Gene Expression Signatures from Three Genetically Separable Resistance Gene Signaling Pathways for Downy Mildew Resistance[1][w]

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Resistance gene-dependent disease resistance to pathogenic microorganisms is mediated by genetically separable regulatory pathways. Using the GeneChip Arabidopsis genome array, we compared the expression profiles of approximately 8,000 Arabidopsis genes following activation of three *RPP* genes directed against the pathogenic oomycete *Peronospora parasitica*. Judicious choice of *P. parasitica* isolates and loss of resistance plant mutants allowed us to compare the responses controlled by three genetically distinct resistance gene-mediated signaling pathways. We found that all three pathways can converge, leading to up-regulation of common sets of target genes. At least two temporal patterns of gene activation are triggered by two of the pathways examined. Many genes defined by their early and transient increases in expression encode proteins that execute defense biochemistry, while genes exhibiting a sustained or delayed expression increase predominantly encode putative signaling proteins. Previously defined and novel sequence motifs were found to be enriched in the promoters of genes coregulated by the local defense-signaling network. These putative promoter elements may operate downstream from signal convergence points.

Genetic screens in Arabidopsis have defined a complex network of pathways controlling local immune responses. These appear to be broadly conserved across all plants analyzed to date. Proteins encoded by disease resistance (*R*) genes mediate specific molecular recognition of pathogenic microorganisms and trigger signaling cascades that activate defense reactions (Dangl and Jones, 2001; Hammond-Kosack and Parker, 2003). Members of the largest R protein class feature nucleotide binding sites and Leu-rich repeats (NB-LRR). In dicots, NB-LRR proteins can be subdivided into those expressing either putative coiled-coil (CC) domains or a domain with homology to the cytoplasmic tail of animal signaling proteins called TIR at the N terminus.

R-mediated pathogen recognition is often associated with a localized hypersensitive cell death response (HR) of cells directly in contact with, or very near to, the invading pathogen. In Arabidopsis, mutation analysis has defined several distinct defense signaling pathways (Aarts et al., 1998; McDowell et al., 2000). Some *R* functions require accumulation of salicylic acid (SA; Klessig et al., 2000). Genetic evidence suggests that there are at least two separable *R*-dependent signaling branches. One requires EDS1 and PAD4 (Falk et al., 1999; Feys et al., 2001), proteins with limited homology to lipases, and the other requires NDR1, a protein of unknown biochemical function (Century et al., 1997). The EDS1/PAD4 signaling pathway is typically associated with TIR-NB-LRR proteins, while the NDR1 pathway is typically associated with

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CC-NB-LRR proteins. There are, however, exceptions to this generality (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001).

R functions can differ in their requirement for the genetically defined defense regulators mentioned above. For example, *RPP7* encodes a CC-NB-LRR protein that recognizes the Hiks1 isolate of the oomycete pathogen *Peronospora parasitica* (McDowell et al., 2000). *RPP7* function is SA and *PAD4* independent (McDowell et al., 2000). *RPP7* is also unaffected in transgenic plants expressing *NahG*, a bacterial gene encoding an enzyme that degrades SA (Gaffney et al., 1993; McDowell et al., 2000), *eds16/sid2* (T. Eulgem and J.L. Dangl, unpublished data), a mutant defective in the isochorismate synthase enzyme required for SA biosynthesis (Wildermuth et al., 2001), or *eds5* (J.M. McDowell, unpublished data). *RPP7* resistance does require *SGT1b*, a putative regulator of proteasome-dependent protein degradation (Austin et al., 2002; Azevedo et al., 2002; Tör et al., 2002). *RPP8* encodes a CC-NB-LRR protein that recognizes the *P. parasitica* isolate Emco5. *RPP8* is also SA independent by the criteria defined above (McDowell et al., 1998, 2000) but differs from *RPP7* because it is *SGT1b* independent. In fact, *RPP8* function is unaltered by *ndr1*, *rar1*, *sgt1b*, *eds1*, *pad4*, *sid2*, *NahG*, *npr1*, *ein2*, or *coi1* mutations (McDowell et al., 1998, 2000). *RPP4* encodes a TIR-NB-LRR protein that requires SA accumulation, PAD4, and SGT1b function. *RPP4* was reported to be weakly compromised in cotyledons of *ndr1* and *npr1* mutants (van der Biezen et al., 2002). Yet, under our experimental conditions (see ''Materials and Methods''), we did not observe any reduction of *RPP4* function in these plants (Table I; data not shown).

In addition to *R*-dependent signaling pathways that mediate rapid and strong resistance responses, plants express a basal defense that is pathogen nonspecific (Glazebrook et al., 1996, 1997a, 1997b). Basal defense limits the growth of virulent pathogens. There is genetic overlap between loci required for *R*-mediated and basal defenses, suggesting that they may share components (for review, see Glazebrook et al., 1997a). Disease resistance mediated by SA signaling can additionally require NPR1, a nuclear transported protein required for a significant portion of the overall defense gene activation during systemic acquired resistance (Kinkema et al., 2000).

Differences in global gene expression patterns between incompatible (plant resistant) and compatible (plant susceptible) interactions are quantitative and temporal rather than qualitative (Maleck et al., 2000; Tao et al., 2003). For example, approximately 30 genes were found to be induced to higher expression levels during incompatible than during compatible interactions of Arabidopsis with *P. parasitica* (Maleck et al., 2000). Thus, *R*-dependent signaling accelerates and amplifies the regulation of a large suite of defense genes that largely overlap those induced by the basal defense system. Interruption of *R* signaling by mutation should alter the expression amplitude and/or timing of these genes. Here, we characterize the transcriptional response following stimulation of three genetically separable *R*-gene pathways, comparing resistant Arabidopsis lines to isogenic lines defective in the respective pathways.

We present a comparative analysis of global gene expression patterns triggered by three different *R*-dependent defense pathways: (1) the canonical *RPP4* that is dependent on PAD4, SA accumulation, and SGT1b; (2) the *RPP7* pathway that is dependent on SGT1b but independent of PAD4 or SA accumulation; and (3) the unique *RPP8* pathway that is independent of PAD4, SA accumulation, or SGT1b. Despite the genetic disparity for signaling downstream from these recognition events, we found that all three pathways trigger up-regulation of common sets of target genes, indicating signal convergence upstream of these target genes. *RPP4* and *RPP7* trigger at least two distinct temporal patterns of gene activation, each targeting genes enriched for defined functional categories. Potential binding sites of at least three different types of transcription factors were found to be conserved in promoters of genes coregulated by the local defense-signaling network.

Table I. *Peronospora interactions examined by expression profiling*

Two-week-old seedlings were sprayed with 10^5 spores of the respective *P. parasitica* isolate/mL. The number of sporangiophores/cotyledon ($n \geq 20$) was determined 7 dpi. I, incompatible, C, compatible.

Pathway	Plant Line	*P. parasitica* Isolate	Sporangiophores/ Cotyledon	Interaction
RPP4	Col-0 (*RPP4*)	Emoy2	0.2 ± 0.1	I
RPP4	*ndr1-1*	Emoy2	0.5 ± 0.3	I
RPP4	*npr1-1*	Emoy2	0.1 ± 0.1	I
RPP4	*pad4-1*	Emoy2	14.3 ± 2.2	C
RPP4	*NahG*	Emoy2	7.1 ± 1.4	C
RPP7	Col-0 (*RPP7*)	Hiks1	0.0 ± 0.0	I
RPP7	*rpp7-3*	Hiks1	16.0 ± 1.3	C
RPP7	*sgt1b*	Hiks1	13.2 ± 1.3	C
RPP8	Col-0::*RPP8*	Emco5	0.06 ± 0.04	I
RPP8	Col-0 (*rpp8*)	Emco5	15.9 ± 1.0	C

RESULTS

Definition of Gene Sets Controlled by *RPP4*, RPP7, or *RPP8*

We sought to define sets of genes controlled by three genetically separable defense signaling pathways. We infected wild-type and mutant plants disrupted in *RPP4*-, *RPP7*-, or *RPP8*-mediated resistance with the *P. parasitica* isolates that trigger each of the respective *R* genes (Table I). *RPP8* was originally cloned from accession Landsberg *erecta*. The *rpp8* allele in Columbia (Col-0) does not recognize any known pathogen (McDowell et al., 1998). Because the majority of relevant signaling mutants were derived in Col-0 and

because there is a weak second resistance locus in Landsberg *erecta* against *P. parasitica* Emco5, we used an *RPP8* transgene under the control of its own promoter in Col-0 for these experiments.

Because the *P. parasitica* infection process is asynchronous and because the timing to HR and cessation of pathogen growth is slightly different for each resistance response we assayed, we chose time points based on microscopic observations for each *RPP* gene. For example, infection with *P. parasitica* Emoy2 and *P. parasitica* Hiks1 results in hyphal growth by 48 h postinfection (hpi) during compatible interactions, while *RPP4*- and *RPP7*-dependent HR are clearly visible at this time point during incompatible interactions (Fig. 1). The timing of *P. parasitica* Emco5 infections differs substantially from that of *P. parasitica* Hiks1 and *P. parasitica* Emoy2 infections. *P. parasitica* Emco5 spore germination and hyphal growth are already visible during compatible interactions, at 12 hpi following *P. parasitica* Emco5 infection (Fig. 1), and *RPP8*-mediated HR is clearly detectable at this time point during incompatible interactions (Fig. 1). Thus, regulatory events and physiological responses responsible for the differences between resistant and susceptible outcomes must occur within the first 48 hpi, but certainly occur on different time scales for each interaction. We therefore determined RNA profiles of all plant lines listed in Table I at 0, 12, or 48 hpi with the respective *P. parasitica* isolates using Affymetrix Arabidopsis genome arrays representing one-third of the Arabidopsis genome.

