

Induced ER Chaperones Regulate a Receptor-like Kinase to Mediate Antiviral Innate Immune Response in Plants

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SUMMARY

Mounting an effective innate immune response against pathogens requires the rapid and global reprogramming of host cellular processes. Here we employed complementary proteomic methods to identify differentially regulated proteins early during a plant's defense response. Besides defense-related proteins, constituents of the largest category of upregulated proteins were cytoplasmic- and ER-residing molecular chaperones. Investigating the significance of upregulated ER chaperones, we find that silencing of ER-resident protein disulfide isomerases NbERp57 and NbP5 and the calreticulins NbCRT2 and NbCRT3 led to partial loss of N immune receptor-mediated defense against *Tobacco mosaic virus* (TMV). Furthermore, NbCRT2 and NbCRT3 were required for the expression of a previously uncharacterized induced receptor-like kinase (IRK). IRK is a plasma membrane-localized protein required for N-mediated hypersensitive response, programmed cell death, and resistance to TMV. These data support a model in which ER-resident chaperones are required for the accumulation of membrane-bound or secreted proteins during plant innate immunity.

INTRODUCTION

Almost 20 years ago, the two-dimensional gel electrophoresis (2D-E)-based proteomics method was employed to identify up-regulated defense-related proteins using N immune receptor-containing *Nicotiana tabacum* plants and *Tobacco mosaic virus* (TMV) as a model system (Van Loon et al., 1987). These proteins were later discovered to be highly upregulated in a variety of plants during defense responses and were termed pathogenesis-related (PR) proteins (reviewed by van Loon et al., 2006).

Since van Loon's classical experiments, there have been significant advances in gel-based proteomics techniques. Two-dimensional differential gel electrophoresis (2D-DIGE) has been developed to use different fluorescent dyes to tag different pools of proteins (Minden, 2007). Fluorescent dyes have a high dynamic range for accurate measurement of relative protein abundance levels and allow multiple sets of proteins to be compared. Multiple sets of tagged proteins can be simultaneously separated on a single two-dimensional gel, and identical proteins within the pools comigrate. The combination of internal standards and comigration on a single gel removes a large portion of the variation associated with traditional 2D-E method.

In addition, in-solution proteomics have been developed for large-scale, quantitative proteomics. Most new technologies implement differential tags to compare protein abundance levels from different pools of proteins. Isotopes can be introduced by stable isotope labeling with amino acids in cell culture (SILAC) or by labeling protein extracts using isotope-coded affinity tags (ICATs) (Gygi et al., 1999; Ong et al., 2002). The labeled peptides from different pools of proteins will have different masses on a mass spectrometer, and their relative abundance levels can be measured. A newer method labels proteins with isobaric iTRAQ reagents that have the same mass but different fragmentation patterns on a mass spectrometer (Ross et al., 2004). Differential labeling combined with chromatography techniques, such as strong cation exchange and reverse-phase chromatography, provides a high-throughput method for comparing relative protein abundance levels from different samples.

To investigate the multitude of biological questions in plant innate immunity, we study the N immune receptor from *N. glutinosa* that provides resistance to TMV (Whitham et al., 1994). The N gene is the only cloned TIR-NB-LRR resistance gene to a virus and is functional in multiple *Solanaceae* species, including the model plant *N. benthamiana* (Liu et al., 2002). N recognizes the p50 region of TMV's replicases to signal hypersensitive response programmed cell death (HR-PCD) (Erickson et al., 1999). HR-PCD is observed as lesions at the infection sites and is correlated with the restriction of TMV.

The goal of this study was to employ two complementary advanced proteomics techniques, 2D-DIGE and iTRAQ, to identify components that are differentially regulated directly following

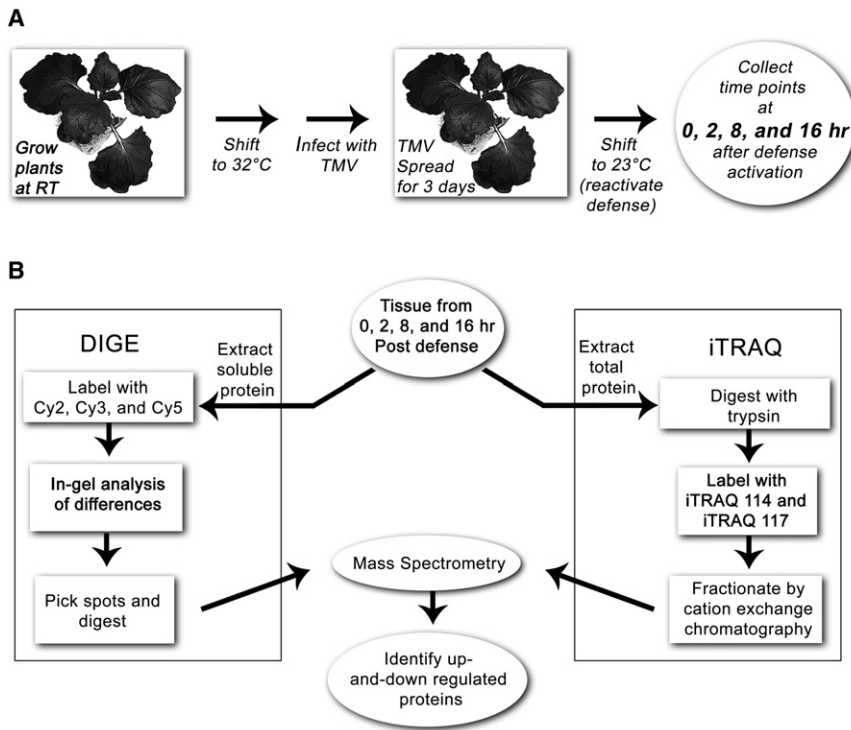


Figure 1. Schematics of Strategy Used for Coordinated Induction of Defense Response and Proteomics Method Workflow

(A) *N. benthamiana* plants with and without the N immune receptor were grown at room temperature (RT, ~22°C–24°C) for 4 weeks (left panel). The plants were shifted to 32°C overnight, and TMV was inoculated on all of the leaves and allowed to replicate for 3 days at 32°C (middle panel). The plants were shifted back to RT and samples were collected at 0, 2, 8, and 16 hr posttemperature shift (left panel).

(B) The DIGE (left) and the iTRAQ (right) workflow is shown. These workflows converged when samples were analyzed by mass spectrometry to identify differentially regulated proteins.

pathogen recognition. Indeed, we found multiple PR proteins and a wide variety of proteins required for defense signaling and reactive oxygen species (ROS) production. More interestingly, we discovered that numerous cytoplasmic and endoplasmic reticulum (ER)-residing chaperones were upregulated. The function of cytoplasmic chaperones during innate immunity has been studied in detail by multiple research groups (Shirasu, 2009). Conversely, very little is known about the function of ER chaperones during innate immunity. Therefore, we investigated the biological significance of upregulated ER chaperones during innate immunity. We show that protein disulfide isomerases (PDIs) and calreticulins (CRTs) are required for the N immune receptor to provide complete defense against TMV. Furthermore, our data suggest that ER chaperones are upregulated during innate immunity to aid in the accumulation of an induced receptor-like kinase (IRK) required for a successful innate immune response.

