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# Induction of *Arabidopsis* Defense Genes by Virulent and Avirulent *Pseudomonas syringae* Strains and by a Cloned Avirulence Gene

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**We developed a model system to study the signal transduction pathways leading to the activation of *Arabidopsis thaliana* genes involved in the defense against pathogen attack. Here we describe the identification and characterization of virulent and avirulent *Pseudomonas syringae* strains that elicit disease or resistance symptoms when infiltrated into *Arabidopsis* leaves. The virulent and avirulent strains were characterized by determining growth of the pathogen in *Arabidopsis* leaves and by measuring accumulation of mRNA corresponding to *Arabidopsis* phenylalanine ammonia-lyase (PAL), β-1,3-glucanase (BG), and chalcone synthase (CHS) genes in infected leaves. The virulent strain, *P. syringae* pv *maculicola* ES4326, multiplied $10^5$-fold in *Arabidopsis* leaves and strongly elicited BG1, BG2, and BG3 mRNA accumulation but had only a modest effect on PAL mRNA accumulation. In contrast, the avirulent strain, *P. syringae* pv *tomato* MM1065, multiplied less than 10-fold in leaves and had only a minimal effect on BG1, BG2, and BG3 mRNA accumulation, but it induced PAL mRNA accumulation. No accumulation of CHS mRNA was found with either ES4326 or MM1065. We also describe the cloning of a putative avirulence (*avr*) gene from the avirulent strain MM1065 that caused the virulent strain ES4326 to grow less well in leaves and to strongly elicit PAL but not BG1 and BG3 mRNA accumulation. These results suggest that the *Arabidopsis* PAL and BG genes may be activated by distinct signal transduction pathways and show that differences in plant gene induction by virulent and avirulent strains can be attributed to a cloned presumptive *avr* gene.**

## INTRODUCTION

Plants mount a coordinated defense response to phytopathogenic microorganisms by accumulating antimicrobial compounds (phytoalexins), by synthesizing hydrolytic enzymes that attack fungi and bacteria, and by producing polyphenolic lignins and hydroxyproline-rich glycoproteins that strengthen their cell walls (for reviews, see Bell, 1981; Darvill and Albersheim, 1984; Collinge and Slusarenko, 1987; Hahlbrock and Scheel, 1987; Lamb et al., 1989; Dixon and Lamb, 1990). Although pathogen-induced genes have been cloned and studied in detail at the molecular level, very little is known about the signal transduction pathways that lead to their activation. For example, it is not known whether genes encoding hydrolytic enzymes like β-1,3-glucanases and genes encoding phytoalexin biosynthetic enzymes are coordinately regulated or are activated by independent mechanisms.

Genetic analysis of host-pathogen interactions can be used to help dissect the process by which plants perceive the presence of a pathogen. For example, many avirulent fungal and bacterial strains differ from virulent ones by one or more avirulence (*avr*) genes that have corresponding "resistance" genes in the host (Flor, 1947, 1971; Ellingboe, 1981; Keen and Staskawicz, 1988). According to one simple formulation of this gene-for-gene model, the specific recognition of an *avr* gene product by a plant resistance gene product leads to a defense response (the so-called hypersensitive response or HR) that is characterized by localized cell necrosis of the infected tissue and induction of certain plant defense-related genes (Lamb et al., 1989).

Although a variety of bacterial *avr* genes have been cloned and shown to decrease significantly the growth of a virulent strain during infection (Staskawicz et al., 1984, 1987; Gabriel et al., 1986; Keen and Staskawicz, 1988; Kobayashi et al., 1990), it has not been demonstrated that transfer of an individual *avr* gene to an otherwise virulent pathogen leads to induction of specific host genes. Moreover, the type of genetic analysis that led to the identification of host resistance genes has not been extended to

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identify the components of the signal transduction pathways that connect the perception of a pathogen to the ultimate expression of genes required to combat its invasion. We are, therefore, developing a new model system that utilizes the infection of the small crucifer *Arabidopsis thaliana* with pathogenic pseudomonads (Davis and Ausubel, 1989; Davis et al., 1989; Schott et al., 1990). The use of *Arabidopsis* to study plant-bacterial interactions is also being pursued by Brian Staskawicz and colleagues at the University of California, Berkeley (Whalen et al., 1991), and our laboratories are cooperating in this effort. Specifically, our laboratory is devising simplified genetic screens in which the induction of the promoters of pathogen-induced genes are monitored in response to well-defined signals such as a cloned bacterial *avr* gene.

To monitor the defense response, we chose the *Arabidopsis* genes encoding phenylalanine ammonia-lyase (PAL) and β-1,3-glucanase (BG) because they belong to two different categories of pathogen-induced genes. BG, in addition to its role in degrading pathogen cell walls, may be involved in the production of oligosaccharide elicitors (Keen and Yoshikawa, 1983; Mauch et al., 1988a, 1988b; Mauch and Staehelin, 1989). PAL, the first committed step in the phenylpropanoid pathway, leads to the biosynthesis of both flavonoid and furanocoumarin phytoalexins and to the biosynthesis of lignins (Collinge and Slusarenko, 1987; Hahlbrock and Scheel, 1989; Lamb et al., 1989).

In this paper, we report the identification of pathogenic *Pseudomonas* strains that elicit readily distinguishable virulent and avirulent phenotypes when infiltrated into *Arabidopsis* leaves. Interestingly, the virulent strain *P. syringae* pv *maculicola* ES4326 and the avirulent strain *P. syringae* pv *tomato* MM1065 had opposite effects on the accumulation of PAL and BG mRNA. Moreover, the virulent strain ES4326, carrying a cloned putative *avr* gene from the avirulent strain MM1065, activated PAL and BG mRNA similarly to strain MM1065, demonstrating that a defined DNA sequence encoding a putative *avr* gene can elicit the expression of host defense genes. These experiments suggest that two different signal transduction pathways are involved in the response of *Arabidopsis* to virulent and avirulent pathogens. The pathway leading to PAL induction may recognize a pathogen-specific signal defined in part by an *avr* gene, whereas the pathway leading to BG induction most likely responds to a signal that is not pathogen specific.

## RESULTS

### Identification of Pathogenic *P. syringae* Strains

As a first step in developing a pathogenesis model, we identified a group of taxonomically related phytopathogenic *Pseudomonas* strains that exhibited virulent or avirulent phenotypes when infiltrated into *Arabidopsis* leaves. A strain was classified as virulent if it elicited disease symptoms (water-soaked lesion with or without chlorosis) after 48 hr when *Arabidopsis* leaves were infiltrated with a bacterial suspension at a titer of $10^5$ colony-forming units (cfu) per milliliter (cfu/mL; for details see Methods). A strain was classified as avirulent if it elicited one of the following resistance responses: no symptoms when leaves were infiltrated at $10^5$ cfu/mL, an HR within 24 hr when the titer of the inoculum exceeded $10^6$ cfu/mL, or chlorosis without other disease symptoms within 72 hr when the titer of the inoculum exceeded $10^6$ cfu/mL. The HR in *Arabidopsis* appears as a necrotic dry lesion that develops within 24 hr, whereas disease symptoms only appear after 48 hr.

