

# Age-Related Resistance in *Arabidopsis* Is a Developmentally Regulated Defense Response to *Pseudomonas syringae*

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Age-related resistance (ARR) has been observed in a number of plant species; however, little is known about the biochemical or molecular mechanisms involved in this response. *Arabidopsis* becomes more resistant, or less susceptible, to virulent *Pseudomonas syringae* (pv *tomato* or *maculicola*) as plants mature (in planta bacterial growth reduction of 10- to 100-fold). An ARR-like response also was observed in response to certain environmental conditions that accelerate *Arabidopsis* development. ARR occurs in the *Arabidopsis* mutants *pad3-1*, *eds7-1*, *npr1-1*, and *etr1-4*, suggesting that ARR is a distinct defense response, unlike the induced systemic resistance or systemic acquired resistance responses. However, three salicylic acid (SA) accumulation-deficient plant lines, *NahG*, *sid1*, and *sid2*, did not exhibit ARR. A heat-stable antibacterial activity was detected in intercellular washing fluids in response to *Pst* inoculation in wild-type ARR-competent plants but not in *NahG*. These data suggest that the ability to accumulate SA is necessary for the ARR response and that SA may act as a signal for the production of the ARR-associated antimicrobial compound(s) and/or it may possess direct antibacterial activity against *P. syringae*.

## INTRODUCTION

The relationship between plant age and disease resistance has been investigated in many plant-pathogen systems (Bateman and Lumsden, 1965; Griffey and Leach, 1965; Hunter et al., 1977; Lazarovits et al., 1981; Ward et al., 1981; Miller, 1983; Chase and Jones, 1986; Reuveni et al., 1986; Pretorius et al., 1988; Koch and Mew, 1991; Chang et al., 1992; Heath, 1993; Rupe and Gbur, 1995). Some plants become more susceptible to certain pathogens as they develop (Miller, 1983); however, susceptibility decreases with increasing leaf age in the rice/*Xanthomonas campestris* pv *oryzae* (Koch and Mew, 1991) and rice/*Pyricularia oryzae* (Roumen et al., 1992) interactions. In contrast, older plants (both young and mature leaves) display increased resistance in the wheat/*Puccinia recondita* f.sp. *tritici* (Pretorius et al., 1988) and tobacco/*Peronospora tabacina* (Reuveni et al., 1986) interactions. When older leaves/plants display increased resistance or reduced susceptibility to pathogens, this form of resistance often is referred to as age-related resistance (ARR).

The actual mechanisms responsible for the different forms of ARR have been studied in a preliminary manner in only a few cases. In cowpea/rust and cereal/rust interac-

tions, an ARR response is thought to be controlled by single resistance genes expressed in adult plants (Roelfs, 1984; Heath, 1993). Ward et al. (1981) and Lazarovits et al. (1981) observed a positive correlation between increasing plant age, glyceollin production, and resistance to *Phytophthora megasperma* var *sojiae* in soybean. A similar correlation was observed for the accumulation of a cotton phytoalexin in response to *Verticillium albo-atrum* infection (Bell, 1969), constitutive accumulation of terpenoids in older cotton plants (Hunter et al., 1977), or capsidiol accumulation in mature pepper plants in response to *Phytophthora capsici* (Hwang, 1995). The positive correlation observed between the production of defense-associated compounds in older leaves and plants displaying ARR suggests that the various forms of ARR may be controlled developmentally. It also is possible that, in some cases, ARR may result from the accumulation of toxic compounds during the life cycle of the plant.

A number of genes with possible defense functions are expressed late in plant development, when ARR usually is observed, suggesting that they may be involved in ARR. For example, some pathogenesis-related (PR) and PR-like genes are upregulated during flower development and senescence (Fraser, 1981; Lotan et al., 1989; Buchanan-Wollaston, 1994; Hanfrey et al., 1996; Butt et al., 1998; Quirino et al., 1999). Older leaves of flowering tobacco accumulate specific PR proteins (PR-1, PR-2, and PR-3), and this correlates with increased resistance to viral and fungal pathogens (Fraser, 1972; Takahashi, 1972; Reuveni et al., 1986; Wyatt et al., 1991). A subsequent study demonstrated that the

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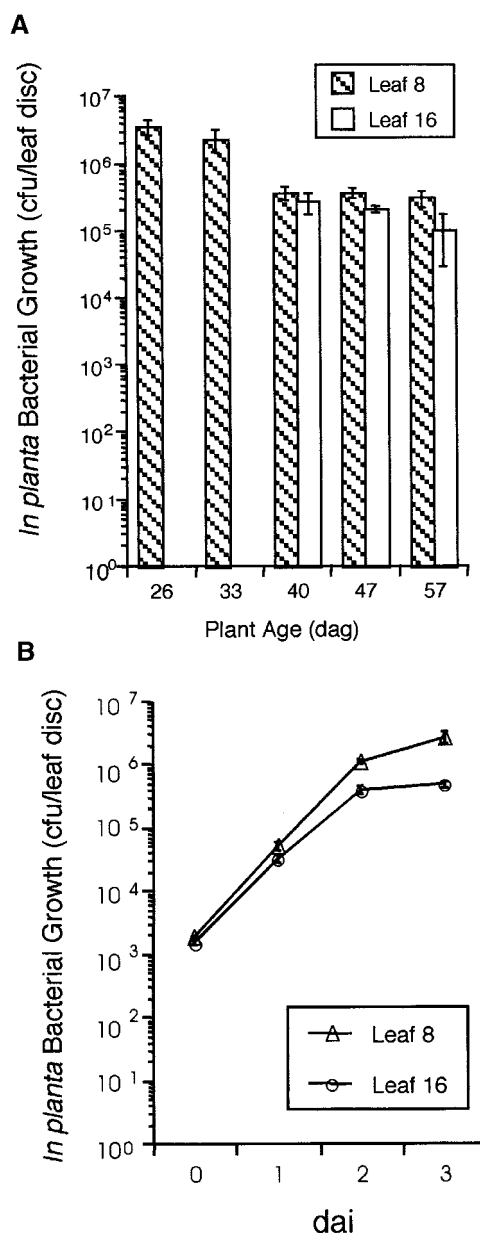
ARR response in tobacco also was associated with a five-fold increase in endogenous salicylic acid (SA) (Yalpani et al., 1993). This example of ARR in tobacco resembles the systemic acquired resistance (SAR) response.

