Effector Proteins of the Bacterial Pathogen *Pseudomonas syringae* Alter the Extracellular Proteome of the Host Plant, *Arabidopsis thaliana*[*]

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In plants, potential pathogenic bacteria do not enter the host cell. Therefore, a large portion of the molecular interaction between microbial pathogen and host occurs in the extracellular space. To investigate potential mechanisms of disease resistance and susceptibility, we analyzed changes in the extracellular proteome, or secretome, using the *Arabidopsis-Pseudomonas syringae* pathosystem. This system provides the possibility to directly compare interactions resulting in basal resistance, susceptibility, and gene-specific resistance by using different genotypes of *Pseudomonas* on the same host. After infecting suspension-cultured cells of *Arabidopsis* with the *Pseudomonas* strain of interest, we isolated protein from the cell culture medium representing the secretome. After one-dimensional gel separation and in-gel digestion of proteins, we used iTRAQ (isobaric tags for relative and absolute quantitation) labeling in conjunction with LC-MS/MS to perform relative quantitative comparisons of the secretomes from each of these interactions. We obtained quantitative information from 45 *Arabidopsis* proteins that were present in all three biological experiments. We observed complex patterns of accumulation, ranging from proteins that decreased in abundance in the presence of all three bacterial strains to proteins that specifically increased or decreased during only one of the interactions. A particularly intriguing result was that the virulent bacteria (*e.g.* a susceptible interaction) caused the extracellular accumulation of a specific subset of host proteins lacking traditional signal peptides. These results indicate that the pathogen may manipulate host secretion to promote the successful invasion of plants. *Molecular & Cellular Proteomics 8:145–156, 2009.*

As sessile organisms, plants require effective defense mechanisms for protection against the plethora of potential microbial pathogens to which they are exposed. Plants and animals have developed basal resistance mechanisms with a number of similarities (1, 2). Basal defense responses are initiated by the detection of conserved microbial features called general elicitors or microbe-associated molecular patterns (MAMPs).[1] MAMPs are molecules that are intrinsic to the lifecycle of the potential pathogen, and in bacteria these include flagellin (3), EF-Tu (4), and lipopolysaccharides (5). In plants, MAMP recognition by receptor-like kinases initiates rapid phosphorylation/dephosphorylation of proteins, a transient increase in cytosolic calcium, and transcription of defense-related genes, ultimately manifesting in resistance against the bacterial invader (6–8).

For pathogens to successfully invade the host, they need to overcome basal resistance mechanisms. Gram-negative bacteria have evolved a type III secretion system (TTSS) to inject numerous bacterial effector proteins into the plant cell that suppress MAMP-triggered, basal resistance responses (9, 10). Effector proteins target a range of processes in the host cell, including activation of mitogen-activated protein kinases (11), RNA metabolism (12), and protein secretion (13). The importance of a functional TTSS for bacterial virulence is illustrated by the fact that normally virulent bacteria defective in TTSS lose pathogenicity (14).

During co-evolution of host and pathogen, plants have developed another layer of defense based on detection of bacterial effector proteins (15, 16). This defense response involves direct or indirect recognition of effectors in the plant cell by resistance proteins (*R* proteins), resulting in restoration of host resistance against the invading microbe. As a consequence, effector proteins recognized by the host and resulting in resistance are called avirulence proteins (Avr proteins). This model for race-specific resistance based on pairwise association of an *R* gene in the host with the cognate *avr* gene from the bacterium has been described as the "gene-for-gene" concept of plant disease resistance (17).

The *Arabidopsis thaliana-Pseudomonas syringae* pathosystem is a model for studies of innate immunity, susceptibility,

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[1] The abbreviations used are: MAMP, microbe-associated molecular pattern; EF, elongation factor; TTSS, type III secretion system; TTE, type III effector protein; *Pst*, *P. syringae* pv. *tomato*; iTRAQ, isobaric tags for relative and absolute quantitation; R, resistance; Avr, avirulence; RT, room temperature; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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and gene-for-gene resistance and has been used for transcriptomics and proteomics studies of these responses (18–21). By studying responses to different *P. syringae* pv. *tomato* (*Pst*) strains, this pathosystem provides the possibility to dissect mechanisms specific for each type of interaction. *Pseudomonas* strains unable to deliver effectors into plant cells, such as *Pst* DC3000 *hrpA*$^-$ (*hrpA*$^-$), cannot establish an infection. In this case, basal resistance of the host is sufficient to restrict pathogen growth. In contrast, treating plants with the virulent strain *Pst* DC3000 with a full TTSS leads to a successful infection because basal resistance of the host is overcome by type III effectors (TTEs) of the pathogen. Finally gene-for-gene resistance can be studied using bacterial strains expressing single avirulence genes, such as *Pst* DC3000 *avrRpm1* (*avrRpm1*), for which the host has the cognate resistance gene, in this case the *RPM1 R* gene in *Arabidopsis* ecotype Landsberg erecta (22–25).

Because bacterial pathogens never enter the cytoplasm of the plant cell, protein secretion from the host cell is likely to play an important role in resistance. Secretome analyses (proteomics analyses of secreted proteins) have been reported using isolated MAMPs (26, 27), but no secretome study has been performed using intact pathogens. Although studies using individual MAMPs are informative, they will only reveal changes in protein secretion in response to the single stimulus without taking into account the full sum of effects of other proteins or small molecules that are produced by the invading organism during host-microbe interactions. Therefore, we infected *Arabidopsis* (ecotype Landsberg erecta (28)) suspension cell cultures with the three *Pst* strains described above to analyze qualitatively and quantitatively extracellular proteins accumulating during distinct *Arabidopsis-Pseudomonas* interactions. As expected, we found subsets of extracellular proteins that were secreted during basal resistance, suppressed by TTSS, or restored by gene-for-gene resistance. However, the interactions of pathogen genotypes with the host also revealed surprising results. The most striking discovery was that the virulent pathogen, *Pst* DC3000, reproducibly caused the extracellular accumulation of a specific subset of host proteins. These results reveal a previously uncharacterized step in the infection process and demonstrate the need for performing studies addressing complex organismal interactions.

EXPERIMENTAL PROCEDURES

Cell Cultures and Pathogen Infection—Suspension-cultured cells of *A. thaliana* ecotype "Landsberg erecta" were maintained as described previously (28) and used for experiments 8 days after subculture. Before treatment with bacteria, 300 ml of cell suspension culture was washed three times with 25 ml of "minimal medium" (1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 20 μM ferric citrate, 43 μM H_3BO_4, 12 μM $MnSO_4$, 1.2 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.5 μM H_2MoO_4, 2% sucrose supplemented with Murashige and Skoog vitamin solution (Sigma); pH was adjusted to 5.0 with 5 mM MES) and resuspended in 75 ml of the same medium. After 1-h incubation in an incubator shaker (model G25, New Brunswick Scientific) at 21 °C at 170 rpm, bacteria were added to a final A_{600} of 0.3. *P. syringae* pv. *tomato* strains DC3000, DC3000 (*avrRpm1*), and DC3000 (*hrpA*$^-$) were cultivated overnight in L medium (10 g/liter Bacto tryptone, 5 g/liter yeast extract, 5 g/liter NaCl, 1 g/liter glucose, 100 μg/ml rifampicin, 25 μg/ml kanamycin). Before infection, bacteria were pelleted by centrifugation, and medium was removed. Cells were washed in 1 ml of 10 mM $MgCl_2$ and resuspended in the same medium, and A_{600} was determined.

Estimation of Cell Death with Evans Blue Staining—For determination of cell death, 2 μl of 1% Evans blue solution was added to 200 μl of cell suspension culture and incubated for 10 min at room temperature (RT) on a shaker. One milliliter of medium was added, and the suspension was centrifuged to pellet the cells. Medium was removed, and the washing step was repeated five times. Dead cells were counted under the microscope and plotted as a percentage of the total cell number.

