Quantitative Proteomic Analysis of Bean Plants Infected by a Virulent and Avirulent Obligate Rust Fungus*

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Plants appear to have two types of active defenses, a broad-spectrum basal system and a system controlled by R-genes providing stronger resistance to some pathogens that break the basal defense. However, it is unknown if the systems are separate entities. Therefore, we analyzed proteins from leaves of the dry bean crop plant Phaseolus vulgaris using a high-throughput liquid chromatography tandem mass spectrometry method. By statistically comparing the amounts of proteins detected in a single plant variety that is susceptible or resistant to infection, depending on the strains of a rust fungus introduced, we defined basal and R-gene-mediated plant defenses at the proteomic level. The data reveal that some basal defense proteins are potential regulators of a strong defense weakened by the fungus and that the R-gene modulates proteins similar to those in the basal system. The results satisfy a new model whereby R-genes are part of the basal system and repair disabled defenses to reinstate strong resistance. Molecular & Cellular Proteomics 8:19–31, 2009.

Basidiomycetes rust fungi are obligate biotrophs that are notorious plant pathogens. They have evolved closely with their hosts, but not with the plants we have chosen as models. Thus, there is no model rust-plant system. Consequently, contemporary understandings of plant disease resistance are not proportionally drawn from research on rusts, some of the most devastating pathogens.

This was not always the case. Many years ago, Flor (1) conceived the gene-for-gene disease resistance theory by evaluating flax rust. The basic tenet of the theory is that if a gene product (effector) of the pathogen is recognized by a plant’s disease resistance R-gene product, a defense response is triggered that renders the pathogen avirulent. A traditional phenotype for gene-for-gene resistance is a hypersensitive reaction (HR)1 yielding complete or nearly complete restriction of pathogen spread, often culminating in cell death at the sites of infection. Gene-for-gene resistance is known by many other terms such as vertical, monogenic, and race-specific resistance, and effector-triggered immunity. Molecular mechanisms consistent with the theory were first delineated in model systems (2, 3), but Flor’s genetic observation was finally confirmed at the proteomic level, revalidating the importance of rusts in testing disease resistance mechanisms (4).

Flor’s theory also states that pathogens not detected by R-genes remain virulent, and plants infected with virulent pathogens are susceptible to disease (1). But this does not mean that susceptible plants do not mount a defense response. Research on Arabidopsis thaliana infected with virulent bacteria led to the discovery of genes that coordinate defenses independent of R-genes (5). These defenses are known as broad-spectrum, horizontal, polygenic, general, or basal resistance and include pathogen-associated molecular pattern-triggered immunity. The basal defense system confers effective resistance that can halt pathogen colonization. However, when this system is suppressed by pathogen effectors, infected plants display the phenotype of expressing weak and insufficient defense responses relative to an R-gene-mediated reaction (2, 3).

Attempts to molecularly define R-gene and basal defenses have revealed unforeseen intersections, with each involving the production of oxidative intermediates at the onset of infection (6, 7), mitogen-activated protein-like kinase signaling (8, 9), and overlapping signal transduction processes leading to the accumulation of salicylic acid (SA), phytoalexins (tox-

1 The abbreviations used are: HR, hypersensitive reaction; SA, salicylic acid; PR, pathogenesis-related; LC-MS/MS, liquid chromatography-tandem mass spectrometry; dai, days after inoculation; hpi, hours post-inoculation; EF1a, elongation factor 1 a; CW, cell wall; FA, fatty acid; GST, glutathione-S-transferase; ADP, adenosine 5’-diphosphate; EST, expressed sequence tag.
ins), and pathogenesis-related (PR) proteins (10). Other processes are initiated as well, including proteolysis (11, 12), fortification of cell walls (12, 13), and expression of many of the same genes (14–16). Even programmed cell death, once thought to be the final step and clear indicator of gene-for-gene resistance (17), may be part of defense responses not specifically regulated by R-genes (18, 19), especially against rusts (20).

It was proposed that because of these similarities, R-gene-mediated defenses are more intense versions of a basal response, kinetically and stochastically overwhelming the pathogen with most of the same defenses deployed in basal resistance reactions (14). Hence, a contemporary model for quantitative disease resistance states that R-genes help plant cells fight pathogens that have overcome basal defenses by amplifying the suppressed basal defenses beyond a threshold that confers effective resistance to a level that elicits HR (3).

It remains unknown whether basal and R-gene-mediated defenses constitute distinct mechanisms. Because gene expression experiments, including those used to justify contemporary disease resistance models, do not evaluate the proteins that prompted the gene expression changes in the first place and because proteins drive many critical defense responses, a proteomics investigation of basal and R-gene-mediated defenses is needed to further understand different components of plant disease resistance responses. In our studies, we focused on Phaseolus vulgaris, a worldwide crop plant that produces the staple edible dry bean. P. vulgaris is plagued by its own rust fungus, Uromyces appendiculatus. There is race-specific resistance to U. appendiculatus in P. vulgaris, but the diversity among races is so great that significant yield loss still occurs. Thus, cultivar improvement would benefit from a better understanding of the molecular mechanisms of general defense and gene-for-gene resistance. For reasons already explained, biology would also benefit from a detailed proteomic analysis of disease resistance to a rust pathogen. Thus, our aim was to discover the changes in the bean leaf proteome during the course of a rust infection using high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS). The difficulty of this task was compounded by the lack of specific genomic reference resources needed to distinguish bean and rust proteins. So, we created customized genomic databases and performed repeated LC-MS/MS to maximize the detection of a wide variety of proteins. By statistically analyzing protein accumulation, we dissected the proteomic changes in susceptible and resistant plants. The data are consistent with a model whereby R-genes repair a basal defense system that is inherently strong.