To identify bacterial pathogens of *Arabidopsis*, we screened *P. syringae* pv *maculicola* (*Psm*), *P. syringae* pv *tomato* (*Pst*), and *P. cichorii* pathogenic strains by infiltrating them into *Arabidopsis* ecotype Columbia leaves. As detailed elsewhere (K. Davis, E. Schott, and F. Ausubel, manuscript submitted for publication), we found that all of the *P. cichorii* strains tested elicited a resistance response, all of the *P. syringae* pv *maculicola* strains caused disease, and the *P. syringae* pv *tomato* strains either caused disease or elicited a resistance response. We chose the *Psm* strains 4326 and 5034 and the *Pst* strain DC3000 for further study because they were among the most virulent strains tested and because they elicited reproducible disease symptoms. We chose the avirulent *Pst* strain JL1065 for further study because it elicited a reproducible resistance response and because a genomic library of this strain had been constructed previously (see Methods). We did not use the *P. cichorii* strains for the experiments reported here even though some of them elicited a strong HR. The *P. cichorii* strains belong to a highly divergent taxonomic group, whereas the *Pst* and *Psm* strains are closely related (Schaad, 1988).

We isolated spontaneous streptomycin-resistant derivatives of *Psm* 5034 and 4326 (MM5034 and ES4326, respectively) and a spontaneous nalidixic acid-resistant derivative of *Pst* JL1065 (MM1065) to facilitate the monitoring of bacterial growth in *Arabidopsis* leaves. The parental and drug-resistant derivatives elicited identical pathogenicity symptoms (data not shown). Strain *Pst* DC3000 is rifampicin resistant. As shown in Figure 1, when *Psm* ES4326 or *Pst* DC3000 were infiltrated into *Arabidopsis* Columbia leaves at $10^6$ cfu/mL, a water-soaked lesion with a spreading chlorosis appeared after 48 hr. Figure 2 shows that *Psm* ES4326 multiplied $10^4$-fold to $10^5$-fold within 96 hr after infiltration of leaves at $10^5$ cfu/mL. Growth of *Pst* DC3000 was similar (data not shown). Strain *Psm* MM5034 elicited the same disease symptoms as *Psm* ES4326 and *Pst* DC3000, as shown in Table 1, but multiplied 10-fold less in leaves than the other two strains (data not shown).

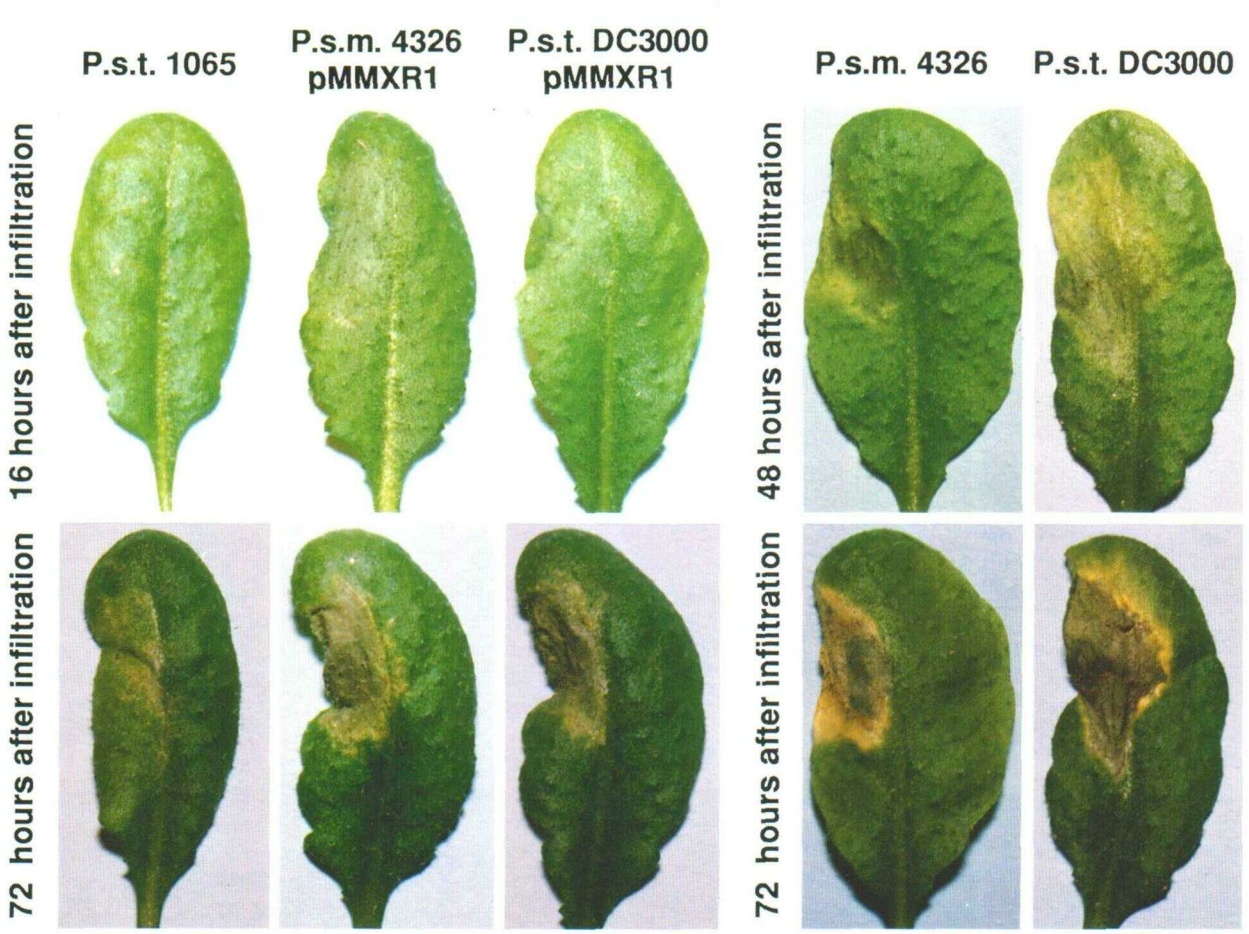


**Figure 1.** Infiltration of *Arabidopsis* Leaves with Virulent and Avirulent *P. syringae* Strains.

The middle of one side of *Arabidopsis* ecotype Columbia leaves was infiltrated with approximately 3 $\mu$L of a bacterial suspension, as described in Methods. The titer of the inoculum was $10^6$ cfu/mL for all strains except for *Pst* MM1065 (P.s.t. 1065), which was $10^7$ cfu/mL. For each strain, the early time point represents the time at which symptoms first appeared, except for the case of MM1065, where no symptoms appeared until 72 hr. P.s.t. 1065, *P. syringae* pv *tomato* strain MM1065; P.s.m. 4326, *P. syringae* pv *maculicola* strain ES4326; P.s.t. DC3000, *P. syringae* pv *tomato* strain DC3000.