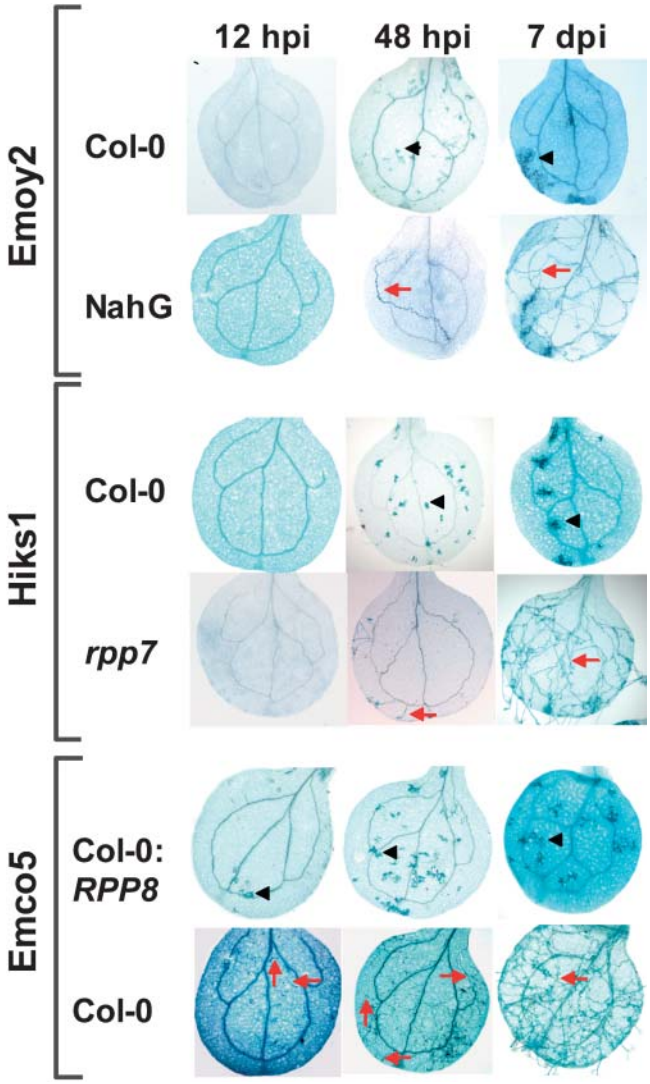

Figure 1. Temporal progression of *P. parasitica* infection. Trypan blue-stained cotyledons of 2-week-old wild-type, mutant, or transgenic seedlings after infection with *P. parasitica* isolates Emoy2, Hiks1, or Emco5 at the indicated time points. dpi, days postinfection. Trypan blue stains HR sites (orange arrowheads) and *P. parasitica* hyphae (red arrows) dark blue.

For each experimental condition, we performed three independent biological repetitions on approximately 50 seedlings per genotype per repetition and pooled equal portions of the corresponding total RNA preparations. Hence, the data from each chip reflect the average of three independent biological experiments covering approximately 150 plants and thousands of interaction sites. Expression data generated for the analysis of each *RPP* signaling pathway were examined separately.

As inclusion criterion for further analysis, we demanded that a given probe set (oligonucleotide probes representing a defined gene) display at least two independent ≥2.5-fold expression differences within the experiments for each *RPP* signaling pathway. These conservative inclusion criteria will underestimate the total number of genes responding to each *P. parasitica* infection and will miss expression changes unique to one treatment at one time point. But this treatment will ensure that the included genes are likely to be true positives (Maleck et al., 2000). We selected 88 genes that meet these criteria in comparisons of Col-0 wild-type plants with isogenic *ndr1.1*, *npr1.1*, *pad4.1*, or *NahG* at 0, 12, or 48 hpi with *P. parasitica* Emoy2 (the *RPP4* set; Supplemental Table Ia). Similarly, 72 genes were selected that exhibit at least two ≥2.5-fold expression differences between Col-0 wild-type plants and *rpp7-3* or sgt*1b* at 0, 12, or 48 hpi with *P. parasitica* Hiks1 (the *RPP7* set; Supplemental Table Ib). Finally, 182 genes were selected displaying at least one 2.5-fold expression difference between a transgenic Col-0:*RPP8* line (McDowell et al., 1998) and Col-0 plants at 0, 12, or 48 hpi with *P. parasitica* Emco5 (the *RPP8* set; Supplemental Table Ic). For this last example, the lack of additional loss of resistance Col-0 mutants altering *RPP8* function forced us to adopt this simpler inclusion regimen.

The asynchronous nature of *P. parasitica* infection probably strengthens the robustness of inclusion for those genes that are in our data set. Pathogen-induced gene expression changes are most dramatic in plant tissue directly in and around the infection site (Schmelzer et al., 1989; Eulgem et al., 1999; Kirsch et al., 2001; Rushton et al., 2002). Because we extracted

RNA from homogenized aerial seedling tissues, the expression changes detected in our experiments constitute the average of infected and uninfected cells in the aerial tissues at the time point of harvest. As depicted in Figure 1, most plant cells are not in contact with pathogen structures and may not respond. Thus, our observed expression changes must underestimate the expression changes in and near infection sites that one can achieve with uniform stimuli. However, the selection criteria for the inclusion of genes in our data sets were as stringent as (and therefore more conservative than) those typically applied in experiments where more uniform responses are achieved throughout entire plants (e.g. after stimulation with chemicals, abiotic stress, or high titer bacterial infections; Maleck et al., 2000; Kreps et al., 2002; Glazebrook et al., 2003; Tao et al., 2003).

The three datasets defined above (Supplemental Table I) were separately subjected to hierarchical clustering, using average linking (Eisen et al., 1998). The resulting clustergrams defined two categories of genes controlled by each *RPP* pathway: (1) genes showing elevated expression during all incompatible interactions relative to all compatible interactions at one or more time points (*RPP4*, *RPP7*, or *RPP8* elevated; Supplemental Table II), and (2) genes showing reduced expression during all incompatible interactions relative to all compatible interactions at one or more time point (*RPP4*, *RPP7*, or *RPP8* reduced; Supplemental Table II). These genes are controlled by the respective *RPP* pathways since genetic disruption (or absence) of each pathway alters their expression characteristics.

Importantly, the expression levels for these genes are typically not altered in the mutants that affect another of the three *RPP* pathways assayed. For example, *RPP4* elevated genes are consistently more strongly expressed following infection of *P. parasitica* Emoy2 resistant Col-0 plants than in *P. parasitica* Emoy2 susceptible *pad4* mutants or *NahG* plants, but their expression is not altered following infection of mutants like *ndr1* or *npr1* that are irrelevant for *RPP4* function in our tissues. This strict correlation strengthens their definition as *RPP4* controlled genes.

The *RPP* response clustergrams in Figure 2 reveal several interesting characteristics of *RPP4*, *RPP7*, or *RPP8* elevated genes. Engagement of any of the three *R* genes induces elevated expression of target genes at 12 and/or 48 hpi (represented in Fig. 2 by red signal in 12 and 48 hpi columns). The *pad4* mutation and *NahG* transgene have a more pronounced effect on *RPP4* elevated genes at 48 hpi, while the *rpp7* and *sgt1b* mutations predominantly affect *RPP7* elevated genes by 12 hpi. The absence of *RPP8* in Col-0 plants mainly affects *RPP8* elevated genes by 12 hpi.

This general trend may reflect PAD4 and SA action in *RPP4* signaling at a later stage than SGT1b, RPP7, and RPP8 in their respective regulatory cascades. This is consistent with genetic and biochemical analyses of *pad4* and *sgt1b* with respect to other defense signaling events (Rusterucci et al., 2001; Aviv et al., 2002; Tör et al., 2002). Expression of the *NahG* transgene clearly has a more profound effect on *RPP4* elevated genes than the *pad4* mutation, as it more efficiently blocks *P. parasitica*-dependent elevated expression at 12 hpi. Yet by 48 hpi expression profiles of *pad4* and *NahG* plants are almost identical. This may suggest the existence of early signaling events that are independent of *PAD4* but affected by *NahG*.

Interestingly, each of the *RPP* response pathways appears to influence transcriptional response leading to elevated target gene expression in uninfected plants. A significant number of genes are differentially expressed in defense-compromised backgrounds prior to infection, compared to resistant lines (see 0 hpi columns of Col-0/*pad4* and Col-0/*NahG* [Fig. 2A], Col-0/*rpp7* and Col-0/*sgt1b* [Fig. 2B], and Col-0:*RPP8*/Col-0 [Fig. 2C]). Comparisons of Col-0 to Col-0:*RPP8* are particularly striking in this regard. These results suggest that NB-LRR proteins might have a constitutive regulatory activity. Another, less likely possibility is that the *RPP8* transgene insertion causes these transcriptional changes.

