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Stage-Specific Suppression of Basal Defense Discriminates Barley Plants Containing Fast- and Delayed-Acting *Mla* Powdery Mildew Resistance Alleles

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Nonspecific recognition of pathogen-derived general elicitors triggers the first line of plant basal defense, which in turn, preconditions the host towards resistance or susceptibility. To elucidate how basal defense responses influence the onset of *Mla* (mildew resistance locus *a*)–specified resistance, we performed a meta-analysis of GeneChip mRNA expression for 155 basal defense–related genes of barley (*Hordeum vulgare*) challenged with *Blumeria graminis* f. sp. *hordei*, the causal agent of powdery mildew disease. In plants containing the fast-acting *Mla1*, *Mla6*, or *Mla13* alleles, transcripts hyper-accumulated from 0 to 16 h after inoculation (hai) in both compatible and incompatible interactions. Suppression of basal defense–related transcripts was observed after 16 hai only in compatible interactions, whereas these transcripts were sustained or increased in incompatible interactions. By contrast, in plants containing wild-type and mutants of the delayed-acting *Mla12* allele, an early hyper-induction of transcripts from 0 to 8 hai was observed, but the expression of many of these genes is markedly suppressed from 8 to 16 hai. These results suggest that the inhibition of basal defense facilitates the development of haustoria by the pathogen, consequently delaying the onset of host resistance responses. Thus, we hypothesize that the regulation of basal defense influences host-cell accessibility to the fungal pathogen and drives allelic diversification of gene-specific resistance phenotypes.

Additional keywords: innate immunity, pathogen-associated molecular patterns, timing of resistance response.

Nonself recognition is an indispensable system for eukaryotes in preventing microbial attack. In plants, perception of pathogen-derived general elicitors, which are similar to pathogen-associated molecular patterns (PAMP), triggers nonspecific basal defense (Kim et al. 2005; Navarro et al. 2004; Nürnberger et al. 2004; Zeidler et al. 2004; Zipfel et al. 2004), while recognition of pathogen avirulence effectors initiates gene-specific resistance (Dangl and Jones 2001; Martin et al. 2003). Both pathogen-derived general elicitors and avirulence gene effectors are released during pathogen infection, and recent experimental evidence indicates that basal and host-specific mechanisms of innate immunity are linked (Caldo et al. 2004; Kim et al. 2005; Navarro et al. 2004).

General elicitor or PAMP-induced defense is an important component of the total immune response. Hyperactivation of basal defense, in most cases, is one of the consequences of an *R*-mediated pathogen recognition leading to resistance (Caldo et al. 2004; Nimchuk et al. 2003). By contrast, suppression of basal defense leads to pathogen establishment (Abramovitch and Martin 2004; Caldo et al. 2004; Hückelhoven 2005; Panstruga 2003). In addition, it has been suggested that suppressor molecules of general defense influence specificity in many plant-pathogen interactions (Bushnell and Rowell 1981; Heath 1981; Shiraishi et al. 1994). Taken together, these support the hypotheses that basal defense is one of the initial targets of suppression by the pathogen and that the host-specific resistance conceivably evolved from R proteins guarding the plant against effector molecules that trigger basal defense suppression (Alfano and Collmer 2004; Caldo et al. 2004; Kim et al. 2005).

Powdery mildew of barley, caused by *Blumeria graminis* f. sp. *hordei*, is an ideal system to explore the interactions of obligate fungal biotrophs with their cereal hosts. Specific recognition in barley–*B. graminis* f. sp. *hordei* interactions is triggered in a gene-for-gene manner by genes designated *Ml* (mildew-resistance loci) (Jørgensen 1994; Schulze-Lefert and Vogel 2000; Wise 2000). Approximately 30 distinct resistance specificities have been identified at the *Mla* locus on chromosome 5 (1H) (Halterman and Wise 2004; Halterman et al. 2001, 2003; Jørgensen 1994; Shen et al. 2003; Zhou et al. 2001), and well-defined stages of powdery mildew disease development provide multiple possibilities to interrogate the regulation of host genes in response to *Mla*-specified incompatible and compatible barley–*B. graminis* f. sp. *hordei* interactions (Clark et al. 1993; Ellingboe 1972; Jørgensen 1988; Kunoh 1982). The *Mla1*, *Mla6* and *Mla13* encoding coiled-coil-nucleotide binding-leucine-rich repeat alleles trigger a rapid and absolute resistance, while *Mla7* and *Mla12* trigger a delayed and intermediate response. In rapid resistance, fungal growth is terminated on or before haustorium formation, leading to a single cell death (Boyd et al. 1995; Kruger et al. 2003; Wise and Ellingboe

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*The *e*-Xtra logo stands for "electronic extra" and indicates the HTML abstract available on-line contains supplemental material describing basal defense-related genes and showing timecourse expression patterns of anthranilate *N*-benzoyltransferase in *Mla1* and *Mla6* alleles that is not included in the print edition.

1983). By contrast, in delayed resistance, termination of fungal growth occurs after the formation of haustorium and secondary hypha, leading to death of the infected as well as surrounding cells (Boyd et al. 1995; Freialdenhoven et al. 1994; Kruger et al. 2003). Although the physiology of the infection process is well established (Green et al. 2002), the molecular mechanisms that differentiate the onset of *Mla*-specified rapid and delayed resistance remain unclear.

Parallel gene-expression profiling has become an integral tool to interrogate the molecular mechanisms underlying biological phenomena. With the increasing availability of a large number of microarray data sets in public repositories, it is beneficial to use comparative meta-profiling strategies to draw conclusions that span multiple experiments (Ghosh et al. 2003; Moreau et al. 2003; Pellegrino et al. 2004; Rhodes et al. 2002, 2004; Stevens and Doerge 2005; Wang et al. 2004). In addition, data integration from related expression profiling experiments is critical to the success of the large investment made on genomics studies (Cope et al. 2004). Here, we perform cross-experiment analysis of transcript accumulation of genes involved in basal defense and determine how the accumulation of this early-induced defense differentiates responses in barley plants infected with *B. graminis* f. sp. *hordei*. In genotypes containing the fast-acting *Mla1*, *Mla6*, and *Mla13* alleles, highly parallel and significant hyper-accumulation of 207 basal defense-related transcripts was observed in both incompatible and compatible interactions up to 16 h after inoculation (hai), coinciding with germination of *B. graminis* f. sp. *hordei* conidiospores and formation of appressoria. After 16 hai, these transcripts are sustained or increased in incompatible interactions but are significantly down-regulated in compatible interactions (Caldo et al. 2004). In plants containing wild-type and mutant forms of the delayed-acting *Mla12* allele, a similar hyper-induction of transcripts is observed from 0 to 8 hai; however, mRNA expression is markedly suppressed from 8 to 16 hai, before re-induction from 16 to 32 hai. We hypothesize that suppression of basal defense may precondition epidermal cells to become accessible to the pathogen and, thus, may influence the mechanisms of *Mla*-specified rapid and delayed resistance, analogous to mammalian innate immunity, which influences the expression of the acquired immune response (Fearon and Locksley 1996).