**EXPERIMENTAL PROCEDURES**

Inoculations—Two primary leaves from 24, 10-day-old P. vulgaris cv. Early Gallatin plants were inoculated with a liquid suspension of uredospores of U. appendiculatus race 41, 49, or water and placed in an 18 °C dew chamber for 12 h and then moved to a 23 °C growth chamber with fluorescent lighting (21). Inoculum was adjusted to produce 2–4 pustules per leaf surface cm². One of each inoculated plant was kept as an indicator to ensure successful infection. Experiments in which indicator plants did not develop symptoms at 10 days after inoculation (dai) or did not display desired infection density were abandoned. Three biological replicate experiments were evaluated.

RT-PCR—Leaves were collected at 24 and 72 h post-inoculation (hpi). Ten ng DNase-treated total RNA from three biological replicates of inoculated and mock-inoculated leaves was used with TaqMan One-step RT-PCR (Applied Biosystems, Foster City, CA) reagents for quantitative analysis in an Mx3000P machine (Stratagene, La Jolla, CA) according to the manufacturers’ instructions. Primers and 6-carboxyfluorescein 5’ end-labeled probes were designed from the sequences using Primer Express software (Applied Biosystems) and are as follows: U. appendiculatus actin primers CTGTTTTCTTCTCATCTGTG, GCCTTTGGGCCCCATTTCA, and probe GTGCCCTGCAC-CAAGGTGAT; U. appendiculatus elongation factor 1a (EF1a) primers TGCTGCTGTGCTTTCCATGCA, CAAATTTGGGACATTCTCTT, and probe TGCCCTGCAAATTGATACCTC; U. appendiculatus RTPI primers CGTCCGGAATCTGTGTTGG, GTCGACCTGTCGTTATGATT, and probe ATGCTGCTGTTCCAGATGCCTTCTTC; P. vulgaris EF1a primers GAATGTTGATGCTGTTGTTTTG, TCTCCACGCCTCTTGTGAC, and probe CAAAGTGTGAGCCATGCCTCCAC. Expression levels were interpolated from standard curves with a correlation coefficient of 0.9 or greater.

cDNA Library Construction—RNA was extracted from leaves inoculated with race 41 or race 49 and collected at 6, 12, 15, 24, 48, 72, 96, and 120 hpi. The RNA from the 6–24 hpi time points were pooled as were the RNA from the 48–120 hpi time points, producing 4 RNA populations. Poly(A)+ RNA and tagged primers were used for cDNA synthesis. cDNAs were size-selected (more than 500 bp). RNA from germinating uredospores and mock-inoculated control plants were used to make a control library. PCR products of the control library were used as a driver for subtraction against single-strand circles of the primary library. Unhybridized circles were used to generate the subtracted library (22). Normalization was similarly performed. Compared with the primary library of 8.5 × 10⁶ clones, the final library had ~3% fewer clones and fewer short inserts (i.e. 150 to 200 b). Average insert size was ~1.2 kb.

DNA Sequencing—DNA from 20,000 bacterial colonies was sequenced on an ABI 3730xl machine. Phred was used to convert the electropherogram files to base and quality files (23, 24), and vector sequence was removed. The sequences were assembled using CAP3 (25), and the consensus sequences used in BLAST searches are described below.

Database Generation—10,309 assembled contig and singleton DNA sequences were compared by BLAST against A. thaliana cDNA and protein datasets, NCBI’s non-redundant datasets (protein and nucleotide), the Phakopsora pachyrhizi genome, soybean ESTs, Genbank ESTs, and the genome survey sequences dataset, as well as TIGR’s (Rockville, MD) ESTs for P. vulgaris and Phaseolus coccineus. This process distinguished 8,689 plant and 466 fungal sequences. The remaining 1,154 sequences had neither sufficient DNA nor protein identity to known fungal or plant sequences and were labeled unknown. The “plant” and “unknown” ESTs, 10,880 TIGR P. vulgaris ESTs, 8,201 P. coccineus TIGR ESTs, and 3,984 ESTs specific to U. appendiculatus race 41 and other rust fungi (21) were searched against the NCBI non-redundant protein database. The reading frame from the best BLASTX alignment was used to redirect a single translation for each EST (21). The match information was appended to the sequence to aid annotation. If there was no match, then the longest translatable open reading frame of all 6 frames was appended and denoted as a hypothetical translation. The derived protein sequences and a list of common contaminants were used for Mascot searching (33,064 entries; Supplemental Table S1).
Protein Preparation—Inoculated plant leaves were harvested at 24 and 72 hpi, making for 6 experimental treatments (including the controls). Proteins were extracted from leaves as previously outlined (26). About 4 g leaves were ground in liquid nitrogen and then homogenized in a buffer (100 mM Tris–HCl, pH 8.5; 5 mM dithiothreitol, 1 mM EDTA, and 1% plant proteinase inhibition mixture (Sigma–Aldrich, St. Louis, MO) with a glass grinder. The homogenate was centrifuged at 1000 × g for 10 min, producing a cell wall (CW) pellet and supernatant. The supernatant was centrifuged at 30,000 × g for 1 h producing the crude membrane/organellar pellet (P30) and supernatant (S30). The CW pellet was washed one time in homogenization buffer. The CW and P30 pellets were resuspended (8 μl urea; 100 mM Tris–HCl pH 8.5; 5 mM dithiothreitol; 2% dodecyl–[β]-maltoside), and the CW samples were vortexed, whereas the P30 samples were sonicated for 30 s. After 30 min incubation at room temperature, both sets of fractions were centrifuged for 15 min at 14,000 × g, and the supernatant was retained. The resulting CW, P30, and S30 fractions were filtered through 0.45-μm filters to remove protein retention by polyvinylidene difluoride membranes (Millipore, Bedford, MA). Proteins were precipitated in 25% trichloroacetic acid, washed in acetone, and resolubilized in 8 μl urea/100 mM Tris–HCl, pH 8.5. Protein concentrations were assayed with a 2 D Protein Quant kit (GE Healthcare). 500 μg protein from each fraction was reduced, carboxyamidomethylated, and digested with trypsin (27). The digested samples were desalted using solid phase extraction with SPEC-PLUS PT C18 columns (Varian, Lake Forest, CA).