Extraction and Separation of Extracellular Proteins—After 10 h of incubation with the bacteria, plant cells were separated from the medium by filtering through three layers of Miracloth. Plant cells were immediately frozen in liquid nitrogen and stored at −80 °C until further use. The filtrate was centrifuged (45,000 × *g*, 5 min, RT) to remove bacteria, and protein in the supernatant was extracted by adding 0.5 volume of Tris-buffered phenol (pH 8.0) and mixing vigorously. After 30 min on ice, phase separation was performed by centrifugation (12,000 × *g*, 10 min, RT). The phenol phase was back-extracted twice with 1 volume of back extraction buffer (0.1 M Tris-HCl, pH 8.0, 20 mM KCl, 10 mM EDTA, 0.4% (v/v) β-mercaptoethanol). After addition of 5 volumes 0.1 M NH_4OAc in methanol, protein was precipitated overnight at −20 °C. Protein was recovered by centrifugation (45,000 × *g*, 1 h, 4 °C), and the pellet was washed twice with 0.1 M NH_4OAc in methanol and twice with 80% acetone. Protein was kept as an acetone suspension at −20 °C until further use. Protein quantification was performed using the BCA assay (Sigma) with BSA as standard. Proteins were separated by SDS-PAGE using precast NuPAGE® 12% BisTris gels with MES running buffer (Invitrogen). Proteins in the gel were stained with colloidal Coomassie overnight using a modified method after Neuhoff *et al.* (29). Gels were destained in water, and each lane was divided into six strips with a razor blade. Resulting gel slices were subjected to tryptic digestion overnight in 100 mM triethylammonium bicarbonate buffer. After digestion, peptides were eluted with 0.1% acetic acid and acetonitrile. The concentration of organic solvent was decreased by drying for 20 min in a vacuum concentrator, and subsequently peptides were dried in a freeze-drier overnight. After reaching complete dryness, peptides were stored at −80 °C until further use.

Quantitative Mass Spectrometric Analysis Using iTRAQ Reagent—For quantification by mass spectrometry, peptides were labeled with iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). After labeling for 2 h at RT, peptides from corresponding gel pieces of each treatment were combined, and the concentration of organic solvent was reduced using a vacuum concentrator. Subsequently peptides were dried in a freeze-drier and stored at −80 °C until further analysis. Lyophilized peptides were dissolved in 1% acetonitrile and 0.1% formic acid for MS analysis. Peptides were applied to a precolumn (C_{18} PepMap100, LC Packings, Camberley, UK) connected to a self-packed C_{18} 8-cm analytical column (BioBasic resin, ThermoElectron; Picotip, 75-μm inner diameter, 15-μm tip, New Objective, Woburn, MA) and eluted into the nanoelectrospray ion source of a quadrupole time-of-flight mass spectrometer (Q-ToF2, Micromass UK Ltd., Manchester, UK) controlled by MassLynx version 1.4. A fully automated chromatography run was carried out with the mass spectrometer operating in data-dependent mode. The buffer solutions used for chromatography were 0.1% formic acid (buffer A) and 100% ACN, 0.5% formic acid (buffer

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B). The reverse-phase gradient was developed with the following profile: $t = 0$, B = 2%; $t = 60$, B = 40%; $t = 70$, B = 80%. The MS survey scan covered the range 400–1600 *m/z* with a threshold of 20 counts/s and a scan time of 2 s. For MS/MS spectra the *m/z* range was 50–2500 with a scan time of 2 s. Charge state selection was enabled with a peak detection window of 4 Da, the advanced charge state components were 1000, and a tolerance of 0.3 Da and a peak extraction window of 4 Da were used. Dynamic exclusion was enabled for 30 s after acquisition with a tolerance of 1.5 Da. Dynamic collision energies were used depending upon charge state.

LC-MS/MS Data Analysis—ProteinLynx Global Server version 2.1.5 was used to extract both combined and individual MS/MS data as pkl files using default settings. Fragment ion spectra (as combined pkl files) were searched using the MASCOT search tool (version 2.1, Matrix Science Ltd., London, UK) against weekly updates of the UniProt database (3,023,461 sequences in April 2006) with taxonomy limited to *A. thaliana* and *P. syringae* (63,787 sequences actually searched). The following MASCOT search parameters were used: peptide mass tolerance, 0.35 Da; fragment mass tolerance, 0.25 Da; trypsin cleavage with a maximum of three missed cleavages; fixed modification, iTRAQ on lysine and N termini; and variable modification, oxidation of methionine. False positive rates were calculated using the decoy option provided by MASCOT for each experiment independently resulting in rates between 0.94 and 1.33% (peptide matches above identity threshold). Quantitation of the *m/z* 114–117 peak areas was performed with the i-Tracker program (30). A requirement of the i-Tracker program is that data are presented in either .mgf or .dta format and must contain uncentroided data. To facilitate this, the data from the Q-ToF2 was processed using tools publicly available from the Seattle Center for Proteomics. First the massWolf program was used to convert the raw data from the Q-ToF2 into mzXML format. The resulting mzXML data file was then converted to .mgf format using the program mzXML2other. The .mgf file was then processed with i-Tracker to obtain the reporter ion peak areas. For further analysis, data were imported into Microsoft Excel. Peptides identified with MASCOT scores less than 20 were removed. For peptides identified multiple times in the same analysis, the one with the highest MASCOT score was used for quantitation. To identify peptides that match to multiple members of a protein family amino acid sequences were searched against an *Arabidopsis* and *P. syringae* database (The Institute for Genomic Research ATH1, version 5.0, containing 26,207 protein-coding genes and 3786 pseudogenes, created June 2004; Ref. 31) to exclude ambiguous peptides. Proteins identified with two or more unique peptides were included for further quantitative analysis. The iTRAQ values of all peptides from one protein in a treatment were averaged, and S.D. was calculated for every experiment. To check data for statistically significant differences, the values were compared between treatments using a pairwise Student's *t* test. Ratios of treatments to control were calculated to obtain relative quantitative comparisons. For complete presentation of these data see supplemental Table 1. In addition, ratios of *Pst* DC3000 to *Pst* (*hrpA*$^-$) and *Pst* (*avrRpm1*) to *Pst* DC3000 are given. Mean values and S.D. were calculated from three independent biological replicates, and statistical significance was tested as described above.

Immunoblot Analysis—Immunoblot analysis was performed with extracellular protein samples obtained as described above. Cytosolic proteins were prepared by grinding ~0.5 g of plant cells with 1 ml of protein extraction buffer (100 mM sodium phosphate, 5% glycerol, 0.5% polyvinylpyrrolidone, 5 mM DTT, 1 mM EDTA, 1 mM PMSF, 10 μM leupeptin, pH 7.0) with a pestle and mortar on ice. After two consecutive centrifugation steps (16,000 $\times$ *g*, 5 min, 4 °C and 16,000 $\times$ *g*, 120 min, 4 °C), the cleared supernatant was frozen in liquid nitrogen and stored at −80 °C until further use. Protein concentration was determined using the BCA assay (Sigma) with BSA as standard. For immunoblotting of ubiquitin, 20 μg of total protein/lane was separated using a 12% NuPAGE BisTris gel with MES buffer (Invitrogen) and blotted on a PVDF membrane. As a positive control, bovine ubiquitin (Biomol, Exeter, UK) was used. Immunoblots were incubated with ubiquitin (P4D1) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and detection was performed using a chemiluminescent substrate (Pico, Pierce) and x-ray films (Fujifilm, Bedford, UK). For analysis of methionine synthase AtMS1, 20 μg of total protein was separated with a 4–12% NuPAGE BisTris gel with MES buffer and electroblotted on a nitrocellulose membrane (Amersham Biosciences). AtMS1-specific antibodies were obtained from Stephane Ravanel, Grenoble, France, and detection was performed as described previously (32).

RESULTS

Establishing Treatment Conditions with Pathogens—Previously *Arabidopsis* suspension cell cultures were shown to undergo gene-for-gene-specific resistance responses such as the formation of nitric oxide and induced cell death when infected with *Pseudomonas* expressing the avirulence gene, *avrRpm1* (33). Because this cell culture system is amenable to isolation of large amounts of secreted proteins, we wanted to examine accumulation patterns of extracellular proteins during *Arabidopsis-Pseudomonas* interactions using *Arabidopsis* suspension cell cultures challenged with three different *Pst* genotypes: DC3000, DC3000 (*avrRpm1*), and DC3000 (*hrpA*$^-$) representing virulent, avirulent, and non-pathogenic strains, respectively. To reduce the abundance of proteins secreted more generally during cell growth, *Arabidopsis* cells were thoroughly washed before onset of infection, and the medium was exchanged from full cultivation medium to a minimal medium containing no nitrogen with a pH of 5 (for details see "Experimental Procedures") that we found to promote the efficiency and speed of bacterial infection. Next we investigated the timing of the induced cell death during a strong gene-for-gene resistance response to minimize the possibility of our studies being complicated by cellular leakage of proteins. Using Evans Blue staining to indicate cell viability at 10, 24, and 48 h after infection with the three *Pst* strains, the pattern of host cell death was consistent with the anticipated genotypic interaction (Fig. 1). Although no significant cell death was observed in the control or DC3000 (*hrpA*$^-$)-treated cells throughout the experiment, nearly 70% of plants cells died within 24 h of infection with the avirulent strain *Pst* (*avrRpm1*) and continued to nearly 100% by 48 h. In contrast, infection with the virulent strain DC3000 resulted in delayed cell death with only 20% of cells dead after 24 h and increasing to about 60% by 48 h after infection. Because none of the treatments resulted in significant cell death 10 h postinfection in three separate biological treatments, we chose this time point for our proteomics study of secreted proteins.

