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Quantitative and Temporal Definition of the Mla Transcriptional Regulon During Barley–Powdery Mildew Interactions

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Abstract

Barley *Mildew resistance locus a* (*Mla*) is a major determinant of immunity to the powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei*. Alleles of *Mla* encode cytoplasmic- and membrane-localized coiled-coil, nucleotide binding site, leucine-rich repeat proteins that mediate resistance when complementary avirulence effectors (AVRa) are present in the pathogen. Presence of an appropriate AVRa protein triggers nuclear relocalization of MLA, in which MLA binds repressing host transcription factors. Timecourse expression profiles of plants harboring *Mla1, Mla6*, and *Mla12* wild-type alleles versus paired loss-of-function mutants were compared to discover conserved transcriptional targets of MLA and downstream signaling cascades. Pathogen-dependent gene expression was equivalent or stronger in susceptible plants at 20 h after inoculation (HAI) and was attenuated at later timepoints, whereas resistant plants exhibited a time-dependent strengthening of the transcriptional response, increasing in both fold change and the number of genes differentially expressed. Deregulation at 20 HAI implicated 16 HAI as a crucial point in determining the future trajectory of this interaction and was interrogated by quantitative analysis. In total, 28 potential transcriptional targets of the MLA regulon were identified. These candidate targets possess a diverse set of predicted functions, suggesting that multiple pathways are required to mediate the hypersensitive reaction.

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Quantitative and Temporal Definition of the *Mla* Transcriptional Regulon During Barley–Powdery Mildew Interactions

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Barley *Mildew resistance locus a* (*Mla*) is a major determinant of immunity to the powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei*. Alleles of *Mla* encode cytoplasmic- and membrane-localized coiled-coil, nucleotide binding site, leucine-rich repeat proteins that mediate resistance when complementary avirulence effectors (AVR_a) are present in the pathogen. Presence of an appropriate AVR_a protein triggers nuclear relocalization of MLA, in which MLA binds repressing host transcription factors. Time-course expression profiles of plants harboring *Mla1*, *Mla6*, and *Mla12* wild-type alleles versus paired loss-of-function mutants were compared to discover conserved transcriptional targets of MLA and downstream signaling cascades. Pathogen-dependent gene expression was equivalent or stronger in susceptible plants at 20 h after inoculation (HAI) and was attenuated at later timepoints, whereas resistant plants exhibited a time-dependent strengthening of the transcriptional response, increasing in both fold change and the number of genes differentially expressed. Deregulation at 20 HAI implicated 16 HAI as a crucial point in determining the future trajectory of this interaction and was interrogated by quantitative analysis. In total, 28 potential transcriptional targets of the MLA regulon were identified. These candidate targets possess a diverse set of predicted functions, suggesting that multiple pathways are required to mediate the hypersensitive reaction.

The obligate fungal biotroph, *Blumeria graminis* f. sp. *hordei*, is the causal agent of powdery mildew on barley (*Hordeum vulgare* L.) (Bushnell 2002). The genetics and physiology of this host-pathogen interaction have been well characterized, beginning with a report by Biffen (1907). Recognition of *B. graminis* f. sp. *hordei* is mediated by several loci distributed throughout the barley genome and designated *Ml* (*Mildew resistance locus*) (Jørgensen 1994). Most well-known is *Mla*, located on the short arm of chromosome 1H, with approximately 30 alleles that mediate resistance when corresponding AVR_a effectors are present in the pathogen (Jørgensen 1994). In contrast to the genetic structure of *Mla* with multiple alleles at a single locus, AVR_a genes are scattered throughout the *B. graminis* f. sp. *hordei* genome, with the cloned AVR_{a10} belonging to a diverse family encoding proteins that lack secretion signals (Ridout et al. 2006; Skamnioti et al. 2008). Sequenced alleles of *Mla* encode cytoplasmic- and membrane-localized coiled-coil (CC), nucleotide binding site, leucine-rich repeat proteins (Halterman and Wise 2004; Seeholzer et al. 2010; Shen et al. 2003) that translocate into the nucleus after recognition of a corresponding AVR_a effector from *B. graminis* f. sp. *hordei*. Nuclear localization of AVR_a is required to mediate the hypersensitive reaction (HR) (Shen et al. 2007), which may be dependent on the direct interaction between appropriate MLA and AVR_a proteins (Seeholzer et al. 2010). After recognition, the CC domain of MLA binds the transcription factors WRKY1 and WRKY2 (WRKY1/2) (Shen et al. 2007). Thus, the translocation of MLA and subsequent interaction with WRKY1/2 is expected to remodel the transcriptional landscape leading to the HR.

The physical association of MLA with transcription factors indicates that activation, inhibition, or modulation of gene expression may be required to mediate the HR in epidermal cells breached by fungal appressoria. In this mRNA-based model for induction of the HR, the identification of the primary downstream transcriptional targets of MLA would make it possible to address several central questions, such as what are the genes and pathways that initiate the HR and which molecular processes are involved in mediating this cell death program. While investigations conducted prior to knowledge of MLA localization were able to link the kinetics of several well-known pathogenesis-related (*PR*) and other genes with the development of *B. graminis* f. sp. *hordei* on the host, they were limited in their ability to causally associate the regulation of gene expression to *Mla*-meditated resistance (Collinge et al. 2002; Eichmann et al. 2006; Gjetting et al. 2007).

Previously, we investigated the reprogramming of the barley transcriptome in response to powdery mildew infection by utilizing three near-isogenic lines of barley (harboring different alleles of *Mla*) and two contrasting isolates of *B. graminis* f.

GeneChip data is available in ArrayExpress database under accession numbers E-TABM-82 (BB2 Sultan-5 experiment) and E-TABM-142 (BB10 Manchuria experiment).

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sp. *hordei* (Caldo et al. 2004). Our primary objective at that time was to identify conserved genes whose expression patterns differed across incompatible (i.e., *Mla6-AVR$_{a6}$*, *Mla13-AVR$_{a13}$*) versus compatible (i.e., *Mla6-AVR$_{a13}$*, *Mla13-AVR$_{a6}$*) interactions. A total of 22 host genes were identified at a threshold *P* value <0.0001 and false discovery rate (FDR) of 7%, for which increased expression was observed from 0 to 16 h after inoculation (HAI) in all interactions relative to the 0 HAI timepoint (Caldo et al. 2004). From 20 to 32 HAI, these genes displayed divergent expression between incompatible and compatible interactions, with greater expression almost always in the incompatible interaction (Caldo et al. 2004). In a subsequent experiment, relaxation of the stringency paired with correlation to the original pattern extended this list to an additional 134 genes ($P < 0.01$) (Caldo et al. 2006). Functional analysis of three of these genes (*chorismate synthase*, *anthranilate synthase α subunit 2*, and *chorismate mutase 1*) demonstrated that they were necessary for penetration resistance against *B. graminis* f. sp. *hordei* (Hu et al. 2009). In contrast, another gene, *Blufensin1* (*Bln1*), encoding a small peptide induced by a broad range of fungal pathogens, was shown to negatively impact defense (Meng et al. 2009). Neither silencing nor overexpression of these four genes suppressed the HR mediated by MLA, suggesting their roles are sufficiently downstream of MLA signaling such that they do not compromise the HR (Bent and Mackey 2007).

Here, we describe an alternative approach that takes advantage of paired wild-type and loss-of-function mutant alleles of *Mla.* Initially, we establish a comprehensive index of temporal gene expression patterns after inoculation with *B. graminis* f. sp. *hordei*. Interestingly, the overall transcriptional response was equivalent or stronger at 20 HAI in compatible interactions; however, this response was not quantitatively sustained over time as compared with incompatible interactions. By identifying the conserved quantitative differences between three wild-type and mutant pairings at 16 HAI, we predict a set of candidate genes that are hypothesized to be transcriptional targets of the MLA-mediated HR.

RESULTS

Concept and experimental design.

Our early experiments focused on distinguishing the transcriptional differences between incompatible and compatible interactions using alleles of *Mla* and complementary isolates of *B. graminis* f. sp. *hordei* (Caldo et al. 2004). Specifically, we used a matrix of three near-isogenic barley lines with introgressed *Mla1*, *Mla6*, or *Mla13* alleles, each challenged with the contrasting powdery mildew isolates 5874 (containing *AVR$_{a1}$* and *AVR$_{a6}$*) and K1 (containing *AVR$_{a1}$* and *AVR$_{a13}$*) (Caldo et al. 2004, 2006). Since there were no noninoculated tissues in this experiment, we were restricted to evaluating divergent responses between different *Mla-AVR$_a$* pairings.

