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## Abstract

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## Disciplines

Agricultural Science | Agriculture | Plant Pathology

## Comments

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# Transcript Profiling in Host–Pathogen Interactions\*

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## Key Words

cluster analysis, genetical genomics, meta-analysis, MIAME, microarray, plant ontology, signaling cascades

## Abstract

Using genomic technologies, it is now possible to address research hypotheses in the context of entire developmental or biochemical pathways, gene networks, and chromosomal location of relevant genes and their inferred evolutionary history. Through a range of platforms, researchers can survey an entire transcriptome under a variety of experimental and field conditions. Interpretation of such data has led to new insights and revealed previously undescribed phenomena. In the area of plant–pathogen interactions, transcript profiling has provided unparalleled perception into the mechanisms underlying gene-for-gene resistance and basal defense, host vs non-host resistance, biotrophy vs necrotrophy, and pathogenicity of vascular vs nonvascular pathogens, among many others. In this way, genomic technologies have facilitated a system-wide approach to unifying themes and unique features in the interactions of hosts and pathogens.

## INTRODUCTION

Research hypotheses can now be addressed in the context of entire developmental or biochemical pathways under a large array of experimental or field conditions. Access to complete genomic sequences, coupled with rapidly accumulating data related to RNA and protein expression patterns, have made it possible to determine comprehensively how genes contribute to complex phenotypes. The genetic inheritance of transcript profiles can be used to map the chromosomal location of relevant, regulatory loci. In addition, comparison of gene expression networks among model and crop species can be used to infer function as well as evolutionary history, based on expression patterns shared with genes of known function (166).

Interesting topics for plant pathologists include the mechanisms underlying gene-for-gene resistance and basal defense, host vs nonhost resistance, biotrophy vs necrotrophy, or pathogenicity of vascular vs nonvascular pathogens, among many others. As the different expression technologies as well as the importance of statistical design in large-scale biology have been well described (26, 93, 104, 115), our approach focuses on key systems as examples of how transcript profiling has been utilized effectively to drive biological discovery in host-pathogen interactions.

With the increasing availability of a large number of microarray data sets in public repositories, it is also beneficial to use comparative meta-profiling strategies to draw conclusions that bridge multiple experiments (53, 97, 112, 116, 117, 131, 151). Differences in design and conduct of the experiment should be acknowledged up front to avoid misleading conclusions (62). New information can be derived from further mining of published data, and distinct gene expression patterns can be used as a point of reference for meta-analysis involving several experiments (20, 103).

## Moving from Model Systems into Crops: Not Just for *Arabidopsis* Anymore

Parallel expression profiling, once thought to be available only to large research groups with substantial infrastructure, can now be used by small groups and individual laboratories. A myriad of microarray platforms now exist for most organisms through commercial and public sources. Off-the-shelf, high-density DNA arrays are now available for barley, wheat, rice, maize, sugarcane, grape, citrus, poplar, tomato, *Arabidopsis*, *Brassica*, and cotton, among others. In addition to using these arrays to monitor the expression of thousands of transcripts in parallel, many projects are accessing this technology for both host and pathogen through community-driven development of multispecies arrays, such as soybean/*Phytophthora sojae* (root rot)/*Heterodera glycines* (soybean cyst nematode), *Medicago truncatula*/*Medicago sativa*/*Sinorhizobium meliloti*, *Fusarium graminearum* (scab), and rice/*Magnaporthe grisea* (rice blast). Also, many companies now collaborate with investigators to produce custom arrays (or the oligonucleotides for spotting) at a reasonable cost. This enabling technology provides the opportunity to investigate the regulation of entire pathways in both hosts and pathogens under uniform experimental conditions.

## EXPERIMENTAL DESIGN CONSIDERATIONS

### Different Designs to Answer Different Questions

Typically, one is interested in mRNA transcripts that are significantly accumulated or diminished under particular sets of conditions or treatments. A “treatment” is anything that perturbs the transcript profile in reference to the control condition, be it a plant or pathogen genotype, a mutant, a time course, temperature, or light conditions, etc.

In host-pathogen interactions, it is particularly useful to use a time course combined with alternate plant genotypes or pathogen isolates to elicit a response that will enable a hypothesis to be tested in relation to the historical knowledge base for the system being evaluated. At the very least, the experiment should be designed such that alternative hypotheses can be assessed. Consideration must be given to the effects of environment (growth chamber, greenhouse, or field), host genotype (isogenic or diverse), pathogen strains (again, isogenic or diverse) and inoculation technique.

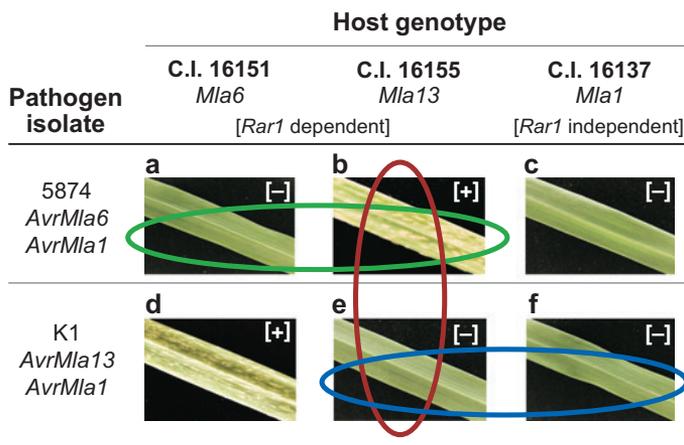
Ultimately, the particular use of expression profiling depends on the question being asked. If expression data are used to draw broad conclusions about the biology of a system, such as host-pathogen interactions (93), rigorous statistical design, replication, and analysis are essential. On the other hand, one can also lend support for downstream functional analysis by assaying thousands of transcripts in response to a particular gene or knockout mutation. Whatever the question being asked, or the system being interrogated, a thorough discussion with your local statistics expert is a prerequisite to planning expression profiling or other experiments in large-scale biology.

### Use Cases: Multiple Attack and Counter Attack Strategies

Three examples, focusing on powdery mildews, illustrate contrasting uses for parallel expression profiling. The first case addresses mechanisms by which pathogens suppress host defenses in order to establish basic compatibility or biotrophy: What are the mechanisms that make a plant susceptible or resistant? Caldo and associates (20, 21) investigated differences in global transcript accumulation among incompatible and compatible interactions between barley and the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei*. Several design considerations were incorporated to facilitate data analysis and interpretation: (a) multiple contrasting

host genotypes, (b) alternate pathogen isolates, (c) selection of a time course based on historical data on the kinetics of fungal infection, and (d) rigorous statistical standards (blocking, randomization, replication) (104). They utilized a  $3 \times 2$  matrix consisting of three near-isogenic barley lines, harboring introgressed *Mla6*, *Mla13*, and *Mla1* CC-NBS-LRR resistance alleles, challenged with contrasting *B. graminis hordei* isolates 5874 (*AvrMla6*, *AvrMla1*) or K1 (*AvrMla13*, *AvrMla1*), and harvested at 0, 8, 16, 20, 24, and 32 h after inoculation (hai). Similar to a classic “quadratic check” (49), the  $3 \times 2$  matrix design enabled at least two independent host-isolate combinations to be assessed for each question being asked (Figure 1).

One of the most significant nonparallel profiles represents a coregulated cluster of >150 genes that are significantly up-regulated in both incompatible and compatible interactions up to 16 hai, coinciding with germination of *B. graminis hordei* conidia and formation of appressoria.



**Figure 1**

Quadratic check design to assess differences in transcript accumulation among incompatible and compatible interactions between barley (*Hordeum vulgare* L.) and the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei*. Four contrasts were used to search for genes associated with incompatibility vs compatibility as determined by plant genotype (A vs B, D vs E; e.g., circled in green) or by pathogen isolates (A vs D, B vs E; e.g., circled in red). Two contrasts were used to test for genes associated with *Rar1* independence vs *Rar1* dependence (A vs C, E vs F; e.g., circled in blue) (20, 21). C.I. = Cereal introduction.

**Hai:** hours after inoculation

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**PAMP:**  
pathogen-associated-  
molecular-patterns

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Down-regulation of these transcripts occurs after 16 h only in compatible interactions, during contact between fungal haustoria and host epidermal cells, whereas these transcripts are sustained or increase in incompatible interactions (**Figure 2d**) (20, 21). Many of these genes, such as those shown in **Figure 2c**, are involved in nonspecific, PAMP (pathogen-associated-molecular-patterns)-induced, basal defense pathways. However, in incompatible interactions governed by the tested *Mla* alleles, recognition of specific pathogen effectors leads to the maintenance of increased expression. By contrast, in the absence of the MLA and/or corresponding effector proteins, time-point specific up- and down-regulation of transcripts are observed that predict compatibility and implicate a miscoordination of plant transcriptome reprogramming as compared to incompatible interactions (M. Moscou, R. Caldo & R. Wise, unpublished data). These results are consistent with the hypothesis that host regulators of basal resistance are the targets of pathogen effectors. Recent work on nuclear localization of MLA interactors indicates that

two of these basal resistance regulators are a pair of transcription factors, *HvWRKY1* and *HvWRKY2* (127). *Arabidopsis* homologues of *HvWRKY1/2*, *AtWRKY18/40* appear to be involved in a feedback repression system as a control feature of basal defense (127).

The second case addresses the molecular basis for nonhost resistance (46, 92, 140). *Arabidopsis* does not support the growth and asexual reproduction of *B. graminis bordei*. Conidia will germinate and form appressoria, but the majority will not penetrate the epidermal cell wall and papillae (168). However, *Arabidopsis* is a host to the powdery mildews *Erysiphe cichoracearum* and *E. orontii*. The *Arabidopsis pen3-1* mutant allows increased penetration by *B. graminis bordei*. PEN3 encodes the putative ATP binding cassette (ABC) transporter PDR8 (130). *pen3-1* is less resistant to the necrotroph *Plectosphaerella cucumerina*, as well as to two additional nonhost biotrophs, *E. pisi* (pea powdery mildew) and *Phytophthora infestans* (potato late blight). However, *pen3-1* is resistant to *E. cichoracearum*.

To investigate gene expression in host and nonhost interactions, Stein

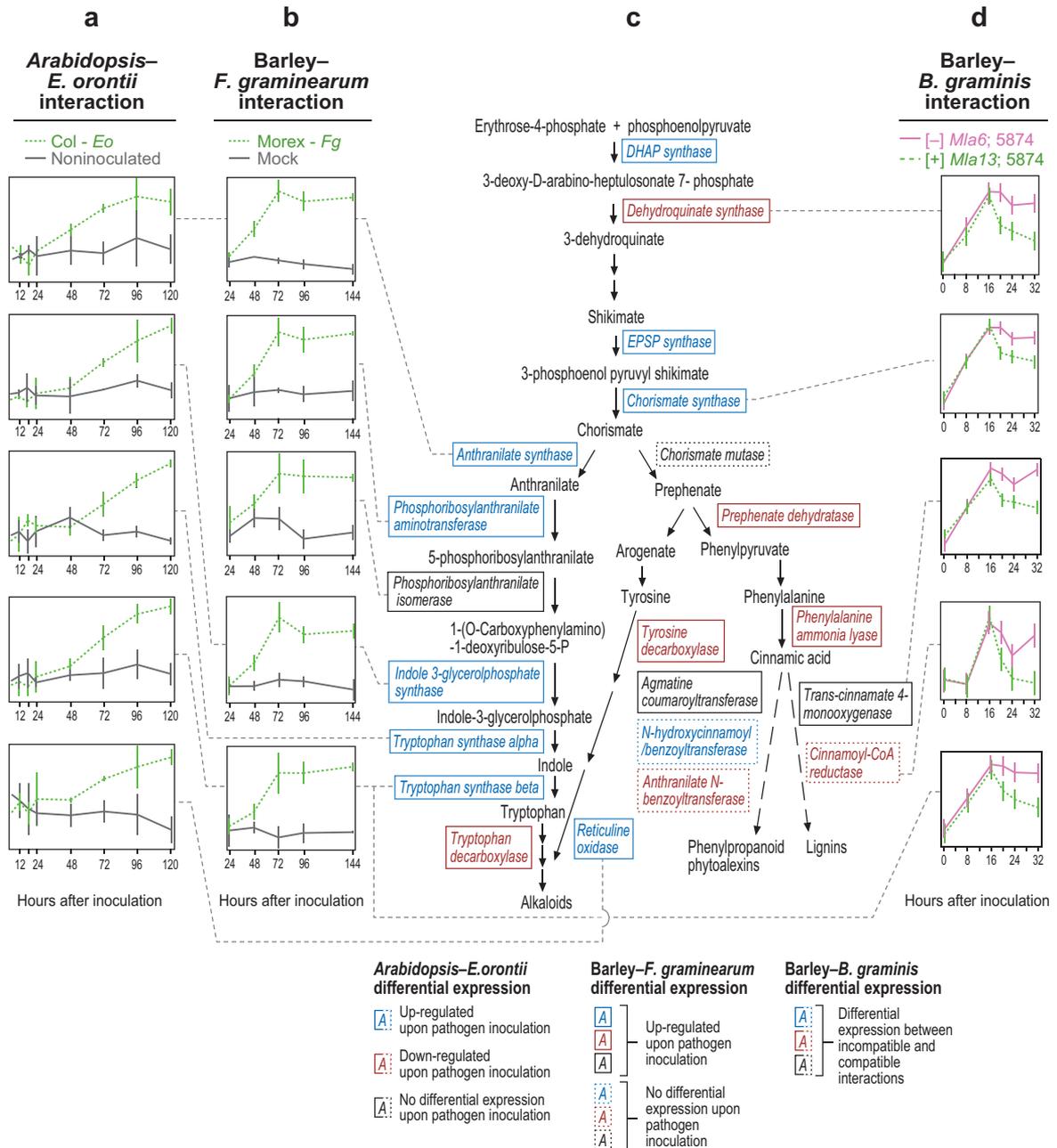
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**Figure 2**