In contrast to these virulent strains, when *Pst* MM1065 was infiltrated at $10^5$ to $10^6$ cfu/mL, no visible symptoms appeared within 72 hr (Table 1) and it multiplied less than 10-fold (Figure 2). When infiltrated at $10^7$ cfu/mL, however, *Pst* MM1065 elicited a mild chlorosis after 72 hr, as shown in Figure 1, and when *Pst* MM1065 was infiltrated at $10^8$ cfu/mL, a visible HR appeared within 24 hr (data not shown).

We reasoned that the most versatile strain for an *Arabidopsis* model system would be one that was also virulent on a variety of crucifers. Choosing such a strain would allow us to test whether *Arabidopsis* as well as plants taxonomically related to *Arabidopsis* could recognize the same presumptive cloned *avr* genes. Therefore, we tested the virulence of strains ES4326, DC3000, MM5034, and MM1065 on radish, turnip, and cabbage. As shown in Table 1, *Psm* ES4326 appeared to be the most virulent among these strains and we chose it for further analysis.

Similarly, the most versatile ecotype of *Arabidopsis* for a model system would be one that was highly susceptible to *Psm* ES4326. To determine whether other *Arabidopsis* ecotypes were more susceptible to *Psm* ES4326 than Columbia (the most commonly used ecotype), we infiltrated *Psm* ES4326 into 29 additional geographically diverse *Arabidopsis* isolates (see Methods for a list of ecotypes tested). Among these 30 ecotypes, 27, including Columbia, appeared to be equally susceptible hosts (data not shown).

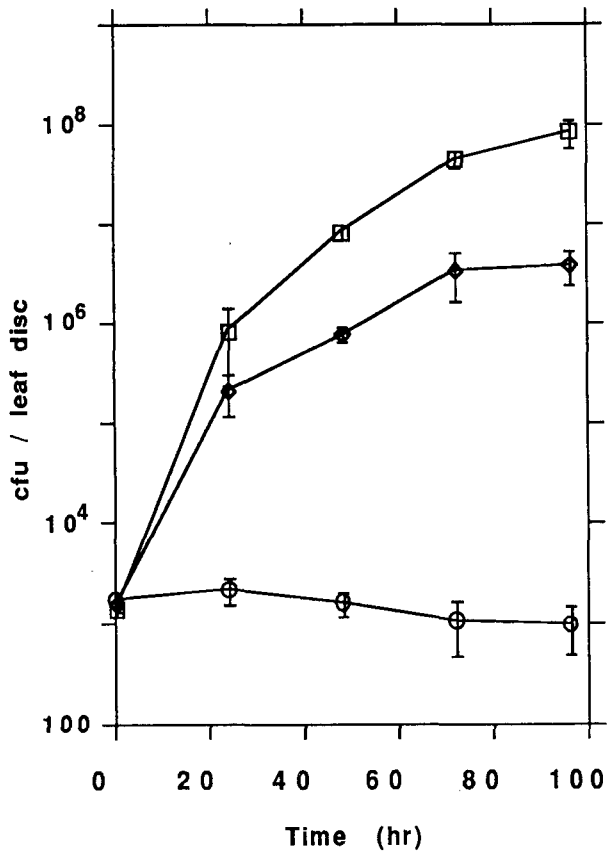


**Figure 2.** Growth of Virulent and Avirulent *P. syringae* Strains in *Arabidopsis*.

Five-week-old *Arabidopsis* Columbia seedlings were infiltrated with approximately 10 $\mu$L of a bacterial suspension containing $10^5$ cfu/mL. At the indicated times, 0.5-cm$^2$ leaf discs at the site of infection were harvested and the number of viable bacteria in each disc was determined. For ES4326/pMMXR1, tetracycline was omitted from the plating media to avoid underestimating the number of cells (see Methods). The results, shown with error bars corresponding to the standard deviation, represent the average of five independent measurements. □, *Psm* ES4326; ○, *Pst* MM1065; ◇, ES4326/pMMXR1.

For the experiments reported in the rest of this paper, we chose *Psm* ES4326 and *Pst* MM1065 as virulent and avirulent pathogens, respectively, and *Arabidopsis* ecotype Columbia as the host.

### Cloning an *avr* Gene from *P. syringae* pv *tomato*

To determine whether the different symptoms elicited by the virulent strains ES4326, DC3000, and MM5034 and by the avirulent strain MM1065 could be attributed to specific *avr* loci in *Pst* MM1065, a genomic library of *Pst* MM1065 DNA constructed in the broad host range cosmid vector pLAFR3 was conjugated into *Psm* ES4326 (see Methods for details). ES4326 transconjugants were screened for ones that failed to elicit disease symptoms on *Arabidopsis* ecotype Columbia when infiltrated at $10^5$ cfu/mL. Among 350 transconjugants tested, one transconjugant was found that did not elicit disease symptoms, and the cosmid in this strain, pLM22B, was isolated and studied further. When ES4326/pLM22B was infiltrated at $10^6$ cfu/mL, a visible necrotic HR-like response appeared within 16 hr (data not shown).

Cosmid pLM22B, which carries a 23.5-kb chromosomal insert, reduced the growth of *Psm* ES4326 in ecotype Columbia approximately 50-fold 4 days post-inoculation (data not shown). To delimit the presumptive *avr* gene(s) on pLM22B, we constructed in pLAFR3-76 a series of subclones of the 23.5-kb pLM22B insert. One subclone, pMMXR1, carried an 8.7-kb XhoI-EcoRI fragment and conferred the same phenotype as pLM22B when transferred to *Psm* ES4326. No sequences outside of this 8.7-kb fragment had any visible effect on the virulence of *Psm* ES4326. Additional subcloning of the 8.7-kb XhoI-EcoRI fragment further delimited the sequences that would confer an avirulent phenotype on *Psm* ES4326 to a 2.95-kb BamHI fragment (see Methods for details). Figures 1 and 2 show, respectively, that ES4326/pMMXR1 elicited a visible necrotic HR-like lesion on *Arabidopsis* leaves 16 hr post-inoculation when infiltrated at $10^6$ cfu/mL and that pMMXR1 reduced the growth of the virulent *Psm* strain ES4326 approximately 50-fold. Plasmid pMMXR1 had no effect on the growth rate of *Psm* ES4326 in King's B or M9 media in vitro (data not shown). These data indicate that pMMXR1 carries a presumptive *avr* gene.

The putative *avr* gene carried on pMMXR1 also reduced the growth of *Pst* DC3000 and *Psm* MM5034 by 50-fold to 100-fold (data not shown). However, neither DC3000/pMMXR1 (Figure 1) nor MM5034/pMMXR1 (not shown) elicited a visible HR as strong as that elicited by ES4326/pMMXR1 at 16 hr. After 72 hr, the phenotypes elicited by ES4326/pMMXR1 and DC3000/pMMXR1 were similar (Figure 1). The rapid appearance of a readily discernible HR by ES4326/pMMXR1 was a second reason that we chose *Psm* ES4326 as a standard virulent strain, in addition to the fact that ES4326 was more virulent on the crucifers tested than *Psm* MM5034 or *Pst* DC3000.