RESULTS

Coordinated Induction of an N Immune Receptor-Mediated Defense Response

Differentially regulated proteins during an innate immune response should be investigated in the context of the whole organism, because immune receptors, such as N, fail to function in protoplasts and cell culture (Beachy and Murakishi, 1973; Otsuki et al., 1972). Therefore, we studied an N-mediated defense response to TMV within whole *N. benthamiana* plants. One difficulty of studying defense responses in whole organisms is that during a normal infection only a small number of cells are infected, which results in a mixed population of uninfected and infected cells. To overcome this, we exploited the tempera-

ture-sensitive nature of N-mediated resistance (Fraser and Loughlin, 1980) to coordinate a defense response in every cell (Figure 1A). Plants were shifted to 32°C to fully inactivate N and to allow TMV to spread without inducing a defense response. Plants were then shifted to room temperature to initiate a coordinated defense response in every cell. Tissue was collected at 0, 2, 8, and 16 hr postinitiation of defense (herein referred to as T = 0 hr, T = 2 hr, T = 8 hr, T = 16 hr). Plants without N did not initiate a defense response and were used as a control for any effects caused by TMV infection or the temperature shift. We used the same tissue samples in the two complementary methods, DIGE and iTRAQ (Figure 1B).

Quantification of Soluble Proteins by DIGE during Defense Response

For DIGE, soluble proteins were extracted from plants with and without the N immune receptor at T = 0 hr, T = 2 hr, T = 8 hr, and T = 16 hr postinduction of defense (Figure 1B). Wild-type plant extracts were labeled with Cy3, and N-containing plant extracts were labeled with Cy5. Extracts from N-containing plants at T = 0 hr were labeled with Cy2 to serve as an internal control. A representative 2D-DIGE gel (T = 16 hr) was cropped to contain only spots identified by MS (Figure 2A and Table 1). Red spots indicate upregulated proteins, green spots indicate downregulated proteins, and yellow spots indicate no change. Spot volumes and relative abundance levels of comigrating proteins were determined using the software package Decyder MS. Only spots that showed a 1.5-fold change were considered to be differentially regulated and subjected to MS. Protein identification was conducted using SEQUEST, the Trans-Proteomic Pipeline, and our in-house *Nicotiana* species database (for details, see the Experimental Procedures and the Supplemental Data available online). From this analysis, we identified 24 spots that were up- or downregulated at least 1.5-fold, had a protein probability score of ≥ 0.99 , and were identified by at least two unique peptides (Table 1). Eleven spots with a 1.5-fold change in regulation did not result in a significant identification. The proteins with a change in regulation were sorted into four major

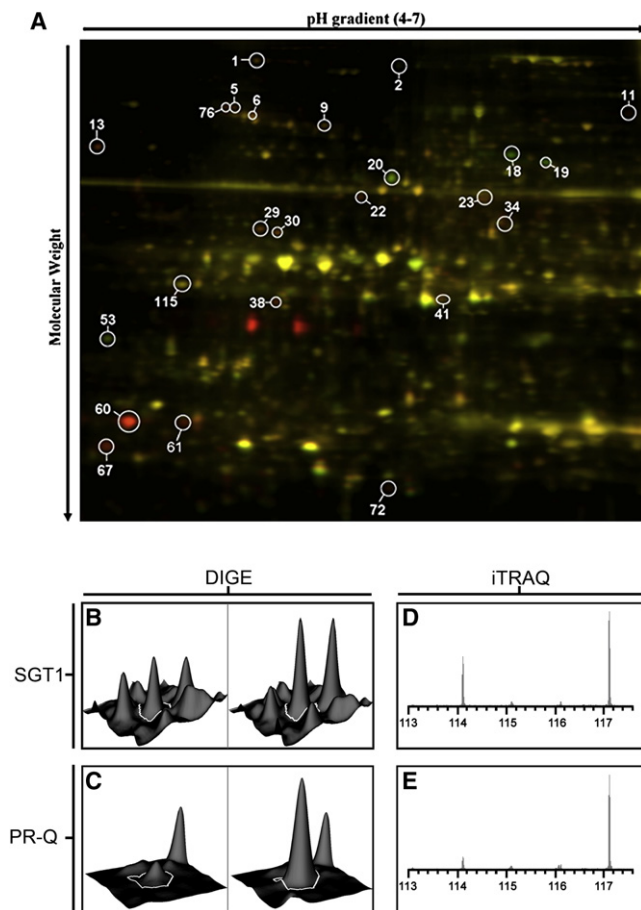


Figure 2. Representative Illustration of Differentially Regulated Proteins from DIGE and iTRAQ Analyses

(A) A cropped DIGE gel image. The first dimension is pH via isoelectric focusing, and the second dimension is molecular weight via SDS-PAGE. Green spots are downregulated, and orange/red spots are upregulated. Yellow spots exhibit no change in regulation. The circled numbered spots were identified by mass spectrometry (Table 1).

(B–E) Three-dimensional volumetric models for DIGE spots were created and measured (circled white) with Decyder MS (B and C) and compared with centroided iTRAQ data displayed with Comet Viewer (D and E) from the time point T = 8 hr. (B) and (D) represent quantification of SGT1, and (C) and (E) represent the quantification of PR-Q. The right section of DIGE panels (B and C) and the peak at 114.1 in iTRAQ panels (D and E) represent proteins or peptides from plants not undergoing a defense response. The left section of DIGE panels (B and C) and the peak at 117.1 in iTRAQ panels (D and E) represent proteins or peptides from plants undergoing a defense response.

categories: proteins previously implicated as defense related, proteins required for the production or regulation of ROS and hormones, molecular chaperones, and general metabolic proteins.

Quantification of Proteins in Whole-Cell Lysates by iTRAQ during Defense Response

Since our DIGE analysis only investigated the soluble proteome during a defense response, we conducted iTRAQ analysis with the exact same tissue samples and time points to obtain global quantification data (Figure 1B). Proteins extracted from plants

without N (no defense response) were labeled with iTRAQ reagent 114, and proteins extracted from plants containing N (defense response) were labeled with iTRAQ reagent 117 (see the Experimental Procedures and the Supplemental Data). Multi-dimensional protein identification technology (MuDPIT) was performed on a QSTAR XL MS and identified 368 proteins at T = 2 hr (175 single hits) and 775 proteins at T = 8 hr (492 single hits). Ratios (114/117) were calculated using the LIBRA module of the TPP software.

iTRAQ 114/117 ratios for 9124 peptides that showed >90% probability of identification confidence were graphed to choose up- and downregulation cutoff values (Figure S1). Similar methods have been used previously for iTRAQ and ICAT experiments (Boersema et al., 2009; Pawlik et al., 2006). Of the peptides, 91% fell between -1.5 and 1.5 ratios of 114/117 and were designated as having no change in regulation. Approximately 7.8% and 1.3% of the peptides were up- and downregulated >1.5-fold, respectively. This corresponds to a total of 82 differentially regulated proteins, with only 37 proteins meeting our stringent identification criteria (Table 2). More specifically, eight proteins were up- or downregulated in iTRAQ at both T = 2 and T = 8 hr. Eleven and 18 proteins were up- or downregulated in iTRAQ at only T = 2 or T = 8 hr, respectively. An additional 45 proteins were differentially regulated, but their identifications were supported by only one unique peptide (Table S1). Eleven differentially regulated proteins identified in iTRAQ were also identified in DIGE (Figures 2B–2E and Table S2). The ratios of DIGE and iTRAQ were highly similar and have a Pearson correlation of 0.89 for both time points, partially validating these two independent approaches.

Contrasting Regulation of Defense-Related Proteins during Viral and Bacterial Innate Immunity

Similar to the classic experiments that originally identified PR proteins (Van Loon et al., 1987), our DIGE and iTRAQ analyses also discovered PR proteins, PR-Q, PRp27, and PR-R. Furthermore, we observed an upregulation of HSR203J that was previously shown to be transcriptionally upregulated during the HR-PCD (Takahashi et al., 2004). In addition, we observed an upregulation of alanine aminotransferase (AlaAT), which was shown to be transcriptionally upregulated in *Capsicum annuum* undergoing a TMV-P₀- or *Xanthomonas campestris*-induced HR-PCD (Kim et al., 2005). Our data suggest that the transcriptional upregulation of HSR203J and AlaAT also results in an upregulation of their protein abundance and that it occurs early on during HR-PCD.