Overlapping and conserved gene expression in diverse host-pathogen interactions. Selected time-course expression profiles of differentially expressed genes annotated to the shikimate pathway leading to the biosynthesis of aromatic amino acids. (a) *Arabidopsis* response to *Erysiphe orontii* infection. Three independent replications of 4-week/old Columbia wild-type plants were infected with *E. orontii* cultures and leaves number 7 to 10 were harvested at 6, 12, 18, 24, 48, 72, 96, and 120 hai [data downloaded from Pathogenomics Integrated Microarray Database System (IMDS) (<http://ausubellab.mgh.harvard.edu/imds/>)]. (b) Transcript profiling of barley infected with *Fusarium graminearum* (12). Four biological replications of Morex spikes infected with *F. graminearum* isolate Butte 86 and mock water control were sampled at 24, 48, 72, 96, and 144 hai (12); Accession no. BB9 (<http://plexdb.org/>). (c) In *Arabidopsis-E. orontii* interactions, genes designated in blue are up-regulated, genes designated in red are down-regulated, and genes in black show no differential expression (see **Supplementary Table 1**: Follow the Supplemental Material link from the Annual Reviews home page at <http://www.annualreviews.org>). In barley-*F. graminearum* interactions, genes encircled by solid lines are up-regulated upon pathogen inoculation and genes encircled by dotted lines are not differentially expressed. In barley-*B. graminis bordei* interactions, all designated genes are differentially expressed. (d) Subset of genes identified as differentially expressed among compatible and incompatible interactions in barley challenged with *B. graminis* f. sp. *bordei*. Incompatible and compatible interactions were generated by all pairwise combinations of the C.I. 16151 and C.I. 16155 near-isogenic lines (containing *Mla6* and *Mla13* resistance alleles, respectively) and the two *B. graminis bordei* isolates, 5874 (*AvrMla6*, *AvrMla1*) and K1 (*AvrMla13*, *AvrMla1*). Three independent replications of *B. graminis bordei* inoculated first leaves were harvested 0, 8, 16, 20, 24, and 32 hai (20, 21); Accession no. BB4 (<http://plexdb.org/>). Y axes on graphs designate relative expression.

and colleagues (130) utilized contrasting host (*E. ciburacearum*) and nonhost (*B. graminis bordei*) powdery mildews to examine differences in expression among wild-type and *pen3-1* plants one day after inoculation (dai) using the Affymetrix *Arabidopsis* ATH1

GeneChip. Of the 22810 probe sets (genes) on the array, 4240 were considered differentially expressed between the *E. ciburacearum* and *B. graminis bordei* infections at a *p* value of <0.001. As expected, genes were both induced and repressed by pathogen



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**dai:** days after inoculation

**SA:** salicylic acid

**JA:** jasmonate acid

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challenge. *pen3-1*-mediated resistance to *E. cichoracearum* was salicylic acid (SA) dependent. Likewise, SA pathway genes, such as *PAD4*, *SID2*, *EDSI*, and *EDS5*, as well as downstream SA pathway markers, were up-regulated in *pen3-1* plants as compared to the wild type. In addition, plants inoculated with *B. graminis bordei* produced a more dramatic up- or down-transcript response than *E. cichoracearum*, consistent with earlier experiments with the AFGC (*Arabidopsis* Functional Genomics Consortium) cDNA arrays aimed at dissecting the SA or jasmonate (JA)/ethylene (ET) defense pathways in host and nonhost interactions (168). Up-regulation of genes in the SA pathway suggested that *pen3-1*-mediated resistance to *E. cichoracearum* is likely caused by an enhanced activation of the SA pathway (130). Stein and colleagues (130) hypothesize that *Arabidopsis* plants may respond more dramatically to penetration by *B. graminis bordei* because it cannot suppress host basal defenses as well as *E. cichoracearum* can.

The third case concerns host genes correlated with the establishment of biotrophy. Powdery mildews are biotrophic pathogens that keep host cells alive in order to acquire nutrients, while minimizing tissue damage. However, many outcomes are possible when thousands of powdery mildew conidia land on the same leaf. For example, one conidiospore may succeed during the infection process and form a functional haustorium, whereas another may not. Therefore, there are likely different changes in the transcriptome of individual cells depending on whether penetration was successful. To investigate the transcript changes in individual infected cells, Lyngkjaer and colleagues have developed a system to micromanipulate single barley epidermal cells (55).

The key to this system is that individual resistant and infected epidermal cells of susceptible barley can be distinguished easily by microscopic observation of *B. graminis bordei*-challenged barley leaves at 18 hai (55). Subsequently, the contents of these and noninoculated control cells were collected for mRNA

extraction, amplification, and hybridization to the IPK (Gatersleben) barley PGRC1 10k cDNA array (56, 129). Hierarchical clustering and multidimensional scaling (31) were used to visualize the relationships between samples. Notably, up-regulation of sucrose synthase was exclusive to infected cells (56). In addition, two hexose transporters were up-regulated in both resistant and susceptible cells as well as genes associated with sucrose transport. Analogous findings have been observed in inoculated as compared to noninoculated control samples from seedling leaves [(20); accession BB2 (<http://plexdb.org/>)], where sugar transport-related genes are reported to be significantly up-regulated after challenge with *B. graminis bordei*. dsRNAi single cell silencing (42) of these predicted sugar transporters produced a more resistant phenotype, suggesting that the pathogen regulates these plant genes to manipulate sugar availability for biotrophic growth (73, 119; R. Caldo, M. Moscou, G. Fuerst, T. Bancroft, D. Nettleton & R. Wise, unpublished data), as has been predicted from observations of *Arabidopsis* infected with *E. cichoracearum* (50). By contrast, silencing of genes encoding predicted UDP-glucose dehydrogenase and alkaline/neutral invertase produce a more susceptible phenotype, suggesting that sucrose partitioning is triggered upon pathogen recognition. To identify host genes important for establishment of powdery mildew infection, Lyngkjaer and colleagues are currently analyzing expression data from a time course (12–48 hai) of individual haustoria-containing barley cells (M. Lyngkjaer, personal communication). This analysis will have far-reaching implications on deciphering genes involved in the establishment of biotrophy.

The three cases presented emphasize that the inoculation and tissue harvest methods used are dependent on the question being addressed. In plant-bacterial interactions, for example, the hypothesis that mesophyll cell responses contribute to the differentiation

between vascular and nonvascular bacterial pathogens might be tested by introducing bacterial suspensions into the mesophyll apoplast through stomates using a syringe-infiltration technique, and then harvesting the infiltrated tissue for transcript profiling. The mechanism of bacteria-induced changes in stomatal aperture, on the other hand, might be better addressed by profiling changes in the transcriptome of guard cells following a dip or spray inoculation, and harvesting by laser capture microdissection (LCM) (15, 19, 84, 101, 107). An approach creatively and carefully tailored to the question being addressed can have extraordinary predictive power. Thus, parallel expression technologies can be applied to almost any question; the key is to design the best set of experiments around the advantages (or disadvantages) of a particular biological system.

### Levels of Significance, False Discovery Rate, and Biological Significance: Reaching the High Hanging Fruit

The first pass at the data is normally used to extract the most significant patterns or pathways using a stringent cutoff and allows the construction of a model describing the biological framework. This statistical cutoff is normally associated with a low False Discovery Rate (FDR) or  $q$  value (132). However, one should assess a user-imposed cutoff against the biological system that is being evaluated such that, in the end, some of the most interesting genes or patterns of gene expression are not eliminated. These may be harder to extract because they are part of a group with a higher FDR. However, it can be useful to mine the data set in steps, first with a stringent cutoff, then with successively more relaxed criteria to extract other genes that match (or challenge) the original model.

For example, in a series of investigations to mine data sets for genes involved in barley-powdery mildew interactions, Caldo and associates (21) originally used a cutoff  $p$  value

$<0.0001$ , associated with an FDR  $<7\%$  (132). This analysis was conducted to extract the major significant clusters of differentially regulated genes in conjunction with the kinetics of *B. graminis hordei* infection. Subsequently, they considered a more relaxed threshold  $p$  value of  $<0.001$  and identified 81 additional genes (20). Although the FDR associated with the  $p$  value  $<0.001$  was 20%, by evaluating the individual time course expression graphs, they extracted 28 of these 81 genes that displayed the same pattern of expression as the first 22 identified in the primary analysis (21). To go even further, they collected the most significant 500 genes with  $p$  values  $<0.01$  and performed cluster analysis of mean signal intensities, grouping 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  $<0.0001$  (21) and the subsequent 28 genes identified above ( $p$  values  $<0.001$ ), which had identical time course expression patterns. Twenty-one of the predicted genes from cluster 3 have annotations associated to the shikimate pathway leading to the biosynthesis of secondary metabolites (Figure 2c).

In addition to relaxing the FDR or identifying additional genes that fit a certain profile, it is also possible to re-examine data in light of selected gene families or functional groups. For example, Carr and colleagues (23) reanalyzed the data of Huang and associates (72) with a specific gene set in mind. The original intent of the Huang et al. (72) experiment was to examine the effects of *Arabidopsis* defense pathway mutants on the expression of defense-related genes in compatible host-virus interactions. However, Carr and colleagues were interested in the behavior of the eight HSP100 gene family members that were all also represented on the array. They utilized the data to determine that only HSP101 was induced significantly by the viruses used in the study, and that its expression was independent of the defense signaling pathway mutants used in that

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**LCM:** laser capture microdissection

**FDR:** False Discovery Rate

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study. Based on this reanalysis of the data, they concluded that HSP101 and other heat shock proteins induced by viruses are regulated by a pathway independent of the defense-related genes.

## BIOLOGICAL SYSTEMS

### Plant-Fungal Interactions: Comparative Analysis of Stealth and Brute Force

Biotrophic fungi and oomycetes, which require a living host for nutrients, must avoid recognition and activation of host defenses until they can complete their life cycles. If a host resistance gene is present, then rapid recognition results in defense responses that inhibit further pathogen ingress. Many genes that are induced during defense responses are also induced during compatibility, albeit with different kinetics (20, 21, 42, 48). A good example of this is the shikimate pathway shown in **Figure 2c**. Enzymes in this pathway catalyze reactions leading to the production of aromatic amino acids and to secondary metabolites associated with plant protection such as alkaloids, phenylpropanoid phytoalexins, and lignins.

As illustrated in **Figure 2** and accompanying **Supplementary Table 1**, mRNA transcript abundance is indicated for biotrophic interactions between *Arabidopsis* and *E. orontii* as well as between barley and *B. graminis bordei*. In **Figure 2d**, the expression of selected genes is compared in the compatible and incompatible interactions. These data encompass the early times after infection and illustrate the general increase in expression of genes in the shikimate pathway over the first 16 hai regardless of interaction types. After 16 hai corresponding to haustoria formation, the profiles of these genes diverge, with their levels remaining high in incompatible but falling in the compatible interactions.

The compatible *Arabidopsis*-*E. orontii* interaction reveals patterns different from those

observed in the barley-*B. graminis bordei* interaction (**Figure 2a**). Genes significantly induced in the shikimate pathway in *Arabidopsis* were not induced as early as in the barley-*B. graminis bordei* interaction, but instead, were induced later (24–48 hai). It would be interesting to know if these genes are also induced later in the compatible barley-*B. graminis bordei* interaction. Finally, the compatible interaction between barley and *F. graminearum* shows a profile similar to that of the *Arabidopsis*-*E. orontii* interactions (**Figure 2b**), although *F. graminearum* behaves as a hemibiotroph, where genes in the shikimate pathway are induced in the 48–72 hai interval corresponding to a period of rapid fungal growth and accumulation of a nonselective toxin (12). The increased expression of these genes is likely a general defense mechanism that is enhanced by the *R-Avr* interaction.

*B. graminis bordei* infects epidermal cells of barley, but also induces local acquired resistance in wheat, a nonhost (18, 45). Thus, for this pathogen it is interesting to consider the effects of penetration upon the epidermal cells versus the underlying mesophyll cells. Bruggmann and colleagues (18) assayed incompatible responses of wheat in RNA samples enriched for epidermis or mesoderm. Infection by *B. graminis bordei* caused significant changes in transcript accumulation in both epidermis and mesophyll tissues, demonstrating that systemic signaling occurs. As expected, a large number of defense proteins were induced, but most had a greater fold change in the mesophyll than in the epidermis. The expression of Sec61 alpha subunit (AY044237), calreticulin, and cyclophilin-encoding genes, which are genes involved in protein secretion, were induced most strongly in mesophyll cells in concert with the greater expression of the pathogenesis-related genes. Further analysis of data published by Caldo and colleagues (20, 21) revealed induction of a suite of genes involved in protein secretion during barley-*B. graminis bordei* interactions.

Similarly, genes encoding several secretory proteins are induced in *Arabidopsis* defense responses to *Pseudomonas syringae*; these proteins are necessary for secretion of defense proteins and systemic acquired resistance (SAR) (150). Thus, the concomitant induction of secretory and defense genes occurs in cereals as well as *Arabidopsis*, suggesting a highly conserved mechanism in plant defenses.

