invertase. Invertases catalyse the cleavage of sucrose to glucose and fructose, and invertase gene induction has previously been observed in plants challenged by fungal pathogens (Fotopoulos *et al*., 2003). The fact that photosynthesis and photosynthetic gene expression has been shown to be repressed in plants following pathogen attack (Berger *et al*., 2004; Herbers *et al*., 2000) suggests that plant cells around the site of infection may be reliant on alternative sources of carbohydrates to satisfy the increased metabolic demand on infected leaf tissue. Therefore, induction of these two genes could serve to replenish and maintain sufficient sugar levels in such tissue.

DR genes representing possible signal transduction components

Eight *DR* genes encode putative signal transduction components, including a 14-3-3 protein, several protein kinases, a leucine-rich repeat (LRR) protein and a B-box-type zinc finger protein. Interestingly, among this group of *DR* genes are all three cluster 1 members, and their early induction in the barley–*P. teres* incompatible interaction suggests that they could be amongst the first group of genes that are involved in triggering the resistance-associated defence response immediately downstream of pathogen recognition. 14-3-3 proteins have previously been shown to regulate the activities of a number of proteins potentially involved in disease resistance, including H^+-ATPases and several classes of protein kinases (Roberts, 2003), and the 14-3-3 protein-encoding gene transcript isolated in this study was also induced in barley leaves inoculated with the pathogens responsible for powdery mildew (Brandt *et al*., 1992) and scald (K. Oldach, personal communication). Protein kinases are known to play an important role in activating plant defence responses following pathogen recognition (Romeis, 2001). Two kinase-encoding clones share homology with receptor-like kinases (RLKs) and several *R* genes have been shown to encode RLKs (Song *et al*., 1995; Sun *et al*., 2004). A casein kinase was also found to be up-regulated during the rice–*Magnaporthe grisea* (Rao *et al*., 2002) and olive–*Spilocaea oleagina* (Benitez *et al*., 2005) interactions. LRR proteins function in a number of signal transduction pathways and play an important role in mediating protein–protein interactions (Kobe and Deisenhofer, 1994). The LRR domain is a structural feature of four of the eight major classes of plant R proteins (Chisholm *et al*., 2006), and genes encoding other proteins with LRR domains have also been found to be induced in plants following pathogen infection (Hipskind *et al*., 1996; Jung *et al*., 2004).

DR genes involved in calcium signal perception

Three *DR* genes, *HvPtr1*, *HvPtr10* and *HvPtr24*, encode proteins putatively involved in Ca^{2+} signal perception. Ca^{2+} signalling is known to initiate cellular responses to a diverse range of developmental cues and environmental challenges, and increases in cytosolic Ca^{2+} levels have previously been reported in plants following pathogen attack (Olivain *et al*., 2003). *HvPtr1* shares homology with a gene encoding a protein kinase, *NPH1/PHOT1*, that encodes one of the major photoreceptors of the signal transduction pathway for phototropism (Briggs and Christie, 2002). The finding that Arabidopsis PHOT1 is also involved in the downstream activation of Ca^{2+}-permeable channels (Baum *et al*., 1999) suggests that a similar protein in barley may be involved in regulating the flow of Ca^{2+} in plant cells following pathogen attack. The fact that *HvPtr1* was up-regulated within 3 hai in the net blotch resistant genotype suggests that early recognition of specific Ca^{2+} signatures may be necessary to establish an effective defence response against *P. teres*. The differential expression of *HvPtr10* and *HvPtr24*, which encode proteins known to bind Ca^{2+}, at later time points, supports the involvement of Ca^{2+} in regulating the defence response. CBL proteins contain multiple Ca^{2+} binding domains and one *CBL* gene family member has been shown to play a crucial role in transducing and coordinating Ca^{2+}-mediated plant signalling and adaptation responses during cold, drought and salt stresses (Albrecht *et al*., 2003). Similarly, the copines are a group of Ca^{2+}-dependent, membrane-binding proteins, with structural domains that suggest they may be involved in defining Ca^{2+} signalling specificity and mediating protein–protein interactions (Creutz *et al*., 1998).