Isolation and Identification of Secreted Proteins—To collect the extracellular proteins, plant cells were first filtered away from the medium, and then the bacteria were removed by centrifugation. The medium was concentrated by phenol ex-

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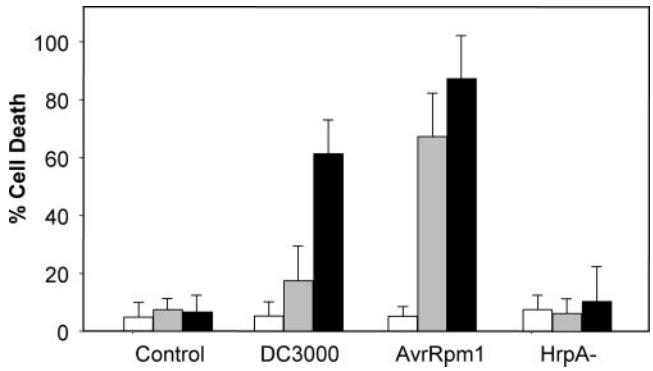

FIG. 1. **Timing of cell death in *Arabidopsis* cells depends on the *P. syringae* pv. *tomato* strain used for infection.** The percentage of dead cells 10 (*white bars*), 24 (*gray bars*), and 48 h (*black bars*) after no infection (*Control*) or infection with *Pst* DC3000 (*DC3000*), *Pst* DC3000 expressing *avrRpm1* (*AvrRpm1*), or *Pst* DC3000 lacking a functional type III secretion system (*HrpA−*) is shown. Cell death was measured by staining cells with Evans Blue and counting the number of cells taking up the dye. The experiment was performed at least three times with similar results. Results shown are means ± S.D.

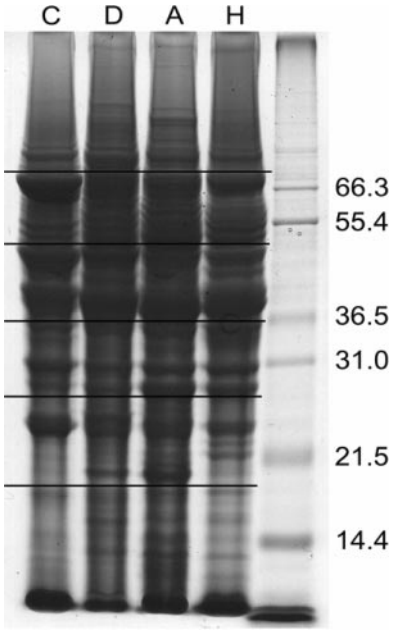

FIG. 2. **Separation of extracellular proteins by SDS-PAGE.** Proteins from the extracellular medium were collected after no infection (*C*) or infection with *Pst* DC3000 (*D*), *Pst* DC3000 expressing *avrRpm1* (*A*), or *Pst* DC3000 lacking a functional type III secretion system (*H*). After separation, the gel was stained with colloidal Coomassie. *Lines* indicate where the gel was cut to obtain gel slices.

traction followed by methanol precipitation of proteins. Total proteins from the supernatant were separated by SDS-PAGE and stained with colloidal Coomassie (Fig. 2). Visual inspection of the gels revealed a number of differences in protein patterns between treatments particularly in the low molecular mass range. To analyze these differentially accumulating protein bands quantitatively, we used an isobaric labeling reagent (iTRAQ, Applied Biosystems) in conjunction with LC-MS/MS. To decrease sample complexity, the SDS-PAGE gels were cut into six pieces per lane (as shown schematically in Fig. 2), and each gel slice was subjected to in-gel tryptic digestion. Gel-eluted peptides from each treatment were labeled with one of the iTRAQ reagents (*e.g.* in one of the biological experiments, all control peptides were labeled with the 114 reagent, all DC3000 peptides were labeled with the 115 reagent, etc.) before pooling peptides from all four treatments. The iTRAQ-labeled peptides were analyzed by LC-MS/MS using a Q-ToF2 (Micromass UK Ltd.) and MASCOT software. Filter criteria for identification of proteins were a peptide MASCOT score of over 20 and two or more peptides per protein. Additionally all identified peptide sequences were searched against an *Arabidopsis* protein database (The Institute for Genomic Research ATH1) to exclude peptides not unique to a single protein. These criteria were applied to the data sets of three independent biological experiments, and only proteins identified in all three experiments were used for further analysis.

Although about 91 *Arabidopsis* proteins were identified by these criteria in at least one of the experiments, a total of 45 proteins were found reproducibly in all three experiments (a Venn diagram showing the number of proteins identified in each experiment is provided as supplemental Fig. 1). In addition, the secretome contained proteins originating from *Pseudomonas* (listed in supplemental Table 1), but because we did not observe any consistent quantitative changes in these proteins, we did not pursue them further. *Arabidopsis* proteins found in all three experiments are listed in Table I. With only a few exceptions, the peptides identified unique proteins. In two cases (glyceraldehyde-3-phosphate dehydrogenase and peptidyl-prolyl cis-trans isomerase) where highly conserved paralogues made it impossible to differentiate between two proteins, both possible gene accessions are provided. In the case of purple acid phosphatase PAP10, different gene models exist under The *Arabidopsis* Information Resource entry At2g16430. Our results agree with only one gene model (At2g16430.2) as indicated in Table I. The final exception was ubiquitin. In *Arabidopsis*, at least 14 ubiquitin-containing genes exist whose nucleotide sequences translate to the peptides identified with 100% identity. Therefore, we refer to this identification as ubiquitin without indicating a specific gene accession. Protein predictions based on genomic sequences of 24 of the identified extracellular proteins contain N-terminal sequences coding for a signal peptide, suggesting that these extracellular proteins traveled through the secretory pathway. Although the remaining do not appear to be "classically" secreted proteins, 11 of these have been reported previously in extracellular cell wall preparations of *Arabidopsis* cells or tissue in other studies as indicated in Table I.

For most proteins, the calculated molecular mass of the predicted proteins was in relative agreement with the apparent mass based on their migration on SDS-PAGE gels (see

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TABLE I
Complete list of identified proteins
Proteins identified with two or more peptides in each of three independent biological replicates are shown. AGI, *Arabidopsis* Gene Index.