RESULTS

Experimental concept and design.

Basal defense is part of all plant-pathogen interactions; yet, little is known about its influence on the onset of gene-specific resistance. A previous experiment conducted on near-isogenic lines containing fast-acting *Mla1*, *Mla6* and *Mla13* alleles (Caldo et al. 2004) demonstrated that basal defense-associated mRNA accumulation is dependent on the kinetics of powdery mildew infection and that its modulation influences the outcome of particular gene-for-gene interactions towards incompatibility or compatibility. To further elucidate how basal defense responses modify the onset of *Mla*-specified resistance, we extended the analysis of general elicitor perception from the Caldo et al. (2004) investigation to another large expression-profiling experiment involving variants of cultivar Sultan 5 harboring the delayed-acting allele, *Mla12*. In addition to Sultan 5, we examined its EMS (ethyl methane sulfonate)–derived *mla12*-M66 loss-of-function *Mla12* allele (Shen et al. 2003; Torp and Jørgensen 1986), the NaN_3-derived *rar1-1* (M82) and *rar1-2* (M100) mutants (Freialdenhoven et al. 1994; Jørgensen 1996; Shirasu et al. 1999; Torp and Jørgensen 1986), and *rom1*, a restorer of *Rar1*-independent, *Mla12*-specified resistance (Freialdenhoven et al. 2005). The experiment conducted on Sultan 5 and its derived mutants was based on a split-split plot design described by Caldo et al. (2004), except for the parallel inclusion of control noninoculated plants, with barley first leaves harvested at 0, 8, 16, 20, 24, and 32 h after inoculation with *B. graminis* f. sp. *hordei* isolate 5874 (*AvrMla1*, *AvrMla6*, *AvrMla12*) (discussed below). The Sultan 5 experiment consisted of 180 Barley1 GeneChip hybridizations (5 genotypes × 6 timepoints × 2 inoculation treatments × 3 biological replications), and the Caldo et al. (2004) study consisted of 108 hybridizations (3 genotypes × 6 timepoints × 2 isolates × 3 biological replications) resulting in a total of 96 treatment combinations for the two experiments. To alleviate cross-experiment variability, both studies were conducted under identical conditions. Because of the differences in genetic background of Manchuria-type isolines containing *Mla1*, *Mla6*, and *Mla13* and variants of Sultan 5 harboring *Mla12*, each experiment was treated independently, although analyzed side-by-side, and interpretation of results was based on gene-expression data within each experiment (Stevens and Doerge 2005).

Initial analysis strategy.

In our previous analysis (Caldo et al. 2004), we focused on 22 highly significant genes identified from contrasting incompatible (*Mla*/*AvrMla*) and compatible (*Mla*/*avrMla*) interactions. These selected genes had a cutoff P value that was <0.0001, a false discovery rate (FDR) that was <7%, and displayed highly synchronized patterns of transcript upregulation among all incompatible and compatible interactions up to approximately 16 hai, coinciding with germination of *B. graminis* f. sp. *hordei* conidiospores and formation of appressoria. By contrast, significant divergent expression was observed from 16 to 32 hai, during membrane-to-membrane contact between fungal haustoria and host epidermal cells, with notable suppression of most transcripts identified as differentially expressed in compatible interactions. These observations were consistent with the hypothesis that these 22 genes encode proteins that are involved in nonspecific basal defense pathways.

The multiple-test situation described in the split-split plot design of the Caldo et al. (2004) study also allows the total number of differentially expressed genes to be estimated by analyzing the distribution of P values. To begin our cross-experiment, we used the histogram-based method described by Mosig et al. (2001) and identified 1,432 differentially expressed genes among incompatible vs. compatible interactions over the period of 0 to 32 hai (Fig. 1). Then, to ultimately extract the most biologically meaningful results, we used timecourse expression patterns that correlated to the kinetics of fungal infection as our criteria for gene selection.

Keeping in mind our model described above, we first considered the threshold P value of <0.001 and identified 81 genes in the comparison of incompatible and compatible interactions. Although the FDR was higher (20%), by evaluating the individual timecourse expression graphs, we found that 28 of these 81 genes also showed the same pattern of expression as the first 22 highly significant genes identified by Caldo et al. (2004). To then extract the majority of coexpressed genes, we collected the most significant 500 with P values that were <0.01 and performed cluster analysis of mean signal intensities (Fig. 2). As shown in Figure 2A, this analysis grouped the genes into three major clusters based on their expression profiles. Of the three main clusters, cluster 3 contained 207 genes, including 21 out of the original 22 identified by threshold P values of <0.0001 (Caldo et al. 2004) and the subsequent 28 genes identified above ($P < 0.001$), which had identical timecourse patterns of expression. Notably, 20 predicted genes from cluster 3 had annotations associated to the shikimate pathway leading to the biosynthesis of secondary metabolites (Fig.

2B, right side). Because of the observed coexpression of these 207 genes in regards to the kinetics of fungal infection, they were selected as a point of reference to analyze basal defense-related expression in plants with fast-acting (*Mla1*, *Mla6*, and *Mla13*; Caldo et al. 2004) and delayed-acting (Sultan 5 loss-of-function mutants and *Mla12*) resistance alleles.

Transcript accumulation shows evidence of nonspecific pathogen recognition by the plant.

