

Discovery of ADP-Ribosylation and Other Plant Defense Pathway Elements Through Expression Profiling of Four Different *Arabidopsis*–*Pseudomonas R-avr* Interactions

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Submitted 20 September 2007. Accepted 10 January 2008.

A dissection of plant defense pathways was initiated through gene expression profiling of the responses of a single *Arabidopsis thaliana* genotype to isogenic *Pseudomonas syringae* strains expressing one of four different cloned avirulence (*avr*) genes. Differences in the expression profiles elicited by different resistance (*R*)–*avr* interactions were observed. A role for poly(ADP-ribosylation) in plant defense responses was suggested initially by the upregulated expression of genes encoding NUDT7 and poly(ADP-ribose) glycohydrolase in multiple *R-avr* interactions. Gene knockout plant lines were tested for 20 candidate genes identified by the expression profiling, and *Arabidopsis* NUDT7 mutants allowed less growth of virulent *P. syringae* (as previously reported) but also exhibited a reduced hypersensitive-response phenotype. Inhibitors of poly(ADP-ribose) polymerase (PARP) disrupted *FLS2*-mediated basal defense responses such as callose deposition. *EIN2* (ethylene response) and *IXR1* and *IXR2* (cellulose synthase) mutants impacted the *FLS2*-mediated responses that occur during PARP inhibition, whereas no impacts were observed for *NPRI*, *PAD4*, or *NDR1* mutants. In the expression profiling work, false-positive selection and grouping of genes was reduced by requiring simultaneous satisfaction of statistical significance criteria for each of three separate analysis methods, and by clustering genes based on statistical confidence values for each gene rather than on average fold-change of transcript abundance.

Additional keywords: elf18, flg22, flagellin, PARG, *Pseudomonas syringae* pv. *glycinea*, *P. syringae* pv. *tomato*.

Plant disease resistance is mediated by both preformed defenses and inducible defense responses (Lucas 1998). Effective

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*The e-Xtra logo stands for “electronic extra” and indicates that eight supplemental figures and supplemental methods material are published online.

disease resistance often can be attributed to plant disease resistance (*R*) genes whose products elicit defense responses following recognition of the presence of particular effector or avirulence (*avr*) proteins expressed by pathogens (Dangl and Jones 2001; Jones and Dangl 2006). The diverse array of defense responses controlled by *R* gene action includes cell wall reinforcement; synthesis of phytoalexin compounds, defense peptides, and antimicrobial enzymes; release of signaling molecules that activate defenses in neighboring and distant cells; and activation of the hypersensitive cell death response (HR) (Dangl and Jones 2001; Lucas 1998; Martin et al. 2003; van Loon et al. 2006). Plants induce some of these same mechanisms of resistance, albeit less strongly, against virulent pathogens or in response to nonpathogenic microbes or microbe-associated molecular patterns (MAMPs) such as flagellin (Alfano and Collmer 2004; Conrath et al. 2006; Jones and Dangl 2006; Zipfel and Felix 2005). It is becoming clear that there is significant interplay between basal defenses, *R-avr*-triggered defense responses, and defense suppression by pathogens (Bent and Mackey 2007; Chisholm et al. 2006; Shen et al. 2006). In addition, it is likely that many antimicrobial compounds and processes activated as part of the plant defense response remain to be discovered.

The extent to which different *R-avr* interactions activate similar or different defense responses is not completely understood. Individual plants express multiple *R* genes with specificities for different strains of viruses, bacteria, oomycetes, fungi, nematodes, or insect pests, and individual plant genomes include a few hundred *R* gene-like sequences (Meyers et al. 2003; Michelmore 2000). Several distinct structural classes of *R* gene products can be distinguished on the basis of sequence. Most *R* genes studied to date encode putative intracellular proteins with nucleotide binding site and leucine-rich repeat motifs (NBS-LRR proteins) (Hammond-Kosack and Parker 2003; Jones and Dangl 2006; Meyers et al. 2003). These NBS-LRR proteins can be differentiated into further structural subclasses, which may activate overlapping but distinct plant responses.

Overlaps and distinctions between *R* and *avr* pathways have been supported by mutational studies (Feys and Parker 2000; Glazebrook 2001; Hammond-Kosack and Parker 2003; Martin et al. 2003). Some genes, such as *NPRI* in *Arabidopsis*, play a central role in the elicitation of multiple plant defense responses. Others, such as *NDR1*, *EDSI*, *PBS2*, *PAD4*, and *RARI*,

identify divergence in *R* gene signaling that correlates with the *R* gene involved. Different *R* gene-mediated signal transduction pathways may converge downstream to induce a common set of plant defense responses. Alternatively, the different *R* gene pathways may utilize some distinct signaling components and induce different defense responses (Eulgem et al. 2004; Sato et al. 2007).

Pseudomonas syringae pathovars, like many other gram-negative bacterial pathogens of plants or animals, use a type III secretion system to deliver virulence effectors into host cells (Alfano and Collmer 2004). When these effector proteins are recognized by *R* gene-mediated processes, they are defined as *avr* gene products. The four *P. syringae* *avr* genes used in this study (*avrRpt2*, *avrRpm1*, *avrPphB*, and *avrRps4*) and their cognate *Arabidopsis* *R* genes (*RPS2*, *RPM1*, *RPS5*, and *RPS4*) have been cloned and are known to elicit defense responses in an *R-avr*-dependent fashion (Bent et al. 1994; Debener et al. 1991; Dong et al. 1991; Gassmann et al. 1999; Grant et al. 1995; Hinsch and Staskawicz 1996; Jenner et al. 1991; Mindrinos et al. 1994; Warren et al. 1998; Whalen et al. 1991). These *R* genes all encode NBS-LRR proteins; *RPS2*, *RPM1*, and *RPS5* are predicted to have a coiled-coil near the N-terminus and *RPS4* has a predicted toll interleukin 1 receptor (TIR) domain. Whole-genome phylogenetic analyses indicate that *RPS2*, *RPM1*, and *RPS5* are diverse representatives of two of the four major classes of coiled-coil R proteins (Meyers et al. 2003).

Distinct strains of *P. syringae* previously have been used to study plant defense responses. Of these, *P. syringae* pv. *tomato* strain DC3000 (referred to subsequently as DC3000) is virulent on many genotypes of *Arabidopsis* as well as tomato and other host species (Cuppels 1986; Whalen et al. 1991). *P. syringae* pv. *glycinea* race 4 strain (referred to subsequently as R4) is virulent on soybean but, on *Arabidopsis* Col-0, it is non-virulent in that it multiplies poorly and neither causes disease nor elicits an HR (Yu et al. 1998). Despite differences in virulence on *Arabidopsis*, both DC3000 and R4 can deliver the same *avr* gene products and elicit defense responses in *Arabidopsis* that are mediated by the same *R-avr* pairings. This provides an experimental opportunity to investigate the extent to which host responses are the same or different when the eliciting *avr* signal is delivered by virulent as opposed to nonvirulent pathogen strains.

To initiate the present study, isogenic *P. syringae* strains and a single genotype of *Arabidopsis* were used to compare the transcript profiles induced by four different *R-avr* pairings. A limited transcript-profiling experiment was carried out to identify candidate genes for further functional study, rather than to establish definitive expression profiles. Because different statistical tests identified markedly different sets of genes as exhibiting significant changes in transcript level, a conservative data analysis approach was used that relied on multiple statistical tests. For each *R-avr* interaction, sets of genes were identified that exhibited consistent upregulation 14 h after inoculation. Genes upregulated in three or more of the tested *R-avr* interactions provided a reproducible candidate gene set. Gene knockouts of 20 candidate genes provided evidence for contributions to plant defense by four of these genes and suggested a role for poly(ADP-ribosylation) in plant-pathogen interactions.

RESULTS

Expression profiling using two isogenic systems for elicitation of four different *R- or avr*-mediated defense responses.

We utilized an isogenic system to compare plant responses mediated by four different *R* genes, resulting in the identification of numerous plant genes that are differentially expressed

in response to different *R-avr* interactions. Leaves of *Arabidopsis* ecotype Col-0 were inoculated with isogenic strains of two pathotypes of *P. syringae* that differed only by the expression of one of four cloned *avr* genes (*avrRpt2*, *avrRpm1*, *avrPphB*, or *avrRps4*). The four *avr* gene products were delivered individually by *P. syringae* pv. *tomato* strain DC3000 and *P. syringae* pv. *glycinea* strain R4. As controls, plants were either mock inoculated with solution containing no bacteria, inoculated with the same strain of *P. syringae* carrying the plasmid with no *avr* gene, or left untreated. Unless otherwise noted, transcript abundance was analyzed relative to the abundance in mock-inoculated control samples. A low titer of bacterial inoculum (1×10^5 CFU/ml) was used that, for DC3000, results in symptoms resembling those observed following natural infections. This inoculation titer avoided abnormal mass-activation of defense responses in all cells; however, a corollary is that changes in pathogen-induced gene expression were substantially diluted because many host cells were not in direct contact with pathogen (Turner and Novacky 1974). Leaf samples were harvested 14 h after pathogen inoculation. The relative levels of approximately 8,300 *Arabidopsis* gene transcripts were determined using AG1 Affymetrix *Arabidopsis* Gene Chip arrays.

