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Isolation of Arabidopsis Genes That Differentiate between Resistance Responses Mediated by the *RPS2* and *RPM1* Disease Resistance Genes

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The Arabidopsis disease resistance gene *RPS2* is involved in recognition of bacterial pathogens carrying the avirulence gene *avrRpt2*, and the *RPM1* resistance gene is involved in recognition of pathogens carrying *avrRpm1* or *avrB*. We identified and cloned two Arabidopsis genes, *AIG1* and *AIG2* (for avrRpt2-induced gene), that exhibit *RPS2*- and *avrRpt2*-dependent induction early after infection with *Pseudomonas syringae* pv *maculicola* strain ES4326 carrying *avrRpt2*. However, ES4326 carrying *avrRpm1* or *avrB* did not induce early expression of *AIG1* and *AIG2*. Conversely, ES4326 carrying *avrRpm1* or *avrB* induced early expression of the previously isolated defense-related gene *ELI3*, whereas ES4326 carrying *avrRpt2* did not. The induction patterns of the *AIG* genes and *ELI3* demonstrate that different resistance gene-*avr* gene combinations can elicit distinct defense responses. Furthermore, by examining the expression of *AIG1* and *ELI3* in plants infiltrated with a mixed inoculum of ES4326 carrying *avrRpt2* and ES4326 carrying *avrRpm1*, we found that there is interference between the *RPS2*- and *RPM1*-mediated resistance responses.

INTRODUCTION

The outcome of an interaction between a plant and a pathogen is often determined by the presence of resistance genes in the plant host that correspond to pathogen avirulence (*avr*) genes in a gene-for-gene relationship (Flor, 1971). Infection of a plant by a pathogen that carries an *avr* gene for which the plant carries a corresponding resistance gene results in the rapid activation of a number of inducible defense responses. These responses include an oxidative burst, reinforcement of cell walls by lignification, the production of antimicrobial compounds, such as phytoalexins and hydrolytic enzymes, and programmed cell death, known as the hypersensitive response (HR), in the region of the infection (reviewed in Lindsay et al., 1993; Dixon et al., 1994; Staskawicz et al., 1995). When the infecting pathogen lacks *avr* genes that are recognized by the host, no HR results, induction of other defense responses is much slower, and disease ensues.

A ligand-receptor model has been proposed to explain the specificity of resistance gene-*avr* gene interactions (reviewed in Gabriel and Rolfe, 1990). According to this model, resistance genes encode receptors that bind elicitors directly or indirectly produced by the corresponding *avr* genes. Elicitor-bound receptors are postulated to activate a signal transduction pathway leading to activation of the HR and induction of defense gene expression. Because different *avr* gene-resistance gene pairs generally activate similar defense responses, a corollary of this model has been the assumption that downstream components of this signal transduction pathway are shared by different *avr* gene-resistance gene pairs.

Our laboratory and others have developed a system to study plant resistance gene-*avr* gene interactions in the crucifer Arabidopsis by using the bacterial pathogen *Pseudomonas syringae* (Debener et al., 1991; Dong et al., 1991; Whalen et al., 1991). *P. syringae* pv *maculicola* strain ES4326 is virulent on Arabidopsis ecotype Columbia (Col-0). Introduction of the cloned *avr* gene *avrRpt2* into ES4326 allows recognition of the pathogen by Col-0 and elicitation of the HR. Arabidopsis mutants that no longer mount an HR in response to *avrRpt2* have been isolated (Kunkel et al., 1993; Yu et al., 1993) and used to map and clone the corresponding resistance gene, *RPS2* (Bent et al., 1994; Mindrinos et al., 1994). Similarly, resistance loci corresponding to the cloned *avr* genes *avrRpm1* and *avrB* have been mapped (Debener et al., 1991; Innes et al., 1993), and mutational analysis has demonstrated that both of these *avr* genes correspond to the same resistance gene, *RPM1* (*RPS3*) (Bisgrove et al., 1994). *RPM1* has recently been isolated by map-based cloning (Grant et al., 1995).

The predicted RPS2 and RPM1 proteins are members of a family of resistance gene products containing a nucleotide binding site and leucine-rich repeats (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995). Other genes that encode members of this family include the tobacco mosaic virus resistance gene *N* (Whitham et al., 1994) and the flax rust resistance gene *L6* (Lawrence et al., 1995). It is not known

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whether RPS2, RPM1, and other proteins in this family are the receptors predicted by the ligand–receptor model. The elicitors produced by the corresponding *avr* genes are also not known. Little is known about the signal transduction pathways leading from activation of these resistance gene products to induction of the HR and other defense responses.

By using *P. s. maculicola* ES4326 carrying *avrRpt2* or *avrRpm1* to infect wild-type and *rps2* or *rpm1* mutant plants, respectively, it is possible to examine the effects of the signal transduction pathway initiated by the interaction of single resistance and *avr* genes without the complications introduced by differences in plant or pathogen genetic background. In this study, we set out to isolate defense-related genes that are specifically induced in an interaction between resistant plants and avirulent bacteria. Such genes would be useful as reporters for the downstream events in resistance.