Whalen et al. (1991) also describe the cloning and characterization of a presumptive *avr* gene, *avrRpt2*, from *Pst* 1065. DNA gel blot analysis showed that pMMXR1 and pABL18 (Whalen et al., 1991; a plasmid that carries *avrRpt2*) contain overlapping regions of the *Pst* 1065 genome (data not shown). Moreover, *avrRpt2* is located within the 2.95-kb BamHI fragment of pMMXR1 that we have shown confers an avirulent phenotype on *Psm* ES4326 (data not shown). In the remainder of this paper, we refer to the presumptive *avr* gene carried on pMMXR1 as *avrRpt2* to conform to the nomenclature used by Whalen et al. (1991).

**Table 1.** Elicitation of Disease Symptoms or Resistance Response by Virulent and Avirulent *P. syringae* Strains

| Strain | *Arabidopsis* cv Columbia (16 HAI[a]) | *Arabidopsis* cv Columbia (72 HAI) | Radish cv Champion (96 HAI) | Turnip cv Shogoin (96 HAI) | Turnip cv Just Right (96 HAI) | Cabbage cv Early Jersey (96 HAI) |
|---|---|---|---|---|---|---|
| ES4326 ($10^5$ cfu/mL) | − | ++ | ++ | ++ | ++ | + |
| MM5034 ($10^5$ cfu/mL) | − | ++ | + | NT | + | ++ |
| DC3000 ($10^5$ cfu/mL) | − | ++ | + | NT | + | NT |
| MM1065 ($10^5$ cfu/mL) | − | − | − | − | − | − |
| MM1065 ($10^7$ cfu/mL) | − | Chl | Chl | Chl | Chl | Chl |
| ES4326/pMMRX1 ($10^6$ cfu/mL) | HR | HR | HR | ++ | HR | NT |
| MM5034/pMMRX1 ($10^6$ cfu/mL) | Weak HR | Weak HR | NT | NT | NT | NT |
| DC3000/pMMRX1 ($10^6$ cfu/mL) | Weak HR | HR | NT | NT | NT | NT |

Definition of symbols: ++, strong disease symptoms typified by a water-soaked lesion that becomes necrotic with spreading chlorosis; +, weak disease symptoms typified by a limited water-soaked lesion with mild chlorosis; −, no symptoms; Chl, chlorosis with no other disease symptoms or with occasional dry necrotic areas; HR, visible dry necrotic lesion with no or limited chlorosis that covers the entire infiltrated area; weak HR, visible scattered dry necrotic lesions within the area of infiltration; NT, not tested.

[a] HAI, hours after infiltration.

If ES4326/pMMXR1 elicited an HR because *Arabidopsis* ecotype Columbia carries a resistance gene corresponding to *avrRpt2*, then an *Arabidopsis* ecotype that lacked this resistance gene might be susceptible to ES4326/pMMXR1. To investigate this possibility, we infiltrated ES4326/pMMXR1 into the 26 *Arabidopsis* ecotypes in which ES4326 elicited disease symptoms. ES4326/pMMXR1 elicited an HR on 25 of the 26 ecotypes and elicited disease-like symptoms on a single ecotype, Kashmir (Kas). The growth of ES4326 was similar in Columbia and Kashmir ($10^5$-fold); however, growth of ES4326/pMMXR1 in Kashmir ranged from $10^2$-fold to $10^5$-fold from plant to plant.

Because we did not identify an *Arabidopsis* ecotype that was unambiguously susceptible to ES4326/pMMXR1, we also tested ES4326/pMMXR1 on several turnip cultivars. As shown in Figure 3 and summarized in Table 1, pMMXR1 caused a 50-fold reduction in growth of *Psm* ES4326 in the turnip cultivar Just Right but had no effect on the growth of ES4326 in the turnip cultivar Shogoin. These results suggest that Just Right carries a resistance gene corresponding to *avrRpt2* that is lacking in Shogoin. Additional genetic analysis of these turnip cultivars is currently underway to establish whether Just Right carries a single resistance gene corresponding to *avrRpt2*.

## Cloning the *Arabidopsis* BG Genes

We previously cloned an *Arabidopsis* gene encoding PAL, the first committed step in flavonoid, furanocoumarin, and lignin biosynthesis, and demonstrated that PAL mRNA is induced after infiltration of *Arabidopsis* leaves with avirulent *P. syringae* and *P. cichorii* strains but not with most virulent strains (Davis et al., 1989; K. Davis, E. Schott, and F. Ausubel, manuscript submitted for publication). To determine whether a hydrolytic enzyme such as BG that degrades fungal and bacterial cell walls is also induced as part of the *Arabidopsis* defense response, we cloned the *Arabidopsis* BG gene(s) to monitor levels of BG mRNA in infected leaves. A recombinant library of *Arabidopsis* DNA (ecotype Landsberg) constructed in λFIX (Voytas and Ausubel, 1988) was screened with a heterologous tobacco (*Nicotiana tabacum*) BG cDNA clone (pGL43; Mohnen et al., 1985). As shown in Figure 4, all of the sequences in the *Arabidopsis* genome that hybridized to the tobacco BG cDNA were located on a single λFIX clone called λAtBG. Subclones of λAtBG that hybridized to pGL43 were constructed, and a total of 6 kb from the BG region was sequenced (data not shown).

The sequence analysis showed that *Arabidopsis* contains three adjacent BG genes, BG1, BG2, and BG3, that are clustered within a 12-kb region and are oriented in the same direction (Figure 4). BG1, BG2, and BG3 each share 60% to 70% sequence identity with the tobacco BG gene (Shinshi et al., 1988) and 65% to 80% identity with each other. The details of the structure of the three BG genes will be published elsewhere. The DNA sequences of BG1, BG2, and BG3 have been deposited in GenBank (accession numbers M58462, M58463, and M58464).