Different analytical methods produced substantially different results.

Array data initially were processed and analyzed using the Affymetrix MAS software package. Reproducibility of transcript abundance data between treatment replicates was confirmed but fold-change values derived from comparisons of two treatments were less reproducible (Supplemental Fig. S1). This emphasized the need for statistical analysis methods that extended beyond average fold-change to identify those genes whose transcript level is reproducibly altered.

When we used the data set containing two biological replicates of each treatment to identify genes that exhibit a significant transcript abundance change following any given treatment, different commonly used analytical approaches and software packages identified partially overlapping but substantially different sets of genes (Fig. 1). Genes identified by one data analysis method as having exhibited a significant change in transcript abundance often were not detected as significant by another analysis method. The genes identified by one method were not a subset of those identified by a second method, which might be expected if one analysis method was simply more stringent than another. This implies that both false-positives and false-negatives are frequent when lists of genes with a significant transcript abundance change are generated using common expression profile data analysis methods, as also has been reported by others (discussed below).

Data filtering and grouping based on the intersection of statistical criteria.

We reduced the false-positive (type I error) selection or grouping of genes by requiring satisfaction across multiple significance criteria such as average fold-change, GeneSpring *P* value, dChip lower bound of confidence interval for fold-change (LBFC), Affymetrix difference call, and analysis of variance (ANOVA). The complete results of these analyses for all probe sets on the arrays, as well as the raw data for this entire study, are available on the University of California Davis and ArrayExpress websites (cited below).

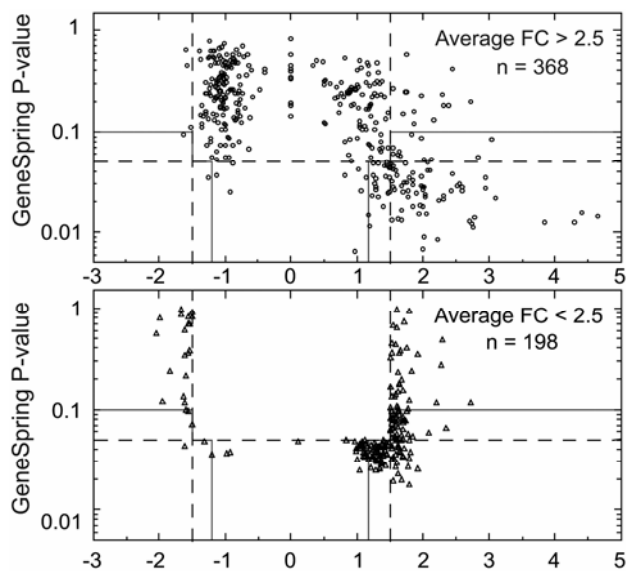
Data were sorted based on the GeneSpring *P* value, dChip LBFC, and ANOVA rather than on average transcript abundance fold-change (discussed below and in supplemental data). A master list of genes exhibiting altered transcript abundance in response to at least one *R-avr* interaction compared with

either mock- or DC3000- (with no *avr* transgene) inoculated plants was established (Supplemental Table S1). Induced expression was validated by RNA blot analysis and reverse-transcription polymerase chain reaction (RT-PCR) for a small subset of these genes (Supplemental Fig. S2 and discussed below). Due to the fact that some genes are identified by multiple probe sets, this set of 234 probe sets represents 220 genes.

To group genes into sets that exhibited similar regulation in response to different *R-avr* interactions, we established qualitative bins that each encompassed a defined quantitative range of significance values (dChip LBFC and GeneSpring *P* values). Each transcript initially was marked as increased, decreased, or unchanged for each treatment using the cutoff values that had first been applied in data filtering to generate the 234-probe-set master set. The relative reliability of the conclusion (level of statistical significance) was noted by the darkness of the gold or blue shading for increase and decrease, respectively (Fig. 2). Genes then were clustered manually based on the strength of statistical support for the conclusion that transcript abundance changed, rather than basing clustering on the transcript abundance fold-change values.

Transcripts altered by wild-type DC3000 or R4 are almost all altered by DC3000+*avr* or R4+*avr* strains as well.

In general, the *P. syringae* pv. *glycinea* R4 strains elicited a much weaker response than the DC3000 strains. Our conservative procedure revealed few or no genes that were elevated by DC3000 or R4 alone (with no *avr* transgene) compared with the mock control. When a less stringently determined list of 558 probe sets was examined, nearly all of the genes detectably activated by virulent DC3000 at 14 h after infection also were activated by *R-avr* interactions (Supplemental Table S2). Consistent with this observation, very few genes were identified whose abundance in an *R-avr* interaction was different from



dChip: Lower Bound of Confidence Interval for Fold-Change

Fig. 1. Different software packages identify substantially different sets of 'significant' genes. A representative example is shown, presenting data for transcript levels in plants inoculated with DC3000 *avrRpt2*⁺ relative to the levels for control mock-inoculated plants from the same experiments. Each data point represents one gene. The upper graph includes all 368 genes that exhibited an average fold-change (for two biological replicates) ≥ 2.5 as determined by Affymetrix MAS. In the lower graph, genes are presented if they were not already in the upper graph but GeneSpring *P* value was < 0.05 or dChip LBFC value was $\geq |1.5|$. Genes were excluded from the figure if $|AvDiff_{exp} - AvDiff_{control}| < 100$.

the abundance during infection with isogenic bacteria lacking a *avr* transgene. This is a primary reason why mock-inoculated plants were used as the control in our analyses below. The few genes that were upregulated in three or more *R-avr* interactions compared with DC3000 with no *avr* transgene also were identified on our candidate gene list when mock-inoculated plants were used as the control.

Different *R-avr* interactions produce overlapping but distinct expression profiles.

Our expression profiling study revealed sets of genes within the 220-gene master set that were induced by multiple *R-avr* interactions and other sets of genes that were activated by specific *R-avr* interactions. The relative number of genes in each tested *R-avr* interaction that exhibited altered transcript levels 14 h after inoculation is summarized in Figure 2. In terms of the overall number of genes exhibiting elevated transcript levels, *RPS2-avrRpt2* $>$ *RPM1-avrRpm1* $>$ *RPS5-avrPphB* $>>$ *RPS4-avrRps4*. *RPS4-avrRps4* interactions produced a minimal response that was more similar to the DC3000 (no *avr*) treatment than it was to the other three *R-avr* interactions. This latter result is not surprising given that *RPS4* is known to be active but minimally effective in the Col-0 genetic background (Gassmann et al. 1999; Hinsch and Staskawicz 1996; Yu et al. 1998).

Inspection of the data revealed a number of genes for which the statistical test for transcript level change was above the significance cutoff for one or more *R-avr* interactions, yet close to

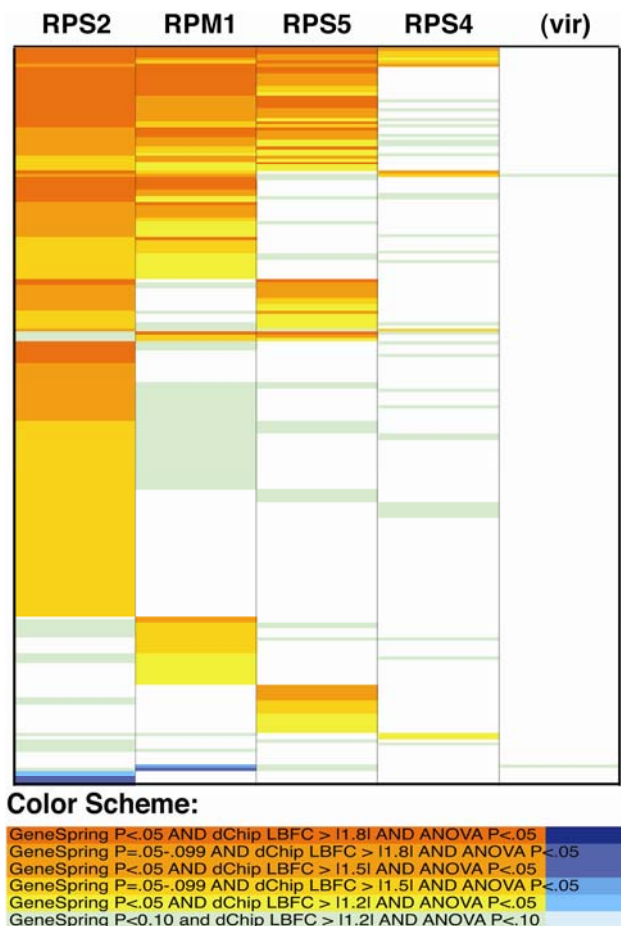


Fig. 2. Extent of co-expression of 234 probe sets that revealed significant upregulation in at least one treatment. Color intensities represent strength of conclusion that the transcript was more (orange) or less (blue) abundant in treatment than in the control, as described in the color key. Vertical height of a colored area reflects the number of genes in that area (figure derived from a vertical list of 234 probe sets).

but just below the cutoff for another *R-avr* interaction. To reduce type II errors (false characterization of such genes as “differently regulated in the tested *R-avr* interactions”), the 220-gene master list was resorted after identifying those genes that satisfied a less stringent cutoff (GeneSpring $P < 0.10$ and dChip LBFC $> |1.2|$ for any of the DC3000/mock-inoculation comparisons). The resulting data are presented in Supplemental Table S3. The overall conclusion remained that the different *R-avr* treatments produced different expression profiles.