**COII-dependent signaling networks in host-necrotroph interactions.** *Arabidopsis-Botrytis cinerea* and *Arabidopsis-Alternaria brassicicola* are models for host-necrotroph interactions. *B. cinerea* causes disease in wild-type *Arabidopsis* ecotypes, and disease is enhanced in *coi1* mutants. *A. brassicicola* does not cause disease in wild-type *Arabidopsis* ecotypes, but *coi1* mutants are susceptible. Local responses of wild-type and mutant plants to both pathogens have been investigated using the Affymetrix *Arabidopsis* 8K GeneChip (1, 145), and systemic responses to *A. brassicicola* were investigated using a cDNA microarray containing 2375 *Arabidopsis* ESTs that were biased toward defense and signal transduction (121). In the *B. cinerea* experiments, samples were collected at times corresponding to penetration (24 hai), early colonization (36 hai), and late infection (60 hai). The *A. brassicicola* samples were collected at 12, 24, and 36 hai, all corresponding to early times in infection. In these experiments, 462 *B. cinerea*-induced genes (BIGs) and 645 *A. brassicicola*-induced genes (AIGs) were identified within the first 36 hai in wild-type plants. The *coi1* mutation, which is defective in jasmonic acid (JA) perception, affected the expression of nearly one third of the 462 BIGs and two fifths of the 645 AIGs. Many of the affected genes were associated with JA/ET-mediated defense such as *PDF1.2*, *HEL1*, and antioxidants as well as JA biosynthetic enzymes. These data suggest a link between *COII*-dependent genes and defense against necrotrophic pathogens. In the case of *B. cinerea*, induction of these effector genes was also accompanied by in-

creased expression of at least 30 regulatory transcription factors, of which 27 were *COII*-dependent (1). Analysis of mutations in 14 of these genes identified two transcription factors (WRKY70 and ZFAR1) that regulate responses that protect *Arabidopsis* against *B. cinerea* infection. These data demonstrate the important role of JA-/ET-mediated defenses in protecting plants against necrotrophs and provide insight into the complex networks that regulate this defense.

**Profiling fungal gene expression within the host.** In addition to differential gene expression occurring within host cells, it is necessary to consider the gene expression of pathogens within the infection site as well. A study to monitor expression of genes in *Colletotrichum graminicola*, causal agent of maize anthracnose stalk rot, used laser capture microscopy (LCM) in combination with fluorescent AmCyan protein-tagging, to produce samples for microarray analysis (135). This approach identified over 8000 genes as significantly expressed in *C. graminicola* as early as 2 days after inoculation, which is an early stage of infection with relatively little fungal biomass accumulation. Studies with another comparable host-pathogen interaction at 2 days after inoculation, but not employing LCM, led to the identification of only about 900 expressed fungal genes. Thus, LCM clearly provided for greater enrichment of fungal mRNA and an order of magnitude increase in power to detect fungal mRNA transcripts. Comparison of gene expression between in vitro grown cultures and in planta grown cells showed significant up-regulation of secreted proteins, perhaps signifying the production of effectors and other proteins required for pathogenicity. It would be interesting to determine how the maize cells collected in these samples were also responding to *C. graminicola* in these samples.

Both and colleagues found many patterns of coordinate expression among *B. graminis hordei* genes in defined metabolic pathways

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**SAR:** systemic acquired resistance

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from cDNA microarray profiling experiments monitoring the infection cycle in barley (14). This allowed an assessment of the metabolic status of the fungus during asexual development as it infected the host plant. Genes encoding several glycolytic enzymes are significantly up-regulated as mature appressoria form, and in the infected epidermis, which contain fungal haustoria. Concomitantly, host plants show up-regulation of sugar transport and utilization-related genes after powdery mildew infection, providing a source for nutrient acquisition (50, 56; R.A. Caldo, unpublished data).

Obligate biotrophic rust fungi infect host tissue via intercellular mycelia that form haustoria within the living plant cells. Jakupovic and colleagues (77) identified genes expressed during biotrophic growth of the bean rust *Uromyces fabae* by EST sequencing of a haustorium-specific cDNA library. Several of the *Uromyces* ESTs were identical to the *in planta* induced genes (PIGs) identified in earlier studies (65). Virus-encoded sequences were identified, providing evidence for two RNA mycoviruses in *U. fabae*. Subsequent microarray experiments revealed many cDNAs that were significantly expressed in rust-infected leaves as compared to germinated urediniospores, suggesting a shift in rust gene expression between germination and the biotrophic stage of development.

To monitor the ascomycete *B. cinerea*, a broad-spectrum plant pathogen, in real-time infection conditions, Gioti and associates (54) infected *Arabidopsis* leaves with *B. cinerea* and assayed transcript accumulation using a custom macroarray. Seven percent of *B. cinerea* genes were differentially expressed during infection, and 27 genes were significantly up-regulated *in planta*. Two of the genes, trichodiene oxygenase and pentalenene synthase, had already been associated with fungal pathogenicity, whereas eight have unidentified functions. The 27 genes were clustered into three groups; the first group showed maximal expression at the early stage following fungal penetration, the second showed maxi-

mal expression at the outset of the colonization of plant leaves, and the third showed maximal expression when the colonization of plant leaves was completed. A gene homologous to FKBP12 proteins was identified from cluster three and was confirmed to be a pathogenicity determinant via gene disruption.

The genomes of several filamentous fungi have recently been completely sequenced, making possible genome-wide expression analysis. Guldener and colleagues took advantage of the genome sequence of *F. graminearum*, the causal organism of *Fusarium* head blight of wheat and barley, to design a whole-genome (18 K) Affymetrix GeneChip (64). To establish a baseline set of gene expression data, *F. graminearum* GeneChips were interrogated with RNA isolated from fungus grown in culture under three nutritional regimes (141), in addition to *in planta* growth in infected barley (12). During the barley infection time course 7132 *Fusarium* probe sets were called present, even though the fraction of fungal transcripts in the total RNA from infected plants is quite low, notably during the early stages of infection.

### Plant-Bacterial Interactions: Effectors and Their Effects

Host transcript profiling in studies of the interactions of plants with bacteria has shed light on plant defense and on processes that result in disease. Partly because many plant-bacterial pathosystems are readily manipulated, studies conducted on these systems also have contributed greatly to our understanding of SAR and induced systemic resistance (ISR) that are important in a broad array of plant pathogen interactions. Profiling of pathogen transcripts has aided in the identification of bacterial virulence factors, and in exploring the effect of plant signals and compounds on pathogen global transcriptional behavior. Functional analysis of specific bacterial effector proteins delivered into the host cell during infection has been empowered by comparative transcript profiling of the host responses

to strains with and without the effectors, owing also to the genetic tractability of bacterial pathogens. A dominant theme from such studies has been the ability of many effectors to suppress defenses in plants triggered early in the interaction by molecules such as flagellin and lipopolysaccharide (LPS), referred to as pathogen-, or more generally, microbe-associated-molecular-patterns (MAMPs). Additionally, in the case of a class of transcription activator–like (TAL) effectors, host targets have been identified that establish new paradigms for effector function and disease resistance.

**Profiling plant *R* gene-mediated responses to pathogenic bacteria.** A number of studies have yielded global views of the transcriptional response of plants undergoing *R* gene–mediated defense against pathogenic bacteria. Mysore et al. (98) utilized GeneCalling technology in tomato to define the gene expression changes in resistance to bacterial speck, and the specific contributions of the *R* gene *Pto* and the gene *Prf*, which is required for *Pto* function. They defined an early role for *Prf* in the response pathway, and also identified changes dependent on *Prf* but not *Pto*, suggesting a distinct, independent role for *Prf* in pathogen recognition. In a subsequent study that used a variety of transcript profiling techniques and resources (including subtractive-suppressive hybridization and cDNA microarrays), it was shown that overexpression of *Pto* induced gene expression changes similar to those observed during immune responses in animals (99). To gain insight into the molecular basis of tomato resistance to *Xanthomonas axonopodis* pv. *vesicatoria* strains expressing the effector AvrRxv, which is governed by three, nondominant resistance genes, Bonshtein et al. used a similar profiling approach (13). Others have obtained global transcript profiles of plants undergoing *R* gene–mediated responses toward identifying genes that might enhance resistance when expressed highly in genetically engineered or selectively bred plants (146).

In a study of interactions of *Arabidopsis* with *P. syringae*, Tao and colleagues (137) examined global gene expression patterns in responses of susceptible plants to a virulent strain (a compatible interaction), responses of plants carrying either of two *R* genes (*Rpm1* or *Rps2*) to corresponding avirulent strains (incompatible interactions), and plants exhibiting non-host resistance to a strain that normally infects bean (incompatible interaction). The analysis showed overall strong similarity among the responses mediated by the two different *R* genes and in nonhost resistance, despite genetically well-defined differences in respective signaling pathways. The study also revealed that the differences among responses in incompatible and compatible interactions were largely quantitative, analogous to observations of Caldo and associates with barley-*Blumeria* interactions (20, 21) and Eulgem and colleagues on *Arabidopsis*-*Peronospora* interactions (48).

**Understanding pathogen induction and counteraction of basal plant defense.** Even in the absence of *R* gene–mediated defense, pathogens encounter basal plant defense triggered by the MAMPs that they present (87). Transcript profiling experiments have elucidated plant basal defense, and are proving useful in revealing how bacterial pathogens overcome it. de Torres and colleagues (36) compared transcript profiles in *Arabidopsis* plants following inoculation with a type III secretion system (T3SS)–deficient (nonpathogenic) strain, an avirulent strain, or a virulent strain of *P. syringae*. The authors defined discriminate responses of the plant to each inoculum and correlated these to well-characterized physiological responses in defense. These data provided an inventory of markers for basal defense and evidence at the level of host gene expression that the virulent strain counteracts basal-defense responses in a T3SS-dependent fashion. The study also clearly indicated that type III effectors do not play a role in the early (up to 2 hai) responses of the plant, and that in the interaction with the

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**LPS:**

lipopolysaccharide

**MAMP:**

microbe-associated-molecular-pattern

**TAL:** transcription activator–like**T3SS:** type III secretion system

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avirulent strain, *R* gene-mediated responses begin within 3 hai and affect gene expression globally.

Desaki and colleagues (39) analyzed gene expression changes in cultured rice cells in response to LPS from pathogenic and non-pathogenic bacterial strains and fungal chitin fragments using an Agilent 22 K rice (60 mer) oligonucleotide microarray. They observed a correlation among gene expression changes elicited by these distinct MAMPS, suggesting that the signaling pathways activated by the respective receptors converge. These changes included up-regulation of defense genes and genes involved in the generation of reactive oxygen. Unexpectedly, and different from the case in *Arabidopsis*, LPS also induced genes implicated in programmed cell death, typical of responses mediated by gene-for-gene interactions.

In a more recent study, Truman and associates (142) extended the results of de Torres and colleagues by examining 2, 4, and 12 hai and by using the Affymetrix 22 K ATH1 GeneChip. They identified the set of genes induced independent of type III effector activity (i.e., prior to 2 hai), and defined type III effector-dependent modulation of host transcript levels (at 12 hai). One fifth of the T3SS-modulated genes were genes induced by the bacteria at 2 hai. This modulation included repression of genes encoding putative extracellular receptors and up-regulation of protein phosphatases, suggesting a T3SS-mediated, coordinated suppression of the plant's pathogen recognition and signaling capacities. The data also provided insight into other possible functions of the T3SS, including enhancing availability of nutrients and water to the pathogen, and enhancing plant resistance to stress, which may play a role in preventing premature death and desiccation of infected tissue. A study by Thilmony and colleagues (138) (published at the same time as the study by Truman and associates) provided a similar view of basal defense induction and repression during infection, noting in particular the abundance of genes encoding tran-

scription factors, signaling proteins, and proteins associated with secretion or present in the cell wall. Further, this study dissected the interrelated changes in transcript abundance mediated by MAMPs, type III effectors, and the phytotoxin coronatine by judicious use of selected mutant strains of *P. syringae*. The authors also recorded early plant responses to *Escherichia coli* and found a strong correlation with basal defense responses elicited by *P. syringae*, again highlighting the conserved response of plants to MAMPs.

### **Connecting the dots in systemic acquired resistance.**

Transcript profiling experiments provide a snapshot or a series of snapshots of global transcript levels. These snapshots can aid in the discovery of genes putatively involved in a process and identification of a set of coregulated genes that may represent part of a regulatory network (88). Yet even from a series of snapshots, it may be difficult to discern hierarchical, cause-and-effect relationships among changes in transcript levels for different genes. Wang and associates (149) overcame this limitation to define regulatory nodes in SAR in *Arabidopsis* by using an elegant experimental set-up. Fusing the transcription cofactor NPR1, essential for SAR, to the glucocorticoid receptor (GR) allowed control over entry of NPR1 into the nucleus. Carefully timed application of the protein translation inhibitor cycloheximide ensured that genes observed to be induced upon NPR1 entry into the nucleus represented direct targets of this factor. They showed that the translocation of NPR1 promoted not only defense gene expression, but also the protein secretion pathway genes required for SAR in *Arabidopsis*. They further discovered that eight members of the WRKY family of transcription factors are directly regulated by NPR1. Using iterative genetic analysis and transcript profiling, they characterized the functions of five of these factors, and determined their positions in the SAR regulatory network. In addition, they used the *ab initio* motif prediction software, MEME,

to identify *TL1*, a conserved *cis*-regulatory element in the upstream sequence of these genes (see section on Promoter analysis). This study highlights the potential for transcript profiling to unravel complex signaling networks that operate downstream of major genes identified through traditional genetics (105).

### Elucidating priming of transcriptional responses in induced systemic resistance.

ISR is a potentiation of defense responses to pathogens due to prior colonization of roots by certain strains of non-pathogenic *Pseudomonas* spp. (rhizobacteria) (30). Rhizobacteria were observed not to induce per se defensive phytoalexin production, but to “prime” the plant for enhanced phytoalexin production (relative to non-ISR-induced plants) in response to subsequent pathogen challenge (144). The generality of this observation with respect to other defenses was supported by transcript profiling of *Arabidopsis* leaves following colonization of roots by an ISR-inducing strain of *Pseudomonas fluorescens* (147). This study showed no consistent response in leaves in the expression of any of approximately 8000 genes assayed prior to pathogen challenge, but markedly enhanced expression of 81 defense-related genes following pathogen inoculation, relative to noninduced, pathogen-challenged plants.