To determine if the 207 coexpressed transcripts identified above accumulated in response to pathogen inoculation in the Sultan 5 experiment, we performed a test for differential expression patterns over time between noninoculated and inoculated plants, using a contrast statement in SAS (Statistical Analysis Software). A total of 155 of the 207 genes in Sultan 5 were identified as differentially expressed with *P* values below 0.05 and an estimated FDR of 1.5% (Storey and Tibshirani 2003). Of these 155 differentially expressed genes, 46 (29.7%) had a predicted function in cellular metabolism, 22 (14.2%) had a predicted function in the shikimate pathway or secondary metabolism, and 37 (24%) are of unknown function (Table 1). Transcript accumulation of these genes was induced at the very early stages of infection, specifically at 8 hai. A test of fixed effects showed nonsignificant genotype, genotype × treatment, genotype × time, and genotype × treatment × time interactions for most of the genes (data not shown), indicating a high degree of similarity in the mRNA expression among wild type and mutants derived from Sultan 5. These results indicate that the expression profiles of these selected genes are similar among all genotypes and most likely are due to the perception of general elicitors.

Basal defense expression in delayed-acting *Mla12* plants and derived mutants is similar at 0 and 16 hai.

To analyze time-specific responses to pathogen inoculation, we performed condition clustering of the different treatment-factor combinations in the Sultan 5 and Caldo et al. (2004) experiments, using the Spearman correlation based on the 155 differentially expressed genes identified in the analysis above. The Spearman correlation was used to measure the correlation of ranks of data values rather than correlation between the actual data values themselves, which is required in this clustering, as the conditions being compared were derived from two separate experiments. Since our objective here was to compare responses to pathogen inoculation, conditions involving noninoculated plants (Sultan 5 experiment) were excluded.

As shown in Figure 3, three major clusters resulted from this analysis, one cluster consisting of 0-h timepoints and the other two clusters distinguishing between delayed and rapid responses after pathogen inoculation. In cluster 1, all responses at the 0-h timepoint immediately after inoculation clustered together regardless of genotype × isolate combination, indicating similarity in transcription profiles for all genotypes prior to infection. Also, in cluster 1, expression profiles at 16 hai in the delayed-acting *Mla12* and Sultan 5 loss-of-function mutants were equivalent to the responses at 0 hai for all genotypes used in the two experiments. This is worth noting, because 16 hai is the timeframe when membrane-to-membrane contact is made between fungal haustorium and the host cell. In cluster 2, expression profiles at each timepoint for delayed-acting *Mla12* and Sultan 5 loss-of-function mutants clustered together regardless of the interaction types. This result is also consistent with the above statistical analysis that shows the fixed effects involving genotypes are nonsignificantly different for most genes. In cluster 3, plant responses in incompatible and compatible interactions at the early stages clustered together, while grouping of responses at the later stages is influenced by genotypes and isolates, consistent with the findings of Caldo et al. (2004). Taken together, these results show that important differences in the expression patterns of the basal defense-related genes occurred at the early stages of infection in barley plants containing fast- and delayed-acting *Mla* alleles.

Suppression of basal defense coincides with haustorial formation.

Following the cluster analysis above, we examined the expression profiles for the first 32 h after pathogen inoculation for each of the 155 selected genes. Figure 4 illustrates the mRNA expression of a gene encoding a putative B12D protein, one of the 155 basal defense-related genes that differentiate responses of plants containing rapid- (*Mla1*, *Mla6*, and *Mla13*; Caldo et al. 2004) and delayed-acting (*Mla12* and Sultan 5 loss-of-function mutant) alleles. In barley lines containing *Mla1*, *Mla6*, and *Mla13*, mRNA expression of most selected genes displayed significant upregulation from 0 to 16 hai, coincident with termination of fungal growth. These genes also displayed steady upregulation at the later stages of infection in incompatible interactions, whereas suppression of transcript levels occurs after 16 hai in compatible interactions (Caldo et al. 2004). By contrast, in plants containing wild-type and mutant alleles of *Mla12*, an early upregulation was observed but with significant suppression of transcript levels from 8 to 16 hai, coincident with attempted fungal penetration. Using a threshold *P* value of <0.05 for upregulation from 0 to 16 hai in the Caldo et al. (2004) experiment and downregulation from 8 to 16 hai in the Sultan 5 experiment, 27 genes were identified in the analysis of C.I. 16151 (*Mla6*) and Sultan 5 (*Mla12*), 26 genes in the analysis of C.I. 16137 (*Mla1*) and Sultan 5, and 21 genes in the analysis of C.I. 16155