Genes upregulated by different *R-avr* interactions include a core set of genes upregulated by three or more of the tested *R-avr* interactions.

In all, 38 genes exhibited elevated transcript levels for three or more *R-avr* interactions tested (Fig. 3). Supplemental Table S4 is a less stringently defined set of 71 genes that exhibited elevated transcript levels in three or more different DC3000+*avr* treatments. A number of genes not previously associated with defense were revealed, providing interesting candidates for further study.

Knockout study identifies genes that impact susceptibility to *P. syringae* pv. *tomato*.

Of the genes that showed significantly upregulated transcript levels in three or more *R-avr* interactions, those that had not been studied previously with respect to plant–pathogen interactions were targeted for gene knockout experiments. SALK T-DNA insertion *Arabidopsis* lines were obtained and homozygous mutant lines were generated for the 20 of the 71 genes marked as such. Pathogen growth within leaves then was measured for virulent *P. syringae* pv. *tomato* DC3000 and for DC3000 expressing *avrRpt2*. This candidate gene approach yielded four genes for which mutants showed significantly altered susceptibility to virulent DC3000 in multiple independent tests (Supplemental Fig. S3); At4g12720 (*NUDT7*, encoding a nudix hydrolase), At4g23700 (*CHX17*, encoding a cation/H⁺ exchanger), At4g39950 (*CYP79B2*, encoding a cytochrome P450), and At4g29740 (*CKX4*, encoding a cytokinin oxidase). Overall, roles in plant disease resistance cannot be ruled out for the other genes that were mutationally tested;

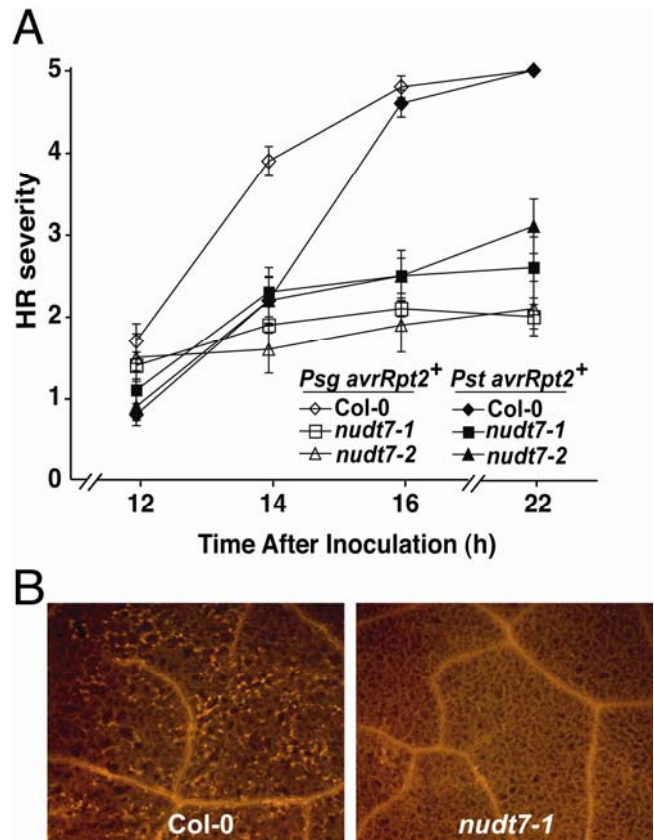


Fig. 4. The *nudt7*⁻ mutants display a diminished hypersensitive response (HR) in response to avirulent *Pseudomonas syringae* pv. *tomato*. **A**, HR-associated tissue collapse in leaves of Col-0, *nudt7-1*, and *nudt7-2* plants inoculated on one side of the leaf with either *P. syringae* pv. *glycinea* R4 + *avrRpt2* or *P. syringae* pv. *tomato* DC3000 + *avrRpt2* at 5×10^7 CFU/ml. Approximately 10 leaves per genotype were scored for severity of HR on a scale from 0 (no collapse) to 5 (100% collapse) over a time course ranging from 12 to 22 h. **B**, Autofluorescence detection of HR cell death in leaf mesophyll tissue of wild-type Col-0 (left) or *nudt7-1* mutant (right) 24 h after inoculation with *P. syringae* pv. *glycinea* R4 (*avrRpt2*) at 1×10^6 CFU/ml.