SAR is an inducible defense response that leads to broad-spectrum systemic resistance after an initial "immunizing" infection (Hammerschmidt, 1999) and is associated with SA accumulation and *PR-1* expression in both inoculated and systemic tissue (Kuc, 1982; Ward et al., 1991; Yalpani et al., 1991; Uknes et al., 1992, 1993; Cameron et al., 1999). In the course of studying the SAR response, we observed that older *Arabidopsis* plants become more resistant to normally virulent *Pseudomonas syringae* pv *tomato* (*Pst*) regardless of SAR induction. Preliminary experiments suggested that ARR in the *Arabidopsis/Pst* system was different from the SAR response. We also considered the possibility that ARR could be a form of induced systemic resistance (ISR) resulting from contact with soil microbes during the course of our experiments. ISR occurs in plants colonized with nonpathogenic plant growth-promoting rhizobacteria such that they become resistant to subsequent infection with virulent pathogens (reviewed by Van Loon et al., 1998). ISR occurs in transgenic *NahG* plants (Delaney et al., 1994), which cannot accumulate SA, suggesting that, unlike the SAR response, SA accumulation is not required for ISR (Pieterse et al., 1998). Moreover, functional jasmonate and ethylene signaling pathways are necessary for the ISR response in *Arabidopsis* (Pieterse et al., 1998). In this work, we present data that suggest that ARR is distinct from the ISR and SAR pathways in that the NPR1 gene product is not required. However, our results also suggest that the ability to accumulate SA is a necessary component of the ARR pathway.

## RESULTS

### In Planta *Pst* Growth Is Reduced in Older *Arabidopsis* Plants

Wild-type *Arabidopsis* ecotype Columbia (Col-0) plants of different ages were inoculated with  $10^6$  colony-forming units (cfu)/mL of virulent *Pst*. Older plants became less susceptible, or more resistant, to virulent *Pst*, as demonstrated by a 10-fold reduction in bacterial growth between 30 and 40 days after germination (dag) (Figure 1A). In other experiments, plants displayed a 100-fold reduction in bacterial growth in a more gradual manner over 50 days (Figure 2A). Mature plants exhibiting ARR were symptomless or slightly chlorotic at the site of inoculation compared with young plants, which displayed typical chlorotic water soaking over the entire leaf. This ARR response also has been observed in ecotypes Wassilewskija and Bensheim and in response to a different pathovar, *P. syringae* pv *maculicola* (*Psm*), as demonstrated by a 100-fold reduction in bacterial growth in mature plants compared with young plants (data not shown).



**Figure 1.** In Planta Growth of Virulent *Pst* in Leaves 8 and 16 for 57 Days.

**(A)** Leaves 8 and 16 were inoculated with  $10^6$  cfu/mL virulent *Pst* at  $\sim 1$ -week intervals from 26 to 57 dag. In planta bacterial growth was monitored 3 days after inoculation (dai) and is presented as the mean of five samples  $\pm$ SE. In planta bacterial growth in leaf 8 at 26 dag was significantly different from in planta bacterial growth in leaves 8 and 16 at 40, 47, and 57 dag, as determined by Student's *t* test ( $P \leq 0.05$ ).

**(B)** In planta bacterial growth in mature Col-0 plants (42 dag) was measured over 3 dai ( $10^6$  cfu/mL virulent *Pst*) in leaves 8 and 16 and is presented as the mean of five samples  $\pm$ SE.

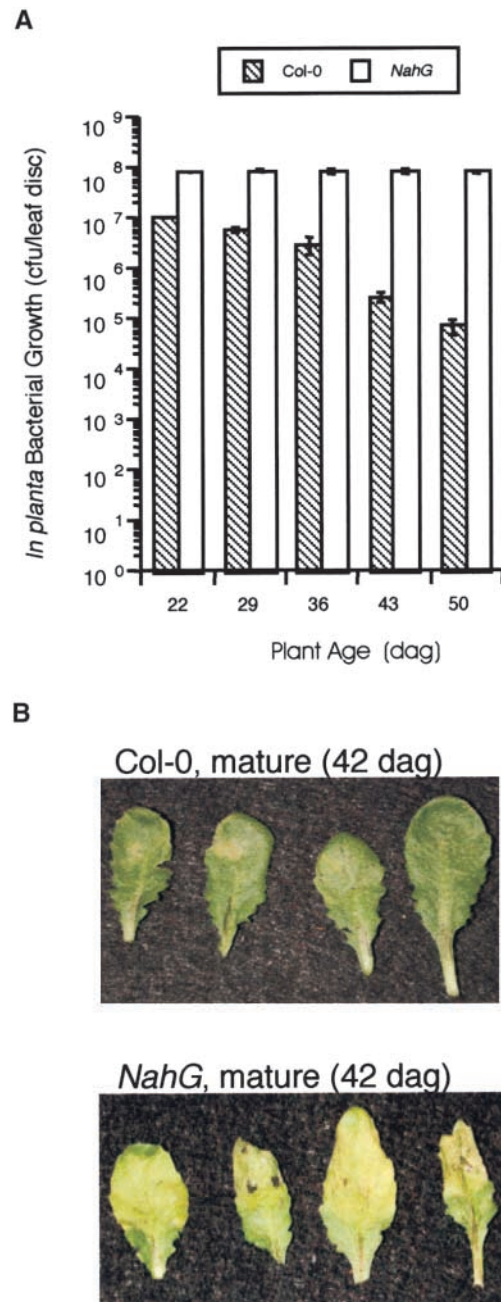
The experiments in both **(A)** and **(B)** were repeated two additional times with similar results.

All ARR experiments were performed using overnight *P. syringae* cultures grown to midexponential log phase to ensure that similar bacterial numbers were inoculated into plants at each time point throughout each experiment.

To prove that the reduction in bacterial growth in older plants is not caused by potential differences in inoculum concentration, ARR experiments were conducted on plants whose growth was synchronized so that young and mature plants were inoculated on the same day with the same inoculum. ARR also was observed, such that mature Col-0 supported at least 10-fold less bacterial growth than young Col-0 inoculated at the same time (Figure 3). Additionally, differences in leaf morphology between young and mature plants could affect the in planta bacterial concentration immediately after inoculation (day 0) of plants of different ages and therefore the final bacterial concentration on day 3 after inoculation. This was not observed, because similar levels of bacteria were detected in leaves 8 to 12 of plants of different ages immediately after inoculation (day 0) with  $10^6$  cfu/mL *Pst* (average of five replicates  $\pm$ SD [22 dag,  $304 \pm 48$  cfu/leaf disc; 28 dag,  $612 \pm 54$  cfu/leaf disc; 40 dag,  $619 \pm 63$  cfu/leaf disc; 49 dag,  $669 \pm 49$  cfu/leaf disc]). Young plants (22 dag) displayed a twofold lower bacterial concentration immediately after inoculation, perhaps because the cells in young expanding leaves are tightly packed with fewer intercellular spaces (Esau, 1977; Donnelly et al., 1999). It is interesting that young plants still support vigorous bacterial growth (Figures 1A and 2A) even when twofold fewer bacteria are inoculated into leaves. These results indicate that the reduced bacterial growth observed in older plants is not attributable to variation in inoculum concentrations or the number of bacteria infiltrated into young versus mature leaves.