## Induction of the BG and PAL Genes by Virulent and Avirulent *P. syringae* Strains

Utilizing DNA sequence data for the *Arabidopsis* BG genes, restriction fragments located within each of the three genes (Figure 4) that do not cross-hybridize were chosen for use as gene-specific probes. Figures 5 and 6 show that infiltration of *Arabidopsis* leaves with the virulent *Psm* strain ES4326 at a low ($10^6$ cfu/mL) or a high ($10^7$ cfu/mL) titer resulted in a fivefold to 10-fold accumulation of BG3

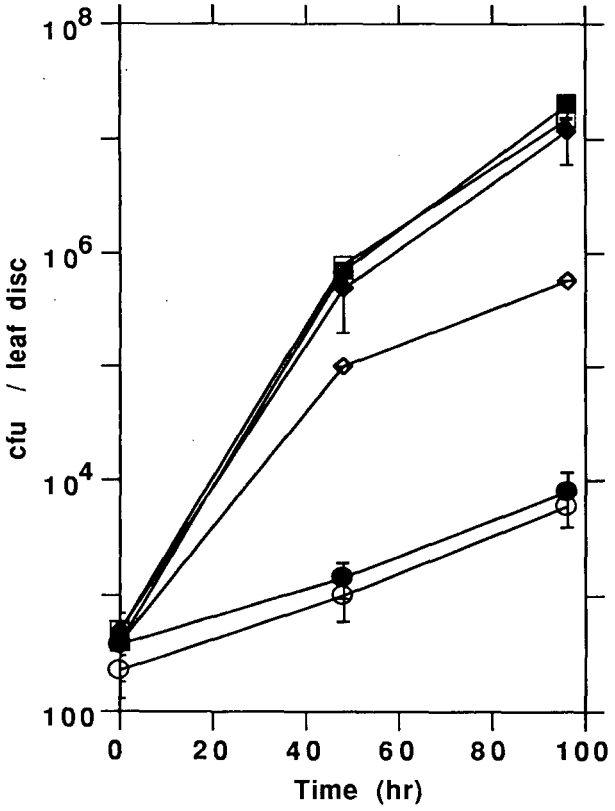


**Figure 3.** Growth of Virulent and Avirulent *P. syringae* Strains in Two Turnip Cultivars.

Turnip plants were infiltrated as described in Methods with a bacterial suspension containing $10^5$ cfu/mL. At the indicated times, growth of the bacterial strains was determined as described in the legend to Figure 2. The results, shown with error bars corresponding to the standard deviation, represent the average of five independent measurements. Open symbols correspond to cultivar Just Right and closed symbols correspond to cultivar Shogoin. Squares, *Psm* ES4326; circles, *Pst* MM1065; diamonds, ES4326/pMMXR1.

mRNA over the course of 48 hr. However, infiltration with a high titer of the avirulent *Pst* strain MM1065 only had a modest effect on BG3 mRNA accumulation. Infiltration with a low titer of *Pst* MM1065 had no effect. Similar results were obtained with BG1 (data not shown). Figures 5 and 6 show that BG2 was also activated fivefold to 10-fold by the virulent strain ES4326 but, unlike BG1 and BG3, appears to be much more sensitive to activation by the avirulent strain at a low dose. In control experiments in which leaves were inoculated with 10 mM $MgCl_2$, none of the BG genes was induced.

In contrast to the results obtained with the BG genes, significant accumulation of PAL mRNA occurred only after infiltration with the avirulent strain *Pst* MM1065 but not with the virulent strain *Psm* ES4326 (Figure 5). PAL mRNA accumulation was transient and dependent on the titer of the initial inoculum. As shown in Figure 6, PAL mRNA levels increased fivefold to 10-fold 6 hr after infiltration with the avirulent strain *Pst* MM1065. Within 12 hr after infiltration, PAL mRNA declined to almost the basal level.

We observed the rapid induction of PAL mRNA by avirulent strains and the slow induction of BG mRNA by virulent strains in more than 10 independent experiments in which *Arabidopsis* ecotype Columbia was infiltrated with a variety of *P. syringae* and *P. cichorii* strains (data not shown; K. Davis, E. Schott, and F. Ausubel, manuscript submitted for publication).

To determine whether the *Arabidopsis* chalcone synthase (CHS) gene is coordinately induced with the PAL gene, we probed the RNA gel blots shown in Figure 5 with an *Arabidopsis* CHS probe (pCHS3.9; Feinbaum and Ausubel, 1988) but did not detect any increase in CHS mRNA after infiltration with *Pst* MM1065 or *Psm* ES4326 (data not shown).

### Induction of PAL and BG mRNA by ES4326/pMMXR1

Several experiments have shown that single bacterial *avr* genes are capable of evoking an HR as defined by localized rapid necrosis and limited growth of the pathogen in the infected plant (Staskawicz et al., 1984; Keen and Staskawicz, 1988). To determine whether the presence of the presumptive *avr* gene, *avrRpt2*, carried on pMMXR1 was sufficient to affect the level of expression of individual defense-related genes, we isolated RNA from *Arabidopsis* leaves infiltrated with ES4326/pMMXR1. As shown in Figures 5 and 6, ES4326/pMMXR1 elicited the accumulation of PAL mRNA similarly to the elicitation by the avirulent strain *Pst* MM1065. Moreover, ES4326/pMMXR1 was also similar to *Pst* MM1065 with respect to the accu-

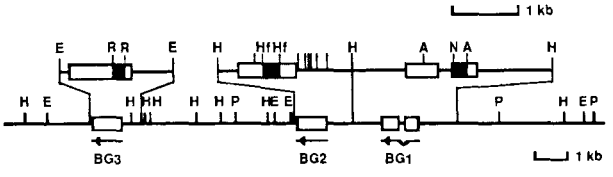


**Figure 4.** Linear Map of the *Arabidopsis* β-1,3-Glucanase Genes Contained on λAtBG.

Regions that encode β-1,3-glucanase core enzyme sequences are shown as open boxes. The direction of transcription of each gene is indicated by an arrow. The restriction fragments that were used as gene-specific probes are shown as solid boxes. Regions that were sequenced are shown in the expanded map. A, AvaII; E, EcoRI; H, HindIII; Hf, HinfI; N, NcoI; P, PstI; R, RsaI. HinfI sites in the HindIII fragment containing the BG2 gene are also indicated as short vertical ticks.

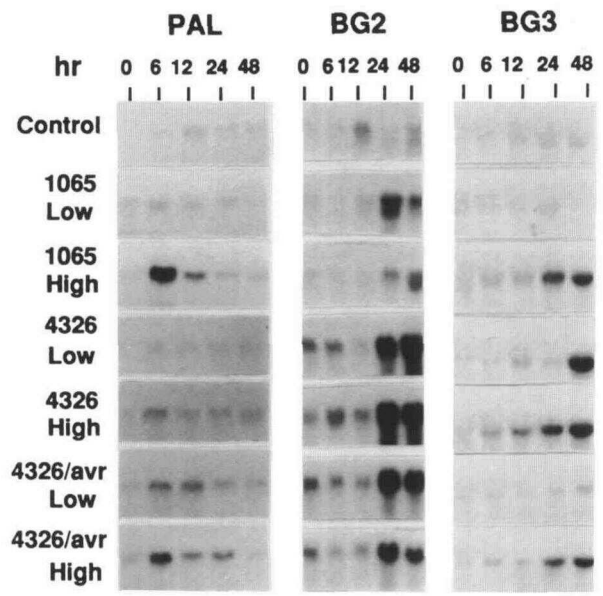


**Figure 5.** RNA Gel Blot Analysis of RNA Samples from *Arabidopsis* Ecotype Columbia Leaves Infiltrated with Virulent and Avirulent *P. syringae* Strains at a High ($10^7$ cfu/mL) or Low ($10^6$ cfu/mL) Titer.