AG#	TIGR Gene Annotation	DC+avrRpt2/mock					DC+avrRpt1/mock					DC+avrPphB/mock					DC+avrRps4/mock					DC (no avr) / mock				
		GeneSpring FC	dChip LBFC	ANOVA P	FC	P	GeneSpring FC	dChip LBFC	ANOVA P	FC	Prob	dChip LBFC	ANOVA P	FC	P	dChip LBFC	ANOVA P	FC	P	dChip LBFC	ANOVA P					
At4g39030	enhanced disease susceptibility 5 (Eds5)	5.94	0.016	5.60	4.41	0.008	4.15	0.015	3.69	2.48	0.011	5.95	0.006	5.65	2.77	0.003	2.44	0.047	2.31	1.51	0.048	1.99	0.097	1.92	1.20	0.148
At2g04430	MutT/nudix family protein (NUDT5)	6.40	0.014	5.46	4.65	0.006	3.10	0.036	2.72	2.06	0.018	2.81	0.034	2.98	2.04	0.044	2.36	0.058	2.06	1.79	0.042	1.61	0.203	1.77	1.25	0.141
At2g05940	similar to auxin-regulated dual specificity cytosolic kinase	3.31	0.022	3.64	3.10	0.004	2.62	0.033	2.69	2.35	0.009	2.42	0.061	3.02	1.80	0.012	2.19	0.052	2.30	1.52	0.016	1.85	0.105	2.11	1.33	0.059
At3g24230	receptor-related protein kinase	3.92	0.023	2.93	2.63	3E-05	2.81	0.031	2.45	1.64	6E-05	3.83	0.016	2.83	1.61	6E-05	2.54	0.045	2.13	1.43	2E-04	2.09	0.072	1.82	1.15	1E-04
At3g24230	phytoalexin-deficient 4 protein (pad4)	4.18	0.028	3.13	2.60	2E-05	2.72	0.051	2.06	1.72	2E-04	3.19	0.038	2.20	1.87	9E-05	2.36	0.076	1.81	1.52	3E-04	1.74	0.200	1.41	1.07	0.002
At4g34480	glycosyl hydrolase family 17	2.63	0.053	2.52	2.12	2E-05	2.87	0.049	2.54	2.13	2E-05	2.75	0.051	2.43	2.10	2E-05	2.39	0.076	2.19	1.86	4E-05	1.51	0.308	1.49	1.29	0.001
At2g30770	cytochrome P450 71A13	10.48	0.013	6.27	4.30	4E-04	9.40	0.016	6.15	4.49	4E-04	5.88	0.035	4.16	3.07	0.001	2.18	0.157	1.54	1.03	0.017	1.87	0.335	1.66	1.08	0.052
At3g50770	calmodulin-related protein	2.78	0.026	2.90	2.42	0.004	3.05	0.017	3.03	2.33	0.003	2.46	0.032	2.65	1.98	0.007	1.76	0.098	1.67	1.10	0.205	1.72	0.119	1.67	1.06	0.131
At2g18690	expressed protein	3.55	0.023	3.07	2.32	0.001	3.09	0.031	2.72	2.20	0.002	2.63	0.039	2.50	1.68	0.004	1.79	0.128	1.63	1.19	0.03	1.70	0.231	1.64	0.87	0.047
At4g39950	cytochrome P450 79B2	3.98	0.009	2.67	2.02	2E-04	4.26	0.014	2.93	2.55	1E-04	3.28	0.015	2.18	1.67	5E-04	1.65	0.133	1.27	1.08	0.011	1.40	0.128	1.33	1.04	0.012
At1g02920	glutathione transferase, putative	2.78	0.029	2.34	1.98	1E-04	2.41	0.036	2.15	1.88	2E-04	2.29	0.040	2.06	1.56	4E-04	1.31	0.332	1.29	1.04	0.028	1.47	0.270	1.37	1.04	0.032
At4g36990	heat shock transcription factor 4 (HSF4)	3.15	0.026	2.97	2.59	0.003	2.51	0.039	2.35	2.03	0.004	2.56	0.032	2.41	1.50	0.005	1.79	0.107	1.70	1.32	0.021	1.90	0.120	1.89	1.17	0.029
At2g31865	poly(ADP-ribose) glycohydrolase-related (PARG)	3.06	0.035	3.53	2.95	0.002	2.74	0.047	2.34	1.91	0.003	2.30	0.077	2.93	1.65	0.001	1.83	0.148	1.98	1.58	0.007	2.01	0.111	2.04	1.71	0.021
At2g11900	light repressible receptor protein kinase, putative	2.59	0.009	3.03	2.23	0.006	1.97	0.022	2.48	1.91	0.006	1.62	0.058	2.21	1.62	0.031	1.02	0.918	1.42	1.08	0.034	1.39	0.205	1.92	1.17	0.014
At1g18000	glucose-6-phosphate(1)phosphate-translocator, putative	3.21	0.041	3.48	2.10	0.004	3.23	0.035	3.06	1.86	0.006	3.03	0.040	2.69	1.46	0.01	1.41	0.330	1.33	0.80	0.374	1.71	0.176	2.09	1.17	0.04
At1g18570	myb family transcription factor	3.01	0.027	4.41	2.94	2E-06	2.16	0.055	3.40	2.35	8E-06	2.76	0.030	4.85	3.22	9E-07	1.42	0.269	2.49	1.64	4E-06	1.25	0.502	2.13	1.42	2E-04
At4g12720	MutT/nudix family protein (NUDT7)	3.32	0.018	2.50	1.99	0.002	2.29	0.045	1.89	1.70	0.009	2.86	0.026	2.39	1.95	0.002	2.03	0.066	1.68	1.34	0.012	1.61	0.173	1.39	1.19	0.103
At5g54840	SGP1 monomeric G-protein	2.99	0.035	2.93	1.97	0.003	2.15	0.071	2.65	2.00	0.013	2.71	0.035	2.80	1.91	0.002	1.77	0.133	2.03	1.46	0.029	1.52	0.318	1.87	1.31	0.024
At1g24140	metallo proteinase -related	2.99	0.019	2.49	1.96	1E-03	2.31	0.039	2.00	1.59	0.002	3.42	0.011	2.87	1.83	6E-04	2.07	0.074	1.83	1.08	0.005	1.47	0.228	1.38	1.08	0.016
At4g29740	cytokinin oxidase family (CKX4)	3.05	0.021	2.81	2.32	1E-04	2.64	0.027	2.42	1.69	2E-04	2.55	0.035	2.57	1.75	1E-04	1.87	0.085	1.87	1.38	5E-04	1.43	0.245	1.59	1.04	6E-04
At2g29460	glutathione transferase, putative	2.96	0.028	2.39	2.00	5E-04	2.42	0.037	1.99	1.66	0.001	2.62	0.026	2.33	1.73	8E-04	1.31	0.345	1.18	0.93	0.084	1.66	0.130	1.45	1.14	0.021
At4g35110	expressed protein; pEARL1 4	2.86	0.024	2.45	1.98	0.008	2.98	0.021	2.39	1.72	0.014	2.88	0.023	2.63	1.62	0.008	1.71	0.123	1.46	1.22	0.04	1.21	0.658	1.30	0.96	0.219
At3g46900	zinc finger protein (ZAT7)	4.62	0.014	4.50	2.79	0.002	3.34	0.063	2.82	1.92	0.011	2.76	0.040	3.15	1.47	0.008	2.34	0.061	2.38	1.39	0.015	1.76	0.147	1.72	1.13	0.067
At5g24900	heat shock protein hsc70 (hsc70.2) (hsp70-2)	4.00	0.029	3.94	2.61	0.01	2.72	0.060	2.51	1.64	0.04	3.36	0.035	3.32	1.87	0.004	1.37	0.392	1.39	0.86	0.243	1.32	0.450	1.31	0.84	0.5
At2g21830	expressed protein; CGI-131 protein	4.35	0.013	2.13	1.52	1E-05	4.45	0.015	2.37	2.02	8E-06	3.93	0.014	2.33	2.01	2E-05	1.87	0.087	1.10	0.89	2E-04	1.39	0.318	1.04	0.87	0.003
At2g04400	3-kinase phosphatase family (IGPS)	2.67	0.022	2.12	1.78	0.002	2.37	0.034	2.12	1.97	0.003	2.12	0.044	2.16	1.73	0.007	1.66	0.113	1.38	1.27	0.009	1.56	0.175	1.52	1.10	0.031
At4g21380	receptor-related serine/threonine protein kinase ARK3	2.90	0.026	2.26	1.78	0.004	2.63	0.028	2.42	1.84	5E-05	2.58	0.032	2.27	1.52	1E-04	2.09	0.061	1.68	1.38	1E-04	1.56	0.201	1.58	1.25	0.002
At2g38860	(YL55) protease1 (pfp1)-like protein	2.35	0.034	2.40	1.69	0.016	2.31	0.037	2.33	1.65	0.01	2.28	0.038	2.37	1.72	0.013	1.70	0.104	1.70	1.28	0.045	1.56	0.161	1.69	1.28	0.182
At1g30900	spot 3 protein and vacuolar sorting receptor (AIELP6)	2.59	0.031	2.09	1.75	2E-04	2.49	0.031	1.80	1.54	6E-04	2.53	0.031	1.98	1.48	0.001	2.01	0.060	1.62	1.31	8E-04	1.64	0.144	1.42	1.20	0.011
At4g33300	disease resistance protein (CC-NBS-LRR class), putative	2.31	0.032	1.84	1.57	0.006	2.18	0.037	1.83	1.52	0.01	2.02	0.048	1.88	1.30	0.009	1.73	0.087	1.47	1.28	0.037	1.61	0.131	1.34	1.06	0.083
At4g12800	ACC synthase 6	2.55	0.042	2.44	1.54	0.024	2.05	0.075	2.04	1.68	0.032	3.69	0.016	3.42	2.30	0.01	1.95	0.144	2.01	0.83	0.045	1.43	0.371	1.50	0.73	0.167
At2g15390	xyloglucan fucosyltransferase, putative	3.01	0.032	2.36	1.53	0.015	2.50	0.053	1.97	1.65	0.02	2.68	0.045	2.42	1.40	0.049	1.58	0.232	1.62	1.27	0.094	1.15	0.085	1.54	1.20	0.174
At1g61140	SNF2 domain-containing protein	2.49	0.020	2.25	1.55	9E-04	1.98	0.046	2.01	1.29	0.001	2.11	0.034	2.30	1.22	1E-03	1.80	0.069	1.67	1.38	9E-04	1.44	0.201	1.52	1.20	0.006
At4g34150	C2 domain-containing protein	2.43	0.035	2.05	1.45	0.002	2.29	0.047	1.98	1.63	0.008	2.16	0.052	2.16	1.81	0.017	1.77	0.106	1.39	1.00	0.055	1.34	0.380	1.34	0.96	0.146
At1g61120	terpene synthase/cyclase; similar to S-linalool synthase	3.53	0.030	1.53	1.27	0.005	4.32	0.012	1.94	1.54	0.002	3.79	0.027	1.52	1.27	0.005	1.91	0.099	1.07	0.93	0.014	1.65	0.198	1.01	0.86	0.035
At2g38470	WRKY family transcription factor	3.52	0.022	2.57	1.32	0.015	2.72	0.047	2.34	1.30	0.023	3.86	0.018	3.15	2.10	0.011	2.15	0.084	1.83	1.17	0.046	1.64	0.214	1.54	0.92	0.087