### Identification of candidate virulence factors through pathogen transcript profiling.

As analysis of the host transcriptome has been highly informative, analysis of the pathogen transcriptome has also been fruitful, identifying genes involved in pathogenesis that other methods have failed to uncover. Using such an approach with the causal agent of citrus variegated chlorosis, *Xylella fastidiosa*, de Souza et al. (35) took advantage of attenuation in pathogenicity that takes place in strains of this pathogen following serial passage in axenic culture. By comparing gene expression profiles of a passaged, nonpathogenic

strain with a freshly isolated strain, differences were observed in expression of genes involved in adhesion and environmental adaptations, revealing pathogen attributes potentially important in disease. Microarray analysis of differential gene expression in the soft rot pathogen *Erwinia chrysanthemi* harvested from the apoplast of African violet leaves and compared to the pathogen grown in vitro revealed induction of known and new candidate virulence factors, as well as changes in gene expression likely important in adaptation to the environment in planta (108). In bacteria in which key regulators of pathogenicity have been characterized, transcript profiling of wild-type and regulatory mutant strains has defined the “regulon” consisting of all the targets of the regulator. This approach was used to characterize SalA-regulated genes involved in production of the phytotoxin syringomycin by *P. syringae* pv. *syringae*, a pathogen of bean (86). The same approach, followed by functional characterization by deletion mutagenesis, was used to uncover novel virulence loci and type III effector proteins in *X. axonopodis* pv. *vesicatoria* (106), an important pathogen of pepper and tomato. In this study, the authors took advantage of a mutation that results in a constitutively active form of HrpG, an activator of the hypersensitive reaction and pathogenicity (*hrp*) gene-encoded type III secretion (T3SS).

Microarrays have facilitated identification of type III effectors in *P. syringae* pv. *tomato* also (171), as well as examination of global transcriptional reprogramming that is coordinated with activation of the *hrp* pathway (83). In these studies, the authors took advantage of a *hrp*-inducing growth medium and loss-of-function mutations in the key *hrp* regulators *hrpRS* and *hrpL*. In the latter study, a number of loci with putative T3SS-independent function were found to be *hrp*-activated, indicating potential roles in infection, and several genes involved in common metabolic functions showed *hrp*-dependent down-regulation, reflecting a potential metabolic cost to initiating disease.

**Effector-mediated suppression of plant defenses.** In addition to delineating host defense responses triggered by *R* gene-mediated recognition of type III effectors, transcript profiling has propelled investigation of the virulence contributions of these proteins. Using a custom cDNA microarray, Hauck et al. (69) discovered that AvrPto of *P. syringae* pv. *tomato* suppresses the expression of a suite of genes in *Arabidopsis* putatively involved in a cell-wall based defense response that limits the growth of T3SS-deficient strains. Observed suppression of cell-wall callose deposition and enhancement of population growth of a *brp* mutant of *P. syringae* pv. *tomato* in transgenic plants expressing AvrPto corroborated these transcript profiling results. Later, a role for ethylene signaling in the virulence activity of AvrPto and another *P. syringae* pv. *tomato* effector with similar avirulence activity, AvrPtoB, was demonstrated with the aid of a tomato cDNA microarray (28). As part of a set of experiments to elucidate the virulence function of AvrPtoB, de Torres et al. (36) examined the response to AvrPtoB of several genes identified in previous transcript profiling studies (103) as pathogen induced genes (PIGs) or genes up-regulated in response to the flg22 peptide of flagellin. They found that AvrPtoB effectively suppresses basal but not *R*-gene-mediated defenses. This study exemplifies the value of global transcript profiling in honing in on useful markers for smaller scale analyses. Select genes from earlier global profiling studies, examined by qRT-PCR, were also used as markers in further elucidation of the suppressor activities of AvrPto and AvrPtoB toward MAMP-induced basal defenses in *Arabidopsis* (70). This study demonstrated suppression early in the MAMP response signaling pathway, affecting MAPKKK activation and likely occurring at the membrane.

**Novel effector functions and new types of resistance paradigms.** In addition to suggesting new hypotheses, transcript profiling often provides specific leads to major discov-

eries. This has been the case with respect to functions of the transcription activator-like (TAL) effectors of *Xanthomonas* spp. A cDNA-AFLP experiment in bacterial spot of pepper identified transcripts produced in response to AvrBs3, a TAL effector protein required for induced host mesophyll cell hypertrophy (91). The genes included auxin-induced and expansin-like genes, suggesting a role in hypertrophy development and demonstrating a tight link between effector function and gene induction that suggested active manipulation of host gene expression by the effector.

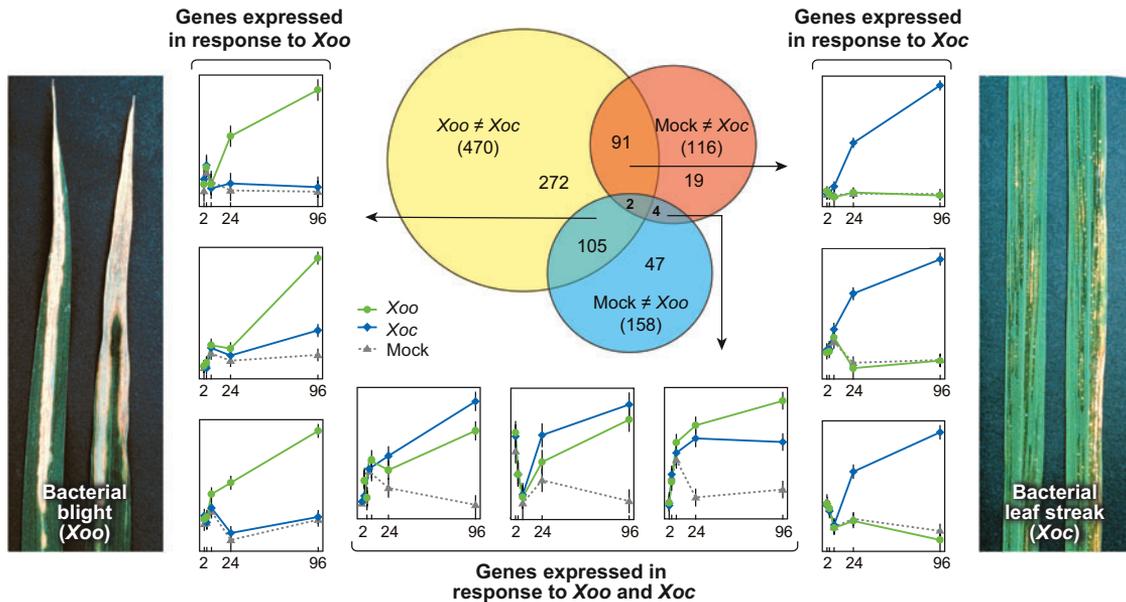
In bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae*, individual members of the large family of TAL effectors contribute to virulence in different strains (160). Host transcript profiles in response to wild-type *X. oryzae* pv. *oryzae* strains were compared to profiles in response to strains with mutations affecting specific TAL effectors or strains expressing heterogenous effectors, with a custom Affymetrix rice GeneChip (63, 159). These comparisons enabled the identification of (potentially direct) transcriptional targets of these bacterial proteins. In the case of effector PthXo1, which is a major virulence determinant in strain PXO99A, *Os8N3*, a rice gene with similarity to a gene involved in nodulation in legumes, was identified as a target (159). Up-regulation of *Os8N3* accounted for the virulence function of PthXo1, and RNA interference in *Os8N3* expression revealed a role for this gene normally in pollen development (159). It was later demonstrated that *Os8N3* is allelic to the recessive *R* gene *xa13* (25), which is not induced during the interaction. This discovery revealed a new paradigm for disease resistance that explains the recessive nature of some *R* genes: lack of inducibility required for full susceptibility.

Another effector-*R* gene interaction operates inversely. The *Xa27* gene for resistance to bacterial blight and its nonfunctional recessive allele *xa27* encode identical proteins. In this case, *Xa27* is activated by the pathogen and provides resistance. Activation is dependent on the effector AvrXa27, an observation

originally gleaned from a global transcription profiling experiment. *Xa27* represents an evolutionary foil to effector-mediated host transcriptional reprogramming that positions a targeted promoter in front of a resistance gene (63).

**Prospects.** As illustrated in **Figure 3**, profiling with the publicly available Affymetrix rice GeneChip representing 51279 transcripts revealed that closely related bacterial pathogens of rice that infect either through the xylem or via invasion of the mesophyll apoplast, causing distinct diseases, trigger markedly distinct patterns of gene expression (D. Niño-Liu, R. Caldo, T. Bancroft, D. Nettleton, R. Wise &

A. Bogdanove, unpublished data). These differences strongly suggest a role for host response in tissue specificity in plant bacterial interactions. This experiment highlights the potential for dissecting the complexities of bacterial pathogenesis of plants by choosing pathosystems tailored to specific lines of inquiry. This type of experiment and others that take full advantage of the genetic tractability of bacterial pathogens, and availability of genomic data for many, suggest that transcript profiling will continue to be a powerful approach for exploratory and hypothesis-driven research to identify host and pathogen genes and gene functions that determine the outcome of bacterial-plant interactions.



**Figure 3**

Patterns of differential gene expression in rice in response to vascular and nonvascular bacterial pathogens. Shown are the results of comparative transcript profiling with the Affymetrix GeneChip® Rice Genome Array of whole rice leaves responding over 96 h to a compatible strain of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which causes bacterial blight by invading the xylem, to a compatible strain of *X. oryzae* pv. *oryzicola* (*Xoc*), which causes bacterial leaf streak by colonizing the mesophyll apoplast, or to a mock inoculum. The Venn diagram includes probe sets that were significantly different ( $q \leq 0.3$ ) after a mixed linear model analysis of pairwise comparisons and their interaction over time and shows the total numbers of probe sets representing genes expressed uniquely in response to *Xoo* or *Xoc*, or in response to both *Xoo* and *Xoc*. Patterns of expression in response to the three treatments are shown for three representative genes in each of these classes. Values represent least-squares means of signal intensities from four independent repetitions. Y-axis, gene expression level; x-axis, time. Standard error is indicated by vertical bars.

## Plant-Virus Interactions

### Compatible viruses induce plant defense and stress responses.

Transcriptome analyses of virus-host interactions have mostly focused on the general question of the effects of viral infection on the mRNA transcript abundance of the susceptible host [see (156) and references therein]. Direct comparisons of microarray data from host-virus interactions are complicated by the use of different hosts, various technology platforms, and a lack of studies involving extensive time courses, but some interesting themes have nevertheless emerged with respect to both induced and suppressed genes. The first is that compatible viruses, like bacteria and fungi, induce responses resembling basal plant defenses (128, 156), but the recognition mechanism of this interaction is likely different from that of bacteria and fungi. Defense-like responses of compatible hosts to viruses are dependent on SA and require upstream signaling components such as *EDS1* (72). Plant defense genes induced by *Oilseed rape mosaic virus* (ORMV; genus *Tobamovirus*) and *Cucumber mosaic virus* (CMV; genus *Cucumovirus*) had differing requirements for *NPR1*, which functions downstream of SA. Increased expression of most defense-related genes was not dependent upon *NPR1* although the levels of many were reduced in an *npr1* mutant. Virus-induced expression of a few genes, especially *PR-1*, was absolutely dependent upon *NPR1*. Thus, compatible plant viruses induce defense-like responses through a SA-dependent pathway that is largely independent of *NPR1*, with the exception of *PR-1* and a few other genes. Also related to defense and stress, a spectrum of heat shock proteins is induced by diverse viral infections (124, 155), by an as-yet unknown mechanism that is independent of SA (23).

### Down-regulation of genes with potential roles in plant growth and development.

A more recent theme is the preferential down-regulation of cell wall modification genes

and plastid genes (128, 161). Two unrelated viruses, *Turnip mosaic virus* (TuMV, genus *Potyvirus*) and *Rice dwarf virus* (RDV, genus *Phytoreovirus*), were shown to cause a decrease in the expression of genes with known or potential function in the expansion of cell walls in *Arabidopsis* and rice, respectively. The predicted proteins have functional annotations including xyloglucan endotransglycosylase/hydrolase (XTH), pectin methyl esterase (PME), and expansin (EXP). Because the expression of such genes is positively correlated with plant cell growth and expansion, their decreased expression may well be directly related to the stunted growth of infected *Arabidopsis* and rice plants. The reduced expression of genes annotated as having functions in plastids and in particular chloroplasts is also intriguing, because this is expected to underlie the chlorotic symptoms that frequently accompany viral infections. The fact that expression of these genes is decreased in a monocot and dicot host in response to two very distinct viruses identifies another potentially conserved property of viral infections. Studies are needed that address the mechanism(s) responsible for decreased expression of these genes. Potential mechanisms include interference with plant hormone biosynthesis and signaling (57, 109, 110) as well as the activities of pathogen-induced small RNA species and viral RNA silencing suppressors (81, 102).

