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ORIGINAL PAPER

Parallel expression profiling of barley–stem rust interactions

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Abstract The dominant barley stem rust resistance gene *Rpg1* confers resistance to many but not all pathotypes of the stem rust fungus *Puccinia graminis* f. sp. *tritici* (*Pgt*). Transformation of *Rpg1* into susceptible cultivar Golden Promise rendered the transgenic plants resistant to *Pgt* pathotype MCC but not to *Pgt* pathotype QCC. Our objective was to identify genes that are induced/repressed during the early stages of pathogen infection to elucidate the molecular mechanisms and role of *Rpg1* in defense. A messenger ribonucleic acid expression analysis using the 22K Barley1 GeneChip was conducted in all pair-wise combinations of two isolines (cv. Golden Promise and *Rpg1* transgenic line G02-448F-3R) and two *Pgt* pathotypes (MCC and QCC) across six time points. Analysis showed that a total of 34 probe sets exhibited expression pattern differences between Golden Promise (susceptible) and G02-448F-3R (resistant) infected with *Pgt*-MCC. A total of 14 probe sets exhibited expression pattern differences between *Pgt*-MCC (avirulent) and *Pgt*-QCC (virulent) inoculated onto G02-448F-3R. These differentially expressed genes were activated during the early infection process, before the hypersensitive response or fungal growth inhibition occurred. Our analysis provides a list of candidate signaling components, which can be analyzed for function in *Rpg1*-mediated disease resistance.

Keywords Barley · Disease-resistance · Expression profiling · Microarray data · Rust resistance · *Puccinia graminis* f. sp. *tritici*

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Introduction

Stem rust, caused by the fungus *Puccinia graminis* f. sp. *tritici* (*Pgt*), has historically been a devastating barley disease in the USA. The resistance gene *Rpg1* in barley is effective against many pathotypes of *Pgt* (Steffenson 1992). It was bred into North American barley cultivars to control the stem rust epidemics that plagued the Northern Great Plains of the USA during the first half of the twentieth century. Since 1942, *Rpg1* has protected barley from significant stem rust losses, except for a minor epidemic caused by pathotype *Pgt*-QCC in 1989–1991. Race QCC is virulent on barley cultivars carrying the *Rpg1* gene (Jin et al. 1994).

A large body of intensive research over the past 75 years established *Pgt* as a model system for the rusts (Staples 2000). However, relatively little is known about the molecular basis of either resistance in barley or of virulence in *Pgt* during their interaction. Such knowledge may be crucial to combat future stem rust epidemics and to engineer improved disease resistance and durability in crop plants. The measurement of expression levels of thousands of genes at once by microarray analysis offers an opportunity to probe overall transcript patterns in particular organs, tissues, or cells. Microarray data provide an overview of gene expression patterns and can identify gene targets for further study. For these reasons, microarray-based studies of plant–pathogen interactions are becoming

more common. The recently developed Affymetrix Barley1 GeneChip (Close et al. 2004) that contains at least 21,439 genes is a platform technology that facilitates extensive and globally comparable transcript profiling experiments in this crop. The Barley1 GeneChip has been used in transcript-based cloning (Zhang et al. 2006), as an efficient approach for large-scale physical mapping, and to investigate gene expression patterns during barley development (Druka et al. 2006). Another common use for the GeneChip has been the study of plant–pathogen interactions, such as barley–*Blumeria graminis* (Caldo et al. 2004, 2006) and barley–*Fusarium graminearum* (Boddu et al. 2006) pathosystems.

Specific recognition in the barley–*Pgt* interaction is triggered in a gene-for-gene manner by the *Rpg1* gene. The introduction of a single copy of the *Rpg1* gene into the highly susceptible cv. Golden Promise by transformation rendered it highly resistant to pathotype *Pgt*-MCC (Horvath et al. 2003) but not to *Pgt*-QCC, showing that *Rpg1* functioned with the same specificity as in the parent cv. Morex. The *Rpg1* transgenic line (G02-448F-3R) differs from its isogenic cv. Golden Promise only in the presence of one copy of *Rpg1* and the selectable marker gene *bar*.

From the pathogen's perspective, a number of developmental processes are required for *Pgt* to successfully invade barley, including germination of urediniospores, elongation of germ tubes, formation of appressoria, formation of substomatal vesicles, formation of haustorium mother cells and haustoria, formation of secondary infection hyphae, and subsequent colonization of host tissue. An overview of the rust infection process during the first 36 h is provided in Fig. 1 (Lin et al. 1998; Sellam and Wilcoxson 1976). We are primarily interested in the early stages of barley–*Pgt* interaction leading to resistance.

The perception of the *Pgt* fungus by barley and the ability of the pathogen to avoid or overcome the host's defenses imply a complex, dynamic network of communication between these organisms, which may be modified through the different phases of their interaction. The induction and signal transduction of defense responses or the development of cell types specific to the interaction require up- or downregulation of many genes. We conducted messenger ribonucleic acid (mRNA) expression profiling using the Barley1 GeneChip to study barley

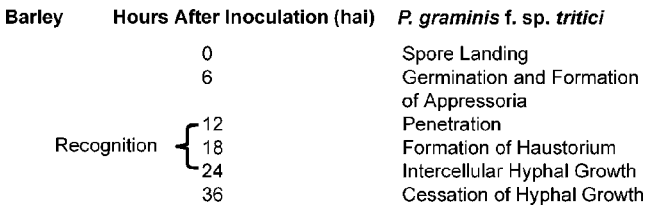

Fig. 1 Schematic representation of timing of events in the incompatible interaction between barley and *Puccinia graminis* f. sp. *tritici* (Adapted from Lin et al. 1998; Sellam and Wilcoxson 1976)

responses to the stem rust pathogen *Pgt*. In this study, we provide a comprehensive examination of gene transcript accumulation in barley during the early stages of infection by *Pgt*. This work may provide clues regarding the molecular mechanisms of compatibility and incompatibility in the barley–*Pgt* interaction.