A surprising result from this study was the downregulation of polyphenol oxidase (PPO) and chloroplastic drought-induced stress (CDSP34/fibrillin) proteins that were previously shown to be transcriptionally upregulated during bacterial innate immune responses (Langenkamper et al., 2001; Thipyapong et al., 2004). PPOs are released from disrupted chloroplasts and oxidize phenolic compounds to quinones (Mayer, 2006). PPO overexpression enhanced defense against *Pseudomonas syringae* (Li and Steffens, 2002; Thipyapong et al., 2004). Surprisingly, instead of an upregulation, we observed a downregulation of PPO and CDSP-34/fibrillin (Tables 1 and 2 and Table S2). These results show apparent differences in defense responses to bacteria and viruses.

Table 1. Identification of Differentially Regulated Proteins in DIGE

Spot	Protein ID	Protein Name	Regulation Factor			Protein Prob.	Number of Peptides	
			T = 2	T = 8	T = 16		Unique	Total
Defense-Related Proteins								
60	Nsp SEQ08864318	Acidic chitinase (PR-Q)	4.14	4.65	7.43	1.00	8	9
22	Nb TC14345, Nb TC14659	Alanine aminotransferase (AlaAT)	1.71	2.12	1.95	1.00	6	6
67	Nb TC12269	Thaumatococcus-like protein (PR-R)	2.36	2.03	4.24	1.00	2	2
61	Nb TC12034	Cysteine proteinase aleuran type ^a	1.85	1.93	2.63	1.00	4	4
1	Nb ES888160, Nb CN746487	Cell division cycle protein 48 homolog (CDC48)	1.67	1.52	1.56	1.00	7	7
19	Nb TC16011	Polyphenol oxidase (PPO)	-1.37	-1.49	-1.82	1.00	5	5
53	Nt TC15963, Nt TC14739	Fibrillin, plastid-lipid associated, chloroplastic	1.02	-1.64	-1.63	1.00	6	6
18	Nb TC16011	Polyphenol oxidase (PPO)	-2.05	-2.21	-2.79	1.00	8	8
Reactive Oxygen Species and Hormones								
72	Nb TC13502	Superoxide dismutase [Fe]	1.63	1.36	1.91	1.00	5	6
11	Nb TC14818, Nt TC14027	Phenylalanine ammonia-lyase (PAL)	1.26	1.17	1.58	0.98	2	2
115	Nt TC14135, Nt TC141531, Nt TC14952, Nt TC19272	Cytosolic ascorbate peroxidase	t1.12	-1.55	1.05	1.00	7	8
Chaperones								
13	Nb CK295775, Nb TC11094	Protein disulfide isomerase (NbERp57)	2.33	1.74	2.26	1.00	2	2
29	Nb TC10793, Nsp SEQ08864501	SGT1	1.79	1.61	1.94	1.00	11	11
30	Nb TC10793, Nsp SEQ08865322	SGT1-like protein ^b	2.06	1.50	1.59	1.00	8	8
5	Nb TC14285, Nb TC10790, Nb TC13962, Nb TC15716	Heat shock protein 70 (HSP70)	2.14	1.46	1.87	1.00	6	8
6	Nsn CL582Contig1	Peptidyl-prolyl isomerase, ROF1-like ^c	1.69	1.32	1.35	1.00	14	14
9	Nb CK296023, Nb CK296024	Peptidyl-prolyl isomerase, ROF1-like	1.54	1.42	1.40	1.00	10	10
76	Nt TC15468, Nt TC14168, Nt TC25840, Nt TC14368, Nt TC14597	Luminal-binding protein 5 precursor (GRP 78-5)	1.39	1.34	2.04	1.00	2	3
34	Nb TC11072, Nb TC11617	Similar to Calreticulin-3 (CRT3)	1.10	1.36	1.57	1.00	2	6
Metabolism								
38	Nb TC14111, Nb TC13364, Nb TC 13881	Similar to fructose-bisphosphate aldolase, chloroplastic	1.76	1.75	2.39	1.00	3	3
41	Nb TC12594	Malate dehydrogenase, cytoplasmic	1.64	1.55	1.30	0.99	3	4
23	Nsn CL954Contig1	Similar to 6-phosphogluconate dehydrogenase	1.27	1.22	1.75	1.00	11	11
2	Nt TC37429, Nt TC39557	Pyruvate phosphate dikinase, chloroplastic	1.03	-1.60	-1.56	1.00	3	4
20	Nb TC13065	Inositol-3-phosphate synthase	1.05	t1.37	-1.77	1.00	3	3

“Protein ID” is the EST number in which Nb = *N. benthamiana*, Nt = *N. tabaccum*, Nsp = RefSeq protein, and Nsn = *Nicotiana species* contig from NCBI ESTs. “Regulation Factor” is the ratio of no defense response to N-mediated defense response. The “Protein Prob.” generated by ProteinProphet is the probability that the protein is correctly identified. The “Number of Peptides” refers to the quantity of unique peptides and the total number of peptides supporting the protein identification.

^a Also has ID Nsp|SEQ08864318, which is most likely contamination from the neighboring spot.

^b Also has ID for Nb|TC10793, but Nsp|SEQ08865322 shows nondegenerate support.

^c Contains a significantly weaker ID for Hsp70 (three unique peptides).

Regulation of Chloroplastic Proteins Required for ROS Accumulation

Among the early hallmarks of a defense response are bursts of ROS such as hydrogen peroxide (H₂O₂), superoxide (O₂⁻), and nitric oxide (NO) (Delledonne et al., 1998; Lamb and Dixon, 1997). As a result, a large percentage of the identified upregulated

proteins in our study have been implicated previously during the production or modulation of ROS (Tables 1 and 2).

Interestingly, chloroplast-generated ROS are induced by a SIPK/Nt4/WIPK MAP kinase pathway and play a crucial role during TMV-induced HR-PCD (Liu et al., 2007). The induction of H₂O₂ occurs in a light-dependent manner, suggesting that it

is a product of the photosynthetic electron transport (PET) chain of photosystem I (PS I). The PET chain can result in the photoreduction of O_2 to O_2^- , which can then be converted to H_2O_2 by superoxide dismutase (SOD). We observed an upregulation of components of PS I in iTRAQ and SOD in DIGE at $T = 2$ hr, which correlates with the early burst of H_2O_2 during HR-PCD (Tables 1 and 2). H_2O_2 also may be formed by the partial water reduction by photosystem II (PS II) (Fine and Frasch, 1992), and indeed, we found that the manganese-containing 23 kDa protein of the PS II oxygen-evolving complex was upregulated at $T = 8$ hr in iTRAQ. Furthermore, ascorbate peroxidase, which uses ascorbate to detoxify H_2O_2 , was downregulated at $T = 8$ hr in DIGE. All of these changes in regulation would culminate in an increase in H_2O_2 levels during a defense response.

Upregulation of Plant Immune Receptor-Associated Cytoplasmic Chaperones

Multiple heat shock proteins (HSPs), and their associated cofactors, interact with immune receptors to modulate the accumulation or folding of immune receptor complexes (reviewed by Shirasu, 2009). Interestingly, we discovered an upregulation of SGT1, HSP90, and HSP70. SGT1 has cochaperone features including tetratricopeptide repeats found in HSP70/HSP90-organizing proteins (Dubacq et al., 2002). HSP90 directly associates with the N immune receptor and SGT1 (Liu et al., 2004). Silencing of HSP90 or SGT1 results in a loss of N-mediated immunity to TMV (Liu et al., 2004; Peart et al., 2002). Similarly, HSP90 is required for the function of the RPM1, RPS2, and Rx immune receptors (Hubert et al., 2003; Lu et al., 2003a; Takahashi et al., 2003). HSP90 was upregulated at $T = 2$ hr after the initiation of a defense response (Table 2).