NPR1 is a key regulator of systemic disease resistance (Cao et al., 1997; Ryals et al., 1997; Dong et al., 2001; Mou et al., 2003). We previously demonstrated that its importance in systemic acquired resistance is reflected by its regulatory effect on a large number of defense-related genes (Maleck et al., 2000). NPR1 is not necessary for resistance to *P. parasitica* Emoy2 and *RPP4*-dependent up-regulation of defense genes (Table I). We nevertheless observed that the majority of genes activated by *RPP4* exhibited elevated expression levels in *npr1-1* before infection (Fig. 2A). One possible explanation for this may be that NPR1 acts as a negative regulator of some defense genes. This is consistent with recent reports on repression of some defense responses by NPR1 or *NPR1*-dependent TGA transcription factors (Spoel et al., 2003; Zhang et al., 2003). Derepression of such genes in *npr1-1* may partly be responsible for elevated *P. parasitica* Emoy2 resistance in this mutant as compared to other mutants with defects in SA signaling, such as *pad4*.

RPP4 and *RPP7* Simultaneously Trigger Two Distinct Temporal Patterns of Gene Expression

To examine the kinetic behavior of *RPP4*, *RPP7*, and *RPP8* elevated genes defined in Figure 2 in detail, we further categorized them using *k*-means clustering (GeneSpring, Silicon Genetics; see "Materials and Methods"). *k*-means clustering is a nonhierarchical clustering algorithm that assigns each gene to one of a user-defined number of clusters based on its distance to the centroid of each cluster (Knudsen, 2002). We used this method because it allowed us define common qualitative patterns in gene expression changes over time. Normalized mRNA levels (not expression ratios) detected by probe sets representing 54 *RPP4*, 50

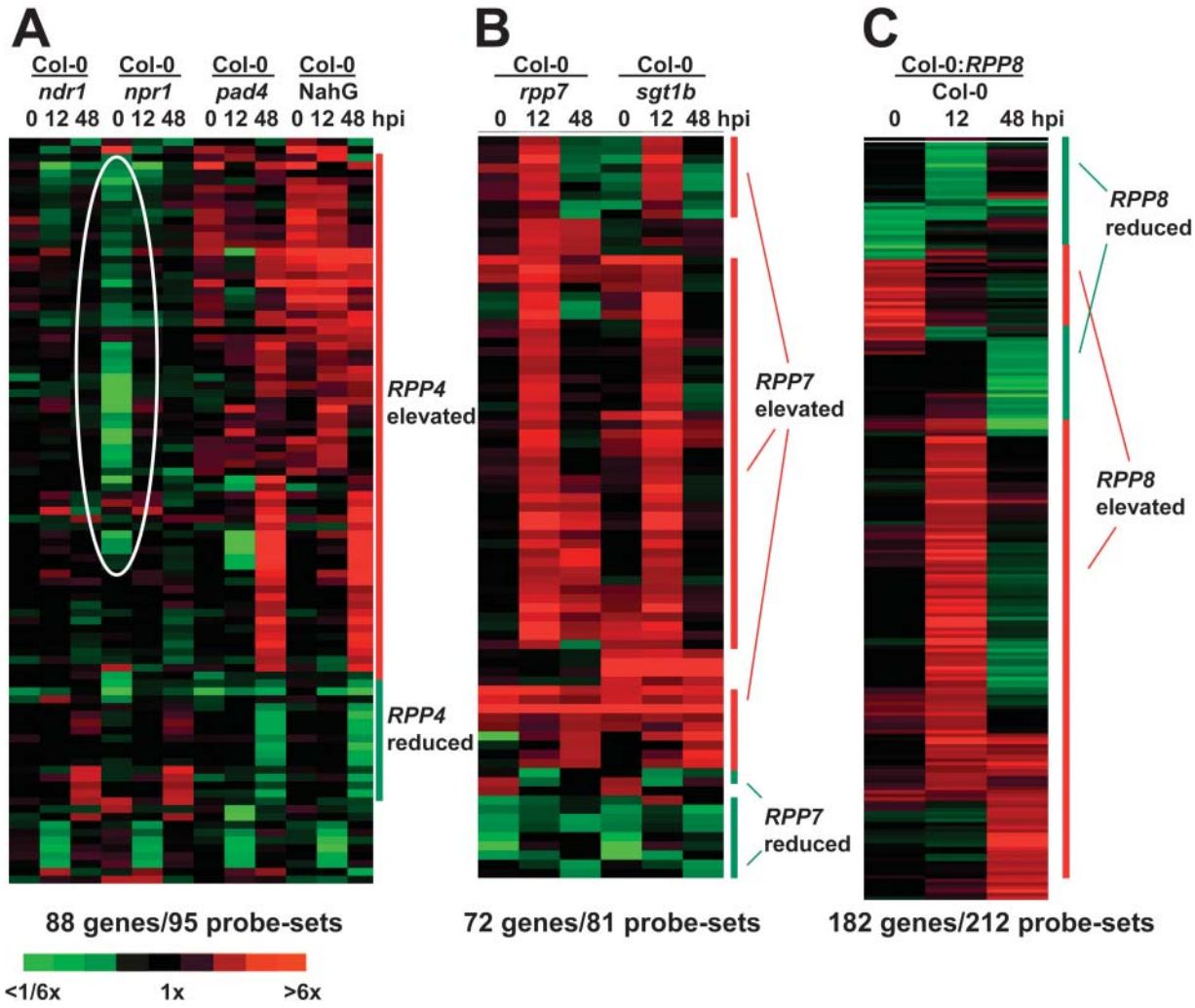

Figure 2. Profiling of *RPP4*-, *RPP7*-, and *RPP8*-mediated gene expression responses defines sets of *R*-associated genes. Hierarchical clustergrams with 88 genes (A; represented by 95 probe sets) that show at least two ≥2.5-fold expression differences between Col-0 and *ndr1*, *npr1*, *pad4*, or *NahG* after inoculation with *P. parasitica* Emoy2, 72 genes (B; represented by 81 probe sets) that show at least two ≥2.5-fold expression differences between Col-0 and *rpp7* or *sgt1b* mutants after inoculation with *P. parasitica* Hiks1, or 182 genes (C; represented by 212 probe sets) that show at least one ≥2.5-fold expression difference between Col-0:*RPP8* and Col-0 after inoculation with *P. parasitica* Emco5. Expression ratios are displayed at the indicated time points postinfection; red, positive ratios; green, negative ratios. As illustrated by the color bar in the lower left corner, the brightest color intensity represents a ≥6-fold expression difference. Clusters defining genes more strongly expressed in resistant as compared to susceptible plants (elevated) and genes less strongly expressed in resistant as compared to susceptible plants (reduced) are marked by red and green bars, respectively. Signals suggesting derepression of defense genes in *npr1* are encircled. Signal intensities of all genes represented in this figure are listed in Supplemental Table I. In addition, signal intensities of *RPP4*, *RPP7*, or *RPP8* elevated and reduced genes are listed separately in Supplemental Table II.

RPP7, or 135 *RPP8* elevated genes were separately clustered into three sets of *k*-means clusters based on their temporal pattern of expression (Supplemental Fig. 1). We defined a set of genes that display a pronounced mRNA increase between 0 and 12 hpi, after which the expression levels decline or remain constant (early and transient; Table II). By contrast, we found a second set of genes that exhibit a substantial expression increase between 12 and 48 hpi (late or sustained; Table II). In both cases, the timing or amplitude of the expression response is altered in the appropriate susceptible mutant plants (Supplemental Table III). While we appreciate the limited utility of defining temporal patterns of gene regulation based on a two time points, we use these terms to group genes roughly according to apparently shared temporal expression patterns. These groupings proved useful in the subsequent definition of both common putative regulatory elements and common putative cellular functions (see below).

Table II. *Early/transient or late/sustained up-regulation genes controlled by RPP4 or RPP7*

Genes showing an early/transient or late/sustained up-regulation by *RPP4* and/or *RPP7* as defined in Supplemental Figure 1. Some of these genes are also controlled by *RPP8*. Only probe sets that could be clearly assigned to Arabidopsis Genome Initiative (AGI) numbers were included in this table. Gene Ontology (GO) annotations were retrieved from TAIR. We selected for each gene the GO annotation most informative to describe its molecular function and manually assigned it to one of three categories: (1) unclear, (2) defense execution or metabolism, and (3) signaling/transcription. *, *RPP* genes triggering elevated expression.