Here, we describe the isolation of two genes, designated *AIG1* and *AIG2* (for avrRpt2-induced gene), that show *RPS2*- and *avrRpt2*-dependent induction. Because of the similarities in structure between RPS2 and RPM1 and the proteins encoded by other resistance genes in this family, we expected that *AIG1* and *AIG2* would be activated in a variety of *avr*–resistance gene interactions and would be markers for the HR. Unexpectedly, we found that ES4326 carrying *avrRpm1* or *avrB* did not induce early expression of *AIG1* and *AIG2*. In addition, we found that early expression of the pathogenesis-related gene *PR1* was induced by ES4326 carrying *avrRpt2* but not by ES4326 carrying *avrRpm1*. Conversely, ES4326 carrying *avrRpm1* or *avrB* induced early expression of a different defense-related gene, *ELI3*, which was not induced by *avrRpt2*. These results demonstrate that there can be differences between resistance responses mediated by different resistance gene–*avr* gene combinations. Furthermore, examination of *AIG1* and *ELI3* expression after infiltration of plants with a mixed inoculum of ES4326 carrying *avrRpt2* and ES4326 carrying *avrRpm1* revealed interference between the *RPS2*- and *RPM1*-mediated resistance responses.

RESULTS

Two Novel Pathogen-Induced Genes Isolated by Differential mRNA Display

We used the differential mRNA display technique (Liang and Pardee, 1992) to identify genes that show *RPS2*- and *avrRpt2*-dependent induction. Wild-type (Col-0) and *rps2* mutant plants (strain *rps2-101C*) were infiltrated with *P. s. maculicola* ES4326 carrying *avrRpt2*. Total RNA was isolated from tissue harvested at 3 and 9 hr after infiltration and used as a template for reverse transcription and the polymerase chain reaction (PCR), as described in Methods. We examined the PCR products produced by 40 primer pairs and investigated 23 potential differentially amplified bands. The reamplified PCR products were used to probe blots of total RNA from Col-0 and *rps2* plants isolated at several time points after infiltration with $MgSO_4$ solution, ES4326, or ES4326 carrying *avrRpt2*. Only six of these 23 probes detected transcripts on total RNA blots. Of these six, two hybridized to transcripts that were more highly expressed in Col-0 after infiltration with ES4326 carrying *avrRpt2* than in Col-0 after infiltration with ES4326 or in *rps2-101C* after infiltration with either strain. The two differentially expressed genes were named *AIG1* and *AIG2*.

The reamplified *AIG1* PCR product was ~1.3 kb, which is unusually long for a differential display product. The *AIG1* PCR product hybridized to a single transcript of ~1.4 kb that was strongly expressed at 6 and 9 hr after infection of Col-0 with ES4326 carrying *avrRpt2* but was not strongly induced until 12 to 24 hr after infection with ES4326, as shown in Figure 1. The mean fold difference in *AIG1* expression between leaves inoculated with ES4326 carrying *avrRpt2* and leaves inoculated with ES4326 at 6 hr was 10.1 (SD = 6.2) in seven independent experiments. This early expression of *AIG1* is dependent on the presence of the resistance gene *RPS2* because it was not seen in the *rps2-101C* mutant after infection with ES4326 carrying *avrRpt2* (Figure 1).

We were unable to isolate a cDNA clone that corresponded to the *AIG1* PCR product from two independent cDNA libraries, and we were unable to clone the amplified cDNA product by any of several different methods. Therefore, we suspect that the full-length *AIG1* cDNA clone may be toxic to bacteria. However, a genomic DNA clone containing *AIG1* was isolated from a library of Col-0 DNA cloned into λ-GEM11. The sequence obtained from the genomic clone was used to design primers to sequence the *AIG1* PCR product and obtain the 5′ end of the cDNA. The complete *AIG1* cDNA sequence (GenBank accession number U40856) contains a 1062-bp open reading frame beginning with a methionine codon. The computer program BLAST (Altschul et al., 1990) detected no significant similarity between the deduced amino acid sequence of *AIG1* and any known protein. *AIG1* most likely represents a single-copy gene, because an *AIG1* probe hybridized at high stringency to only one band in digests of Col-0 DNA with BamHI, BglI, ClaI, EcoRI, EcoRV, HindIII, and XbaI. One weakly hybridizing band was observed in most digests, suggesting that there is another Arabidopsis gene with some sequence similarity with *AIG1* (data not shown).

The amplified *AIG2* product was 0.5 kb, and it hybridized to a single band of ~0.7 kb on RNA blots. Figure 1 shows that the *AIG2* transcript is more highly expressed in Col-0 at 6 and 9 hr after infection with ES4326 carrying *avrRpt2* than after infection with ES4326, and this early expression of *AIG2* also depends on *RPS2*. The mean fold difference in *AIG2* expression between leaves inoculated with ES4326 carrying *avrRpt2* and leaves inoculated with ES4326 at 6 hr was 1.8 (SD = 0.5) in five independent experiments. *AIG2*, like *AIG1*, was induced at 12 to 24 hr after infection with the virulent strain.