AGI no.	Annotation	No. pep[a]	Signal peptide[b]	Category[c]	Molecular mass	Apparent molecular mass	Identified as cell wall-associated
					kDa	*kDa*	
	Ubiquitin	3–5		misc	8.6	<18	44
At1g13440/At3g04120	Glyceraldehyde-3-phosphate dehydrogenase C subunit (GAPDH)	12–15	n	prim	41.7	35–50	27, 50
At1g14890	Invertase/methylesterase inhibitor family protein	2–3	y	cw	23.3	18–25	
At1g23190	Putative phosphoglucomutase	2–7	n	prim	70.0	50–75	
At1g35720	Annexin (ANNAT1)	2–5	n	mem	39.5	35–50	51
At1g45145	Thioredoxin (TRX5)	4–5	n	redox	15.2	<18	51
At1g56070	Putative elongation factor EF-2	3–4	n	prim	102.8	>75	27, 50
At1g63770	Putative aminopeptidase	5–11	n	misc	108.3	>75	
At1g68560	α-Xylosidase (ATXYL1)	2–4	y	cw	110.7	>75	44, 52
At1g74010	Putative strictosidine synthase	2–4	y	sm	38.1	25–35	53
At1g76160	Multicopper oxidase type I family protein (SKS5)	2–7	y	cw	62.3	50–75	44, 51
At1g78380	Glutathione transferase (GSTU19)	7–10	n	redox	29.1	18–25	
At1g78830	Curculin-like lectin family protein	5–7	y	cw	50.3	25–35	27, 44, 51–53
At1g79550	Putative phosphoglycerate kinase	4–8	n	prim	47.4	35–50	
At2g01820	Putative receptor-like protein kinase	3–5	y	misc	102.0	50–75	53
At2g02990	Ribonuclease (RNS1)	6–7	y	misc	28.4	18–25	51
At2g05920	Putative subtilisin-like serine protease	2–6	y	cw	86.4	50–75	44, 51
At2g16060	Non-symbiotic hemoglobin 1 (AHB1)	5–8	n	redox	20.9	18–25	
At2g16430	Purple acid phosphatase (PAP10)	5–7	y	cw	42.9	50–75	27, 52, 53
At2g17760	Aspartyl protease family protein	3–4	y	def	59.1	50–75	
At2g21130/At4g38740	Peptidyl-prolyl cis-trans isomerase	2	n	redox	21.1	18–25	
At2g32520	Putative carboxymethylenebutenolidase	2–5	n	sm	28.4	18–25	51
At2g36530	Enolase	3–11	n	prim	53.5	35–50	27, 50
At2g43570	Putative class IV chitinase	6–7	y	def	33.0	25–35	
At2g46880	Purple acid phosphatase (PAP14)	3–5	y	cw	47.5	50–75	
At3g18280	Lipid transfer protein	2	y	mem	11.7	<18	
At3g28940	AIG2 protein-like	2	n	def	21.4	18–25	51
At3g52880	Peroxisomal monodehydroascorbate reductase isoform 1 (MDAR 1)	2–5	n	redox	52.4	35–50	
At3g54420	Class IV chitinase (AtEP3)	3–5	y	def	31.1	25–35	51
At3g55440	Triose-phosphate isomerase	3–10	n	prim	30.1	25–35	51, 52
At4g09320	Nucleoside-diphosphate kinase I (NDPK1)	2–3	n	prim	17.4	<18	51
At4g25100	Superoxide dismutase (FSD1)	2–5	n	redox	23.1	18–25	
At4g25810	Putative xyloglucan endotransglucosylase/ hydrolase (XTH23)	7	y	cw	34.2	25–35	51, 52, 54
At4g26690	Putative glycerophosphodiesterase 2 (GPDL2)	2–3	y	mem	88.6	>75	
At4g34180	Putative cyclase	5	y	sm	30.6	25–35	51, 54
At4g34480	Putative glucan endo-1,3-β-glucosidase 7	4–8	y	cw	40.5	35–50	52
At5g06720	Peroxidase 53 (ATPA2)	2–4	y	redox	36.3	35–50	51, 54
At5g06860	Polygalacturonase-inhibiting protein 1 (PGIP1)	9–11	y	def	40.6	35–50	27, 44, 51–53
At5g17920	Cobalamin-independent methionine synthase (MS1)	4–9	n	prim	84.5	50–75	
At5g19440	Cinnamyl-alcohol dehydrogenase-like protein	3–4	n	sm	39.7	35–50	
At5g44130	Putative fasciclin-like arabinogalactan protein (FLA13)	2–5	y	cw	28.1	50–75	53
At5g51480	Multicopper oxidase type I family protein (SKS2)	5–7	y	cw	69.3	50–75	
At5g59880	Actin-depolymerizing factor 3 (ADF3)	2–4	n	misc	15.5	<18	51
At5g64120	Peroxidase 71 (ATP15a)	11–13	y	redox	35.9	25–35	51, 54
At5g67360	Subtilisin-like protease (ARA12)	13–16	y	cw	84.8	50–75	44, 52, 54

[a] Number of peptides identified in three biological replicates.
[b] Prediction results of SignalP 3.0 (55). y, yes; n, no.
[c] Functional category according to Fig. 3: misc, miscellaneous; prim, primary metabolism; cw, cell wall; mem, membrane; redox, redox-related; sm, secondary metabolism; def, defense.

Table I). However, the apparent masses of three proteins were substantially different from the theoretical mass. One protein, a putative fasciclin-like arabinogalactan protein (At5g44130) with a theoretical mass of 28 kDa was found in the mass range of 50–75 kDa. This shift to a higher mass is likely to result from glycosylation. Two proteins, a curculin-like lectin family protein (At1g78830) and a putative receptor-like kinase (At2g01820), migrated at masses much lower than expected,

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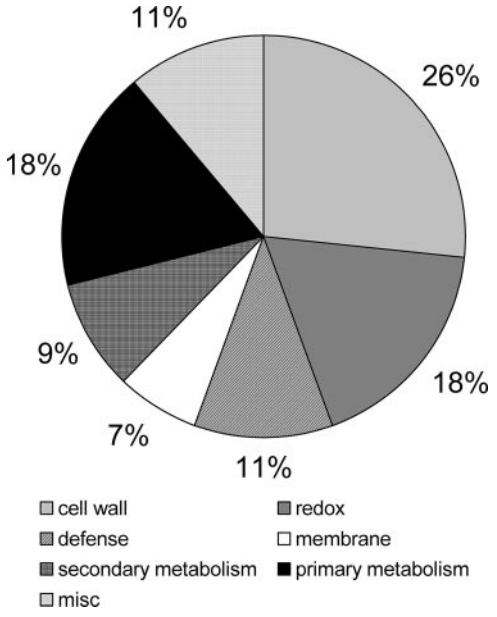

FIG. 3. **Distribution of functional categories of the identified extracellular proteins.** A total of 45 proteins were identified in all three independent experiments. The percentages of proteins of the functional categories are shown.

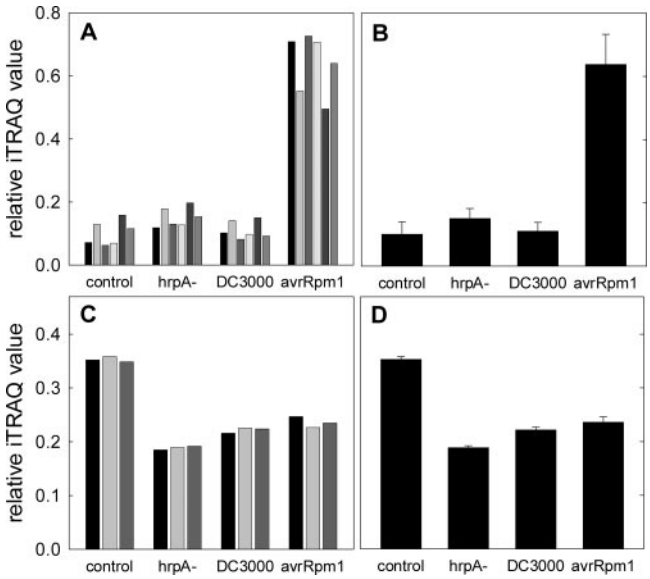

FIG. 4. **Relative abundance of peptides of two purple acid phosphatases (PAP10 (*A* and *B*) and PAP14 (*C* and *D*)) is shown.** In *A* and *C*, relative iTRAQ values of the four treatments for all peptides identified are presented, demonstrating little variability of abundance among the peptides of a particular protein. In *B* and *D*, mean values for the protein in each treatment are shown with S.D. These mean values were used to calculate accumulation and suppression rates shown in Table II.

raising the possibility of proteolytic processing. Indeed peptides from the receptor-like kinase were found only from the N-terminal part of the protein but not from the putative transmembrane or kinase domain, indicating cleavage and release of the leucine-rich repeat-containing domain. However, peptides identified from the curculin-like lectin spanned the complete sequence of the protein, indicating that discreet proteolytic processing/maturation is not responsible for lower apparent molecular mass.

Classification of proteins according to their annotated functions is shown in Fig. 3. As expected, the most highly represented class of proteins (26%) is involved in cell wall biosynthesis and degradation. Another large group (18%) consists of proteins involved in redox regulation processes. As will be discussed below, a surprising finding was that 18% of all proteins belong to primary metabolism.

Quantitative Comparisons of Secreted Proteins—To compare abundance of the proteins between treatments, we used the iTRAQ value of each peptide from a protein (Fig. 4, *A* and *C*) to determine the average iTRAQ value for the protein (Fig. 4, *B* and *D*). As illustrated by the results of the two proteins in Fig. 4, the quantitative values for each peptide from a protein were very consistent, indicating the reproducibility of the iTRAQ quantitation in these experiments. These total averages for a protein were used to calculate ratios between treatments. Ratios were calculated for each biological experiment to compare all three treatments *versus* control, DC3000 *versus* *hrpA*$^-$, or *avrRpm1* *versus* DC3000 (complete values for each experiment are provided in supplemental Table 2). To control for potential variation in the iTRAQ reagents, we swapped labels in each of the three biological experiments.

The quantitation of proteins from one representative experiment is shown in Table II. In many cases, the accumulation pattern of a protein was highly confident ($p < 0.05$) in all three experiments. However, proteins were included in a group if the p values were less than 0.05 in at least two of the three experiments with a similar trend in the third experiment. In addition, accumulation patterns were required to be supported by a similar confidence criteria when directly comparing ratios of DC3000 *versus* *hrpA*$^-$ (D/H in Table II) or *avrRpm1* *versus* DC3000 (A/D in Table II). All other proteins not meeting these criteria for consistency in accumulation patterns are listed in Table II under the heading No significant pattern of change. The complete data set of iTRAQ values of proteins and comparative ratios including statistical analysis from all three experiments is presented in supplemental Table 2.

