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ORIGINAL PAPER

Exploiting regulatory variation to identify genes underlying quantitative resistance to the wheat stem rust pathogen *Puccinia graminis* f. sp. *tritici* in barley

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Abstract We previously mapped mRNA transcript abundance traits (expression-QTL or eQTL) using the Barley1 Affymetrix array and 'whole plant' tissue from 139 progeny of the Steptoe × Morex (St/Mx) reference barley mapping population. Of the 22,840 probesets (genes) on the array, 15,987 reported transcript abundance signals that were suitable for eQTL analysis, and this revealed a genome-wide distribution of 23,738 significant eQTLs. Here we have explored the potential of using these mRNA abundance eQTL traits as surrogates for the identification of candidate genes underlying the interaction between barley and the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici*. We re-analysed quantitative 'resistance phenotype' data collected on this population in 1990/1991 and identified six loci associated with barley's reaction to stem rust. One of these coincided with the major stem rust resistance locus *Rpg1,* that we had previously positionally cloned using this population. Correlation analysis between phenotype values for rust infection and mRNA abundance values reported by the 22,840 GeneChip probe sets placed *Rpg1,* which is on the Barley1 GeneChip, in the top five candidate genes for the major QTL on chromosome 7H corresponding to the location of *Rpg1*. A second co-located with the *rpg4/Rpg5* stem rust resistance locus that has been mapped in a different population and the remaining four were novel. Correlation analyses identified candidate genes for the *rpg4/Rpg5* locus on chromosome 5H. By combining our data with additional published mRNA profiling data sets, we identify a putative sensory transduction histidine kinase as a strong candidate for a novel resistance locus on chromosome 2H and compile candidate gene lists for the other three loci.

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Introduction

Phenotypic variation is frequently determined by regulatory regions of DNA sequence that affect mRNA abundance

(reviewed in Wray 2007). By subjecting variation in mRNA abundance observed in individuals from meiotic mapping populations to genetic analysis, each mRNA can be treated as a heritable, quantitative trait. Subsequently, the chromosomal loci that regulate the abundance of specific mRNAs in a given biological sample can be identified as expression quantitative trait loci (eQTL). The methodology to dissect complex traits by simultaneously measuring the expression of many genes across a genetically defined population was suggested and experimentally tested by Damerval et al. (1994). Later, by combining the power of large scale mRNA profiling platforms, full genome sequences, high throughput genotyping technologies and precision phenotyping, modelling of integrated interaction networks underlying complex traits has become possible (Chesler et al. 2005; Schadt et al. 2005). To date, behavioural and neuro-anatomical traits (Chesler et al. 2003; Kerns et al. 2005; Matthews et al. 2005), hematopoietic stem cell turnover (Bystrykh et al. 2005; de Haan et al. 2006), susceptibility to obesity (Schadt et al. 2005), hypertension (Hubner et al. 2005), type 2 diabetes mellitus (Yaguchi et al. 2005) and carcinogenesis in animals (Mariappan et al. 2006), somatic embryogenesis (Decook et al. 2005) and stress response in Arabidopsis (Kliebenstein et al. 2006; West et al. 2007), lignification in trees (Street et al. 2006; Kirst et al. 2005), digestibility in maize (Shi et al. 2007) and seed development in wheat (Jordan et al. 2007) are examples of phenotypic traits analysed by combining large scale mRNA profiling with classical genetic analyses.

We are interested in understanding the molecular genetics of stem rust resistance in cereals to develop cultivars with wide-spectrum and durable resistance. *Rpg1* is a stem rust resistance gene in barley that is effective against many, but not all, races of the stem rust pathogen *Puccinia graminis* f. sp. *tritici* (*Pgt*). The gene is present in all barley cultivars grown in the Upper Midwest region of North America and has protected the crop from epidemics for over 60 years. Some stem rust races, such as *Pgt*-QCC and *Pgt*-TTKS (Ug99) can overcome *Rpg1* mediated resistance (B. Steffenson, unpublished), and one can predict devastating epidemics if such races could establish themselves over a large region, not only in the Great Plains of the USA, but worldwide (http://www.globalrust.org/ and http://www.nature.com/news/archive/070122.html). Fortunately, genes at the *rpg4*/*Rpg5* locus confer resistance to these dangerous races (B. Steffenson and Y. Jin, unpublished). Using the Steptoe × Morex (St/Mx) population as a base map, *Rpg1* was mapped and cloned (Brueggeman et al. 2002) and functional analyses are now in progress to determine how the gene functions in conferring resistance. In parallel, experiments to clone *rpg4*/*Rpg5* genes (Druka et al. 2000) and other genes involved in the barley-stem rust interaction (Zhang et al. 2006) are being carried out. Such studies could lead to the development of novel control strategies using host resistance. The knowledge gained from these stem rust investigations in barley may also potentially be applied to wheat because of the conserved synteny between the basic genomes of the two species (Gale and Devos 1998). This is particularly critical with respect to *Pgt*-TTKS (Ug99), a recently emerged race that is capable of attacking most of the world's wheat cultivars (Kolmer 2005).

The St/Mx DH population was originally generated to identify genetic factors underpinning malting quality and yield (Kleinhofs et al. 1993), but has also been used extensively to map a range of agronomic, developmental and disease resistance traits. We have previously performed a genome-wide genetic analysis of mRNA transcript abundance in the St/Mx population, investigated the genetic complexity of transcriptional regulation using eQTL analysis and, from a subset of the data, generated ~6,000 gene-based genetic markers that segregate in this population (Luo et al. 2007; Potokina et al. 2007). In these studies 15,987 of the Barley1 GeneChip probe sets were suitable for eQTL analysis, reporting 23,738 eQTLs (Potokina et al. 2007). When we initiated this project our original hypothesis was that the potential number of gene related eQTLs would represent informative surrogates that would help us deconstruct traditional phenotypic 'traits' into an unspecified number of functionally consistent and genetically tractable components. Here, we report the use of previously collected data on quantitative phenotypic variation in the St/Mx population in response to pathogen infection combined with eQTL analysis to identify candidate genes associated with stem rust resistance in barley.