Experimental methods

Plant materials

Barley cv. Golden Promise (CIho 13841) was used in this study. It is a stem rust-susceptible line that lacks *Rpg1*. G02-448F-3R is highly resistant transgenic line genetically engineered with *Rpg1* in a cv. Golden Promise genomic background. Plants were grown in a growth chamber at 20–22°C and 14-h photoperiod (160-W very-high-output [VHO] fluorescent and 60-W incandescent lamps) for a week before inoculation.

Fungal isolates

Pathotypes *Pgt*-MCC (avirulent for *Rpg1*) and *Pgt*-QCC (virulent for *Rpg1*) were used in this study. Spores of each pathotype were increased on a susceptible host McNair 701 (CItr 15288) line E (PI 357308), collected, desiccated at room temperature, and stored at −80°C until needed. On the day of inoculation, the rust spores were heat shocked at 45°C for 10 min and allowed to rehydrate for 4 h at room temperature and humidity.

Experimental design

Incompatible and compatible barley–stem rust interactions were generated by all pair-wise combinations of the Golden Promise (CIho 13841) and G02-448F-3R isogenic lines and the two *Pgt* pathotypes MCC and QCC. For each replication, individual genotypes were planted in six separate flats (20.7 cm deep×3.8 cm wide) using steamed field soil and 200 Metro Mix (containing equal amount of vermiculite, peat moss, perlite, and sand mix; #1 Sunshine Mix, Fisons, Vancouver, Canada). Each experimental flat consisted of six rows of ten seedlings, with rows randomly assigned to one of six harvest times in hours after inoculation (0, 6, 12, 18, 24, and 36 hai). Seedlings were grown to the one-leaf stage, 7 days (PO:0007094) after planting. Inoculations were performed using a rust inoculator pressured (27.5 kPa) by an air pump. The rate of inoculum applied was about 0.25 mg spores per plant. Inoculated plants were placed in a mist chamber in the dark at 21±1°C (100% relative humidity) for 16 h. Then the plants were exposed to light (120–160 μmol photon·m^{-2}·s^{-1}) for 2 h with mist and

allowed to dry slowly for another 3 h without mist before being placed in the incubation environment (23±1°C) under 160-W VHO fluorescent and 60-W incandescent lamps. Ten fully expanded primary leaves from each flat were collected at each time point, carefully folded, and placed in a 15-ml Falcon tube filled with RNA Later solution (Ambion, Texas, USA). The entire experiment was repeated three times in a standard split-split-plot design with 72 experimental units.

RNA isolation

Total RNA was isolated using the hot (60°C) phenol/guanidinium thiocyanate method (Close et al. 2004). Trizol-like reagent contained 38% saturated phenol, pH 4.3, 1 M guanidine thiocyanate, 0.1 M sodium acetate, pH 5.0, and 5% glycerol (Fisher Scientific, Pittsburg, PA). RNA was further purified using the RNeasy Midi kit (Qiagen, Valencia, CA).

Target synthesis and GeneChip hybridization

Target synthesis and GeneChip hybridization, washing, staining, and scanning were performed at the Molecular Biology Core at Washington State University. Microarray output was examined visually for excessive background noise and physical anomalies. The default MAS (Microarray Suite 5.0, Affymetrix, California, USA) statistical values were used for all analyses. All probe sets on each array were scaled to a mean target signal intensity of 125, with the signal correlating to the amount of transcript in the sample. An absolute analysis using MAS was performed to assess the relative abundance of the 22,792 represented transcripts based on signal and detection (present, absent, or marginal). The resulting data from the absolute analysis were exported into Microsoft Excel and then imported into EDGE software (EDGE, Seattle, WA).

Data analysis

After the expression data was loaded into EDGE, a covariate file was created and loaded into EDGE. The covariate file contains information about the experimental design (Table 3), such as which biological group comes from which array. Then, a "between class" analysis was performed to assess the evidence for a difference in expression over time between the two biological groups. In our analysis, we performed comparison between group "Golden Promise" and group "G02-448F-3R" and also comparison between group "MCC" and group "QCC." The number of permutations used in the significance calculation is 200; all runs, conducted several times, gave reproducible similar results. We also specified a natural cubic spline type in fitting the longitudinal model (Leek et al. 2006).

After EDGE ran the analysis based on our specifications, the "Differential Expression Results" menu was displayed. We selected a Q value less than 5% and a P value less than 0.0001 to display the genes that meet the significance threshold. Then, the final list of differentially expressed genes was exported to an Excel file.

Cluster analysis

Patterns of gene expression were identified using unsupervised cluster analysis within the set of differentially expressed transcripts that met the requirements detailed previously. Clustering algorithms allow for the separation of distinct patterns of expression based on the similarity of expression profiles between different genes. In this analysis, a hierarchical clustering algorithm utilizing a smooth correlation with the default parameters was utilized to isolate distinct, nonrepetitive patterns of expression within the time course. Clustering analysis was conducted by GeneSpring 5.1 software (Silicon Genetics, Redwood City, CA).

Data access

All detailed data and protocols from these experiments have been deposited in the Plant Expression Database (PLEXdb; http://www.plexdb.org). PLEXdb is a unified public resource for gene expression for plants and plant pathogens, including previous BarleyBase (BB; Shen et al. 2005). Files are categorized under accession number BB49 and can be downloaded at the Download Center as batch files in CSV, CEL, DAT, or expression data formats under "Browse BarleyBase Experiment Data."

Results

Transcriptome comparison between Golden Promise and *Rpg1* Transgenic Line without fungus inoculation

A Golden Promise line was converted from highly susceptible to highly resistant by transformation with a single copy of the *Rpg1* gene. Sequencing the plant deoxyribonucleic acid (DNA) at the transferred-DNA junction of the transgene and Southern hybridization of this ^{32}P-labeled flanking sequence to barley genomic DNA suggested that the transgene was inserted into a repetitive region, presumably not disrupting any genes (Horvath et al. 2003). To determine the changes in gene expression that might have taken place upon introduction of a single copy of the *Rpg1* transgene, we first compared transcriptomes of Golden Promise and the *Rpg1* transgenic line G02-448F-3R at 0 h time point. Gene expression data were obtained from six independent biological replicates (Table 1).