AGI No.	Probe Set IDs	Gene Product	GO Annotations	Functional Category	*RPP**
		Genes Showing Early/Transient Up-Regulation			
At1g65970	15116_f_at	Peroxiredoxin TPx2	Antioxidant activity	Defense/metabolism	7, 8
At2g15390	12642_at	Putative xyloglucan fucosyltransferase	Fucosyltransferase activity	Defense/metabolism	4
At2g29460	19640_at	Putative glutathione S-transferase	Glutathione transferase activity	Defense/metabolism	7, 8
At2g43510	19171_at	Putative trypsin inhibitor	Trypsin inhibitor activity	Defense/metabolism	7, 8
At2g43620	18928_at	Putative endochitinase	Chitinase activity	Defense/metabolism	4, 7
At2g45220	20269_at	Putative pectinesterase	Pectinesterase activity	Defense/metabolism	4, 7, 8
At3g26830	14248_at	PAD3, cytochrome P450 emb|CAA50677.1	Indole phytoalexin biosynthesis	Defense/metabolism	4, 7, 8
At3g49120	14638_s_at	Peroxidase	Peroxidase activity	Defense/metabolism	4, 8
At3g54640	17487_s_at, 14672_s_at	Trp synthase α-chain	Trp synthase activity	Defense/metabolism	4, 8
At5g05730	20291_s_at, 12889_s_at	Anthranilate synthase alpha subunit	Anthranilate synthase activity	Defense/metabolism	4
At5g39580	18946_at	Peroxidase ATP24a	Peroxidase activity	Defense/metabolism	4, 8
At5g57550	18968_at, 18969_g_at	Endoxyloglucan transferase	Xyloglucan:xyloglucosyl transferase activity	Defense/metabolism	7, 8
At5g64120	17413_s_at	Peroxidase	Peroxidase activity	Defense/metabolism	4
At3g50770	13217_s_at, 17500_s_at	Calmodulin-like protein	Calcium ion binding	Signaling/transcription	7
At4g17500	12904_s_at, 16063_s_at	EREBP 1	Transcription factor activity	Signaling/transcription	4, 7
At4g33050	19182_at	Protein with calmodulin binding motif	Calmodulin binding	Signaling/transcription	4, 7
At4g36990	16105_s_at	Heat shock transcription factor 4	Transcription factor activity	Signaling/transcription	7, 8
At4g01870	13656_at	Protein of unknown function	Molecular function unknown	Unclear	7
At1g17740	15629_s_at	Phosphoglycerate dehydrogenase	Molecular function unknown	Unclear	4
AT1G27020	18235_at	Unknown	Molecular function unknown	Unclear	4
At2g30140	14614_at	Putative glucosyltransferase	UDP-glycosyltransferase activity	Unclear	4, 8
At2g38860	18255_at, 15866_s_at	Similarity of pfpI-like protein (protease)	Molecular function unknown	Unclear	4
At4g12480	16150_s_at	pEARLI 1	Lipid transport	Unclear	7
At4g15610	17899_at	Unknown	Molecular function unknown	Unclear	7, 8
At4g17470	13949_s_at	Thioesterase like protein	Palmitoyl-hydrolase activity	Unclear	7
At5g13490	15978_at	Adenosine nucleotide translocator	ATP:ADP antiporter activity	Unclear	4
At5g24780	15125_f_at	Vegetative storage protein, VSP1	Acid phosphatase activity	Unclear	7, 8
		Genes Showing Late/Sustained Up-Regulation			
At1g05300	19718_at	Putative Fe(II) transport protein	Cation transporter activity	Defense/metabolism	4
At1g75040	16153_s_at, 14636_s_at	Thaumatin-like protein	PR5	Defense/metabolism	4
At2g14610	14635_s_at, 17128_s_at	PR-1-like protein	Molecular function unknown	Defense/metabolism	4, 8
At4g08870	17187_at	Arginase	Hydrolase activity	Defense/metabolism	7, 8

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

AGI No.	Probe Set IDs	Gene Product	GO Annotations	Functional Category	*RPP**
At5g42980	13189_s_at	Thioredoxin	Thiol-disulfide exchange intermediate activity	Defense/metabolism	7, 8
At5g64120	17413_s_at	Peroxidase	Response to oxidative stress	Defense/metabolism	7 (4 early up)
At1g21250	15616_s_at	wall-associated kinase 1	Kinase activity	Defense/metabolism	4, 7, 8
At1g28370	14232_at	ERF11	Transcription factor activity	Signaling/transcription	7
At1g33960	12879_s_at, 17544_s_at	AIG1	Nucleotide binding (GO:0000166)	Signaling/transcription	7, 8
At1g68050	14196_at	F-box protein FKF1/ADO3, AtFBX2a	Ubiquitin-protein ligase activity	Signaling/transcription	4
At1g69490	18590_at	NAC domain transcription factor	Transcription factor activity	Signaling/transcription	7
At1g72930	18003_at	Toll/interleukin-1 receptor-like protein	Signal transduction (GO:0007165)	Signaling/transcription	4
At2g40080	18272_at	Similar to RNA polymerase subunit PB2	Positive regulation of circadian rhythm	Signaling/transcription	4
At2g41090	17917_s_at	Calcium binding protein (CaBP-22)	Calcium ion binding	Signaling/transcription	4, 8
At2g46430	17499_s_at	Cyclic nucleotide gated channel, CNGC3	Ion channel activity/ calmodulin binding	Signaling/transcription	4, 7, 8
At3g56710	14148_at	Sigma factor A binding protein	Protein binding	Signaling/transcription	4, 7
At4g11280	12891_at, 12892_g_at, 16817_s_at	ACC synthase 6	Ethylene biosynthesis/ response to external stimulus	Signaling/transcription	7, 8
At4g21380	16360_at	Receptor-like Ser/Thr protein kinase ARK3	Kinase activity/receptor activity	Signaling/transcription	4
At5g04340	15665_s_at	Putative c2h2 zinc finger transcription factor	Transcription factor activity	Signaling/transcription	7
At5g52310	15611_s_at	Similar to RNA polymerase subunit PB2	Response to abiotic stimulus	Signaling/transcription	4
At1g31580	16439_at	Transmembrane protein with similarity to CD8 C	Response to biotic stimulus	Unclear	4
At1g76960	14096_at	Putative transmembrane protein	Molecular function unknown	Unclear	4, 8
At2g14560	14704_s_at, 15846_at, 15847_g_at	Unknown	Molecular function unknown	Unclear	4, 7, 8
At3g22240	14691_at, 14709_at	Unknown	Molecular function unknown	Unclear	7, 8
At4g14400	20429_s_at	Transmembrane protein with ankyrin repeats	Protein binding	Unclear	4, 7, 8
At4g35480	17047_s_at	RING-H2 finger protein RHA3b	Molecular function unknown	Unclear	4
At5g10760	14145_at	CND41, chloroplast nucleoid DNA binding protein	Proteolysis and peptidolysis	Unclear	4

RPP4 and *RPP7* Early/Transient Genes Predominantly Encode Proteins That Execute Defense Reactions

The majority of both *RPP4* and *RPP7* early/transient genes encode proteins putatively involved in metabolic processes (approximately 75% of all genes that were assigned to the classes defense/metabolism or signaling/transcription in Table II), many of which are typically associated with defense. Genes with predicted signal transduction or gene regulation functions are much less represented (approximately 20%). Several *RPP4* and *RPP7* early/transient genes encode cell wall modifying enzymes such as pectin esterase and endoxyloglucan transferase, as well as peroxidases whose enhanced expression may be related to oxidative cross-linking of cell wall components.

Plant defense responses can involve synthesis of phytoalexins, secondary metabolites with potential anti-microbial function. In Arabidopsis, the indole-derivate camalexin that accumulates during pathogen infections can act as a phytoalexin in vitro (Glazebrook and Ausubel, 1994; Rogers et al., 1996; Slusarenko and Schlaich, 2003). Biosynthesis of camalexin requires the cytochrome P450 monooxygenase CYP71B15

encoded by *PAD3* (Zhou et al., 1999). Radiolabeled tracer experiments suggested that anthranilate as well as indole are precursors of camalexin (Zook and Hammerschmidt, 1997; Zook et al., 1998), and it was speculated that camalexin synthesis may involve a Trp synthase α-chain as well as the *PAD3* product converting shikimate pathway derived indole-3-glycerolphosphate via indole to camalexin (Zook et al., 1998; Zhou et al., 1999). A Trp synthase α-chain gene (*TSA1*) and *PAD3* exhibit pronounced *RPP4*- and *RPP7*-mediated early/transient up-regulation. The standard correlation of the *TSA1* expression profile to that of *PAD3* over all *P. parasitica* treatments is 0.89. A gene encoding an anthranilate synthase α-subunit that catalyzes a step in the shikimate pathway is also coregulated with *TSA1* and *PAD3* (standard correlation to *PAD3* profile over all *P. parasitica* treatments = 0.82). *PAD3* is required for *RPP4* function, while *RPP7* function is only modestly reduced in a *pad3/pad1* double mutant (Glazebrook et al., 1997). Hence, this early/transient pattern of gene activation initiated by *RPP4* and *RPP7* consists of genes whose functions are potentially involved in stopping pathogen growth. Some of these genes are also up-regulated by *RPP8* (Table II), providing additional support for their potential importance in disease resistance against *P. parasitica*.

RPP4 and *RPP7* Late/Sustained Genes Predominantly Encode Putative Regulatory Proteins

Surprisingly, the majority of both *RPP4* and *RPP7* late/sustained up-regulated genes (approximately 75% of all genes that were assigned to the classes defense/metabolism or signaling/transcription in Table II) appear to be involved in signaling or gene regulation, whereas genes putatively involved in metabolism are much less represented in this set (Table II). Several members of this category have Ca^{2+}-binding motifs and may therefore act downstream from cellular Ca^{2+} fluxes. A large body of evidence points to a role of Ca^{2+} fluxes in defense signaling (Jabs et al., 1997; Zimmermann et al., 1997; Kim et al., 2002), but direct genetic evidence for a contribution of Ca^{2+} fluxes to disease resistance is still lacking. Five of 27 late/sustained genes are also constitutively expressed in the presence of *RPP8* (At2g46430, At4g14400, At1g21250, At2g14560, and At2g41090 show at least 2.5 times higher expression levels in Col-0:*RPP8* as compared to Col-0). Increased expression of these five genes is strictly associated with disease resistance mediated by three different *RPP* genes and may therefore control processes executing shared defense functions (see ''Discussion'').