A group of proteins showed no significant change after bacterial treatment (Table II, No significant pattern of change). These proteins all contain predicted signal peptides for classical secretion through the endoplasmic reticulum and Golgi (as indicated by a "y" under the SignalP heading). Many proteins of this class have apparent roles in cell wall metabolism and are likely general components related to cell growth and expansion. A second group of proteins is suppressed in the presence of all three genotypes of bacteria. These proteins may be suppressed by MAMP perception or by other

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TABLE II

Summary of quantitative data

Ratios of all three bacterial treatments to control and of DC3000 to *hrpA*$^-$ and *avrRpm1* to DC3000 from one representative experiment are shown (H, *Pst* DC3000 (*hrpA*$^-$); D, *Pst* DC3000; A, *Pst* DC3000 (*avrRpm1*); C, control). Classification of proteins required $p \leq 0.05$ in all three experiments for the predominant abundance pattern except where noted. *p* values were calculated using a paired Student's *t* test. AGI, *Arabidopsis* Gene Index; y, yes; n, no.

AGI no.	Annotation	SignalP	H/C	D/C	A/C	D/H	A/D
No significant pattern of change							
At1g14890	Invertase/methylesterase inhibitor family protein	y	0.69	1.23	1.03	1.78	0.84
At4g26690	Putative glycerophosphodiesterase 2 (GPDL2)	y	1.32	0.87	0.63	0.66	0.73
At5g06860	Polygalacturonase-inhibiting protein 1 (PGIP1)	y	0.67	0.70	1.17	1.04	1.68
At5g44130	Putative fasciclin-like arabinogalactan protein (FLA13)	y	0.98	0.78	0.60	0.80	0.76
At5g51480	Multicopper oxidase type I family protein (SKS2)	y	1.19	0.89	1.13	0.74	1.27
At5g64120	Peroxidase 71 (ATP15a)	y	0.70	2.20	1.48	3.16	0.67
Suppressed by all bacteria							
At1g74010	Putative strictosidine synthase[a]	y	0.63	0.73	0.65	1.16	0.89
At2g01820	Putative receptor-like protein kinase	y	0.57	0.37	0.33	0.65	0.91
At2g02990	Ribonuclease (RNS1)	y	0.38	0.39	0.50	0.71	1.41
At2g17760	Aspartyl protease family protein[a]	y	0.66	0.50	0.43	0.76	0.86
At2g46880	Purple acid phosphatase (PAP14)	y	0.53	0.63	0.67	1.17	1.06
At3g18280	Lipid transfer protein	y	0.44	0.47	0.38	1.08	0.80
At4g34480	Putative glucan endo-1,3-β-glucosidase 7	y	0.24	0.32	0.39	1.33	1.22
At5g06720	Peroxidase 53 (ATPA2)	y	0.39	0.37	0.30	0.93	0.82
At5g67360	Subtilisin-like protease (ARA12)	y	0.30	0.30	0.27	0.97	0.92
Induced by MAMPs/suppressed by TTEs							
At2g43570	Putative class IV chitinase	y	5.78	1.63	1.35	0.28	0.83
At4g25810	Putative xyloglucan endotransglucosylase/hydrolase (XTH23)	y	2.07	0.57	0.70	0.27	1.22
Induced by MAMPs/suppressed by TTEs/restored by gene-for-gene resistance							
At1g68560	α-Xylosidase (ATXYL1)[a]	y	1.35	0.70	1.43	0.52	2.04
Suppressed by TTEs							
At3g54420	Class IV chitinase (AtEP3)	y	1.19	0.55	0.54	0.46	0.99
At4g34180	Putative cyclase	y	1.28	0.66	0.56	0.52	0.84
Induced by TTEs							
At1g78380	Glutathione transferase (GSTU19)	n	0.51	5.34	2.33	10.57	0.44
At3g52880	Peroxisomal monodehydroascorbate reductase 1 (MDAR 1)[a]	n	0.90	2.77	2.99	3.07	1.08
At3g55440	Triose-phosphate isomerase[a]	n	0.79	2.89	3.85	3.65	1.33
At4g09320	Nucleoside-diphosphate kinase I (NDPK1)[a]	n	0.63	2.82	3.93	4.51	1.39
At4g25100	Superoxide dismutase (FSD1)	n	0.63	2.32	2.47	3.68	1.07
At5g19440	Cinnamyl-alcohol dehydrogenase-like protein	n	0.84	2.50	3.59	2.97	1.44
Suppressed by MAMPs/induced by TTEs/suppressed by gene-for-gene resistance							
At1g13440/At3g04120	Glyceraldehyde-3-phosphate dehydrogenase C (GAPDH)	n	0.21	2.11	0.39	10.26	0.18
At1g23190	Putative phosphoglucomutase[a]	n	0.41	2.25	1.49	5.48	0.66
At1g56070	Putative elongation factor EF-2[a]	n	0.55	1.44	0.39	2.60	0.27
At1g63770	Putative aminopeptidase[a]	n	0.72	1.69	0.60	2.36	0.36
At2g36530	Enolase[a]	n	0.36	3.38	0.93	9.51	0.28
At5g17920	Cobalamin-independent methionine synthase (MS1)	n	0.39	1.98	0.79	5.03	0.40
Induced by gene-for-gene resistance							
At1g35720	Annexin (ANNAT1)[a]	n	0.98	1.39	3.71	1.43	2.66
At1g45145	Thioredoxin (TRX5)	n	2.14	2.31	17.75	1.08	7.70
At1g76160	Multicopper oxidase type I family protein (SKS5)[a]	y	0.43	0.61	2.10	1.44	3.42
At1g78830	Curculin-like lectin family protein	y	1.72	1.30	2.75	0.76	2.12
At1g79550	Putative phosphoglycerate kinase	n	1.01	1.22	5.21	1.21	4.29
At2g05920	Putative subtilisin-like serine protease[a]	y	1.20	1.09	2.08	0.90	1.91
At2g16060	Non-symbiotic hemoglobin 1 (AHB1)	n	0.71	1.90	5.41	2.69	2.85
At2g16430.2	Purple acid phosphatase (PAP10)	y	1.50	1.05	5.62	0.70	5.35
At2g21130/At4g38740	Peptidyl-prolyl cis-trans isomerase[a]	n	1.24	1.37	9.13	1.10	6.69
At2g32520	Putative carboxymethylenebutenolidase	n	1.23	2.97	9.25	2.42	3.11
At3g28940	AIG2 protein-like	n	1.59	2.53	18.53	1.60	7.32
At5g59880	Actin-depolymerizing factor 3 (ADF3)	n	0.84	1.80	7.18	2.15	3.99
	Ubiquitin	n	0.82	2.17	5.00	2.66	2.31

[a] $p \leq 0.05$ in two of three experiments.

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factors from the bacteria but are not further regulated by TTEs or gene-for-gene resistance. As was the case for the proteins not changing in abundance, all proteins in this class also contain predicted signal peptides.

Two extracellular proteins showed the highest accumulation after treatment with the *Pst hrpA*$^-$ mutant, suggesting a possible role in MAMP-mediated defense against pathogens. This hypothesis is supported by the finding that bacteria with a TTSS (*i.e. Pst* DC3000 or *Pst* (*avrRpm1*)) suppress the accumulation of these proteins. The fact that *avrRpm1*-expressing bacteria do not restore the extracellular accumulation of these proteins indicates that these proteins may play a role in basal defense but not in gene-for-gene resistance.

Another group of proteins was regulated mainly by TTEs. After treatment with both *Pst* DC3000 or *Pst* (*avrRpm1*), two proteins were consistently down-regulated, and six proteins accumulated. The suppressed proteins both have traditional signal peptides for secretion, indicating that these proteins may be involved in constitutive defense in the cell wall as opposed to MAMP-induced defense. Surprisingly all six proteins that accumulated in the extracellular space in response to TTEs do not contain signal peptides. A number of these proteins appeared to demonstrate reduced accumulation in the MAMP-induced responses (*i.e.* DC3000 *hrpA*$^-$). This result indicates that these proteins accumulate extracellularly to a limited extent under normal conditions but are suppressed by MAMP responses and induced by TTEs, suggesting that they may play a role in pathogenicity. In addition, the complex patterns of accumulation suggest that these proteins without a signal peptide are not released by passive mechanisms but are secreted in a controlled manner.