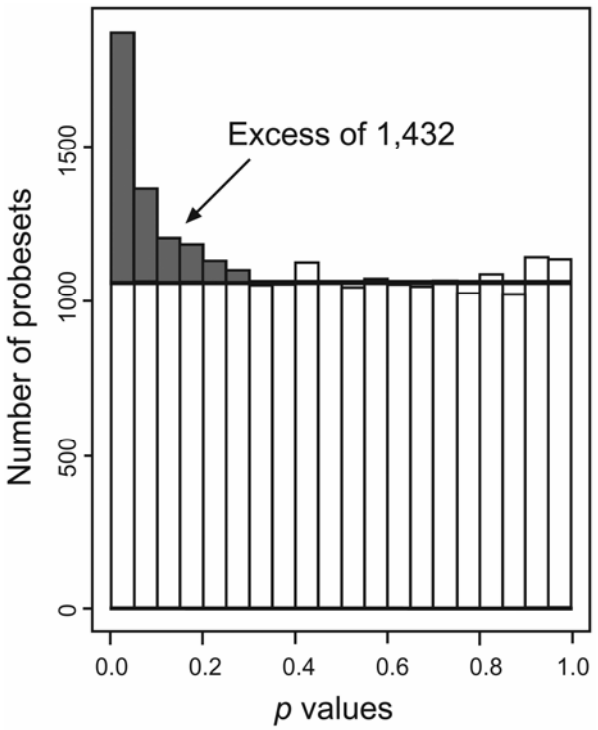
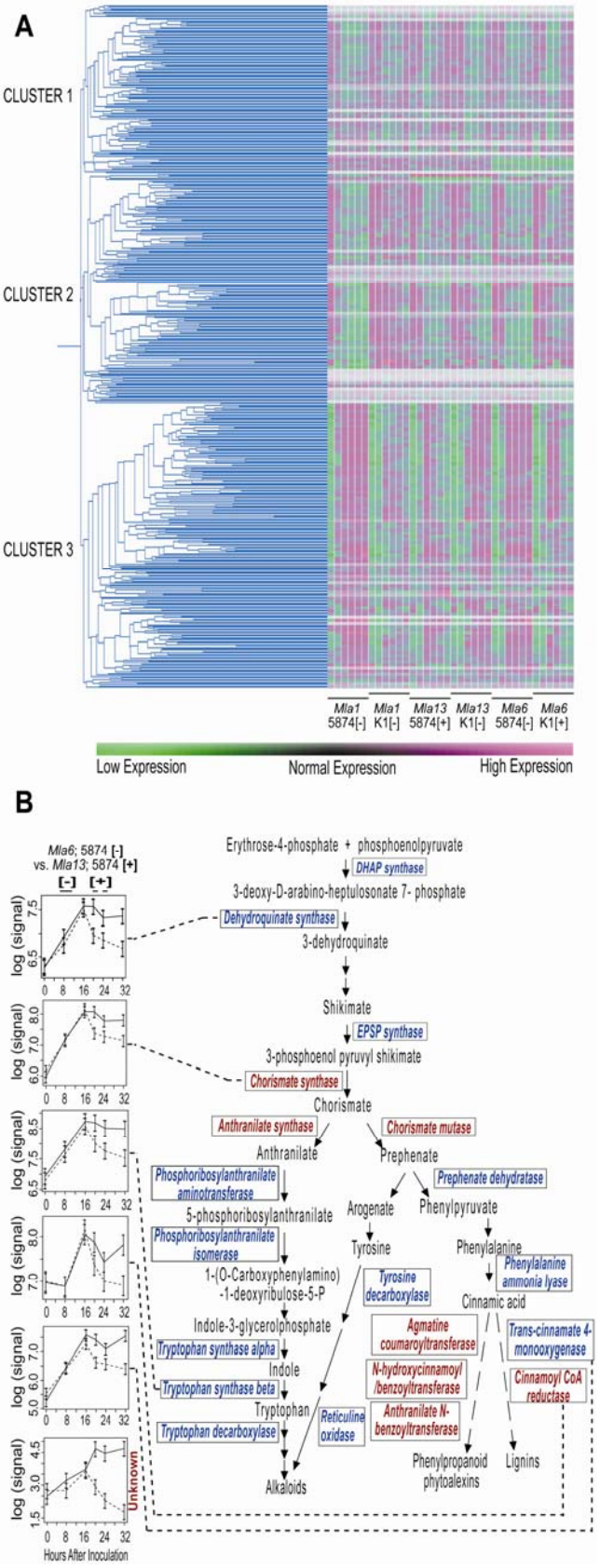


Fig. 1. Estimate of the number of differentially expressed genes based on histogram of *P* values as described by Mosig and associates (2001). Histogram of 22,840 *P* values for the comparison of incompatible and compatible interactions in the Caldo et al. (2004) study were distributed into 20 bins. The nonshaded area below the horizontal line designates the uniform distribution associated to non–differentially expressed genes. The shaded region of the histogram corresponds to an excess of 1,432 genes that had time-specific differences in the expression between compatible and incompatible interactions.

(*Mla13*) and Sultan 5. Of these, 21 genes were consistently up-regulated from 0 to 16 hai in all plants containing fast-acting *Mla* alleles and also were significantly down-regulated from 8 to 16 hai in plants containing the slow-acting *Mla12* allele and mutant derivatives (Table 2; Fig. 4).



Sequence identities revealed that seven of the 21 differentially expressed genes at 16 hai are putatively involved in the shikimate pathway. Notably, three of these seven genes, cinnamoyl-CoA reductase, anthranilate *N*-benzoyl transferase, and agmatine coumaroyltransferase, are implicated in phenylalanine metabolism leading to the synthesis of lignins and phenylpropanoid phytoalexins. The other four genes (anthranilate synthase alpha 2 subunit, phosphoribosylanthranilate isomerase, and two corresponding to tryptophan synthase beta-subunit) are involved in tryptophan biosynthesis in the anthranilate synthase branch of the shikimate pathway (Table 2). Only four of the total 37 basal defense-related genes that were annotated as "unknown" had significant differential expression at 16 hai in plants undergoing rapid and delayed resistance.

DISCUSSION

Biological questions addressed via meta-analytic approaches.

Expression profiling data sets deposited in public repositories are good substrates for continued analyses, as they contain thousands of datapoints that can and should be re-interrogated (Pellegrino et al. 2004). In this regard, gene-expression patterns from published results can be the focus of more detailed analysis as well as being utilized as a point of reference for meta-analysis involving several experiments. Experimental differences should be acknowledged up front to avoid misleading conclusions (Grütsmann et al. 2005); however, careful selection of studies driven by a particular biological question makes possible access to previously undescribed phenomena.

In this study, we focused on the analysis of basal defense responses induced by nonspecific pathogen recognition in two independent but related parallel expression experiments. Although the results of the two experiments were analyzed side-by-side (Stevens and Doerge 2005), they were treated independently, because of the differences in genotypic background and other possible variations in experimental conditions. The focal point of this analysis was based on data that indicated that basal defense expression in the host was dependent on the kinetics of pathogen infection. Due to its nonspecific nature, the perception of general elicitors from the same pathogen isolate regardless of plant genotypic background leads to the induction of host general defense. These basal defense responses are, in turn, potential targets for suppression by the pathogen effector molecules to establish successful infection (Alfano and Collmer 2004; Caldo et al. 2004; Espinosa and Alfano 2004). Thus, differences in the patterns of expression of such general elicitor-triggered responses should provide insights into how pathogens respond or counterattack early plant-defense mechanisms.

Fig. 2. Basal defense-related genes differentially expressed among incompatible and compatible barley–powdery mildew interactions involving fast-acting *Mla* (mildew resistance locus *a*) alleles. **A,** Cluster analysis of the top 500 differentially expressed genes (Caldo et al. 2004). Genes with *P* values below 0.01 were collected, and a data matrix of mean signal intensities in incompatible (–) and compatible (+) interactions of the identified 500 genes was uploaded in GeneSpring 6.2. Low (green), normal, and high (magenta) expressions in the heat map were based on the GeneSpring 6.2 color scheme. Hierarchical clustering was performed using the Pearson correlation. Individual timepoints, within a genotype-isolate interaction from 0 to 32 h after inoculation, are separated by white lines. **B,** A total of 20 genes represented by 24 Barley1 probe sets had annotations related to the shikimate pathway. Genes designated in red were identified via a *P* value < 0.0001 (Caldo et al. 2004), whereas additional genes identified through cluster analysis are designated in blue.