HSP70 homolog that was upregulated in DIGE (Table 1) is similar to the *Arabidopsis* HSC70 homologs that associates with SGT1 and are upregulated during a defense response (Noel et al., 2007). SGT1 is thought to function as a cochaperone of HSP90 and HSP70/Hsc70. Two peptidyl-prolyl isomerases called immunophilin rotamase FKBP (ROF1) were upregulated in DIGE and could act as an HSP90/HSP70-organizing protein like SGT1 (Table 1). ROF1 and other immunophilins have been shown to directly interact with HSP90 in both plants and animals (Harrell et al., 2002; Owens-Grillo et al., 1996). Other FKBP, similar to ROF1, bind to the glucocorticoid receptor (GR) and dynein to aid in the movement of GR from the cytoplasm to the nucleus (Harrell et al., 2002).

Upregulation of ER-Resident Multichaperone Complexes during Defense

One of the most intriguing results from this study was the discovery that a large number of ER-resident chaperones were upregulated during N-mediated defense. These include PDIs, ERp57, P5, calreticulin 3 (CRT3), glucose-regulated protein 78 (GRP78), and luminal-binding protein 5 (BiP5). Interestingly, the function of these proteins has not been examined extensively during plant innate immunity. Since the cytoplasmic chaperones HSP90, HSP70, and SGT1 have crucial functions during a defense response, we hypothesized that ER-resident chaperones, which were similarly upregulated, might be another important class of proteins for defense. Hence, the rest of our study

focused on these ER chaperones and their function during plant innate immunity.

PDIs function during the folding and the formation of disulfide bonds in the ER (reviewed by Christis et al., 2008). We discovered that three PDI homologs were among the highest upregulated proteins in our study (Tables 1 and 2). Nt|TC19383 contains two PDI-b domains sandwiched by two PDI-a domains, which suggests it is a homolog to canonical PDI. The second PDI was homologous to ERp57 and was represented by the ESTs Nb|TC11094 and Nb|CK295775 that overlap by 163 nucleotides with 97% identity. Indeed, we were able to amplify the full-length sequence of NbERp57 from *N. benthamiana*, suggesting they are transcribed from the same gene. The third PDI, Nt|TC14539, had a domain structure similar to P5 and was identified with one unique peptide in iTRAQ at $T = 8$ hr (Table S1).

We also observed an upregulation of *N. benthamiana* calreticulin 3 (NbCRT3) homolog (Table 1). Calreticulin is a lectin-like chaperone that interacts with ERp57 and mediates the association of ERp57 with its substrates (Maattanen et al., 2006). They function together during the proper folding of ER-resident proteins. In addition, we discovered another chaperone, GRP78-5, that was upregulated in iTRAQ (Table 2). A multichaperone complex that includes PDI, P5, GRP78, GRP94, and ERp72 was found to function during the folding of interferon- γ in the ER of human cell lines (Vandenbroeck et al., 2006). In this study, we discovered that three out of five homologs of these chaperones were upregulated during a plant innate immune response.

To verify the upregulation of some of these chaperones, we performed western blot analyses. Analysis with CRT antibodies showed a strong upregulation of CRT as early as $T = 2$ hr (Figure 3, top panels), even though the DIGE analysis suggests that CRT3 is only significantly upregulated at $T = 16$ hr. The CRT antibodies detected three bands, a nonspecific calnexin (top), NbCRT2 (middle), and NbCRT3 (bottom). Although there are three CRTs in *Arabidopsis*, currently only homologs to CRT2 and CRT3 are represented in the available *Nicotiana* sequence information. Analysis with HSP90 antibodies showed a slight upregulation at $T = 2$ hr and stronger upregulation at $T = 8$ hr (Figure 3, middle panels). These data are interesting because HSP90 transcripts were not upregulated during a defense response in our previous study (Liu et al., 2004); hence, the increase in HSP90 must occur on the posttranscriptional level. Our analysis with Hsc70 antibodies indicates no change during a defense response (Figure 3, lower panels). This may be due to the high homology between members of the HSP70/Hsc70 superfamily. Consistent with this, we observed a fairly large variation for the abundance ratios of different HSP70 homologs. Furthermore, other homologs to HSP70 showed no change in regulation, such as Nb|TC16565 (seven unique peptides; data not shown).

The Biological Significance of Upregulated ER Chaperones

To determine the biological significance of the upregulation of PDIs and CRTs, we used *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) (Liu et al., 2002) to knock down the expression of ERp57, P5, CRT3, and CRT2. After approximately 10 days of silencing, the plants were infected with either TMV-U1 tagged with GFP (TMV-U1-GFP) or untagged

Table 2. Identification of Differentially Regulated Proteins in iTRAQ

Protein ID	Protein Description	T = 2	T = 8	T = 2			T = 8		
		Ratio ± SD	Ratio ± SD	Prob	Uniq	Total	Prob	Uniq	Total
Defense-Related									
Nb TC16574	Putative secretory protein, PRp27	1.92 ± 0.00	5.70	0.99	1	2	1.00	1	1
Nb TC12269	Osmotin-like precursor, PR-R	1.81	4.59	0.95	1	1	1.00	1	1
Nt TC14089	Acidic endochitinase Q precursor, PR-Q	2.94 + 0.01	5.35 ± 0.02	0.99	1	3	1.00	2	4
Nt TC19270	Alanine aminotransferase (AlaAT)	1.48 + 0.04	2.02 + 0.01	1.00	2	4	1.00	1	5
Nt TC34209	Alanine aminotransferase (AlaAT)	1.63	1.42	0.95	1	1	1.00	1	1
Nb TC12663	Cell death-associated protein, HSR203J		3.68 + 0.02				1.00	2	3
Nt TC14739	Light-induced fibrillin, chloroplast precursor		-1.54 + 0.01				1.00	3	9
Nsn CL1Contig116	Polyphenol oxidase (PPO)		-1.55 + 0.03				1.00	3	7
Nt TC13992	Polyphenol oxidase (PPO)	-1.84 + 0.01	-1.77 + 0.03	1.00	3	7	1.00	2	5
Hormone and Reactive Oxygen Species Related									
Nsp SEQ08862687	1-aminocyclopropane-1-carboxylate oxidase, ACO3	1.66 + 0.05	1.65 + 0.03	1.00	2	4	1.00	4	7
Nb TC13762	Glutathione S-transferase	1.96 + 0.03		1.00	2	4			
Nt TC15374	1-aminocyclopropane-1-carboxylate oxidase	1.66 + 0.05		1.00	2	4			
Nb TC14976	3-ketoacyl-CoA thiolase	1.59 + 0.01	1.25 + 0.01	1.00	3	8	1.00	2	6
Nt TC24578	Plastid enolase	1.59 + 0.03	1.41 + 0.01	0.99	1	4	1.00	1	2
Nsp SEQ08860921	Photosystem I P700 chlorophyll a apoprotein A1	1.58 + 0.01		1.00	5	30			
Nt TC28453	Photosystem I P700 chlorophyll a apoprotein A2	1.54 + 0.02	0.95 + 0.01	1.00	3	11	1.00	1	5
Nb TC14470	Glutaredoxin	1.18 + 0.06	2.52 + 0.07	0.99	1	3	0.99	1	6
Nt TC15495	23 kDa of photosystem II oxygen-evolving complex		1.60 + 0.02				1.00	3	11
Chaperones									
Nb TC11094	Protein disulfide isomerase (NbERp57) ^a	2.18 + 0.01	2.15 + 0.01	0.99	1	6	1.00	1	2
Nb CK295775									
Nt TC19383	Protein disulfide isomerase (NbPDI)	1.70 + 0.08	1.95 + 0.01	1.00	2	7	1.00	1	2
Nb NP880339	Molecular chaperone HSP90	1.68 + 0.02	1.40 + 0.03	1.00	3	4	0.99	1	3
Nsn CL5673Contig1	Glucose-regulated protein 78 homolog, GRP78	1.57 + 0.02		1.00	3	7			
Nt TC14539	Protein-disulfide-isomerase (NbP5) ^b		2.31				1.00	1	1
Nsp SEQ08864501	SGT1	1.38	1.50	0.99	1	1	1.00	1	1
Nt TC16057	Protein-disulfide reductase	-1.63 + 0.02	-1.67 + 0.03	1.00	2	12	1.00	2	4
Metabolism									
Nsp SEQ08863512	Threonine deaminase	1.87 + 0.01	1.14 + 0.01	0.99	1	5	1.00	4	6
Nb TC12835	Alcohol dehydrogenase class III-like	1.76 + 0.04	1.04 + 0.01	1.00	2	4	0.99	1	4
Nt TC18425	Aldose 1-epimerase family protein		2.02 + 0.03				1.00	2	4
Nb TC16522	Phosphoenolpyruvate carboxylase	1.45 + 0.02	1.67 + 0.03	0.99	1	2	1.00	2	3
Nsn CL1Contig5601	Adenosylhomocysteine nucleosidase	1.38 + 0.03	1.54 + 0.03	1.00	3	12	1.00	2	6
Nt TC14301	Isocitrate dehydrogenase (NADP[+])	1.14 + 0.03	1.55 + 0.02	1.00	5	7	1.00	2	5
Nt TC34179	Starch synthase		-2.45 + 0.04				1.00	2	3
Other									
Nb TC14479	RNA-binding protein		1.67				1.00	2	2
Nb TC11893	Dynamin-like protein	1.49	1.50 + 0.02	0.99	1	1	1.00	2	3