ARR also could be the result of transplantation stress and the active oxygen species produced by mechanical damage (Yahraus et al., 1995). Therefore, the ARR response was monitored in plants sown directly to soil or in plants that were transplanted at the seedling stage from medium to soil. ARR developed in a similar manner with both growth methods in repeated experiments (data not shown), indicating that transplantation-induced mechanical damage and the associated active oxygen species production are not involved in the ARR response.

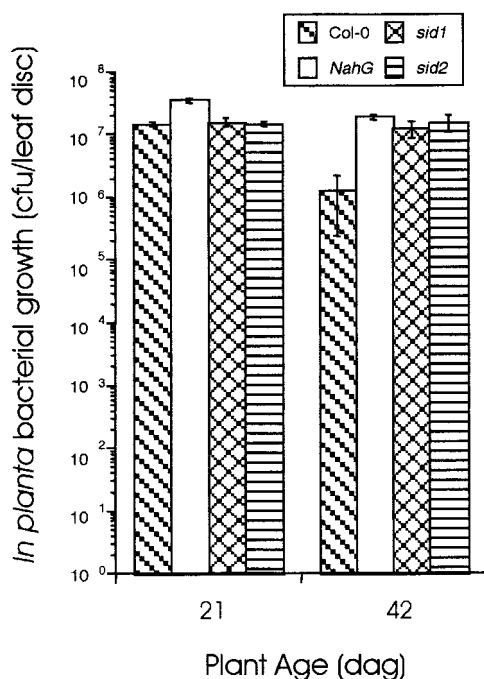
Young and mature leaves on older plants were followed in a number of experiments to determine if the ARR response in Arabidopsis is a whole-plant phenomenon affected by the overall age of the plant or whether it is a leaf-specific response pertaining to individual leaf age. For example, if ARR is governed by the developmental state of individual leaves, young leaves on mature plants would not exhibit ARR. Leaf 8 was chosen to represent an older leaf because it is formed well after the transition from juvenile to adult phase (Telfer et al., 1997; Donnelly et al., 1999) and is macroscopically visible at 14 dag (and therefore present throughout the experiment). Additionally, it is one of the leaves (leaves 8 to 12) normally inoculated in our infection experiments. Leaf 16 represents a younger leaf that becomes macroscopically



**Figure 2.** In Planta Bacterial Growth in Arabidopsis for 50 Days in *NahG* Versus *Col-0*.

**(A)** *Col-0* and *NahG* plants were inoculated with  $10^6$  cfu/mL virulent *Pst* at  $\sim$ 1-week intervals from 22 to 50 dag. In planta bacterial growth was monitored 3 dai and is presented as the mean of five samples  $\pm$ SD. A significant difference (Student's *t* test,  $P < 0.00003$ ) was observed between in planta *Pst* growth in young *Col-0* plants (22 dag) compared with mature *Col-0* plants (43 and 50 dag). This experiment was repeated at least three additional times with similar results.

**(B)** Mature (43 dag) *Col-0* and *NahG* leaves inoculated with  $10^6$  cfu/mL virulent *Pst* and photographed 3 dai.



**Figure 3.** In Planta Bacterial Growth in Col-0, *NahG*, *sid1*, and *sid2*.

Col-0, *NahG*, *sid1*, and *sid2* plants were inoculated with  $10^6$  cfu/mL virulent *Pst* at 21 and 42 dag. In planta bacterial growth was monitored 3 dai and is presented as the mean of five samples  $\pm$ SD. A significant difference (Student's *t* test,  $P < 0.001$ ) was observed in *Pst* growth in corresponding young versus mature Col-0 plants. This experiment was repeated two additional times with similar results.

visible at  $\sim$ 23 dag. In planta bacterial growth was reduced 10-fold in leaf 8 on mature plants (40, 47, and 57 dag) compared with young plants (26 dag) (Figure 1A). At 40 dag, leaf 16 supported similarly low in planta bacterial growth, and this was reduced further at 47 and 57 dag. These data suggest that the age of the plant, rather than individual leaf age, regulates the expression of ARR.

Leaves 8 and 16 are morphologically different: leaf 16 has a narrow leaf blade with more trichomes compared with leaf 8. To determine if these morphological differences could account for the observed reduction in in planta bacterial growth in leaf 16, the growth of *Pst* during 3 days in leaves 8 and 16 was monitored in mature plants (50 dag). There was no significant difference in leaf 8 and 16 bacterial levels immediately after inoculation (day 0); therefore, differences in leaf morphology did not affect the number of bacteria that were infiltrated successfully into the leaves (Figure 1B). In both leaves 8 and 16, bacterial growth was reduced similarly over 3 days, but to a greater extent in leaf 16 compared with leaf 8 (Figure 1B). These data suggest that a stronger ARR response correlates positively with the length of time a leaf spends on a plant that is expressing ARR.

### ARR Response Differs from the SAR and ISR Response Pathways

There is increasing evidence that the signal transduction pathways leading to different types of disease resistance share common components (Dong, 1998; Maleck and Dietrich, 1999). This prompted us to examine a number of Arabidopsis defense response mutants to determine if these gene products are required for a successful ARR response. The *npr1-1* mutant is defective for SAR and is more susceptible to virulent pathogens (Cao et al., 1994; Delaney et al., 1995). Interestingly, mature versus young *npr1-1* plants exhibited a 10-fold reduction in in planta bacterial growth, even though they were more susceptible to *Pst* than was Col-0 (Table 1). This finding suggests that an intact SAR pathway is not required for the ARR response. Moreover, functional NPR1 is required for the ISR response and ARR occurs in *npr1*, suggesting that ARR is not a form of ISR.