In plants containing fast-acting *Mla* alleles, an upregulation of mRNA expression of most basal defense-related genes was observed in compatible and incompatible interactions at the early stages of powdery mildew infection. The peak of transcript accumulation was attained at 16 hai and continuously up-regulated in incompatible interactions at the later stages of infection, which coincides with the timing of the termination of fungal growth (Figs. 2 and 4; Caldo et al. 2004). By contrast, suppression of basal defense in compatible interactions was observed after 16 hai, which may be necessary for the formation of haustoria (Caldo et al. 2004). However, in plants containing wild type and mutants impacting the delayed-acting *Mla12* allele, a biphasic transcript accumulation was observed in many of these basal defense-related genes, which includes those involved in the later steps of the shikimate pathway (Fig. 4; Table 2). The first phase of mRNA induction was observed from 0 to 8 hai, followed by marked suppression at 16 hai, corresponding to attempted fungal penetration.

Table 1. Predicted functional classification of 155 differentially expressed genes[a] among inoculated and noninoculated Sultan 5 (*Mla12*) plants

Predicted functional classification[b]	Number of genes	Percentage
Cellular metabolism	46	29.7
Shikimate pathway/secondary metabolism	22	14.2
Electron transport	13	8.4
Cell transport	8	5.2
Signal transduction	8	5.2
Defense response	5	3.2
Protein biosynthesis	3	1.9
Nucleic acid binding	3	1.9
Oxidative response	3	1.9
Protein degradation	2	1.3
Cellular component	2	1.3
Protein secretion	2	1.3
Transcription factor	1	0.6
Unknown	37	23.9
Total	155	100.0

[a] Based on p value < 0.05 in the comparison between inoculated and noninoculated Sultan 5 (*Mla12*) plants from 0 to 32 h after inoculation.
[b] Functional classification based on Uniprot BlastX results.

Further confirmation of the timecourse expression profiles from plants with fast-acting *Mla* alleles was also possible by investigating additional barley–powdery mildew data sets. Indeed, similar expression for plants with fast-acting alleles was observed in another independent timecourse expression experiment consisting of 144 Barley1 GeneChip hybridizations (R. A. Caldo and R. P. Wise, *unpublished data*). *B. graminis* f. sp. *hordei* isolate 5874–inoculated C.I. 16151 (*Mla6*) and C.I. 16137 (*Mla1*) plants displayed consistent upregulation of all 21 basal defense-related genes that were the final focus of the analysis described here. In addition, RNA blot analysis of barley cv. Pallas isolines demonstrated stage-specific suppression of phenylalanine ammonia lyase, one of the basal defense genes identified in this study, in plants harboring the delayed-acting *Mla12* allele but not the fast-acting *Mla1* allele (Kruger et al. 2003).

Powdery mildew haustoria develop from 14 to 18 hai. Coincident with haustorial formation, suppression of basal defense occurred at 16 hai in all plants containing the delayed-acting

