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Flor Revisited (Again): eQTL and Mutational Analysis of NB-LRR Mediated Immunity to Powdery Mildew in Barley

Roger P. Wise
United States Department of Agriculture, rpwise@iastate.edu

Priyanka Surana
Iowa State University, psurana@iastate.edu

Gregory S. Fuerst
Iowa State University, gsfuerst@iastate.edu

Ruo Xu
Iowa State University

Divya Mistry
Iowa State University, divyam@iastate.edu

See next page for additional authors

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Abstract

Genes encoding early signaling events in pathogen defense often are identified only by their phenotype. Such genes involved in barley-powdery mildew interactions include Mla, specifying race-specific resistance; Rar1 (Required for Mla12-specified resistance1), and Rom1 (Restoration of Mla-specified resistance1). The HSP90-SGT1-RAR1 complex appears to function as chaperone in MLA-specified resistance, however, much remains to be discovered regarding the precise signaling underlying plant immunity. Genetic analyses of fast-neutron mutants derived from CI 16151 (Mla6) uncovered a novel locus, designated Rar3 (Required for Mla6-specified resistance3). Rar3 segregates independent of Mla6 and Rar1, and rar3 mutants are susceptible to Blumeria graminis f. sp. hordei (Bgh) isolate 5874 (AVRa6), whereas, wild-type progenitor plants are resistant. Comparative expression analyses of the rar3 mutant vs. its wild-type progenitor were conducted via Barley1 GeneChip and GAIIx paired-end RNA-Seq. Whereas Rar1 affects transcription of relatively few genes; Rar3 appears to influence thousands, notably in genes controlling ATP binding, catalytic activity, transcription, and phosphorylation; possibly membrane bound or in the nucleus. eQTL analysis of a segregating doubled haploid population identified over two-thousand genes as being regulated by Mla (q value/FDR=0.00001), a subset of which are significant in Rar3 interactions. The intersection of datasets derived from mla-loss-of-function mutants, Mla-associated eQTL, and rar3-mediated transcriptome reprogramming are narrowing the focus on essential genes required for Mla-specified immunity.

Keywords

eQTL, transcript profiling, immunity, resistance signaling, barley, Blumeria graminis

Disciplines

Agricultural Science | Agriculture | Bioinformatics | Biostatistics | Computational Biology | Plant Pathology | Systems and Communications

Comments

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Authors

Roger P. Wise, Priyanka Surana, Gregory S. Fuerst, Ruo Xu, Divya Mistry, Julie A. Dickerson, and Dan Nettleton Dr.

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REVIEW

Flor Revisited (Again): eQTL and Mutational Analysis of NB-LRR Mediated Immunity to Powdery Mildew in Barley

Roger Wise[1, 2, 3], Priyanka Surana[2, 3], Greg Fuerst[1, 2], Ruo Xu[4], Divya Mistry[3, 5], Julie Dickerson[3, 5] and Dan Nettleton[3, 4]

[1] *Corn Insects and Crop Genetics Research Unit, U.S. Department of Agriculture-Agricultural Research Service, Iowa State University, Ames, Iowa 50011, USA*

[2] *Department of Plant Pathology & Microbiology, Center for Plant Responses to Environmental Stresses, Iowa State University, Ames, Iowa 50011, USA*

[3] *Bioinformatics and Computational Biology Graduate Program, Iowa State University, Ames, Iowa 50011, USA*

[4] *Department of Statistics, Iowa State University, Ames, Iowa 50011, USA*

[5] *Department of Electrical and Computer Engineering, Iowa State University, Ames, Iowa 50011, USA*

Abstract

Genes encoding early signaling events in pathogen defense often are identified only by their phenotype. Such genes involved in barley-powdery mildew interactions include *Mla*, specifying race-specific resistance; *Rar1* (*Required for Mla12-specified resistance1*), and *Rom1* (*Restoration of Mla-specified resistance1*). The HSP90-SGT1-RAR1 complex appears to function as chaperone in MLA-specified resistance, however, much remains to be discovered regarding the precise signaling underlying plant immunity. Genetic analyses of fast-neutron mutants derived from CI 16151 (*Mla6*) uncovered a novel locus, designated *Rar3* (*Required for Mla6-specified resistance3*). *Rar3* segregates independent of *Mla6* and *Rar1*, and *rar3* mutants are susceptible to *Blumeria graminis* f. sp. *hordei* (*Bgh*) isolate 5874 (AVR_{a6}), whereas, wild-type progenitor plants are resistant. Comparative expression analyses of the *rar3* mutant *vs*. its wild-type progenitor were conducted *via* Barley1 GeneChip and GAIIx paired-end RNA-Seq. Whereas *Rar1* affects transcription of relatively few genes; *Rar3* appears to influence thousands, notably in genes controlling ATP binding, catalytic activity, transcription, and phosphorylation; possibly membrane bound or in the nucleus. eQTL analysis of a segregating doubled haploid population identified over two-thousand genes as being regulated by *Mla* (q value/FDR=0.00001), a subset of which are significant in *Rar3* interactions. The intersection of datasets derived from *mla*-loss-of-function mutants, *Mla*-associated eQTL, and *rar3*-mediated transcriptome reprogramming are narrowing the focus on essential genes required for *Mla*-specified immunity.