The contribution of the Arabidopsis phytoalexin, camalexin, was determined by testing *pad3-1* for the ARR response. The *pad3-1* mutant was chosen because it accumulates very little camalexin in response to *Pst* but exhibits a wild-type response to both avirulent and virulent *Pst* (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). The *pad3-1* mutant displayed ARR to the same degree as Col-0 (Table 1). The *eds7-1* mutant also was tested for the ARR response because it exhibits enhanced disease susceptibility to both *Pst* and *Psm* but is wild type for SAR (including *PR-1* expression) (Rogers and Ausubel, 1997). As with the *npr1-1* and *pad3-1* mutants, the *eds7-1* mutant plants also displayed wild-type levels of ARR (Table 1).

**Table 1.** In Planta Bacterial Growth in Young and Mature Arabidopsis Mutants

Genotype	In Planta Bacterial Growth	
	Young Plants	Mature Plants
Col-0	$6.1 \times 10^6 \pm 1.5 \times 10^5$	$3.0 \times 10^5 \pm 1.0 \times 10^4$
<i>npr1</i>	$1.6 \times 10^7 \pm 2.7 \times 10^5$	$1.7 \times 10^6 \pm 1.7 \times 10^5$
Col-0	$9.9 \times 10^6 \pm 4.4 \times 10^5$	$9.5 \times 10^5 \pm 2.9 \times 10^4$
<i>pad3-1</i>	$9.8 \times 10^6 \pm 1.2 \times 10^6$	$9.3 \times 10^5 \pm 3.5 \times 10^4$
Col-0	$6.1 \times 10^6 \pm 1.7 \times 10^5$	$2.8 \times 10^5 \pm 3.3 \times 10^4$
<i>eds7-1</i>	$1.3 \times 10^7 \pm 4.8 \times 10^5$	$2.2 \times 10^5 \pm 5.6 \times 10^4$
Col-0	$9.9 \times 10^6 \pm 4.4 \times 10^5$	$9.5 \times 10^5 \pm 2.9 \times 10^4$
<i>etr1-4</i>	$9.7 \times 10^6 \pm 1.1 \times 10^6$	$1.2 \times 10^6 \pm 2.8 \times 10^5$

Various Arabidopsis mutants plus wild type (Col-0) were inoculated with virulent *Pst* at  $10^6$  cfu/mL. In planta bacterial growth was determined at 3 dai and is presented as cfu/leaf disc  $\pm$ SE. Each Col-0/mutant experiment was repeated three times with similar results. Young plants ranged in age from 28 to 30 dag, and mature plants ranged in age from 52 to 62 dag.

<sup>a</sup>In planta bacterial growth in old plants was significantly different from that in corresponding young plants ( $P \leq 0.05$ , Student's *t* test).

<sup>b</sup>In planta bacterial growth in old plants was significantly different from that in corresponding young plants ( $P \leq 0.001$ , Student's *t* test).



The *etr1-4* mutant (which is defective in ethylene signaling and ISR) was examined to determine if ARR requires a functional ethylene signaling pathway and to confirm that ARR is not an ISR response to the potential accumulation of microbes in the soil during the experiment. The *etr1-4* plants also were capable of expressing ARR, as demonstrated by a 10-fold reduction in in planta bacterial growth in mature compared with young *etr1-4* (Table 1).

### ARR Does Not Occur in *NahG* Plants

*NahG* plants accumulate little SA during defense responses, display enhanced disease susceptibility to some virulent pathogens, and are compromised in their ability to establish SAR (Delaney et al., 1994; Vernooij et al., 1994). *NahG* plants were tested to determine if SA accumulation is required for the ARR response. As demonstrated in Figure 2, *NahG* plants displayed chlorotic disease symptoms and supported vigorous bacterial growth throughout the 50-day experiment. In contrast, wild-type Col-0 plants were almost completely symptomless and displayed increasing resistance to *Pst* (100-fold reduction) during the course of the experiment (Figures 2A and 2B). These results demonstrate that *NahG* plants do not exhibit the ARR response, suggesting that SA accumulation is required to manifest ARR. To confirm these findings and to ascertain whether *NahG* is ARR defective as a result of the inability to accumulate SA or some other effect on phenylpropanoid metabolism of the *NahG* transgene (Cameron, 2000; Maleck et al., 2000), two other SA accumulation mutants were tested for their ability to manifest ARR. The *sid1* and *sid2* (SA induction-deficient) mutants were chosen because they contain single recessive mutations and accumulate little SA in response to pathogen inoculation. Moreover, they are more susceptible to both virulent and avirulent *Pst* and *Peronospora parasitica* than wild-type plants but less susceptible than *NahG* lines (Nawrath and Métraux, 1999), suggesting that these mutants affect fewer aspects of phenylpropanoid metabolism than does *NahG*.

Both *sid1* and *sid2* supported vigorous in planta bacterial growth in young and mature plants in a manner similar to *NahG*, unlike wild-type Col-0, which displayed a typical ARR response (Figure 3). These observations suggest that the ARR-defective phenotype observed in *NahG* and *sid* plants is attributable to the inability to accumulate SA.

### Accumulation of *PR-1* and *SAG-13* Transcripts during ARR

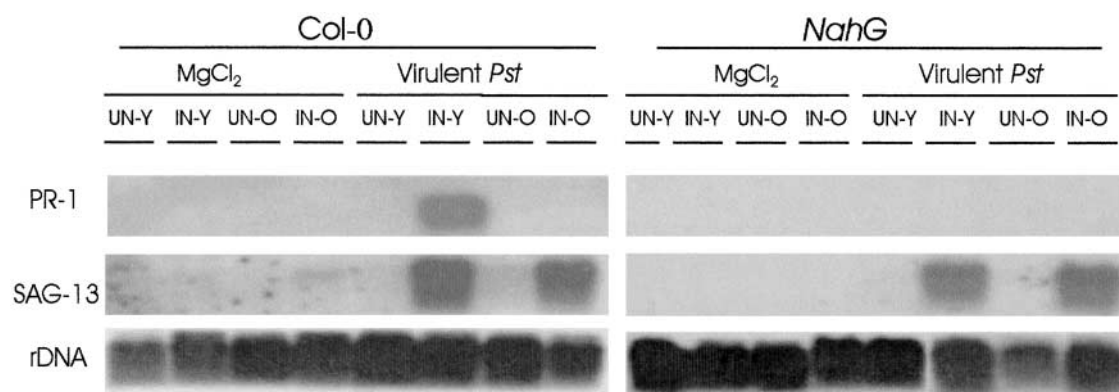
The SAR-defective *npr1-1* mutant expresses little *PR-1*, *PR-2*, or *PR-5* in response to pathogens (Cao et al., 1994) but does exhibit ARR, suggesting that *PR* gene expression and a functional SAR pathway are not required for the ARR response. Therefore, it was intriguing to discover that *NahG*, which also is compromised for *PR-1* gene expression and