Table 2. Continued

Protein ID	Protein Description	T = 2	T = 8	T = 2			T = 8		
		Ratio ± SD	Ratio ± SD	Prob	Uniq	Total	Prob	Uniq	Total
Nb TC12569	SEC14-like cytosolic factor protein		-1.46 ± 0.01				1.00	2	6
Nt TC17631	Hydrolase, alpha/beta-fold family protein		-1.96 ± 0.02				1.00	5	10
Nsp SEQ08863023	CYP81B2v2 cytochrome p450	1.22	3.23 ± 0.05	0.99	1	1	1.00	2	2

“Protein ID” is the EST number in which Nb = *N. benthamiana*, Nt = *N. tabacum*, Nsp = RefSeq protein, Nsn = *Nicotiana species* contig from NCBI ESTs. “Regulation Factor” is the ratio of no defense response to N-mediated defense response. “SD” is the standard deviation of the ratio calculated from the total number of peptides by the LIBRA module. The “Protein Prob.” generated by ProteinProphet is the probability that the protein is correctly identified. The “Number of Peptides” refers to the quantity of unique peptides and the total number of peptides supporting the protein identification.

^a Nb|TC11094 and Nb|CK295775 form a contig, representing the same protein. Nb|TC11094 was identified in T = 2 hr, and Nb|CK295775 was identified in T = 8 hr.

^b Nt|TC14539 identification is supported by only one unique peptide in T = 8 hr. No SD means that ratio was calculated from one peptide.

TMV-U1. The loss-of-resistance phenotype was observed as spreading HR-PCD to the upper uninoculated leaves. The partial knockdown of components required for N-mediated resistance to TMV results in a loss of viral containment to the inoculated leaf but does not fully disrupt HR-PCD. As a result, we observe HR-PCD where TMV spreads. For example, silencing the N gene (our positive control) results in spreading HR-PCD (Figure 4A, column 2). The causation of spreading HR-PCD by the movement of TMV into the upper uninoculated leaves was verified by semiquantitative reverse transcriptase (RT) PCR (Figure 4B, column 2). We discovered that silencing NbERp57, NbP5, NbCRT3, and NbCRT2 resulted in partial movement of TMV (Figure 4 and Table S3). TMV-U1 moved in 56% of ERp57-, 71% of P5-, 80% of CRT3-, and 73% of CRT2-silenced plants using 26–40 biological replicates over six independent experimental replicates (Figure S2 and Table S3). TMV moved in 100% of N-silenced plants and 6.45% of the vector-silenced

plants. Hence, the TMV movement in NbERp57, NbP5, NbCRT3, and NbCRT2 was statistically greater than our vector-silenced control but less than N-silenced plants, suggesting the silencing partially disrupts N-mediated resistance to TMV. The partial disruption of resistance may be caused by the partial knockdown of transcripts, since mRNA levels were only reduced 75.7% ± 3.1% for NbERp57, 60.4% ± 7.8% for NbP5, 78.1% ± 3.7% for NbCRT3, and 45.0% ± 5.5% for NbCRT2 (Figure S2). On the protein level, NbCRT2 abundance was reduced but still detectable in silenced plants (Figure 4C, lanes 7–9). In the NbCRT3-silenced plants, NbCRT3 protein was decreased to undetectable levels (Figure 4C lanes 4–6), which may have resulted in the more penetrant loss-of-resistance phenotype. Collectively, these results indicate that NbERp57, NbP5, NbCRT3, and NbCRT2 are required for the N immune receptor to activate a complete defense response.

Calreticulin Is Required for the Accumulation of an Induced Receptor-like Kinase

We hypothesized that one possible function for the upregulation of ER-resident chaperones was to aid in the folding or processing of membrane proteins required for plant innate immunity. A recent study suggests that ER-resident chaperones are required for the active accumulation of the receptor-like kinase, EFR, which is required for PAMP-triggered innate immunity (PTI) (Saijo et al., 2009; Nekrasov et al., 2009). Previously, our lab identified an IRK that was upregulated ~5-fold within 2 hr during an N-mediated defense response in microarray studies (data not shown). We confirmed the rapid upregulation of NbIRK during a defense response using RT-PCR (Figure 5A). Like EFR, IRK is a plasma membrane-localized leucine-rich repeat (LRR) receptor-like kinase (Figure 5B). IRK shows highest homology to the *Arabidopsis* receptor-like kinase, At4g23740, and contains five extracellular LRR repeats and an intracellular kinase domain (Figure S3).

To determine if IRK is required for N to function, we used VIGS to knock down IRK expression. Indeed, silencing IRK resulted in a loss of resistance to TMV in ~77.3% of the silenced plants in two independent experiments. Similar to above, we observed the loss-of-resistance phenotype as spreading HR-PCD to the upper leaves (Figure 5C), and TMV movement to upper leaves was confirmed by RT-PCR (Figure 5D). To further characterize the requirement of IRK, we conducted an HR-PCD assay by expressing TMV-p50 effector. In the VIGS-vector control, HR

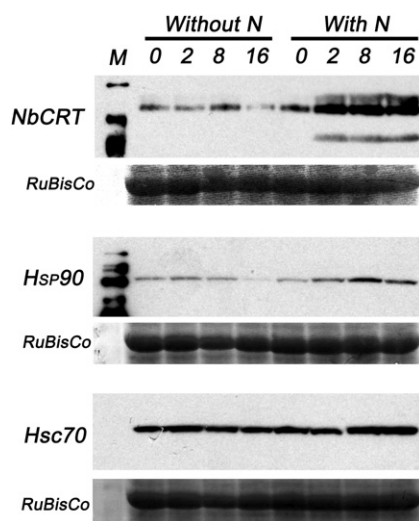


Figure 3. Validation of Upregulated Chaperones during Defense Response

Western blot analyses were conducted with CRT, HSP90, or HSC70 antibodies using protein extracted from 0, 2, 8, and 16 hr treated plant tissue without N that did not undergo a defense response and plant tissue with N immune receptor that did undergo a defense response. Protein concentration was normalized using a Bradford assay, and Coomassie-stained RuBisCo was used as a loading control. The protein ladder was Magic Marker XP (M).