Five micrograms of RNA was loaded in each lane and the gels were stained with ethidium bromide to ensure that equal amounts of RNA were loaded. In addition, the amounts of PAL and BG mRNA in each lane were adjusted by normalizing to the amount of an unknown transcript that was not affected relative to total RNA by bacterial inoculation. The unknown transcript hybridized to an uncharacterized sequence adjacent to the *Arabidopsis* PAL gene on plasmid pAtPalRI.

mulation of BG3 and BG1 (data not shown) mRNA. That is, there was significantly less accumulation of these mRNAs after infiltration with ES4326/pMMXR1 than with *Psm* ES4326 (Figures 5 and 6). In contrast to BG3, significant induction of BG2 occurred with ES4326/pMMXR1 (Figures 5 and 6). The entire experiment shown in Figure 5 was repeated twice with similar results.

We also carried out PAL induction studies using the plasmid pABL18 described by Whalen et al. (1991) that also carries *avrRpt2*. As expected, we found that ES4326/pABL18 elicited PAL mRNA with the same kinetics and to the same extent as ES4326/pMMXR1 (data not shown).

## DISCUSSION

We developed a system for studying plant pathogenesis that utilizes infection of *Arabidopsis* with pathogenic pseudomonads. *Arabidopsis* offers several advantages compared with other plants that have been used previously to study plant-pathogen interactions. Because of its small stature, fast generation time, copious production of tiny (25 $\mu$g) seeds, and small (100 Mb) genome (Redei, 1975; Koornneef et al., 1983; Meyerowitz, 1987, 1989; Chang et al., 1988; Nam et al., 1989), it should be possible to identify genetically genes that regulate the defense response and then clone these genes by using techniques such as chromosome walking (Hauge et al., 1990), gene tagging (Feldmann et al., 1989), or genomic subtraction (Straus and Ausubel, 1990).

To develop this system, we first studied a variety of pathogenic *P. syringae* strains that infect *Arabidopsis*. We chose *Psm* ES4326 and *Pst* MM1065 as a virulent and avirulent pair of strains, respectively, because they are closely related and because *Psm* ES4326 is highly virulent on a variety of crucifers. Second, to monitor induction of individual defense-related genes, we cloned three physically adjacent *Arabidopsis* BG genes. Third, we observed that virulent and avirulent strains have different effects on the amounts and kinetics of PAL and BG mRNA that accumulate during an infection. Fourth, we cloned an 8.7-kb fragment from the genome of *Pst* MM1065 that carries a putative *avr* gene and showed that when this DNA fragment was transferred into the virulent strain *Psm* ES4326 on plasmid pMMXR1, it caused *Psm* ES4326 to become avirulent.

### Cloning a Presumptive *avr* Gene

When the presumptive *avr* gene *avrRpt2*, carried on pMMXR1, was transferred to the virulent strain *Psm* ES4326, ES4326/pMMXR1 did not acquire an avirulent phenotype equivalent to the avirulent *Pst* strain MM1065 from which pMMXR1 was derived. First, ES4326/pMMXR1 elicited a visible resistance response 16 hr after infiltration at $10^6$ cfu/mL, whereas *Pst* MM1065 only elicited a visible resistance response 72 hr after infiltration with $10^7$ cfu/mL. Second, ES4326/pMMXR1 grew $10^3$-fold, whereas *Pst* MM1065 only grew 10-fold at most. These results suggest that the phenotype of the resistance response depends upon the genetic background in which a specific *avr* gene is expressed and that the appearance of a necrotic HR-like response is not necessarily correlated with the lack of bacterial growth. The fact that ES4326/pMMXR1 is more virulent than *Pst* MM1065, as determined by growth in *Arabidopsis* leaves, suggests the presence of additional *avr* genes in *Pst* MM1065. Indeed, preliminary results indicate that we have cloned an additional *avr* gene from *Pst* MM1065, and we are currently constructing derivatives of *Psm* ES4326 that carry both putative *avr* genes to test whether they have an accumulative effect on growth. Results and conclusions similar to ours have been published previously for the *Xanthomonas campestris* pv *vesicatoria* interaction with bean (Whalen et al., 1988). Whalen et al. (1991) also reported similar results with the *Arabidopsis P. syringae* system.

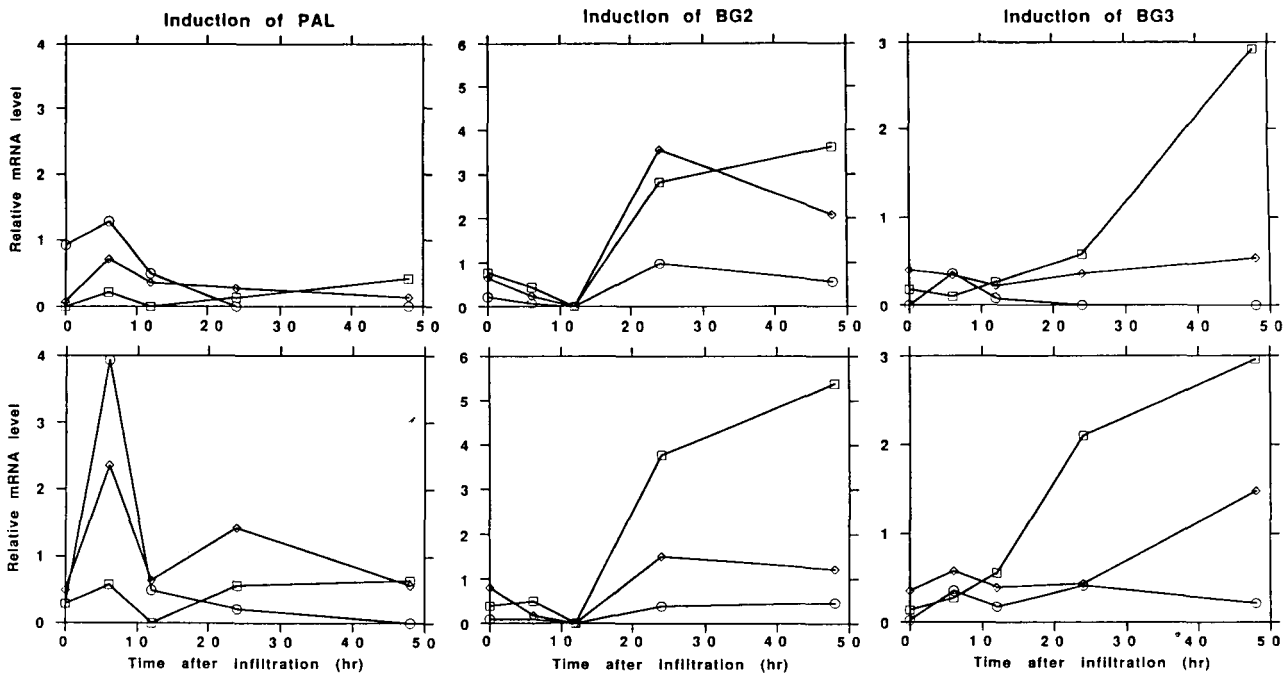


**Figure 6.** Kinetics of PAL, BG2, and BG3 mRNA Accumulation in *Arabidopsis* Leaves Infiltrated with Virulent and Avirulent *P. syringae* Strains.