Table 1 Experimental design

Plants	Pathotypes	Time point (hai)	Replicates	Interaction
G02-448F-3R	MCC	0, 6, 12, 18, 24, 36	3	Incompatible
Golden Promise	MCC	0, 6, 12, 18, 24, 36	3	Compatible
G02-448F-3R	QCC	0, 6, 12, 18, 24, 36	3	Compatible
Golden Promise	QCC	0, 6, 12, 18, 24, 36	3	Compatible

G02-448F-3R is an *Rpg1* transgenic line that differs from cv. Golden Promise only by a single copy of *Rpg1* and the selectable marker *bar* gene inserted by transformation. The experiment was conducted in three independent biological replications using a standard split-split-plot design with replications as blocks, pathotype as the whole-plot factor, plant genotype as the split-plot factor, and time as the split-split-plot factor. At 0 time point, there were six (3+3) independent biological replicates for Golden Promise and G02-448F-3R.

There were a total of 15 upregulated and 9 downregulated genes in the *Rpg1* transgenic line compared with parent cv. Golden Promise at 0 h time point using a twofold change threshold (Table 2; Fig. 2).

Analysis strategy for expression profile comparison after infection

To study patterns of gene expression during pathogen infection of barley by *Pgt*, we chose to track changes in gene expression between a compatible and an incompatible interaction during the early stages of infection. We are interested in the genes that are responsible for initiating the resistance cascade. Consequently, we posed the question: What genes are induced and/or repressed in the early stages of infection (i.e., sampling times at 0–36 hai) of resistant and susceptible isolines? The experimental design employed is shown in Table 1. The two isogenic barley lines, a highly resistant transgenic line G02-448F-3R containing a single copy of *Rpg1* in cv. Golden Promise background and the susceptible wild type cv. Golden Promise, were challenged with either *Pgt* pathotype MCC or QCC, which are avirulent and virulent for *Rpg1*, respectively (Fig. 3).

Table 2 Genes significantly upregulated or downregulated (more than twofold) at 0 h time point in the *Rpg1* transgenic line G02-448F-3R compared to parent cv. Golden Promise identified by microarray analysis

Affymetrix probe set ID	Gene annotation	Fold change
Upregulated genes		
Contig12031_at	MADS box-like protein	10.72
Contig270_at	Seed storage protein	3.882
Contig3381_s_at	Subtilisin–chymotrypsin inhibitor 2	3.28
Contig7384_s_at	Putative zinc finger protein	2.898
Contig9248_s_at	Hypothetical protein	2.705
Contig14719_s_at	Ubiquitin-conjugating enzyme	2.637
Contig20418_at	Hypothetical protein	2.617
HD04G07u_s_at	Putative protease inhibitor	2.588
rbaal5k15_s_at	Hypothetical protein	2.54
HD07M22r_s_at	Proteinase inhibitor	2.49
Contig3380_s_at	Subtilisin–chymotrypsin inhibitor 2	2.386
Contig23505_at	Hypothetical protein	2.373
AB011266_at	Nicotianamine synthase 4	2.343
Contig15511_at	Putative protein	2.215
HV_CEa0002I05r2_at	Putative 2-Hydroxyisoflavanone dehydratase	2.081
Downregulated genes		
HVSMEm0003G16r2_at	Putative cytochrome P450	3.832
Contig5345_s_at	Expressed protein	2.869
Contig13474_at	Putative peptide deformylase	2.761
HV_CEa0002E17r2_s_at	Superoxide dismutase	2.504
Contig19393_at	Aquaporin 2	2.439
Contig6788_at	Copper chaperone homolog CCH	2.29
Contig17275_at	PG1-related cluster	2.219
Contig2716_s_at	Ferritin	2.186
Contig2894_s_at	Chloroplast inner envelope membrane protein	2.089