LC-MS/MS—Fifty-four LC-MS/MS runs were performed, which consisted of 1 run each for the leaf CW, P30, and S30 fractions from each of 6 treatments organized into 3 biological replicates (9 separate analyses per treatment). The tryptic peptides for each fraction were separated on home-made biphasic columns prepared from 365 outer diameter × 75 inner diameter fused silica with a 5-μm tip and packed with reverse phase C18 resin followed by strong cation exchange resin (28, 29). Separation followed a 12-step elution procedure consisting of stepwise increasing concentrations of salt solution followed by increasing gradients of organic mobile phase (30). Solvent flow of 200 nl/min was controlled with a Surveyor HPLC pump (Thermo Fisher Scientific, Waltham, MA) and a T-split junction where 1,800 V electricity was also applied (31). The eluent was electrosprayed directly into the orifice of an LCQ-Deca XP ion trap mass spectrometer (Thermo Fisher Scientific) controlled by Xcalibur 1.3 software (Thermo Fisher Scientific). A parent-ion scan was performed over the range of 400–1600 m/z, and MS/MS was performed on the three most intense parent ions. Automated peak recognition and dynamic exclusion were enabled.

Peptide Sequence Interpretation—Tandem mass spectrum data files were extracted from the raw data with Bioworks 3.2 (Thermo Fisher Scientific) using the parameters 400–3500 mass range, 1 group scan, 1 minimum group count, and 15 minimum ion counts. The spectra collected for all fractions and replicates for a treatment were combined, yielding 6 data files containing ~500,000 spectra apiece. Spectra were searched with Mascot 2.1 (32). Search parameters were for trypsin digests, 1 possible missed cleavage, fixed amino acid modification [+57, Cys], averaged mass values, ± 1.5 Da parent ion mass tolerance, and ± 0.8 fragment ion mass tolerance. Mascot output was processed by PANORAMICS, a probability-based program that determines the likelihood that peptides are correctly assigned to proteins (33). PANORAMICS first considers all peptide matches made by Mascot and calculates the probability that these matches are correct. Then, when computing the probability of a protein or group of proteins matched with the same set of identified peptide sequences, PANORAMICS takes into account the probabilities for both distinct peptides and shared peptides in a coherent manner and distributes the probabilities of shared peptides among all related proteins. The Mascot Ion Score, the database size, and the length and charge state of each peptide sequence are incorporated into the probability model. The probability that protein identification is not correct (false-positive rate) is 1 minus the calculated protein probability and agrees with false-positive rates that can be deduced by reverse database searching (33). Peptide sequence matches, Mascot scores, protein group probabilities, and other relevant data are provided (Supplemental Tables S2–S7).

Statistical Analysis—Matches made to plant proteins and fungal proteins were separated. Plant proteins that were found in each of three treatments for each time point were kept if the probability for a given protein in at least one of the treatments exceeded the 0.95 probability threshold and exceeded 0.80 in the others. Similarly, some researchers evaluate proteins identified with peptides with ±1% false-positive rates at the peptide level (34, 35). There were 1,155 proteins at the 24-h time point and 1,181 proteins at the 72-h time point that satisfied our requirements. The numbers of spectra contributing to the identification of shared and distinct peptides assigned to a protein group for each treatment were summed, as described previously (21). Because the PANORAMICS probability model dictates that shared peptides in a protein group contribute significantly to protein groups with high probabilities, spectra assigned to shared peptides were counted equally as those assigned to distinct peptides. A G-test was used to assess the statistical differences of the spectral counts per treatment/time point (36), with the hypothesis being that the spectral count of any protein A was equal among three treatments. The corresponding p value was calculated from χ2 distribution with 2 degrees of freedom. The Benjamini and Hochberg correction was applied such that only a protein with p value less than 0.05/j, where j is the rank of the protein in the list of t proteins sorted in ascending order by p value, was considered as having differential spectral counts across a treatment/time point.