The reamplified *AIG2* PCR product was used to probe a cDNA library produced from Col-0 infected with the aviru-

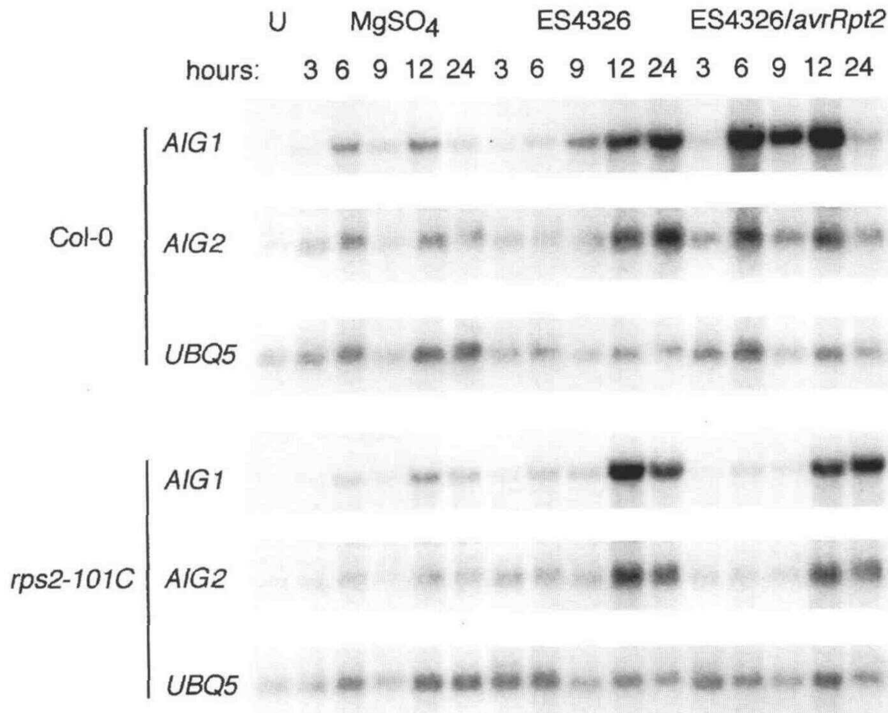

Figure 1. Expression of *AIG1* and *AIG2* in Plants Infiltrated with Virulent and Avirulent *P. syringae*.

Col-0 and *rps2-101C* plants were infiltrated with $MgSO_4$ solution, *P. s. maculicola* ES4326, or *P. s. maculicola* ES4326 carrying *avrRpt2* (ES4326/*avrRpt2*), and tissue was harvested at 3, 6, 9, 12, and 24 hr after infection. Expression of *AIG1* and *AIG2* is compared with that of the ubiquitin control probe *UBQ5*. The 9-hr lane for Col-0 inoculated with ES4326/*avrRpt2* is underloaded. Quantitated data from similar experiments are graphed in Figures 2A and 2B. U, untreated plant.

lent pathogen *P. syringae* pv *tomato* MM1065 (G. Yu and F.M. Ausubel, unpublished results), and two cDNA clones of 583 and 711 bp were isolated. The longer clone, *AIG2-3*, contains a 513-bp open reading frame starting with a methionine codon. As was the case for *AIG1*, the computer program BLAST (Altschul et al., 1990) detected no significant similarity between the deduced amino acid sequence of this open reading frame and any known protein. The shorter cDNA clone lacked 67 bp in the 3′ untranslated region of the putative transcript, apparently due to use of an alternative polyadenylation site, and lacked 61 bp at the 5′ end of the putative transcript. The GenBank accession number of the *AIG2* cDNA sequence is U40857.

AIG2 is most likely a member of a small gene family, because the *AIG2-3* clone hybridized at high stringency to two bands in digests of Col-0 DNA with BamHI, BglII, ClaI, EcoRI, and HindIII, and to three bands in EcoRV and XbaI digests. In each digest, one to three bands that hybridized at low intensity were also observed, suggesting that there may be other Arabidopsis genes with sequence similarity with *AIG2* (data not shown). The genes that hybridize at high stringency to the *AIG2-3* clone are closely linked, because two large NdeI bands that are polymorphic between Col-0 and Landsberg *erecta* cosegregated in 95 recombinant inbred lines (see below).

Restriction fragment length polymorphisms between the Col-0 and Landsberg *erecta* ecotypes were used to map the *AIG1* and *AIG2* genes, using a collection of recombinant inbred lines (Lister and Dean, 1993). On the recombinant inbred map posted to the Arabidopsis data base AAtDB (Cherry et al., 1992) on June 29, 1995 (accessible at http://probe.nalusda.gov:8300/cgi-bin/dbrun/aatdb?find+Map+Recombinant_Inbred*), *AIG1* maps to chromosome 1 at 77.2 centimorgans, between the markers mi423a and *RPS18b*. *AIG2* maps to chromosome 3 at 68.2 centimorgans, between *GL1* and mi413.

Early *AIG1* and *AIG2* Expression Is Not Induced by Two Other Cloned Avirulence Genes, *avrRpm1* and *avrB*

To determine whether early expression of *AIG1* and *AIG2* occurs in other resistance gene–*avr* gene interactions, we examined *AIG1* and *AIG2* expression after infection with