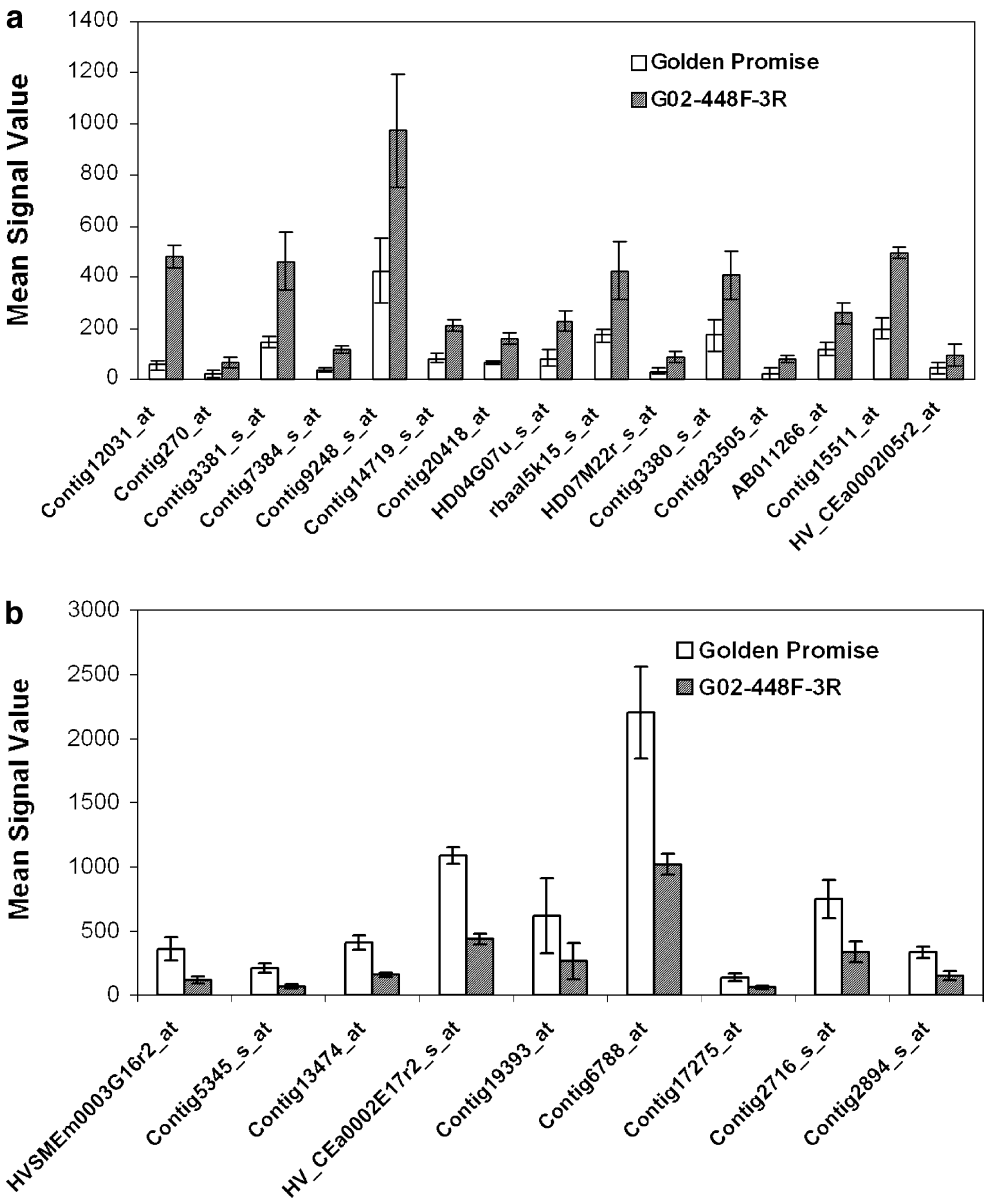

Fig. 2 Expression patterns of 15 upregulated genes (**a**) and nine downregulated genes (**b**) in the stem rust-resistant *Rpg1* transgenic line G02-448F-3R compared to the susceptible parent cv. Golden Promise. The mean signal values and the standard errors were calculated based on six independent biological replicates

We were primarily interested in genes (other than *Rpg1*) whose expression might be used to distinguish incompatible from compatible interactions in barley lines isogenic for *Rpg1*. Two different analyses were conducted to achieve the goal: (1) by comparing the two isogenic lines challenged with *Pgt*-MCC and (2) by comparing the effects of avirulent and virulent *Pgt* pathotypes, MCC and QCC, respectively, inoculated onto the *Rpg1* transgenic line.

Because the regulation of gene expression is a dynamic process, the arrays were collected over a time course, allowing us to study the dynamic behavior of gene expression and characterize changes in gene expression over time, instead of just a single time point. The software EDGE (Storey and Tibshirani 2003; Storey et al. 2005) is specifically designed for time course experiments; expression over time is modeled flexibly, and statistical significance is calculated while accounting for sources of dependence over time. It is suitable for our data analysis.

In the first comparison, we further restricted our attention to genes that were specifically induced by MCC, not QCC. Because we did not include a mock control, we eliminated the genes that showed similar changes in response to challenge by both pathotypes. The genes that were differentially expressed (P value less than 0.0001) between the two host lines only by MCC challenge are the genes of interest. In the second comparison, we selected for genes that are only differentially expressed between MCC and QCC inoculated onto the *Rpg1*-carrying transgenic line G02-448F-3R.

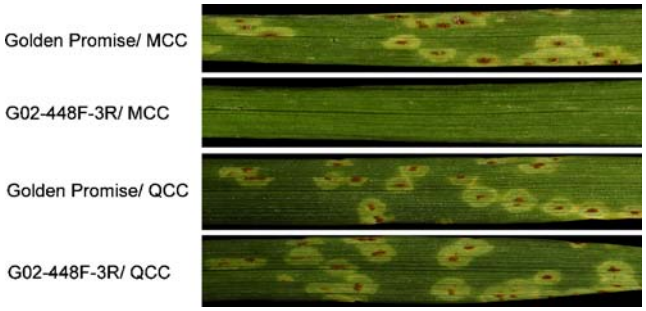

Fig. 3 Disease reaction of *Rpg1* transgenic line G02-448F-3R compared to parent cv. Golden Promise. Golden Promise is susceptible to both pathotypes *Pgt*-MCC and *Pgt*-QCC, whereas G02-448F-3R is resistant to *Pgt*-MCC but susceptible to *Pgt*-QCC

Barley genes differentially expressed in a *Rpg1*-dependent manner

Data analysis identified that 34 probe sets were differentially expressed between Golden Promise and the *Rpg1* transgenic line after *Pgt*-MCC challenge over time (Table 3; Fig. 4). These selected genes had a cutoff *P* value of less than 0.0001 and a false discovery rate of less than 5% (Storey and Tibshirani 2003). Classification of these genes by predicted function is shown in Table 3. Twenty-six of the 34 differentially expressed genes are predicted to function in cellular metabolism, signal transduction, regulation of gene expression, and plant defense. Eight genes

Table 3 Genes differentially expressed in the comparison of the *Pgt*-MCC pathotype-infected resistant transgenic line G02-448F-3R and susceptible parent cv. Golden Promise at the significance level of 0.0001