SAR, was unable to manifest ARR. To understand these results, *PR-1* expression was monitored in young and mature Col-0 and *NahG* plants. As expected, *PR-1* was expressed weakly at 1 dai in young Col-0 plants (data not shown) but was expressed strongly at 3 dai (Figure 4). In contrast, *NahG* plants did not express *PR-1* when young or mature (Figure 4). Interestingly, mature Col-0 plants that displayed ARR (100-fold reduction in *Pst* growth compared with young Col-0; Figure 2A) did not express *PR-1* in response to virulent *Pst* inoculation at 1 dai (data not shown) or 3 dai (Figure 4). Additionally, there was no expression of *PR-1* in mature mock-inoculated plants, indicating that ARR is not caused by constitutive *PR-1* gene expression in mature leaves, unlike the ARR response in tobacco (Fraser, 1972; Takahashi, 1972; Reuveni et al., 1986; Wyatt et al., 1991; Yalpani et al., 1993). Similar results also were obtained for *PR-5* (data not shown). Clearly, Arabidopsis plants respond differently to the same pathogen at different stages of development. Moreover *PR-1* gene expression does not correlate with the ARR response. This may explain why *npr1-1* plants exhibited ARR even in the absence of *PR-1* expression (Cao et al., 1994).

Because ARR occurs in mature Arabidopsis plants that have not begun to flower, it is possible that ARR is associated with the early stages of senescence. The expression of *SAG-13*, a molecular marker for the early prechlorotic stages of senescence (Weaver et al., 1998), was monitored in the same Col-0 and *NahG* plants described above. *SAG-13* was not expressed in uninoculated or mock-inoculated leaves of Col-0 or *NahG* (young or old). Therefore, it appears that mature Col-0 plants have not yet entered even the early stages of the senescence program. Little *SAG-13* expression was observed in uninoculated leaves of *Pst*-infected plants; however, *SAG-13* transcripts accumulated in leaves inoculated with *Pst* in both Col-0 and *NahG*. This is not unexpected, because it has been observed that some pathogens induce the synthesis of ethylene, and thus senescence (Stall and Hall, 1984), or pathogen-induced chlorophyll destruction (chlorosis) may induce the senescence program (Quirino et al., 2000). These results correspond to the observations that both total protein and carbohydrate (soluble hexoses) levels remain elevated throughout the experimental period (data not shown), suggesting that ARR is not the result of senescence-associated nutrient reduction that could negatively affect in planta bacterial growth.

### Stress Induces an ARR-Like Response in Young Plants

Observations made during the course of these studies led us to hypothesize that certain stressful conditions might induce an ARR-like response in developmentally younger plants. For example, an episode of mild drought, in which the soil dries out in the absence of plant wilting, was correlated with reduced in planta bacterial growth (twofold to sixfold reduction) compared with control plants of the same age (20 to 28 dai;



**Figure 4.** PR-1 and SAG-13 Transcript Accumulation in Young and Mature Col-0 and *NahG* Plants.

Col-0 and *NahG* plants were mock inoculated or inoculated with  $10^6$  cfu/mL virulent *Pst* at 28 dag (young [Y]) and 50 dag (old [O]). Three days after inoculation, leaves were monitored for PR-1, SAG-13, and rDNA transcript accumulation by RNA gel blot analysis in both uninoculated (UN) and inoculated (IN) leaves collected from mock-inoculated and *Pst*-inoculated plants. This experiment was repeated three times with similar results.

data not shown). Additionally, like many *Arabidopsis* researchers (Scholl et al., 1998), we fertilize seedlings once during seedling transfer to soil to standardize plant growth procedures in the laboratory. This fertilization regimen produces healthy plants that do not appear to be limited for nutrients and do not initiate flowering during the experimental period. However, it has been reported that poor nutrition causes stress-induced premature flowering (Martinez-Zapater et al., 1994; Scholl et al., 1998). To determine if limited fertilization can accelerate development and affect the timing of the ARR response, the one-time fertilization regimen was compared with a once per week regimen. Plants that received just one application of fertilizer as seedlings supported reduced levels of bacterial growth as both young and mature plants compared with those fertilized once per week; however, plants fertilized once per week still exhibited ARR (Figure 5). These results suggest that even mild nutrient limitation can affect the level of in planta growth of *Pst* in *Arabidopsis*. We also observed that constantly wet soil sometimes supports algae growth and that plants grown under these conditions exhibit reduced in planta bacterial growth when young (21 to 28 dai) and often flower prematurely (data not shown). These data suggest that various stresses can accelerate *Arabidopsis* development, and this correlates with the appearance of an ARR-like response in young plants.

#### Plants Displaying ARR Produce an Intercellular Antimicrobial Compound

*Pseudomonas* species reside in plant intercellular spaces (Collmer and Bauer, 1994) and therefore may be subject to attack by secreted plant antimicrobial compounds. An in vitro assay for the detection of antimicrobial activity in intercellular washing fluids (IWFs) was used to determine if ARR-

competent plants produce intercellular antimicrobial compounds. An inhibition assay consisting of a short incubation of *Pst* with IWF followed by plating on appropriate medium was chosen to quantify the number of viable *Pst* and to make efficient use of the limited quantities of IWF (15 to 25  $\mu$ L/50 mature leaves), thus allowing sufficient experimental replication. IWFs collected from young Col-0 or *NahG* leaves (mock or *Pst* inoculated) did not inhibit the growth of *Pst* in the in vitro assay (Table 2, experiment 1). Only IWFs collected from mature Col-0 plants inoculated with *Pst* and displaying ARR significantly inhibited the growth of *Pst*, by 20% in experiment 1 and 46% in experiment 2 (Table 2). Antibacterial activity was not observed in IWFs from ARR-defective *NahG* plants (Table 2, experiment 1 or 2). Boiling treatment did not alter the inhibitory activity of IWFs from inoculated mature Col-0 plants (Table 2, experiment 2). Similar levels of bacterial inhibition (20% to 50%) have been observed by others who, like us, used dilute plant IWFs or extracts rather than purified compounds (Smith, 1982; Rauscher et al., 1999; Brader et al., 2001). IWF samples with antibacterial activity were concentrated 10- to 30-fold to increase the level of inhibition observed; unfortunately, antibacterial activity was abolished by this procedure. Overall, these data suggest that ARR is an induced response to *Pst* infection, because antibacterial activity was observed in IWFs from *Pst*-inoculated mature Col-0 but not in IWFs collected from mature mock-inoculated plants.