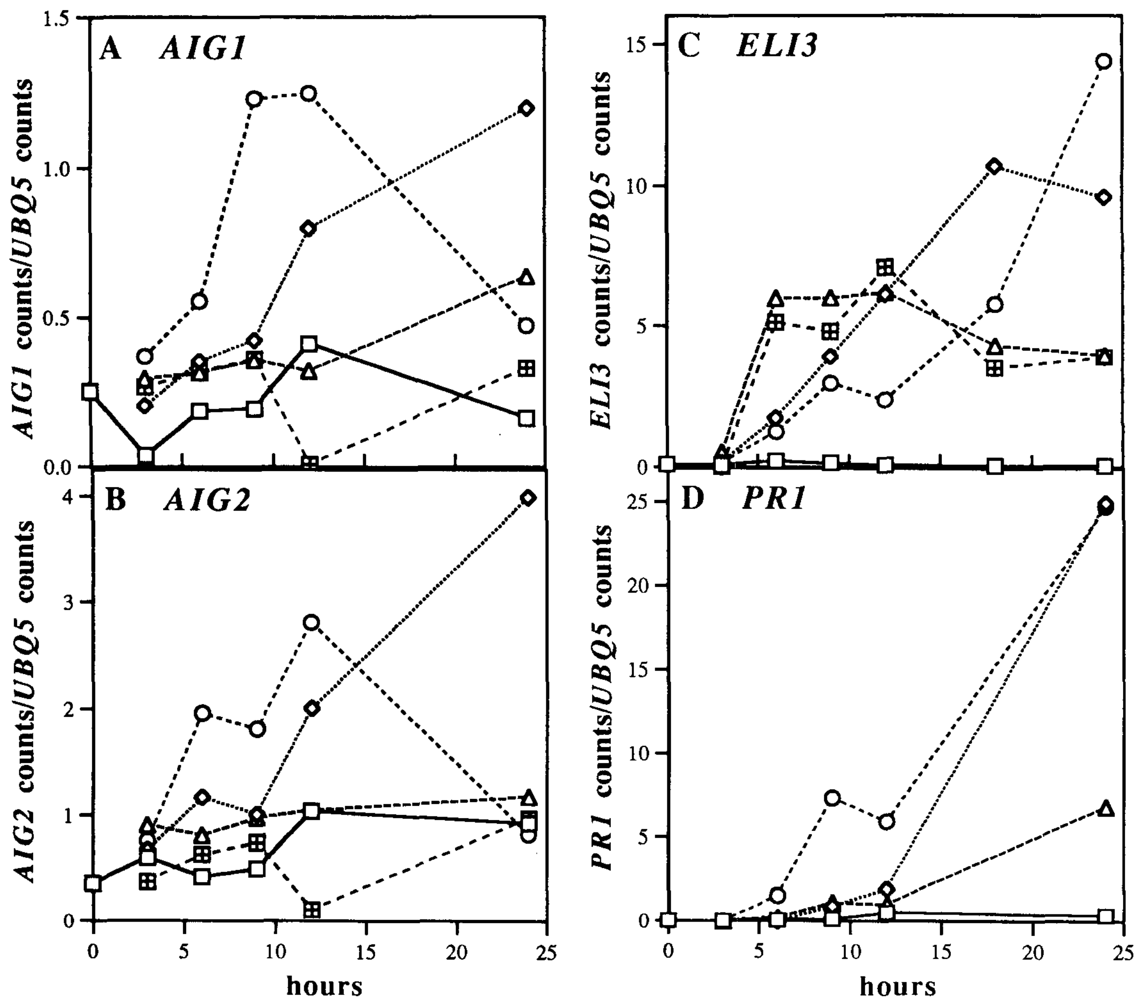

Figure 2. Expression of *AIG1*, *AIG2*, *ELI3*, and *PR1* in Col-0 Plants in Response to Virulent and Avirulent *P. s. maculicola*.

Blots of total RNA were hybridized with the indicated probes, and the bands were quantitated on a PhosphorImager. The signal for each lane was normalized to the counts obtained for a ubiquitin (*UBQ5*) control probe, and the resulting values were plotted. In each case, squares indicate the $MgSO_4$ control; diamonds, *P. s. maculicola* ES4326; circles, *P. s. maculicola* ES4326 carrying *avrRpt2*; and triangles, *P. s. maculicola* ES4326 carrying *avrRpm1*. In **(A)** to **(C)**, crossed squares indicate *P. s. maculicola* ES4326 carrying *avrB*.

(A) *AIG1* expression.

(B) *AIG2* expression.

(C) *ELI3* expression.

(D) *PR1* expression.

ES4326 carrying either of the cloned *avr* genes, *avrRpm1* or *avrB*. These *avr* genes correspond to the resistance gene *RPM1* (Bisgrove et al., 1994). Interestingly, as shown in Figures 2A and 2B, *AIG1* and *AIG2* expression was not induced at 6 and 9 hr by *avrRpm1* or *avrB*. The experiments were repeated, with similar results.

A possible explanation for the apparent differential activation of *AIG1* and *AIG2* by *avrRpt2* on the one hand and *avrRpm1* and *avrB* on the other stems from the observation that *P. s. maculicola* ES4326 carrying *avrRpm1* or *avrB* produced an HR in 5 to 6 hr, whereas ES4326 carrying *avrRpt2* produced an HR in 12 to 16 hr at the doses used in these experiments. We hypothesized that gene induction might occur more rapidly in response to bacteria carrying *avrRpm1* and *avrB*, and an earlier induction of *AIG1* and *AIG2* in those interactions might have been missed in the time course shown in Figures 2A and 2B. We therefore examined *AIG1* expression during the period 0.5 to 9 hr after infection. Figure 3 shows that *AIG1* was not

induced by ES4326 carrying *avrRpm1* or ES4326 carrying *avrB* at any time after infection. Therefore, an early activation of *AIG1* had not been overlooked in the experiments shown in Figure 2.