Here, we included noninoculated tissue to generate a comprehensive index of differential steady-state expression levels in response to powdery mildew infection, used a single isolate of *B. graminis* f. sp. *hordei*, and incorporated several loss-of-function mutants of *Mla* (Fig. 1). The use of three incompatible (*Mla-AVR$_a$*) versus compatible (*mla-AVR$_a$*) pairings leveraged the diversity inherent in this host-pathogen interaction. These combinations represent fast versus intermediate resistance-gene kinetics (i.e., *Mla1* and *Mla6*, pathogen ingress halted at haustorial development, in contrast to *Mla12*, which allows limited hyphal growth), *Rar1*-dependent (*Mla6* and *Mla12*) versus *Rar1*-independent (*Mla1*), and Manchuria (*Mla1* and *Mla6*) versus Sultan-5 (*Mla12*) genetic backgrounds (Boyd et al. 1995; Meng et al. 2009; Shen et al. 2003; Torp and Jørgensen 1986; Wise and Ellingboe 1983; Zhou et al. 2001). Utilizing these reagents, we set out to predict conserved transcriptional targets of MLA that mediate the HR upon its translocation into the nucleus (Shen et al. 2007).

As illustrated in Figure 1, our experimental material consisted of three barley lines carrying the resistance alleles *Mla1* (CI 16137), *Mla6* (CI 16151), and *Mla12* (Sultan-5) and their corresponding loss-of-function mutants, *mla1-m508*, *mla6-m9472*, and *mla12-m66*, respectively (Meng et al. 2009; Moseman 1972; Torp and Jørgensen 1986; Shen et al. 2003; Zhou et al. 2001). A split-split-plot design with three independent biological replications was used to obtain expression measurements (Kuehl 2000). Genotype was the whole-plot treatment factor, and each whole-plot experimental unit consisted of a pair of trays of the same genotype (one inoculated and one noninoculated) side-by-side in a growth chamber during the timecourse after inoculation. The split-plot experimental units were the individual trays that were randomly assigned to inoculation with *B. graminis* f. sp. *hordei* 5874 (*AVR$_{a1}$*,

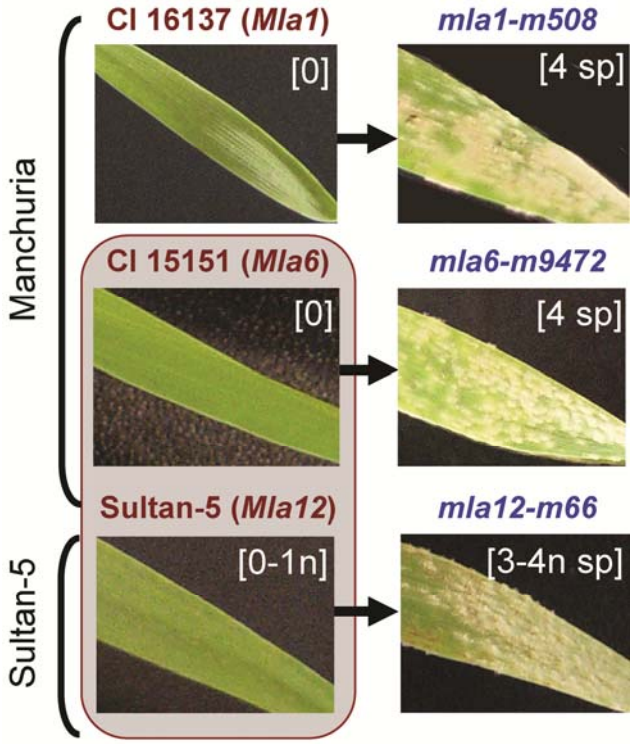

Fig. 1. Seedling phenotypes and experimental design. Transcript profiling was based on a split-split-plot design with replications as blocks, genotype as the whole plot factor, inoculation treatment as the split-plot factor, and time as the split-split-plot factor. Seven-day-old seedlings of wild-type *Mla1* (CI 16137), *Mla6* (CI 16151), *Mla12* (Sultan-5), and their corresponding loss-of-function mutants, *mla1-m508*, *mla6-m9472*, and *mla12-m66*, respectively, were inoculated with *Blumeria graminis* f. sp. *hordei* isolate 5874 (*AVR$_{a1}$*, *AVR$_{a6}$*, and *AVR$_{a12}$*) or noninoculated. Fifteen first leaves were harvested at 0, 8, 16, 20, 24, and 32 h after inoculation (HAI). One Barley1 GeneChip was used for each of the 216 split-split-plot experiment units, corresponding to six genotypes × two inoculation treatments × six timepoints × three replications. Wild-type derived mutant seedling infection types are shown 7 days after inoculation. Lines containing *Mla1* and *Mla6* are introgressed into Manchuria background, whereas *Mla12* and its mutant are derived from Sultan-5. Cells labeled in red (wild type) and blue (mutant) are incompatible and compatible, respectively, when inoculated with *B. graminis* f. sp. *hordei* 5874. Gray highlight indicates *Rar1*-dependant genotypes. An infection type of 0 to 2 is considered to be resistant ("–" designates incompatible or no sporulation), whereas an infection type of 3 to 4 is considered to be susceptible ("+" designates compatible or abundant sporulation). 0 = immune; 0-1n = few to several small necrotic flecks (0.5 mm); 4sp = completely susceptible.

AVR_{a6}, and AVR_{a12}) or to noninoculated control. The split-split-plot experimental units were rows of plants within trays randomly assigned to the harvest times 0, 8, 16, 20, 24, and 32 HAI. The analysis we present here is based on gene expression data from six genotypes × six timepoints × two inoculation treatments × three replications, collected using 216 Barley1 GeneChips (Close et al. 2004).

Defining the set of barley genes responsive to *B. graminis* f. sp. *hordei* inoculation.

Fold-change estimates and associated *P* values for differential expression between inoculated and noninoculated treatments for each timepoint were determined using a mixed linear model. The model included fixed effects for genotype, treatment (*B. graminis* f. sp. *hordei* 5874 or noninoculated), timepoint, and all interactions among these factors, as well as random effects for replication and the corresponding interactions of genotype × replication (whole-plot), genotype × treatment × replication (split-plot), and genotype × time × replication (split-split-plot) (Kuehl 2000; Wolfinger et al. 2001). FDR were estimated using the histogram-based technique as described by Nettleton and associates (2006). In summary, controlling the FDR at 0.0001, 0.01, and 1% found 3,810, 6,846, and 15,149 genes differentially expressed between inoculated and noninoculated plants for at least one timepoint among the wild-type genotypes, respectively. The large number of genes identified as differentially expressed is a result of strong statistical power, the examination of multiple timepoints, and most significantly, the strong transcriptional response of barley to powdery mildew infection. To avoid loss of information by selecting an arbitrary FDR cutoff, we incorporated all three FDR cutoffs for subsequent analyses.

A total of 54 timecourse patterns of expression define the transcriptional response of barley to *B. graminis* f. sp. *hordei* infection.

We identified composite patterns separately for both incompatible and compatible interactions, based on conserved differential gene expression within the sets of wild-type *Mla* and derived mutant lines, respectively. Each timepoint was evaluated for differential expression between inoculated and noninoculated treatments using a voting approach that considered all three genotypes at each of the three FDR levels, for a total of nine comparisons. A consensus designation of induced (+) or suppressed (–) was recorded only when the differential expression was present in at least four of the nine comparisons (discussed below). Based on the selected FDR, the directions of any significant changes (+ or –) were always the same within a gene and timepoint so that the consensus designation of + or – represents the direction of change common to all significant differences within a gene for a given timepoint. If the gene was not differentially expressed based on four of nine criterion, it was given a 0. As illustrated in Figure 2, ternary representations of these consensus designations were used to graphically depict the composite patterns of 3,876 genes. For example, a gene upregulated at the 20 and 24 HAI timepoints would be represented as 000++0. Conversely, if the same gene was downregulated only at those two timepoints, the representation would be 000—0. This approach has the advantage of removing genotype-specific expression and leverages several FDR to identify conserved sets of genes that have particular differential expression responses to inoculation with the 5874 isolate (AVR_{a1}, AVR_{a6}, AVR_{a12}) of *B. graminis* f. sp. *hordei*.