Affymetrix probe set ID	Predicted function	Classification	Organism
Upregulated genes			
Contig11773_at	Unknown	Unknown	
Contig9172_at	Probable germin protein 4	Defense	*O. sativa*
Contig3383_at	Subtilisin–chymotrypsin inhibitor 2	Defense	*H. vulgare*
Contig1675_s_at	Jasmonate-induced protein 1	Defense	*H. vulgare*
Contig19854_at	Cinnamyl alcohol dehydrogenase	Lignin synthesis	*L. perenne*
Contig10838_at	Putative threonine synthase	Cellular metabolism	*O. sativa*
Contig1942_at	Putative mono- or diacylglycerol acyltransferase	TAG synthesis	*O. sativa*
Contig16535_at	3-Methylcrotonyl CoA carboxylase biotin-containing subunit	Cellular metabolism	*O. sativa*
rbaal17b01_s_at	Unknown	Unknown	
HT11O15u_s_at	Putative mono- or diacylglycerol Acyltransferase	TAG synthesis	*O. sativa*
Contig9094_at	Osmotin-like protein	Defense	*O. sativa*
HU14N23u_s_at	Unknown	Unknown	
Contig11397_s_at	Unknown	Unknown	
Contig21116_at	Unknown	Unknown	*O. sativa*
Contig6582_at	Phi-1 protein	Cellular metabolism	*O. sativa*
Contig14377_at	Glycerophosphoryl diester phosphodiesterase-like	Cellular metabolism	*O. sativa*
HM05N11r_at	cinnamyl alcohol dehydrogenase	Lignin synthesis	*L. perenne*
Contig3319_at	Expressed protein	Unknown	*O. sativa*
Contig3814_at	Putative delta 1 pyrroline-5-carboxylate synthetase	Cellular metabolism	*O. sativa*
Contig1791_x_at	Adenosylhomocysteinase	Cellular metabolism	*H. vulgare*
Contig14679_at	Xylanase inhibitor	Defense	*H. vulgare*
Contig2864_s_at	Beta-glucosidase homolog	Defense	*A. thaliana*
Contig13422_at	Putative dioxygenase	Stress induced	*O. sativa*
Contig5924_s_at	Protein kinase	Signal transduction	*O. sativa*
Contig8361_at	RNA helicase	Signal transduction	*V. radiata*
Downregulated genes			
HW08H20u_at	Putative peroxidase	Oxidative stress	*O. sativa*
Contig3636_at	Leucine-rich repeat protein LRP	Defense	*O. sativa*
Contig25762_at	Unknown	Unknown	*O. sativa*
Contig3235_at	Methylenetetrahydrofolate reductase	Cellular metabolism	*Zea mays*
Contig5059_s_at	RNase S-like protein	Defense	*H. vulgare*
Contig25699_at	Putative membrane protein	Unknown	*A. thaliana*
Contig9747_at	Putative peptidyl-prolyl *cis–trans* isomerase	Protein folding	*O. sativa*
HV_CEb0010I11r2_at	Small GTP-binding protein	Signal transduction	*O. sativa*
Contig3667_s_at	myb-Related protein	Cellular metabolism	*H. vulgare*

Twenty-five genes with predicted function were upregulated in the transgenic line compared to Golden Promise. Nine genes with predicted function were downregulated in the transgenic line compared to Golden Promise.

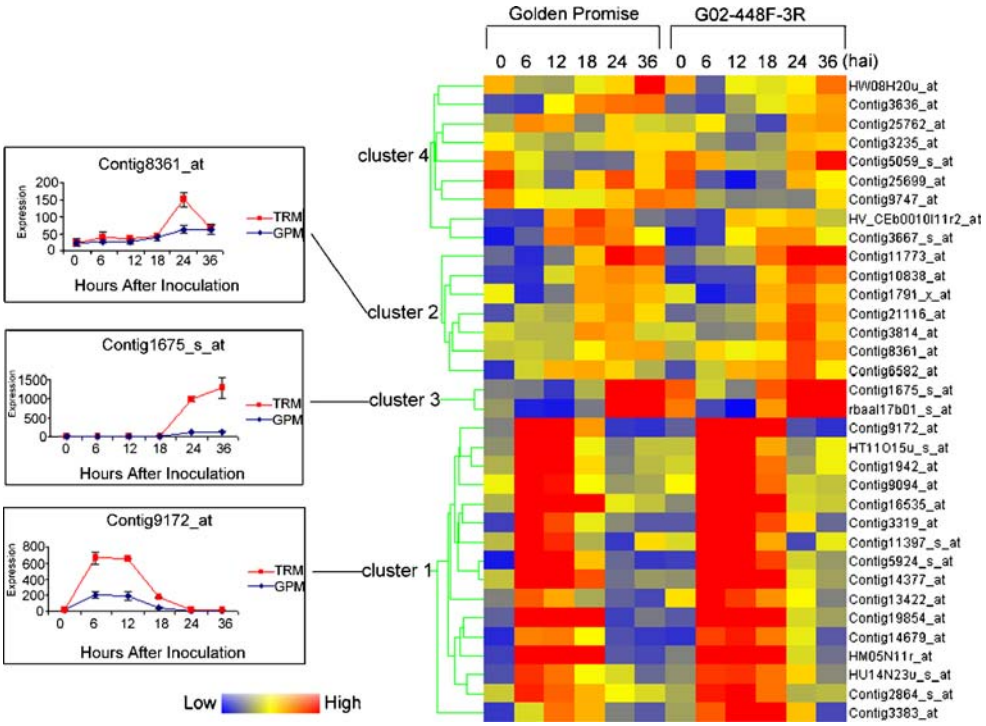

Fig. 4 Expression profile of 34 probe sets differentially expressed between incompatible and compatible interactions with *Pgt*-MCC in isolines differing in the presence or absence of the *Rpg1* gene. Golden Promise (*GPM*) is susceptible to *Pgt*-MCC. G02-448F-3R (*TRM*) is the *Rpg1* transgenic line in a Golden Promise genomic background, which is highly resistant to *Pgt*-MCC. Mean signal values were used to determine the similarities of expression profiles through cluster analysis. Hierarchical clustering was performed with the GeneSpring 5.1 software. The expression profiles of typical members of each cluster are presented in graph format. Cluster 4 could not be represented by a single graph because the individual expression profiles are too diverse

were of unknown function; three of these eight shared significant similarities to annotated sequences in the rice genome, and one had similarity to an annotated sequence in the *Arabidopsis* genome.