ELI3 Is Induced Early by *avrRpm1* and *avrB* but Not by *avrRpt2*

Early expression of the *Arabidopsis* defense-related gene *ELI3* has previously been shown to be induced by *P. syringae* strains carrying *avrRpm1* (Kiedrowski et al., 1993) and *avrB* (Mittal and Davis, 1995). Therefore, we examined *ELI3* expression in response to ES4326 carrying *avrRpt2*. Figure 2C shows that *ELI3* was more highly expressed at 6 hr after infection with ES4326 carrying *avrRpm1* and ES4326 carrying *avrB* than with the virulent strain. The differences in expression between the avirulent and virulent strains were smaller than those observed by Kiedrowski et al. (1993) in a different strain background, but we observed a mean 3.1-fold difference (SD = 1.1) in *ELI3* expression at 6 hr with *avrRpm1* in seven experiments and a mean 3.6-fold difference (SD = 1.8) in *ELI3* expression at 6 hr with *avrB* in six experiments. However, as shown in Figure 2C, we did not observe early *ELI3* expression with ES4326 carrying *avrRpt2*. The experiment was repeated, with similar results. Examination of *ELI3* expression in *rpm1* mutant plants demonstrated that the early induction of *ELI3* was dependent on *RPM1* (data not shown).

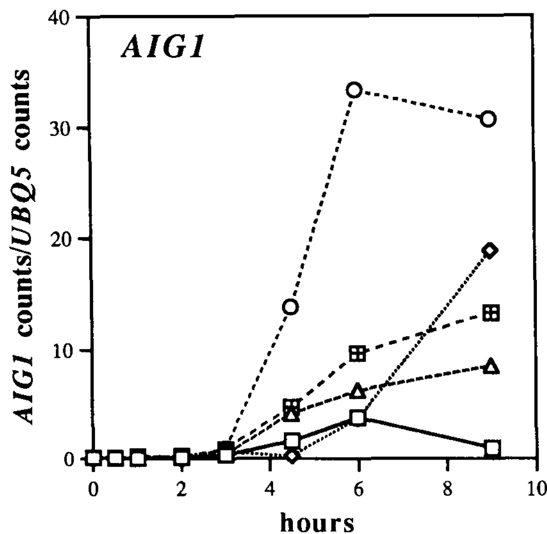

Figure 3. Expression of *AIG1* from 0.5 to 9 Hr in Col-0 in Response to Virulent and Avirulent *P. s. maculicola.*

Blots of total RNA were hybridized with the *AIG1* probe, and the bands were quantitated on a PhosphorImager. The signal for each lane was normalized to the counts obtained for a ubiquitin (*UBQ5*) control probe, and the resulting values were plotted. Squares indicate the $MgSO_4$ control; diamonds, *P. s. maculicola* ES4326; circles, ES4326 carrying *avrRpt2*; triangles, ES4326 carrying *avrRpm1*; crossed squares, ES4326 carrying *avrB*.

PR1 Is Induced at 9 and 12 Hr by *avrRpt2* but Not by *avrRpm1* or *avrB*

The Arabidopsis *PR1* gene has previously been shown to be expressed earlier in an interaction with ES4326 carrying *avrRpt2* than with ES4326 (Greenberg et al., 1994). Therefore, we examined the expression of *PR1* in response to ES4326 carrying *avrRpm1*. Figure 2D shows that *PR1* is more highly expressed at 9 and 12 hr after infiltration with ES4326 carrying *avrRpt2* than after infiltration with ES4326. However, *PR1* was not more highly expressed at 9 and 12 hr in response to ES4326 carrying *avrRpm1*. The experiment was repeated, with similar results.

Mixed Inoculation Reveals Interference between *RPS2*- and *RPM1*-Mediated Gene Induction

One explanation for the differences in gene induction caused by strains carrying *avrRpt2* and *avrRpm1* is that *RPS2* and *RPM1* activate different signal transduction pathways. If this were the case and the two pathways did not interact, inoculation with a mixture of ES4326 carrying *avrRpm1* and ES4326 carrying *avrRpt2* would be expected to induce both *AIG1* and *ELI3*. To test this model, we examined *AIG1* and *ELI3* expression in leaves inoculated with a 1:1 mixture of ES4326 carrying *avrRpm1* and ES4326 carrying *avrRpt2* at 5×10^6 colony-forming units (cfu)/mL concentrations of each bacterial strain (10^7 cfu/mL total bacteria). In the same experiments, we also examined *AIG1* and *ELI3* expression in leaves inoculated with 5×10^6 cfu/mL ES4326 carrying *avrRpm1* or ES4326 carrying *avrRpt2*. This experimental design provided an equal number of bacteria carrying *avrRpt2* or *avrRpm1* in the mixed inoculations and in the controls. As in previous experiments (Figure 2), Figure 4A shows that expression of *AIG1* was induced by ES4326 carrying *avrRpt2* but not by ES4326 carrying *avrRpm1*, and Figure 4B shows that the opposite was true for *ELI3*. However, the mixture of the two strains elicited the induction of *ELI3* but not *AIG1*, even though there were equal numbers of bacteria carrying *avrRpt2* in single and mixed inocula. The presence of bacteria carrying *avrRpm1* apparently blocked the *avrRpt2*-dependent induction of *AIG1*. These experiments were repeated twice, with similar results.