Clustering—Relative accumulation differences were determined for 413 proteins having differential spectral counts across treatments at the 24-h time point and 296 proteins having differential spectral counts across treatments at the 72-h time point. The sum of the spectral count for any protein A from an infected plant normalized by the sum of all protein spectral counts at that time point (1,155 or 1,181 proteins) was divided by the normalized sum of the spectral count for protein A in the control plant at that time point. This established a ratio regarding the accumulation of a protein with respect to infection with race 41 or 49 in relation to mock-inoculated control plants. The Log2 transformed ratio pairs were clustered by affinity propagation using default values (37) (Supplemental Tables S8 and S9). Input values for similarities based on squared error and the median of similarities were computed with a custom C program. Clusters and the relative accumulation changes were visualized with heat maps made with TreeView. Accumulation differences of ~8 orders of magnitude (Log2) were evaluated by this method. Although some protein accumulation differences appear to be quite small in the TreeView heat maps, they are statistically validated (see Supplemental Tables S8 and S9 for ratios).

RESULTS

Infections on Susceptible and Resistant Plants Are Distinct—P. vulgari cv. Early Gallatin plants contain the dominant Ur-4 gene that confers resistance to avirulent U. appendiculatus race 49 but not to virulent race 41 (38, 39). Resistance is marked by the development of local lesions, plant cell death, and little or no fungal sporulation at 10–14 dai (Fig. 1A). These are the hallmarks of classical gene-for-gene resistance and indicate an HR. Susceptibility to race 41 is
differentiated by the presence of sporulating uredia, the asexual fruiting bodies that emerge from leaves 10–14 dai (Fig. 1B). Examination of two different races eliciting distinct phenotypes on one host genomic background is sufficient for resolving two different reactions, one that severely restricts fungal growth and one that does not. Here, resistant plants are those inoculated with race 49, and susceptible plants are those inoculated with race 41.

Inoculated and control leaves were harvested at 24 and 72 hpi. We reiterate that one fungal strain is virulent and the other is avirulent on Early Gallatin, which by their very definitions means that the growth of one is much more impeded by the plant than the other. There is no way to dissect growth potential without separating each strain from its inherent virulent/avirulent attributes or taking it away from its host, at which point the fungus would no longer be a pathogen in either case. Therefore, the time points we investigated were associated with a number of distinct features of fungal development, and it was assumed that the time points we investigated were associated with unique plant responses as well. By 24 hpi, the fungus produced haustoria in both susceptible and resistant plants as indicated by the presence of RNA for the haustoria marker RTP1 (40) (Fig. 1C). The haustorium is a specialized cell structure that infects leaf cells and absorbs nutrients for the fungus. Secreted haustorial proteins likely induce the plant to produce nutrients for the fungus, but they are also likely to dismantle early plant defense responses to allow successful infection. Inadvertently, the secreted proteins may also trigger a resistance response in plants with R-genes that recognize the effectors. By 72 hpi, the fungus continued to colonize susceptible plants (Fig. 1C) and began to produce uredia (21). By contrast, there were reduced amounts of *U. appendiculatus* translation elongation factor and RTP1 transcripts in resistant plants at 72 hpi (Fig. 1C). Thus, the 72-h time point likely coincided with plant responses favorable for rust development in susceptible plants and/or with secondary defenses with respect to the 24-h time point in both susceptible and resistant plants.

As points of clarification, although we fully expect there to be proteomic changes in the plant prior the 24-h time point, we did not choose to analyze a 6-h time point, for example, because it is our experience that under such short incubation times in the dew chamber infections rates are insufficient for whole-leaf proteomic analysis. A 12-h time point is a nighttime collection given a work hour inoculation, which produces confounding circadian rhythm effects with respect to disease resistance. Therefore, the 24-h time point was the most suitable early time point for our proteomic experimentation and was fully justified by the RT-PCR analysis, which showed that both fungal strains were producing a haustorial transcript and suggested that the plant should be reacting to the infection at that time.

Identification of Proteins Using a High-throughput Workflow—We performed differential centrifugation on detergent-treated plant leaf extracts (26). Proteins from crude cell wall, organelle/membrane, and soluble subcellular fractions were analyzed by LC-MS/MS (30). Three biological replicates of each fraction were analyzed to reduce random sampling effects and increase the statistical confidence associated with reproducible detection of proteins (41). For each of the six treatments (i.e. two inoculations and one control over two time points), spectra from all fractions and replicates were combined.

Tandem mass spectra were interpreted by matching them to protein sequences. Because species-specific protein sequence information is best suited for interpreting peptide tandem mass spectra (21) and reduces the number of false matches as well (42), we constructed a customized database comprising translated sequences from a subtracted, normalized cDNA library of race 41 and 49 infected Early Gallatin leaves and from public *Phaseolus* spp. ESTs. The database

![Fig. 1. Rust pathology on Early Gallatin leaves. A, local lesions elicited by race 49 12 dai. B, uredia (pustules) of race 41 12 dai. C, relative accumulation of RNA for *U. appendiculatus* actin (*Ua* actin), translation elongation factor EF1α (*Ua* EF1α), and haustoria marker RTP1 (*Ua* RTP1), and *P. vulgaris* EF1α (*PV* EF1α). Error bars show experimental deviation (3 trials). ND, not detected.](image-url)
also contained translated ESTs from *U. appendiculatus* and other rusts (21) (Supplemental Table S1). Peptide sequences from this custom database that matched spectra were assembled into probability-based, non-redundant protein complements (33) (Supplemental Tables S2–S7).