Materials and methods

Stem rust infection phenotyping

Each of the St/Mx DH lines was challenged with the stem rust fungus race *Pgt*-MCC in five replications over 2 years (1990 and 1991) as seedling plants in the greenhouse according to the methods of Steffenson et al. (1993). Phenotypic scores were made 12–14 days after inoculation according to the infection type (IT) scale of Stakman et al. (1962) as modified by Miller and Lambert (1965) (Fig. 1a). Under the Stakman system, IT 0 indicates no visible infection; only a necrotic "fleck" (i.e., hypersensitive response) with no sporulation; IT 1 designates a minute uredinium (i.e., sporulating pustule) surrounded by necrosis; IT 2 designates a small uredinium often surrounded by chlorosis; IT 3 designates a moderate sized uredinium sometimes surrounded by chlorosis; and IT 4 designates a large uredinium. Since barley exhibits chlorosis in association with most ITs (excluding IT 0 and IT 1), Miller and Lambert

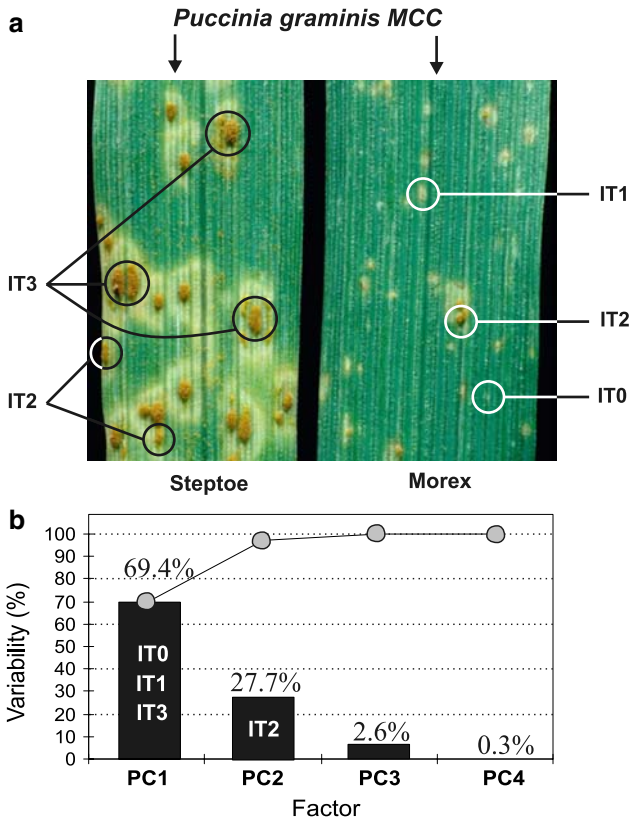


Fig. 1 Quantitative analysis of the stem rust fungus infection. **a** Phenotypes of the parents, Steptoe and Morex 14 days after inoculation with *Pgt*-MCC. **b** PCA results showing the percentage variation associated with the first four PC's. The IT types superimposed on PC1 and PC2 are based on co-location of QTL for PC1 and IT0, IT1 and IT3 and PC2 and IT2. Adjusted frequencies of individual ITs were used as input values for PCA (see Table S1 and S2)

modified the Stakman system and classified ITs 2, 3 and 4 on the basis of uredinium size alone. Barley often exhibits a mixture of different ITs on a single plant—the "mesothetic" reaction described by Stakman et al. (1962) (Fig. 1a). ITs on the St/Mx DH lines were recorded according to prevalence (Table S1 and S6 in Electronic supplementary material). In most cases, the one or two most common ITs comprised over 75% of the total observed and were used to assign the general binary classes of resistant and susceptible. ITs 0, 1 and 2 were considered indicative of host resistance (i.e., a low infection type), whereas IT 3 and 4 were indicative of host susceptibility (a high infection type). The classic "diamond shaped" uredinium of IT 4 was not observed on plants in the St/Mx population.

mRNA profiling

Preparation and dissection of the embryo-derived tissues [whole plant (Plant Ontology term PO:0000003) at the development stage 1.05-coleoptile emerged from seed (plant Growth Ontology term GRO:0007056)] has been described previously (Luo et al. 2007). Trizol RNA isolation, RNeasy clean up and standard labelling and hybridization protocols were used. Detailed descriptions of these procedures can be found under the Array Express (http://www.ebi.ac.uk/aerep/?) protocol P-MEXP-4631 (Parkinson et al. 2005). The RNA labelling and GeneChip processing was at Iowa State University GeneChip facility (http://www.biotech.iastate.edu/facilities/genechip/Genechip.htm).

Correlation analyses

The CEL files from 150 Genechips were imported into Genespring version 7.3 (Agilent Technologies, Santa Clara, CA). For calculation of the integrated probe signal, the gcRMA algorithm was used (Irizarry et al. 2003). Data were normalised according to Genespring's recommendations for the analysis of Affymetrix GeneChips. The expression data of 11 DH lines were removed after error checking; discrepancies with genotyping data were found leaving 139 lines in the final analysis (Luo et al. 2007). To calculate maximal LRS for each of the 22,840 Barley1 GeneChip probe sets, the custom program QTL Reaper was used (http://sourceforge.net/projects/qtlreaper.) For mapping individual phenotypic and expression traits, the Interval Mapping function of WebQTL (www.genenetwork.org) or QTL Cartographer (Wang et al. 2007) were used. The Pearson product–moment correlations were performed by using the 'Correlation' function from WebQTL.

Genotyping

The linkage map presented here was generated as part of two barley association mapping projects in the UK (http://www.agoueb.org) and USA (http://barleycap.coafes.umn.edu/) (Rostoks et al. 2006a; Hayes and Szucs 2006). To create the genotype file, we used data from a pilot barley Illumina Oligo Pool Assay (POPA1) that employs GoldenGate BeadArray technology (Illumina, San Diego, CA) to test 1,536 barley SNP markers in each of the 150 St/Mx DH lines. Polymorphic SNPs were integrated into the existing RFLP map using Map Manager QTX (ver. 0.27) software (Manly and Olson 1999). A final map was generated after removing co-segregating markers (leaving a single marker per locus) and correcting apparent single marker double recombination events. The genotype file is available from the GeneNetwork (http://www.genenetwork.org/genotypes/SXM.geno).