DISCUSSION

We isolated two novel Arabidopsis genes, *AIG1* and *AIG2*, that show *RPS2*- and *avrRpt2*-dependent induction at early time points after infection with *P. s. maculicola* ES4326 carrying

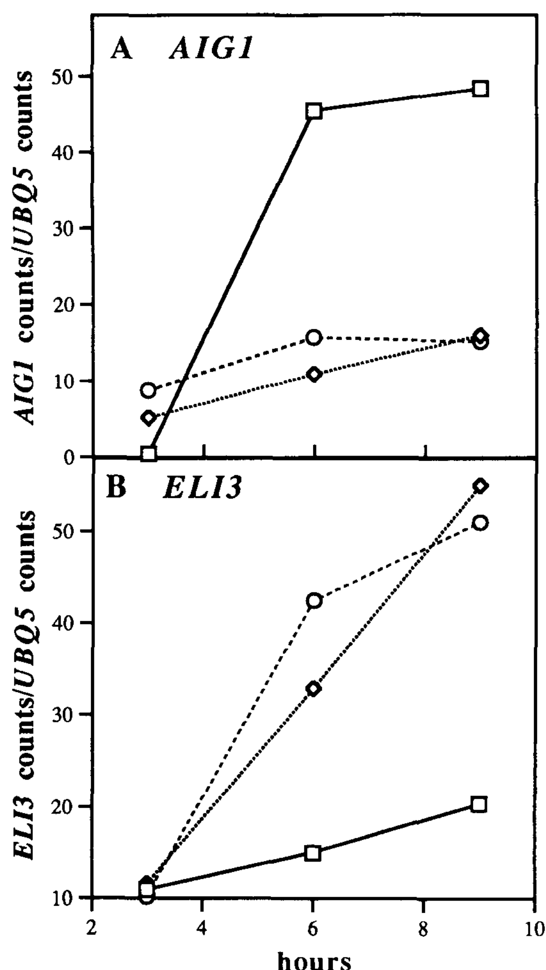

Figure 4. Expression of *AIG1* and *ELI3* in Response to Mixed Inocula.

Blots of total RNA were hybridized with the indicated probes, and the bands were quantitated on a PhosphorImager. The signal for each lane was normalized to the counts obtained for a ubiquitin (*UBQ5*) control probe, and the resulting values were plotted. In each case, squares indicate the inoculum was *P. s. maculicola* ES4326 carrying *avrRpt2*; diamonds, ES4326 carrying *avrRpm1*; and circles, a 1:1 mixture of ES4326 carrying *avrRpt2* and ES4326 carrying *avrRpm1*.
(A) *AIG1* expression.
(B) *ELI3* expression.

avrRpt2. Both of these genes are also induced at 12 and 24 hr after infection with the virulent strain (ES4326). Late induction of defense responses in an interaction with a virulent pathogen is often observed (Dixon and Lamb, 1990). Because the sequences of these genes provide no clue to their functions, it is unclear what roles *AIG1* and *AIG2* play in plant defense.

Interestingly, *AIG1* and *AIG2* are not induced early by ES4326 carrying either of two other *avr* genes, *avrRpm1* and *avrB*, which correspond to the resistance gene *RPM1*. In addition, the *PR1* gene shows early induction by ES4326 carrying *avrRpt2* but not by ES4326 carrying *avrRpm1*. Conversely, *ELI3* is induced early by ES4326 carrying *avrRpm1* or *avrB* but not by ES4326 carrying *avrRpt2*. One model to explain these results is that *RPS2* and *RPM1* activate different signal transduction pathways leading to gene induction. This model is contrary to the conventional model of resistance gene action in which different resistance genes are postulated to feed into the same downstream pathway leading to the HR. The observation that the *RPS2* and *RPM1* pathways involve the differential activation of *AIG1* and *AIG2* versus *ELI3* might be considered surprising, given that *RPS2* and *RPM1* have such a high degree of structural similarity (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995). However, there is no a priori reason to assume that each resistance gene product could not stimulate several downstream pathways that activate various aspects of the defense response. A common pathway might lead to the HR, whereas other pathways could be specific to particular resistance genes. One possibility is the existence of different downstream effectors that interact with RPS2 and RPM1. Kinetic or quantitative differences in signal strength between different resistance genes might also cause differences in downstream pathway activation, perhaps through different levels of modification of a downstream effector.

The data in Figures 1 and 2 show that after infection with ES4326 carrying *avrRpt2*, *AIG1* and *AIG2* mRNA accumulation first increases and then drops after the appearance of the HR. Therefore, it is possible that the HR negatively regulates *AIG1* and *AIG2* expression. Thus, an alternative explanation of our data is that *avrRpm1* activates a signal transduction pathway leading to *AIG1* and *AIG2* expression but that the rapid induction of the HR by *avrRpm1* prevents the expression of *AIG1* and *AIG2*. This is in contrast to the explanation proposed above in which *AIG1* and *AIG2* are activated as a result of a signal cascade mediated by *avrRpt2–RPS2* but not by *avrRpm1–RPM1*. On the other hand, the more rapid induction of an HR by *avrRpm1* does not explain the activation of *ELI3* by *avrRpm1* but not by *avrRpt2*. Moreover, the kinetics of *PR1* mRNA accumulation that we observe (Figure 2D) are more consistent with a model of gene induction by an *avrRpt2*-specific pathway, because *PR1* is not downregulated after the appearance of the HR.