The *RPP4*, *RPP7*, and *RPP8* Pathways Converge in the Up-Regulation of Overlapping Sets of Genes

To uncover commonalities among the responses triggered by the three pathways examined, we performed hierarchical clustering combining all experimental conditions represented by the 21 expression ratios in Figure 2, A to C. Hierarchical clustering was performed in two dimensions (dimensions of genes and experimental conditions) with 419 genes (549 probe sets) that show at least one 2.5-fold expression difference over all 21 comparisons (Fig. 3; Supplemental Table IV). Requiring only one 2.5-fold expression difference for inclusion in this analysis allows the broadest comparisons but at a probable cost to the robustness of any gene expression ratio change that occurs only once in Figure 3. Our goal, however, was to identify common patterns, not to ascribe meaning to expression changes of single genes in single treatments.

Clustering in the dimension of experimental conditions clearly separated treatments into two sets, A and B (defined by the first node of the dendogram in Fig. 3). Strikingly, the expression profiles associated with conditions that appear to have a strong impact on *RPP4*-, *RPP7*-, or *RPP8*-dependent increases in gene expression occur within set A (*pad4* 48 hpi and *NahG* 12 and 48 hpi infected with *P. parasitica* Emoy2; *rpp7* 12 hpi and *sgt1b* 12 hpi with *P. parasitica* Hiks1 as well as Col-0 12 hpi with *P. parasitica* Emco5; asterisks in Fig. 3, top). These six key conditions affect two largely overlapping sets of genes, clusters I and II, defined by distinct nodes in the dendogram of genes (Fig. 3, marked by red bars; Supplemental Table V). Many genes within these two clusters are commonly up-regulated by two or all three of the examined pathways, strongly supporting convergence of *RPP4*-, *RPP7*-, and *RPP8*-dependent signaling.

Interestingly, a large number of these genes are also affected simply by the presence of intact *RPP4*, *RPP7*, or *RPP8* signaling pathways in uninfected tissue, and conditions that define this constitutive activity (all the 0 hpi ratios) also cocluster within set A (Fig. 3). Furthermore, the vast majority of genes controlled by *RPP4*, *RPP7*, and *RPP8* exhibit elevated expression in *npr1-1* plants (green signal in Col-0/*npr1* 0 hpi column) and may be controlled by NPR1-dependent repression. Hence, *P. parasitica*-induced elevated expression (at 12 and/or 48 hpi) correlates with derepression in *npr1*. In support of these findings, 67% of our cluster I and II genes are included in the set defined by Tao et al. (2003) to be differentially expressed following infection with *Pseudomonas syringae*. These genes typically exhibited elevated expression during incompatible interactions involving *avrB/RPM1* and *avrRpt2/RPS2* interactions in that dataset (compared to compatible interactions; data not shown).

There are also some displacements between the responses of mutations that affect a particular *RPP* response and the experimental conditions dendogram in Figure 3. For example, as described above, *NahG* blocks *RPP4*-triggered gene expression more efficiently than *pad4* at 12 hpi. Perhaps reflecting this, these two conditions are separated by the first node in the dendogram. Alternatively, this separation may

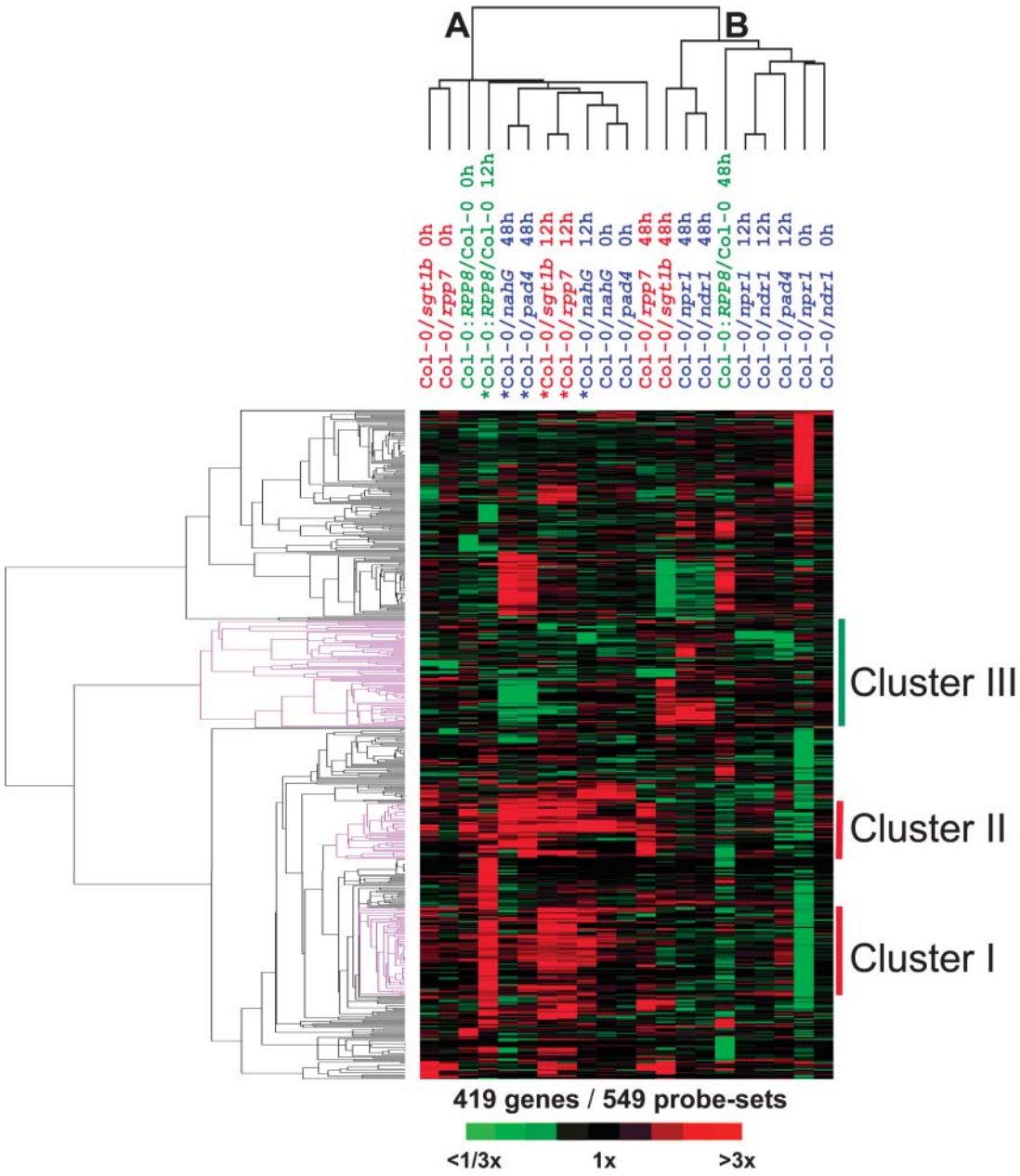

Figure 3. *RPP4*, *RPP7*, and *RPP8* control common sets of target genes. Hierarchical clustering in gene and treatment dimensions with 549 probe sets representing 419 genes that show at least one 2.5-fold expression difference over all displayed 21 experimental comparisons. Maximal color intensity represents a 3-fold or higher expression difference. Treatments of the *RPP4* set (*P. parasitica* Emoy2 infections) are in blue; treatments of the *RPP7* set (*P. parasitica* Hiks1 infections) are in red; and treatments of the *RPP8* set (*P. parasitica* Emco5 infections) are in green. Two clusters comprising genes that are commonly up-regulated by *RPP4*, *RPP7*, and/or *RPP8* signaling activities are marked by red bars (clusters I and II). A cluster of genes showing reduced expression in resistant plant lines is marked by a green bar (cluster III). The dendogram above the clustergram represents the relatedness of the overall expression pattern between the different experimental conditions. The shorter the branches that connect two given conditions, the more closely related are the expression profiles associated with them. Experimental conditions that have the strongest impact on *RPP4*, *RPP7*, or *RPP8* elevated genes (*pad4* 48 hpi, *NahG* 12 hpi and 48 hpi, *rpp7* 12 hpi, *sgt1b* 12 hpi, and Col-0 [*rpp8*] 12 hpi) are labeled with asterisks. The dendogram to the left of the clustergram represents the relatedness of expression patterns of individual genes and gene clusters. Branches corresponding to clusters I, II, and III are highlighted in light purple. Some higher order branches were cut off to reduce complexity of the figure. Signal intensities for treatments and probe sets represented in this figure are listed in Supplemental Table IV. In addition, data for all cluster I, II, and III genes are separately listed in Supplemental Table V.

reflect the recently described pleiotropy of *NahG* (Heck et al., 2003; van Wees and Glazebrook, 2003). Expression profiles of *rpp7* and *sgt1b* at 48 hpi are also separated by this node. It is clear that *sgt1b* affects expression of a larger set of genes than *rpp7* (e.g. genes within cluster IIII), consistent with recent reports of a broad role in cellular signaling for Sgt1b (Gray et al., 2003).