DR genes involved in detoxification mechanisms

Six *DR* genes identified in this study may be involved in regulating plant cell detoxification mechanisms. *P. teres* reportedly produces

at least three phytotoxins that incite many of the symptoms associated with net blotch disease (Weiergang *et al*., 2002). In addition, accumulation of toxic reactive oxygen species has been reported in barley cultivars infected with *Ptt* (Able, 2003). Therefore, it is possible that successful activation of a plant detoxification system may play a major role in determining the outcome of the interaction between plant and fungus. Among this group of genes is *HvPtr23*, which encodes a putative multidrug and toxin efflux (MATE) protein. In addition, the putative major facilitator superfamily protein encoded by *HvPtr7* is also a MATE family member. In plants, three MATE family members have previously been implicated in defence responses (Diener *et al*., 2001; Nawrath *et al*., 2002; Simmons *et al*., 2003), including *EDS5*, which is an important component of salicylic acid-dependent *R* gene-mediated signalling in Arabidopsis (Nawrath *et al*., 2002). The *NorM* gene from *Vibrio parahaemolyticus* is the only biochemically characterized MATE family member to date and is responsible for pumping antimicrobial agents out of bacterial cells in exchange for sodium (Morita *et al*., 2000). It is possible that HvPtr23 or HvPtr7 may function in similar ways by exporting *P. teres* toxins out of plant cells in exchange for essential nutrients. Two more *DR* genes potentially associated with detoxification mechanisms, *HvPtr12* and *HvPtr15*, encode putative aldehyde dehydrogenases (ALDHs). These enzymes catalyse the oxidation of various toxic aldehydes to carboxylic acids (Perozich *et al*., 1999). Aldehydes accumulate as a result of lipid peroxidation caused by oxidative stress and increasing ALDH activity may represent one defence strategy that plants can use to prevent cell death caused by oxidative stress, thereby combating hemibiotrophic pathogens such as *P. teres* that rely on dead tissue in order to survive during the latter stages of their infection cycle. The potential involvement of plant ALDHs as components of defence responses to biotic stress is highlighted by studies which have shown that *ALDH* gene transcripts accumulate differentially in plants responding to various abiotic stress treatments (Kirch *et al*., 2001; Ozturk *et al*., 2002). In one report, over-expression of an *ALDH* gene in Arabidopsis resulted in transgenic plants displaying improved tolerance to oxidative stress (Sunkar *et al*., 2003).

DR genes involved in general defence responses

Three *DR* genes encode putative defence components, of which two are PR proteins. *HvPtr26* shares homology with a gene encoding a PR-1 protein that was also expressed earlier and more strongly in powdery mildew resistant compared with susceptible barley lines infected with *B. graminis* (Peterhänsel *et al*., 1997; Schultheiss *et al*., 2003). Schultheiss *et al*. (2003) reported that transgenic barley lines over-expressing PR-1 limited penetration of *B. graminis* on the host, so it is possible that elevated levels of PR-1, especially in the epidermal cell layer, may also play a role in arresting *P. teres* development. *HvPtr4* is similar to a chitinase-encoding gene. Chitinases belong to the PR-3 family of PR proteins and chitinase protein activity and transcript levels have been shown to increase in a number of plant–pathogen interactions (Wang *et al*., 2005). Like PR-1, transgenic plants producing elevated levels of chitinase have also displayed improved resistance to fungal pathogens (Melchers and Stuiver, 2000). The only gene with a unique expression profile, *HvPtr28*, encodes an iron/ascorbate-dependent oxidoreductase. This putative redox enzyme may be involved in ethylene biosynthesis as its deduced amino acid sequence is also similar to that of an ethylene-forming enzyme. The possible involvement of ethylene in conferring resistance to *P. teres* is interesting as this plant hormone has been widely implicated in the activation of plant defence responses (Wang *et al*., 2002). In addition, the nearest barley homologue to *HvPtr28* was found to encode a putative redox enzyme, HCP1, that interacts with the *Brome mosaic virus* coat protein (Okinaka *et al*., 2003), which could suggest that a similar interaction may occur between the *HvPtr28* gene product and *P. teres*-derived elicitors to inhibit infection.