Composite patterns for incompatible and compatible interactions were grouped into three main categories, based on the timing of differential expression, early (0 to 16 HAI), late (20 to 32 HAI), or a mixture of these responses (Fig. 2; Supplementary Table S1). Upregulation predominates both the early and mixed responses to challenge with powdery mildew in both incompatible (650 genes) and compatible (515 genes) interactions. In contrast, genes that are downregulated were poorly represented in early and mixed-response categories, with 93 and 90 genes in incompatible and compatible interactions, respectively. More genes were downregulated in late responses, with 832 and 981 upregulated and 1,511 and 1,624 genes downregulated among wild-type and mutant lines, respectively. In general, incompatible and compatible interactions had similar representation of patterns, which resulted from the selection of early timepoints in the interaction of barley and powdery mildew, before the onset of massive fungal growth.

Of the 54 composite patterns observed, 52 contain only combinations of two designations, i.e., '+' with 0 or '–' with 0 (Fig. 2), indicating that trends of induction or suppression do not reverse within this timecourse. Not shown in Figure 2 are the two composite patterns that are mixtures of both induction and suppression across the timecourse. However, each of these is represented only by a single gene, which suggests that these patterns may be exceptions to the apparent rule that the direction of differential expression does not reverse. If these are indeed exceptions, one might expect the representative genes to lack clear roles in disease defense. This may be the case, since Barley1_11076 (incompatible: +000–0; compatible: +00—0) is predicted to be a Ca^{2+}-dependent nuclease, while Barley1_50237 (incompatible: +00000; compatible: +000–0) has sequence similarity to the *defective embryo and meristems* gene in tomato (Keddie et al. 1998).

Relation of composite patterns to infection kinetics.

Several characteristics distinguish incompatible and compatible responses. First, there are almost twice as many genes upregulated in incompatible (153 genes) versus compatible (84 genes) interactions in early response patterns (Fig. 2). Second, the most represented patterns in incompatible interactions were 00000+ (323 genes) and 00000– (832 genes) for late responses, while these same two classes are considerably reduced (147 and 326 genes, respectively) in compatible interactions. Accompanying this reduction is an increase in four of the remaining six late response patterns (0000++, 000+00, 0000+0, 000++0) for compatible as compared with incompatible interactions. The effect was exactly mirrored with the downregulated patterns, indicating that late response gene expression was occurring even later in incompatible interactions regardless of its direction (Fig. 2). The late responses at the 24 and 32 HAI timepoints are both after penetration of epidermal cells by *B. graminis* f. sp. *hordei* and the formation of haustoria. Thus, susceptible plants were responding earlier than their resistant counterparts, and this transcriptional outcome coincides with the physical interaction at the perihaustorial interface between plant cell and fungal pathogen.

Integration of fold change into timecourse patterns of expression reveals an earlier response in compatible interactions.

In order to understand the magnitude of the response to powdery mildew invasion, we integrated fold change as a measure of the quantitative effect occurring at each timepoint in inoculated versus noninoculated seedlings. We used only those genes with consensus patterns in either the wild-type or mutant genotypes, or both, and selected fold change ranges of 2 to 3, 3 to 4, 4 to 5, 5 to 10, and greater than 10, to broadly categorize induction or suppression of transcript accumulation at each timepoint. As induction or suppression is consistent, fold change refers to inoculated versus noninoculated for induced genes, and conversely, noninoculated versus inoculated for suppressed genes.

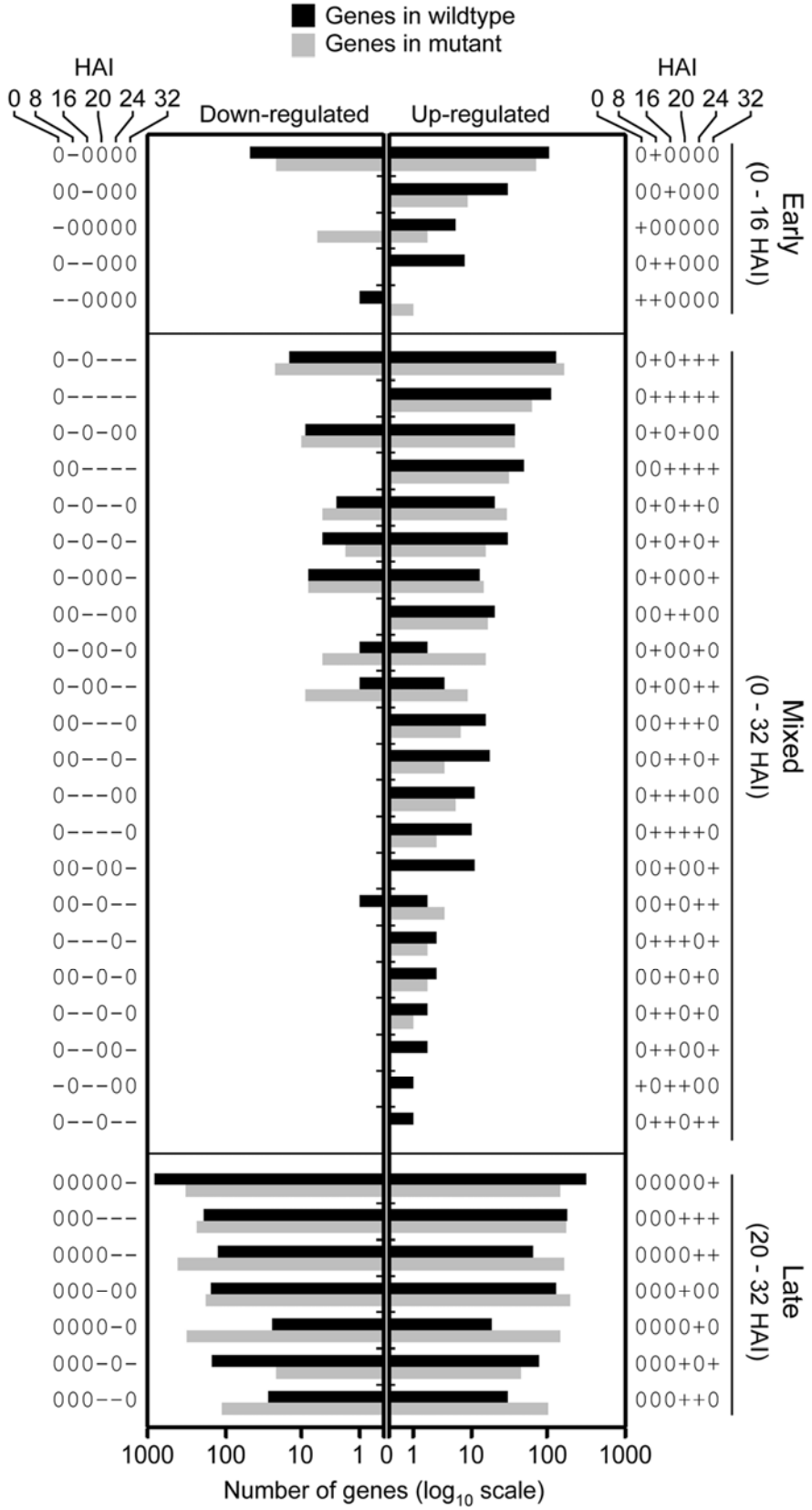

Fig. 2. Distribution of consensus expression patterns in incompatible and compatible interactions. Patterns represent a consensus of differential expression in at least four of nine genotype × false discovery rate comparisons, with induced or suppressed gene expression in inoculated plants relative to noninoculated plants for a given timepoint, represented by either a '+' or '–', respectively. If the gene is not differentially expressed based on the consensus, it is given a '0'. Concatenation of all six timepoints (0, 8, 16, 20, 24, and 32 h after inoculation [HAI]) provides the patterns shown on the borders. The number of genes with consensus patterns in incompatible (*Mla-AVR$_a$*) or compatible (*mla-AVR$_a$*) interactions are illustrated by black and gray horizontal bars, respectively. The horizontal axis is $\log_{10}$-scale. Patterns were ordered based on the total number of genes from incompatible and compatible interactions within each response category (early [0 to 16 HAI], mixed, and late [20 to 32 HAI]).

As illustrated in Figure 3 (an alternate presentation is shown in Supplementary Figure S1), both incompatible and compatible interactions exhibited a several-fold increase in differentially expressed genes by 8 HAI, indicating that a substantial nonspecific response occurs regardless of the presence or absence of MLA. By 20 HAI, two major patterns in differential expression distinguish the two interaction types. Incompatible interactions revealed an ever-increasing set of genes that are both differentially expressed and intensifying in their relative fold change between 20 and 32 HAI. In contrast, compatible interactions show an early induction of gene expression with a high fold change at 20 HAI, but this effect either weakens gradually with time (*mla1-m508*) or the larger fold change categories (5 to 10 and >10) stabilize (*mla6-m9472* and *mla12-m66*).