Another possible explanation for the different effects of *avrRpt2* and *avrRpm1* or *avrB* on downstream gene induction could be that an elicitor produced directly or indirectly by *avrRpt2* induces *AIG1* and *AIG2* through a second pathway, perhaps through a second receptor or an effect on metabolism, rather than by interaction with the resistance gene. Similarly, elicitors produced by *avrRpm1* and *avrB* could activate *ELI3* through an alternate pathway. This explanation is not implausible, because it has been demonstrated that *avrRpm1* can act as a virulence factor in Arabidopsis ecotypes that do not carry *RPM1* (Ritter and Dangl, 1995), indicating that an *avrRpm1*-dependent product has a pathogenesis-related function that could generate a signal. However, because *AIG1* and *AIG2* are not activated by *avrRpt2* in an *rps2* mutant and similarly because *ELI3* is not activated by *avrRpm1* in an *rpm1* mutant, it is necessary to postulate that any secondary

pathways involved can activate these genes only when the resistance pathway is also activated. Such models cannot be ruled out by our work; however, they are more complex than models that postulate that the *RPS2* and *RPM1* signal transduction pathways differ.

Differences in resistance reactions mediated by different resistance genes have been observed before at the physiological and microscopic level in various systems, and this variation may also correlate with differences in gene induction. For instance, in a study of the response of resistant tomato leaves to the *Cladosporium fulvum* Avr9 and Avr2 elicitors, it was found that Avr9 but not Avr2 induced supraoptimal stomatal opening, resulting in leaf dehydration (Hammond-Kosack and Jones, 1995). In the interaction of Arabidopsis with *Peronospora parasitica*, many pairs of resistance genes and fungal *avr* genes have been defined, and several morphologically distinct phenotypes of hypersensitive lesions have been observed in different incompatible interactions (Holub et al., 1994). Microscopic studies of many plant–fungal interactions have shown that the action of different resistance genes inhibits fungal colonization at various stages of infection (Mansfield, 1990). In the *P. syringae*–Arabidopsis system, the timing of the HR mediated by *avrRpt2* or *avrRpm1* is different, and characteristic timing of the HR has also been noted in other resistance gene–*avr* gene interactions (Mansfield et al., 1994).

Despite the differences we have described between *RPS2*- and *RPM1*-mediated gene induction, the interference between the two pathways that we have observed when *avrRpt2*- and *avrRpm1*-containing strains were coinoculated suggests that there may be competition for a component that is shared by the two pathways. Ritter and Dangl (1996) have also observed interference between the *RPS2*- and *RPM1*-mediated resistance pathways, and they have demonstrated that this interference is dependent on the relative numbers of *avrRpt2*- and *avrRpm1*-containing bacteria inoculated.

METHODS

Bacterial Strains and Plasmids

Pseudomonas syringae pv *maculicola* strain ES4326 has been described by Dong et al. (1991). The cloned avirulence (*avr*) genes used were *avrRpt2* on plasmid pLH12 (Whalen et al., 1991), *avrRpm1* on plasmid pK48 (Debener et al., 1991) or p*AvrRpm1* (Innes et al., 1993), and *avrB* on plasmid pPSG0002 (Staskawicz et al., 1987) or pVB01 (Innes et al., 1993). *P. syringae* strains were grown at 28°C in King's B medium (King et al., 1954), with the addition of 10 μg/mL tetracycline or 50 μg/mL kanamycin as required.

Growth of *Arabidopsis thaliana* and Hand Infiltration of *Pseudomonas* Strains

The Arabidopsis ecotype Columbia (Col-0) mutant strains *rps2-101C* (Yu et al., 1993) and *rps3-2* (carrying a mutation in the gene now termed *RPM1*) (Bisgrove et al., 1994) have been described. Arabidopsis plants were grown essentially as described by Greenberg and Ausubel (1993), except that seedlings germinated in a greenhouse were placed in a Conviron (Asheville, NC) growth chamber (20°C, at 90% relative humidity) after 7 to 10 days. *P. syringae* suspensions in 10 mM $MgSO_4$ were hand infiltrated into Arabidopsis leaves by using a 1-mL syringe without a needle, as described by Dong et al. (1991). For inoculation of plants for RNA preparation, a dose of 10^7 colony forming units (cfu)/mL (OD_{600} = 0.1) was used, except for preparation of RNA for differential mRNA display, when a dose of 5×10^6 cfu/mL was used, and in mixed inoculation experiments, as described in the text.

Differential mRNA Display

The differential mRNA display procedure was performed using a modification of the original procedure of Liang and Pardee (1992), essentially according to the instructions in the RNAmap kit (GenHunter, Brookline, MA). Briefly, the RNA samples to be compared were reverse transcribed with an anchored oligo(dT) primer and then amplified by polymerase chain reaction (PCR) with the same anchored oligo(dT) primer and an arbitrary 10-mer to produce a characteristic set of bands for each primer pair. In the modified procedure, four degenerate anchored oligo(dT) primers were used for reverse transcription and PCR ($T_{12}VA$, $T_{12}VC$, $T_{12}VG$, and $T_{12}VT$, where V represents a mixture of A, C, and G), instead of using the 12 possible anchored oligo(dT) primers separately. Reverse transcription, PCR, and separation of labeled products on a 6% polyacrylamide gel were performed according to the instructions for the RNAmap kit. The arbitrary 10-mers used for PCR were obtained from GenHunter and Operon Technologies (Alameda, CA). PCR products were labeled with ^{35}S-dATP (Du Pont–New England Nuclear, Boston, MA).