Sequencing and probe level analysis

The probe and exemplar sequences of the Affymetrix Barley1 GeneChip were downloaded from the Affymetrix'

web-site (www.affymetrix.com), processed using custom perl scripts and assembled using the Gap4 module from the Staden sequence analysis package (Bonfield et al. 1995). PCR primers were designed using Primer3 (Rozen and Skaletsky 2000). To extract individual probe signals, the CEL files from 139 Genechips were entered into a normalised relational MySQL database. Signal values of all 22 individual probes were extracted using optimized queries written in SQL.

Data access

The mRNA database ['Barley1 Embryo gcRMA SCRI (Dec06)'] and the stem rust infection type scores ['Phenotypes' database entries "Disease resistance, *P. graminis*, infection type 0" (or 1, 2, 3)] used in this study are accessible through the Genenetwork (www.genenetwork.org).

Results

Quantitative trait loci controlling resistance to stem rust in barley

To simplify the mapping and then successful isolation of *Rpg1* by Brueggeman et al. (2002), quantitative stem rust infection type (IT) scores (see "Materials and methods") were transformed into a binary score of resistant or susceptible for each DH line. Our primary objective was to investigate whether any underlying (residual) quantitative phenotypic variability could be interrogated in this population. We re-examined the original quantitative phenotypic data scores to calculate the frequencies of each of the ITs on the DH lines (Table S1 and S2). We then performed principal components analysis (PCA) using the frequencies of the individual ITs as input values. We used both the principal component (PC) and individual infection type (IT) scores as trait values for both QTL mapping and correlation analysis (Table S3).

Decomposition of the IT frequencies into principal components revealed that the first two PCs explained 97% of the variability (Fig. 1b). Linkage mapping using scores of all four PCs identified QTLs on barley chromosomes 7H (two loci), 2H, 3H and 5H (two loci) (Fig. 2). Linkage mapping using the scores for each of the individual IT types revealed that the major PC1 QTL on chromosome 7H was supported by the major QTL from analysis of ITs 0, 1 and 3. QTL analysis of each of these IT's identified a strong, single QTL near the telomere on chromosome 7HS, with Morex providing increased resistance against IT 0 and IT 1 and Steptoe increasing susceptibility in IT 3. The significant QTL of PC2 co-located with the major IT 2 QTL and mapped to the centromeric region of chromosome 2H. Since the Morex allele at this locus increased resistance, the 2H locus may encode a gene/genes that enhance stem rust resistance in the St/Mx population. This locus is at a different location from the previously described *Rpr1* locus (required for *Puccinia* Resistance 1) (Zhang et al. 2006). PC3 and PC4 accounted for only 2.6 and 0.3% of the residual variance, but by mapping their PC scores, several significant and/or suggestive QTLs were identified on 7H, 3H and 5H (Fig. 2). PC3 and PC4 QTL did not co-locate with any of the individual IT type QTL.

Use of the major stem rust resistance gene *Rpg1* as a test for eQTL based candidate gene selection

In an attempt to identify genes associated with the quantitative resistance phenotypes, we performed correlation analysis using frequencies of each IT with the integrated signals (a measure derived from use of all 11 pairs of probes in a probe set) of all 22,840 Barley1 GeneChip probe sets. The top 100 probe sets identified by this analysis correlated $>|0.4|$ ($P < 10^{-7}$) with the infection types IT 0, IT 1 and IT 3. For IT 2, correlations of $>|0.3|$ ($P < 10^{-5}$) were observed for the top 100 genes. In all groups, the number of positive and negative correlations were approximately equal.

The top 40 correlates with ITs 0, 1 and 3 included probe set AF509747.1_at (Table S4), which represents the *Rpg1* gene (Brueggeman et al. 2002). For all three of these ITs, the correlation rank of *Rpg1* transcript abundance increased if allelic effects were taken into account. Thus, when correlating IT3 (susceptibility to infection) against the 22,840 mRNA abundance values across 139 recombinant lines, *Rpg1,* annotated as a protein kinase, ranks fifth amongst the negatively correlated genes (Table 1). Had we been trying to clone *Rpg1* based on this eQTL approach, according to the functional annotations of these top five genes, we would have prioritised two, HVSMEl0003E22r2_at and AF509747.1_at (*Rpg1*), for more detailed characterisation.

We then looked at the patterns of expression of these two genes across a publicly available atlas of developmental gene expression (uninfected tissues) from the barley genomic reference strain, cv. Morex (Druka et al. 2006). Analysis of HVSMEl0003E22r2_at showed that across all 15 developmentally and ontologically distinct tissues it accumulated at moderately high level in most, except leaf. Since stem rusts are foliar pathogens, it would have been difficult (but not impossible) to interpret such transcript abundance profile as a strong candidate for *Rpg1*. We would therefore have focused on AF509747.1_at which of course we now know is *Rpg1*. *Rpg1* mRNA accumulates at a low level and has a relatively low variance in both the St/Mx population and across different tissues and genotypes with no particular tissue or obvious genotype preference (data not shown). However, the variation in *Rpg1*