Key words: eQTL, transcript profiling, immunity, resistance signaling, barley, *Blumeria graminis*

INTRODUCTION

The interactions between plants and obligate plant pathogenic fungi are a counter-balancing act. As the pathogen attempts to maximize nutrient siphoning to enable colonization, the host restricts nutrient loss while minimizing the cost of defense. To establish biotrophy, the fungus must penetrate cell walls and establish haustoria, which function in nutrient

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Correspondence Roger Wise, E-mail: rpwise@iastate.edu

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exchange between the host and pathogen. In addition, to survive and reproduce, the pathogen needs to keep the invaded tissue alive and photosynthetically productive. Initially, plants activate non-specific PAMP-triggered immunity (PTI) to limit penetration and haustorial development (Hu *et al.* 2009). These responses include the transcription of thousands of stress-related genes, as well as the induction of antimicrobial metabolites and peptides during early stages of pathogen invasion (Wise *et al.* 2007). However, plants typically will engage effector-triggered, *R*-gene mediated defenses (ETI) to restrict nutrient loss, often by local programmed cell death (Jones and Dangl 2006; Bent and Mackey 2007). Thus, a variety of defense strategies are deployed, enabling hosts to mount a sufficient but not unnecessary response.

The obligate fungal biotroph, *Blumeria graminis* f. sp. *hordei*, is the causal agent of powdery mildew on barley (*Hordeum vulgare* L.) (Bushnell 2002). Effector-triggered recognition of *B. graminis* f. sp. *hordei* by the barley host is mediated by several *Ml* (*mildew resistance*) loci distributed throughout the genome (Jørgensen 1994). *Mla* is positioned on the short arm of chromosome 1H (Wei *et al.* 2002), with approximately 30 specificities that mediate variable resistances when corresponding AVR_a effectors are present in the pathogen (Shen *et al.* 2003; Halterman and Wise 2004; Seeholzer *et al.* 2010).

Mla alleles encode nuclear and cytoplasmic localized coiled-coil, nucleotide binding site, leucine -rich repeat (CC-NBS-LRR) proteins (Halterman and Wise 2004; Shen *et al.* 2007; Seeholzer *et al.* 2010). These CC-NBS-LRR MLA proteins translocate into the nucleus after recognition of a corresponding AVR_a effector from *B. graminis* f. sp. *hordei*, where it associates with the HvWRKY1 and HvWRKY2 host transcription factors (TFs) (Shen *et al.* 2007). These WRKY TFs interact with MLA and MYB6 to regulate resistance against *B. graminis* f. sp. *hordei* (Chang *et al*. 2013). Additionally, WRKY10, -19, and -28 act positively to control the outcome of *Mla*-mediated immunity (ETI), as well as basal defense (Meng and Wise 2012).

Additional genes involved in barley-powdery mildew interactions include *Rar1* (*Required for Mla12-specified resistance1)* (Shirasu *et al*. 1999) and *Rom1* (*Restoration of Mla-specified resistance1*) (Freialdenhoven *et al*. 2005), as well as *Sgt1* and *Hsp90* (Shirasu 2009). The HSP90-SGT1-RAR1 complex appears to function as chaperone in MLA-specified resistance (Shirasu 2009), however, much remains to be discovered regarding the precise signaling underlying plant immunity (Gassmann and Bhattacharjee 2012). We therefore hypothesized that we could narrow the focus by utilizing the intersection of datasets derived from *mla*-loss-of-function mutants, *Mla*-associated eQTL, and transcriptome reprogramming mediated by a newly discovered locus, *rar3* (*required for Mla6-specified resistance3*), in order to determine essential genes required for *Mla*-specified immunity.