Mapping of *DR* genes in the barley genome

The observations made from the genetic mapping of *HvPtr* genes corroborate those of recent studies in which certain *DR* genes have been shown to co-localize with QTL or *R* loci associated with resistance to a wide range of diseases or insect manifestations in pepper (Pflieger *et al*., 2001), potato (Trognitz *et al*., 2002), rice (Liu *et al*., 2004; Wang *et al*., 2001; Wisser *et al*., 2005), wheat (Faris *et al*., 1999) and barley (Marcel *et al*., 2007). The genetic mapping also showed that ten of the 21 mapped *HvPtr* genes mapped to within 15 cM of at least one other *HvPtr* gene, indicating that some *DR* genes associated with the barley–*P. teres* interaction may be closely linked. Comparable map positions for at least two different *DR* genes were observed on chromosomes 1H, 2H, 5H and 7H—with the 7H group of genes located in the region of the *Rpt4* locus. Again, this observation is in accordance with those reported in the aforementioned studies. There was no correlation between individual genes grouped to a particular expression cluster and their map location in relation to other members of the same cluster. A number of the *HvPtr* genes also co-localize with regions conferring resistance to a number of other barley diseases, including scald, CCN, powdery mildew, BYMV, leaf rust and SSLB. The possible co-localization of *HvPtr* genes with genetic loci known to confer resistance to several barley diseases indicates that individual chromosomes may harbour defined genetic regions that confer quantitative or qualitative resistance to multiple pathogens. Recently, Wisser *et al*. (2005) reported that a number of chromosomal segments in rice appear to be associated with broad-spectrum quantitative disease resistance.

The generation of the SSH libraries and the subsequent expression profiling and mapping of *DR* genes involved in both barley–*Ptt* and barley–*Ptm* incompatible interactions has laid the foundation to investigate the barley–net blotch pathosystem in more detail. The production of transgenic lines that over-express/silence the identified *DR* genes, in particular, will facilitate experimentation to evaluate any link between their induction and map locations in relation to quantitative disease resistance against the barley net blotch and other fungal pathogens.

EXPERIMENTAL PROCEDURES

Plant material, fungal inoculation and sampling

Barley cultivars CI9214 and B87/14 were grown at 17 °C under fluorescent light in a controlled-environment chamber. It has been reported that CI9214 carries the SFNB resistance gene *Rpt4* (Williams *et al*., 1999) and is resistant to most *Ptm* and some *Ptt* isolates (Tekauz, 1990; Williams *et al*., 1999). Plants at the seedling stage were inoculated with a spore suspension of South Australian *Ptm* isolate 43/96 and *Ptt* isolate 19/98 (kindly provided by Hugh Wallwork, SARDI). CI9214 was resistant and B87/14 was susceptible to both isolates. Inoculum was prepared to a concentration of 1×10^4 conidia/mL in 0.01% Tween-20 and applied at a rate of approximately 0.8 mL per plant. Mock-inoculated plants were treated using the same conditions, but with a water solution containing only 0.01% Tween-20. After inoculation, all plants were kept at 19 °C in the dark for 24 h at 100% relative humidity and then under a 12-h light regime with alternating temperatures of 24 and 19 °C in the light and dark, respectively. The abaxial side of the epidermis from infected and control leaves was peeled at 1, 3, 6, 12, 24, and 48 hai and immediately frozen in liquid nitrogen. To reduce the impact of biological variation, ten epidermal samples per time point were pooled. Some plants from each cultivar were kept in the controlled-environment chamber for 14 days as controls to confirm the efficiency of the inoculation.

Suppression subtractive hybridization (SSH)

Total RNA was isolated from frozen epidermal tissue harvested at 24 hai using Tri Reagent (Molecular Research Centre, Cincinnati, OH) following the manufacturer's instructions. The Super SMART PCR cDNA synthesis kit (BD Biosciences Clontech, Palo Alto, CA) was then used to generate tester and driver double-stranded cDNA from approximately 0.5 μg total epidermal RNA according to the manufacturer's protocol. Two populations of tester and driver cDNA were generated—one each for the NFNB- and SFNB-associated SSH. In both subtractions, cDNA derived from infected CI9214 (resistant) and B87/14 (susceptible) epidermis at 24 hai was the source of tester and driver cDNA, respectively. Following cDNA synthesis, SSH was performed using the Clontech PCR-select cDNA subtraction kit (BD) as per the manufacturer's instructions.