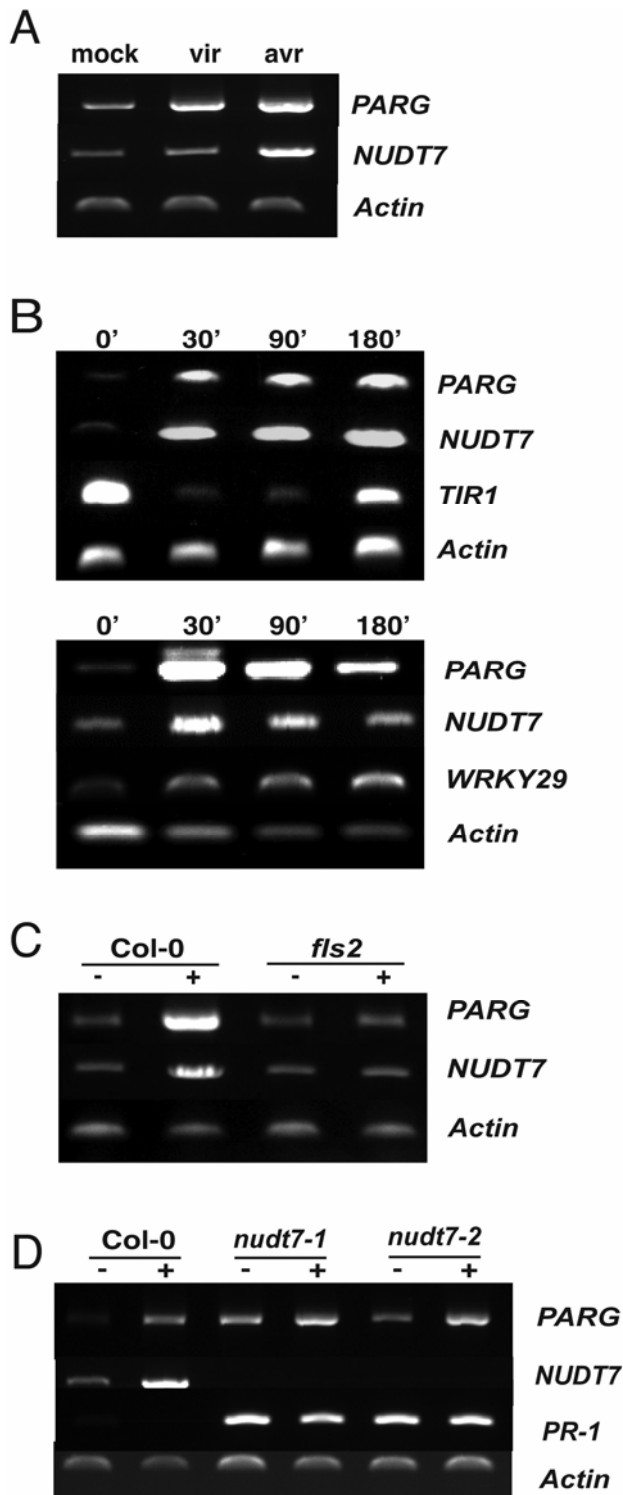


Fig. 5. *NUDT7* and poly(ADP-ribose) glycohydrolase (*PARG*) gene expression is upregulated upon treatment with flg22 and is dependent upon functional FLS2. Semiquantitative reverse-transcription polymerase chain reaction was performed on RNA extracted from **A**, 5-week-old *Arabidopsis* vacuum infiltrated with 10 mM MgCl₂ (mock) or with *Pseudomonas syringae* pv. *tomato* DC3000 (virulent) or *P. syringae* pv. *tomato* DC3000 + *avrRpt2* (avirulent) at 1 × 10⁵ CFU/ml or **B**, 2-week-old *Arabidopsis* seedlings treated with 10 μM flg22 peptide over a time course ranging from 0 (water-treated control) to 180 min. Two independent biological experiments are shown. *TIR1* gene expression is negatively regulated by flagellin treatment (Navarro et al. 2006) while *WRKY29* gene expression serves as a positive control (Asai et al. 2002). **C**, Wild-type or *fls2* mutant seedlings treated with (+) or without (-) 10 μM flg22 for 2 h. **D**, Wild-type or *nudt7* mutant seedlings treated with (+) or without (-) 2.5 μM flg22 for 2 h.

however, the four genes for which an altered plant response to pathogens was observed are targets for future study.

Poly(ADP-ribose)ation-related gene expression and plant defense responses.

Two plant lines carrying independent *nudt7* SALK T-DNA mutant alleles identified from the knockout study exhibited elevated resistance to virulent DC3000 (data not shown). Similar results with *Arabidopsis nudt7* mutants and virulent pathogens subsequently were published by three other groups (Bartsch et al. 2006; Ge et al. 2007; Jambunathan and Mahalingam 2006). We observed an additional defense-associated phenotype: a diminished HR (Fig. 4). Leaves initially were tested with high titers of avirulent *P. syringae* expressing *avrRpt2* to monitor macroscopically visible collapse (Fig. 4A) and the reduced HR then was confirmed using autofluorescence microscopy to track death of individual mesophyll cells in response to lower, more biologically realistic inoculum titers (Fig. 4B). In multiple independent experiments, HR cell death was not entirely absent but was greatly attenuated relative to wild-type *Arabidopsis* Col-0. In addition, an increased sensitivity to inoculation damage was noted for the *nudt7* mutants in multiple experiments in which a plastic syringe with no needle was used to gently introduce pathogen suspensions into leaf mesophyll tissue role in plant defense.

NUDT7 has been shown to exhibit ADP-ribose pyrophosphatase activity in vitro (Jambunathan and Mahalingam 2006; Ogawa et al. 2005; Olejnik and Kraszewska 2005). ADP-ribose pyrophosphatase converts ADP-ribose into AMP and ribose 5-phosphate, thus limiting pools of reactive (and hence toxic) free ADP-ribose (Ogawa et al. 2005). Poly(ADP-ribose) glycohydrolase (*PARG*) is one of the major enzymes responsible for generating free ADP-ribose in response to the activation of poly(ADP-ribose)ation (discussed below). Strikingly, we discovered that At2g31865 (*PARG*) is one of the few other genes whose transcript levels reliably increased upon infection with avirulent *P. syringae* pv. *tomato* during multiple *R-avr* interactions (Fig. 3, confirmed in Fig. 5A).

The expression of *NUDT7* and *PARG* genes during plant innate immune responses also was examined. The flagellin-based peptide flg22 is a potent elicitor of plant basal defenses (Felix et al. 1999; Gomez-Gomez et al. 1999). Expression of *NUDT7* and At2g31865/*PARG* mRNA is upregulated upon flg22 treatment (Fig. 5B). This upregulation by flg22 treatment was dependent upon the presence of the flagellin receptor FLS2, because neither transcript accumulated in *fls2* mutant seedlings treated with flg22 (Fig. 5C).

In *nudt7* mutants, *PARG* gene expression was constitutively elevated (Fig. 5D). Note that pathogenesis-related (PR)-1 expression also was elevated in untreated aseptically grown *nudt7* mutants from the same experiment (Fig. 5D). Mutant *nudt7* plants still exhibited flg22 responses (seedling growth inhibition and callose deposition) (Supplemental Fig. S4).

Inhibition of poly(ADP-ribose) polymerase also alters defense responses.

The above results suggested further examination of poly(ADP-ribose)ation processes during plant defense responses. Poly(ADP-ribose)ation is a post-translational protein modification that is known to influence human ischemia, inflammation, plant light stress, and other cellular stresses (Burkle 2005; Gagne et al. 2006; Hunt et al. 2004). Protein poly(ADP-ribose)ation is carried out by poly(ADP-ribose) polymerases (PARPs) (note that the glycohydrolase activity of *PARG* counters PARP by removing these poly(ADP-ribose) polymers). Mutational analysis of PARPs is difficult because *Arabidopsis*, humans, and other eukaryotes carry multiple

PARP genes; however, commercially available inhibitors such as 3-aminobenzamide (3-AB) often are used to disrupt PARP activity (Jagtap and Szabo 2005). These inhibitors have been shown to be specific in their ability to inhibit PARP activity and not other NAD⁺-consuming enzymes (Banasik et al. 1992; Hunting et al. 1985; Purnell and Whish 1980; Rankin et al. 1989), and have been used successfully in *Arabidopsis* and other plants (Berglund et al. 1996; De Block et al. 2005; Panda et al. 2002). We observed that PARP inhibition by 3-AB blocked flg22-induced callose deposition in *Arabidopsis* seedlings (Fig. 6A). Treatment with a separate defense elicitor, the bacterial EF-Tu-derived peptide elf18, also elicits callose deposition (Zipfel et al. 2006). As for flg22, callose deposition in response to elf18 was blocked by treatment with 3-AB (Fig. 6A).

In addition, PARP inhibition converted one or more FLS2-mediated responses into an overtly toxic response (Fig. 6B). Seedling growth inhibition is common in plants that have continuously activated defenses and is a widely used assay for plant innate immune responses such as FLS2 activation by flagellin or flg22 (Chinchilla et al. 2007; Dunning et al. 2007; Felix et al. 1999; Gomez-Gomez and Boller 2000; Sun et al. 2006). However, flg22 elicited dramatic and much more severe seedling growth inhibition in the presence of PARP inhibitor, and did so only if the plants carried a functional *FLS2* (Fig. 6B and C). In the absence of flg22, 3-AB-treated seedlings did not grow as well as untreated seedlings (Fig. 6C) but growth in 3-AB + flg22 was highly toxic (Fig. 6B and C). The loss of flg22-induced callose deposition in the presence of 3-AB, together with the *FLS2*-dependent toxicity of multiday growth in 3-AB + flg22, suggests that *FLS2*-mediated responses are partially disrupted and unfavorably diverted by inhibition of poly(ADP-ribosyl)ation.

The toxicity that PARP inhibition causes during *FLS2*-mediated responses apparently does not act through NPR1, salicylate, or NDR1-mediated pathways, because *Arabidopsis npr1-2*, *pad4-1*, (Glazebrook et al. 1996), and *ndr1-1* (Century et al. 1995) mutations did not relieve this response (Fig. 6D). However, *ein2-1* mutants (Guzman and Ecker 1990) displayed wild-type responsiveness to flg22 alone but displayed significantly less seedling toxicity than wild-type plants in the presence of 3-AB + flg22 (Fig. 6D). This suggests that ethylene responses are partially responsible for the toxicity that *FLS2*-mediated responses cause when PARP has been inhibited. Isoxaben-resistant *Arabidopsis ixr* mutants (Desprez et al. 2002; Scheible et al. 2001) also were tested, because these cellulose synthase mutants resist isoxaben-induced callose and lignin deposition. Intriguingly, the *ixr* mutants were noticeably more sensitive than the wild type to the toxicity of *FLS2*-mediated responses in the presence of PARP inhibition (Fig. 6E), even after data normalization to account for the smaller size of untreated *ixr* mutants, suggesting a linkage between poly(ADP-ribosyl)ation and the regulation of cell wall modification during plant defense responses.