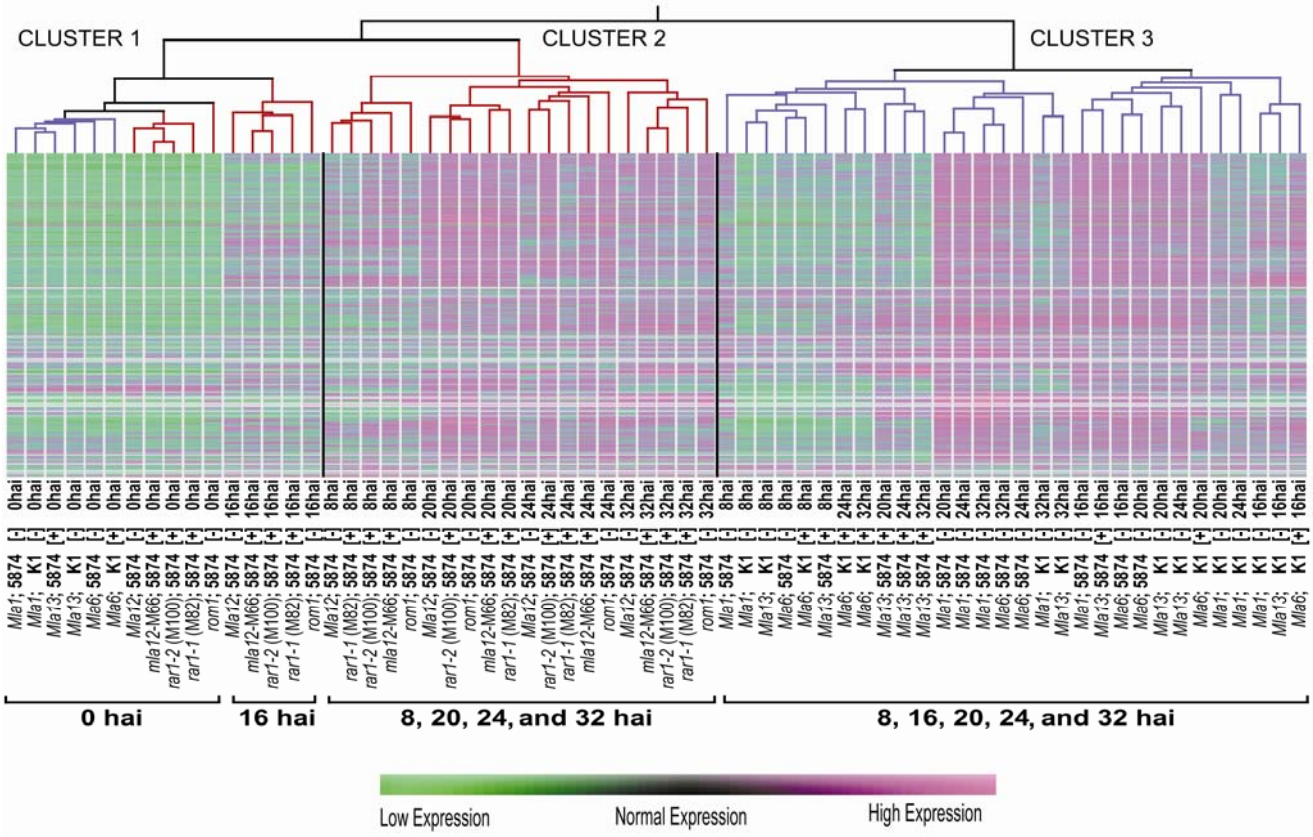


Fig. 3. Time-specific expression of 155 differentially expressed genes in plants containing fast- and delayed acting *Mla* (mildew resistance locus *a*) alleles. Clustering of responses based on a combination of 66 conditions from the study by Caldo et al. (2004) and the Sultan 5 experiment reported here. Mean expression was computed using Microsoft Excel 2002. A data matrix was constructed based on 155 genes that were found to be differentially-expressed between inoculated and noninoculated Sultan 5 plants. Data were uploaded in GeneSpring 6.2, and clustering was performed using the Spearman correlation. Purple lines in the cluster tree are conditions from the experiment involving fast-acting *Mla* alleles, while red lines in the cluster tree are conditions from the experiment involving wild type (*Mla12*) and mutants of Sultan 5. The GeneSpring 6.2 heat-map color scheme was used, with green designating low expression and magenta designating high expression for the 155 genes.

Mla12 allele but only after 16 hai in susceptible plants containing fast-acting *Mla* alleles. Likewise, chemical inhibition of genes related to phenylpropanoid metabolism (phenylalanine ammonia lyase and cinnamoyl CoA dehydrogense), which are downstream of the shikimate pathway, increased *B. graminis* f. sp. *hordei* haustorial formation and led to the suppression of *Mla1*-mediated powdery mildew resistance (Kruger et al. 2002; Zeyen et al. 1995). Results of these previous inhibition studies, therefore, support the involvement of basal defense suppression in conditioning host cells to allow formation of fungal haustoria, conceivably contributing to the delay in the timing of resistance responses.

Haustorial development is critical in pathogenesis because only this specialized structure has direct contact with the host

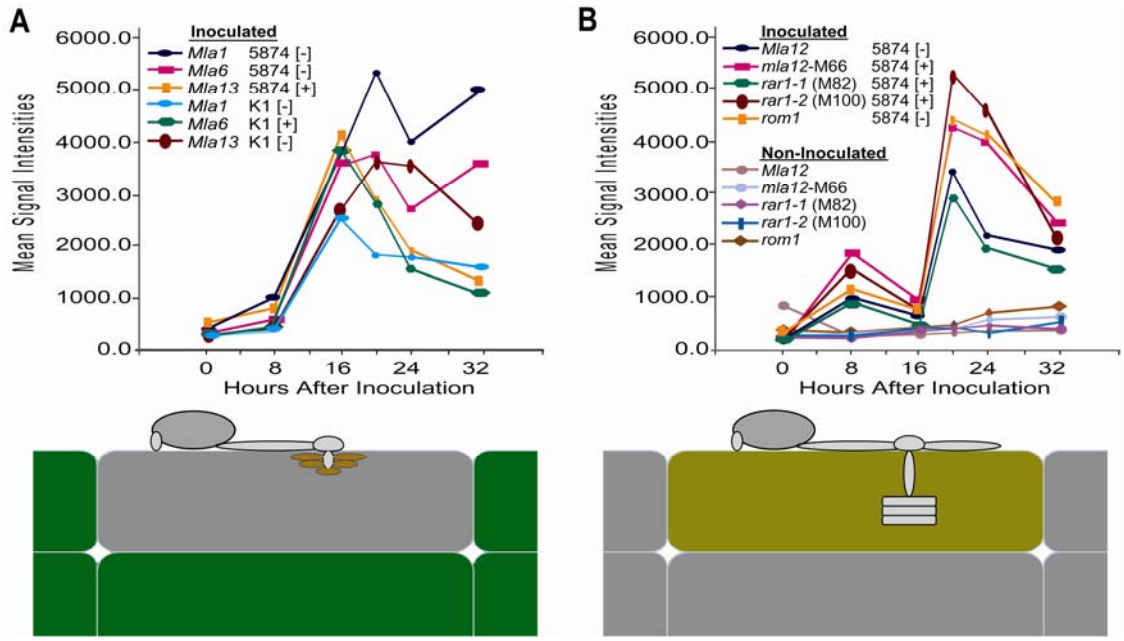


Fig. 4. Representative differential expression of 155 basal defense-related genes in plants undergoing *Mla* (mildew resistance locus *a*)–specified rapid and delayed resistance. **A,** mRNA expression detected by Contig8605_s_at, a gene encoding a predicted B12D protein in plants containing fast-acting *Mla* alleles. Normalized mean signal intensities were plotted from 0 to 32 h after inoculation (hai) in incompatible (–) and compatible interactions (+) from the experiment by Caldo et al. (2004), using Microsoft Excel 2002. The peak of mRNA expression is coincident with the termination of fungal growth leading to single-cell death. **B,** Transcription profiles of the same gene in panel A for plants containing delayed-acting *Mla12* allele. Normalized average signal intensities were calculated from three independent replications. Gene expression was plotted from 0 to 32 hai in inoculated and noninoculated wild type and mutants of Sultan 5, using Microsoft Excel 2002. A total of 21 basal defense-related genes were consistently up-regulated from 0 to 16 hai in all plants containing fast acting *Mla* alleles and also were significantly down-regulated from 8 to 16 hai in plants containing the slow-acting *Mla12* allele (*P* value <0.05).