### Spatial analysis of host responses to viral infection using a microarray approach.

The analysis of host responses to viral infection is complicated by the obligate intracellular nature of these pathogens. Viruses are frequently introduced into hosts through mechanical or vector-mediated inoculation procedures that make it impossible to predict where the initially infected cells might be. Symptoms may be used to track the progress of the virus, but they occur long after the viral infection has been initiated. Thus, normal sampling procedures involve grinding up whole leaves, resulting in loss of spatial information and dilution of interesting early

gene expression changes. To overcome this problem, in part, Yang et al. (161) used an infectious clone of TuMV tagged with GFP (TuMV-GFP) to identify infection foci and specifically dissect them away from noninfected tissue. As shown in **Figure 4**, four zones were defined macroscopically based on the size of the infection foci at 5 days after inoculation and the 1.2 mm diameter of a micropunch. Zone 0 was at the center of the foci, zone 1 was near the periphery, and zone 2 was next to the periphery. Zone 3 was not directly in contact with any GFP fluorescent cells and contained nearly undetectable levels of virus. Using this strategy they observed that defense- and stress-like responses were localized to infection foci and generally occurred in proportion to the amount of virus accumulation. There was very little evidence for altered host gene expression in advance of the TuMV-GFP infection front.

This sampling strategy enabled new sets of up- (ribosomal proteins, protein turnover) and down-regulated genes (cell wall modification proteins described in the previous section) to be identified that had not been previously observed, presumably because the dissection enriched for cells at similar stages of infection and undergoing similar host responses. The infection of *Arabidopsis* by TuMV also induces the mRNA expression of at least one homolog of nearly all ribosomal proteins belonging to either the 40S or 60S subunits (161). Whether this reflects a generally increased demand on the host cells for protein synthesis or a specific response initiated by TuMV to enhance its pathogenicity remains to be determined. Investigation into mechanisms by which viruses modify translation within susceptible hosts will be an interesting area for future study.

### **Plant-Nematode Interactions: What's Mine Is Mine; What's Yours Is Mine**

Plant parasitic nematodes present some unique questions regarding mechanisms of pathogenesis and plant defense. They en-

gage in highly localized interactions with their hosts that result in the development of specialized cells at the feeding sites. Host cells are transformed into giant cells in root knot nematode (RKN) infections or into syncytia in cyst nematode (CN) infections. The molecular mechanisms underlying this dramatic reprogramming of root cell fate to acquire functions that benefit nematodes are not well understood at this time, and these events are good targets for expression profiling studies. *Arabidopsis* was utilized to profile host responses to beet cyst nematode (BCN; *Heterodera schachtii*) and root-knot nematode (RKN; *Meloidogyne incognita*) and nonhost responses to soybean cyst nematode (SCN; *H. glycines*) (67, 78, 114, 157). BCN and RKN both establish characteristic feeding sites and complete their life cycles in *Arabidopsis* roots, whereas SCN initially penetrates *Arabidopsis* roots but fails to successfully establish a feeding site. Soybean microarrays have been used to profile soybean responses to SCN infections (3, 75). Ithal and colleagues used the Affymetrix soybean/*P. sojae*/*H. glycines* GeneChip to simultaneously assay 37,500 soybean and 7431 SCN transcripts, measuring mRNA transcript abundance of both host and pathogen (75). The distinct morphology of the SCN feeding cells has also enabled the application of laser capture microscopy (LCM) to isolate syncytia for gene expression analyses (76). The increasing numbers of studies are revealing common host responses as well as distinct mechanisms of pathogenesis between CN and RKN, some of which are highlighted in the following sections.

**Cell walls come unglued.** One similarity is the degree to which cell wall modification proteins are differentially regulated by nematode infection. The cell wall modification proteins include expansins (EXPs), pectin esterases (PEs), xyloglucan endotransglycosylase/hydrolases (XTHs), and extensins (EXTs). Genes with these annotations are primarily up-regulated in susceptible BCN, SCN, and RKN interactions, although there

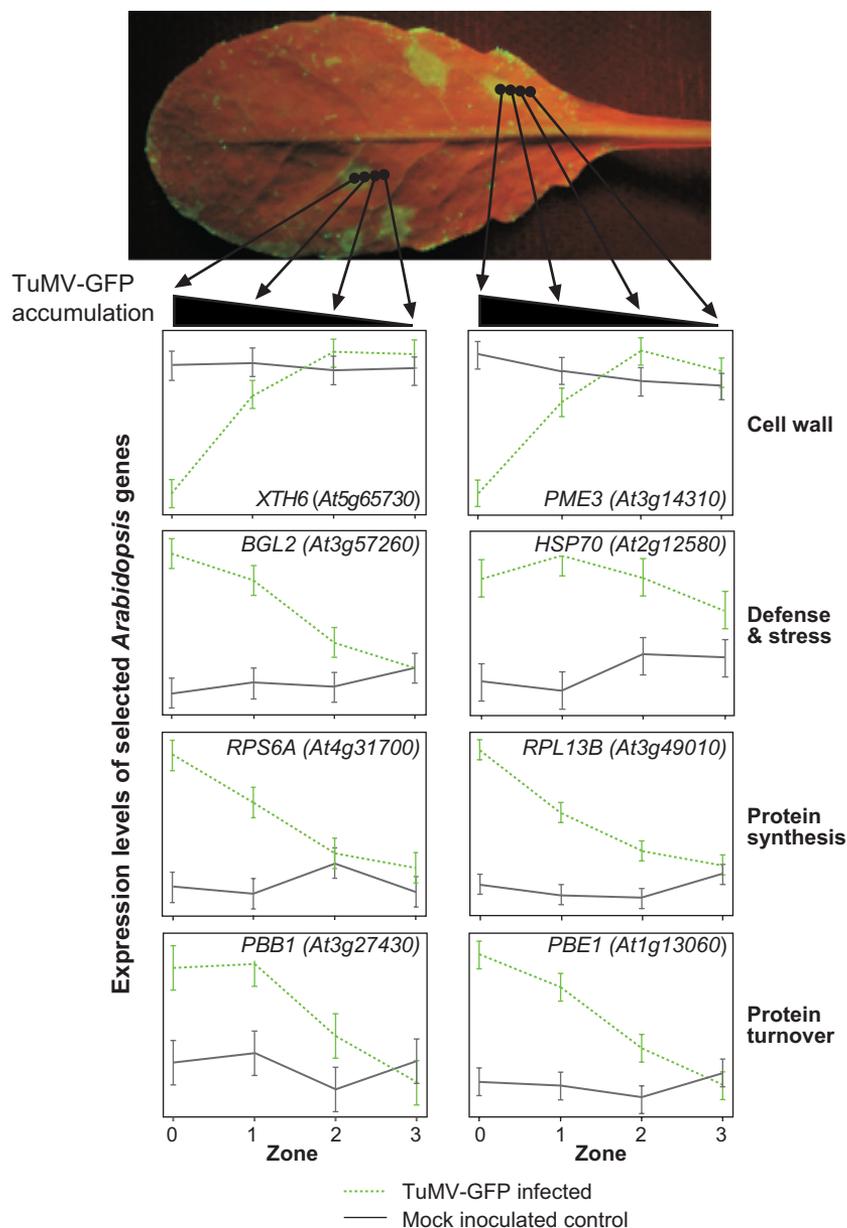
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**RKN:** root knot nematode

**BCN:** beet cyst nematode

**SCN:** soybean cyst nematode

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**Figure 4**

Gene expression profiling in dissected TuMV-GFP infection foci. The top panel illustrates TuMV-GFP infection foci at 5 days after inoculation and how the foci were dissected into four zones for gene expression analyses (161). The accumulation of TuMV-GFP across the four zones is indicated by the arrowheads pointing to the solid triangles. The plots represent gene expression profiles of eight different genes representing four distinct functional classes of plant genes across the four zones in mock-inoculated (*solid lines*) and TuMV-infected (*green, dashed lines*) samples. The functional classes are indicated to the right of each pair of representative genes. Zone 0 represents the region of highest virus accumulation and greatest change in expression of most host genes, whereas zone 3 represents the lowest virus accumulation and least change in gene expression for most host genes.

is some differential regulation among the various family members (75, 76, 78, 157). The increased expression of these cell wall modification proteins is usually positively correlated with increased cell growth. A detailed characterization of the expression of the 32-member *Arabidopsis* expansin gene family was performed for BCN (157). This study combined microarray analyses, semiquantitative RT-PCR, PCR screening of a cDNA library of 5–7-day-old syncytia, and promoter GUS fusions. The authors used the Affymetrix ATH1 GeneChip much in the way an RNA gel blot was used in the past to examine the expression patterns of a specific set of genes. The biological samples used for microarray analyses were derived from microaspirated syncytial cytoplasm that enriched for expansin expression in syncytia. These analyses showed that at least 10 of the 32 gene family members were differentially regulated at 5 and/or 15 dai (8 up, 2 down) and identified candidate genes for further functional analyses.

**Plant defense responses to nematodes.** An interesting distinction between CN and RKN revealed by comparison of different microarray experiments is the differential induction of plant defense-related genes. Pathogenesis-related genes, glutathione S-transferases, and other defense-associated genes, such as members of the phenylpropanoid pathway, are induced by CNs in their susceptible hosts. For example, LCM analysis of SCN feeding sites demonstrates that phenylpropanoid pathway genes are induced early in the infection (0 to 2 dai); however, later in infection (10 dai), their expression is not significantly different from that of the control cells (76). By contrast, in RKN infection of *Arabidopsis*, expression of these genes remains unchanged or becomes down-regulated, suggesting a potential ability of the RKNs to suppress host defense responses (78). CNs migrate through cells, whereas RKNs migrate between cells. Although the much less destructive intercellular migration path of RKN suggests a reason why they do not cause induc-

tion of defense genes, their down-regulation suggests active suppression of host defense responses. Jammes and colleagues (78) found that 17 of 21 WRKY transcription factors were down-regulated as were genes involved in phenylpropanoid biosynthesis and the defense regulatory *PAD4* lipase. The nonhost interaction between *Arabidopsis* and SCN was not associated with an induction of defense-related mRNA transcripts, suggesting that well-characterized defense responses are not involved in preventing SCN from successfully infecting *Arabidopsis* roots (114).

### **Nematode feeding sites as nutrient sinks.**

Similar to the biotrophic powdery mildews, nematodes up-regulate suites of genes that are predicted to make the feeding site a sink for nutrient up-take and utilization. Two functional groups of genes that are highlighted here are host ribosomal proteins and transporter proteins. RKN infection is accompanied by a dramatic increase in the mRNA abundance for ribosomal proteins belonging to either the 40S or 60S rRNA subunits. Increased mRNA accumulation of these genes was observed at 7 dai, which was the earliest time point used in that study (78). The up-regulation of the ribosomal proteins indicates a substantial increase in capacity for protein synthesis and would be consistent with the dramatic reprogramming that occurs within the host cells.

Proteins involved in transport across host plasma membranes have also been identified as differentially regulated in both CN and RKN infections (67, 76). Hammes et al. (67) used the *Arabidopsis* ATH1 GeneChip for a targeted analysis of the expression of most known or predicted transporter proteins in response to RKN. Interest in the transporter proteins derives from the theory that the giant cells are symplastically isolated from their neighboring cells and thus the expression of genes encoding transporter proteins would be predicted to be dramatically altered. In this experiment, reliable expression measurements were obtained for 634 of the estimated 805

transporter genes represented on the ATH1 GeneChip. Statistical analyses identified 50 transporter genes that were differentially expressed (FDR = 0.2,  $p < 0.016$ ), with 20 being down-regulated at 1, 2, and 4 weeks after inoculation (wai); 15 up-regulated at all three times; and 15 up at 1 and/or 2 wai and down at the subsequent time point(s). One interesting trend observed in this study was the induction of amino acid transporters. The sucrose transporter *AtSUC1* was also induced in giant cells, suggesting a mechanism for accumulation of the osmolyte, sucrose. The induction of sucrose and amino acid transporters has interesting implications, because it suggests that nematodes actively alter host cells to enhance their nutritive qualities. Hammes and associates (66) went on to characterize a particular amino acid transporter, *AtCAT6* (At5g04770). This gene was not identified in their list of 50 genes in the 2005 microarray study, but nevertheless is significantly induced by RKN. An inspection of the 2005 data demonstrated that *AtCAT6* was differentially expressed in that experiment, and would have been detected if less stringent statistical criteria had been used. However, lowering the statistical stringency is accompanied by the problem of increasing the number of false discoveries. This observation demonstrates the utility of analyzing and reanalyzing data sets using different statistical criteria, which could also be coupled with other expression pattern analysis based on a prototypical gene of a particular functional category. To examine the function of *AtCAT6* in the *Arabidopsis*-RKN interaction, two alleles of *AtCAT6* containing T-DNA insertions were tested for their effects on RKN infection, but were found to have none. Hammes and associates (66) speculated that other amino acid transporters compensated for the *AtCAT6* deficiency or that there are some other redundant functions that supply giant cells with adequate amounts of amino acids. Combining the *atcat6* mutants with combinations of mutations in other amino acid transporters will be valuable in addressing their requirement in nematode feeding.

**Profiling nematode gene expression within the host.** Because the Affymetrix Soybean GeneChip contains both SCN and soybean probe sets, Ithal et al. (75) were able to examine the changes in SCN mRNA transcript abundance over their infection time course. One particularly interesting observation was the decreasing expression of most parasitism genes that are expressed in the esophageal gland over the infection time course. Most of the down-regulated parasitism genes may only be needed early in the infection process such as for migration and initiation of the feeding site. For example, SCN-encoded cell wall modification proteins are presumably needed early to aid in migration. The microarray studies also informed new functional annotation to genes not previously associated with the esophageal gland or parasitism, i.e., down-regulated genes that shared expression profiles with genes known to be expressed in the esophageal gland and possessing amino-terminal signal peptides could be new parasitism genes.