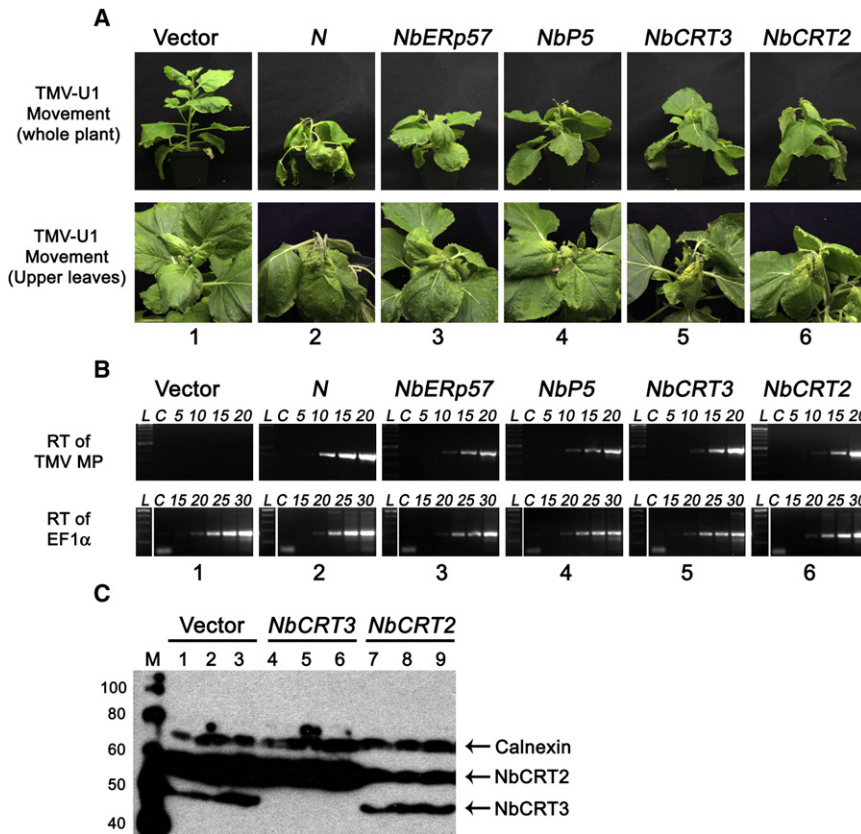


Figure 4. ER Chaperone Function Is Required for N Immune Receptor-Mediated Resistance to TMV

(A) *N*-containing *N. benthamiana* plants were infiltrated with *Agrobacterium* containing VIGS-vector control, VIGS-*N*, VIGS-*NbERp57*, VIGS-*NbP5*, VIGS-*NbCRT3*, and VIGS-*NbCRT2*. VIGS-silenced plants were infected with TMV-U1 approximately 10 days after the introduction of silencing constructs. Pictures were taken of the whole plant (top row) and the upper leaves (bottom row). HR-PCD spread with the movement of TMV, which was characterized by a collapse of tissue and yellowish-brown death. TMV did not move in the VIGS-vector control (column 1) and moved strongly in VIGS-*N* (column 2). Movement was observed in the VIGS-*NbERp57*-, VIGS-*NbP5*-, VIGS-*NbCRT3*-, and *NbCRT2*-silenced plants (columns 3–6).

(B) TMV movement from inoculated leaves into upper uninoculated leaves was verified using semiquantitative RT-PCR of the TMV movement protein (MP) (top row). TMV-MP was detected in the VIGS-*NbERp57*, VIGS-*NbP5*, VIGS-*NbCRT3*, and VIGS-*NbCRT2* (columns 3–6) plants, but less when compared to VIGS-*N* (column 2). No TMV-MP was detectable in the VIGS-vector control (column 1). Levels of EF1α were used as a quantity control (bottom row).

(C) Western blot analyses were conducted with CRT antibodies using protein extracted from three biological replicates of VIGS-vector control (lanes 1–3)-, VIGS-*NbCRT3* (lanes 4–6)-, and VIGS-*NbCRT2* (lanes 7–9)-silenced plants. Calnexin (top band) is unaffected by the silencing. NbCRT2 (middle band) is greatly reduced in the VIGS-*NbCRT2* plants. The NbCRT3 (bottom band) is undetectable in the VIGS-*NbCRT3* plants.

proceeded normally as PCD leading to tissue collapse (Figure 5E, left panels). In the VIGS-IRK-silenced plants, HR was attenuated to yellowing of the tissue with very little cellular collapse (Figure 5E, right panels). These results suggest that IRK is required for early signaling during the initial HR progression triggered by the N immune receptor.

Since IRK plays an important role during innate immune responses, we investigated the requirement of CRT3 and CRT2 for the accumulation of IRK. For this, we expressed IRK-citrine in vector-, CRT3-, and CRT2-silenced plants. IRK protein was greatly reduced in CRT3-silenced plants and partially reduced in CRT2-silenced plants (Figure 5F). This suggests that both CRT3 and CRT2 have a function during the folding and accumulation of IRK. As a control, we expressed the known plasma membrane-localized protein RIN4-citrine. The accumulation of RIN4-citrine was not affected by silencing of CRT3 or CRT2 (Figure 5F). These results suggest that one of the reasons CRT3 and CRT2 are induced early during a defense response is to fold plasma membrane-localized proteins required for innate immunity.

DISCUSSION

We implemented DIGE and iTRAQ proteomic methods to identify proteins that are upregulated early during a defense

response. Indeed, we identified previously discovered defense proteins that constitute the machinery and enzymes required for the rapid burst of ROS and defense hormone signals. This study also showed that cytoplasmic molecular chaperones that were previously shown to be required for plant innate immunity are upregulated early during a defense response. More importantly, we have demonstrated that components of ER-resident multichaperone complexes are similarly upregulated and are required for a successful defense response. We propose that the upregulated ER chaperones function during the folding and subsequent accumulation of proteins required for innate immunity. To test this model, we analyzed the expression of a receptor-like kinase, IRK, and discovered that it requires CRTs to accumulate. Furthermore, IRK is required for the N immune receptor to fully mount HR-PCD and subsequent defense against TMV.

Proteomics in Organisms without Sequenced Genomes

The lack of a full genome sequence and a complementary protein database for the model plant species *N. benthamiana* makes it extremely challenging to identify proteins by MS. As a result, we created a high-quality protein database in this study for large-scale proteomic experiments in *N. benthamiana*. In our iTRAQ experiments, searching our new database resulted in an approximately 16% increase in identifications compared to