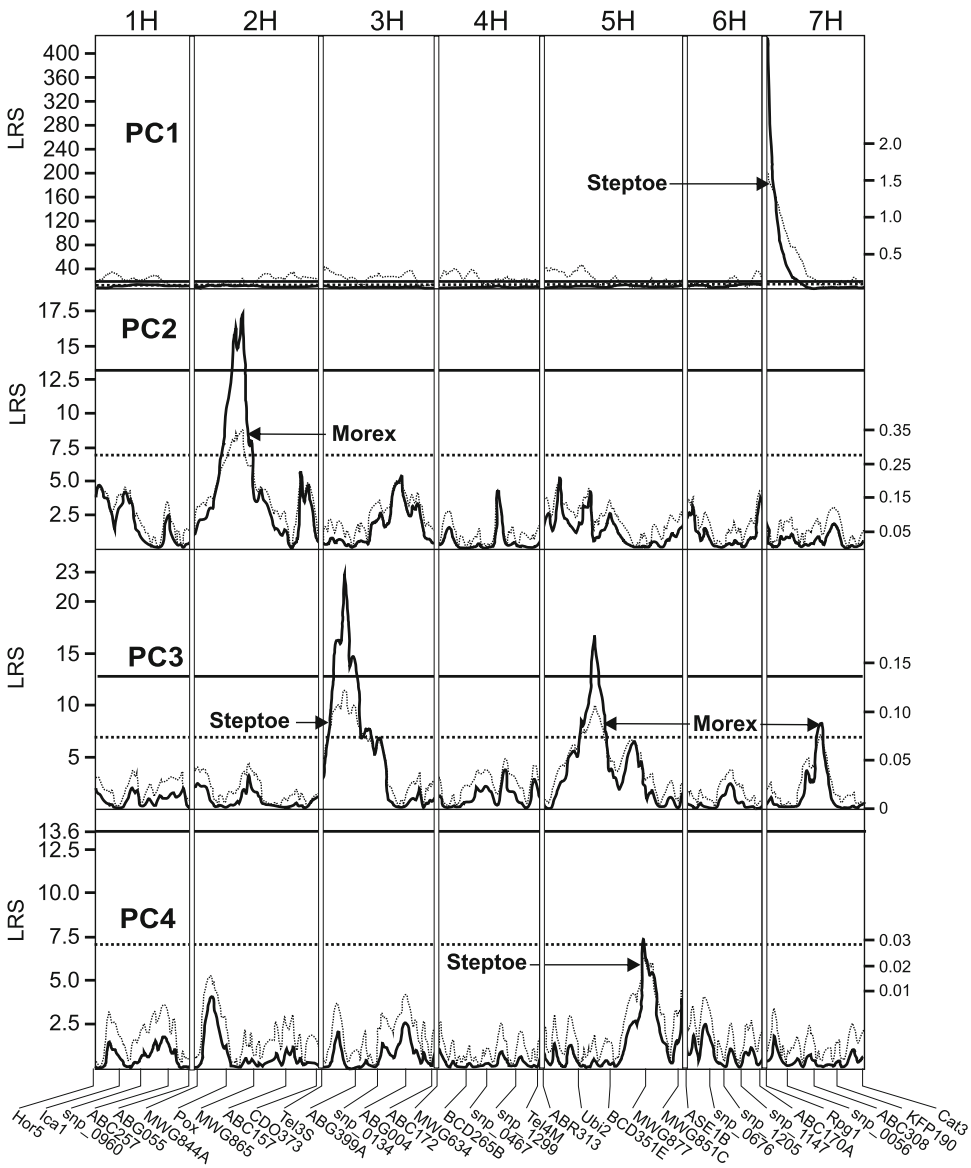


Fig. 2 Interval mapping of PC1–PC4. *Bold line* with the corresponding scale on the left shows the Likelihood Ratio Statistic (LRS) profile. The *thin line* shows the allelic effect with the scale on the right (indicated by an *arrow* and label for each significant QTL). *Horizontal lines* show upper (*solid*) and lower (*dotted*) LRS significance thresholds that were determined using 1,000 permutation tests. *Vertical lines* delimit chromosomes

transcript abundance across the St/Mx population approaches a normal distribution that is suitable for eQTL analysis. Interval mapping using *Rpg1* transcript abundance values revealed a single peak near the telomere of chromosome 7H (LRS = 98.6, $P < 10^{-6}$) coinciding with both its structural gene location and QTLs for IT 0, 1 and 3 (data not shown).

There is a possibility that the variation leading to such strong association is not due to differences in transcript abundance, but rather to the presence of one or more SNPs between the St and Mx genotypes and sequences of the probes represented on the Barley1 array. To investigate this possibility, we re-sequenced the Steptoe and Morex alleles across the GeneChip probe binding regions (to our knowledge the publicly available Steptoe sequence is not complete). We found several SNPs between Steptoe and Morex, one corresponding to the 12th nucleotide of probe #8 (Fig. 3). Despite the presence of this SNP, no obvious probe #8 polymorphism can be seen when the signals from individual probes were inspected across all 139 segregating lines (Fig. 3). Submitting the probe #8 signal alone to linkage analysis did reveal a locus coinciding with *Rpg1*, suggesting that we may have simply genotyped the population based on alternative SNP alleles. However, the sequences of the other probes were perfectly complementary to both Steptoe and Morex. Two of these also showed significant association with *Rpg1*, with perfectly matching probe #10 having even stronger association (LRS = 127) than probe #8 (LRS = 40). Thus, we conclude that the integrated probe set signal (i.e., derived from all informative probes in a probeset) primarily reflects mRNA abundance differences. The observed differences, are only very slightly, if at all enhanced by the additional effect of a SNP between probe (#8) and one of its target alleles (Fig. 3). *Rpg1/rpg1*

Table 1 Top 20 correlates of the IT 3 phenotype ($P < 10^{-6}$)

Probe set ID	Annotation	Pearson's *r*
Contig10452_at	Reverse transcriptase	−0.77
Contig12634_at	Beta-14-mannan synthase	0.76
Contig6348_at	Expressed protein	0.76
HS07M18u_s_at	Beta-14-mannan synthase	0.75
Contig3141_at	Acyl-coenzyme A oxidase 1.2 peroxisomal	0.74
Contig18035_at	Sulfotransferase	0.74
Contig14185_at	NB-ARC protein	0.73
HVSMEl0003E22r2_at	NB-ARC protein	−0.73
Contig9996_at	Kelch repeat-containing F-box protein	−0.72
Contig6006_s_at	Hypothetical protein	0.72
Contig3140_at	Acyl-coenzyme A oxidase 1.2 peroxisomal	−0.71
Rbags12l21_at	DNA methyltransferase DMT106	0.70
AF509747.1_at	*Rpg1*	−0.70
Contig6347_at	IQ calmodulin-binding protein	−0.69
HS16G07u_at	None	−0.69
Contig15882_s_at	Alpha-dioxygenase	−0.68
Contig11156_at	Signal peptide peptidase	−0.68
Contig2434_at	Acid phosphatase	−0.67
Contig4695_at	RNA recognition motif	0.65
Contig3141_s_at	Acyl-coenzyme A oxidase 1.2 peroxisomal	−0.65