To determine the overall pattern of expression of the identified genes, we used a hierarchical clustering algorithm to construct a hierarchical tree using the transcript level per time point of the 34 differentially expressed genes in *Rpg1*-dependent compatible and incompatible interactions (Fig. 4). mRNA levels of the 34 differentially expressed genes clustered into four groups. The genes in the first cluster showed the same basic divergent expression between incompatible and compatible interactions as early as 6 and 12 hai and started to decline at 18 hai. In the resistant *Rpg1* transgenic line, these genes start to be upregulated around 6 hai and continued to diverge at 12 and 18 hai. This time period is coincident with fungus appressoria formation, penetration, and first haustorium formation. This result indicates that resistance to stem rust in barley, as conditioned by the *Rpg1* gene, may be expressed as early as appressoria formation above the stomata. The genes in the second cluster reached an expression maximum of 24 hai in the incompatible interaction and then declined at 36 hai, corresponding to haustorium formation and intercellular hyphae growth of the fungus. The transcripts of genes in the third cluster accumulated later at a higher level in the incompatible interaction, around 24 to 36 hai, a time when the hypersensitive response was more abundant and colony growth was partially inhibited. Therefore, 25 genes (Table 2; cluster nos. 1, 2, and 3 in Fig. 4) showed higher induction during the incompatible interaction, while nine genes (HW08H20u_at, Contig3636_at, Contig25762_at, Contig3235_at, Contig5059_s_at, HV_CEb0010I11r2_at, Contig25699_at, Contig3667_s_at, and Contig9747_at) in cluster no. 4 showed higher expression levels during the compatible interaction.

Barley genes differentially expressed in response to MCC and QCC

Because the *Pgt* pathotype QCC is virulent on barley cultivars carrying *Rpg1*, microarray experiments were conducted to evaluate the gene expression patterns of the *Rpg1* transgenic line G02-448F-3R, which was inoculated with MCC and QCC separately. We used the same analysis strategy to determine variation in transcript abundance in the same host genotype that was infected with different pathotypes. As reported by Rostoks et al. (2004), a time course experiment with Morex seedlings infected with the incompatible stem rust pathotype MCC did not show any significant differences in the *Rpg1* expression level compared to mock-treated controls. In agreement with this finding, our microarray data showed that the *Rpg1* transcript levels were not significantly different between *Pgt*-MCC and *Pgt*-QCC inoculated onto the *Rpg1* transgenic line across six time points (data not shown). Our results showed that 14 probe sets had *P* values less than

Table 4 Genes differentially expressed in the comparison of the *Rpg1* transgenic line G02-448F-3R infected with *Pgt*-MCC or *Pgt*-QCC

Affymetrix probe set ID	Predicted function	Classification	Organism
Upregulated genes			
Contig4402_s_at	Pathogenesis-related protein 10	Defense	*H. vulgare*
HV_CEa0009C05r2_s_at	Unknown	Unknown	
Contig5838_at	Glutathione S-transferase 2	Oxidative stress	*A. tauschii*
Contig6008_s_at	Glutathione S-transferase GST 34	Oxidative stress	*Zea mays*
Contig3383_at	Subtilisin–chymotrypsin inhibitor 2	Defense	*H. vulgare*
Contig3211_at	Blue copper-binding protein homolog	Electron transport	*T. aestivum*
Contig19593_at	Unknown	Unknown	*O. sativa*
Contig14915_at	Calmodulin-binding protein	Signal transduction	*O. sativa*
Contig16985_at	Putative ABA-responsive protein	Stress related	*O. sativa*
Contig3017_at	Oxalate oxidase	Defense	*H. vulgare*
Contig8489_s_at	Monooxygenase 2 (MO2)	Cellular metabolism	*A. thaliana*
Contig17916_at	Putative lignostilbene-alpha, beta-dioxygenase	Cellular metabolism	*O. sativa*
Contig4405_x_at	Pathogenesis-related protein PR-10a	Defense	*O. sativa*
Downregulated genes			
Contig8896_s_at	Cysteine proteinase	Pathogenesis	*A. thaliana*

Thirteen genes with predicted functions were upregulated during the MCC infection compared with the QCC infection, while only one gene was down regulated during the MCC infection compared with the QCC infection.

0.0001 for the comparison of expression patterns across incompatible and compatible interactions (Table 4). This set of 14 genes was associated with a false discovery rate less than 5% (Storey and Tibshirani 2003). The 14 genes that are differentially expressed during MCC and QCC infection include two PR-10 proteins, two glutathione-S-transferases (GSTs), a blue copper-binding protein homolog, calmodulin-binding proteins, and genes with unknown functions. Thirteen of these genes had a higher expression level in the incompatible MCC interaction than in the compatible QCC interaction. Only one gene, Contig8896_s_at, encoding a cysteine proteinase, was expressed at higher levels after QCC infection than MCC infection. As shown in Fig. 5, mRNA levels of the 13 differentially expressed genes clustered together, while Contig8896_s_at belongs to another cluster by itself.

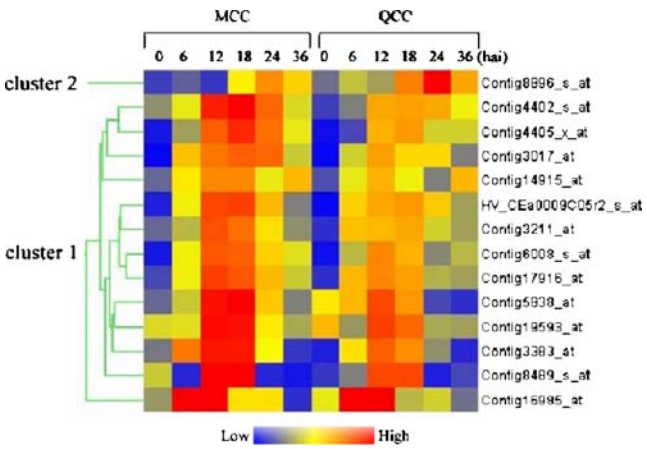

Fig. 5 Expression profile of 14 probe sets differentially expressed in the comparison of the *Rpg1* transgenic line (G02-448F-3R) infected with *Pgt*-MCC or *Pgt*-QCC, avirulent and virulent pathotypes, respectively. Mean signal values were used to determine the similarities of expression profiles through cluster analysis. Hierarchical clustering was performed with the GeneSpring 5.1 software

In cluster no. 1, the pattern of mRNA accumulation was highly similar in both incompatible and compatible interactions up to 12 h after infection and divergent thereafter. This is particularly interesting because 12 h after infection is when the fungus starts to enter through the stomata. This result also suggests that the resistance protein detects pathogen invasion at a very early stage, as early as the start of the fungal penetration.