Approximately 1,400 proteins were identified in each treatment, and more than 3,000 proteins were identified overall (protein identification false-positive rate < 5%). Among them, 62% were identified specifically with Early Gallatin ESTs. Approximately 70 fungal proteins were identified among the mock-inoculated controls. Since these plants were not infected, quality matches between a plant spectrum and a non-target fungal sequence were made as a result of there being a high degree of conservation between a non-target fungal protein sequence and a related plant sequence (e.g. ATP synthases and ribosomal sequences). Thus, the rate of non-target matching was 5%. 68 to 97 fungal proteins were identified in different infected plants, and ~22 of those overlapped with those found in controls. We suspect that the reason why very few additional fungal proteins were detected in infected plants is because so little fungal biomass accumulated at the two time points in relation to plant biomass. Fungal protein data was not included in the following analyses but is in the Supplemental Material (S2–S7) (protein name prefix "RUST").

Quantitative and Differential Proteomic Analysis—Spectral counting can correlate linearly with protein abundance (41) and can provide accurate estimates of the relative levels of proteins between samples comparatively to radiolabeled quantification (43). So, we summed the number of spectra matched to the peptides for the proteins in each treatment (21). Because of random sampling effects associated with LC-MS/MS, it was difficult to determine whether the absence of any protein was a result of a specific treatment or a by-product of the chance of not detecting it. Therefore, quantitative analysis was limited to 1,155 and 1,181 proteins that were detected across the treatments for the early and late time points, respectively. Per this requirement, there remained many proteins that were detected under one condition but not another. These data for which no quantitative information could be produced, but which have relevant qualitative information regarding the proteomics of beans, are included in Supplemental Tables S2–S7.

We applied a statistical G-test for multi-conditional comparison to identify differentially accumulating proteins (36). The advantage of the G-test is that spectral datasets for replicates can be combined rather than treated separately. This reduces MS/MS random sampling errors by increasing the numbers of spectra associated with peptides, which in turn favors statistically reproducible protein identification. Analysis indicated that 413 proteins at the 24-hpi time point and 296 proteins at the 72-hpi time point had statistically different spectral counts across respective treatments. The proteins without statistically different spectral counts (742 proteins at 24 hpi and 885 proteins at 72 hpi) can be considered either as proteins, the amounts of which did not fluctuate significantly or as proteins for which more data needs to be collected in order to determine whether fluctuations existed.

The proteins with statistically different spectral counts were examined further. Normalized spectral counts from infected plants were compared with controls, and the ratios revealed over or under-accumulation of a protein in an infected plant. The \( \log_2 \) transformed value pairs (race 41/control and race 49/control) were clustered by affinity propagation to reveal sets of proteins exhibiting significantly similar accumulation properties. Forty-four clusters emerged from the early time point, and 32 clusters emerged from the late time point (Supplemental Tables S8 and S9). The clusters were categorized into six groups. The potential function for each significant protein was deduced based on similarity to other known proteins (Supplemental Tables S8 and S9). Together, the cluster analysis and functional descriptions reveal many proteins with a variety of different functions as being co-regulated with respect to basal defense and differentially regulated with respect to *Ur*-mediated disease resistance responses. Our interpretation of this analysis will follow after this subsection.

Independent of the clustering, a separate, generalized functional categorization was performed on proteins with statistically significant spectral counts. Gene Ontology classifications were assigned to the proteins and the proteins were categorized according to Bevan et al. (Supplemental Tables S8 and S9) (44). Proteins categorized as having functions in disease/defense (11%), energy (16%), metabolism (19%), protein destination and storage (14%), and protein synthesis (15%) predominated the 24-h time point (Supplemental Tables S8 and S9). Since the genome for *P. vulgaris* is unresolved, it is uncertain whether this is over- or under-representation for these categories. Further complicating generalized functional categorization interpretation is the likely inherent bias to the types of proteins identified because matches were predicated on a compound, non-uniform database derived from ESTs from several independent projects (including ours) where certain classes of molecules could have been over- or under-represented depending on tissue, treatment, or time in which RNA was collected. Nevertheless, similar percentages of functional categories were observed at the 72-h time point with there being slightly less for disease/defense (7%) and metabolism (14%), and slightly more for energy (22%) and protein destination and storage (17%). Given the relatively small number of proteins in each category compared with the few hundred proteins analyzed, the unresolved status of the *P. vulgaris* genome, and the EST database derived from multiply biased experiments, it is difficult to clearly assess whether these slight differences in functional category percentages between the 24- and 72-h time points are significant.

The potential cellular localization of each protein was also deduced based on its Gene Ontology classification (Supplemental Tables S8 and S9). 13 and 16% of the proteins found...
Proteomics of Disease Resistance to Rust

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**Group 1**

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**Group 2**
at the 24- and 72-h time points, respectively, were thought to be chloroplast proteins, 5 and 7% thought to be central to the nucleus, 5 and 6% to mitochondria, 9 and 11% to ribosomes, and 20 and 18% to other cytoplasmic components. There was uncertain location for 32 and 27% of the proteins found at the 24- and 72-h time points, respectively. We point out that this potential localization is predicated on the deduced function of the protein and not on specific proteomic analysis of CW, P30, and S30 subfractions. For this study, subfractionation methodology was crude and was only intended to help provide a broader survey of proteins. No attempts were made to assess the relative purity of the subfractions, which was required if one were to use proteomics to make precise claims of subcellular localization.