DISCUSSION

A single host genotype and isogenic *P. syringae* strains were used to compare the *Arabidopsis* transcript profiles induced by four different *R-avr* pairings. Multiple significance criteria were utilized to reduce false-positive inclusion of genes that were not reliably regulated by a given *R-avr* interaction. The identified genes can serve as targets for future signal transduction studies, promoter dissection, gene knock-out work, or other studies. In the present study, an apparent role for poly(ADP-ribosyl)ation in plant-pathogen interactions was revealed.

Data analysis challenges in expression profiling studies.

We found that different data analysis packages identified quite different sets of genes from the same initial experimental data (Fig. 1). Some of the differences may have arisen because the data set contained two rather than three biological replicates of each treatment, three being more commonly preferred. However, the poor agreement between different expression profile data analysis methods has been examined in the bioinformatics literature (Abruzzo et al. 2005; Jeffery et al. 2006; Millenaar et al. 2006; Nadon and Shoemaker 2002; Shedden et al. 2005; Slonim 2002; Smyth et al. 2003; Stafford and Brun 2007). Multiple statistical tests have been recommended to generate a more refined list of differentially expressed genes (Abruzzo et al. 2005; Kannangara et al. 2007; Millenaar et al. 2006). We did this using commonly accepted analysis methods: GeneSpring *P* value, dChip lower bound of confidence interval for fold-change, and ANOVA. These analysis methods use not only different statistical models but also different forms of input data derived from the same original raw data.

Differentiation among *R-avr* interactions.

One goal of the present study was to identify genes that were robust indicators of a given *R-avr* interaction. The time point 14 h after inoculation was chosen for sampling based on prior published studies of *Arabidopsis* responses to *P. syringae*. Occurring well after initial plant responses to handling, after the pathogen has adapted to the plant interior and initiated type III secretion-mediated suppression of defenses (approximately 2 to 3 h after inoculation), and after the earliest *R* gene-mediated defense signaling events have occurred, the 14-h time point represents a time at which plant defense responses are well underway. However, it also is known that plant responses to different *R-avr* interactions can arise at different rates and with different intensities (as measured, for example, by PR-1 gene induction or strength of restriction of pathogen growth). Macroscopic differences in the plant response as well as different levels of economic disease control have been observed historically with many different *R-avr* gene pairings (McDowell et al. 2000; McIntosh et al. 1995; Minsavage et al. 1990). Specific to the present experiments, *RPM1-avrRpm1* responses frequently have been reported to arise more rapidly than *RPS2-avrRpt2* responses and both of these responses are stronger than the response of ecotype Col-0 mediated by *RPS4-avrRps4* (Ritter and Dangl 1996; Yu et al. 1998). Hence, it is not surprising that substantial differences were observed in the expression profiles for the different *R-avr* interactions. After this study and other expression profiling studies, it remains difficult to identify canonical genes whose expression distinguishes particular *R-avr* interactions. However, the data from the present study emphasize that, even when isogenic host and pathogen strains are used in a controlled environment, the plant responses mediated by different *R-avr* interactions can differ dramatically.

Overlap in responses to virulent and avirulent pathogen.

A much smaller number of transcripts showed significant abundance change during *R-avr* interactions when the compatible interaction with DC3000 (no *avr*) was used as the baseline or control rather than using mock-inoculated plants as the control. Many of the transcripts elevated during the *R-avr* interaction also were elevated, but to a lesser extent, in response to DC3000 with no *avr*, as has been reported previously (Tao et al. 2003). This corroborates previous observations that virulent and avirulent strains of a pathogen often induce similar PR genes and other defense-associated responses, but the responses occur more rapidly or more strongly at early time-points in reaction to avirulent strains than they do to virulent

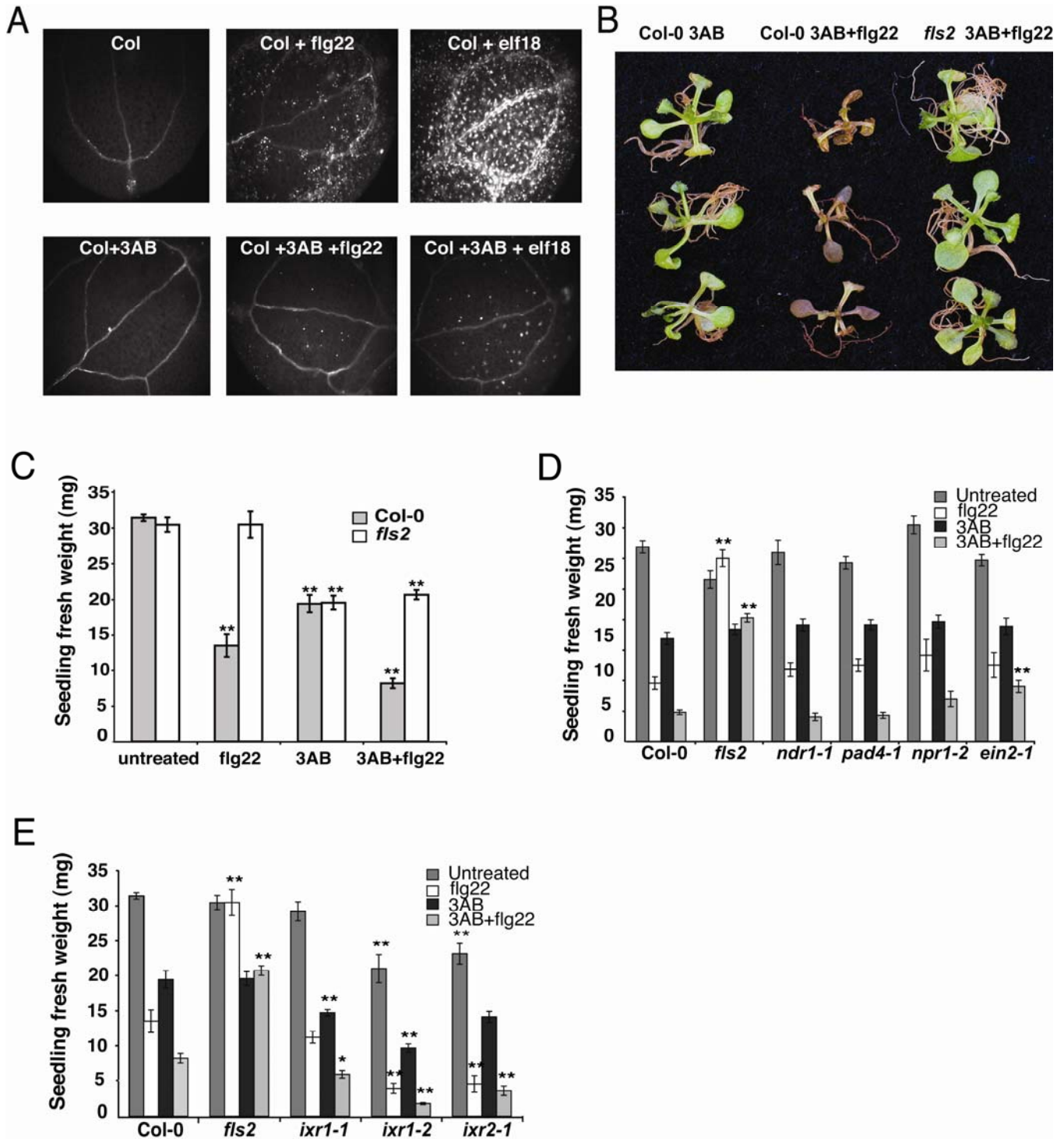


Fig. 6. Inhibitors of poly(ADP-ribosyl)ation impact plant basal defense responses to flg22. **A**, Ten-day-old *Arabidopsis* seedlings treated with 0.6% dimethyl sulfoxide (DMSO) with or without 5 mM 3-aminobenzamide (3-AB) for 24 h. Seedlings then were treated with 2.5 μ M either flg22, elf18, or water for 24 h, fixed with aniline blue, and examined for autofluorescent callose staining. **B**, Wild-type or *fls2* seedlings were grown in liquid Murashige-Skoog (MS)/sucrose for 10 days, at which time 3-AB was added to the growth medium (to a final concentration of 5 mM), followed 2 h later by addition of flg22 (to a final concentration of 10 μ M) or water. Pictures were taken after 10 additional days of growth. **C**, Wild-type or *fls2* seedlings were grown in liquid MS/sucrose for 10 days, at which time 0.6% DMSO with or without 3-AB was added to the growth medium (to a final concentration of 2.5 mM), followed 24 h later by addition of flg22 (to a final concentration of 10 μ M) or water. Seedling weight was recorded after 10 days of additional growth in this solution. The experiment was repeated on four separate dates; asterisks summarize results across these four dates for analysis of variance (ANOVA) tests of similarity of means between the treatment and untreated control plant of the same genotype (Tukey's simultaneous test; ** = $P < 0.001$; no asterisk = $P > 0.05$). **D** and **E**, Experiments performed as described for C using the designated *Arabidopsis* Col-0 mutant plant lines. Asterisks summarize ANOVA results across three separate experiments (exception: for the 3-AB-only treatment, experiments with *ndr1-1* and *npr1-2* were done twice). To account for differences in growth of untreated seedlings of the different genotypes, graphs show actual seedling weight data for one experiment; however, ANOVA tests used, as input data, the weight for each seedling normalized to the within-experiment average weight of the untreated controls of the same genotype. ANOVA tests were for similarity of means between each genotype and the wild-type control subjected to the same flg22 or 3-AB treatment (Tukey's simultaneous test; * and ** = $P < 0.05$ and 0.001 , respectively; no asterisk = $P > 0.05$).