It is unexpected that loss-of-function *mla* mutants would exhibit earlier induction or suppression of gene expression in response to inoculation. Early activation may represent a miscoordination in transcriptome reprogramming, in which the plant is initiating several pathways in an inappropriate order, thus providing a window of opportunity for the pathogen. Alternatively, in the absence of MLA, *B. graminis* f. sp. *hordei* may activate these genes deliberately as a means of disarming the plant. Regardless of the mechanism, the transcriptional cascades observed at 20 HAI implicate the time between 16 and 20 HAI as a key turning point in reprogramming the defense transcriptome.

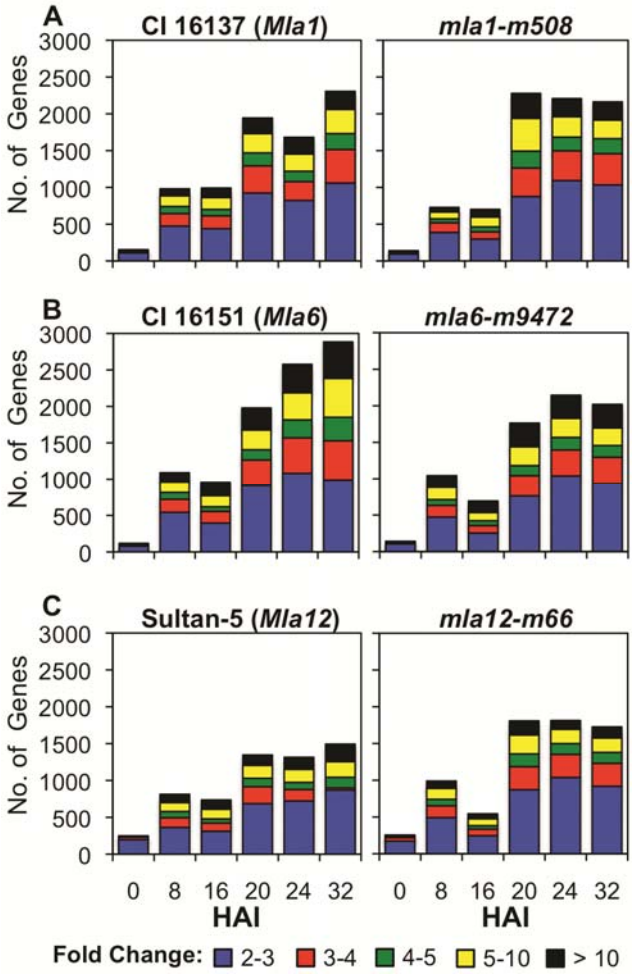

Fig. 3. A stronger transcriptional response occurs at 20 h after inoculation (HAI) in loss-of-function *mla* as compared with wild-type plants. Fold change between inoculated and noninoculated plants was derived for genes with a consensus expression pattern, as shown in Figure 2, at each timepoint. Probe sets not meeting the fold-change cutoff of 2 at any timepoint were not included in this figure. Bar plots show the numbers of genes in fold-change categories of 2 to 3 (blue), 3 to 4 (red), 4 to 5 (green), 5 to 10 (yellow), and >10 (black) for barley lines containing wild-type and respective mutant alleles **A,** *Mla1, mla1-m508*; **B,** *Mla6, mla6-m9472*; and **C,** *Mla12, mla12-m66.*

Identification of the MLA transcriptional regulon.

The in vitro interaction of the CC domain of MLA with the WRKY1/2 transcription factors, coupled with the observation of in planta physical association in the nucleus using Förster resonance energy transfer corroborates MLA as an interactor with WRKY1/2 (Shen et al. 2007). Both transcription factors are repressors of basal defense in compatible interactions with *B. graminis* f. sp. *hordei*, suggesting that the inhibitory function

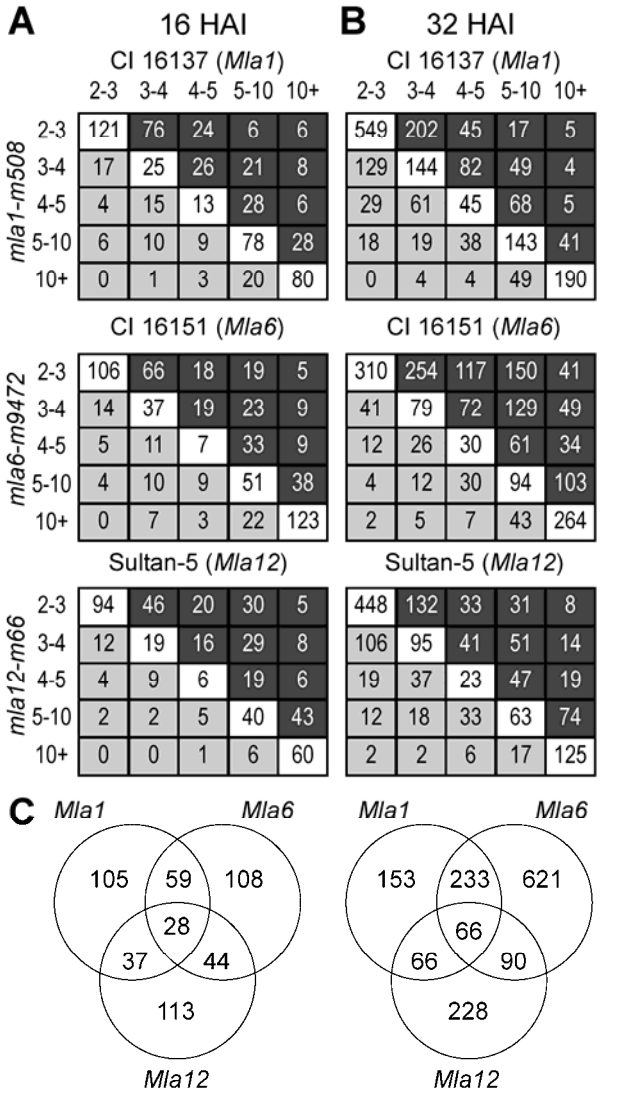

Fig. 4. Quantitative suppression of gene regulation correlates with resistance at both 16 and 32 h after inoculation (HAI). Intersection of genes between wild-type and mutant pairings for all fold change categories at **A,** 16 and **B,** 32 HAI. The upper right portion (dark gray) of individual matrices correspond to genes with suppressed expression in mutant as compared with wild-type alleles of *Mla,* whereas the lower left portion (light gray) of individual matrices correspond to genes having enhanced expression in mutant compared with wild-type alleles of *Mla*. **C,** Venn diagrams illustrating conserved genes at the intersections of the three wild-type versus mutant pairs, for 16 (left) and 32 (right) HAI. Numbers correspond to quantitatively suppressed genes displayed in the upper right portion (dark gray) of panels A and B.

of MLA provides gain-of-function expression of WRKY1/2 repressed targets. Though it is unclear if these two transcription factors are the only targets of MLA and whether or not the nature of WRKY1/2 transcriptional regulation after interaction with MLA is completely inhibitory, our paired analysis of wild-type and mutant lines provides a comprehensive template for identifying candidate genes that belong to the MLA transcriptional regulon.

We focused on 16 HAI, as it coincides with establishment of the perihaustorial interface between host epidermal cells and *B. graminis* f. sp. *hordei* haustoria, nuclear relocalization of MLA, and the major transcriptional cascades observed at 20 HAI. Hence, it represents an ideal timepoint for detecting primary transcriptional targets of MLA and its interactors. We hypothesized that genes in the MLA transcriptional regulon would have quantitative effects between incompatible and compatible interactions and that this effect would be conserved among all three wild-type and mutant pairs. To accomplish this, we compared genes at 16 HAI between each wild-type and mutant pair for the previously defined fold change categories (2 to 3, 3 to 4, 4 to 5, 5 to 10, and >10). It follows that we can identify genes with lower fold change in mutant as compared with wild type or the reverse by observing the off-diagonal enrichment of differentially expressed genes. The results are summarized in Figure 4A. Suppression of regulation in the mutant as compared with wild type is shown in the upper right quadrant (dark gray) and induction in the mutant as compared with wild type in the lower left quadrant (light gray). The diagonal represents genes conserved within a fold change category between a wild-type and mutant pair. Interestingly, we found that all three paired wild-type and mutant alleles of *Mla* have between 2.7 to 5.4 times as many genes suppressed in *mla* relative to *Mla* as compared with the reverse. Specifically, 229 genes were suppressed in *mla1-m508* as opposed to 85 in CI 16137 (*Mla1*), 239 genes were suppressed in *mla6-m9472* versus 85 genes in CI 16151 (*Mla6*), and lastly, 222 genes were suppressed in *mla12-m66* versus 41 in Sultan-5 (*Mla12*).