A proteinase inhibitor in response to pathogen attack

In comparison of the *Rpg1* isolines infected with the avirulent pathotype MCC and infection of the *Rpg1* transgene with either compatible (QCC) or incompatible (MCC) pathotypes, a probe set, Contig3383_at, differentiated compatibility and incompatibility in the barley–stem rust interaction (Fig. 6). Contig3383_at is a gene predicted to encode a protein with homology to a subtilisin–chymotrypsin inhibitor. This gene was expressed at a low level before fungus infection (Fig. 2). At 6 hai, the gene was induced in both compatible and incompatible interactions but at a higher level in the incompatible interaction. The expression level kept increasing at 12 hai and reached a peak at 18 hai in the incompatible interaction. In the compatible interaction, it peaked at 12 hai and declined to a lower level at 18 hai. In both cases, the expression level was much lower in the compatible vs incompatible interaction. This gene could be a signature that differentiates *Rpg1*-dependent compatibility and incompatibility.

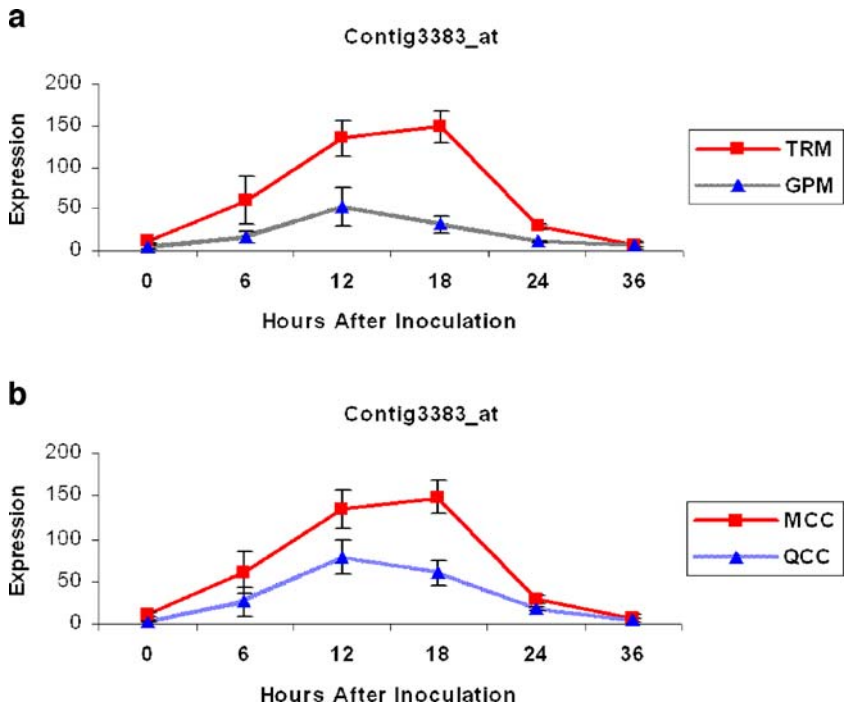

Fig. 6 Transcript accumulation pattern of Contig3383_at (encoding a subtilisin–chymotrypsin inhibitor) at different time points after infection. *Red*, incompatible interaction; *blue*, compatible interaction. **a** Comparison between Golden Promise (*GPM*) and *Rpg1* transgenic line (*TRM*) infected with *Pgt*-MCC. **b** Comparison of the *Rpg1* transgenic line infected with avirulent pathotype MCC or virulent pathotype QCC

Discussion

Durable resistance to stem rust, such as that provided by *Rpg1*, is highly desirable for crop breeding. We took a mRNA profiling approach to examine the barley–*Pgt* interaction. This study identified genes showing differential expression patterns between compatible and incompatible interactions.

The development of *Pgt* on resistant and susceptible barley cultivars was found to be similar to that on resistant and susceptible wheat (Sellam and Wilcoxson 1976). There were no differences among cultivars in urediniospore germination, appressorium formation, or penetration. However, growth of the pathogen was restricted in tissues of resistant cultivars in comparison to the susceptible cultivars. Furthermore, fewer uredinia formed in resistant than in susceptible cultivars. This suggests that resistance to stem rust in barley, as conditioned by the *Rpg1* gene, is expressed after penetration has occurred and the hyphae begin to grow in the intercellular space. Yet, how and when the signal is perceived by the host and transduced is still poorly understood. Therefore, we focused on genes that accumulated preferentially in the incompatible reaction before 36 hai, which might lead to disease resistance.

Analysis of the transcriptome at the 0-h time point identified 15 genes that were upregulated and nine genes that were downregulated in the transgenic line compared to its isoline, Golden Promise. Four of the upregulated genes are predicted to be protease inhibitors and one an ubiquitin-conjugating enzyme (Table 2). Although the significance of this observation is not clear at this time, our laboratory has shown that protein degradation by the proteasome pathway is involved in *Rpg1*-mediated disease resistance and that the RPG1 protein undergoes a basal level of polyubiquitination in uninfected plants (Nirmala et al. 2007). It is possible that introduction of the *Rpg1* gene could have altered protein degradation and the ubiquitination complex gene expression.