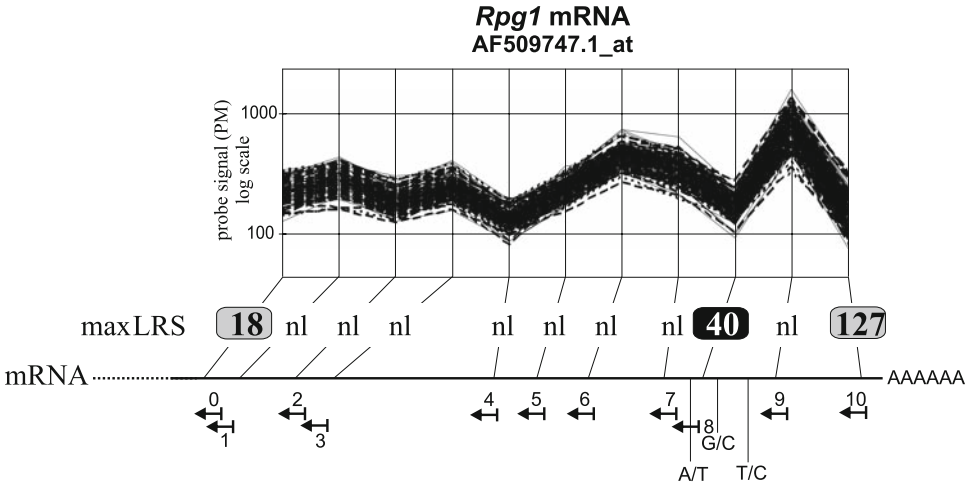


Fig. 3 Individual probe level linkage analysis of *Rpg1* mRNA abundance. The multiple-line graph shows signal values of the individual Perfect Match (PM) probes of contig AF509747.1_at (*Rpg1*) across 139 SM DH lines. Probe #8 contains a SNP (A/T) between St and Mx at position 12 in the 25 nt long probe. Below the graph, the physical distribution of individual probes are shown across the gene and the results of allele sequencing (A/T etc.) and linkage mapping (maxLRS) of the individual probes is depicted. The 'maxLRS' is the maximal LRS determined by using the Interval Mapping function. In this case the LRS peaks always coincided with that of integrated signal. 'nl' signifies no linkage

therefore exhibits an allelic imbalance in steady state mRNA transcript abundance between Steptoe and Morex.

QPgt.StMx-2H: a new partial stem rust resistance locus

Rpr1, is a locus required for *Rpg1*-dependent resistance to stem rust. *Rpr1* was identified as a fast neutron induced *Pgt*-MCC susceptible mutant of cv. Morex (wild type *Rpg1*). The locus was characterised initially by examining differential gene expression between the mutant and wild type and is located on chromosome 4H (Zhang et al. 2006). We did not find any evidence for phenotypic variation in the St/Mx population associated with this locus. However, over 30% of the variation we observed was attributed to genetic factors other than *Rpg1*. The majority of the residual variation was accounted for by PC2, was revealed as a

single QTL located near the centromere of chromosome 2H, and was supported by the major QTL for IT2 (Fig. 2). Unfortunately, over 400 probe sets reported significant eQTL peaks coinciding with PC2 (and IT 2) and a direct prediction of a small number of candidate genes for this locus was not feasible. We therefore explored the publicly available gene expression data set that was used to characterise the *Rpr1* locus as a route towards the identification of putative candidate genes (Zhang et al. 2006).

Our analysis of the data set of Zhang et al. (2006) highlighted Contig13680_s_at, a putative sensory transduction histidine kinase (Fig. 4a) that was highly down-regulated in uninoculated primary leaves of *rpr1*. This was because Contig13680_s_at was also included in the set of eQTLs underlying IT 2 on chromosome 2H where it was revealed as a single strong QTL peak (LRS = 345) (Fig. 4b) with high expression correlated with resistance. We identified multiple SNPs between Morex and Steptoe alleles in this gene, but none coincided with any of the GeneChip probes (not shown). Every probe of Contig13680_s_at reported a strong eQTL at the same location as the integrated signal eQTL. Using the SNP polymorphisms, we mapped the histidine kinase gene to the same location as its mRNA QTL on 2H, suggesting its mRNA abundance is regulated in *cis*- in the St/Mx population.

Two completely independent experimental approaches [eQTL analysis (this work) and transcriptional cloning (Zhang et al. 2006)] have converged on this sensory transduction histidine kinase as being involved in the barley/stem rust interaction. We consider it a strong candidate for *QPgt.StMx-2H*. Taken together, the data from our study and that of Zhang et al. (2006) would also support an interaction between *QPgt.StMx-2H* and *Rpr1*. However, we did not observe any *trans*-eQTL effect on Contig13680_s_at and no resistance QTL were detected at the locus on chromosome 4H. An obvious explanation would be that the St/Mx population is fixed for *Rpr1*. Despite the strong candidacy of the sensory transduction histidine kinase, another group of transcripts associated with disease resistance and programmed cell death (PCD) was also identified in the list