To control for the case that suppression may be associated with genotype-specific effects or minor differences that met the threshold cutoff but are not biologically relevant, we iden-

Table 1. Candidates for *Mildew resistance locus a* (*Mla*) transcriptional regulon based on quantitative suppression in three wild-type versus mutant pairings at 16 h after inoculation

		Consensus expression patterns		BLASTn or BLASTx reference information			
Probe Set	**Contig No.[a]**	**Wild type**	**Mutant**	**Organism**	**Accession/locus**	**E-score**	**Annotation[b]**
Contig5537_at	5537	0+++++	0+++++	*Oryza sativa*	Os07g01560.2	2.00E-69	Monosaccharide transport protein
Contig11154_at	11154	0+++++	0++++0	*Hordeum vulgare*	Os02g03410.2	0	Calcium-dependent protein kinase
Contig10860_at	10860	0+++++	0+0+++	*O. sativa*	Os03g58980.1	5.00E-42	Germin-like protein
Contig12724_at	12724	0+++++	0+0+++	*O. sativa*	Os03g03034.4	1.00E-122	Flavonol synthase/flavanone 3-hydroxylase
Contig14304_at	14304	0+++++	0+0+++	*O. sativa*	Os10g38470.1	8.00E-84	Glutathione *S*-transferase
Contig2639_at	2639	0+++++	0+0+++	*O. sativa*	Os05g05680.1	1.00E-156	1-aminocyclopropane-1-carboxylate oxidase
EBem10_SQ002_I10_s_at	29452	0+++++	0+0+++	*O. sativa*	Os01g59660.2	0	Transcription factor–GAMYB
Contig3568_at	3568	0+++++	000+++	*H. vulgare*	Os02g17940.6	1.00E-131	Iron/ascorbate-dependent oxidoreductase
Contig26368_at	26368	0++++0	0+++00	*O. sativa*	Os06g11450.1	5.00E-08	RING-H2 finger protein ATL3B precursor
Contig9422_at	9422	0+++0+	000+0+	*O. sativa*	Os01g07870.1	1.00E-132	Multidrug resistance-associated protein MRP2 / ABC transporter
Contig4942_at	4942	0+++00	000+00	*O. sativa*	Os02g32520.2	0	Early responsive to dehydration 1 (ERD1) protein
Contig8949_at	8949	0++000	000+00	*O. sativa*	Os03g17310.1	0	Calcium-transporting ATPase, endoplasmic reticulum-type
HF22G17r_at	34443	0++000	000+00	*H. vulgare*	BU989685	1.00E-128	Unknown
Contig11285_at	11285	0+0+++	0+0+++	*O. sativa*	Os04g37600.1	1.00E-138	Phosphate carrier protein
HV_CEb0004O15r2_s_at	39931	0+0+++	0+0+++	*O. sativa*	Os10g38470.1	8.00E-09	Glutathione *S*-transferase
Contig5876_at	5876	0+0+++	000+0+	*O. sativa*	Os01g08110.1	0	Flavonol-3-*O*-glycoside-7-*O*-glucosyltransferase 1
Contig12286_s_at	12286	0+0+0+	000+00	*O. sativa*	Os06g35700.1	0	Reticuline oxidase precursor
HVSMEb0007D15r2_at	41025	00++++	00++++	*O. sativa*	Os06g45570.1	4.00E-15	VQ motif family protein
HVSMEf0001H14r2_at	42024	00++++	00++++	Not applicable	n.a.	n.a.	Unknown
Contig15548_at	15548	00++++	000+++	*O. sativa*	Os05g25210.1	1.00E-148	Chaperone protein dnaJ-related
Contig3744_s_at	3744	00++++	000+++	*O. sativa*	Os04g58850.1	5.00E-71	Harpin-induced protein 1 domain containing protein
Contig8468_at	8468	00+++0	00++00	*O. sativa*	Os01g38980.2	1.00E-117	Calmodulin binding protein
Contig24439_at	24439	00+++0	000+00	*O. sativa*	Os02g43430.1	6.00E-68	Protein kinase, putative
Contig13091_s_at	13091	00++0+	000+00	*O. sativa*	Os06g50390.1	7.00E-16	Aspartic-type endopeptidase/ pepsin A
Contig10615_at	10615	00+000	000000	*O. sativa*	Os03g07400.1	3.00E-36	Unknown protein
HV_CEa0009C05r2_s_at	39626	000+0+	0000++	*O. sativa*	OJ1117_G01.1	1.00E-138	Expressed protein
Contig21659_s_at	21659	0000++	0000++	*O. sativa*	Os11g37700.1	1.00E-36	PDR-type ABC transporter
Contig2170_at	2170	0000++	0000++	*Triticum aestivum*	Q01482	9.00E-08	WIR1A protein

[a] Affymetrix Barley1 GeneChip contig numbers.

[b] Consensus annotation based on six BLASTn or BLASTx methods: i) BLASTx UniProt: Uniref90 (July 09), ii) BLASTn The Institute for Genomic Research (TIGR) plant transcript assemblies: TIGR TA *Hordeum vulgare* release 2, iii) BLASTx rice genome: TIGR release 6.1, iv) BLASTx National Center for Biotechnology Information (NCBI) reference sequence: NCBI RefSeq rel-35, v) BLASTn DFCI *Hordeum vulgare* gene index: HvGI (Dana Farber Cancer Institute) release 10, and vi) BLASTx *Arabidopsis thaliana* genome: TAIR release 9.

tified those genes that were conserved in all three wild-type versus loss-of-function mutant pairs. Shown as a Venn diagram in Figure 4C, 28 genes were suppressed at 16 HAI in all compatible interactions relative to incompatible interactions (Table 1; Supplementary Table S2). Annotation of these genes revealed a broad set of functional roles, involving redox homeostasis, signal transduction, energy transfer, proteolysis, protein folding, transport, ethylene biosynthesis, protein degradation, defense, transcription, and several genes with unknown function. Strikingly, all genes identified are induced after inoculation with *B. graminis* f. sp. *hordei*, with a diversity of patterns that include early, mixed, and late responses. Thus, increased expression was associated with wild-type *Mla*, as compared with the paired *mla* mutant for all 28 genes. As candidates of the MLA transcriptional regulon, this implicates MLA as an activator of gene expression (Shen et al. 2007). Though indirect, this asso-

Table 2. Downstream targets associated with resistance or susceptibility based on quantitative suppression in three wild-type versus mutant pairings at 32 h after inoculation