Cloning and differential screening using macroarrays

Products of the second PCR from both NFNB- and SFNB-associated subtractions were directly inserted into the pCR®2.1-TOPO® plasmid vector (Invitrogen, Carlsbad, CA) and transformed into chemically competent *Escherichia coli* strain DH5α cells. Positive colonies were picked and grown in 96-well microtitre plates in 200 μL Luria-Bertani (LB) medium containing ampicillin (100 mg/L). The cDNA inserts were then amplified by colony PCR using a PTC-100 thermocycler (MJ Research, Waltham, MA) and M13 forward and reverse primers to check for the presence and size of individual inserts. The final reaction volume of 25 μL comprised a mixture containing 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 μM of each primer, 0.5 U of *Taq* DNA polymerase, and 1 μL of bacterial culture. The PCR was effected using the following cycling conditions: 1 min at 95 °C, 40 s at 94 °C, 35 s at 57 °C, 1 min at 72 °C, then 35 cycles of 40 s at 94 °C, 35 s at 52 °C and 1 min at 72 °C, and a final extension step of 10 min at 72 °C. All PCR products were electrophoresed on a 1.2% agarose gel, stained with ethidium bromide, then visualized under UV light.

For differential screening, SSH clones were arrayed in duplicate onto Hybond-N+ nylon membranes (Amersham Biosciences, Little Chalfont, UK) as described in Clontech's PCR-select differential screening kit protocol. Two identical membranes were prepared for each SSH library. They were hybridized with [α-^{32}P] dCTP-labelled probes generated from unsubtracted and subtracted cDNAs, then washed as described also in the aforementioned protocol. Washed membranes were placed in cassettes containing phosphor imaging plates (BAS-MP 2040S; Fujifilm, Minato, Tokyo, Japan) at room temperature for 24 h. The imaging plates were scanned using a STORM 860 phosphorimager (GE Healthcare) and the resulting hybridization signals were inspected visually.

DNA sequencing and sequence analysis

Cycle sequencing reactions were prepared using the Big Dye Terminator Version 3.1 labelling kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocol but using one-eighth of the recommended reaction mix. DNA sequencing was performed on an ABI 3700 DNA Sequencer (PE Applied Biosystems) and DNA sequences were edited using Vector NTI software (Invitrogen) to remove sequencing ambiguities and vector sequences. The edited sequences were used to query the public nucleotide and amino acid sequence databases at the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Sequence comparisons were carried out

using the basic local alignment search tool (BLAST) algorithms, BLASTn and BLASTx (Altschul *et al.*, 1990). Similarity to a known sequence was considered as significant if the obtained BLAST score had an E-value of less than 1×10^{-5}. For clones that had insufficient coding information from the insert sequence alone to predict functions using BLASTx, the amino acid sequence encoded by the best barley EST match was queried against the non-redundant GenBank protein database to determine putative function. Clones that did not show significant similarities to barley ESTs were queried against a database of genomic sequences from 25 fungi available from the Broad Institute (http://www.broad.mit.edu).

Q-PCR and data analysis

To prepare cDNA template for Q-PCR, RNA preparations from epidermal peels harvested 1, 3, 6, 12, 24 and 48 hai were treated with RNase-free *DNase* I (Ambion, Austin, TX) to remove residual DNA before first-strand synthesis. cDNA was synthesised from 0.5 μg of total epidermal RNA using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR primers were designed specifically to the nucleic acid sequence of each respective SSH clone using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Q-PCR was performed in an RG 3000 Rotor-Gene Real Time Thermal Cycler (Corbett Research, Sydney, Australia). The final reaction volume of 12 μL comprised a mixture containing 1× QuantiTect SYBR Green PCR reagent (Qiagen, Valencia, CA), 0.4 μM of each forward and reverse gene-specific primer, and 1 μL of a 1:10 dilution of transcribed single-stranded cDNA. The PCR was effected using the following cycling conditions: 15 min at 95 °C, followed by 35 cycles of 20 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and 15 s at the optimal data acquisition temperature for each gene-specific product (approximately 2 °C below the T_m of the specific PCR product), which was initially determined by heating the reaction products from 70 to 99 °C and analysing the resulting melt curve. All Q-PCR reactions were carried out in duplicate and melt curve analysis was performed at the end of each run to confirm that there was no signal from non-specific binding products. No template controls were included in each run to test for possible contamination of assay reagents. All gene-specific primers used for Q-PCR are listed in Supporting Information Table S2.