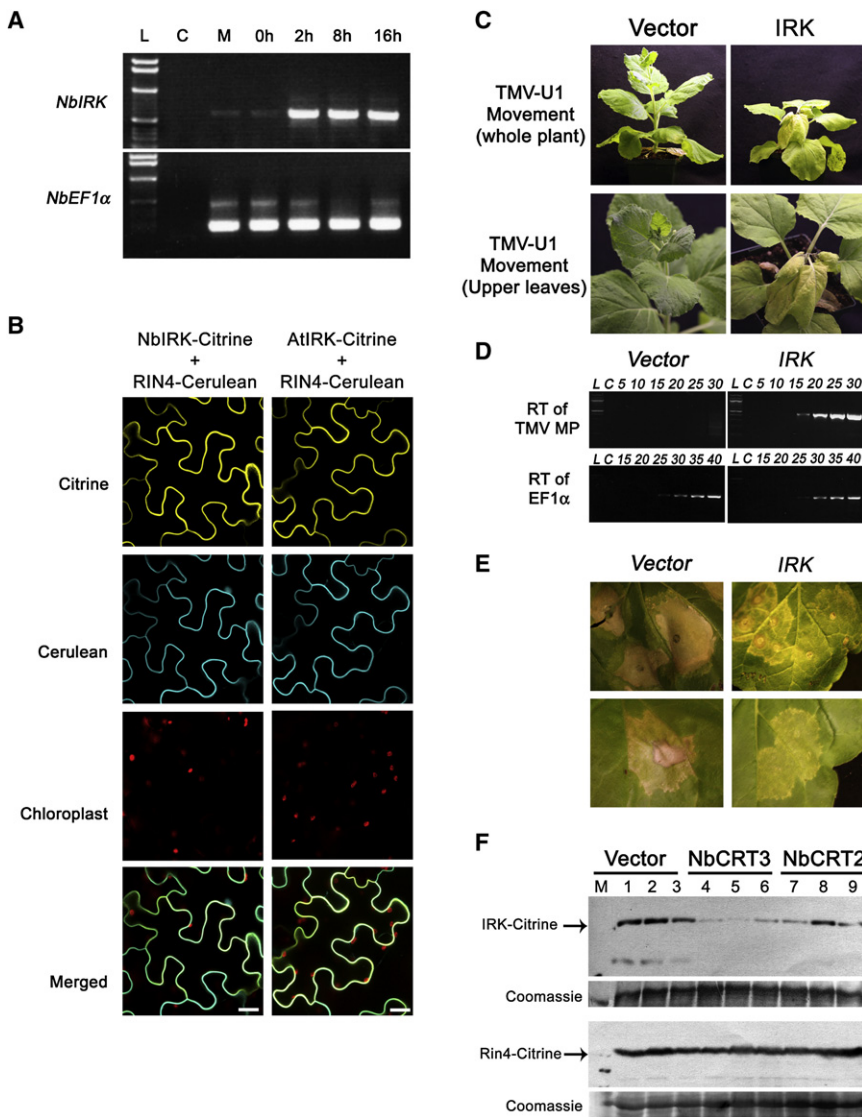


Figure 5. Plasma Membrane-Localized IRK Function Is Required for Defense Response, and Accumulation Is Dependent on CRT

(A) The upregulation of NbIRK was verified by RT-PCR. There is a strong increase in NbIRK expression between 2 and 16 hr after the induction of defense (top panel). No RT (C) and an uninfected mock tissue (M) were used as controls. Levels of EF1 α were used as a quantity control (bottom panel).

(B) Confocal images of NbIRK-citrine (left column) or AtIRK-citrine (right column) coexpressed with RIN4-cerulean. NbIRK-citrine and AtIRK-citrine are colocalized with RIN4-cerulean around the border of the cell in the plasma membrane. Chloroplasts autofluoresce red. Scale bar represents 20 μ m.

(C) *N*-containing *N. benthamiana* plants were infiltrated with *Agrobacterium* containing VIGS-vector control or VIGS-*IRK*. VIGS-silenced plants were infected with TMV-U1 approximately 10 days after the introduction of silencing constructs. Pictures were taken of the whole plant (top row) and the upper leaves (bottom row). HR-PCD spread with the movement of TMV, which was characterized by a collapse of tissue and yellowish-brown death in the upper uninoculated leaves of VIGS-*IRK* (right column). TMV did not move in the VIGS-vector control (left column).

(D) Movement of TMV was verified using semi-quantitative RT-PCR of TMV-MP (top panels). TMV-MP was detected in the VIGS-*IRK* plants (right column), but not in the VIGS-vector control (left column). Levels of EF1 α were used as a quantity control (bottom panels).

(E) HR-PCD assay in VIGS-vector (left column) and VIGS-*IRK* (right column) silenced *N. benthamiana* plants. TMV-p50 was expressed via *Agrobacterium* transient expression. Two independent replicates show decreased HR in *IRK*-silenced plants compared to the VIGS-vector control.

(F) IRK-citrine and RIN4-citrine were expressed in plants silenced with VIGS-vector control (lanes 1–3), VIGS-*NbCRT3* (lanes 4–6), and VIGS-*NbCRT2* (lanes 7–9). Protein was extracted from

three independent biological replicates, and immunoblot analysis was performed with GFP antibodies. The relative quantity of IRK-citrine is strongly reduced in VIGS-*NbCRT3* and partially reduced in VIGS-*NbCRT2*. Quantity of RIN4-citrine was not affected. Protein concentration was normalized using a Bradford assay, and Coomassie staining of the membrane was used as a loading control.

searching a six-frame translated database composed of ESTs from *N. benthamiana*, *N. tabacum*, *Lycopersicon esculentum*, *Capsicum annuum*, *Solanum tuberosum*, and *Petunia hybrida* (data not shown). We also developed a relational database system that can store, compare, and sort proteomic data. These tools will provide the framework for future proteomic experiments, such as identification of protein complex components by tandem affinity purification.

Cytoplasmic Multichaperone Complexes Are Rapidly Upregulated during an Innate Immune Response

Multichaperone complexes have been implicated in both plant and animal defense against pathogens. In this study, we show that HSP90, SGT1, and HSP70 homologs are upregulated early during a defense response. SGT1 directly binds to HSP90 and to

HSC70, a close homolog to HSP70, possibly bridging their chaperone functions (Noel et al., 2007; Takahashi et al., 2003). SGT1 is also transcriptionally upregulated during defense responses to *Hyaloperonospora parasitica* (Azevedo et al., 2006), suggesting that the upregulation of SGT1 is not specific to N immune receptor responses. HSP90 also has been implicated during effector-triggered immunity (ETI) by multiple R immune receptors, including N (Hubert et al., 2003; Liu et al., 2004; Takahashi et al., 2003). Interestingly, HSP90 associates with N, MLAs, and I-2 immune receptors (Bieri et al., 2004; de la Fuente van Bentem et al., 2005; Liu et al., 2004). Along with HSP90, SGT1 interacts with the cochaperone RAR1 (required for *Mla12* resistance), functions additively or independently during RPP5 immune receptor-mediated defense (Austin et al., 2002; Azevedo et al., 2002), and regulates barley MLA levels (Bieri et al., 2004).

Originally, HSP90, RAR1, and SGT1 were thought to function together during the accumulation of R immune receptors. However, it is now clear that different immune receptors have different requirements for HSP90, RAR1, and SGT1 during immune receptor accumulation and activation. HSP90, RAR1, and SGT1 are required for the accumulation of the Rx immune receptor; furthermore, the association of SGT1 and HSP90 was required for that accumulation (Azevedo et al., 2006; Boter et al., 2007). Conversely, SGT1 antagonized immune receptor accumulation by RAR1, possibly by guiding them to host cellular degradation machinery (Holt et al., 2005). This is supported by specific mutations in HSP90.2 that suppress the *rar1* mutant phenotype, suggesting that in *rar1* mutants, HSP90 is not properly regulated, resulting in a disruption of immune receptor accumulation (Hubert et al., 2009). Furthermore, SGT1 is required for N immune receptor accumulation (Mestre and Baulcombe, 2006).

The varying requirements of HSP90 and RAR1 may be caused by different pools of HSP90 having different functions. For example, SGT1 may not be the only HSP90/HSP70-organizing protein involved in defense. In this study, we discovered an upregulation of two ROF1-like proteins that are known to function with HSP90, which may partially account for the varying requirements for these chaperones during innate immunity. ROF1 associates with HSP90 and dynein, which is required for the retrograde movement of the GR along microtubules from the cytoplasm to the nucleus (Galigniana et al., 2001). Furthermore, these associations and functions are conserved between plants and animals (Harrell et al., 2002). The function of the ROF1/HSP90 chaperone complex during the maturation and localization of immune receptors should be investigated further.