strains (Lucas 1998). Experiments performed using *P. syringae* pv. *glycinea* R4 identified only a small set of significantly regulated genes, nearly all of which also were identified in the DC3000 experiments. Note again that DC3000 (lacking an *avr* transgene) is virulent on *Arabidopsis* ecotype Col-0 whereas R4 (lacking an *avr* transgene) is not virulent on this host. In the present study, we were left with the impression that R4 is less effective at delivering *R*-activating *avr* signals, possibly as a quantitative rather than a clearly qualitative difference.

ADP-ribosylation and defense responses.

Follow-up study of 20 candidate genes identified from the expression profiling experiments revealed an association between ADP-ribosylation and defense responses. *NUDT7* is one of at least four *Arabidopsis* nudix hydrolases with ADP-ribose pyrophosphatase activity (Jambunathan and Mahalingam 2006; Ogawa et al. 2005; Olejnik and Kraszewska 2005). *NUDT5* also was upregulated in our study (Fig. 3); however, its product apparently does not exhibit ADP-ribose pyrophosphatase activity (Ogawa et al. 2005). When *nudt5* knockout lines were tested, we did not detect defense alteration. In addition to the upregulated expression of *NUDT7* that we observed in three *R-avr* interactions (Fig. 3), other studies have noted elevated *NUDT7* gene expression in response to flg22 treatment (Navarro et al. 2004), and *PAD4*- and *EDS1*-dependent regulation in response to *P. syringae* expressing *avrRPS4* (Bartsch et al. 2006). A leading hypothesis is that *NUDT7* contributes to the removal of toxic ADP-ribose (Bartsch et al. 2006; Ogawa et al. 2005). Several studies in addition to this study have now identified increased restriction of virulent pathogen growth in *nudt7* mutants (Bartsch et al. 2006; Ge et al. 2007; Jambunathan and Mahalingam 2006).

We observed two additional phenotypes not reported in other *nudt7* studies: a significant reduction in the growth of avirulent *P. syringae* pv. *tomato* in *nudt7* mutants and a clear and reproducible loss of HR in *nudt7* mutants (Fig. 4). Our observation of a reduced HR in *nudt7* mutants contrasts with a report of a normal HR phenotype for *nudt7* mutants (Ge et al. 2007). The plants used by Ge and colleagues for HR tests were grown in near-aseptic conditions to avoid plant growth phenotypes and constitutive activation of PR gene expression, whereas the plants utilized in our HR study were grown in communal growth rooms and exhibited reduced plant size and constitutive PR-1 gene expression relative to wild-type plants. In our work, there was some variability in the extent of HR reduction across multiple experiments but, in each instance, the HR produced by the *nudt7* mutants was significantly attenuated relative to the wild type. This interesting association between constitutive PR expression and reduction of HR cell death also has been observed with other genotypes (for example, with *Arabidopsis dnd* mutants); however, the correlation is not absolute because other constitutive PR mutants do not exhibit a loss of HR (Yu et al. 1998).

A gene encoding a second component of the poly(ADP-ribosylation) process, *PARG*, also was identified as being reliably upregulated by three or more *R-avr* interactions and in response to flg22 treatment (Figs. 3 and 5). Only one *PARG* gene exists in mammals and insects, whereas there are two apparent *PARG* genes located in tandem on *Arabidopsis* chromosome 2 (At2g31865, identified in this study, and At2g31870). A third *PARG* gene may exist (At2g31860); however, it does not have any expressed sequence tags (EST) or cDNAs associated with it and, therefore, may not be expressed (Hunt et al. 2004). The *Arabidopsis tej* mutation was identified as a lesion in At2g31870 and the protein encoded by the wild-type gene appears to be a regulator of the circadian oscillator in

Arabidopsis (Panda et al. 2002). Very little is known about At2g31865, the *R-avr*-induced *PARG* gene. At2g31865 does not have a probe set on the more recent 22,000-gene Affymetrix *Arabidopsis* chip despite clear EST evidence for the existence of this gene and its transcript and, hence, would remain unnoticed by researchers relying on data from the 22,000-gene chip. *PARG* is the only known enzyme that can counter the catalytic activity of PARP enzymes. The upregulation of *PARG*/At2g31865 during defense responses may contribute to recovery efforts in response to pathogen infection. In a separate expression profiling study, *PARG* and *NUDT7* were among the many genes upregulated in response to *P. syringae* pv. *phaseolicola*, a nonhost pathogen, but not by virulent *P. syringae* strains (Tao et al. 2003). Enzymes that impact poly(ADP-ribosylation) may be a component of the response that is downregulated by type III-secreted effectors that determine *P. syringae* host range.

Poly(ADP-ribosylation) of proteins occurs in response to a variety of cellular processes, including DNA repair and transcription, cell cycle regulation, apoptosis, and regulation of telomere length (Burkle 2005; Gagne et al. 2006; Hunt et al. 2004; Oei et al. 2005; Schreiber et al. 2006; Ziegler 2000). PARPs use β -NAD⁺ as a substrate, attaching ADP-ribose residues to specific protein acceptors to form branched chains of ADP-ribose polymers. Hence, PARP activity can directly modify target protein function. This process results in the consumption of significant quantities of NAD⁺ and generates substrate that *PARG* then can act on to release toxic ADP-ribose. Under excessive DNA damage, PARP-1 becomes overactivated, leading to massive consumption of NAD⁺ and ultimately resulting in necrotic cell death (Burkle 2005; Koh et al. 2004). It may be very relevant that PARP is involved in the cell-death response of cultured soybean cells subjected to oxidative stress by treatment with H₂O₂ (Amor et al. 1998). When levels of poly(ADP-ribosylation) are decreased via PARP inhibition, plants become less sensitive to drought and high light stress (De Block et al. 2005; Vanderauwera et al. 2007). This particular outcome was attributed mainly to prevention of high NAD⁺ consumption. In *Catharanthus roseus* tissue culture, increases in phenylalanine ammonia-lyase activity were prevented by addition of an inhibitor of poly(ADP-ribose) polymerase (Berglund et al. 1996). We have located no other inquiries into the role of poly(ADP-ribosylation) in plant defense.

Poly(ADP-ribosylation) was further implicated in plant defense responses when treatment with 3-AB, an extensively characterized PARP inhibitor, altered flg22-induced responses. Treatment of seedlings with PARP inhibitor blocked both flg22- and elf18-induced callose deposition (Fig. 6A) and caused FLS2-mediated flg22 responses to become nonproductive and toxic (Fig. 6B and C). This response arose independent of *NPR1*, *PAD4*, and *NDR1* (Fig. 6D). However, ethylene apparently contributes to the toxic response because *ein2* mutants were significantly less damaged than wild-type seedlings (Fig. 6D). The increased sensitivity of *ixr* (cellulose synthase) mutants to FLS2 signaling under PARP inhibition (Fig. 6E) suggests that poly(ADP-ribosylation) may impact the regulation of cell-wall modification during plant defense responses. This may arise due to poly(ADP-ribosylation) of specific proteins or, alternatively, via modulation of NAD⁺ pools. NAD⁺/NADH ratios impact the level and type of cell wall matrix biosynthetic activity (Seifert 2004).

Additional points about *NUDT7* bear mentioning. First, the *NUDT7* product has been shown to preferentially hydrolyze both ADP-ribose and NADH substrates in vitro (Jambunathan and Mahalingam 2006; Ogawa et al. 2005; Olejnik and Kraszewska 2005), and it remains possible that the defense

upregulation of *NUDT7* expression or the defense phenotypes of *nudt7* mutants are more related to impacts on NAD⁺/NADH pools than to removal of free ADP-ribose. Second, the elevated *PARG* expression in *nudt7* mutants may be specifically regulated by pathogen-induced alterations in PARP activity or by changes in ADP-ribose or NAD⁺ pools. Alternatively, *PARG* may simply be among a larger number of defense-related genes (including PR-1) that are upregulated in *nudt7* mutants, which also carry elevated levels of salicylic acid (Bartsch et al. 2006; Jambunathan and Mahalingam 2006). Third, Ge and associates (2007) found no constitutive elevation of PR-1 expression in *nudt7* mutants grown in isolation, leading them to conclude that mutation of *nudt7* potentiates plants for a heightened response after exposure to pathogenic and nonpathogenic microorganisms. However, when we grew *nudt7* seedlings under sterile conditions, we observed constitutive PR-1 expression (Fig. 5D). Potentiation of defenses and constitutive defense expression may both be present in *nudt7* mutants.