Early Basal Defense Responses in both Susceptible and Resistant Plants—We began our analysis with the assumption that proteins exhibiting similar accumulation patterns in Early Gallatin leaves inoculated with race 41 or 49 may be part of the Ur-4-independent basal defense system. Four proteins with increased accumulation in both susceptible and resistant plants at 24 hpi were PR proteins including one similar to PR1, an inhibitor of bean rust differentiation (45), and chitinase, which is likely to be detrimental to chitin-based cell walls of rust germlings (Fig. 2, Group 1). Similar PR proteins are general markers for basal defense response induction, and their presence indicates coordinated SA-based defenses (10). These proteins accumulated to nearly equivalent levels in both susceptible and resistant plants. Thus, some parts of the SA defense response appeared to be just as strong in susceptible plants as in resistant plants. In conclusion, our data support the idea that basal defenses were triggered in susceptible and resistant plants at 24 hpi.

Clustering linked 86 other proteins with accumulations that increased alongside the PR proteins in both susceptible and resistant plants (Fig. 2, Group 1), suggesting that many are also “pathogenesis-related” or part of co-regulated basal defenses. The presence of thioredoxin, other antioxidants, and MPBQ methyltransferase, an enzyme producing lipid peroxidation scavenging tocopherol, indicate that the plants were either initiating the production of reactive oxidative species to fight the pathogen directly (46) or were responding to oxidative intermediates produced as a result of cell wall or membrane damage (47, 48). There was an increased amount of a lipolytic enzyme with a GDSL-motif, reminiscent of a lipase that creates fatty acid (FA)-based signals from oxidatively stressed membranes that may trigger downstream defense responses (49). There was an increased amount of \( \beta \)-mannosidase, possibly implicit in cell wall fortification, another defense response (50). And there were increased amounts of coatamers, suggestive of induced mechanistic action of vesicle secretion triggered by oxidative intermediates which may subsequently deliver antimicrobials to sites of infection at the plasma membrane (19). Interestingly, several proteins in Group 1 are similar to proteins that confer immunity to non-adapted pathogens (12, 19, 46). Based on our data, there may be an overlap between proteins involved in the basal defenses and those conferring insusceptibility, broadly defined by the term “non-host” resistance.

We detected a number of membrane-bound proteins with likely defense roles in susceptible and resistant plants. There was a protein with homology to an A. thaliana R-gene, a membrane transport nodulin-26 protein (51), a plasma membrane intrinsic protein similar to others involved in biotic stress responses (52), and an annexin-like protein similar to those that regulate cell death in animals (53). Along this line, we found increased amounts of an MLO1-like membrane spanning protein. MLO is a negative regulator of defense and cell death (54), thus its increase suggests enhanced suppression of defense and cell death. Since part of any analysis of a biotrophic relationship must consider the likelihood that the pathogen is subjugating host defenses to preserve its own fitness, it is also possible that the accumulation of the MLO1-like protein was actuated by the fungus to prevent defense responses detrimental to it. Either way, regulation of cell death, mostly thought to be relegated to R-gene defenses, may be part of basal defenses.

There was another set of 112 proteins whose accumulations generally decreased, most by nearly equivalent levels, as a result of inoculation with either virulent or avirulent rusts at 24 hpi (Fig. 2, Group 2). A decrease of some of these proteins may lead to increased basal defense responses. For example, there were lipoxigenases, the decreased amounts of which may lead to repression of the jasmonic acid defense pathway which is an agonist of SA defenses (55). Since free FAs may accumulate as a result of decreased amounts of lipoxigenase, these FAs may be diverted to a pathogen-inducible dioxygenase pathway known to be activated during a strong HR in other plants (56). Although many of these decreases point to negative regulation of proteins to inhibit rust infection, other decreases may have a positive effect—the plant may decrease the amounts of proteins involved in sugar and amino acid metabolism to retard fungal growth. In addition, the role of the fungus in altering plant protein accumulation must be considered. For example, the fungus may have decreased the accumulation of glutathione-S-transferase (GST)-like In2-1, phospholipid glutathione peroxidase, gamma-glutamylcysteine synthetase, and dehydroascorbate reductase to stall detoxification and antioxidant defense to
produce an environment more favorable for biotrophy. Likewise, the fungus may have reduced the accumulation of an ADP-ribosylation factor to deregulate a vesicle trafficking-coordinated strong immune response (12).

Late Basal Defense Responses—Only 35% of the proteins found at 24 hpi in susceptible and resistant plants were discovered at 72 hpi. For example, most of the proteins involved in sugar biosynthesis/metabolism, the accumulations of which decreased at 24 hpi, were no longer observed at 72 hpi. These early patterns may partially reflect the ability of the rust fungus to deregulate some early basal defenses, whereas the later ones reflect the plant’s countermeasures or a systematic deployment of defense over time.

Defense-related proteins, the accumulations of which rose specifically by 72 hpi, included distinct GSTs and other proteins that probably mobilized cellular toxins (Fig. 2, Group 1). At the same time, there were increased amounts of enzymes such as O-methyltransferase, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECS), cyclase, and isopentenyl pyrophosphate isomerase that produce isoprenoids, flavonoids, and phytoalexins that are part of SA-mediated disease resistance (57). A few additional PR proteins were also activated at 72 hpi.