Cluster III comprises 79 genes exhibiting reduced expression levels in plants with intact *RPP4*, *RPP7*, or *RPP8* signaling (Fig. 3; Supplemental Table V). Expression of these genes is elevated in Arabidopsis lines defective in the respective pathways. This effect is most pronounced in *NahG* and *pad4* at 48 hpi following *P. parasitica* Emoy2 infection, but members of this cluster show the same trend in the other set A conditions. These genes may be commonly down-regulated by all three defense pathways. Alternatively, their expression may be directly or indirectly induced by the growth of *P. parasitica* in the susceptible plant lines. Several genes involved in photosynthesis and primary metabolism are present in this set (Supplemental Table V).

Figure 4 shows temporal expression profiles of cluster I and II genes from Figure 3. Expression levels (not ratios) normalized to a median of 1 over all tested conditions and time points are presented. Members of cluster I (59 genes, 64 probe sets) predominantly exhibit a pattern of *RPP4*, *RPP7*, and *RPP8* early/transient up-regulation. Most *RPP4* (14 of 16) and *RPP7* (9 of 16) early/transient genes as defined in Supplemental Figure 1 are included in cluster I. Genes in cluster II (38 genes, 46 probe sets) predominantly exhibit a pattern of *RPP4*- and *RPP7*-triggered late/sustained up-regulation, and most *RPP4* (14 of 18) and *RPP7* (10 of 14) late/sustained genes as defined in Supplemental Figure 1 are included in cluster II (Supplemental Table II). Although the timing of *P. parasitica* Emco5 infections is different from that of *P. parasitica* Emoy2 and *P. parasitica* Hiks1, some members of cluster II show also a late/sustained pattern after triggering of the *RPP8* pathway.

Our combined analysis of all three *RPP* signaling pathways identified two gene sets, illustrated as clusters I and II in Figures 3 and 4, that are commonly targeted by the *RPP4*, *RPP7*, and *RPP8* pathways. Members of each of these two clusters exhibit a defined pattern of temporal expression, early/transient or late/sustained, again indicating that the local defense pathways we examined converge upstream of two distinct temporal patterns of defense-associated gene expression.

Several Genes Tightly Coregulated with *PAD4* Encode Signaling Proteins

PAD4 is a regulator of SA biosynthesis (Zhou et al., 1998; Jirage et al., 1999). Thus, one could expect a subset of *RPP4*-controlled genes that are up-regulated downstream of PAD4 but upstream or independent of SA accumulation. These should be affected in their *RPP4*-triggered expression in *pad4-1* plants but not or less affected in *NahG*. Interestingly, the *PAD4* gene itself shows this type of expression pattern (Fig. 5; normalized mRNA levels; not expression ratios). *PAD4* autoregulation has been reported before (Jirage et al., 1999). The *pad4-1* mutation is a single nucleotide exchange in the coding region leading to a functionally compromised protein but not a shortened transcript (Jirage et al., 1999). The probe sets representing *PAD4* on the chip we used (14249_I_at and 14250_r_at) detect the 3′ end of the *PAD4* transcript, which appears not to be altered in *pad4-1*. *PAD4* exhibits a strong and sustained up-regulation in Col-0, *ndr1*, *npr1*, and *NahG* (Fig. 5). In *pad4-1* plants, there is an early expression increase, but the sustained expression is blocked. Interestingly, we defined a set of seven *PAD4* coregulated genes (correlation coefficient $\geq$0.85; Fig. 5). *EDS1*, encoding a defense signaling component that acts in the same pathways as PAD4 (Feys et al., 2001), is one of them. Its sustained up-regulation is blocked in *pad4* but not in *NahG*. Both *EDS1* and *PAD4* encode putative lipases that were shown to physically interact (Feys et al., 2001). Strict coregulation of a variety of other genes encoding interacting signaling proteins has been shown before (Cooper et al., 2003) and appears to be a common principle in signal transduction processes (Marcotte et al., 1999). Some of the other genes coregulated with *PAD4* encode proteins with putative signaling functions (Fig. 5) that we predict will participate in *EDS1*/*PAD4*-dependent regulatory processes.

Sets of Coregulated *RPP4*, *RPP7*, and *RPP8* Target Genes Contain Known and Novel Conserved Promoter Motifs

The high degree of coregulation of early/transient and late/sustained genes suggests common regulatory mechanisms for each of these gene sets. We used the Gibbs Sampling algorithm AlignACE (Hughes et al., 2000) to search for conserved sequence motifs in the promoters of cluster I (early/transient) and cluster II (late/sustained) genes defined in Figure 3 and 4. Functional cis-elements on plant promoters are typically found within the first 1 kb upstream from the translation start site (Rombauts et al., 2003), and we previously used this cutoff to identify cis-elements enriched in pathogen coregulated gene clusters (Maleck et al., 2000). Therefore, we downloaded 1 kb upstream from the inferred translational start site for each gene on this chip (The Arabidopsis Information Resource [TAIR], http://www.arabidopsis.org/tools/bulk/sequences/index.html). Since most known cis-elements consist of 6 to 12 bp, each promoter set was subjected to a series of AlignACE runs varying the parameter width from 12 down to 6. Based on the frequencies of potentially conserved motifs in 1 kb upstream sequences from all Arabidopsis genes as a reference, we calculated *P* values using the Poisson

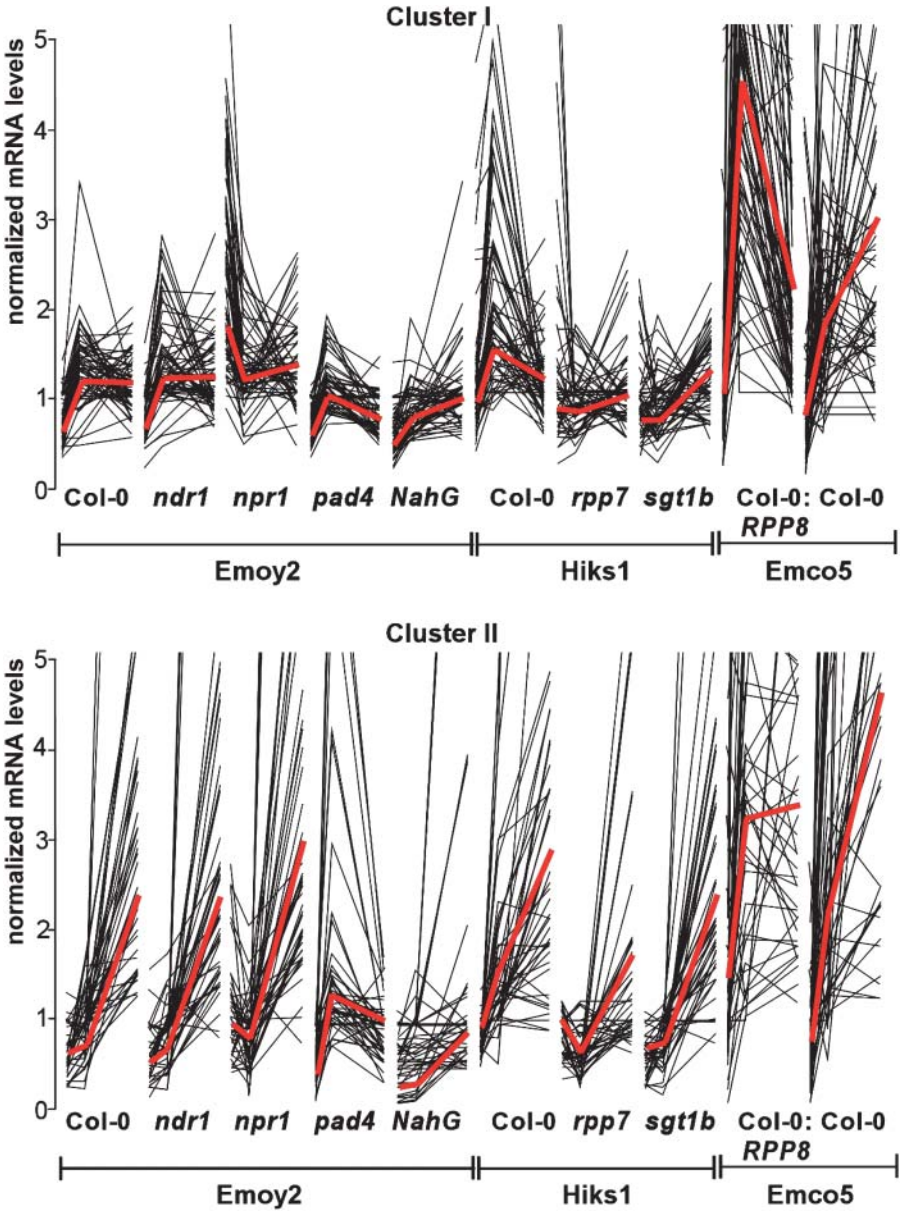

Figure 4. Temporal expression profiles of cluster I and II genes. Normalized mRNA levels (not ratios) for cluster I and II genes as defined in Figure 3 at 0, 12, or 48 hpi with the respective *P. parasitica* isolate. Highlighted in red is the weighted average of each gene set (weighted by a control factor for each gene; see "Materials and Methods").

distribution to describe the likelihood of the observed sequence conservation occurring by chance.