STRATEGY

Natural variability and loss-of-function *mla* mutants

Our early experiments focused on distinguishing the transcriptional differences between incompatible and compatible interactions using alternate alleles of *Mla* and complementary isolates of *B. graminis* f. sp. *hordei* (Caldo *et al.* 2004). To do this, we took advantage of a modified "quadratic check" design (Flor 1955), consisting of three near-isogenic barley lines, with introgressed *Mla1*, *Mla6* or *Mla13* alleles, each challenged with the contrasting powdery mildew isolates 5 874 (containing AVR_{a1} and AVR_{a6}) and K1 (containing AVR_{a1} and AVR_{a13}). One particular analysis identified a highly co-regulated cluster of >160 defense-related genes that are significantly up-regulated in both incompatible and compatible interactions, coinciding with germination of *B. graminis* conidia and formation of appressoria (Caldo *et al.* 2004, 2006). Later, during establishment of the perihaustorial interface between penetrating *Bgh* and host epidermal cells, divergent expression occurs, in which lower accumulation of these transcripts is observed in compatible interactions compared to paired incompatible interactions. A significant fraction of these genes are associated with PAMP-triggered,

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basal defense (Chisholm *et al.* 2006; Bent and Mackey 2007). These results, as well as others, established a regulatory link between basal defense and *R*-gene mediated resistance (Holt *et al.* 2005; Kim *et al.* 2005; Shen *et al.* 2007; Wise *et al.* 2007).

Recently, in an analysis outlined by Moscou and colleagues (Moscou *et al*. 2011a), an alternative approach was used that took advantage of paired wild-type and loss-of-function mutant alleles of *Mla*. By identifying the conserved quantitative differences between three wild-type and mutant pairings at 16 and 32 h after inoculation (HAI) with *B. graminis* f. sp. *hordei*, candidate genes that are hypothesized to be transcriptional targets of the MLA-mediated hypersensitive reaction (HR) were predicted (Moscou *et al.* 2011a).

These predictions were taken a step further by incorporating a genetical genomics, or expression quantitative trait (eQTL), strategy, where one investigates gene expression on a population level (Hansen *et al.* 2008). In this case, a barley doubled haploid population segregating for *Mla* (as well as *MlLa*) was utilized as described in the Results below, enabling the use of linkage and network analyses to identify key regulators of gene expression, based on the experimental parameters (Jansen and Nap 2001; Rockman and Kruglyak 2006; Williams *et al.* 2007).

Genetical genomics – genetic analysis of transcriptome data

The key to our understanding of biological phenomena is the interpretation of controlling factors essential to the regulation of gene networks. The co-expression of genes that make up a biochemical or metabolic pathway associated with a particular trait can be controlled by a number of features, e.g, promoter or enhancer sequences in upstream, downstream or intron regions of the genes themselves. Additionally, groups of physically linked genes may be regulated globally by chromatin remodeling, where nucleosomes are temporarily displaced, providing access to chromosomal regions by auxiliary transcription factors (Zhu 2003).

Quantitative trait locus (QTL) mapping finds statistical associations between genotypes and phenotypes, allowing regions of the genome harboring allelic differences that cause variation in the phenotype to be identified; these regions are called QTLs (Mackay 2001). Transcript abundance of a single gene is a quantitative trait and its regulation can be genetically interrogated. This is often called genetical genomics, or eQTL mapping because the phenotypes in question are the expression of individual genes (Jansen and Nap 2001; Rockman and Kruglyak 2006; Chen and Kendziorski 2007). Thus, while comparative analysis of expression data has led to elucidation of significant co-expression networks, identification of the ***regulators*** of those networks has been limited; here, the use of eQTL analysis is advantageous.

Loci that regulate expression of genes or networks can be *cis*- or *trans*-acting eQTL. A *cis*-eQTL is defined as a segregating difference in mRNA or protein expression that maps at (or very close to) the gene that produces the transcript or protein whose regulation is being investigated. In contrast, a *trans*-eQTL is a segregating allelic difference affecting the transcript and/or protein levels of an unlinked gene. A region of the genome that controls the expression of many genes is referred to as an eQTL hotspot (Chesler *et al.* 2005). One can imagine that an allelic difference in nucleosome structure could lead to the identification of a *cis*-eQTL hotspot, but most chromosomal regions identified as hotspots tend to act in *trans*, regulating more than 1 000 genes in some cases (West *et al.* 2007). Identification of eQTL hotspots is an effective way to begin building gene networks, especially if one can identify the gene variants that modulate a cluster of mRNAs and proteins associated with a biological process of interest, for example, disease defense traits (Mozhui *et al.* 2008).