ER Chaperone Complexes Are Required for N-Mediated Immunity to TMV

In this study, we show that multiple components of ER chaperone complexes are upregulated during a defense response. PDI chaperone complexes function during the formation of disulfide bonds between cysteines, which is often the rate-limiting step of protein folding (Creighton et al., 1995; Hatahet and Ruddock, 2007). More generally, they function with other ER molecular chaperones like CRTs, BiPs, GRP78, and GRP94 to form functionally competent proteins. Although the exact roles of PDIs during innate immunity were previously unknown, recent research suggests that ER chaperones may have a general role during all forms of plant innate immunity. The basal immune receptor, EFR, requires ER chaperones to accumulate during PTI (Saijo et al., 2009; Nekrasov et al., 2009). The rapid transcriptional upregulation of a PDI homolog during fungal-induced HR (Ray et al., 2003) and viral-induced HR (this study) suggests ER chaperones function early during ETI. Following PTI and ETI, plants induce systemic acquired resistance (SAR), which provides enhanced resistance to incoming pathogens. During SAR, ER chaperones, including PDIs, CRTs, and GRP94, are primary target genes of NPR1 and are necessary for the secretion of PR proteins (Wang et al., 2005).

We discovered three distinct homologs to PDIs: archetypal PDI, ERp57, and P5. The identified plant NbERp57 is the closest homolog to mammalian ERp57 and was one of the most upregulated proteins in our study. ERp57 and other PDIs function with CRT in the ER as multichaperone molecular complexes required

for the proper conformational folding and function of proteins (reviewed by Christis et al., 2008). If a protein does not fold properly, it is removed by ER-associated degradation (ERAD) machinery (Anelli and Sitia, 2008). Hence, ERp57 and CRT may regulate the folding and maturation of immune receptors or other factors required for innate immunity. The N immune receptor does not localize or fold in the ER, and disruption of ER chaperones had no effect on the accumulation of N (data not shown). However, confocal microscopy experiments examining other *Arabidopsis* NB-LRR immune receptors found many of them localizing to the ER (data not shown), and hence they may require ER chaperones for maturation.

Silencing of *NbCRT2* and *NbCRT3* led to a partial loss of resistance that was more penetrant than silencing the PDIs, NbERp57, or NbP5. However, the PDI family is quite large in *N. benthamiana*, and consequently there may be more functional redundancy compared to CRTs where there are only two homologs. Alternatively, the stronger phenotype may be caused by the dual function of CRTs as molecular chaperones and as calcium buffers. The C domain of calreticulin can store a large percentage of total ER calcium (Molinari et al., 2004) and may function during the initial calcium burst during HR-PCD. In the future, we would like to measure calcium levels in CRT-silenced plants during HR-PCD and what effect silencing may have on the rate of HR-PCD onset.

An Induced Receptor-like Kinase IRK Is Required for Innate Immunity

To test the possible role of ER chaperones during the folding of proteins required for innate immunity, we chose to study the function of CRTs rather than PDIs, for two reasons. First, an antibody that recognizes both forms of CRT was available. This allowed us to confirm the upregulation of CRTs and measure the level of silencing in VIGS experiments. Second, there are only two CRT homologs, making it a fairly easy protein family to study. Since our data suggested that CRT function is not required for the folding of the N immune receptor, we then took a candidate approach for substrates of CRT. IRK was our top candidate because it is transcriptionally upregulated and is a plasma membrane protein; hence, it is processed in the ER. As expected, disrupting CRT3, and to some extent CRT2, resulted in a decrease in the expression of IRK. This supports our model that CRTs may be upregulated to aid in the folding and accumulation of factors required for HR-PCD and defense. Alternatively, it is possible that silencing CRTs, PDIs, and IRK indirectly suppresses the plant innate immune system. Irrespective of direct or indirect role, the identification of IRK as a plasma membrane-localized receptor-like kinase required for viral ETI is an important discovery. Our data show that IRK is necessary both for the progression of HR-PCD and for the N to provide complete resistance to TMV. Future studies will be aimed at how a cytoplasmic and nuclear localized N immune receptor-mediated recognition of viral effector (Burch-Smith et al., 2007; Caplan et al., 2008) signals to activate or function with a plasma membrane-localized IRK.

In summary, this research has opened up two related, but distinctly different, areas of research in plant innate immunity. First, we have implicated the upregulation and requirement of ER chaperones during a defense response to TMV. Since the upregulation is downstream of pathogen recognition, we

hypothesize that the requirement of ER-resident chaperones may be a general, but crucial, requirement for innate immunity to a variety of pathogens. Hence, it will be exciting to see what other plant immune receptors require ER chaperones to function. Second, this study led to the identification and initial characterization of IRK, a RLK required for innate immunity. IRK requires ER-resident chaperones to accumulate and currently has an unknown function during a defense response. Future elucidation of IRK's biological role during plant defense will lead to new insights into how HR progresses and leads to innate immunity.

EXPERIMENTAL PROCEDURES

DIGE, Image Analysis, and Protein Identification

Protein was extracted from plants with or without the N gene at T = 0 hr, T = 2 hr, T = 8 hr, and T = 16 hr postinduction of a defense response (see the [Supplemental Experimental Procedures](#) for details). 2D-DIGE was conducted by Applied Biomics, Inc. (Hayward, CA). Wild-type plant extracts were labeled with Cy3, and NN plant extracts were labeled with Cy5 or with Cy2. N-containing plant extracts at T = 0 hr were labeled with Cy2 as an internal control. The expression of N-containing and wild-type plants was compared at T = 2 hr, T = 8 hr, and T = 16 hr. The samples were run on a single 2D-PAGE gel where the first dimension was performed by isoelectric focusing with pH 4–7 gel strips. The second dimension was conducted on a SDS-polyacrylamide gel. The Cy2-, Cy3-, and Cy5-labeled proteins were measured using excitation/emission wavelength of 488/520 nm for Cy2, 532/580 nm for Cy3, and 633/670 nm for Cy5. The relative expression levels were determined using the program Decyder MS (GE Healthcare). Identification of DIGE samples were performed by the LCQ Deca XP Plus ion trap mass spectrometer (Thermo Fisher Scientific) at the Midwest Bio Services, Inc., KS (see the [Supplemental Experimental Procedures](#) for details).

iTRAQ and MuDPIT Analysis

Protein was extracted with ProteoPrep (Sigma-Aldrich) chaotropic membrane extraction buffer 3 from plants with or without the N gene at T = 2 hr and T = 8 hr. Protein was precipitated with 4 volumes of ice-cold 100% acetone at -20°C for 1 hr and then washed three times with 80% acetone. Of total protein extract in digestion buffer, 100 μg was reduced, blocked, and digested with 10 μg of trypsin overnight according to the manufacturer's protocol (Applied Biosystems). Samples from plants with and without the N gene were labeled with iTRAQ 117 and iTRAQ 114 reagents (Applied Biosystems), respectively. The samples were pooled and purified by strong cation exchange columns (Applied Biosystems), and labeled proteins were eluted with 5% ammonium hydroxide in 30% methanol. MuDPIT analysis was performed on a QSTAR XL mass spectrometer with a nanospray II ion source (ABI, CA) at The State University of New York at Albany (see the [Supplemental Experimental Procedures](#) for details).

Protein Identification Search Strategy

A high-quality Nicotiana protein database (DKDB v7.1) was created and will be available upon request (see the [Supplemental Experimental Procedures](#) for details). DIGE and iTRAQ data were not prefiltered. Proteins were identified using a pipeline consisting of SEQUEST, TPP, and a custom relational database (see the [Supplemental Experimental Procedures](#) for details).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, three tables, and three figures and can be found with this article online at [http://www.cell.com/cell-host-microbe/supplemental/S1931-3128\(09\)00324-2](http://www.cell.com/cell-host-microbe/supplemental/S1931-3128(09)00324-2).

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