Table 2. Basal defense-related genes (total of 21) differentially-accumulated[a] in plants containing fast- and delayed-acting *Mla* alleles at 16 h after powdery mildew infection

Affymetrix probe set ID	Barley1 GeneChip exemplar	Predicted function[b]	Predicted functional classification[c]	Organism	E-value
Contig11064_at	11064	Phosphoribosylanthranilate isomerase	Downstream shikimate	*A. thaliana*	5e-72
Contig14426_at	14426	Cinnamoyl-CoA reductase	Downstream shikimate	*P. balsamifera*	8e-58
Contig15413_at	15413	Putative anthranilate N-benzoyltransferase	Downstream shikimate	*O. sativa*	2e-47
Contig3904_at	3904	Tryptophan synthase beta-subunit	Downstream Shikimate	*S. bicolor*	0.0
Contig20247_at	20247	Agmatine coumaroyltransferase	Downstream shikimate	*H. vulgare*	9e-37
HK05P12r_s_at	35069	Tryptophan synthase beta-subunit	Downstream shikimate	*S. bicolor*	0.0
HY07P02u_at	48443	Anthranilate synthase alpha 2 subunit	Downstream shikimate	*O. sativa*	2e-67
Contig9990_at	9990	Putative pectin methylesterase	Defense	*O. sativa*	1e-166
Contig11518_at	11518	Protein kinase family	Signal transduction	*A. thaliana*	0.0
Contig3166_at	3166	Ras-related GTP-binding protein	Signal transduction	*O. sativa*	1e-110
Contig3208_at	3208	Blue copper binding protein	Electron transport	*H. vulgare*	3e-62
Contig11969_at	11969	Aldehyde 5-hydroxylase	Cellular metabolism	*L. styraciflua*	1e-110
Contig15946_at	15946	Putative serine palmitoyltransferase	Cellular metabolism	*O. sativa*	3e-42
Contig4897_at	4897	Putative esterase	Cellular metabolism	*O. sativa*	0.0
Contig8057_at	8057	Ubiquitin family protein	Protein metabolism	*A. thaliana*	1e-159
Contig24175_a	24175	Transporter-related	Cellular transport	*A. thaliana*	3e-31
Contig9341_at	9341	Putative Na+/K+/Cl cotransport protein	Cellular transport	*O. sativa*	0.00
Contig26363_at	26363	Unknown	Unknown	–[c]	–
Contig13189_at	13189	Hypothetical protein	Unknown	*O. sativa*	1e-11
Contig6995_at	6995	Unknown	Unknown	*O. sativa*	1e-150
Ebpi01_SQ005_A03_at	29950	Unknown	Unknown	–	–

[a] Based on *P* value < 0.05 upregulation from 0 to 16 h after inoculation (hai) in plants containg fast-acting *Mla* alleles and downregulation from 8 to 16 hai in plant containing delayed *Mla* allele.

[b] BarleyBase/PLEXdb annotations were based on the consensus of multiple searches. NCBI/TIGR/ATH1 searches were performed using Harvest:Barley assembly 25, and best BLASTX nonredundant was performed using HarvEST:Barley assembly 31.

[c] No organism designated for genes with nonsignificant E value.

cell to acquire plant nutrients for further fungal growth (Freialdenhoven et al. 1994; Panstruga 2003; Schulze-Lefert and Panstruga 2003). Thus, it is reasonable to hypothesize that inhibition of early-induced innate immunity preconditions the cell to allow the pathogen to develop a haustorium, resulting in delayed mechanisms of barley powdery mildew resistance. Previous studies have shown that suppressor molecules not only inhibit host defense responses but also condition host cell accessibility to pathogens (Hayami et al. 1982; Kohmoto et al. 1987; Kunoh 2002; Shiraishi et al. 1994). In addition, it has been suggested that suppressors might act as determinants of pathogen specificity by establishing basic compatibility through suppression of host general resistance (Shiraishi et al. 1994). Because of the differences in fungal development, which appear to be influenced by suppression of early basal defense, specific avirulence effectors may be released at different stages, triggering variation in the timing of the hypersensitive response for fast and delayed *Mla*-specified resistance (Kruger et al. 2003).

The level of R protein accumulation offers an alternative hypothesis to explain the differences in the timing of host-specific resistance. Shen et al. (2003) showed that overexpression of the *Mla12* allele can change the kinetics of infection from an intermediate to a rapid response, similar to those conferred by *Mla1* or *Mla6*, suggesting that the level of R protein is rate limiting for the onset of resistance (Bieri et al. 2004; Holt et al. 2005). MLA proteins accumulate to a higher level in *Rar1*-independent as compared with *Rar1*-dependent genotypes. In *rar1* mutants, MLA protein accumulation is impaired, compromising resistance function in *Rar1*-dependent types (Bieri et al. 2004). Because of the differences in R protein accumulation, impairment of MLA protein levels in *rar1* backgrounds may still be above the threshold level of activation for *Rar1*-independent but not in *Rar1*-dependent types, leading to the idea of the "threshold model" (Bieri et al. 2004; Holt et al. 2005). In relation to this model, we speculate that the modulation of general elicitor-induced defense responses affects the threshold of activation of MLA12 proteins or key components of R signaling, leading to the evolution of delayed resistance. This hypothesis supports the finding of Holt et al. (2005) on a potential linkage of *Rar1* (and most likely R protein accumulation) and basal defense. Consistently, in mammalian immunity, innate mechanisms have an influence on the expression of acquired immune response by modulating the threshold level of adaptive antigen-recognition receptors and by inducing key costimulatory molecules and cytokines (Fearon and Locksley 1996; Girardin et al. 2002; Medzhitov and Janeway 1998). Taken together, the effect of suppressors that target early induction of basal defense likely influences intracellular mechanisms of pathogen recognition, triggering the timing of effective resistance responses.

Cross-experiment analysis of basal defense responses has advanced our understanding of the mechanisms that influence the onset of resistance responses. Cellular conditioning through basal defense accumulation appears to be important in modulating host accessibility and inaccessibility to the pathogen. Thus, the interplay of nonspecific and specific host recognition of pathogen-derived molecules, depending on the nature of microbial infection, conceivably triggers the diversification of *R* gene–mediated resistance phenotypes.

MATERIALS AND METHODS

Fungal isolates.

Blumeria graminis f. sp. *hordei* isolate 5874 (Torp et al. 1978; Wei et al. 1999; *AvrMla1*, *AvrMla6*, *AvrMla12*) was propagated on *Hordeum vulgare* cv. Manchuria (C.I. 2330) in controlled growth chambers at 18°C (16 h light and 8 h dark).

Plant material.