		Consensus expression patterns		BLASTn or BLASTx reference information			
Probe Set	**Contig no.[a]**	**Wild type**	**Mutant**	**Organism**	**Accession/locus**	**E-score**	**Consensus Annotation[b]**
Resistant Association							
Contig7933_at	7933	0+++++	0+0+++	*Oryza sativa*	Os03g04110.1	2.00E-93	LysM domain-containing GPI-anchored protein precursor
Contig10585_at	10585	0+++++	0+0+++	*O. sativa*	Os06g12250.1	1.00E-145	Sphingolipid C4-hydroxylase SUR2
Contig10887_at	10887	0+++++	0+0+++	*O. sativa*	Os04g11820.1	0	White-brown complex homolog protein
Contig12794_at	12794	0+++++	0+0+++	*O. sativa*	TC179812	1.00E-129	Expressed protein
Contig9422_at	9422	0+++0+	000+0+	*O. sativa*	Os01g07870.1	1.00E-132	ABC transporter family protein
Contig6539_s_at	6539	0+0+++	0+0+++	*O. sativa*	Os02g33110.1	1.00E-145	Cell wall invertase
Contig14426_at	14426	0+0+++	0+0+++	*O. sativa*	Os09g25150.3	8.00E-54	Cinnamoyl-CoA reductase
Contig24409_at	24409	0+0+++	0+0+++	*O. sativa*	Os06g15760.1	1.00E-32	Eukaryotic aspartyl protease domain containing protein
Contig406_at	406	0+0+++	0+0++0	*O. sativa*	Os08g38900.1	1.00E-102	Caffeoyl-CoA O-methyltransferase
Contig18290_at	18290	0+0+++	0+000+	*Hordeum vulgare*	TC170053	1.00E-111	Unknown
Contig406_s_at	406	0+0+++	000+++	*O. sativa*	Os08g38900.1	1.00E-102	Caffeoyl-CoA O-methyltransferase
Contig3563_at	3563	0+0+++	000+++	*O. sativa*	Os02g17940.6	5.00E-62	Leucoanthocyanidin dioxygenase
Contig6967_at	6967	0+0+++	000+0+	*O. sativa*	Os11g47820.1	0	Glucan endo-1,3-beta-glucosidase precursor
Contig4676_at	4676	0+0++0	0+0++0	*O. sativa*	Os02g08100.1	0	4-coumarate coenzyme A ligase
Contig14427_at	14427	0+0+00	0+0000	*O. sativa*	Os06g35650.1	1.00E-138	Reticuline oxidase-like protein precursor
HVSMEf0019O11r2_at	42715	0+0000	0000+0	*O. sativa*	Os12g04120.1	6.00E-19	Phosphoglycerate mutase
Contig14713_at	14713	00++++	0+0+++	*O. sativa*	Os01g15029.1	5.00E-16	Unknown
Contig15493_at	15493	00++++	0+0+++	*O. sativa*	Os06g14490.	3.00E-31	Calmodulin-binding heat-shock protein
Contig13615_at	13615	00++++	00++0+	*O. sativa*	Os02g07690.1	4.00E-26	VQ domain containing protein
Contig16910_at	16910	00++++	00++0+	*H. vulgare*	TC182261	0	Weakly similar to UniRef100_Q3HTK6 Cluster
Contig3564_s_at	3564	00++++	000+0+	*O. sativa*	Os02g17940.6	1.00E-128	Leucoanthocyanidin dioxygenase
Contig20294_at	20294	00++++	000+0+	*O. sativa*	Os09g15330.2	5.00E-53	Transporter family protein, putative, expressed
Contig14032_at	14032	00++00	000+00	*O. sativa*	Os12g06180.1	4.00E-91	HVA22
Contig15715_at	15715	000-0-	000---	*O. sativa*	Os02g54360.1	8.00E-57	Enzyme of the cupin superfamily protein
Contig10150_at	10150	000-0-	0000--	*H. vulgare*	TC169848	0	Weakly similar to UniRef100_Q2IMJ3 cluster
Contig14570_at	14570	000-0-	000000	*O. sativa*	Os10g33250.2	6.00E-56	WAX2
Contig7663_at	7663	000-00	000-00	*O. sativa*	Os12g01370.1	0	Omega-3 fatty acid desaturase
Contig11163_at	11163	000-00	000000	*O. sativa*	Os10g42620.1	7.00E-63	Cinnamoyl-CoA reductase-related
Contig5942_at	5942	000+++	0+0+++	*O. sativa*	Os04g44870.1	2.00E-64	C2 domain containing protein
Contig4833_at	4833	000+++	0+00++	*O. sativa*	Os03g42110.1	0	Semialdehyde dehydrogenase, NAD binding domain containing protein
Contig1737_at	1737	000+++	000+++	*O. sativa*	Os03g49380.1	0	Lipoxygenase
Contig8900_at	8900	000+++	000+++	*O. sativa*	Os09g32550.2	0	Glucan endo-1,3-beta-glucosidase precursor
Contig9086_at	9086	000+++	000+++	*O. sativa*	Os01g70380.1	0	Serine palmitoyltransferase 2

(*continued on the next page*)

[a] Affymetrix Barley1 GeneChip contig numbers.

[b] Consensus annotation based on six BLASTn or BLASTx methods: i) BLASTx UniProt: Uniref90 (July 09), ii) BLASTn The Institute for Genomic Research (TIGR) plant transcript assemblies: TIGR TA *Hordeum vulgare* release 2, iii) BLASTx Rice genome: TIGR release 6.1, iv) BLASTx National Center for Biotechnology Information (NCBI) reference sequence: NCBI RefSeq rel-35, v) BLASTn DFCI *Hordeum vulgare* Gene Index: HvGI (Dana Farber Cancer Institute release 10, and vi) BLASTx *Arabidopsis thaliana* genome: TAIR release 9.

[c] Annotation based on information from the BluGen website.

ciation and the functional annotations of these targets provide additional support for these genes as candidates for MLA-mediated transcriptional activation.

The signaling cascades at 16 HAI and later timepoints control both the transcriptional and phenotypic outcomes of the barley–*B. graminis* f. sp. *hordei* interaction. It follows that genes associated with resistance at later timepoints may identify downstream targets of this primary signaling cascade or transcriptome reprogramming related to post-resistance mechanisms. We found a similar effect at 32 HAI as compared with 16 HAI, where quantitative suppression of expression in mutant *mla* alleles as compared with wild-type *Mla* predominated all three paired compatible and incompatible interactions. In total, 66 genes were associated with resistance, with 45 genes induced

Table 2. (*continued from the previous page*)

		Consensus expression patterns		BLASTn or BLASTx reference information			
Probe Set	**Contig no.[a]**	**Wild type**	**Mutant**	**Organism**	**Accession/locus**	**E-score**	**Consensus Annotation[b]**
Contig12590_at	12590	000+++	000+++	*O. sativa*	Os01g72530.1	3.00E-49	Calmodulin-related calcium sensor protein
Contig13144_at	13144	000+++	000+++	*O. sativa*	Os11g05880.1	0	Exocyst complex subunit
HVSMEm0005J13r2_at	45474	000+++	000+++	*O. sativa*	Os06g07200.1	1.00E-48	Syntaxin
S0000700018E12F1_s_at	51485	000+++	000+++	*Triticum aestivum*	TC156523	2.00E-84	Alkaline invertase
Contig17964_at	17964	000+++	000+0+	*O. sativa*	Os03g18560.1	7.00E-79	DUF538 domain containing protein
Contig6380_at	6380	000+++	0000++	*O. sativa*	Os11g08100.1	1.00E-179	Eukaryotic aspartyl protease domain containing protein
Contig12360_at	12360	000+0+	000++0	*O. sativa*	Os02g46962.3	7.00E-88	VHS and GAT domain containing protein
Contig10151_s_at	10151	0000--	0000--	*H. vulgare*	TC169848	2.00E-99	Weakly similar to UniRef100_Q2IMJ3 cluster
Contig15701_at	15701	0000--	0000--	*O. sativa*	Os03g59070.1	3.00E-18	Phosphatase, putative, expressed
HY03H15u_s_at	48271	0000--	00000-	*H. vulgare*	TA36935_4513	0	Putative CENP-E like kinetochore protein
Contig20750_at	20750	0000++	000+0+	*O. sativa*	Os03g47034.1	8.00E-19	Unknown
Contig10205_at	10205	00000-	000--0	*O. sativa*	Os04g50710.1	3.00E-36	Pathogenesis-related Bet v I family protein
Contig14611_at	14611	00000-	0000--	*O. sativa*	Os12g37710.1	2.00E-92	PsbP
Contig10822_at	10822	00000-	0000-0	*O. sativa*	Os03g45400.1	8.00E-82	Antitermination NusB domain-containing protein
Contig11917_at	11917	00000-	00000-	*O. sativa*	Os01g10810.1	5.00E-38	Rho termination factor, N-terminal domain containing protein
HF01F13w_s_at	33960	00000-	00000-	*O. sativa*	Os01g65780.4	2.00E-13	Glycosyl transferase
Contig5988_at	5988	00000-	000000	*O. sativa*	Os03g25960.1	1.00E-80	RNA recognition motif containing protein
Contig7032_at	7032	00000-	000000	*O. sativa*	Os04g54810.1	0	Beta-D-xylosidase
Contig12100_at	12100	00000-	000000	*H. vulgare*	BF627497	0	
Contig16570_at	16570	00000-	000000	*O. sativa*	Os02g35230.1	1.00E-42	Unknown
Contig18909_at	18909	00000-	000000	*O. sativa*	Os03g07120.1	1.00E-101	Unknown
Contig25506_at	25506	00000-	000000	*H. vulgare*	BI778169	1.00E-180	Unknown
HVSMEb0011L02r2_s_at	41192	00000-	000000	*O. sativa*	TA44824_4513	1.00E-49	Hypothetical protein
Contig10709_at	10709	00000+	0000++	*O. sativa*	Os06g03580.2	8.00E-55	Zinc RING finger protein
Contig15880_at	15880	00000+	00000+	*O. sativa*	Os12g09000.2	6.00E-76	Phosphomethylpyrimidine kinase/thiamin-phosphate pyrophosphorylase
Contig21643_at	21643	00000+	00000+	*O. sativa*	Os03g04570.1	3.00E-73	Peptide transporter PTR3-A
Contig12469_at	12469	00000+	000000	*O. sativa*	Os03g0188500	1.00E-160	Expressed protein
Contig12629_s_at	12629	00000+	000000	*O. sativa*	Os01g49529.3	1.00E-147	Receptor-like cytoplasmic kinase OsWAK-RLCK
Contig14625_at	14625	00000+	000000	*H. vulgare*	TC174003	0	Unknown
HF06A04r_at	34067	00000+	000000	*H. vulgare*	BU985056	0	Unknown
Contig16710_at	16710	000000	0+0000	*O. sativa*	Os07g18230.1	1.00E-77	Lectin-like receptor kinase
EBro02_SQ004_C14_at	30414	000000	000+00	*H. vulgare*	TA53062_4513	1.00E-142	
Contig12640_at	12640	000000	0000-0	*O. sativa*	Os01g69120.1	1.00E-43	Uncharacterized RNA methyltransferase pc1998
Susceptible Association							
HVSMEm0013N06r2_at	45705	0+00+0	0+000+	*Blumeria graminis*[c]	BI953678	1.00E-88	Hypothetical protein
HO09D16S_at	36134	00+0+0	00++++	*B. graminis*	TC175543	0	Unknown
HO10J02S_at	36228	00+000	000000	*B. graminis*	TC172857	0	Unknown
HVSMEl0002L06r2_at	44740	000--0	0000--	*O. sativa*	Os03g03670.1	3.00E-35	Hypothetical protein
Contig5378_at	5378	0000+0	0000++	*O. sativa*	Os09g12600.1	1.00E-163	Phosphate/phosphate translocator
HVSMEl0014B21r2_at	45091	000000	000+0+	*H. vulgare*	BI949476	1.00E-100	Expressed protein
Contig4174_at	4174	000000	00000+	*O. sativa*	Os05g33130.1	1.00E-109	Chitinase family protein precursor
Contig25983_at	25983	000000	00000+	*B. graminis*	BE214522	0	Hypothetical protein