The data shown in Figure 5 were quantitated by normalizing the number of radioactive counts in each band of the RNA gel blot (obtained using a Betascope 603 Blot Analyzer) with that of the constitutive gene control and then subtracting the number of counts obtained at each time point following infiltration with $MgCl_2$. *Pst* MM1065 (○), *Psm* ES4326 (□), or *Psm* ES4326/pMMXR1 (◇) were used to infiltrate *Arabidopsis* Columbia leaves with a low ($10^6$ cfu/mL; top panel) or a high ($10^7$ cfu/mL; bottom panel) bacterial titer.

Interestingly, the growth of ES4326/pMMXR1 was reduced 50-fold compared with ES4326 in the turnip cultivar Just Right, whereas ES4326 and ES4326/pMMXR1 grew to the same extent as another cultivar, Shogoin. This result suggests that Just Right may carry a resistance gene that specifically recognizes *avrRpt2* and that specific resistance genes may be widespread with the Cruciferae.

### PAL Induction in Response to Virulent and Avirulent Strains

A variety of experiments have shown that genes in the phenylpropanoid pathway such as PAL and CHS are induced to the same extent by virulent and avirulent strains but that avirulent strains elicit a much more rapid response than virulent strains (Bell et al., 1986; Esnault et al., 1987; Lawton and Lamb, 1987; Jahnen and Hahlbrock, 1988; Habereder et al., 1989; Voisey and Slusarenko, 1989; Benhamou et al., 1990a, 1990b). In contrast, our results indicate that the kinetics of PAL induction by the virulent strain *Psm* ES4326 and the avirulent *Pst* MM1065 are the same but that the avirulent strain *Pst* MM1065 is significantly more effective at inducing PAL than the virulent strain *Psm* ES4326. In the published experiment most comparable to ours, Dhawale et al. (1989) showed that a soybean CHS gene was activated more strongly by an avirulent race of *P. syringae* pv *glycinea* than by a virulent race and, as we also observed, that the kinetics of activation were the same in both cases.

### Role of Avirulence Genes in PAL and BG Activation

Our data indicate that a cloned putative *avr* gene, *avrRpt2*, carried on pMMXR1 specifically affects expression of plant defense-related genes. Interestingly, ES4326/pMMXR1 behaved like the avirulent strain *Pst* MM1065 in that it elicited a resistance response, grew to lower levels in leaves, induced the rapid and transient accumulation of PAL mRNA, and failed to induce the gradual accumulation of BG1 and BG3 mRNA. These observations suggest that PAL responds to a signal generated by pMMXR1, whereas the BG1 and BG3 genes appear to be responding to a signal that may be generated by substantial bacterial growth that is likely not pathotype specific.

Unlike BG1 and BG3, ES4326/pMMXR1 elicited BG2 mRNA similarly to ES4326. One explanation for this result is that induction of BG2 is more sensitive to bacterial growth than BG1 and BG3 and that the total number of ES4326/pMMXR1 cells that accumulated during the course of the infection was sufficient to activate BG2 fully.

### Role of the Phenylpropanoid Pathway in the *Arabidopsis* Defense Response

CHS is a key enzyme in the phenylpropanoid pathway downstream of PAL that leads to the synthesis of flavonoid compounds. CHS is induced in elicitor-treated or fungal pathogen-treated bean tissue culture cells or hypocotyls (Collinge and Slusarenko, 1987; Hahlbrock and Scheel, 1989; Dixon and Lamb, 1990) and in soybean leaves infiltrated with an avirulent race of *P. syringae* pv *glycinea* (Dhawale et al., 1989). Because the expression of the *Arabidopsis* CHS gene was not affected by inoculation with virulent or avirulent *P. syringae* strains, we think that it is unlikely that flavonoid compounds are produced as a defense response in *Arabidopsis*. This observation is consistent with the lack of CHS induction that we observed in elicitor-treated *Arabidopsis* tissue culture cells (Davis and Ausubel, 1989). On the other hand, these same tissue culture experiments showed that two other enzymes in the phenylpropanoid biosynthetic pathway specifically involved in the synthesis of lignins, caffeic acid *O*-methyltransferase and peroxidase, were induced after treatment with $\alpha$-1,4-endopolygalacturonic acid lyase elicitor (Davis and Ausubel, 1989). This observation and the rapid induction of PAL by pMMXR1 reported here suggest that products of the phenylpropanoid pathway may play an important role in the *Arabidopsis* defense response.

### Role of BG in the *Arabidopsis* Defense Response

The late induction of BG during the defense response is consistent with a model proposed by Mauch and Staehelin (1989) that states that $\beta$-1,3-glucanase synthesized after infection is deposited into vacuoles and functions as a last line of defense when plant cells are lysed. Mauch and Staehelin also proposed that BG molecules synthesized before infection generate oligosaccharide elicitors that in turn activate defense gene expression. Putting our observations in the context of the Mauch and Staehelin model, it is possible that induction of the *Arabidopsis* BG genes occurs during the course of an infection as a result of the production of oligosaccharide elicitors produced by the degradation of pathogen and/or host cell walls. According to this model, the BG genes would be induced autocatalytically. This hypothesis is supported by the data in Figure 5, which show that infiltration of *Arabidopsis* leaves with high doses of an avirulent strain leads to some activation of the BG genes. Additional evidence in support of this model is the observation that inoculation of *Arabidopsis* leaves with a crude fungal glucan elicitor from cell walls of *Phytophthora megasperma* f sp *glycinea* stimulated the accumulation of BG mRNA (X. Dong, unpublished data).

### Isolation of *Arabidopsis* Mutants in Signal Transduction Pathways Leading to Defense Gene Activation

A major goal of the experiments described in this paper was to characterize readily discernible and highly reproducible phenotypes associated with the infection of virulent and avirulent *P. syringae* in *Arabidopsis* leaves. In the case of infection with an avirulent strain, we sought to correlate the elicitation of a resistance response with a well-defined bacterial DNA fragment to decrease the possibility that *Arabidopsis* is responding to more than one signal. We believe that we have achieved these goals and are now in a position to start screening for *Arabidopsis* mutants that contain lesions in the components of the signal transduction pathways leading to the defense response.

For example, because the HR response elicited by ES4326/pMMXR1 and the pathogenic response elicited by *Psm* ES4326 are separated by at least 24 hr (Figure 1), it is easy to distinguish these phenotypes. This clear-cut temporal separation of the HR and the disease symptoms will facilitate the screening for mutant *Arabidopsis* plants that fail to give an HR after infiltration with ES4326/pMMXR1. Mutations obtained in this manner should define *Arabidopsis* resistance genes.