When the *Rpg1* transgenic line and the susceptible isogenic cv. Golden Promise were infected with MCC, 34 genes were found to have different patterns of expression over time between the respective incompatible and compatible interaction. The genes were grouped according to their expression profiles. Results indicated sequential activation of genes with the genes in the first three clusters accumulating before fungal growth inhibition, implicating them in early events leading to resistance. Immediately downstream of pathogen perception, a battery of responses could be elicited, such as new amino acid synthesis, including methionine, proline, threonine, and leucine. Amino acids are precursors for a variety of secondary metabolites that influence various defense responses. The induction of genes involved in the phenylpropanoid biosynthetic pathway is well documented in other plant–pathogen interactions (Moerschbacher et al. 1988; Caldo et al. 2004). Histological investigation of the interaction of wheat lines carrying the stem rust resistance gene *Sr5* with an incompatible race of *Pgt* showed accumulation of lignin and lignin-like materials, which are associated with the hypersensitive reaction conditioned by the *Sr5* gene (Tiburzy and Reisener 1990). Contig19854_at and HM05N11r_at, both encoding cinnamyl alcohol dehydrogenase, showed increased expression in the incompatible interaction as early

as 6 hai and reached maximum expression at 12 hai, when the fungus started to penetrate through stomata. Other rapidly induced events included increased diacylglycerol levels through some lipid-metabolizing enzymes (Contig1942_at, HT11O15u_s_at, and Contig14377_at), repressed expression of some regulatory proteins, such as guanosine triphosphate-binding protein (HV_Ceb0010I11r2_at), myb-related protein (Contig3667_s_at), and protein kinase (Contig5924_s_at). Activation of a number of defense-related genes, like osmotin-like protein (Contig9094_at), germin protein (Contig9172_at), and xylanase inhibitor (Contig14679_at), also was observed. Our analyses suggest that close communication occurs when a pathogen comes into contact with the plant. Positive and negative feedback loops likely exist both within and between signaling pathways and metabolic pathways to ensure tight coordination of the eventual defense response.

When the *Rpg1* transgenic line G02-448F-3R was inoculated with pathotypes MCC and QCC, 14 genes showed differentially expressed patterns over time. These included two pathogensis-related (PR) proteins 10, Contig4402_s_at and Contig4405_x_at. There are 11 different classes of PR proteins that are induced in a variety of plant species in response to pathogen invasion. A few PR proteins have been shown to possess antimicrobial activity. Studies revealed that the PR-10 class of proteins share homology to a ribonuclease isolated from phosphate-starved ginseng cells, suggesting that PR-10 proteins may possess such activity (Moiseyev et al. 1994). Ribonuclease has been investigated extensively in different host–parasite combinations, including mildew and rust on cereals. Members of this gene family were differentially induced in rice after infection with *Magnaporthe grisea* (McGee et al. 2001). Our results showed that when the G02-448F-3R was inoculated with MCC and QCC, only the mRNA encoding the PR10 protein accumulated higher in the incompatibility reaction, not other classes of PR proteins. PR10 and subtilisin–chymotrypsin inhibitor 2 have similar induction behavior. Both genes showed a rapid and transient activation in the incompatible interaction with the avirulent pathotype MCC, with a maximum at 18 hai. It is interesting to note that in a study of barley defense reactions to a necrotrophic fungus *Rhynchosporium secalis* (Steiner-Lange et al. 2003), *PR-10* and SD10 (putative proteinase inhibitor) are induced rapidly in the epidermis, with substantial amounts of mRNA detectable at 18 hai in the resistant cultivar.

The plant hormone abscisic acid (ABA) plays important roles in many aspects of plant development, in the regulation of stomatal aperture, and in the initiation of adaptive responses to various environmental conditions. Current evidence suggests that ABA affects disease resistance mainly negatively by interfering at different levels with biotic stress signaling. The involvement of ABA in primed callose production is one of the few examples of a positive role of ABA in disease resistance (Rezzonico et al. 1998). It has become increasingly clear that the previously isolated abiotic signaling network that is controlled by ABA and the biotic network that is controlled by salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are interconnected at various levels (Mauch-Mani and Mauch 2005). Contig17916_at, encoding putative lignostilbene-alpha, beta-dioxygenase, showed induction in response to MCC but not QCC. Lignostilbene-alpha, beta-dioxygenase catalyzes a reaction in ABA biosynthesis. Contig16985_at, encoding a putative ABA-responsive protein, also showed early activation in response to MCC. Contig5838_at and contig6008_s_at, both encoding GST, were clustered together with Contig17916_at. Phytohormone treatments such as ethylene, auxins, ABA, methyl jasmonate, and salicylic acid have been shown to induce expression of GST genes (Marrs 1996). Induction of GST expression by so many diverse stimuli implies that plant GSTs are critical in plant response to stress, such as pathogen attack, either by participating in the signal transduction and/or detoxifying harmful compounds produced as a result of a given stress. The activation of these genes suggests that ABA, like other well-studied plant hormones (i.e., salicylic acid, jasmonic acid, and ethylene), is also involved in the plant–pathogen interaction.

The corresponding *Avr* gene of the fungus *Pgt*-MCC has not been cloned; thus, its function inside the plant cell is unknown. The recent discovery that the effectors XopD (Hotson et al. 2003), AvrXv4 (Roden et al. 2004), AvrPphB (Shao et al. 2003), and AvrRpt2 (Axtell et al. 2003) have cysteine protease functions reveals that the proteolysis of host substrates is a conserved strategy employed by diverse pathogens to manipulate host signal transduction (Hotson and Mudgett 2004). It is plausible that the plant cell activates its protease inhibitors to counterattack the pathogen. A subtilisin–chymotrypsin inhibitor, identified in our study, suggests the involvement of a protease in the regulation of *Rpg1*-mediated defense.

An understanding of the barley–*Pgt* interaction transcriptome is an important step in unraveling the complex signaling and response networks that result either in successful infection for the pathogen or resistance for the host. Our data provide us candidate pathogenicity- and resistance-associated genes in this particular pathosystem, many of which are yet to be assigned a function. Because *Barley Stripe Mosaic Virus* (BSMV) has recently been developed as a viral-induced gene silencing (VIGS) vector for monocots (Holzberg et al. 2002), several laboratories have optimized the protocol to assay genes functioning in

barley powdery mildew and wheat leaf rust disease resistance (Hein et al. 2005; Scofield et al. 2005). BSMV-VIGS may also become a valuable tool for the functional analysis of the *Rpg1*-mediated resistance pathway. The differentially expressed genes in our study provide a list of candidate signaling components in the *Rpg1*-mediated pathway, which can be further examined by the BSMV-VIGS system.

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