#### DISCUSSION

*Arabidopsis* plants grown under short daylengths and fertilized once at the seedling stage exhibited ARR to *Pst* and *Psm* in a gradual manner during 8 weeks or more abruptly at

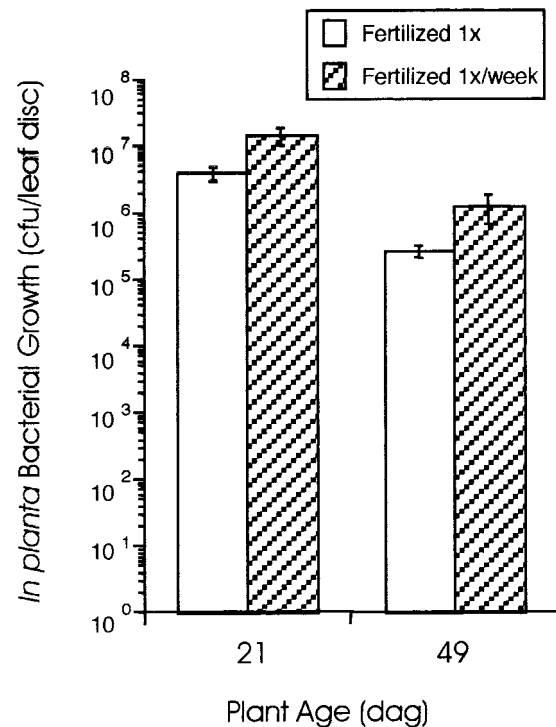
30 to 40 dag. Increasing the fertilization frequency to once per week resulted in a fivefold increase in in planta *Pst* growth in both young and mature plants, but ARR still was observed in mature plants. Therefore, the fertilization regimen significantly affects the level of in planta bacterial growth in Arabidopsis, suggesting that mild nutrient limitation may contribute to the ARR response. This also may explain why ARR is observed during *Pst* infection experiments on a regular basis in our laboratory and highlights the dramatic effects that different fertilization regimens can have on Arabidopsis physiology.

We also observed, but never fully documented, that an ARR-like response was seen in young plants (15 to 21 dag) grown in long day conditions (14 to 24 hr of light) or in plants exposed to stresses such as crowded growth conditions, mild drought, or infestation with thrips or algae. Our empirical observations suggest that these environmental conditions induce Arabidopsis to develop more quickly, as demonstrated by premature flowering in some experiments. This may explain why ARR develops gradually as plants mature in some experiments and more abruptly in experiments that include stress-associated accelerated development. These observations are not unexpected, because stress-induced (poor nutrition, crowded growth conditions, algae growth on soil) transition to flowering has been documented previously (Martinez-Zapater et al., 1994; Scholl et al., 1998). In numerous experiments in which an ARR-like response developed in younger stressed plants, reduced *PR-1* gene expression also was observed (data not shown), suggesting that this response is similar to ARR observed in mature unstressed Arabidopsis plants. The effect of fertilization and other stresses on in planta *Pst* growth in Arabidopsis highlights the major impact that environmental factors have on the level of disease observed in a plant/pathogen interaction (classic disease triangle; Agrios, 1997).

Our studies also indicate that ARR is a whole-plant phenomenon, in that both young and older leaves on mature plants exhibit ARR. Thus, ARR appears to be a developmentally regulated and environmentally sensitive response.

Interestingly, of the mutants tested for their ability to exhibit ARR, all exhibited ARR except the SA accumulation-deficient lines *NahG*, *sid1*, and *sid2*. The *pad3-1* camalexin-deficient mutant (Glazebrook et al., 1997) displayed ARR, indicating that camalexin accumulation is not required for the ARR response. The *eds7-1* mutant was chosen because it is defective in general or horizontal resistance to both *Pst* and *Psm* but displays a normal SAR response (Rogers and Ausubel, 1997). The *eds7-1* mutant line displayed ARR, suggesting that the ARR response is a distinct defense response that does not require a functional EDS7 protein.

The fact that ARR was observed in *npr1-1* plants indicates that *PR-1* gene expression is not necessary for ARR, and this is corroborated by the finding that *PR-1* gene expression is reduced greatly in plants displaying ARR. Both NPR1 and ETR1 are required for the ISR response (Pieterse et al., 1998), and both *npr1-1* and *etr1-4* exhibit ARR,



**Figure 5.** In Planta Bacterial Growth in Plants Fertilized Once Versus Once per Week.

Young (21 dag) and mature (49 dag) Col-0 plants that received fertilizer once at the seedling stage (1×) or were fertilized once per week (1×/week) were inoculated with 10<sup>6</sup> cfu/mL virulent *Pst*. In planta bacterial growth was monitored 3 dai and is presented as the mean of five samples ±SD. A significant difference (Student's *t* test, *P* < 0.05) was observed between in planta *Pst* growth in the corresponding young and mature Col-0 plants. This experiment was repeated two additional times with similar results.

strongly suggesting that ARR is not a form of ISR. Moreover, the early senescence-associated molecular marker *SAG-13* was not expressed in mature plants, suggesting that ARR begins before the onset of the senescence program.

Given the facts that the ARR response is independent of NPR1 function and that *NahG* and *npr1* are defective for SAR, it was surprising to discover that *NahG*, unlike *npr1*, was defective for the ARR response. One could conclude that the ARR response is SA dependent, because *NahG* plants do not accumulate SA in response to infection as a result of the activity of the salicylate hydroxylase transgene (Delaney et al., 1994). However, as discussed elsewhere (Cameron, 2000; Maleck et al., 2000), other components of the phenylpropanoid pathway also may be affected in *NahG* plants. It is possible that in an attempt to compensate for the inability to accumulate SA during pathogen attack, the flux through the phenylpropanoid pathway may be altered in *NahG* plants, reducing the production of other

**Table 2.** IWFs from Plants Displaying ARR Inhibit the Growth of *Pst* in Vitro

IWF Source	In Vitro <i>Pst</i> Growth with or without IWF		
	Col-0	<i>NahG</i>	No IWF
Experiment 1			
Young leaves			
Mock inoculated	1084 ± 36	1101 ± 72	
<i>Pst</i> inoculated	1048 ± 48	1067 ± 54	
Mature leaves			
Mock inoculated	1134 ± 69	1175 ± 186	
<i>Pst</i> inoculated	914 ± 57 <sup>a</sup>	1206 ± 53	
			1084 ± 291 <sup>b</sup>
Experiment 2			
Mature leaves			
Mock inoculated	92 ± 6.1	89 ± 8.8	
<i>Pst</i> inoculated	41 ± 9.5 <sup>a</sup>	90 ± 2.2	
<i>Pst</i> inoculated + B	41 ± 3.5		
			88 ± 5.8 <sup>b</sup>

*Pst* (tetracycline-resistant) bacteria (10  $\mu$ L) were incubated in King's B (KB) medium  $\pm$ IWF for 1 hr and then plated on KB plus rifampicin and tetracycline. The number of cfu/plate was determined 3 days later. IWFs were collected from mock-inoculated and *Pst*-inoculated Col-0 and *NahG* leaves from 4-week-old (young) and 6.5-week-old (mature) *Arabidopsis* plants. IWFs collected from mature *Pst*-inoculated Col-0 leaves were boiled (B) for 10 min before incubation with *Pst*.