and 21 genes suppressed in response to *B. graminis* f. sp. *hordei* challenge (Fig. 4B and C). As summarized in Table 2, an even greater diversity of functional roles was identified, revealing the complexity associated with downstream responses to *B. graminis* f. sp. *hordei* challenge.

So far, our analyses have focused on gene expression associated with resistance, i.e., genes with lower expression in *mla* mutants as compared with wild type. We also identified genes that had greater expression in mutant *mla* versus wild-type *Mla*. The expression of these eight genes was associated with susceptibility at 32 HAI (Table 2). Functional annotation of these genes established that four were fungal in origin (Spanu et al. 2010), arising from several expressed sequence tag libraries derived from barley tissue inoculated with powdery mildew during the design phase of the Barley1 GeneChip (Close et al. 2004). One of the four fungal genes (represented by HVSMEm0013N06r2_at) was the only gene associated with susceptibility at 16 HAI. It is difficult to ascertain the significance of this association, as these few are a subset of those *B. graminis* f. sp. *hordei* probe sets that remain on the Barley1 GeneChip due to stringent filtering of fungal sequences (Close et al. 2004). However, their identification does demonstrate that our approach for detecting quantitative expression differences associated with susceptibility reflects the potential for colonization of *B. graminis* f. sp. *hordei* on the surface of barley leaves.

DISCUSSION

Our molecular understanding of plant-pathogen interactions is most complete at the level of perception. A primary component of this perception is the recognition of pathogen-associated molecular patterns (PAMPs) (Jones and Dangl 2006). PAMP recognition receptors, located at the cell periphery or the plasma membrane, initiate a signal transduction cascade in the host that produces the innate immune response (Jones and Dangl 2006). To counter this, pathogens have within their repertoire a diverse set of effectors that act as inhibitors of PAMP-mediated resistance. Plants have evolved effective countermeasures by elevating the mechanism of perception from nonspecific recognition of PAMPs to specific recognition of AVR effectors via resistance (R) proteins (Jones and Dangl 2006).

In vitro and in vivo interaction assays have pointed to an intermolecular complex composed of MLA, RAR1, SGT1, and HSP90 (Azevedo et al. 2002, 2006; Bieri et al. 2004; Zhang et al. 2008), demonstrating that these components are involved in R protein stability and signal transduction after recognition but not necessarily the initiation of the HR. Yet, the mechanistic link between MLA and WRKY1/2 implicates a role for transcriptional regulation in mediating the HR (Shen et al. 2007). Concordantly, a small fraction of MLA protein in the nucleus of noninoculated plants overexpressing a MLA-YFP fusion was detected (Dangl 2007; Shen et al. 2007). Hence, the quantitative kinetics of MLA localization may determine the qualitative phenotype through a threshold mechanism in which the presence of the appropriate AVR_a effector significantly perturbs the balance of cytoplasmic- and membrane-localized and nuclear-localized MLA. It follows that the fluctuating rates of nuclear import or export may influence the transcript levels of MLA targets. This balance could be altered by the residual presence of unrecognized AVR protein in compatible interactions of the type MLA_x-AVR_y, in which the mispairing of MLA_x with AVR_y affects the localization of MLA, resulting in a compatible interaction but still regulates the same set of genes (Caldo et al. 2004, 2006). The balance could also be perturbed by a null (*mla1-m508* and *mla6-m9472*) or loss-of-function (*mla12-m66*) mutation in the MLA protein, as would be the case in the current experiment involving *Mla* mutants. In this case, the AVR effector does not bind with its intended MLA target, resulting in altered programming of the expected defense cascade.

We identified 28 candidates of the MLA transcriptional regulon by characterizing genes at 16 HAI that were quantitatively upregulated in plants containing three diverse wild-type *Mla* alleles as compared with their respective loss-of-function mutants. Since it is known WRKY1/2 are repressors of basal defense, we hypothesized that MLA acts primarily as an activator of gene expression. It is expected, then, that the candidates of the MLA transcriptional regulon would overlap with basal defense processes, regulated in part by pattern recognition receptor–mediated signaling, functionally associated with defense, or both (Jones and Dangl 2006). Several of the gene-expression patterns and annotations matched our previous expectations. Of the genes involved in redox homeostasis, Contig10860_at is predicted to encode a germin-like protein that has the majority of its sequence information contributed by cDNA libraries of powdery mildew–inoculated tissue. Though it has yet to be characterized in the barley–*B. graminis* f. sp. *hordei* interaction, several other members of this protein family are both positively and negatively associated with resistance (Himmelbach et al. 2010). Their direct involvement in defense has been attributed to their production of hydrogen peroxide from superoxide dismutase or oxalate-oxidase activity, or both (Himmelbach et al. 2010). Genes involved in signal transduction have been analyzed using the same family-wise approach used with germin-like proteins, with the initial characterization of the calcium-dependent protein kinases (CDPK) family (Freymark et al. 2007). The CDPK of barley also modulate resistance to *B. graminis* f. sp. *hordei* in a variety of contexts, such as *mlo* and basal penetration resistance. Identification of *HvCDPK5* (Contig11154_at) in our analysis at 16 HAI implicates its potential role in defense, although this gene was excluded in those tested by Freymark and colleagues (2007), as it did not have a full-length coding sequence.

The quantitative differences observed among wild-type *Mla* and loss-of-function mutants are reminiscent of differences observed between susceptible plants carrying wild-type *Mlo* and resistant *mlo* mutants (Zierold et al. 2005). Moreover, there was an overlap in four (HO06K23, HO12F09, HO03A06, HO15N08) of the 39 genes reported by Zierold and colleagues (2005) as being associated with resistance. In particular, this includes Contig9422_at (HO03A06), a predicted multiple resistance–associated protein-like ABC transporter in which greater expression is associated with resistance at 16, 24, and 32 HAI (Tables 1 and 2). Of the remaining genes, three have been identified previously by their transcriptional profile during the barley-*B. graminis* f. sp. *hordei* interaction. HV_CEb0004O15r2_s_at and Contig12286_s_at are predicted to encode a glutathione *S*-transferase (GST) and a reticuline oxidase precursor, respectively, as correlated kinetically with resistance by Caldo and colleagues (2004, 2006). Contig4942_at (Early Responsive to Dehydration 1 [ERD1] homolog) was identified by Jansen and associates (2005), using a differential screening approach between paired near-isogenic *Mlg* (CI 16139) and *mlg* (CI 16140) plants in cv. Manchuria background. ERD1 is known to be induced in *Arabidopsis thaliana* by water stress and during senescence (Nakashima et al. 1997) and is induced in barley by both *B. graminis* f. sp. *hordei* challenge and treatment with the resistance-inducing chemical benzothiadiazole (Jansen et al. 2005).