For each primer pair, two RNA samples from independent experiments were carried through the reverse transcription and PCR steps, and the resulting products were run side by side on the gel. Only bands that were consistently different between wild-type and *rps2-101C* plants were isolated, thereby eliminating many artifacts. Potential differential products were reamplified, gel purified, labeled with a random priming kit (Boehringer Mannheim, Indianapolis, IN), and used to probe RNA gel blots.

Cloning and Sequencing of *AIG1* and *AIG2*

The reamplified *AIG2* PCR product was used to probe a cDNA library in pBlueDB made from Arabidopsis (Col-0) infected with *P. s.* pv *tomato* strain MM1065 (G. Yu and F.M. Ausubel, unpublished results). pBlueDB is a cDNA cloning vector containing a stuffer with asymmetric BstXI sites cloned into the BamHI site of Bluescript (J. Sheen, Massachusetts General Hospital, Boston, MA, unpublished results). Colonies were lifted onto GeneScreen Plus membranes (Du Pont–New England Nuclear), and hybridizations were performed according to the manufacturer's instructions. The *AIG2* cDNA clones (pAIG2-2 and pAIG2-3) were sequenced using the Sequenase kit (U.S. Biochemical Corp., Cleveland, OH).

An *AIG1* genomic clone was isolated as described above from a Col-0 genomic library cloned into λ-GEM11. This library was constructed by ligation of Col-0 DNA to EcoRI adaptors, size fractionation of the DNA, and ligation of an ~20-kb fraction to vector arms cut with EcoRI (J.T. Mulligan and R.W. Davis, Stanford University, Stanford, CA, unpublished data). Sau3aI and TaqI fragments from this clone were subcloned into pBluescriptII SK+, and clones containing *AIG1* DNA were selected by colony hybridization and sequenced using the Sequenase kit. Sequence information from the genomic DNA was used

to design primers to reamplify and sequence the amplified cDNA product obtained from the differential display procedure. The differential display product was sequenced by a combination of PCR sequencing with the fmol DNA sequencing kit (Promega, Madison, WI) and cloning of smaller reamplified PCR products, using the TA cloning kit (Invitrogen, San Diego, CA) for sequencing by the Sequenase protocol. The 5' end of the coding sequence was obtained by the rapid amplification of cDNA ends PCR technique, using the Marathon kit (Clontech, Palo Alto, CA).

Mapping of *AIG1* and *AIG2*

AIG1 and *AIG2* probes (prepared as described for RNA blots below) detected BamHI and NdeI polymorphisms, respectively, between the Col-0 and Landsberg *erecta* ecotypes. The segregation of these markers was followed in 98 recombinant inbred lines, as described by Lister and Dean (1993).

RNA Isolation and RNA Blot Analysis

For RNA blot analysis, RNA was made by a miniprep procedure, loosely based on that of Altenbach and Howell (1981), in which the entire preparation was performed in microcentrifuge tubes. Briefly, three or four leaves were ground in liquid nitrogen in a mortar with a pestle. The ground tissue was added to a tube containing 0.4 mL of water-saturated phenol and 0.4 mL of RNA extraction buffer (0.2 M Tris, pH 8.0, 0.4 M LiCl, 25 mM EDTA, 1% SDS), vortexed, and placed on ice. After centrifugation, the aqueous phase was removed, reextracted with phenol, and extracted once with 0.5 mL of chloroform. RNA was precipitated by the addition of 40 µL of diethyl pyrocarbonate (DEPC)–treated 3 M sodium acetate and 1 mL of absolute ethanol. The pellet was washed once with DEPC-treated 2 M LiCl by pipetting the sample up and down thoroughly, and the sample was centrifuged 10 min at high speed. The pellet was then resuspended in 0.1 mL of DEPC-treated water, reprecipitated with 3 M sodium acetate and ethanol, and resuspended in 50 µl of DEPC-treated water. The usual yield from the procedure was 15 to 50 µg.

RNA samples (5 µg) were separated on formaldehyde–agarose gels (Ausubel et al., 1995) and transferred to GeneScreen membranes (Du Pont–New England Nuclear), hybridized, and washed according to the manufacturer's instructions. Probes were prepared either by labeling isolated DNA fragments with a random priming kit (*ELI3*, *AIG2*) or by preparing radiolabeled single-stranded probes (*AIG1*, *UBI5*, *PR1*), as described by Greenberg et al. (1994). The *ELI3* fragment comprising nucleotides 142 to 1005 of the cDNA was amplified from Arabidopsis Col-0 genomic DNA, using primers from the parsley *ELI3* sequence (M. Mindrinos and F.M. Ausubel, unpublished results). The template for the *AIG1* probe was derived from the plasmid pLR2, which was constructed by PCR amplification of nucleotides 403 to 1069 of the *AIG1* cDNA and cloning the product into the plasmid pCRII, using the TA cloning kit (Invitrogen). Blots were exposed to PhosphorImager screens and developed by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The counts in each band were quantitated by volume integration and normalized to the counts obtained for ubiquitin.

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