In summary, we utilized isogenic plants and pathogens to identify distinguishing features between the gene expression profiles activated by different *R-avr* stimuli. When one of these leads was pursued, we found that two genes involved in ADP-ribosylation are upregulated during both basal and *R-avr*-elicited defense responses, and that mutation of *NUDT7* and chemical inhibition of PARP activity alter these responses. This suggests that poly(ADP-ribosylation) makes a significant contribution to appropriate plant defense responses.

MATERIALS AND METHODS

Experimental design for expression profiling.

Within a plant block, six or seven pots containing 12 to 16 plants per pot were grown adjacently in the same flat and then individual pots were either mock inoculated with 10 mM MgCl₂ alone, inoculated with one of the five isogenic bacterial strains, or left untreated. Rosette tissues from all plants in a pot were harvested as a single pool 14 h after inoculation. For each chip hybridization, RNA samples were pooled by equal RNA mass from three equivalently treated sets of plants taken from plant blocks grown in separate controlled environment chambers. Each chip hybridization experiment was repeated subsequently using entirely independent RNA samples from a pool of three additional plant sets grown roughly 4 months after the first plant sets. This provided two biological replicates per treatment, with each replicate monitoring RNA from three pooled sets of plants. Experiments using DC3000 and R4 were performed independently. The same growth conditions (soil type, potting system, environmental settings, and so on) were used for all plant sets and all sets were treated at the same time of day using the same pathogen inoculation protocol.

Plant inoculation.

Details of the experimental procedure are available in the electronic supplement. In brief, the wild-type *Arabidopsis* Col-0 accession was grown for 5 to 6 weeks at 22°C with lights on from 9:00 a.m. to 6:00 p.m. (16 seeds per 9-cm pot). *P. syringae* pv. *glycinea* strain R4 and *P. syringae* pv. *tomato* strain DC3000 carried the plasmid *pVSP61* with no insert or with *avrRpt2*, *avrRpm1*, *avrPphB*, or *avrRps4* under control of their native promoters (Hinsch and Staskawicz 1996; Innes et al. 1993; Kunkel et al. 1993; Simonich and Innes 1995). Bacteria were resuspended in 10 mM MgCl₂ at 1 × 10⁵ CFU/ml within 30 min before use in vacuum infiltration (at 8:30 to 8:50 a.m.), and plants were returned to their original growth chambers. Fourteen hours after treatment (10:30 to 10:50 p.m.), rosette leaves were collected by cutting with a razor at the basal stem, immediately frozen in liquid N₂, and stored at -80°C.

RNA isolation and synthesis of biotin-labeled cRNA.

Total RNA was isolated from the plants collected from each pot using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Equal amounts of total RNA from each of the three replications of each treatment were pooled and then further purified using a QIAGEN RNeasy mini kit (Qiagen, Valencia, CA, U.S.A.). Double-stranded cDNA was synthesized according to the Affymetrix GeneChip expression analysis technical manual (Affymetrix, Inc., Santa Clara, CA, U.S.A.) using purified total RNA and the Superscript Choice system (Gibco BRL, Gaithersburg, MD, U.S.A.) with a T7-(dT)₂₄ primer containing a T7 RNA polymerase promoter site (Genset, La Jolla, CA, U.S.A.). Biotinylated complementary RNA (cRNA) was made from the above cDNA using the ENZO BioArray HighYield RNA transcript labeling kit (ENZO, New York) and then fragmented to approximately 35 to 200 nucleotides according to the Affymetrix manual.

Array hybridization, washing, staining, scanning, and initial data analysis.

Fragmented biotin-cRNA (15 μg) with manufacturer-recommended controls and spikes were hybridized to an Affymetrix GeneChip *Arabidopsis* AG1 genome array (Affymetrix, Inc.) for 16 h at 45°C with constant rotation at 60 rpm. Chips were washed and stained using the Affymetrix protocol on an Affymetrix fluidics station. Chips were scanned with an HP argon-ion laser confocal microscope, with a 488-nm emission and detection at 570 nm. Raw fluorescent signals corresponding to hybridization intensities were analyzed with the Affymetrix Microarray Suite (MAS) 4.0 software (Affymetrix). The output from all the hybridizations was scaled globally (“Global Scaling”) so that its average intensity was equal to an arbitrary target intensity of 1,500 to allow direct comparison between different treatments. MAS software also was used to calculate average difference, the primary measure of mRNA abundance (Avg Diff), difference call (Diff Call), fold change, presence call, and other available parameters. Additional analyses used GeneSpring (v. 4.0.4; Silicon Genetics, Redwood City, CA, U.S.A.) and dChip (β-test version; Li and Wong 2001). The Methods Supplement published online contains further details.

RT-PCR.

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). Contaminating DNA was removed using the RNase-Free *DNase* Set (Qiagen). First-strand cDNA was synthesized from DNase-treated RNA using SuperScript III Reverse Transcriptase (Invitrogen). PCR reactions contained the first-strand cDNA template and corresponding gene-specific primer pairs 5'-ATATGCGTCACTGCACGAAG-3' and 5'-GGTAGACAGTGAGGTCATGAGCC-3' (for *PARG/At2g31865*), 5'-ATGGGTACTAGAGCTCAGCAGAT-3' and 5'-GAGAGAAGCAGAGGCTTGGTCC-3' (for *NUDT7*), 5'-GCCTCTCTATCTGGCTCTTGAC-3' and 5'-AGGGCAGCTCTCTGGTCTCGAGTCC-3' (for *TIR1*), 5'-ATGGACGAAGGACACCTAG-3' and 5'-CTTTTCTTTGATTGGATTCTG-3' (for *WRKY29*), 5'-CACATAATTCCCACGAGGATC-3' and 5'-GTAGGTGCTCTTGTTCTTCCC-3' (for *PR-1*), and 5'-AGGTTCTGTCCAGCCATC-3' and 5'-TTAGAAGCATTTCCTGTGAAC-3' (for *Actin-2*).

Knockout lines, bacterial growth assays, and HR assays.

Homozygous SALK T-DNA knockout lines for each of the selected genes were identified as described (Alonso et al. 2003). The *nudt7-1* mutant allele corresponds to SALK_04461 and *nudt7-2* mutant allele corresponds to SALK_104293. Bacterial growth within leaves was quantified 3 days after inoculation using standard procedures as described in the figure leg-

end and by Suarez-Rodriguez and associates (2007). In all but one case, leaf punches from four leaves were pooled and tested by dilution plating for each data point, with four data points per treatment in each experiment. The HR was quantified as described in the figure legend and by Suarez-Rodriguez and associates (2007).

Seedling growth inhibition assays.

Flg22-induced seedling growth inhibition assays (Gomez-Gomez et al. 1999) were performed as described (Pfund et al. 2004). Briefly, approximately 10 *Arabidopsis* seedlings per treatment were grown on 0.5× Murashige-Skoog (MS) agar media supplemented with 2% (wt/vol) sucrose and 1× Gamborg's vitamins for 5 days and then transferred to 24-well plates (1 seedling per well) containing 400 µl of liquid 0.5× MS salts, 2% (wt/vol) sucrose, and 1× Gamborg's vitamins media. Seedlings were then treated as described and fresh weight was recorded 2 weeks later.

Callose deposition.

Approximately six *Arabidopsis* seedlings per treatment were grown on 0.5× MS, 2% (wt/vol) sucrose, and 1× Gamborg's vitamins media for 7 days and then transferred to 24-well plates (one seedling per well) containing 400 µl of liquid 0.5× MS salts, 2% (wt/vol) sucrose, and 1× Gamborg's vitamins media. Seedlings were then treated as described. At 24 h after final treatment, seedlings were fixed overnight in 1% (vol/vol) glutaraldehyde, 5 mM citric acid, and 90 mM Na₂HPO₄ (pH 7.4) and then cleared and dehydrated with 100% ethanol. Callose was visualized using ultraviolet epifluorescence microscopy as described (Gomez-Gomez et al. 1999). Independent experiments were performed three times with similar results.

ACKNOWLEDGMENTS

This project was supported by National Science Foundation award number 9975971 to R. W. Michelmore and A. F. Bent and a Ruth L. Kirschstein National Research Service Award National Institutes of Health postdoctoral fellowship to L. Adams-Phillips. We thank S. Splinter (Gene Expression Center, University of Wisconsin-Madison) for performing microarray hybridizations and R. Doerge and H. Jiang for substantial contributions to the ANOVA analysis.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

University of California Davis's Functional and Comparative Genomics of Disease Resistance Gene Homologs website: niblrrs.ucdavis.edu
 ArrayExpress website: www.ebi.ac.uk/arrayexpress