To examine specifically the enrichment of binding sites of transcription factors known to control defense-related genes, only motifs with TGAC, GCC, ACC, and ACGT core sequences were considered. TGAC-containing sequences are known to interact with multiple types of transcription factors, such as members of the WRKY or TGA-bZIP families (Schindler et al., 1992; Eulgem et al., 2000; Jakoby et al., 2002). Binding sites for PBF2 consist of TGAC preceded by four As or Ts (Desveaux et al., 2002, 2004). The triplets GCC or ACC are frequently present in binding sites of ERF (Ethylene Response Factors)-type transcription factors (Rushton and Somssich, 1998; Rushton et al., 2002). ACGT is present in many binding sites of b-ZIP-type transcription factors (Jakoby et al., 2002).

We examined six sets/subsets of genes, the full sets of genes contained in clusters I and II of Figures 3 and 4 and two subsets derived from each of these clusters. The expression profiles of the genes we included in each subset exhibited a standard correlation of 0.90 or 0.95 to the weighted average profile of the respective cluster (see "Materials and Methods"). Thus, they represent genes with the most closely related expression profiles from within each cluster. If conserved promoter motifs are responsible for the coordinated expression of sets of coregulated genes, then their degree of conservation should correlate with the degree of coordinated target gene expression. Consistent

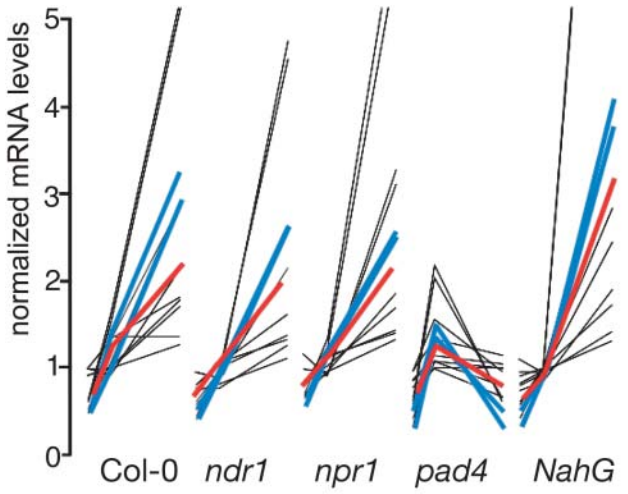

Figure 5. Genes coregulated with *PAD4* are probable functional targets for *RPP4*-dependent regulation. Expression behavior of *PAD4* (represented by the two probe sets in blue) and seven other tightly coregulated genes (correlation coefficient ≥ 0.85). Normalized mRNA levels (not ratios) at 0, 12, or 48 hpi for each indicated plant line are displayed. The weighted average pattern of these genes is shown in red. The individual genes are listed below the graph.

AGI number	probe sets	gene name/function
AT3G52430	14249_i_at, 14250_r_at	*PAD4* / defense signaling
AT3G48090	13241_s_at, 20653_s_at	*EDS1* / defense signaling
At1g33960	12879_s_at	*AIG1* (with Prenyl group binding site & ATP/GTP-binding site motif) / putative signaling protein
At4g12720	13177_at	growth factor like protein / signaling
At1g27350	20032_at	unknown protein
At2g18690	17894_at	unknown protein
At4g14630	20421_at	Germin-like protein 9/ oxalate oxidase
AT5G13200	14139_at	ABA-responsive protein - like

with this, we did not find any significant conservation of TGAC-, GCC/ACC-, or ACGT-containing motifs in the full sets of clusters I and II or in genes showing only a correlation of 0.90 to the weighted average profiles of these clusters. However, we found strong conservation of such motifs in the subsets of genes defined by a correlation coefficient of 0.95 or higher to the weighted averages profiles of clusters I and II (Table III). Motif I is conserved in a subset of cluster I genes. It has a strictly conserved TGAC core sequence and, hence, may interact with WRKY or TGA-bZIP-type factors. Motif III is conserved in the respective subset of cluster II genes. This motif has conserved GCC or ACC core sequences, which are typically present in binding sites of transcription factors of the ERF family.

In addition, we observed strict correlation of a motif containing the invariant palindrome CATG in genes showing a correlation of 0.95 to the average profile of cluster I (Table III, motif II). A search against the plant cis-regulatory elements database PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/) revealed that numerous plant cis-elements contain CATG motifs. We found in PLACE a series of 21 CATG-containing elements mediating responses to auxin, ethylene, abscisic acid, light or developmental stimuli, bound by VP1, EIN3, bZIP-type, or unknown factors. However, beyond their CATG core, none of these elements has any obvious similarity to the motifs we identified.

We did not find conservation of TGAC-, GCC/ACC-, or ACGT-containing motifs in any subsets derived from cluster III or among the set of genes coregulated with PAD4 (Fig. 5). However, a close inspection of all *RPP4* early/transient genes (as defined in Supplemental Fig. 1A) revealed a moderate overrepresentation of one possible PBF2 binding motif (A/TTTTTGAC, P value = 1.5E−3). The PBF2 component AtWHY1 interacts with its binding sites in a transient manner following SA treatment and is required for full defense responses (Desveaux et al., 2004). PBF2 may, therefore, participate in the regulation of the SA-dependent *RPP4* response pathway.

DISCUSSION

We profiled gene expression responses triggered by three different disease resistance signaling pathways. *RPP4* function is compromised by the *pad4-1* mutation as well as the *NahG* transgene, which do not affect *RPP7* or *RPP8* function. A Col-0 derived *rpp4* mutant has not been described yet. The *rpp7-3* and *sgt1b* mutations fully eliminate *RPP7* function (J.M. McDowell, unpublished data; Tör et al., 2002). None of these mutations alters *RPP8* function (B. Holt III and J.L.

Table III. *Promoter motifs conserved in early/transient or late/sustained genes*

Motif	Cluster	Consensus	Observed Frequency[a]	Expected Frequency[b]	*P* Value	No. of Promoters[c]
I	I (correlation = 0.95)	TN (G,T) **TGAC**NNG	0.86/1 kb	0.18/1 kb	1.1 E−5	10/14
II	I (correlation = 0.95)	**CATG**T (C, G) NA	1.21/1 kb	0.25/1 kb	1.9 E−6	8/14
III	II (correlation = 0.95)	(**G, A) CC**AAAA (G, A)	1.6/1 kb	0.49/1 kb	8.5 E−6	10/13

[a]Observed frequency of motifs fitting the consensus sequence per 1 kb of upstream sequence. [b]Average observed frequency in 1-kb sequence stretches upstream of all Arabidopsis genes. [c]Number of promoters of respective gene cluster that have at least one copy of a motif fitting the respective consensus sequence.

Dangl, unpublished data). By comparing gene expression profiles triggered by each *P. parasitica* isolate on resistant lines and comparing these to isogenic susceptible lines, we defined gene sets controlled by three genetically separable *RPP* response pathways (see below). Considerable differences between gene expression profiles from different Arabidopsis ecotypes have been reported recently (Zhu et al., 2001); therefore, we limited our study to Col-0 derived plants.

Technical Validity

We observed very similar expression responses between related conditions (Figs. 2 and 3). Our analyses identified genes whose expression is altered in comparisons of resistant and susceptible lines. We accept that there may be other genes whose transcriptional activity is altered by these infections. We may have missed transcriptional events necessary for resistance mediated by any of the tested resistance specificities. There also may be genes differentially regulated, for example, in *P. parasitica* Emoy2 infected *pad4* that are not altered in *P. parasitica* Hiks1 infected *sgt1b*. However, such transcriptional changes, if they occur, are insufficient in sum to result in resistance. This study was designed to focus on sets of genes whose transcriptional change is strictly correlated with *RPP* function in each case.

We limited our analysis mainly to genes showing elevated expression associated with *RPP*-dependent disease resistance. Yet, there is a cluster comprising 79 genes (Fig. 3, cluster III) whose expression levels are lower in resistant plants than in susceptible mutants. Higher relative expression levels in susceptible mutants might reflect either more intense *P. parasitica*-induced gene expression in these lines or elevated basal transcription in the absence of a putative negative regulatory function of the relevant NBS-LRR R protein. In particular, the majority of *RPP4* reduced genes (Fig. 2; also included in cluster III of Fig. 3) display a pronounced up-regulation in *pad4-1* and *NahG* following infection (data not shown). Many of these genes encode proteins with putative roles in defense, such as chalcone synthase, thaumatin (PR5), and cell wall modifying enzymes. Strong up-regulation of these genes may be triggered by a PAD4 and SA-independent part of the basal defense system responding to extensive growth of *P. parasitica* Emoy2.

Shared and *RPP*-Specific Temporal Patterns of Coregulation

Progression of *P. parasitica* Emoy2 and *P. parasitica* Hiks1 infection events occurs with similar speed. Therefore, the timing of gene expression responses mediated by *RPP4* and *RPP7* can be compared. These pathways converge and trigger two distinct temporal patterns of gene expression. A set of early and transiently up-regulated genes encodes many proteins that might directly fight the invading pathogen. Many of these genes are also controlled by *RPP8*-dependent signaling. Thus, at least one convergence point of signals derived from all three tested *R* genes must exist upstream of these early/transient genes. The second pattern of gene activation triggered by *RPP4* and *RPP7* results in later, or sustained, up-regulation of genes predominantly encoding signaling proteins. Many of these genes are also up-regulated constitutively in the presence of *RPP8*. Hence, *RPP4*, *RPP7*, and *RPP8* derived signals must also converge upstream of late/sustained genes.