A particularly interesting group of proteins are those that are suppressed by MAMP perception, induced by TTEs, and suppressed by gene-for-gene resistance. Therefore, the presence of these proteins in the extracellular medium represents a previously undiscovered connection to the promotion of infection. As was found for the TTE-regulated proteins discussed above, all proteins in this class lack a signal peptide for transport via the classical secretory pathway. Currently we cannot predict whether the presence of these proteins promotes virulence directly or whether their extracellular accumulation is a by-product of the actual virulence-promoting activity of TTEs. However, the suppression of accumulation by two forms of resistance and the reproducible accumulation during the virulent infection indicate that these proteins represent good biomarkers for a successful infection.

The final group consists of proteins associated specifically with gene-for-gene resistance after challenge with *Pst* (*avrRpm1*). In almost all cases, this resistance response is associated with high levels of accumulation in the extracellular space. Most of these proteins do not contain signal peptides. However, three of the proteins contain putative signal peptides. Of these, two proteins, multicopper oxidase type I family protein and polygalacturonase-inhibiting protein, are repressed by both the *hrpA*$^-$ mutant and DC3000 but have the extracellular levels restored by gene-for-gene resistance.

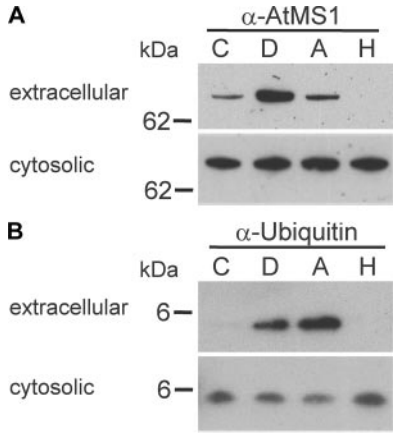

FIG. 5. **Immunoblot analysis for methionine synthase (AtMS1) and ubiquitin.** Total protein (20 μg/lane) from the extracellular medium or cytosol was separated on a 12% NuPAGE BisTris gel (ubiquitin) or 4–12% NuPAGE BisTris gel (AtMS1) with MES buffer and blotted to PVDF (ubiquitin) or nitrocellulose (AtMS1) membrane. *Labels* for the *lanes* are the same as in Fig. 2.

Comfirmation of Results by Immunoblot Analysis—To validate the iTRAQ data, we performed immunoblot analysis of two proteins with different accumulation patterns, cobalamin-independent methionine synthase (AtMS1 (32)) and ubiquitin. The extracellular protein levels of AtMS1 followed the same trend as indicated by the iTRAQ data. Its levels were highest after DC3000 treatment and lowest after *hrpA*$^-$ treatment (Fig. 5*A*). The intracellular level of AtMS1 was similar in all four treatments, suggesting that the differences observed were related to regulation of extracellular protein accumulation as opposed to changes in the overall protein levels. We also investigated the accumulation of ubiquitin. Because ubiquitin was identified only in gel slices corresponding to the lowest apparent molecular mass range, it appeared that the protein was monoubiquitin. Indeed immunoblot analysis detected only a single band of ~6 kDa (Fig. 5*B*). As was observed in the analysis of AtMS1, protein accumulation patterns as determined by immunoblot analysis were comparable to those derived from iTRAQ experiments. The protein was low in control and *hrpA*$^-$-treated samples, but the abundance increased after DC3000 treatment and was highest after *avrRpm1* treatment.

DISCUSSION

Here we report a quantitative LC-MS/MS-based proteomics analysis of extracellular proteins from *Arabidopsis* accumulating after exposure to different genotypes of *P. syringae* pv. *tomato*. We identified 45 proteins from *Arabidopsis* that were present in three independent biological experiments, and these proteins displayed a variety of accumulation patterns depending on whether virulent, avirulent, or type III secretion system-impaired bacteria were used.

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A number of proteins present in the extracellular space of untreated cells consistently decreased after bacterial treatments regardless of the genotype of the bacteria indicating that the response is a general one rather than one related to TTSS. One possibility is that transcription of genes encoding these proteins is down-regulated by MAMPs but is unaffected by TTEs. However, transcripts for none of these proteins were found to be regulated by the MAMP elicitor, flg22, in *Arabidopsis* cell cultures at least within the first 60 min of elicitation (34). Another possibility is that MAMPs activate a protease activity with specificity toward this subset of proteins. This line of reasoning supposes that these proteins would otherwise promote an activity that would benefit the pathogen, so the host removes them. Conversely the bacteria may remove these proteins perhaps by pathogen-derived proteases. In this case, the assumption would be that the proteins have a beneficial activity for the host that the microbe eliminates to promote colonization. In fact, there is no reason to exclude the possibility that both situations may be occurring simultaneously on different subsets of this class of proteins. Consideration of these possibilities illustrates the need for caution in attempting to extrapolate cause-and-effect relationships from these proteomics studies. Clearly these possibilities can only be addressed by understanding both the mechanism(s) leading to the disappearance of these proteins as well as their biological role, and these topics will be the subject of future studies.

At least some TTEs appear to suppress the extracellular accumulation of a number of proteins with classical signal peptides for secretion. These results are consistent with the emerging picture that the accumulation of extracellular proteins are an important component of the host's defense against potential bacterial pathogens (35, 36). To counteract these defenses, bacterial TTEs block the accumulation of many of the extracellular proteins either by blocking the transcriptional activation of genes encoding these proteins (37) or by targeting the host secretion system (13). Interestingly only two of the TTE-inhibited proteins found in this proteomics study, At4g25810 and At3g54420, are transcriptionally regulated during MAMP responses (34), indicating that the remainder may be constitutive extracellularly based defense components.

Another group of proteins are thought to promote resistance by accumulating in the extracellular space specifically during gene-for-gene resistance. This form of resistance in which host proteins recognize the presence of specific TTEs from the pathogen is thought to have evolved in response to the ability of TTEs to suppress basal MAMP-related resistance (15, 16). Interpretation of the gene-for-gene-responsive proteins in the present study, however, is complicated somewhat by the presence of a number of proteins without classical signal sequences. Although extracellular protein samples were isolated before detection of the cell death associated with the hypersensitive response associated with gene-for-gene resistance, the fact that cell death occurs earlier in this genotypic interaction than with the other bacteria raises the possibility that some cellular leakage may be occurring in these samples. However, because only a limited set of proteins was observed rather than a large list of abundant cytosolic proteins, it still seems likely that their accumulation reflects a regulated event rather than nonspecific cell leakage.

An example of how gene-for-gene resistance may be a mechanism for restoring activities blocked or inhibited by the microbe is illustrated by the accumulation of the purple acid phosphatase PAP10 (At2g16430). This class of phosphatases has predicted signal peptides, but their biological role in the extracellular space is unknown. The fact that all bacterial treatments lead to a decrease in the abundance of one of these phosphatases, PAP14 (At2g46880), but that gene-for-gene resistance results in a large accumulation of another member of this gene family indicates that this class of proteins may play an important role in defense. One possibility is that the enzymatic activity of these proteins may not be important in themselves. Purple acid phosphatases are so named because of their purple color in solution, and this color originates from the iron coordinated in the protein. Iron availability is an important limiting factor for the growth and multiplication of the potential bacterial pathogen (38). Therefore, these proteins may be important for sequestering iron from the bacteria.

The identification of extracellular monoubiquitin was interesting in light of a growing number of studies from different organisms in which ubiquitin has been identified outside the cell (39–42), including plant studies on lily (43) and *Arabidopsis* (26, 44). In *Arabidopsis*, ubiquitin is encoded by a multigene family. A single member of this family has an apparent signal peptide, but the sequence conservation of ubiquitin makes it impossible to determine whether the ubiquitin found in our studies originates from this gene or from a number of individual gene family members. Several roles have been proposed for extracellular ubiquitin. Direct evidence for antimicrobial activity of the conserved polypeptide was demonstrated against Gram-positive bacteria and fungi (45, 46). However, we did not observe any inhibiting effect of ubiquitin on growth of Gram-negative *P. syringae* (data not shown). Ubiquitin also functions in cell-cell communications in mammals (47). We used *Arabidopsis* plants expressing 35S:aequorin to investigate whether ubiquitin may activate Ca^{2+} signaling, an early response common to microbial elicitation, but we did not detect any indication that ubiquitin plays a role in cell-cell communication in our system (data not shown). Therefore, we currently do not understand what, if any, role extracellular ubiquitin plays in *Arabidopsis-Pseudomonas* interactions. However, the fact that extracellular accumulation of ubiquitin appears to be a common response in a variety of organisms indicates that a more general role in cellular processes may exist.