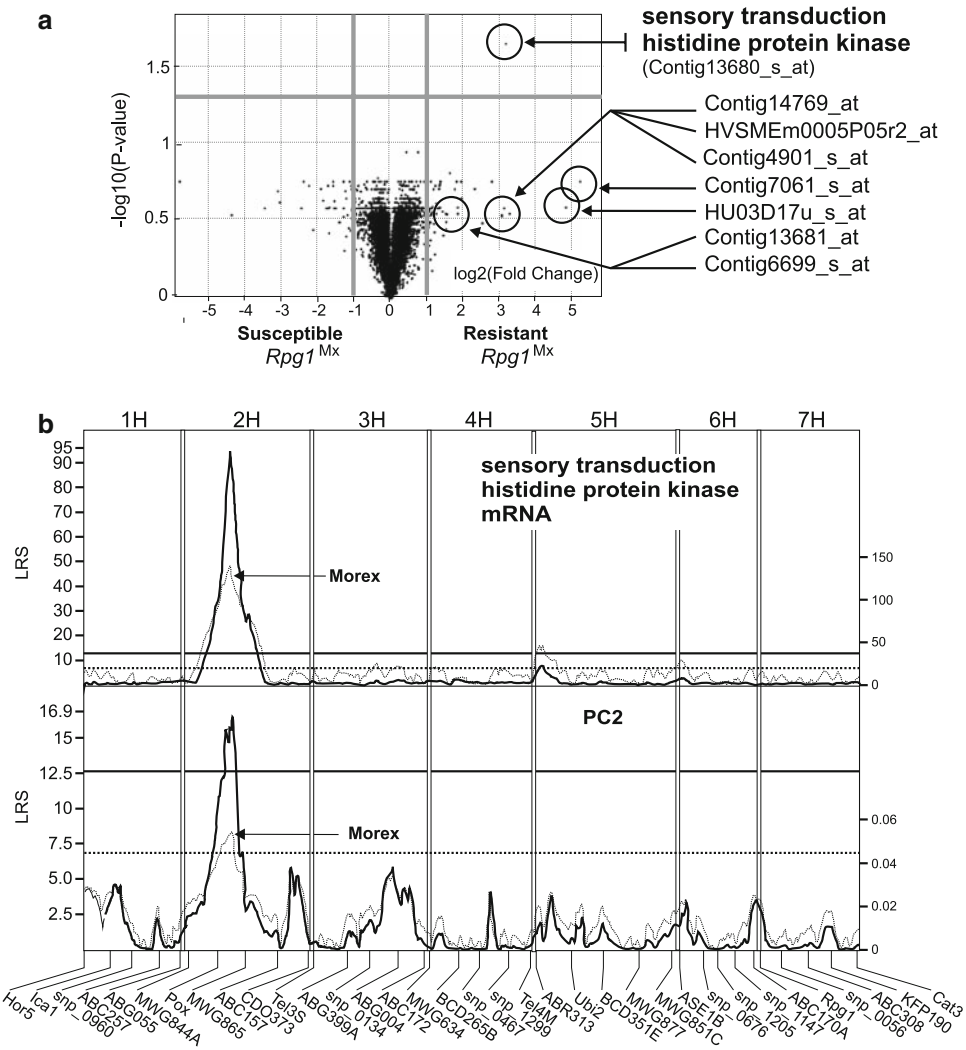


Fig. 4 Identification and linkage analysis of the IT 2 QTL candidate gene. **a** Volcano plots based on a comparison of expression reported by 13,456 GeneChip probe sets from wild type Morex leaves and the 'susceptible to Pgt MCC' *rpr1* mutant line. The *grey lines* delimit 2-fold difference (*vertical*) and 0.05 significance (*horizontal*) levels (Benjamini-Hochberg False Discovery Rate was set at 0.05%) identifying a single probe set, corresponding to a putative sensory transduction histidine protein kinase gene (Contig13680_s_at). Other genes identified by Zhang et al. (2006) are highlighted. **b** Linkage mapping of Contig13680_s_at and IT 2

of probe sets reporting mRNA abundance QTLs at the IT 2 locus (Fig. 5). Many of these genes have been mapped previously to different regions of the barley genome. *QPgt.StMx-2H* therefore putatively represents the location of a *trans*-acting regulator of the pathogen response pathway.

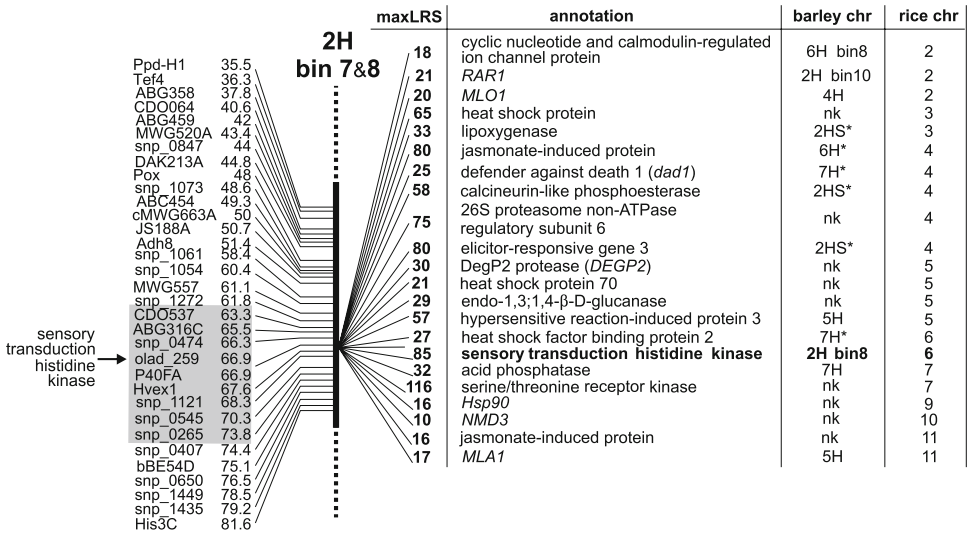
PC3 and PC4 stem rust QTL

PC3 and PC4 QTL combined capture less than 3% of the variation for the stem rust infection phenotype. We realise that from a statistical viewpoint analysis of this data could be considered compromised. Despite this we investigated these two PCs using the same approaches as detailed above. Three significant QTLs were detected for the PC3 phenotype and one for PC4 (Fig. 2). For PC3, to restrict the list of candidates, we limited our search to genes that had all three eQTLs coinciding with those of PC3. Among the top 50 covariates to PC3 ($|r| > 0.29$, $P < 0.0005$), seven had similar QTL profiles. Only two of these, Contig2007_s_at ($|r| = 0.32$, $P = 9.4E\text{-}05$) and Contig3288_x_at ($|r| = 0.31$, $P = 0.0002$), reported eQTL with allelic effects in the same direction as the PC3 QTL, i.e., Steptoe at 3H and Morex at 5H and 7H (Table S4 and Fig. S1 in Electronic supplementary material). The genes underlying these probe sets encoded putative proteins with similarity to small heat shock family proteins (*Hsp17*). These have diverse roles in development and other responses in a broad range of organisms, and in plants have been associated with resistance to tobacco etch virus (TEV) (Welsh and Gaestel 1998; Whitham et al. 2000).

PC4 captures only 0.3% of the phenotypic variation. QTL analysis revealed a single, borderline significant QTL on chromosome 5H, between markers BCD265C and R273 (ARD032) (Druka et al. 2000). Despite the lack of statistical power in this analysis, it is intriguing that two stem rust resistance genes, *rpg4* and *Rpg5* have been mapped previously within this interval using the Steptoe/Q21861 population (Steffenson, unpublished). As the PC4 QTL coincides with the *rpg4/Rpg5* locus, it is possible that *rpg4/Rpg5* has a broader role in stem rust resistance. Thirty-three probe sets reported highly significant eQTL coinciding with the PC4 QTL (Table S5). For 12, their rice orthologs were located on rice chromosome 3, in the region previously found to be syntenic to the barley *rpg4/Rpg5* locus (Druka et al. 2000). Given the biotrophic nature of *Puccinia* infection, and in particular the temperature dependence of resistance conferred by *rpg4*, two of these probe sets were particularly interesting; Contig3878_at which represents the Actin Depolymerising Factor 6 gene (*ADF6*) and Contig3111_at representing Cold stress Regulated protein *COR413-PM1* (Fig. S2).