## **FOLLOW-UP ANALYSIS: WHAT CONSTITUTES VALIDATION?**

### **I Think that Multiple Genes Are Being Expressed: How Do I Convince Myself or An Independent Reviewer?**

Parallel expression profiling experiments have characteristics similar to a large field plot—relatively few observations made on thousands of samples (genes). Variation may derive from multiple and diverse sources. Thus, when expression profiling data alone are used to deduce changes in cellular processes and pathways on a large scale and draw conclusions about the biology of plant-pathogen interactions, careful design (blocking, randomization, and replication) and rigorous statistical analysis are essential (93, 104). If those requirements are met, it is not necessary to further confirm the expression data by using a different technique such as quantitative

RT-PCR or RNA blot analysis. This is particularly true for the many high-quality, commercially available array platforms.

Even if the objective is to survey the landscape and pick candidate genes for functional analysis, by mutation, overexpression, or gene silencing, confirmation of the expression data is not necessary if the experiment is designed and analyzed appropriately. In this scenario, functional characterization of the candidate genes is the ultimate validation of the expression profiling results.

The often inferred necessity of technically validating microarray results is likely a carry-over from when the field of expression analysis was new and based on often quite expensive technology. Investigators were limited in their ability to carry out statistically rigorous experiments due to prohibitive costs of replication, which led to the need to establish that observations were real. Even so, performing a technical replication via qRT-PCR or RNA blot analysis on 0.01% of the genes extracted from a microarray analysis ultimately only confirms data for the selected genes. As more robust platforms are now available, and investigators understand the value of, and can implement, statistically based design and replication, it is generally accepted that microarray data are reliable, and in line with results based on qRT-PCR or RNA gel blot analysis (12, 130). When confirmation is desired (or requested), a practical alternative to further experimentation is to survey existing expression resources, such as EST libraries, microarray databases, and high-throughput sequence data from independent, published experiments (**Table 1**) (12, 21, 56) (see **Figure 2**). Like qRT-PCR, database mining can, and should, be used for corroborating differential expression, although this is not the same as determining function (26). Care should also be taken in the interpretation of gene annotations. Probe sequences are based on the information available at the time of microarray design. This could be based on fully sequenced genomes, such as *Arabidopsis* or rice (plants) or *Magnaporthe*, *Fusarium*, *Phytophthora*, or *Puccinia*

(pathogens). Alternatively, the arrays could be based on assembled contigs from Expressed Sequence Tags (ESTs) or on spotted cDNAs. It is important to understand the benefits and limitations of the chosen platform. Since uncharacterized homologous gene sequences may bias the interpretation when using arrays from organisms without complete genome sequence, candidate genes also need to be confirmed for specificity and accuracy.

## Functional Analysis

Functional characterization of the candidate genes can be accomplished by genetic mutation, over expression, or gene silencing (6, 22, 26, 42, 71, 123, 125). A number of resources needed for reverse genetic and functional analysis of candidate genes are now available for both plants and pathogens. These resources range from knockout lines derived from T-DNA or transposon insertions to full-length cDNA, dsRNA constructs or lines, overexpression lines, and TILLING populations. In addition, sequenced genomes can serve as a reference for many crops, which allow for the identification of key proteins in defense. Translational genomics has become a practical approach, allowing for the use of synergistic experimental approaches that integrate knowledge from both model and crop organisms to propel functional analysis of plant-pathogen interactions.

For crops that may prove difficult and time-consuming to transform, transient methods may be used, such as virus-induced gene silencing (VIGS), single cell gene silencing (TIGS), and overexpression (42). These techniques are amenable to functional analysis of large numbers of genes and have an advantage over stable mutations or transgenic plants in cases when constitutive loss or gain of gene function is lethal. The potential for high-throughput functional analysis by VIGS was illustrated by Lu and colleagues (85). They screened 4992 cDNAs from a normalized library and found 79 silencing constructs that suppressed *Pto*-mediated

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**VIGS:**  
virus-induced gene silencing

**TIGS:** single cell gene silencing

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**Table 1 Expression profiling resources**

Resource	URL	Description
Experimental Design Guidelines		
Microarray Gene Expression Data Society–MGED Society	<a href="http://mged.org/">http://mged.org/</a>	International organization of biologists, computer scientists, and data analysts that aims to facilitate the sharing of microarray data
MIAME	<a href="http://mged.org/Workgroups/MIAME/miame.html">http://mged.org/Workgroups/MIAME/miame.html</a>	Acceptable standards for minimum information about a microarray experiment
Microarray Repositories		
ArrayExpress	<a href="http://www.ebi.ac.uk/arrayexpress/">http://www.ebi.ac.uk/arrayexpress/</a>	Public repository for microarray data
GEO	<a href="http://www.ncbi.nlm.nih.gov/geo/">http://www.ncbi.nlm.nih.gov/geo/</a>	Gene Expression Omnibus: a gene expression/molecular abundance repository
Microarray and Genome Databases		
PLEXdb	<a href="http://plexdb.org/">http://plexdb.org/</a>	Unified public resource for gene expression for plants and plant pathogens
Gramene	<a href="http://gramene.org/">http://gramene.org/</a>	Resource for comparative grass genomics
PlantGDB	<a href="http://plantgdb.org/">http://plantgdb.org/</a>	Cyberinfrastructure for plant (comparative) genomics
The Institute for Genomic Research (TIGR)	<a href="http://plantta.tigr.org/">http://plantta.tigr.org/</a>	Plant transcript assemblies
The Institute for Genomic Research (TIGR)	<a href="http://cpgr.tigr.org/">http://cpgr.tigr.org/</a>	Comprehensive phytopathogen genomics resource
NASCArray	<a href="http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl">http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl</a>	Nottingham <i>Arabidopsis</i> Stock Centre's microarray database
Integrated Microarray Database System (IMDS)	<a href="http://ausubellab.mgh.harvard.edu/imds/">http://ausubellab.mgh.harvard.edu/imds/</a>	<i>Arabidopsis</i> 2010 expression profiling of plant disease resistance pathways
MGOS	<a href="http://www.mgosdb.org/">http://www.mgosdb.org/</a>	<i>Magnaporthe grisea</i> <i>Oryza sativa</i> Interaction database
NSF Rice Array	<a href="http://www.ricearray.org/index.shtml">http://www.ricearray.org/index.shtml</a>	Rice Oligonucleotide Array Project
NSF Maize Array	<a href="http://www.maizearray.org/">http://www.maizearray.org/</a>	Maize Oligonucleotide Array Project
RED	<a href="http://cdna02.dna.affrc.go.jp/RED/">http://cdna02.dna.affrc.go.jp/RED/</a>	Rice Expression database
Rice Pipeline	<a href="http://cdna01.dna.affrc.go.jp/PIPE/">http://cdna01.dna.affrc.go.jp/PIPE/</a>	A unification tool which dynamically collects and compiles data from the National Institute of Agrobiological Sciences (NIAS)
Yale Virtual Center for Cellular Expression Profiling of Rice	<a href="http://130.132.8.83/rc/overview.jsp">http://130.132.8.83/rc/overview.jsp</a>	Whole-genome transcriptional profiles of rice cell types, isolated by laser-capture microdissection
NSF Potato Functional Genomics	<a href="http://www.tigr.org/tdb/potato/">http://www.tigr.org/tdb/potato/</a>	Consortium focused on understanding potato functional genomics
Tomato Expression Database	<a href="http://ted.bti.cornell.edu/">http://ted.bti.cornell.edu/</a>	Tomato microarray expression data and tomato digital expression data
University of Toronto Botany Array Resource	<a href="http://bbc.botany.utoronto.ca/welcome.htm">http://bbc.botany.utoronto.ca/welcome.htm</a>	User-friendly web-based tools for working <i>Arabidopsis</i> functional genomics data
AtGenExpress	<a href="http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm">http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm</a>	International coordinated effort to build an <i>Arabidopsis</i> transcriptome atlas
<i>Arabidopsis</i> 2010: Functional Genomics of Quantitative Traits	<a href="http://elp.ucdavis.edu/">http://elp.ucdavis.edu/</a>	Expression level-polymorphisms of QTLs affecting disease resistance pathways in <i>Arabidopsis</i>

(Continued)

**Table 1 (Continued)**

Resource	URL	Description
<i>Sinorhizobium meliloti</i> Genome Project	<a href="http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/">http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/</a>	Data and annotation for Gram-negative soil and rhizosphere bacterium
MIPS <i>Fusarium graminearum</i> Genome Database	<a href="http://mips.gsf.de/projects/fungi/Fgraminearum.html">http://mips.gsf.de/projects/fungi/Fgraminearum.html</a>	Data and annotation for the causal agent of head blight (scab) of wheat and barley
Analysis Tools		
Bioconductor	<a href="http://www.bioconductor.org/">http://www.bioconductor.org/</a>	Open source project that provides comprehensive tools for microarray data analysis, visualization, and annotation
R	<a href="http://www.r-project.org/">http://www.r-project.org/</a>	Integrated suite of software for data manipulation, calculation, and graphical display
Genevestigator	<a href="https://www.genevestigator.ethz.ch/at/">https://www.genevestigator.ethz.ch/at/</a>	<i>Arabidopsis</i> microarray database and analysis toolbox
Expression Profiler	<a href="http://ep.ebi.ac.uk/EP/">http://ep.ebi.ac.uk/EP/</a>	Analysis and clustering of gene expression data
MPSS Database	<a href="http://mpss.udel.edu/">http://mpss.udel.edu/</a>	<i>Arabidopsis</i> , rice, grape, and <i>Magnaporthe</i> RNA signatures
SIGNAL: Salk Institute Genomic Analysis Laboratory	<a href="http://signal.salk.edu/cgi-bin/RiceGE">http://signal.salk.edu/cgi-bin/RiceGE</a>	RiceGE: Rice Functional Genomics Database
Signal Search Analysis server at ISREC	<a href="http://www.isrec.isb-sib.ch/ssa/ssa.html">http://www.isrec.isb-sib.ch/ssa/ssa.html</a>	
Plant Cis-acting Regulatory DNA Element Database	<a href="http://www.dna.affrc.go.jp/PLACE/signalup.html">http://www.dna.affrc.go.jp/PLACE/signalup.html</a>	PLACE contains known <i>cis</i> -acting regulatory elements and tools for counting known motifs in a sequence
SoftBerry	<a href="http://www.softberry.com">http://www.softberry.com</a>	Bioinformatic software for the analysis of DNA and protein sequences
MotifSampler	<a href="http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html">http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html</a>	Looks for overrepresented sequence strings as compared to a random group
GLAM	<a href="http://zlab.bu.edu/glam/">http://zlab.bu.edu/glam/</a>	Processes data sets iteratively and eliminates incorrect results inherent in stochastic methods
MEME	<a href="http://meme.sdsc.edu">http://meme.sdsc.edu</a>	Multiple Expectation Maximization (EM) for Motif Elicitation
MapMan	<a href="http://gabi.rzpd.de/projects/MapMan/">http://gabi.rzpd.de/projects/MapMan/</a>	User-driven tool that displays large data sets onto diagrams of metabolic pathways or other processes

resistance against *P. syringae* in *Nicotiana benthamiana*. This study implicated HSP90 in plant disease resistance. If a suitable vector is available, VIGS is an effective strategy for crop plants for which a large collection of knockouts does not exist. Dong and associates (41) used the TIGS strategy to screen 389 candidate genes that were found to be up-regulated in host basal defense (167). Of these, 5 of 16 RNAi constructs target-

ing different polyubiquitin proteins enhanced susceptibility to *B. graminis hordei* in barley epidermal cells. Rescue mediated by synthetic monoubiquitin proteins in overexpression constructs implicated an ubiquitination mutant that is blocked in several ubiquitination pathways but retains proteasomal protein degradation. These systems permit the interrogation of microarray-generated hypotheses on a large scale, because they are not limited

by the need to generate and maintain transgenic plant lines.

## OPPORTUNITIES AND POSSIBILITIES

### Meta-Analysis and Comparative Analysis

Analyzing results in the context of similar published work is the logical next step after any experiment. The availability of transcriptional data and tools permits the analysis of a variety of data sets, which may be useful for the generation or validation of hypotheses. As detailed in **Table 1**, research communities have developed on-line tools that greatly facilitate access to the available large data sets.

A recent focus, meta-analysis, involves surveying microarray data across experiments to find the true effect of a treatment across different studies (20, 24, 97, 116, 117). This type of analysis may be particularly useful with standardized oligonucleotide microarrays, where methods and data structures are consistent. By comparing the results of experiments across several labs using the signal log ratio of differentially expressed genes, Stevens & Doerge (131) showed that the combined estimates were more accurate than the estimates from individual experiments. In *Arabidopsis*, the meta-analysis tool at Genevestigator gives the biological researcher access to similar analyses (169) (see section on Expression Databases).