C.I. 16151 (*Mla6*), C.I. 16155 (*Mla13*), and C.I. 16137 (*Mla1*) plants exhibit rapid and absolute resistance responses when challenged by *B. graminis* f. sp. *hordei* isolates that carry cognate *AvrMla6*, *AvrMla13*, and *AvrMla1* genes, respectively (Boyd et al. 1995; Wise and Ellingboe 1983). Sultan 5 loss-of-function mutants *mla12*-M66, *rar1-1* (M82), and *rar1-2* (M100) were generated by EMS and NaN_3 mutagenesis (Torp and Jørgensen 1986). rom1 was generated by NaN_3 mutagenesis of *rar1-2* (M100) (Freialdenhoven et al. 2005). Sultan 5 (*Mla12*) and rom1 exhibit delayed and intermediate resistance to *B. graminis* f. sp. *hordei* 5874 while *mla12*-M66, *rar1-1* (M82) and *rar1-2* (M100) are susceptible.

Experimental design.

Planting, stage of seedlings, harvesting, and experimental design were as described by Caldo et al. (2004), except for the inclusion of noninoculated seedlings. Briefly, two 20 × 30-cm flats per genotype of Sultan 5 (*Mla12*) and derived mutants *mla12*-M66, *rar1-1* (M82), *rar1-2* (M100), and *rom1* were planted in sterilized potting soil. Each experimental flat consisted of six rows of 15 seedlings, with rows randomly assigned to one of the six harvest times. Seedlings were grown in a 20°C controlled glasshouse to 10 cm (first leaf unfolded, GRO:0007060) prior to the application of treatment. Noninoculated plants were processed before the inoculated plants to avoid accidental inoculation. First leaves of noninoculated seedlings in a row assigned for the 0-h timepoint were harvested into liquid nitrogen. Immediately after harvesting, the flats were transferred to the growth chamber at 18°C (16 h light and 8 h dark). For the treated plants, inoculation was performed, starting at 4 PM Central Standard Time (CST), by dusting the plants with a high density of fresh conidiospores (84 ± 19 spores/mm^2). Immediately after inoculation, the row of seedlings designated for the 0-h timepoint was harvested into liquid nitrogen. Similarly, the flats of inoculated plants were also transferred to the growth chamber immediately after harvesting. Noninoculated and inoculated plants were stored side-by-side in randomly assigned positions in the growth chamber. For the 8- to 32-h timepoints, flats were removed one at a time from the growth chamber, and leaves were harvested into liquid nitrogen. Following harvest, flats were immediately returned (approximately 30 s) to randomized positions within the chamber. Lights in the growth chamber were "off" from 4 p.m. to 12 a.m. and were "on" from 12:01 a.m. to 3:59 p.m. CST. 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 (Kuehl 2000). Data were collected from 180 GeneChips, one for each row of seedlings.

Barley1 GeneChip probe array.

The Barley1 GeneChip probe array (part number 900515) is distributed by Affymetrix (Santa Clara, CA, U.S.A.). The array includes 22,792 probe sets derived from worldwide contribution of 350,000 high-quality expressed sequence tags clustered from 84 cDNA libraries in addition to 1,145 barley gene sequences from the National Center for Biotechnology Information nonredundant database (Close et al. 2004). Array annotation information is hosted on the Harvest:Barley and BarleyBase/PLEXdb (Shen et al. 2005) databases.

Target synthesis and GeneChip hybridization.

Total RNA was isolated using a hot (60°C) phenol/guanidine thiocyanate method as described by Caldo et al. (2004). Probe synthesis and labeling followed, using One Cycle and GeneChip IVT labeling protocols based on the Affymetrix manual, and

were performed at the Iowa State University GeneChip Core facility. A total of 15 μg of fragmented cRNA was used to make each hybridization cocktail containing 10% dimethyl sulfoxide, and an equivalent of 10 μg was hybridized to Barley1 GeneChip probe array (Affymetrix #900515; Close et al. 2004). All detailed protocols can be accessed online within the BarleyBase/PLEXdb parallel expression database (Shen et al. 2005).

Normalization and data analysis.

The top 500 differentially expressed genes reported by Caldo et al. (2004) were identified by a *P* value of <0.01 and were analyzed using hierarchical clustering based on Pearson correlation in GeneSpring 6.2 (Silicon Genetics, Redwood City, CA, U.S.A.) software. The average scaled signal intensities (mean of signal intensities that were scaled using marker-assisted selection 5.0 algorithm) were calculated from three replications, using Microsoft Excel 2002. Normalization, data transformation, and mixed linear model analysis (Wolfinger et al. 2001) for the Sultan 5–derived microarray data were patterned after the methods used by Caldo et al. (2004) for 207 selected Barley1 probe sets. The mixed linear model analysis was performed using the SAS mixed procedure. Contrast statements in SAS were made to compare mRNA expression over time in noninoculated and inoculated plants for the individual genotypes. Subsequently, a data matrix was constructed of 155 differentially expressed genes from the Sultan 5 and Caldo et al. (2004) experiments but without the conditions involving noninoculated plants. This data matrix was loaded in GeneSpring 6.2 (Silicon Genetics) for hierarchical clustering using the Spearman correlation.

Histogram-based estimation of differentially expressed genes.

The histogram-based method described by Mosig et al. (2001) was followed to estimate the number of differentially expressed genes (false null hypotheses) by Caldo et al. (2004). The *P* values of 22,840 probesets with interval of (0,1) were partitioned into 20 bins. An iterative algorithm was used to estimate the number of *P* values in excess of the uniform distribution. The excess sum was the estimate of genes with time-specific differences that were not constant across the 32 hai in the comparison of compatible and incompatible interactions.

Data access.

All detailed data and protocols from these experiments have been deposited in BarleyBase/PLEXdb, a MIAME (minimum information about a microarray experiment)–compliant expression database for plant GeneChips (Shen et al. 2005). Files are categorized under accession number BB4 for the 108 GeneChips from the Caldo et al. (2004) study and BB2 for the 180 GeneChip Sultan 5 experiment. Data files have also been deposited in ArrayExpress as accession number E-MEXP-142 for the Caldo et al. (2004) study and E-TABM-82 for the Sultan 5 experiment.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

ArrayExpress repository for microarray data: www.ebi.ac.uk/arrayexpress
BarleyBase database for plant microarrays: barleybase.org
HarvEST EST database-viewing software and Harvest:Barley website: harvest.ucr.edu
PLEXdb database for plant and plant pathogen microarrays: plexdb.org
Uniprot BlastX website: www.pir.uniprot.org