Discussion

Stem rust resistance genes with markedly different characters have been described in barley. One type, represented by the major dominant barley stem rust resistance gene, *Rpg1,* is located near the telomere on barley chromosome 7H. *Rpg1* has been cloned (Brueggeman et al. 2002) and is located in a region where synteny with rice appeared strong at a macro level (Kilian et al. 1995, 1997), but at a micro level was severely disrupted by chromosomal rearrangements (Brueggeman et al. 2002). A remarkable biological property of *Rpg1* is its durability, in that it has controlled stem rust in barley for the past 60+ years. The second type is represented by two other major genes, *rpg4* and *Rpg5,* that are physically closely linked on chromosome 5H (Druka et al. 2004). *rpg4* confers resistance to *P. graminis* f. sp. *tritici* race, *Pgt*-QCC, which can overcome the resistance conferred by *Rpg1*. *rpg4* is highly temperature



maxLRS	annotation	barley chr	rice chr
18	cyclic nucleotide and calmodulin-regulated ion channel protein	6H bin8	2
21	*RAR1*	2H bin10	2
20	*MLO1*	4H	2
65	heat shock protein	nk	3
33	lipoxygenase	2HS*	3
80	jasmonate-induced protein	6H*	4
25	defender against death 1 (*dad1*)	7H*	4
58	calcineurin-like phosphoesterase	2HS*	4
75	26S proteasome non-ATPase regulatory subunit 6	nk	4
80	elicitor-responsive gene 3	2HS*	4
30	DegP2 protease (*DEGP2*)	nk	5
21	heat shock protein 70	nk	5
29	endo-1,3;1,4-β-D-glucanase	nk	5
57	hypersensitive reaction-induced protein 3	5H	5
27	heat shock factor binding protein 2	7H*	6
85	**sensory transduction histidine kinase**	**2H bin8**	**6**
32	acid phosphatase	7H	7
116	serine/threonine receptor kinase	nk	7
16	*Hsp90*	nk	9
10	*NMD3*	nk	10
16	jasmonate-induced protein	nk	11
17	*MLA1*	5H	11

Fig. 5 Genetic linkage map of the vicinity of the *QPgt.StMx-2H* locus and some of the barley genes known to be involved in plant pathogen interaction with eQTL coinciding with the stem rust IT 2 QTL (*grey area*). 'nk' Map position is not known. *Mapping was based on expression profiling data from wheat–barley addition lines (Cho et al. 2005). The 'Bin' designations are used according to Kleinhofs and Graner (2001)

sensitive, being effective at relatively low temperatures (17–22°C), but completely ineffective at temperatures above 27°C. *Rpg5* (previously designated as *RpgQ*) is somewhat sensitive to temperature and confers resistance to the rye stem rust pathogen *P. graminis* f. sp. *secalis*, (*Pgs*), a *forma specialis* of stem rust that is virulent on barley carrying *Rpg1*.

As *Rpg1* has previously been analysed in detail (Brueggeman et al. 2002; Horvath et al. 2003; Nirmala et al. 2006), we were able to take advantage of a good genetic understanding of this host/pathogen interaction which allowed us to simulate how eQTL analysis could have contributed to its isolation. We previously generated an eQTL dataset from the St/Mx population (Luo et al. 2007; Potokina et al. 2007). As this dataset was derived from uninfected whole plant tissues, we hypothesised that it may be useful for investigating underlying ('preformed' or non-induced) resistance mechanisms as well as informing on the genetics of gene expression in barley more generally. 'Un-induced' eQTL datasets have been used previously to investigate characterised phenotypes. For example, Dixon et al. (2007) recently used un-induced Epstein-Barr virus-transformed human lymphoblastoid cell lines to investigate asthma in 400 asthmatic and non-asthmatic children, identifying a number of genes that are candidates for studying, amongst others, infection and inflammatory conditions. We showed that in our particular example, eQTL analysis would have complemented or even substituted for traditional map-based cloning of *Rpg1*. It would have been especially valuable in this case because rice/barley synteny was of limited use given no rice *Rpg1* ortholog exists in the syntenic region (Brueggeman et al. 2002). The ultimately successful approach, positional cloning, was tedious partially because of suppressed recombination near the gene. Taking these factors into account, correlation of the resistance phenotype with mRNA abundance across genetically defined materials could have been a very successful alternative, identifying in our case two strong and functionally consistent candidates, one of which was *Rpg1*.

Despite this 'proof of principle', we recognise that the described approach will not work for all genes. The heritability of allele specific expression, the trait itself and the extent of linkage disequilibrium at a particular locus will strongly influence the success of correlation-based gene prediction. While *Rpg1* expression does not have obvious tissue preference and its mRNA accumulates at a low level in most tissues throughout barley development, if our focus had been on genes that respond differentially to pathogen infection, then tissue specificity and timing could be an issue. In addition, it is likely that less than half of the barley gene complement is represented on the Barley1 GeneChip, meaning that a large number of potential targets will be missed.