Genes required for *R*-gene mediated resistance

Mutations in genes required to manifest *R*-gene mediated immunity are invaluable resources to interrogate disease-resistance pathways and resistance signaling (Love *et al.* 2008). Genetic analyses of fast-neutron mutants derived from CI 16151 (*Mla6*) uncovered a novel locus, designated *Rar3* (*Required for Mla6-specified resistance3*). *Rar3* segregates

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independent of *Mla6* and *Rar1*, and *rar3* mutants are susceptible to *B. graminis* f. sp. *hordei* isolate 5 874 (AVR_{a6}), whereas, wild-type progenitor plants are resistant. The *rar3* mutant has a normal growth habit, and yields as well as its progenitor.

APPROACH

Identification of genes regulated by *Mla via* eQTL analysis

Segregating doubled haploid lines The 75 Q21861xSM89010 (QSM) doubled haploid barley lines used in this eQTL experiment were derived from a single F_1 plant, and have been maintained *via* single-seed descent (Moscou *et al.* 2011b). Q21861 is completely resistant to isolates of *B. graminis* f. sp. *hordei* that harbor AVR_{a1}, whereas SM89010 confers intermediate resistance when AVR_{La} is present (Steffenson *et al.* 1995). The QSM lines also exhibit a range of incompatible and compatible interactions with stem rust (*Puccinia gramnis)*, leaf rust (*Puccinia hordei*), net blotch (*Pyrenophora teres*), and leaf scald (*Rhynchosporium secalis*) pathogens (Steffenson *et al.* 1995; Steffenson *et al.* 2009). These diseases are also of great economic importance in reducing the yield and quality of barley produced in developing countries (Yahyaoui 2002; Yahyaoui *et al.* 2004).

Experimental design to leverage previous results To take advantage of previous parallel expression data (Caldo *et al.* 2004, 2006; Moscou *et al.* 2011a), we selected 16 and 32 HAI, the time frame after appressorial penetration and formation of haustoria, respectively (Moscou *et al.* 2011a). Two 96-cone trays (5 plants/cell) were grown in a climate-controlled greenhouse using a randomized block design. Each tray contained the same 75 QSM lines plus four replicates each of the Q21861 and SM89010 parents. 7 d after sowing, both experimental blocks were inoculated with a high density of *B. graminis* f. sp. *hordei* isolate 5874 (AVR_{a1}, AVR_{a6}, AVR_{a12}, AVR_{La}) conidiospores (200 cm^{-2}). At 16 and 32 HAI, pools of 5 first seedling leaves were harvested, frozen, and RNA extracted, and hybridized to Affymetrix 22K Barley1 expression arrays.

Expression quantitative trait locus (eQTL) mapping We used a 1 248-cM transcript derived marker (TDM) map previously established for the QSM population (Moscou *et al.* 2011b) to perform our eQTL analysis. This QSM map has 1 494 markers, capturing 897 recombination events. Controlling the False Discovery Rate (FDR) at q=0.00001, 3 012 genes (probesets) had a significant eQTL (both *cis* and *trans*) at 16 HAI and 5 293 genes showed significant eQTL at 32 HAI.

Nevertheless, our main objective is to identify major regulators of barley-powdery mildew interactions, i.e., *trans*-eQTL hotspots in response to infection by pathogens (Moscou *et al.* 2011b). Two major *trans*-hotspots were found, associated with *Mla* on chromosome 1H, and also at the distal end of chromosome 2H. Interestingly, the vast majority of the significant eQTL were localized to each of these regions at particular time points: Controlling the q value/FDR at 0.00001, 2 352 of these eQTL were coincident with the *Mla* locus at 32 HAI, and 1 031 mapped to the distal end of chromosome 2H, near *MlLa*, at 16 HAI. Moreover, of the 2 352 eQTL coordinately regulated by *Mla* at 32 HAI, 381 were also under control by the 2H locus at 16 HAI.

eQTL networks reveal the interactions between regulation modules We then took the analysis a step further by using this dataset as a scaffold to construct a barley-powdery mildew eQTL network, by adding a series of previous time-course-expression experiments from PLEXdb (http://www.plexdb.org/), a gene expression resource for plants and plant pathogens (Dash *et al.* 2012). Construction of the network enables each of the identified associations to nucleate a co-expression cluster, extending the biological information for each node (Quigley and Balmain 2009; Bao *et al.* 2010).