Among proteins with pronounced decreased accumulation at 72 hpi were components of thylakoids or proteins that functioned in the chloroplasts, such as lipoxygenases, peptidases, and enzymes for chlorophyll synthesis (Fig. 2, Group 2). Even though other chloroplast proteins appeared in other clusters, predominant clustering here suggests chloroplast deterioration as a result of infection. In resistant plants, deterioration may result from cell death, the visible signs of which first appeared 3 days later, whereas in susceptible plants the decreases may indicate impending tissue deterioration as a result of fungal biotrophy. These observations of chloroplast protein degradation are similar to those seen in other infected plants (58).

Proteins Specifically Regulated during Gene-for-gene Resistance—When the basal resistance response provided by susceptible plants fails to stop the fungus, an HR afforded by an R-gene must either quantitatively supersede a weakened basal response beyond an effective level of resistance (3) or as an alternative hypothesis, repair any part of it that has been deactivated by the pathogen to reinstate an effective level of resistance inherent to the basal defense system (Fig. 3, A and B). We evaluated the same plant variety undergoing either a susceptible response or a resistant response, so there was no genomic difference that accounted for resistance potential. Thus, the differences between susceptibility and resistance lay in the activation and the response of certain sets of proteins under control of Ur-4. At 24 hpi, 49 proteins accumulated to higher levels in the resistant plants but not in the susceptible plants (Fig. 4, Group 3), and 38 proteins significantly decreased in resistant plants but not in susceptible plants (Group 4). These differences point to the resistance governed by Ur-4 activation.

Resistant plants accumulated greater amounts of several proteins with potential roles in conferring strong defense responses associated with HR or cell death. These included a cysteine peptidase similar to VPE, which controls vacuolar proteolysis in tobacco undergoing an HR (17) and serine C-palmitoyltransferase, a sphingolipid synthase the increased activity of which is linked to cell death (59). By 72 hpi in resistant plants, there was increased accumulation of a harpin-binding protein and a zinc metalloproteinase and decreased accumulation of protoporphyrinogen oxidase. Each of these proteins is linked to programmed cell death in different hosts (60–62). Although the differential accumulation of these proteins distinguished resistant plants, we note that the set of basal defense proteins defined earlier also comprised proteins with potential to coordinate cell death processes.
Interestingly, resistant plants differentially accumulated some homologues of proteins that were part of the basal response defined earlier, namely a thioredoxin, esterase/lipase, and plasma membrane protein. Resistant plants also accumulated more proteins involved in phytoalexin synthesis, which is part of the basal, SA-mediated defense pathway—there was more isoflavone reductase, a key enzyme producing isoflavanoid phytoalexins, and there were more 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXPR) and 2-C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (MECS), two enzymes leading to diterpenoid phytoalexin biosynthesis (57). Resistant plants also had more cinnamyl alcohol dehydrogenase (CAD) at 72 hpi. Cinnamyl alcohol dehydrogenase accumulation is important for basal penetration resistance to non-adapted rusts and likely contributes to cell wall lignification and papillae formation (20). Resistant plants accumulated less 9/13 hydroperoxidase and triacylglycerol lipase, both of which are homologous to the lipoxigenases in the set of proteins defining the basal defense response.

These data resolve protein accumulation differences attributed to Ur-4 activation and satisfy the concept that R-genes amplify basal defense responses. However, the data do not fully support the idea that basal and Ur-4-mediated defenses are entirely separate entities because both responses involve many of the same types of proteins, including those linked to cell death processes. Nor do the data fully support the notion that Ur-4 superseded an effective resistance threshold because the proteins that distinguished Ur-4 activation did not accumulate to levels greater than the highest levels of proteins found in susceptible plants that defined the basal defenses. This suggests that no over-amplification was achieved. Hence, the alternative model is a better fit for our data. In other words, it is possible that Ur-4-amplified parts of the strong basal defense deactivated by the rust fungus no further beyond what was required for stopping fungal growth and spread. Such a repair may be a result of the differential accumulation of a specific set of proteins including those seen here: a protein kinase, a TRAF-like zinc finger protein, an ADP-ribosylation-factor-GTPase-activating protein, adaptin/coatamer, aquaporins, and a WD-40 protein with similarity to one involved in brassinosteroid signaling (63).

**Lowered Resistance in Susceptible Plants**—Susceptible beans have equivalent potential to generate a strong resistance response, but do not when infected with race 41, which likely impairs the innate basal resistance. Our experiments were also designed to reveal this possibility and identify sets of proteins the accumulations of which defined susceptibility to race 41 relative to the resistance against race 49. At 24 hpi, 66 proteins had greater accumulation in susceptible plants (Fig. 4, Group 5). Nearly two-thirds of these proteins function in the chloroplast or are implicated in protein translation. Thus, relatively speaking, decreased accumulation of translational elongation factors and chloroplast proteins is a function of disease resistance to *U. appendiculatus* at 24 hpi.