<sup>a</sup>Results are presented as means of five replicates  $\pm$ SD. Student's *t* test indicated a significant difference in Col-0 (mock-inoculated) and Col-0 (*Pst*-inoculated) IWF assays ( $P < 0.006$ ). Both experiments were repeated twice with similar results.

<sup>b</sup>*Pst* alone was followed to monitor the number of bacteria present in each sample in the absence of any IWF.

phenylpropanoid-derived compounds, some of which may be important for ARR. However, both *sid1* and *sid2* are unable to manifest ARR, strongly suggesting that the accumulation of SA is an integral part of the ARR response pathway. Perhaps SA acts as a signal molecule, stimulating the production and secretion of antibacterial compound(s) into the intercellular space, and/or its accumulation contributes to the antibacterial activity observed in plants displaying ARR.

The detection of antibacterial activity in IWFs from plants displaying ARR, but not in ARR-defective *NahG* plants, suggests that antibacterial compound(s) present in the *Arabidopsis* intercellular space are responsible for ARR. The heat-resistant nature of the activity further supports the notion that a low-molecular-mass chemical such as SA or a related phenolic, rather than an antibacterial protein(s), is responsible for ARR. The antibacterial activity was detected only in IWFs from mature plants inoculated with *Pst*, not in IWFs from mature mock-inoculated plants, strongly suggesting that the ARR response in *Arabidopsis* is a developmentally regulated and pathogen-induced response. On the other hand, it is possible that antibacterial compounds accumulate as *Arabidopsis* matures and that these com-

pounds are released by the activity of *Pst* virulence factors secreted into plant cells via the type III secretion system (Galan and Collmer, 1999). This seems unlikely because disease symptoms are suppressed in ARR-competent plants, suggesting that the bacterial type III system has little effect on plant cells displaying ARR.

*Pseudomonas* species are biotrophs that have a necrotrophic phase after a period of multiplication in the plant (Collmer and Bauer, 1994). Quorum sensing or population density-dependent sensing has been demonstrated to be important for the expression of virulence in a number of plant pathogens (Pierson et al., 1998). We postulate that the reductions in chlorosis, necrosis, and bacterial growth observed in plants expressing ARR may be attributable to the inhibition of bacterial multiplication by intercellular antibacterial compound(s), such that the bacterial density remains too low to initiate quorum sensing and the switch to the necrotrophic phase, which includes the production of *Pst* virulence factors and disease.

ARR in tobacco is composed of at least two defense signaling pathways that are activated constitutively in a developmentally regulated manner (Hugot et al., 1999). Older tobacco leaves on flowering plants accumulate SA and PR proteins constitutively (Yalpani et al., 1993), and an intercellular compound toxic to *Phytophthora parasitica* zoospores also is produced, even in *NahG* transgenic lines (Hugot et al., 1999). Earlier work in soybean, cotton, and pepper demonstrated a correlation between ARR and phytoalexin accumulation (Bell, 1969; Ward et al., 1981; Kim et al., 1989), and in *Arabidopsis*, ARR to *P. syringae* appears to be a pathogen-induced response involving an antibacterial activity (this work). Like the complex network of defense pathways available to young plants (Feys and Parker, 2000), it appears that different plant species also use different mechanisms to defend themselves against a variety of pathogens as they mature. Previous studies have not addressed whether ARR provides protection to many pathogens. Future studies will determine if ARR in *Arabidopsis* extends to pathogens other than *Pst* and *Psm*.

ARR appears to be a distinct defense response pathway, unlike SAR or ISR, in that NPR1 and ETR1 functions are not necessary. However, SA accumulation appears to be required for a successful response. Future studies to determine the identity of the ARR-associated antibacterial compound(s) and the role of SA in this developmentally regulated pathogen-induced response will provide insights into this novel pathway and, in addition, contribute to the elucidation of the role of stress in accelerating development and ARR in *Arabidopsis*.

## METHODS

### Plant Material and Growth Conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) plants, along with the Col-0 mutants *npr1-1* (X. Dong, Duke University, Durham, NC), *etr1-4*



(E. Meyerowitz, California Institute of Technology, Pasadena, CA), *pad3-1* (J. Glazebrook, Novartis Agricultural Discovery Institute, San Diego, CA), *eds7-1* (F. Ausubel, Massachusetts General Hospital, Boston), and *sid1* and *sid2* (C. Nawrath, University of Fribourg, Switzerland) and the transgenic *NahG* line (K. Lawton, Syngenta, Research Triangle Park, NC), were used in these studies. Seeds were surface sterilized and germinated on Murashige and Skoog (1962) medium. After 10 days under continuous light, seedlings were transferred to soil (Sunshine Mix No. 1 [Sun Gro Horticulture, Bellevue, WA] moistened with 1 g/L 20-20-20 fertilizer) and grown at 22°C under a 9-hr photoperiod with an average light intensity of 150  $\mu\text{Em}^{-2}\text{sec}^{-1}$  for up to 60 days. Plants treated with a once per week fertilization regimen were grown as described above but received 1 to 2 L of fertilizer (1 g/L 20-20-20) once per week for 3 to 4 weeks and then once every 2 weeks for the remainder of the experiment.