Our primary objective was focussed on the additional, quantitative variation segregating in the St/Mx population. From our re-analysis of the original IT data from the barley–stem rust fungus interaction, we identified several additional 'resistance' QTLs, the largest located on chromosome 2H (Fig. 2). Unfortunately, the relevant genetic interval contained hundreds of eQTL and filtering these for likely functional significance retained a large number of genes. However, one of these, a putative sensory transduction histidine kinase, was previously identified as strongly down regulated in the *Rpg1* suppressor mutant *rpr1*. *rpr1* was identified using fast neutron mutagenesis of cv. Morex (resistant cultivar) followed by screening M2 families for individuals susceptible to race *Pgt*-MCC. *Rpr1* was shown to be required for *Rpg1*-mediated resistance, because *Rpg1* remained unaffected at the DNA sequence, mRNA expression and protein levels in *rpr1* mutants (Zhang et al. 2006). Because fast neutron mutagenesis generates sub-chromosomal deletions, candidate genes for *Rpr1* were identified by expression profiling mutant and wild type plants and analysing genes that have no or reduced expression in the mutated line compared to the control. Several such candidates were mapped and shown to co-segregate with the *rpr1* phenotype on chromosome 4H. In the experiment reported here, we were able to map eQTLs for all of the previously identified *Rpr1* candidate genes (Fig. S3). Two had eQTLs on top of the 4H locus where *rpr1* was previously mapped. In both, the Steptoe allele was the more highly expressed, suggesting they were unlikely candidates for *Rpr1*. However, we did not observe any indication of a trait QTL on 4H in the St/Mx population. The cross used to map *Rpr1* included barley accession Q21861, that, besides *Rpg1*, carries the stem rust resistance genes, *rpg4* and *Rpg5* on chromosome 5H. *rpg4* confers resistance to the *Pgt* pathotype QCC and *Rpg5* confers resistance to *Pgs* isolate 92-MN-90, both virulent for *Rpg1*. Although no obvious interactions between *rpg4* and *Rpg5* with *Rpr1* were found previously, it remains possible that downstream components of an alternative regulatory network underlying the barley and stem rust interaction in Q21861 may overlap with that in cv. Morex. Indeed, some tentative support for this comes from mapping the PC4 phenotype QTL to the *rpg4/Rpg5* locus in the St/Mx population, as well as from mapping the IT2 phenotype independently in subpopulations that carry either rpg1 or Rpg1 alleles (Figure S3). In the latter, the QTL on chromosome 2H disappears when rpg1 (susceptible) lines are used, but was readily detectable in the *Rpg1* (resistant) subpopulation. We interpret this observation as support for an interaction between *QPgt.StMx-2H* and *Rpg1*. In addition, a PC2 QTL mapping to the *rpg4/Rpg5* locus was also revealed in the *rpg1* subpopulation, lending support to the PC4 QTL (given the nomenclature *QPgt.StMx-5H*).

While the sensory transduction histidine kinase (Contig 13680_s_at) is a strong candidate for *QPgt.StMx-2H,* there are many other genes with significant mRNA abundance QTLs at this locus. Indeed a number of these other well characterised genes could be important in determining the outcome of the plant–pathogen interaction. Notable examples are the cyclic nucleotide and calmodulin-regulated ion channel protein (Rostoks et al. 2006b; Day and Greenfield 2003; Orrenius and Nicotera 1994; Balague et al. 2003), *Dad1* (Yamada et al. 2004; Nishii et al. 1999; Kelleher and Gilmore 1997; Yulug et al. 1995; Nakashima et al. 1993), *DegP2* (Haussuhl et al. 2001) and hypersensitive reaction induced protein (Molina et al. 1999; Rostoks et al. 2003) that are all associated with PCD. The phenotypic manifestation of PCD is a hypersensitive reaction followed by localised necrosis that can be either induced by a pathogen or can be constitutive as in case of disease lesion mimic mutants (Jung et al. 2006; Noutoshi et al. 2006; Badigannavar et al. 2002; Hu et al. 1998; Balague et al. 2003). Most interestingly, the mRNA abundance of three genes that are involved in the *Blumeria graminis* (mildew)–barley interaction, *Mlo1* (Buschges et al. 1997), *Rar1* (Shirasu et al. 1999) and *Mla1* (Zhou et al. 2001) also map at the *QPgt.StMx-2H* locus. If, as we suggest, the PC2 QTL on 2H corresponds to the location of a *trans*-eQTL that controls the expression of a group of functionally consistent candidates then it will be important to first assess experimentally the proportion of their structural genes that map elsewhere on the barley genome.

One of the most biologically interesting, while statistically contentious, observations was that the PC4 QTL (*QPgt.StMx-5H*) coincided with the *rpg4/Rpg5* locus. *rpg4* is responsible for temperature dependent recessive resistance to pathotype *Pgt*-QCC and *Rpg5* confers resistance to the rye stem rust pathogen, *P. graminis* f. sp. *secalis*. Both Steptoe and Morex are susceptible to *Pgt*-QCC and *Pgs* isolate 92-MN-90 therefore, different crosses involving the *Pgt*-QCC resistant line Q21861 were used to map *rpg4* and *Rpg5*. Our QTL analysis in St/Mx challenged with *Pgt* - MCC also detected a QTL at this locus and identified several possible candidate genes based on eQTL correlation analyses. Two of these, *Actin Depolymerising Factor 6* (*ADF6,* Contig3878_at) and the cold acclimation protein *COR413-PM1* (Contig3111_at) could potentially explain the temperature dependent resistance conferred by *rpg4*.

ADF gene family members have been mapped physically close to *rpg4/Rpg5* and have been suggested previously as candidate genes (Druka et al. 2004). Parallels between processes involving the actin cytoskeleton can be drawn between those underlying fungal biotrophy and low temperature induced stress. Stem rusts invade plants through stomata and acquire plant energy resources via a haustorial apparatus that comprises several layers of de novo synthesized invaginated host plasma membranes (Schulze-Lefert and Panstruga 2003; Miklis et al. 2007). Trafficking of material for membrane synthesis occurs via re-modelling of the actin cytoskeleton involving *ADF* family members as well as molecular chaperones, like small heat shock proteins (Liang and MacRae 1997; Dalle-Donne et al. 2001; Mounier and Arrigo 2002; Davidson et al. 2002; Xu and Stamnes 2006). Actin cytoskeleton re-organisation is also an integral component in low-temperature signal transduction (Orvar et al. 2000). In wheat, *TaAdf* gene expression level correlates with the capacity to tolerate freezing and may be required for the cytoskeletal rearrangements that occur upon low temperature exposure. *TaAdf* expression was controlled by a locus on wheat chromosome 5A (Ouellet et al. 2001). These arguments, while speculative, are consistent with *ADF* genes being involved in both, temperature response and, directly or indirectly, stem rust resistance.

In conclusion, the phenotypic and eQTL analysis presented here has provided a number of strong candidate genes and some biological insight into the interaction between barley and the stem rust pathogen. Of particular interest is the novel locus on chromosome 2H which explains the expression of a number of functionally consistent resistance response candidate genes. Our data are consistent with this interval containing a master regulator (or *trans*-eQTL) of innate barley fungal disease resistance, a hypothesis we are currently investigating further.

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