The Rotor-Gene 6 software (Corbett Research) was used to establish a standard curve that was generated from the PCR of serial ten-fold dilutions (10^7–10^2 copies/μL) of a purified 122-bp amplicon derived from the cDNA of a barley cyclophilin gene (Burton *et al.*, 2004). The standard curve was formed by plotting copy number (expressed in logarithmic form) versus values of threshold cycle (C_T) and gene-specific transcript quantities were automatically calculated by the supporting software by factoring in the C_T value for each target transcript into the equation of the line of best fit. A fixed fluorescence threshold of 0.1 was used to determine all C_T values. The standard curve derived from the cyclophilin gene transcript was imported into subsequent Q-PCR runs to determine quantities of target gene transcripts. In addition, one cDNA template was used across all Q-PCR experiments to account for differences in PCR efficiency between runs. To normalize for differences in the amount of RNA present in each sample prior to cDNA synthesis, the geometric averaging of eight internal reference gene transcripts (Supporting Information Table S2) by the geNorm program (Vandesompele *et al.*, 2002) was used to generate normalization factors. These were derived from the average expression stability of the three most stably expressed transcripts as determined by geNorm (Supporting Information Fig. S1). The fold changes of differentially expressed genes were calculated by dividing the normalized expression data of the inoculated samples by the data of the corresponding time points obtained for the mock inoculations. Differentially expressed genes were grouped into clusters based on similar expression profiles by visual inspection.

Mapping of candidate *DR* genes by direct PCR, CAPS and RFLP

Four previously described doubled-haploid (DH) mapping populations with existing genetic maps were used in this study: Clipper × Sahara (C*S), Galleon × Haruna nijo (G*HN), Chebec × Harrington (C*H) and Alexis × Sloop (A*S) (Willsmore *et al.*, 2006). To verify the suitability of using these populations for mapping, screens were initially performed to identify polymorphisms between the parents. For SSR and CAPS marker development, ESTs corresponding to candidate *DR* genes were queried against the nucleotide database of the barley gene index available from The Institute for Genomic Research (TIGR) website (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=barley). Unique barley gene indices were then queried against the rice genome database from the NCBI website to identify exon/intron boundaries for genes of interest. Genomic regions spanning introns were amplified by PCR in 25-μL reactions as described for the colony PCR but with the following cycling conditions: 90 s at 95 °C, then 35 cycles of 30 s at 94 °C, 30 s at 62 °C and 60–150 s at 72 °C depending on the expected size of the amplicon, followed by a final extension step of 10 min at 72 °C. Products were sequenced and SNPs/SSRs were identified by visual inspection of the aligned parental sequences. To amplify the detected SSRs, primers flanking the repetitive motif were designed and used in PCR. PCR products were resolved by polyacrylamide gel electrophoresis and visualized by staining with ethidium bromide. The SNP2CAPS program (Thiel *et al.*, 2004) was used to develop CAPS markers from the sequence alignments. For RFLP mapping, Southern hybridization of parental DNA was carried out according to

standard protocols (Sambrook *et al*., 1989) using 10 μg of digested DNA per lane and the restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Xba*I (5 U/μg). Southern filters for the mapping populations were kindly provided by Margaret Pallotta (Australian Centre for Plant Functional Genomics). For two gene markers, polymorphisms were detected after the initial PCR in the form of indels. The Map Manager QTX (version QTXb20; Manly *et al*., 2001) program was used to map *DR* gene markers onto the maps.

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SUPPORTING INFORMATION

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Fig. S1 Chart indicating the average expression stability (M) value of reference genes at each step during stepwise exclusion of the least stable reference gene, according to geNorm analysis

(Vandesompele *et al.*, 2002). Starting from the least stable gene at the left, the genes are ranked according to increasing expression stability, ending with the two most stable genes at the right.

Table S1 Summary of 17 candidate *DR* gene clones showing no differential expression in barley–*Ptt* and barley–*Ptm* incompatible compared with compatible interactions

Table S2 Gene-specific primers, expected PCR product sizes and optimal acquisition temperatures for expression profiling of *DR* genes by Q-PCR

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