The fact that the *Arabidopsis* PAL gene is activated rapidly by ES4326/pMMXR1 but not by *Psm* ES4326 will also facilitate the isolation of mutants that are defective in the signal transduction pathways leading to PAL activation. Moreover, because the PAL and BG genes appear to be activated by different signal transduction pathways, screening for mutants that aberrantly express PAL and BG should yield at least two different categories of mutations.

## METHODS

### Strains, Plasmids, and Media

*Pseudomonas syringae* pv *tomato* strains JL1065 and DC3000 and *P. syringae* pv *maculicola* strains 4326 and 5034 were obtained from B. Staskawicz. A spontaneous nalidixic acid-resistant mutant of *Pst* JL1065 and spontaneous streptomycin-resistant mutants of *Psm* 5034 and 4326 were isolated and called MM1065, MM5034, and ES4326, respectively. *P. syringae* strains were grown at 30°C in King's medium B (King et al., 1954) or in minimal M9 medium (Sambrook et al., 1989), and *Escherichia coli* strains were grown at 37°C in LB medium (Sambrook et al., 1989).

Culture media were supplemented with 15 μg/mL nalidixic acid, 100 μg/mL streptomycin, or 10 μg/mL tetracycline as required.

Plasmid pLAFR3-76 is a derivative of pLAFR3 (Swanson et al., 1988) containing an expanded polylinker (Rahme et al., 1991). Plasmid pCHS3.9 carries the *Arabidopsis thaliana* CHS gene (Feinbaum and Ausubel, 1988), plasmid pGL43 carries a cDNA clone of a tobacco (*Nicotiana tabacum*) β-1,3-glucanase gene (Mohnen et al., 1985), and plasmid pAtPalRI carries the entire genomic sequence of an *Arabidopsis* PAL gene (K. Davis, E. Schott, and F. Ausubel, manuscript submitted for publication) cloned into pSK⁻ (Stratagene, Inc.).

*Arabidopsis* ecotypes No, Col (Columbia), Cvi, Ll, Bus, Ms, Aa, Be, Ge, Ws, Bov, Est, Qes, Hav, Turk-lake, Kas (Kashmir), Mh, Sei, Ba, Greenville, Kindalville, Bur, Nd, Fr, Ag, Ct, Co, and Landsberg erecta were obtained from the *Arabidopsis* Information Service, Frankfurt, Federal Republic of Germany. *Arabidopsis* ecotypes Mv and Jp were isolated from naturalized populations growing in Massachusetts. *Arabidopsis* seedlings were grown in Metro-Mix 200 medium for 2 weeks in a climate-controlled greenhouse (21°C ± 1°C) with supplemental fluorescent lighting (16-hr day) and then transferred to a growth chamber at 20°C with a photoperiod of 12 hr and a light intensity of 100 $\mu E/m^2$ sec. Radish (cv Champion), Turnip (cv Just Right and Shogoin), and cabbage (cv Early Jersey) were grown in the greenhouse as described above.

### Cloning of a *P. syringae* pv *tomato avr* Gene

A library of *Pst* MM1065 DNA in pLAFR3 (kindly provided by L. Rahme) was conjugated into *Psm* ES4326 by triparental mating using pRK2013 in *E. coli* strain MC1061 as the helper plasmid (Ruvkun and Ausubel, 1981). *Psm* transconjugants were selected on King's B medium containing streptomycin and tetracycline. Individual transconjugants were screened for an avirulent phenotype when infiltrated into *Arabidopsis* ecotype Columbia at a concentration of $10^5$ cfu/mL. One cosmid, pLM22B, that conferred an avirulent phenotype to *Psm* ES4326 contained a 23.5-kb insert.

Subclones of pLM22B were generated by digestion with combinations of restriction enzymes. Overlapping subclones covering the entire 23.5-kb insert were conjugated into *Psm* ES4326 from DH5α as described above. One subclone containing an 8.7-kb XhoI-EcoRI fragment conferred an avirulent phenotype to *Psm* ES4326. Subsequently, a 2.95-kb BamHI fragment was identified that also conferred the avirulent phenotype.

### Infection with *Pseudomonas*

*Arabidopsis* plants with well-expanded rosettes but which had not yet bolted (4 weeks to 6 weeks old) were infiltrated with *P. syringae* strains that had been grown overnight in King's B medium and then washed twice with 10 mM $MgCl_2$. Approximately 3 μL to 10 μL of an appropriate dilution (in 10 mM $MgCl_2$) was inoculated into the underside of intact leaves using a 1-mL plastic syringe without a needle (Swanson et al., 1988). Approximately 10 μL was sufficient to infiltrate half an *Arabidopsis* leaf. For RNA isolation, 10 to 20 leaves were collected and frozen in liquid nitrogen. To determine bacterial growth in leaves, leaf discs (0.5 $cm^2$) were made with a cork borer and the bacteria in the leaf tissue extracted by macerating the discs with a plastic pestle in 0.3 mL of 10 mM $MgCl_2$. Serial dilutions were plated on King's B plates containing streptomycin or nalidixic acid. Bacterial titers were determined on the hours indicated, and at least five different samples were taken per time point. We observed that the number of bacterial cells determined by plating ES4326/pMMXR1 on streptomycin media was approximately fourfold higher than the number of cells determined by plating on media containing both streptomycin and tetracycline. We attributed this difference in bacterial count to spontaneous loss of pMMXR1 during growth. Two-week-old turnip, radish, and cabbage plants were infected by vacuum infiltration with a bacterial suspension in 10 mM $MgCl_2$ at $10^5$ cfu/mL.

### Cloning of BG Genes

Twenty positive plaques were obtained after screening $2 \times 10^4$ plaques of a λFIX library of *Arabidopsis* ecotype Landsberg DNA (Voytas and Ausubel, 1988) using the tobacco cDNA clone pGL43 (Mohnen et al., 1985) as a probe. One of the clones, λAtBG, was found to contain all of the *Arabidopsis* sequences homologous to the tobacco clone and was used for further subcloning. Sequence analysis of the subclones showed that λAtBG contained the coding regions for three β-1,3-glucanase genes. All molecular cloning techniques were carried out using standard protocols (Sambrook et al., 1989; Ausubel et al., 1990).

### RNA Isolation and Characterization

RNA was isolated by phenol-sodium dodecyl sulfate extraction and LiCl precipitation (Ausubel et al., 1990). The RNA samples (5 μg) were separated on formaldehyde-agarose gels and transferred to GeneScreen (Du Pont-New England Nuclear) (Davis and Ausubel, 1989). The filters were prehybridized and hybridized in 0.5 M $Na_2HPO_4$, pH 7.2, 7% SDS, 10 mg/mL BSA (Church and Gilbert, 1984) at 60°C. BG1, BG2, BG3, CHS, and PAL fragments were labeled by a random priming reaction (Boehringer Mannheim) and were added to the prehybridization buffer at a final concentration of $1 \times 10^6$ cpm/mL. The filters were hybridized 16 hr to 20 hr and then washed at 65°C for 1 hr with two changes of 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate), 1% SDS.

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