Perhaps one of the most intriguing findings in this study was that TTEs appear to cause the extracellular accumulation

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of numerous host proteins that are predicted to be cytosolic (*i.e.* they do not contain classical signal peptides for secretion via the endomembrane system). Several lines of logic argue that these observations indicate a regulated response as opposed to the occurrence of general leakage of proteins or cell death-dependent release of proteins. First, we intentionally selected a time point for harvesting extracellular proteins that preceded induction of cell death in *Pst* (*avrRpm1*)- or *Pst* DC3000-treated cells. Second, we observed consistent and specific accumulation of proteins during *Pst* (*avrRpm1*) and/or *Pst* DC3000 treatment rather than accumulation of a broad range of cytosolic proteins, indicating that the response was not simply general leakage of cellular contents. Finally we observed specific accumulation patterns following treatment with *Pst* (*avrRpm1*) *versus Pst* DC3000. In fact, the gene-for-gene resistance response of *Pst* (*avrRpm1*) treatment that would be the most likely condition to cause cellular leakage actually reversed the extracellular accumulation of a subset of proteins induced by the virulent *Pst* DC3000 bacteria. Particularly this final point strongly indicates that the extracellular accumulation of certain proteins predicted to be in the cytosol is associated with a pathogenic infection and that part of the gene-for-gene resistance response involves preventing this accumulation.

These interesting observations lead to two questions: 1) how do these proteins exit the cell, and 2) what is the biological significance of their induced extracellular accumulation? In regard to the first question, it seems unlikely that a prokaryotic pathogen has evolved a novel process to preferentially secrete a subset of host proteins. A more likely hypothesis is that the TTEs manipulate an existing cellular process. In mammals it was recently shown that exosomes are involved in the secretion of proteins by a mechanism that is independent of the classical secretion via endoplasmic reticulum and Golgi apparatus (48). Interestingly our study identified proteins similar to those found to be exiting via exosomes such as enolase, phosphoglycerate kinase, and peptidyl-prolyl cis-trans isomerase. Therefore, an intriguing hypothesis is that bacterial virulence proteins may manipulate this process. An added level of complexity must exist even if this hypothesis is correct. Gene-for-gene resistance suppresses the extracellular accumulation of only a subset of TTE-regulated cytosolic proteins, indicating that at least two independent processes must exist to transfer these proteins out of the host cell.

The actual role of these proteins in the extracellular space is speculative. Many proteins of this class were enzymes of primary metabolism such as enolase or triose-phosphate isomerase, so one possibility is that perhaps the microbe invokes the host's own enzymes to assist in metabolic assimilation. However, it is not clear that the low extracellular pH would be conducive to the enzymatic function of many of these proteins. An intriguing possibility is that the pathogen may be using the host's own proteins against it to promote infection. For many bacterial pathogens, enzymes similar to those found in our proteomics study such as glyceraldehyde-3-phosphate dehydrogenase and enolase are found on the surface of the bacteria, and it appears that these enzymes may serve as virulence factors for mammalian pathogens by binding to host proteins such as plasminogen (49). Currently it is not known whether a similar binding of host proteins occurs with *Pseudomonas* in plants; however, it is striking that a conserved strategy appears to exist for a wide range of Gram-negative bacterial pathogens. Moreover gene-for-gene resistance suppresses a portion of this TTE-induced accumulation of host proteins in the extracellular space, indicating that a portion of resistance may involve preventing the pathogen from gaining access to these proteins.

Apart from future work to elucidate the biological function of the various classes of secreted proteins during host-pathogen interactions in plants, the proteins discovered in this proteomics study will be useful to investigate the molecular functions of type III effector proteins from bacteria. We have identified sets of biomarkers that can be used to identify which effector proteins from the pathogen either suppress MAMP-induced secretion during basal defense or induce the extracellular accumulation of host proteins during infection. Once the effector proteins are known, it may be possible to begin unraveling the unusual mechanism by which proteins thought to be cytosolic can exit the cell without using the classical secretion pathway.

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REFERENCES

1. Zipfel, C., and Felix, G. (2005) Plants and animals: a different taste for microbes? *Curr. Opin. Plant. Biol.* **8,** 353–360
2. Nurnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004) Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol. Rev.* **198,** 249–266
3. Felix, G., Duran, J. D., Volko, S., and Boller, T. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18,** 265–276
4. Newman, M.-A., von Roepenack, E., Daniels, M., and Dow, M. (2000) Lipopolysaccharides and plant responses to phytopathogenic bacteria. *Mol. Plant Pathol.* **1,** 25–31