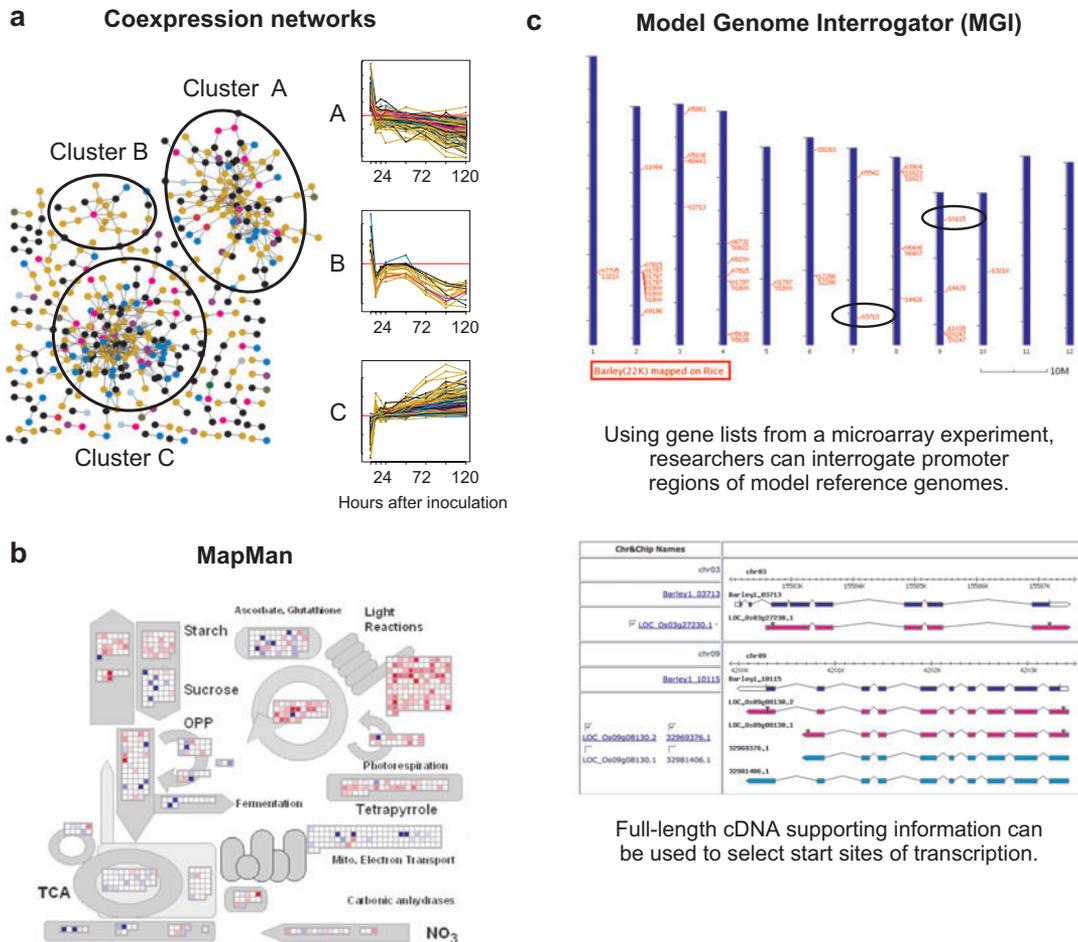
Software that integrates biological information into the analysis of microarray data has become popular recently with the observation that functionally related genes are coexpressed (152). Here we describe three analytical tools that group biologically related genes to increase the power of the analysis. MapMan uses functional “bins” as a tool for the analysis of genes (**Figure 5b**) (139). These hierarchical categories allow for biologically relevant patterns to emerge that might have otherwise been overlooked. Pathways and precomputed mapping files exist for the user to immedi-

ately view their data within biologically relevant groupings. Data can be transcriptional, metabolomic, or proteomic. Gene Set Enrichment Analysis (GSEA) is a less restricted approach that allows the user to group genes based on biological function, chromosomal location, or regulation, to discover patterns that may be overlooked by testing individual gene expression (134). Finally, Wei and associates demonstrated the power of integrating metabolic pathway membership by using pathway-level correlation (PLC) analysis to show that genes within the same pathway are more highly coexpressed with one another (152).

Comparative coexpression analysis extends coexpression analyses within an organism by integrating expression data from multiple organisms (11, 133). *Arabidopsis* has a large set of pathology-related microarray experiments and many genes with unknown function. As shown in **Figure 5a**, gene-coexpression analysis can be used to determine significant patterns that are conserved across a diverse set of conditions, time courses, or taxa. Taking advantage of comparative analyses with similar pathological systems may reveal novel, conserved modes of resistance (**Figure 2**). For example, investigations by Collins and colleagues (29) have raised interesting questions regarding the degree of conservation between monocot and dicot defense; their work highlights the opportunities of using comparative coexpression analysis to study plant-pathogen interactions.

### Promoter Analysis

Promoter analysis can follow any large array experiment conducted in an organism whether promoter sequences are available or not. Investigators first need to determine which of the differentially expressed genes associated with a particular phenomenon are coregulated. The promoter regions of genes with similar expression profiles may contain conserved sequence motifs that explain their coordinated regulation. Methods that



**Figure 5**

Bioinformatic tools for the analysis of microarray data. (a) Gene-coexpression network generated from the *Arabidopsis-E. orontii* experiment described in **Figure 2a**. Nodes and edges represent probe sets and Pearson correlation of at least 0.99, respectively. Graphs of major clusters in the time course are shown on the right. (b) A snapshot of MapMan displaying a NASCArrays Ref. #330 from *Arabidopsis* treated with *P. syringae* pv. *tomato* DC3000 at 12 hai vs mock (37, 139). (c) Model Genome Interrogator at PLEXdb (<http://plexdb.org/>) allows a user to enter a list of probe sets derived from any GeneChip experiment, map the positions onto the sequenced genomes of *Arabidopsis* or rice, visualize gene models and spliced alignments, and retrieve FASTA outputs of genome sequence of 5' and 3' regulatory regions, or specific exons and introns for input into motif-finding software (H. Lu, personal communication; 143). Genes derived from the shikimate pathway in **Figure 2c** are illustrated.

are commonly used for clustering genes by their expression profiles are K-means, self-organizing maps (SOMs), and hierarchical clustering (4, 166). Once clustered, promoter sequences can be analyzed *in silico* via promoter-specific motif-finders, such as the Signal Search Analysis server at ISREC,

FINDPATTERNS program of the Genetics Computer Group, the PLACE database of published motifs found in plant *cis*-acting regulatory DNA elements, or NSITE-PL for the recognition and statistical analysis of plant regulatory motifs (**Table 1**). Other programs, such as MEME, Clover, or TOUCAN2, can

**SOM:**  
self-organizing map

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**SFP:** single feature polymorphism

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be used for the prediction of novel motifs or the analysis of overrepresentation of known motifs (2, 8, 51).

More in-depth analysis may involve an *ab initio* motif-finding program that looks for over-represented sequence strings as compared to a random group such as MotifSampler or GLAM. GLAM, in particular, will iteratively process data sets and eliminate incorrect results inherent in stochastic methods. Additionally, GLAM will eliminate problem sequences, whereas some other *ab initio* programs will force a sequence to fit, resulting in an incorrect identification. Comparative analysis of promoter and other non-coding regions of orthologous genes from closely related species is a powerful approach to motif identification and to find conserved regulatory elements (74).

Maleck and colleagues (88) describe a number of distinct gene clusters that were highly correlated. Promoter analysis was performed on two of the seven clusters, where they observed overrepresentation of WRKY and underrepresentation of TGA transcription factor protein binding sites. For organisms without genome sequence, Model Genome Interrogator at PLEXdb provides mappings for crop plants on to model organisms (**Figure 5c**) (158). Intron, upstream, and downstream sequences can then be extracted based on the model plant's gene model.

### Transcript-Based Cloning

In addition to assessing global gene expression profiles in response to pathogen challenge, DNA microarrays can be used directly to clone genes that are important in host-pathogen interactions. Transcript-based cloning is a high-throughput approach for the identification of mutant alleles via the hybridization of wild-type and mutant mRNA to a microarray. Expression values that are drastically lower in the mutant than in wild type, such as those caused by deletion mutations, are the easiest to characterize (95, 164). Even a single nucleic acid mutation

may be detected, as it could cause loss of expression or alter the stability of transcripts via nonsense-mediated decay (148). An alternative approach, microarray-based cloning, uses genomic DNA hybridization and is practical for plants with small genome sizes and where mutant alleles dramatically alter the transcriptional landscape, creating too many candidates for transcript-based cloning (59).

An early demonstration of transcript-based cloning in plants was the cloning of *DMI3*, a  $Ca^{2+}$ /calmodulin-dependent protein kinase of *Medicago* (96). In barley, Zhang and associates (165) used transcript-based cloning to find a large region containing *Rpp1*, a gene required for *Rpp1*-dependent resistance to stem rust, using mutants derived from fast-neutron bombardment. Fast-neutron mutants are well suited for transcript-based cloning, because the genetic lesions are typically large deletions that increase the likelihood of detecting expression knockouts (5). Transcript-based cloning can be performed with any microarray, but Affymetrix GeneChips, due to their design features, can be particularly sensitive, often allowing detection of single feature polymorphisms (SFPs) and expression level polymorphisms (ELPs) (also see section below) (33, 111, 153).

Candidate genes identified through transcript-based cloning can be characterized further, first by cosegregation analysis, and subsequently with transformation, allelic mutants, or transient assays. Complementation of mutant phenotypes with candidate genes via *Agrobacterium* transformation provides strong evidence and could be used in a screening approach for mutants with large deletions. For mutant plants with large deletions, which are not amenable to *Agrobacterium* transformation, comparative genomics and BAC sequencing can be used with a transient silencing system, such as TIGS (42) or VIGS (71, 123), to characterize candidate genes. When several allelic mutants are available, especially in EMS (ethane methyl sulfonate) and sodium azide-induced mutations, sequencing of

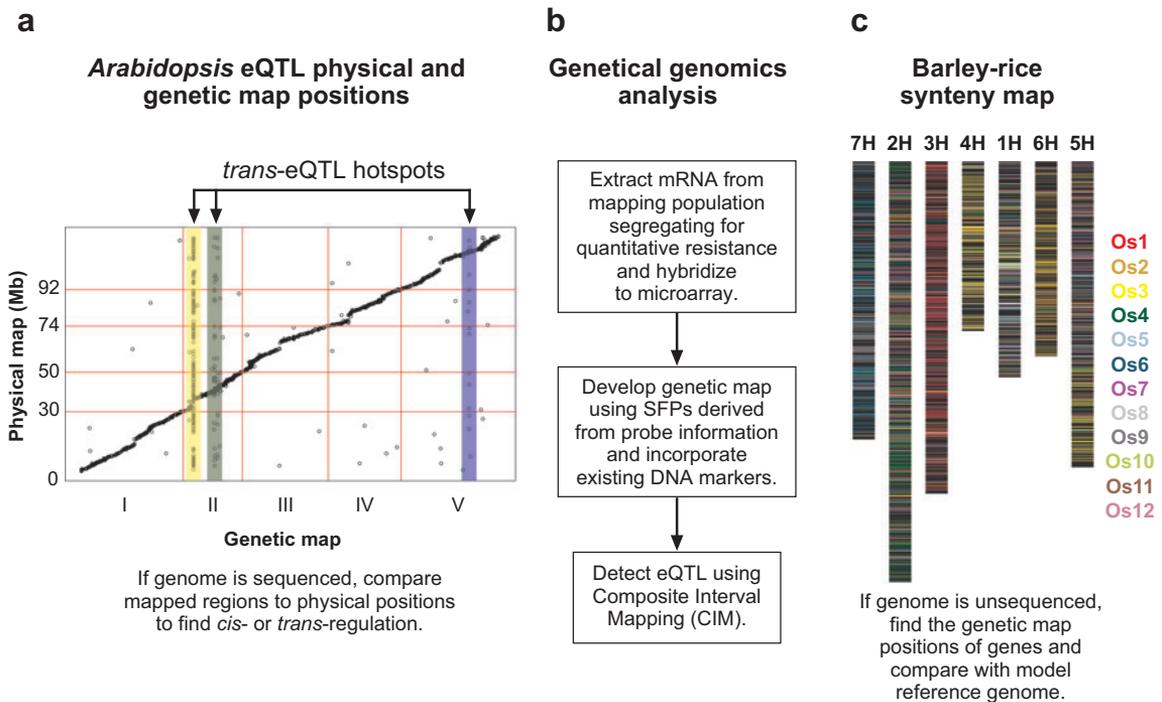
candidate genes may be sufficient to identify the gene responsible for the trait in question.

## Genetical Genomics: Exploiting Natural Variation on a Genome-Wide Scale

Genetical genomics uses natural variation to understand the degree of genetic heritability of gene expression (79). As shown in **Figure 6**, microarrays are used to measure the expression levels of both the mapping population and its parents. Traditional linkage analysis is applied to gene expression data from a map-

ping population for the detection of significant linked regions, termed “expression quantitative trait loci” (eQTLs) (40). Key aspects of experimental design such as size of population, type of population [recombinant inbred lines (RILs), double haploids (DHs), back crosses (BCs)], measurement of quantitative traits, statistical models, and permutation testing have been extensively reviewed (34, 89, 113, 118). The degree of replication depends on the resolution required to answer the biological question: If only major loci are important, a small subset of a mapping population may be sufficient, for example the 30 used by

**eQTL:** expression quantitative trait loci



**Figure 6**

Genetical genomics: using natural variation to understand the genetic control of gene expression in plant-pathogen interactions. (a) Major eQTL for each gene in the *Arabidopsis* Bay-0 and Sha RIL population (ArrayExpress accession E-TABM-62) (153). (b) By applying a pathogen to the population, quantitative resistance can be associated with gene expression. mRNA from each individual in a mapping population is hybridized to a microarray. Genetic maps can be enhanced using markers generated from SFP probe-level data. eQTL analysis is performed on the SFP/DNA marker genetic map using Composite Interval Mapping (CIM). (c) Barley-rice synteny was found using the Steptoe x Morex double haploid (DH) population. Color coding on barley chromosomes identifies the rice chromosome position of best BLASTx of barley genes against TIGR rice protein database (version 4) with stringency cutoff of  $1e-20$  (A. Druka, R. Waugh & M. Kearsey, personal communication). Regions in black represent BLAST hits that did not meet the  $1e-20$  cutoff.

DeCook and colleagues (38). At the other extreme, a high-resolution study of the genetic heritability of gene expression in a cross may examine more than 200 RILs with replicates (154). The authors of this study measured with extreme sensitivity the genetic heritability of gene expression in an *Arabidopsis* Bay-0 and Sha RIL population.