The statistical eQTL analysis discussed above resulted in a list of probesets (genes) and their most significantly associated markers. A subset of this list was chosen using a stringent FDR cutoff of 0.00001 to identify the most significantly associated markers for the probesets. This list was used to first build a disconnected network where nodes represent probesets and markers, and edges represent the statistically significant association between the probesets and

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corresponding marker. In this disconnected network, the connected modules provided information about genetic control that drives gene expression during infection. Additional gene co-expression data is needed to understand how that genetic regulation results in infection phenotype. For the barley powdery mildew system, PLEXdb expression profiling datasets BB2, BB4 and BB10 (Caldo *et al.* 2004, 2006; Moscou *et al.* 2011a) were combined into one dataset. The gene co-expression values for this dataset were calculated using mutual information[1)] measure based on maximum relevance/minimum redundancy (MRMR) according to (Meyer *et al.* 2008). Using mutual information (mi) cutoff of mi⩾0.7 for co-expression, additional edges were added in the eQTL network. The resulting eQTL network thus included information about genetic control of gene expression, as well as gene expression that lead to powdery mildew resistance. Visualization of this eQTL network helped to understand the interactions between various connected modules, i.e., genes co-expressed with genes directly regulated by the two major *trans*-eQTL hotspots, *Mla* and the 2H locus, near *MlLa.*

This is just the first step; further analysis will be required to (1) identify whether edges representing genetic regulation (from initial statistical eQTL analysis) indicate down-regulation or up-regulation of genes, and (2) assign functional annotations to connected modules within the network.

rar3-mediated transcriptome reprogramming

The intersection of datasets derived from *mla*-loss-of-function mutants, *Mla*-associated eQTL, and *rar3*-mediated transcriptome reprogramming are narrowing the focus on essential genes required for *Mla*-specified immunity.

7-d-old seedlings from the *rar3* mutant and wild-type progenitor were inoculated with *B. graminis* f. sp. *hordei* isolate 5 874, harvested at 16 and 32 HAI, and subjected to both Barley1 GeneChip and RNA-Seq analyses. A randomized block design of 4 treatments (2 genotypesx2 time points) with two independent biological replications was used to obtain expression measurements.

These data were used to assess *rar3*-mediated transcriptome reprogramming in both compatible and incompatible interactions in response to challenge with the biotrophic pathogen, *B. graminis* f. sp. *hordei* isolate 5 874. Whereas *Rar1* affects transcription of relatively few genes; *Rar3* appears to influence thousands, notably in genes controlling ATP binding, catalytic activity, transcription, and phosphorylation; possibly membrane bound or in the nucleus.

Interestingly, about half of the genes reprogrammed in the *rar3* mutant, as compared to its progenitor, also intersected with *Mla1* associated eQTL. As described above, 1 031 eQTLs were found to be regulated by the 2H region at 16 HAI, and 2 352 eQTLs were regulated by *Mla* at 32 HAI (0.00001 FDR). 381 of the 1 031 genes associated with the 2H region at 16 HAI transfer control to *Mla* at 32 HAI. Of the 2 352 eQTL that were regulated by *Mla* at 32 HAI, 97 are also dependent on *Rar3*. Moreover, of the 381 that transfer control from the 2H region at 16 HAI to *Mla* at 32 HAI, 33 are also dependent on *Rar3*.

So what are the functions of these genes? Searching the Biological Process category at the Gene Ontology (Gene Ontology Consortium *et al.* 2000) (GO; http://www.geneontology.org/) classified the 97 *Mla*-associated, and *Rar3*-dependent, genes into the oxidation-reduction, transport, metabolic process, and defense/biotic/chitin response categories, and the 381 that transfer control from the 2H region at 16 HAI to *Mla* at 32 HAI into metabolic process, oxidation-reduction, transport and biosynthesis.

CONCLUSION

Active plant defense to microbial attack is highly dependent upon recognition events involving associated gene products in the host and the pathogen. Both perception of general and specific pathogen-associated molecules result in signal transduction cascades ultimately leading to disease resistance.

In order to focus on essential genes required for

[1)] Mutual information is a statistical measure that quantifies dependence or shares information between two random variables. It indicates how much can be known about one variable given the information about the other. This serves as a useful measure to find gene co-expression values because it is not limited to linear dependency relationships between the random variables (i.e., genes or probesets in this case).

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Mla-specified immunity, we intersected datasets derived from *mla*-loss-of-function mutants, *Ml*-associated eQTL, and *rar3*-mediated transcriptome reprogramming. This approach enabled the classification of a subset of genes regulated by *Mla* and required by *Rar3*, within a specific time-frame during the kinetics of *B. graminis* f. sp. *hordei* infection.

The two most significant *trans*-eQTL hotspots are positionally coincident with immunity specified by *Mla* and the chromosome 2H region, respectively. These *Mla*- and 2H eQTL hotspots alter the expression of hundreds of disease responsive genes. Because the eQTL tie together *R*-gene mediated and basal defense networks, we consider the functional identification of their precise roles a key step toward understanding how to achieve durable resistance to fungal pathogens.

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