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5. Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *Plant Cell* **16,** 3496–3507
6. Nurnberger, T., and Lipka, V. (2005) Non-host resistance in plants: new insights into an old phenomenon. *Mol. Plant Pathol.* **6,** 335–345
7. Peck, S. C. (2003) Early phosphorylation events in biotic stress. *Curr. Opin. Plant Biol.* **6,** 334–338
8. Nurnberger, T., and Scheel, D. (2001) Signal transmission in the plant immune response. *Trends. Plant Sci.* **6,** 372–379
9. de Torres, M., Mansfield, J. W., Grabov, N., Brown, I. R., Ammouneh, H., Tsiamis, G., Forsyth, A., Robatzek, S., Grant, M., and Boch, J. (2006) Pseudomonas syringae effector AvrPtoB suppresses basal defence in Arabidopsis. *Plant J.* **47,** 368–382
10. Jamir, Y., Guo, M., Oh, H. S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M. B., Collmer, A., and Alfano, J. R. (2004) Identification of Pseudomonas syringae type III effectors that can suppress programmed cell death in plants and yeast. *Plant J.* **37,** 554–565
11. Zhang, J., Shao, F., Li, Y., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., Chen, S., Tang, X., and Zhou, J.-M. (2007) A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell Host Microbe* **1,** 175–185
12. Fu, Z. Q., Guo, M., Jeong, B. R., Tian, F., Elthon, T. E., Cerny, R. L., Staiger, D., and Alfano, J. R. (2007) A type III effector ADP-ribosylates RNA-binding proteins and quells plant immunity. *Nature* **447,** 284–288
13. Nomura, K., Debroy, S., Lee, Y. H., Pumplin, N., Jones, J., and He, S. Y. (2006) A bacterial virulence protein suppresses host innate immunity to cause plant disease. *Science* **313,** 220–223
14. Lindgren, P. B. (1997) The role of hrp genes during plant-bacterial interactions. *Annu. Rev. Phytopathol.* **35,** 129–152
15. Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006) Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **124,** 803–814
16. Jones, J. D., and Dangl, J. L. (2006) The plant immune system. *Nature* **444,** 323–329
17. Flor, H. H. (1971) Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* **9,** 275–296
18. Jones, A. M., Thomas, V., Truman, B., Lilley, K., Mansfield, J., and Grant, M. (2004) Specific changes in the Arabidopsis proteome in response to bacterial challenge: differentiating basal and R-gene mediated resistance. *Phytochemistry* **65,** 1805–1816
19. Jones, A. M., Thomas, V., Bennett, M. H., Mansfield, J., and Grant, M. (2006) Modifications to the Arabidopsis defense proteome occur prior to significant transcriptional change in response to inoculation with Pseudomonas syringae. *Plant Physiol.* **142,** 1603–1620
20. de Torres Zabela, M., Fernandez-Delmond, I., Niittyla, T., Sanchez, P., and Grant, M. (2002) Differential expression of genes encoding Arabidopsis phospholipases after challenge with virulent or avirulent Pseudomonas isolates. *Mol. Plant-Microbe Interact.* **15,** 808–816
21. de Torres, M., Sanchez, P., Fernandez-Delmond, I., and Grant, M. (2003) Expression profiling of the host response to bacterial infection: the transition from basal to induced defence responses in RPM1-mediated resistance. *Plant J.* **33,** 665–676
22. Tornero, P., Chao, R. A., Luthin, W. N., Goff, S. A., and Dangl, J. L. (2002) Large-scale structure-function analysis of the Arabidopsis RPM1 disease resistance protein. *Plant Cell* **14,** 435–450
23. Andersson, M. X., Kourtchenko, O., Dangl, J. L., Mackey, D., and Ellerstrom, M. (2006) Phospholipase-dependent signalling during the AvrRpm1- and AvrRpt2-induced disease resistance responses in Arabidopsis thaliana. *Plant J.* **47,** 947–959
24. Belkhadir, Y., Nimchuk, Z., Hubert, D. A., Mackey, D., and Dangl, J. L. (2004) Arabidopsis RIN4 negatively regulates disease resistance mediated by RPS2 and RPM1 downstream or independent of the NDR1 signal modulator and is not required for the virulence functions of bacterial type III effectors AvrRpt2 or AvrRpm1. *Plant Cell* **16,** 2822–2835
25. Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A., and Mansfield, J. (2000) The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. *Plant J.* **23,** 441–450
26. Oh, I. S., Park, A. R., Bae, M. S., Kwon, S. J., Kim, Y. S., Lee, J. E., Kang, N. Y., Lee, S., Cheong, H., and Park, O. K. (2005) Secretome analysis reveals an Arabidopsis lipase involved in defense against Alternaria brassicicola. *Plant Cell* **17,** 2832–2847
27. Chivasa, S., Ndimba, B. K., Simon, W. J., Robertson, D., Yu, X. L., Knox, J. P., Bolwell, P., and Slabas, A. R. (2002) Proteomic analysis of the Arabidopsis thaliana cell wall. *Electrophoresis* **23,** 1754–1765
28. May, M. J., and Leaver, C. J. (1993) Oxidative stimulation of glutathione synthesis in Arabidopsis thaliana suspension cultures. *Plant Physiol.* **103,** 621–627
29. Neuhoff, V., Stamm, R., and Eibl, H. (1985) Clear background and highly sensitive protein staining with Coomassie Blue dyes in polyacrylamide gels: a systematic analysis. *Electrophoresis* **6,** 427–448
30. Shadforth, I. P., Dunkley, T. P., Lilley, K. S., and Bessant, C. (2005) i-Tracker: for quantitative proteomics using iTRAQ. *BMC Genomics* **6,** 145
31. Buell, C. R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I. T., Gwinn, M. L., Dodson, R. J., Deboy, R. T., Durkin, A. S., Kolonay, J. F., Madupu, R., Daugherty, S., Brinkac, L., Beanan, M. J., Haft, D. H., Nelson, W. C., Davidsen, T., Zafar, N., Zhou, L., Liu, J., Yuan, Q., Khouri, H., Fedorova, N., Tran, B., Russell, D., Berry, K., Utterback, T., Van Aken, S. E., Feldblyum, T. V., D'Ascenzo, M., Deng, W. L., Ramos, A. R., Alfano, J. R., Cartinhour, S., Chatterjee, A. K., Delaney, T. P., Lazarowitz, S. G., Martin, G. B., Schneider, D. J., Tang, X., Bender, C. L., White, O., Fraser, C. M., and Collmer, A. (2003) The complete genome sequence of the Arabidopsis and tomato pathogen Pseudomonas syringae pv. tomato DC3000. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 10181–10186
32. Ravanel, S., Block, M. A., Rippert, P., Jabrin, S., Curien, G., Rebeille, F., and Douce, R. (2004) Methionine metabolism in plants: chloroplasts are autonomous for de novo methionine synthesis and can import S-adenosylmethionine from the cytosol. *J. Biol. Chem.* **279,** 22548–22557
33. Clarke, A., Desikan, R., Hurst, R. D., Hancock, J. T., and Neill, S. J. (2000) NO way back: nitric oxide and programmed cell death in Arabidopsis thaliana suspension cultures. *Plant J.* **24,** 667–677
34. Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., Boller, T., and Jones, J. D. (2004) The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. *Plant Physiol.* **135,** 1113–1128
35. Wang, D., Weaver, N. D., Kesarwani, M., and Dong, X. (2005) Induction of protein secretory pathway is required for systemic acquired resistance. *Science* **308,** 1036–1040
36. Kalde, M., Nuhse, T. S., Findlay, K., and Peck, S. C. (2007) The syntaxin SYP132 contributes to plant resistance against bacteria and secretion of pathogenesis-related protein 1. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 11850–11855
37. Hauck, P., Thilmony, R., and He, S. Y. (2003) A Pseudomonas syringae type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 8577–8582
38. Expert, D. (1999) Withholding and exchanging iron: Interactions between Erwinia spp., and their plant hosts. *Annu. Rev. Phytopathol.* **37,** 307–334
39. Sakai, N., Sawada, H., and Yokosawa, H. (2003) Extracellular ubiquitin system implicated in fertilization of the ascidian, Halocynthia roretzi: isolation and characterization. *Dev. Biol.* **264,** 299–307
40. Svensson, I., Calles, K., Lindskog, E., Henriksson, H., Eriksson, U., and Haggstrom, L. (2005) Antimicrobial activity of conditioned medium fractions from Spodoptera frugiperda Sf9 and Trichoplusia ni Hi5 insect cells. *Appl. Microbiol. Biotechnol.* **69,** 92–98
41. Sonenshine, D. E., Hynes, W. L., Ceraul, S. M., Mitchell, R., and Benzine, T. (2005) Host blood proteins and peptides in the midgut of the tick Dermacentor variabilis contribute to bacterial control. *Exp. Appl. Acarol.* **36,** 207–223
42. Majetschak, M., Ponelies, N., and Hirsch, T. (2006) Targeting the monocytic ubiquitin system with extracellular ubiquitin. *Immunol. Cell Biol.* **84,** 59–65
43. Kim, S. T., Zhang, K., Dong, J., and Lord, E. M. (2006) Exogenous free ubiquitin enhances lily pollen tube adhesion to an in vitro stylar matrix and may facilitate endocytosis of SCA. *Plant Physiol.* **142,** 1397–1411
44. Boudart, G., Jamet, E., Rossignol, M., Lafitte, C., Borderies, G., Jauneau, A., Esquerre-Tugaye, M. T., and Pont-Lezica, R. (2005) Cell wall proteins in apoplastic fluids of Arabidopsis thaliana rosettes: identification by mass spectrometry and bioinformatics. *Proteomics* **5,** 212–221
45. Kieffer, A. E., Goumon, Y., Ruh, O., Chasserot-Golaz, S., Nullans, G., Gasnier, C., Aunis, D., and Metz-Boutigue, M. H. (2003) The N- and C-terminal fragments of ubiquitin are important for the antimicrobial activities. *FASEB J.* **17,** 776–778

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46. Yoshio, H., Tollin, M., Gudmundsson, G. H., Lagercrantz, H., Jornvall, H., Marchini, G., and Agerberth, B. (2003) Antimicrobial polypeptides of human vernix caseosa and amniotic fluid: implications for newborn innate defense. *Pediatr. Res.* **53,** 211–216
47. Majetschak, M., Krehmeier, U., Bardenheuer, M., Denz, C., Quintel, M., Voggenreiter, G., and Obertacke, U. (2003) Extracellular ubiquitin inhibits the TNF-α response to endotoxin in peripheral blood mononuclear cells and regulates endotoxin hyporesponsiveness in critical illness. *Blood* **101,** 1882–1890
48. Yu, X., Harris, S. L., and Levine, A. J. (2006) The regulation of exosome secretion: a novel function of the p53 protein. *Cancer Res.* **66,** 4795–4801
49. Pancholi, V., and Chhatwal, G. S. (2003) Housekeeping enzymes as virulence factors for pathogens. *Int. J. Med. Microbiol.* **293,** 391–401
50. Slabas, A. R., Ndimba, B., Simon, W. J., and Chivasa, S. (2004) Proteomic analysis of the Arabidopsis cell wall reveals unexpected proteins with new cellular locations. *Biochem. Soc. Trans.* **32,** 524–528
51. Borderies, G., Jamet, E., Lafitte, C., Rossignol, M., Jauneau, A., Boudart, G., Monsarrat, B., Esquerre-Tugaye, M. T., Boudet, A., and Pont-Lezica, R. (2003) Proteomics of loosely bound cell wall proteins of Arabidopsis thaliana cell suspension cultures: a critical analysis. *Electrophoresis* **24,** 3421–3432
52. Robertson, D., Mitchell, G. P., Gilroy, J. S., Gerrish, C., Bolwell, G. P., and Slabas, A. R. (1997) Differential extraction and protein sequencing reveals major differences in patterns of primary cell wall proteins from plants. *J. Biol. Chem.* **272,** 15841–15848
53. Bayer, E. M., Bottrill, A. R., Walshaw, J., Vigouroux, M., Naldrett, M. J., Thomas, C. L., and Maule, A. J. (2006) Arabidopsis cell wall proteome defined using multidimensional protein identification technology. *Proteomics* **6,** 301–311
54. Charmont, S., Jamet, E., Pont-Lezica, R., and Canut, H. (2005) Proteomic analysis of secreted proteins from Arabidopsis thaliana seedlings: improved recovery following removal of phenolic compounds. *Phytochemistry* **66,** 453–461
55. Emanuelsson, O., Brunak, S., von Heijne, G., and Nielsen, H. (2007) Locating proteins in the cell using TargetP, SignalP and related tools. *Nat. Protoc.* **2,** 953–971

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