The genetic control of quantitative disease resistance is a biological question well-suited for the genetical genomics approach (52). Gene expression variation in the plant-pathogen interaction is used to identify *cis*-eQTLs that correspond to resistance QTL and/or *trans*-eQTLs that may coincide with master regulators having genetic variation between two parents (**Figure 6a**) (79). A mapping population can easily be saturated with markers based on SFP or haplotype-specific gene expression [Gene Expression Marker (GEM) or Expression Level Polymorphism (ELP)] (**Figure 6b**) (153).

Genetical genomics is being exploited to dissect the genetic interactions between soybean and its oomycete pathogen *P. sojae*. Tyler and colleagues (B. Tyler, personal communication; <http://soy.vbi.vt.edu>) have used the soybean/*P. sojae*/*H. glycines* Affymetrix GeneChip containing probes for both the host and pathogen to examine the interaction in eight soybean cultivars with varying levels of quantitative resistance. Furthermore, they are currently profiling the interaction in a population of 300 soybean RILs derived from a cross of soybean (*Glycine max*, V71-370) x wild soybean (*Glycine soja*, PI407162), an experiment involving more than 2600 GeneChips. In barley, Druka and colleagues have used grain tissue from the Steptoe x Morex DH population to generate over 23000 eQTLs, which are distributed across all chromosomes (**Figure 6c**) (A. Druka, R. Waugh & M. Kearsey, unpublished data). These major eQTL, which are mostly *cis*-regulated, can be used as molecular markers and utilized for saturation mapping. Expression QTLs segregate in these crosses and provide many opportunities for using genetical genomics to

identify potential regulatory networks in plant defenses.

## High-Throughput Pyrosequencing

A new technology with a robust range of applications is the pyrosequencing platform from 454 Life Sciences Corp (90). From sequencing previously unidentified low expressing cell-specific genes to using the small sequence fragments as expression measures, 454 sequencing represents another important tool for understanding the transcriptomes of hosts and pathogens, and here we highlight some of the fascinating new research. Emrich and associates (47) used LCM to isolate the shoot apical meristem (SAM) tissue from maize, and after subjecting the samples to expression profiling, found that 6% of the total genes were “orphans,” which represent genes not previously identified in any plant. They have continued to characterize gene expression levels in SAM tissues between two different maize inbred lines, B73 and Mo17 (P. Schnable, personal communication). This comparison of different conditions has been attempted on a larger scale with the interaction between *Medicago* and *Phytophthora* (M. Bhattacharyya & S. Cannon, personal communication). They observed average read lengths ranging from 96 to 215 bp, with a total number of reads ranging from 75287 to 165250, where 83% of the sequences have hits to *Medicago* sequences, and the remaining 17% of genes represent novel *Medicago* or *Phytophthora* genes. Both of these data sets were found to have high correlation with existing microarray experiments.

It will be possible to identify differential gene expression between different treatments by using counting statistics. The lack of biological replication makes this technology more appropriate for hypothesis generation or as a complement to microarray results. All massive sequencing technologies show promise for the characterization of small RNAs in plant tissues and to measure the expression of both pathogen and host in an unbiased manner (81, 94, 100). We cite 454

sequencing specifically for the availability of data. Other platforms from companies such as Solexa or Biotage show promise as important technologies, in this rapidly expanding this area of research (82).

## ONLINE REPOSITORIES: HOW DO I ACCESS ALL DATA?

### Philosophy on Data Access and Meta-Analysis of Published Data Sets

High-density, high-genome coverage microarray platforms will be available for most major crops and many pathogens in the near future. Data from large-scale expression experiments are accumulating at an exponential rate. Each hybridization generates up to 170 Mb of raw data, and thus, a replicated multifactor experiment (e.g., genotype  $\times$  treatment  $\times$  time) that uses 100–200 chips will generate 8–17 gigabytes of data (20, 21, 43, 61, 111, 122).

With the advent of increasing amounts of DNA chips for many crop and pathogen species, the general philosophy on data access is to make complete microarray data sets publicly available to researchers through an accepted long-term repository such as NCBI-Gene Expression Omnibus (GEO) (10, 44) or EBI's ArrayExpress (111). Major journals are implementing policies that all parallel expression data be accessible in an appropriate public database prior to publication; some are requiring that reviewers have access to the data for validation purposes. This philosophy is consistent with NCBI or EMBL sequence data submissions, and includes the original raw data files such that independent analyses of the data can be made to extract additional information later. For example, many expression profiling experiments in plant-pathogen interactions follow some sort of time course and thus the same data set could be analyzed to gain inroads into circadian rhythm (68, 120) or the interaction of light-dark periods and pathogen attack, among others.

Users of public databases who download data sets for exploratory or comparative purposes should assign proper credit of the data source in subsequent submissions for publication or public research presentations by citing the database publication or URL, as well as the accession number(s) used to identify the experiment(s) and original manuscript and that the data set was used for. This also adds value to the original data.

### Data Repositories, Expression Database, and Analysis Tools

General repositories, such as GEO (10, 44), Stanford Microarray Database (9, 58), and ArrayExpress (17, 111), act as central data distribution hubs for species ranging from *E. coli* to humans. General repositories make the data available to public users, but because of their large scope and lack of specificity, they do not readily allow searches for specific genes of interest based on sequence information or annotation, or flexible on-line analysis.

For specific hypothesis building, microarray databases that facilitate on-line analysis tend to be the most useful as they contain links to related annotation as well as graphics and tools focused on a specific task. For plants, examples include species-specific resources for *Arabidopsis*, such as Genevestigator (169), GeneFarm (7), AtGenExpress, and NASCArrays (32). Genevestigator was developed for high-throughput gene expression analysis, which allows the user to create a digital northern, trace the expression of a gene through the growth stages of the plant life cycle, or find correlated genes across a variety of factors. NASCArrays and AtGenExpress open their repository of *Arabidopsis* GeneChip data for download and use in relational databases for further analysis. The Rice Expression Database (RED) (162) and the associated Rice Pipeline (163) project compile genomics data (genome sequences, full-length cDNAs, gene expression profiles, mutant lines, *cis* regulatory elements) into one data source with full annotation.

For comparative genomic analysis, the single species databases lack the ability to perform side-by-side comparisons to determine, for example, if similar results have been found for orthologs from other species, or if differences/similarities exist among species for the same treatment. Many currently available expression resources for plants focus almost exclusively on *Arabidopsis* (NASCArrays, Genevestigator) or rice (RED or Rice Pipeline). PLEXdb (Plant Expression Database; <http://plexdb.org/>) (126, 158), a relatively new expression resource, carries on the tradition of Genevestigator but provides transcriptome tools and data access for crop communities that are supported on the Affymetrix and long-oligonucleotide platforms. PLEXdb utilizes a MIAME-compliant data submission process as well as the developing plant and trait ontology ([www.plantontology.org](http://www.plantontology.org)) terms so that experiments can accurately describe development stages and plant tissue types. These terms allow cross-species comparisons based upon common identifiers, facilitating interoperability between existing plant databases. Integrated tools such as Probe Set Annotations, Expression Display, Gene List analysis, Microarray Platform Translator, and Model Genome Interrogator (158) are planned or are currently available for all organisms, experiments, and conditions. Interconnecting links with species-specific genome resources, such as PlantGDB, GrainGenes, Gramene, and TIGR, allow PLEXdb users to perform gene predictions or cross-species comparisons using sequences represented on particular GeneChips (Figure 5c).

While PLEXdb offers many experiments focused on plant-pathogen interaction in crop species as well as *Arabidopsis*, the Pathogenomics Integrated Microarray Database System (IMDS) (<http://ausubellab.mgh.harvard.edu/imds/>) provides detailed searches on stored experimental descriptions and raw microarray data for the NSF *Arabidopsis* 2010 Project, “Expres-

sion Profiling of Plant Disease Resistance Pathways.” Time-course, mutant, and PAMP-induced comparisons are available for treatment of Columbia wild-type seedlings with *E. orontii* (see Figure 2a), *B. cinerea*, *Pseudomonas*, crab-shell chitin and chitin octamer, flg22 and OGs elicitors, Columbia wild-type vs mutant *pmr5*, mutant *pmr6* and double-mutant *pmr5/pmr6*, and many others.

*Magnaporthe grisea*, the causal agent of rice blast disease, is a major deterrent to rice production. The MGOS (*Magnaporthe grisea-Oryza sativa*, <http://www.mgosdb.org/>) database posts the results of an NSF-funded project “Whole Genome Analysis of Pathogen-Host Recognition and Subsequent Responses in the Rice Blast Patho-System.” Several cDNA libraries were constructed from rice at different time points after infection with *M. grisea*. Single-pass DNA sequencing of clones in the libraries subsequently identified ESTs involved in rice defense (80). This information, along with other transcriptome data (60), was used for microarray design. Functional validation will be accomplished via a large collection of gene knockouts to uncover many of the early events in rice-*Magnaporthe* recognition from the perspectives of both the pathogen and host. A NSF renewal project “Community Annotation Database for *M. grisea* and its Interactions with Rice” will open the MGOS database to the research community for gene annotation, microarray data submission, and integration of other rice-*M. grisea* data.

### Controlled Vocabularies

As data from transcript profiling accumulates, it is increasingly important to utilize community-developed ontology terms for plants, plant pathogens, and their genes. The Microarray Gene Expression Data Society (16, 170; <http://mged.org/>) has created MIAME (Minimum Information About a Microarray Experiment, <http://mged.org/Workgroups/MIAME/miame>.

html) guidelines that are needed to enable results of the experiment to be interpreted unambiguously and potentially to reproduce the experiment. The original terms have been extended to plants (136, 170), facilitating cross-comparisons among diverse experiments.

Use of standardized terms will pave the way toward expression data meta-analysis by clearly specifying tissue type and developmental stages. Gene ontology and pathway information will allow functional gene expression analysis with insight on how specific genes are involved in biological processes (<http://www.geneontology.org/>). Ontology information for plants is available from the Plant Ontologies project (<http://www.plantontology.org/>). Ontology information for the pests, pathogens, and nematodes is available from the Fungal Anatomy Ontology Project, Plant-Associated Microbe Gene Ontology, Biotic Environment Ontology, and nematode working groups.

## SYNTHESIS

Transcriptome analysis has fueled a better understanding of host-pathogen interactions through examination of both compatible and incompatible interactions and comparison of responses mediated by different resistance genes. Gene expression studies, especially those involving extensive infection time courses, reveal that an inherent characteristic of successful pathogens is their ability to suppress the host defense responses that they trigger early in the interaction. The expression profiles of genes that reflect this ability thus define specific stages of infection. It appears that *R* genes enable plants to override the suppression of basal defenses and stimulate subsequent massive induction

of potent, broadly effective defenses. *R* gene-mediated defenses appear to be superimposed on the basal defenses, because genes that are induced by pathogen infection in compatible interactions are similar or identical to those induced in incompatible interactions (**Figure 2**).

The signaling networks that control these responses are being unraveled further with the aid of transcript profiling. Early transcriptional targets of regulatory genes have been characterized by studies that have combined microarray analyses with clever experimental design, especially when followed up with functional analyses. While significant advances have been obtained with *Arabidopsis* as a model, the advent of diverse microarrays for crops built upon community-wide sequencing efforts is empowering direct, crop-specific studies. This is especially true for those crop-plant systems for which robust downstream functional genomics platforms are available that permit rapid reverse genetic analysis of statistically significant genes. In addition, identification and characterization of virulence factors also has been accelerated by genomic technologies available for several pathogens.

The exponential growth of data sets from plants and microorganisms presents a plethora of opportunities for comparative analysis. Up till now, parallel expression technology has been mostly applied to experimental systems differing by a particular genotype, time, or pathogen. New genetical genomics applications will make possible the creation of dense expression polymorphism maps and identification of regulatory regions. These resources will promote the understanding of the complex architecture of plant disease defense, which will have long-term value for crop improvement.

## DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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References 20 and 21 highlight the power of experimental design and statistical analysis to interrogate the expression of defense responses in barley to the powdery mildew fungus. One of the first genome-wide demonstrations that gene-for-gene specified resistance is interlinked with PAMP-mediated general defense mechanisms.

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Demonstrated at the level of host gene expression that virulent bacteria counteract plant basal-defense responses with type III-secreted effectors, and provides an inventory of markers for use in subsequent smaller scale analyses.

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Exemplifies the potential of comparative global transcript profiling of host responses to a wild-type and an effector knockout strain of a pathogen to define plant responses elicited specifically by that effector.

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Complete genome GeneChip developed for *F. graminearum*, the causal organism of *Fusarium* head blight of wheat and barley.

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Laser capture microscopy was applied to study soybean gene expression in developing syncytia induced by soybean cyst nematode.

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The authors define the regulon of the *P. syringae* pv. *tomato* T3SS regulators HrpRS and HrpL, showing that it includes novel candidate virulence factors, including T3SS-independent genes.

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A primer on experimental design and statistical analysis strategies for microarray experiments.

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Provides information on the design, analysis, and future applications of genetical genomics for deciphering the genetic control of complex traits.

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Represents one of the earliest studies that attempted to discover the function of unknown genes on a genome scale using gene expression data.

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Demonstrates the power of iterative microarray and genetic analyses to unravel complex regulatory networks.

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West et al. used 211 RILs of an *Arabidopsis* mapping population which found 36871 statistically significant eQTL. They found that most eQTL were controlled in trans and most explained less than 30% of the variation (R<sup>2</sup>) of the gene expression.

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Viral infection foci were dissected to examine host gene expression profiles in infected and noninfected cells.

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## Errata

An online log of corrections to *Annual Review of Phytopathology* articles may be found at <http://phyto.annualreviews.org/>