These convergence points may be mediated by common regulatory molecules such as signaling proteins or small molecule messengers. Reasonable candidates include MAP kinase pathways terminating in the Arabidopsis MPK3 and MPK6 proteins (Asai et al., 2002) and the transcription factors that are presumably the targets of their activities. Tobacco (*Nicotiana tabacum*) orthologs of these MAP kinases were also shown to be involved in defense signaling cascades (Zhang and Klessig, 2001). Members of the large family of WRKY transcription factors were suggested to operate downstream from MPK3 and MPK6 in Arabidopsis (Asai et al., 2002). Consistent with this, we found a potential binding site for WRKY factors conserved in promoters of genes early/transiently up-regulated by the pathways we examined (Figs. 3 and 4, cluster I). Different signaling routes may activate different sets of transcriptional regulators that then target separate or common promoter elements in the genes of each regulon. Alternatively, convergence of defense signals may occur in parallel at multiple points, each controlling only a subset of defense responses. The sets of genes defined here are commonly targeted by more than one of the examined *RPP* signaling pathways and will be important tools for the future dissection of the local defense signaling network.

RPP4 and *RPP7* late/sustained genes predominantly encode signaling proteins. Their roles in the plant immune system are enigmatic. Sustained up-regulation of these genes may control a long-lasting activation of some local defense reactions. However, their elevated expression at a time point (48 hpi), at which the mRNA levels of many genes executing defense reactions have returned to their ground states, is partially unexpected. Thus, another potential role of these late/sustained signaling genes could be to shut down defense responses and to reset the local defense system. Alternatively, their massive transcript increase at later time points may be a result of a delayed activation in tissue surrounding the infection sites, suggesting that these genes play roles in controlling aspects of disease resistance beyond the infection site and perhaps systemically.

Coregulated Responses Feature Enriched Putative Control Elements

We identified promoter motifs strongly conserved in early/transient or late/sustained up-regulated genes. These will serve as a starting point for the cloning of transcription factors potentially participating in the coordinated regulation of these genes. In particular, the novel CATG-containing motif that is enriched in early/transient genes may allow the identification of important transcription factors of the plant immune system. We anticipate that these hypothetical CATG interacting factors may control physiological responses directly affecting pathogen viability, such as camalexin biosynthesis and cell wall modifications.

This analysis also identified two potential binding sites of transcription factors known to regulate defense-related genes. These motifs contain TGAC or GCC/ACC core motifs that are likely to interact with WRKY or ERF-type transcription factors, respectively. Members of each of these families of plant-specific transcription factors have been demonstrated to regulate defense genes (Rushton et al., 1996; Zhou et al., 1997; Eulgem et al., 1999). In each case positions outside the respective core motifs were found to be highly conserved. WRKY- and ERF-type factors are represented by large families in Arabidopsis (Riechmann et al., 2000). Their members typically share a preference for the core motif common to each family's binding site repertoire. The extended conserved motifs that we identified may constitute specific binding sites of individual members within each family. Permutations fitting the consensus sequences of these two conserved motifs are present in promoters of the majority, but not all members, of the respective gene set. Derivatives of the conserved motifs with slightly altered sequences may be present in the remaining promoters. Further analysis may lead to the definition of cis-elements that are targeted by one or more distinct *RPP* response pathways. Future experiments will address whether *RPP4*, *RPP7*, and *RPP8* signaling converges upstream of common cis-elements or if each pathway targets a specific ensemble of regulatory promoter elements. Molecular mechanisms operating at the interface between *R* gene signaling and defense gene regulation are still largely unknown. Our expression profiling data will facilitate systematic studies to uncover basic principles and details of this important regulatory circuit.

MATERIALS AND METHODS

Arabidopsis Lines and *Peronospora parasitica* Isolates

All plants used in this work are in the Col-0 genetic background. The Arabidopsis mutant or transgenic lines *ndr1-1* (Century et al., 1995), *npr1-1* (Cao et al., 1994), *pad4-1* (Glazebrook et al., 1997), *NahG* (Delaney et al., 1994), *sgt1b* (Tör et al., 2002), and Col-0:*RPP8* (McDowell et al., 1998) have been described. In the *rpp7-3* allele, codon 796 (encoding Glu) of *RPP7* is deleted (X.J. Wang and J.M. McDowell, unpublished data). The *Peronospora parasitica* isolates Emoy2, Hiks1, and Emco5 were described previously (Holub and Beynon, 1996).

Infection of Arabidopsis Seedlings, Staining of Cotyledon Tissue, RNA Preparation, and GeneChip Data Generation

P. parasitica was grown and propagated as described previously (McDowell et al., 2000). Arabidopsis seedlings were grown on soil for 14 d in a clean growth chamber (10 h day, 14 h night, 21°C; 100 μE m^{-2} s^{-1}) and sprayed with 100,000 spores/mL of the respective *P. parasitica* isolate. Trypan blue staining of infected Arabidopsis cotyledons was performed as described previously (McDowell et al., 2000). Untreated Arabidopsis seedlings or seedlings at 12 and 48 hpi with the respective *P. parasitica* isolate were shock-frozen in liquid nitrogen. Preparation of total RNA, processing of RNA for GeneChip experiments, hybridization, calculation of signal intensities (average difference values; calculated using Affymetrix Microarray Suite version 4.0; Santa Clara, CA), and overall intensity normalization were performed as described previously (Zhu and Wang, 2000).

GeneChip Data Analysis

Raw data for all chips are deposited at TAIR under the accession number ME00313, according to the MIAME guidelines. Data from each individual chip were normalized against each other by setting their target intensity (average signal intensity) to 100 (Zhu and Wang, 2000). The signals from most of the negative control probe sets were below 25, so we defined signal intensities of 25 (25% of target intensity) as the noise level using our normalization procedure. All signals below 25 were therefore raised to 25 for further data transformation and analysis. The number of false 2-fold expression changes between technical replicates was previously found to be 0.22% using this regime (Zhu and Wang, 2000). This data handling scheme has been used by a variety of authors analyzing diverse biological responses (Harmer et al., 2000; Zhu and Wang, 2000; Zhu et al., 2001; Chen et al., 2002; Kreps et al., 2002; Glazebrook et al., 2003; Laule et al., 2003; Tao et al., 2003). Using Microsoft Excel, average difference values <25 were raised to 25 and ratios of expression levels were calculated. Filtering of expression ratios and hierarchical clustering was performed as described previously (Maleck et al., 2000) using Cluster and Treeview (Eisen et al., 1998). *k*-means clustering was applied to normalized mRNA levels by GeneSpring 3.5 (Silicon Genetics, Redwood City, CA) using standard correlation as a means to calculate distances between expression profiles. The median of all data points for each chip as well as the median of all data points for each gene were set to 1. Subclusters of clusters I, II, and III were defined using GeneSpring by selecting genes with normalized mRNA profiles showing a standard correlation of $\geq$0.90 or $\geq$0.95 to the weighted average profile of the respective cluster. Genes were included in the respective subclusters, if represented by at least one probe set, showing the required correlation to the weighted average. We used the weighted average of each gene set (weighted by a control factor of each gene) for the definition of subclusters. This control factor is a measure of liability and reflects the absolute signal strength. Genes with higher control

values are more reliable. As a result, the weighted average gives less weight to potential noise and outliers (http://www.silicongenetics.com/cgi/TNgen.cgi/GeneSpring/GSnotes/Notes/want_average).

Promoter Analysis

For each member of clusters I, II, and III (Figs. 3 and 4) as well as for all Arabidopsis genes as a reference, 1 kb of genomic DNA sequence upstream from the inferred translational start site was downloaded from the TAIR Web site (http://www.arabidopsis.org/tools/bulk/sequences/index.html). To search for conserved motifs within the promoter sequences of each cluster or subcluster, we used AlignACE (Hughes et al., 2000; http://atlas.med.harvard.edu/). For each promoter set, we performed a series of AlignACE runs with the following set of parameters: number of columns to align = 12 to 6; number of sites to expect = number of promoters in respective input set; fractional background GC content = 0.32 (we found the GC content of Arabidopsis promoters to be approximately 32%). The resulting AlignACE outputs were scanned for conserved motifs containing core sequences of known defense-related transcription factor binding sites as well as the tetramer CATG that we found to be highly enriched in cluster I genes. For each conserved motif containing any of these core sequences, we determined the frequency in all Arabidopsis promoters (expressed as average occurrences/1 kb). Using this value, we calculated the expected frequency of each motif in the set of promoters it was originally derived from. *P* values expressing for each conserved motif the probability for the observed enrichment to occur by chance were calculated by Microsoft Excel using the Poisson distribution with the following set of parameters: number of events = number of occurrences of the respective motif in all promoters of its gene cluster; mean = expected number of occurrences of this motif in this gene cluster based on its average frequency in all Arabidopsis promoters; cumulative = false. Only conserved motifs with *P* values $< 1E-5$ were considered further.

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