### Bacterial Growth and Inoculation Procedures

Avirulent and virulent *Pseudomonas syringae* pv *tomato* (*Pst*) strain DC3000 (rifampicin [rif] and kanamycin [kan] resistant) and *P. syringae* pv *maculicola* (*Psm*) strain 4326 were obtained from Dr. Andrew Bent (University of Wisconsin at Madison) (Whalen et al., 1991). The avirulent *Pst* strain contained the plasmid pV288 harboring the *avrRpt2* gene, whereas the virulent strain contained the same plasmid without the *avrRpt2* gene (pVSP61). Another DC3000 strain (rif and tetracycline [tet] resistant [pL6]; J. Dangl, University of North Carolina, Chapel Hill) was used in the intercellular washing fluid (IWF) experiments. In all experiments, *Pst* or *Psm* from midlog phase overnight cultures shaken at room temperature were diluted to  $10^6$  colony-forming units (cfu)/mL in 10 mM  $\text{MgCl}_2$  and pressure infiltrated into the abaxial sides of leaves 8 to 12 using a needleless 10-mL syringe, filling the intercellular spaces of the entire leaf. Control plants were mock inoculated with 10 mM  $\text{MgCl}_2$ . In planta bacterial growth was determined as described previously with serial dilutions plated on King's B (KB) agar plates containing 100  $\mu\text{g}/\text{mL}$  rif (Sigma) and 50  $\mu\text{g}/\text{mL}$  kan (Sigma) (Wolfe et al., 2000).

### Age-Related Resistance Experimental Design

The growth of *Pst* in planta was monitored for 8 weeks beginning at 22 to 25 days after germination (dag). At each time point (~25, 33, 40, 48, and 57 dag), 20 to 24 plants (four leaves per plant) were inoculated with *Pst* at  $10^6$  cfu/mL and another set of 10 to 14 plants (four leaves per plant) were mock inoculated with 10 mM  $\text{MgCl}_2$ . Leaves were collected at 3 days after inoculation (dai) and frozen for subsequent RNA gel blot analysis. Both inoculated and uninoculated leaves were collected from plants infected with *Pst* or mock inoculated. Leaves also were collected at 3 dai for determination of in planta bacterial growth (eight leaf discs [4 mm diameter] per replicate, five replicates for *Pst*-infected samples and three replicates for the mock control treatment). There was very little *Pst* growth (0 to  $<10^2$  cfu/leaf disc) observed in the negative mock-inoculated control leaves; therefore, these data are not shown. Replicates were averaged, and the SE or SD was calculated. Student's *t* test was used to determine if differences in *Pst* growth between young and mature plants were statistically significant. This experiment was performed on plants germinated first on Murashige and Skoog (1962) agar plates with subsequent transplantation to soil after 10 days of growth in continuous light and also on plants sown directly to soil. The ex-

periment was repeated at least three times for each growing regimen, and similar results were obtained each time (for replicate experiments, see Kus, 1999). Experiments also were conducted in which plant growth was synchronized so that young and mature plants were inoculated on the same day with the same inoculum to determine if differences in inoculum dose were responsible for the reduction in in planta bacterial growth observed in mature plants.

After characterizing age-related resistance (ARR) in Col-0 wild-type plants, young (25 to 30 dag) and old (52 to 62 dag) plants were used to study ARR in the Arabidopsis mutants *npr1-1*, *eds7-1*, *pad3-1*, and *etr1-4* and the transgenic *NahG* line. The various mutant lines were always compared with wild-type plants grown at the same time under the same conditions. Each mutant/Col-0 experiment was repeated three times with similar results each time (for replicate experiments, see Kus, 1999).

To determine whether ARR is a whole-plant response affected by the overall age of the plant or whether it is a leaf-specific response pertaining to the age of individual leaves, experiments similar to those described above were performed. Leaves 8 and 16 were monitored for the ARR response and for leaf blade growth every 2 to 3 days. Both leaves expanded rapidly between 23 and 37 dag, at which time leaf 8 did not expand further, whereas leaf 16 stopped expanding at 43 dag. This experiment was repeated two additional times with similar results.

### Extraction and Analysis of RNA

Uninoculated and inoculated leaves (three leaves per sample) from mock-inoculated plants and plants inoculated with virulent *Pst* ( $10^6$  cfu/mL) were collected at 3 dai and flash frozen in liquid nitrogen. RNA was isolated and analyzed as described previously (Cameron et al., 1999). Arabidopsis cDNA clones for PR-1 (K. Lawton, Syngenta), SAG-13 (R.M. Amasino, University of Wisconsin, Madison), and rDNA (J. Coleman, University of Toronto) were labeled with  $^{32}\text{P}$  by random priming (Random Primer Kit; Amersham). Hybridizations and washes were performed according to Church and Gilbert (1984). This experiment was repeated three times, giving similar results each time (for replicate experiments, see Kus, 1999).

### Collection of IWFs and in Vitro Bacterial Inhibition Assays

Leaves 8 through 12 from young (20 and 25 dag) and mature (40 and 45 dag) plants were infiltrated with either 10 mM  $\text{MgCl}_2$  or  $10^6$  cfu/mL virulent *Pst* (rif and kan resistant). Two days later, leaves were harvested and surface sterilized with 50% ethanol. IWFs were obtained by vacuum infiltrating leaves with sterile distilled  $\text{H}_2\text{O}$  for 25 to 30 min; leaves were surface dried by blotting with absorbent paper or in a salad spinner, and IWFs were collected by centrifugation at 1000g for 30 min at 4°C (Hammond-Kosack, 1992). Fifty mature leaves produced 15 to 25  $\mu\text{L}$  of IWF depending on how effectively the leaves were dried before centrifugation; therefore, some IWF samples might have been more dilute than others. IWFs were collected from young and mature mock- and *Pst*-inoculated leaves and frozen at  $-20^\circ\text{C}$  before use. The IWFs (50  $\mu\text{L}$ ) were incubated with 5 to 10  $\mu\text{L}$  of virulent *Pst* (rif and tet resistant; estimated at  $\text{OD}_{600}$  between 200 and 1000 cfu) and KB broth ( $1 \times$  final concentration) with shaking at room temperature for 1 hr and then plated on KB rif + tet plates. Colonies were counted 3 days after plating, and the percent inhibition of bacterial growth was determined (cfu in IWF from mock-inoculated

leaves minus cfu in IWF from *Pst*-inoculated leaves divided by IWF from mock-inoculated leaves multiplied by 100). *Pst* bacteria were incubated in KB broth because more than 50% died in 10 mM MgCl<sub>2</sub> or sterile water during the 1-hr incubation. Bacterial numbers varied 5- to 10-fold from experiment to experiment (cf. IWFs 1 and 2 in Table 2) because the density of the midlog phase cultures used varied by 5- to 10-fold. A number of controls were included to show that the rif- and kan-resistant *Pst* present in IWFs from *Pst*-inoculated leaves did not grow on the KB rif + tet plates. rif + tet *Pst* growth in the absence of IWF (*Pst* + 4 × KB) was the baseline for normal *Pst* growth. IWFs from mature wild-type plants inoculated with *Pst* were subjected to boiling for 10 min before incubation with rif + tet *Pst* and plating.

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