The diverse biological roles and early induction of these candidates substantiate several hypotheses associated with downstream transcriptional targets of MLA R proteins (Bent and Mackey 2007; Mur et al. 2008; Shen et al. 2007). One hy-

pothesis is based on the interaction of MLA with repressors of basal defense, suggesting that candidate gene targets overlap with genes involved in PAMP-triggered immunity (Jones and Dangl 2006; Shen et al. 2007). Three lines of evidence support an association with PAMP-triggered immunity. First, 17 of the 28 were induced by 8 HAI, implicating their early role in the defense response. Second, functional annotation reflects a direct overlap with known roles in plant-pathogen interactions (e.g., CDPK, GST, germin-like proteins, ATPase). Interestingly, the detection of GST as a significant candidate by Caldo and colleagues (2004) and here suggests that it may be an essential target of MLA regulation or that its expression is hypersensitive to MLA relocalization, or both. Third, activation of gene expression was only observed after MLA nuclear localization. Credibility for the association of these genes with MLA regulation was reinforced by the interrogation of both the 16 and 32 HAI timepoints, at which transcripts of several *B. graminis* f. sp. *hordei* genes present on the Barley1 GeneChip were significantly upregulated on susceptible *mla* mutants.

The generation of a comprehensive index of differentially expressed genes and incorporation of fold change provided a global overview of the transcriptome reprogramming in the context of incompatible and compatible interactions. A significant differential response was observed in plants harboring *mla* loss-of-function mutations, correlating with the establishment of *B. graminis* f. sp. *hordei* haustoria and resulting in a compatible interaction. This response was not sustained, as the number of genes with expression in the upper fold-change categories (5 to 10 and >10) was either unchanged in later timepoints or significantly reduced. This result is somewhat similar to an observation in the grape–powdery mildew interaction, in which resistant varieties had very few differentially expressed genes as compared with susceptible varieties (Fung et al. 2008). In contrast to the grape–powdery mildew system, barley–*B. graminis* f. sp. *hordei* incompatible interactions displayed an ever-increasing expression response. One biological model fitting these observations would implicate MLA as a steady-state suppressor of defense gene activation at 20 HAI, although it is unclear whether this proposed inhibitory role would be in parallel to or distinct from known gene activation (Shen et al. 2007). That said, this model is restricted to transcriptional cascades associated with MLA signaling. An alternative model involves effector-mediated transcriptome reprogramming in the host at 20 HAI and would explain the apparent transcriptional response in plants carrying a loss-of-function *mla* allele.

In considering further work to distinguish between these possibilities, we are aware that a potentially limiting factor has been the use of seedling first leaves (PO:0007094) compared with analyzing mRNA amplified from single cells (Chandran et al. 2010; Gjetting et al. 2004, 2007), distinct epidermal or mesophyll tissues (Zierold et al. 2005), or even nuclear versus mitochondrial or chloroplast subcellular fractions. Even in the absence of such cell sampling approaches, the stronger induction at 20 HAI in loss-of-function *mla* genotypes and identification of candidate genes for the MLA transcriptional regulon provides another regulatory link between innate immunity and the early role of MLA in reprogramming the transcriptional response to powdery mildew.

MATERIALS AND METHODS

Fungal material.

B. graminis f. sp. *hordei* isolate 5874 (Torp et al. 1978; Wei et al. 1999) (AVR_{a1}, AVR_{a6}, AVR_{a12}) was propagated on barley (*Hordeum vulgare*) cv. Manchuria (CI 2330) in a controlled-growth chamber with 16 h (4:00 to 11:59 PM U.S. Central standard time) of light and 8 h (12:00 AM to 3:59 PM) of darkness at 18°C.

Plant material.

CI 16137 (*Mla1*) and CI 16151 (*Mla6*) are near-isogenic lines in the six-row barley cv. Manchuria (Moseman 1972). *Mla* deletion mutants, *mla1-m508* and *mla6-m9472*, are derived from γ-irradiation and fast neutron mutagenesis of CI 16137 and CI 16151, respectively (Meng et al. 2009; Zhou et al. 2001). The two-row barley cv. Sultan-5 harbors the *Mla12* resistance allele and the derived *Mla12* point mutant, *mla12-m66*, was generated by ethylmethane sulfonate mutagenesis (Shen et al. 2003; Torp and Jørgensen 1986). *mla1-m508* was a gift from S. Somerville (University of California, Berkeley, CA, U.S.A.), *mla6-9472* was generated by the Wise lab (United States Department of Agriculture–Agricultural Research Service [USDA-ARS], Iowa State University, Ames, IA, U.S.A.) (Meng et al. 2009), and Sultan-5 and *mla12-m66* were provided by J. Helms Jørgensen (Risø National Laboratory, Roskilde, Denmark). Wild-type *Mla* lines and respective mutants have been maintained by at least 10 generations of selfing, essentially fixing any second-site mutations; however, the lack of *Mla* specificity in the loss-of-function mutants is the primary determinant segregating for susceptibility.

Experimental design.

Planting, stage of seedlings, inoculation, and leaf tissue sampling were followed as described by Caldo and associates (2006). Two 20 × 30–cm trays per genotype of CI 16137, CI 16151, Sultan-5, *mla1-m508*, *mla6-m9472*, and *mla12-m66* were planted in sterilized potting soil. One tray of seedlings was used for noninoculation control and the other was used for inoculation treatment for each genotype. Each experimental tray consisted of six rows of 15 seedling first leaves (PO:0007094), with rows randomly assigned to one of the six harvest times (0, 8, 16, 20, 24, and 32 HAI). The entire experiment was repeated three times in a split-split-plot design with genotype, inoculation type, and harvest time as whole-plot, split-plot, and split-split-plot factors, respectively.

Target synthesis and GeneChip hybridization.

Total RNA was isolated using a hot (60°C) phenol/guanidine thiocyanate method (Caldo et al. 2004, 2006). Probe synthesis and labeling were performed at the Iowa State University GeneChip Core facility (Ames, IA, U.S.A.), using the One Cycle and GeneChip IVT labeling kits. The cRNA was fragmented and was used to make each hybridization cocktail containing 10% dimethyl sulfoxide, and an equivalent of 5 μg was hybridized to Barley1 GeneChip probe arrays (Affymetrix 900515) (Close et al. 2004).

Normalization and mixed linear model analysis.

Normalization, data transformation, and mixed linear model analysis (Wolfinger et al. 2001) were patterned after the methods used by Caldo and colleagues (2004, 2006). The data were split into two datasets based on genetic background, cv. Sultan-5 or Manchuria, for the mixed model analysis. An estimate statement in SAS v9.1 was used to compare transcript levels between inoculated and noninoculated plants of a specific genotype per timepoint (SAS Institute Inc., Cary, NC, U.S.A.). FDR were determined for each estimate using a Python-implementation of the histogram-based algorithm described by Nettleton and associates (2006).

Derivation of consensus patterns.

By insisting on significance in at least four of the nine (three genotypes × three FDR levels) comparisons, we are guaranteed at a minimum that one of the following is true:

i) Significance was obtained at the 0.0001% FDR level for at least one genotype with significance at the 1% level for at least one other genotype,

ii) Significance was obtained at the 0.01% level for at least two of the three genotypes, or

iii) Significance was obtained at the 0.01% level for at least one genotype with significance at the 1% level for the other two genotypes.

Note that if fewer than four significant results were required, it would be possible for a single genotype to determine the "consensus" pattern, which would obviously be undesirable.

Data access.

All original protocols and microarray data are available at the PLEXdb gene expression resource for plants and plant pathogens. Although the biological sampling, RNA extraction and GeneChip hybridizations were performed at the same time, data were uploaded as two experiments based on genetic background. Accession BB2 corresponds to the 180 GeneChips involving Sultan-5 and its derived mutants and BB10 designates the 144 GeneChips coming from Manchuria near-isogenic lines and loss-of-function mutants. GeneChip data files have also been deposited in ArrayExpress with accessions E-TABM-82 (BB2 Sultan-5 experiment) and E-TABM-142 (BB10 Manchuria experiment).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

ArrayExpress database: www.ebi.ac.uk/arrayexpress
BluGen, the *Blumeria* Sequencing Project website: www.blugen.org
Iowa State University GeneChip Core database: www.biotech.iastate.edu/facilities/genechip/Genechip.htm
PLEXdb database: plexdb.org
United States Department of Agriculture National Plant Germplasm System website: www.ars-grin.gov/npgs