There were also 56 proteins that accumulated to much lower levels compared with resistant plants (Fig. 4, Group 6). Many of these proteins are similar to those previously defined as having roles in basal and gene-for-gene defenses. The accumulation of another PR-like protein decreased, suggesting that pathogen effector-driven suppression of some SA-mediated responses had occurred. There was less of another homologue of 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXPR), which may have impacted phytoalexin synthesis at early stages of infection, and less UDP-glucose pyrophosphorylase, which may have affected callose deposition and production of papillae that serve as penetration barriers (64, 65). An adenosine kinase related to those involved in disease resistance in rice (66) was less abundant as were SKP1-ubiquitin ligase and conjugating enzymes, which are key components to some early R-gene-mediated plant defenses (67). These data suggest that the accumulation of proteins potentially involved in both basal and gene-for-gene-mediated defense responses was impacted by race 41. Given recent discoveries that pathogen effector proteins can mimic host proteins, deregulate host defenses, or alter host gene expression (12, 68–70), the rust fungus may also have altered the accumulation of a wide variety proteins in the basal defense system to support its own successful colonization. If true, one may wonder how the plant would have responded had the fungus not been virulent. The answer can be seen in plants challenged with race 49, where Ur-4 was activated. Most of the protein levels in resistant plants accumulated oppositely, but not to levels that would indicate drastic amplification over a hypothetical effective resistance threshold (Fig. 4, Groups 5 and 6). Rather, in resistant plants, the activated Ur-4 gene appeared to sustain proteomic parts of the defense system that was deregulated by race 41, implying that defenses were brought back up rather than raised to a higher level.

**DISCUSSION**

According to our proteomics analysis, some SA-response-driven proteins known to be part of the basal defense system were found to be part of the Ur-4-mediated defenses. This finding further blurs the notion that basal and R-gene-mediated defenses constitute entirely distinct systems. In addition to this, some of the proteomics data regarding the rust-bean interaction is partly inconsistent with a previous disease re-
sistance model (3). Examples are: 1) some proteins possibly regulating cell death or insusceptibility/immunity were clustered among other known basal defense proteins, suggesting that cell death may not be solely relegated to R-gene mediated defense; 2) defense response proteins the accumulations of which decreased in susceptible plants, likely due to the pathogen, were not appreciably induced in resistant plants to a level that would support that a threshold was exceeded; 3) across the board, proteins that distinguished Ur-4-mediated defenses accumulated to levels no higher than proteins that distinguished the basal defense. With respect to the latter two examples, whereas the dynamic range of our method may have restricted the range of our relative accumulation measurements, it seems that Ur-4-mediated defense was not contingent upon excessive protein accumulation beyond the levels that amounted to the basal defense response.

These observations can be used to argue that basal and R-gene-mediated defenses are not mutually exclusive entities and that gene-for-gene resistance does not supersede basal resistance to surpass a special threshold to confer a higher level of defense. In light of evidence that pathogens deploy effectors to dismantle and deregulate host defenses (12, 68, 69), it may be appropriate to reconsider the disease resistance model and correlate the highest degree of effective resistance with the point at which there is no pathogen colonization, as proposed by Cheo (71). This is an important facet that makes our model testable against a reference standard for susceptibility or resistance.

The foundation for the model remains the same—there are several modes of basal defenses, which include passive physiological barriers and multivariant layered active defenses governed by receptors or perceivers (69, 72); active defenses controlled by many different genes representing several defense modes converge into a number of common responses depending on whether the stimulus is external or internal (46). The basal defense system as a whole is conditioned to reach a threshold inherently strong enough to create an incompatible environment for colonization of most potential pathogens (46, 68, 72, 73). Such a response may be tied to insusceptibility and programmed cell death (19, 20).

In the event where pathogens have evolved to overcome basal defenses, susceptible plants exhibit weakened basal defenses. Responding in kind, plants have evolved countermeasures to some, but not all, pathogens (2). In such cases, R-genes have adapted to recognize infections caused by a few pathogens that break basal defenses, but in our model R-genes by themselves do not operate outside the effective resistance potential conditioned by inherently strong basal defenses (Fig. 3B). Our data revealing protein accumulation in susceptible and resistant bean plants are consistent with the idea that Ur-4 acts to restore parts of the basal defense system rather than initiate a separate defense pathway or amplify beyond a level required for effective resistance. In other words, our data follow a model whereby an R-gene repairs any necessary part of the disturbed basal defense system to recondition the potential of a strong response threshold sufficient for sustaining an incompatible reaction.

In addition to our data, an example that satisfies our model is that of AvrPtoB, which elicits an HR and gene-for-gene resistance in tomato plants carrying the R-gene Pto. Mutated AvrPtoB activates Pto-independent cell death, meaning there must be a separate basal receptor for mutant AvrPtoB (69). In essence, Pto functions as a patch to return the inherent strong basal immune defense response in light of functional AvrPtoB. Our model also satisfies explanations for R-gene evolution, innate mechanisms of defense that make plants immune to most potential pathogens (19, 46, 74) and observed stochastic and kinetic defense response differences between susceptible and resistant plants including "spreading" local lesion phenotypes that are part of an R-gene mediated HR (i.e. a leaky patch).

Finally, we reiterate that there are no known rusts adapted to A. thaliana. And although there are data that describe A. thaliana responses to virulent and avirulent bacteria (14–16), we have not attempted to compare our data as a whole across these other datasets because bacteria and rust fungi have drastically different infection mechanisms and because the genomes between the two plants are not readily comparable given that the bean genome is not sequenced. There may be commonalities or differences that are resolvable through additional research. Thus, with respect to beans, one new observation resulting from our data-dependent analysis is that the fluctuation of proteins with roles in regulating FA signaling is a major consequence of rust infection. Such signals may be important to rust resistance. The data also reveal that intricate proteomic dynamics exist in a plant fighting the fungus yet being enticed to support fungal growth at the early stages of infection. Clearly, the increased accumulation of some proteins and the collateral decrease of their homologues or functional analogs indicate that the plant cell performs a delicate balancing act with regard to fighting the pathogen, succumbing to it, and regaining stasis.

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