

# Identification of a Locus in *Arabidopsis* Controlling Both the Expression of Rhizobacteria-Mediated Induced Systemic Resistance (ISR) and Basal Resistance Against *Pseudomonas syringae* pv. *tomato*

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Selected nonpathogenic rhizobacteria with biological disease control activity are able to elicit an induced systemic resistance (ISR) response that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). Ten ecotypes of *Arabidopsis thaliana* were screened for their potential to express rhizobacteria-mediated ISR and pathogen-induced SAR against the leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). All ecotypes expressed SAR. However, of the 10 ecotypes tested, ecotypes RLD and Wassilewskija (Ws) did not develop ISR after treatment of the roots with nonpathogenic *Pseudomonas fluorescens* WCS417r bacteria. This nonresponsive phenotype was associated with relatively high susceptibility to *Pst* infection. The F<sub>1</sub> progeny of crosses between the nonresponsive ecotypes RLD and Ws on the one hand, and the responsive ecotypes Columbia (Col) and Landsberg *erecta* (*Ler*) on the other hand, were fully capable of expressing ISR and exhibited a relatively high level of basal resistance, similar to that of their WCS417r-responsive parent. This indicates that the potential to express ISR and the relatively high level of basal resistance against *Pst* are both inherited as dominant traits. Analysis of the F<sub>2</sub> and F<sub>3</sub> progeny of a Col × RLD cross revealed that inducibility of ISR and relatively high basal resistance against *Pst* cosegregate in a 3 : 1 fashion, suggesting that both resistance mechanisms are monogenically determined and genetically linked. Neither the responsiveness to WCS417r nor the relatively high level of basal resistance against *Pst* were complemented in the F<sub>1</sub> progeny of crosses between RLD and Ws, indicating that RLD and Ws are both affected in the same locus, necessary for the expression of ISR and basal resistance against *Pst*. The corresponding locus, designated *ISR1*, was mapped between markers *B4* and *GL1* on chromosome 3. The observed association between ISR and basal resistance against *Pst* suggests that rhizobacteria-mediated ISR against *Pst* in *Arabidopsis* requires the presence of a single dominant gene that functions in the basal resistance response against *Pst* infection.

Induced disease resistance is the phenomenon that occurs when plants develop an enhanced defensive capacity upon appropriate stimulation. This resistance response is expressed systemically throughout the plant and is effective against a broad spectrum of pathogens (for reviews see Hammer-schmidt and Kuć 1995). The classical way of inducing systemic resistance is by predisposal infection with a necrotizing pathogen. The resulting elevated state of resistance in plant parts distant from the site of primary infection is defined as systemic acquired resistance (SAR) (Ross 1961; reviewed by Ryals et al. 1996). Pathogen-induced SAR is characterized by an early increase in endogenously synthesized salicylic acid (SA) (Malamy et al. 1990; Métraux et al. 1990). SA appears to be an essential signaling molecule in the SAR pathway, because transgenic plants unable to accumulate SA are incapable of developing SAR (Gaffney et al. 1993). Furthermore, SAR is associated with the systemic activation of so-called SAR genes. These include genes that encode pathogenesis-related (PR) proteins (Van Loon 1985; Ward et al. 1991), some of which have *in vivo* antifungal activity and are therefore thought to contribute to the state of SAR (Ryals et al. 1996; Van Loon 1997).

Nonpathogenic rhizosphere-colonizing bacteria have also been shown to induce systemic resistance as well (for review see Van Loon et al. 1998). To differentiate this type of induced resistance from pathogen-induced SAR, the term rhizobacteria-mediated induced systemic resistance (ISR) is used. In *Arabidopsis*, ISR has been studied with the *Pseudomonas fluorescens* strain WCS417r as the inducing agent. With this model system, it was demonstrated that WCS417r-mediated ISR is effective against the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), the fungal root pathogen *Fusarium oxysporum* f. sp. *raphani* (Pieterse et al. 1996; Van Wees et al. 1997), and the fungal leaf pathogen *Peronospora parasitica* (J. Ton, unpublished data), indicating that this type of biologically induced resistance is also effective against different types of pathogens. In contrast to pathogen-induced SAR, WCS417r-mediated ISR is independent of SA accumulation and PR-gene activation (Pieterse et al. 1996). Instead, WCS417r-mediated ISR requires an intact response to the plant hormones ethylene and jasmonic acid (Pieterse et al. 1998). Yet, it is still unknown what defensive

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compounds contribute to the WCS417r-mediated state of enhanced resistance.

Recently, it was demonstrated that the capacity to express WCS417r-mediated ISR in *Arabidopsis* is dependent on the plant genotype. Ecotypes Col and *Ler* were found to be responsive to induction of ISR by WCS417r, whereas ecotype RLD was not (Van Wees et al. 1997). In carnation, cultivar specificity with regard to expression of rhizobacteria-mediated ISR has been reported as well. ISR induced by WCS417r against *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *dianthi*, was clearly expressed in the moderately resistant cultivar Pallas, but less consistently in the susceptible cultivar Lena (Van Peer et al. 1991). This suggests that the level of genetic resistance influences the extent to which ISR is expressed. However, several cases have been reported in which a clear correlation between the capacity to express ISR and basal resistance against the challenging pathogen was absent. For instance, in cucumber it was found that two susceptible cultivars were able to express rhizobacteria-mediated ISR, whereas a resistant cultivar did not (Liu et al. 1995). Moreover, in radish it was demonstrated that both susceptible and resistant cultivars were capable of expressing rhizobacteria-mediated ISR against *Fusarium* wilt (Leeman et al. 1995).

In this study, the genetic basis underlying ecotype specificity of rhizobacteria-mediated ISR in *Arabidopsis* was studied in relation to the level of ecotype-specific basal resistance against *Pst*. Ten *Arabidopsis* ecotypes were tested for their potential to express WCS417r-mediated ISR and their level of basal resistance against *Pst*. We demonstrate that the WCS417r-nonresponsive phenotype correlates with a relatively low level of basal resistance against *Pst*. Furthermore, crosses were made between WCS417r-responsive and WCS417r-nonresponsive ecotypes, after which the resulting F<sub>1</sub>, F<sub>2</sub>, and F<sub>3</sub> progenies were tested for inducibility of ISR by WCS417r, as well as basal resistance against *Pst*. We provide evidence that the potential to express WCS417r-mediated ISR and the relatively high level of basal resistance against *Pst* are both dominant traits that are monogenically inherited and genetically linked.

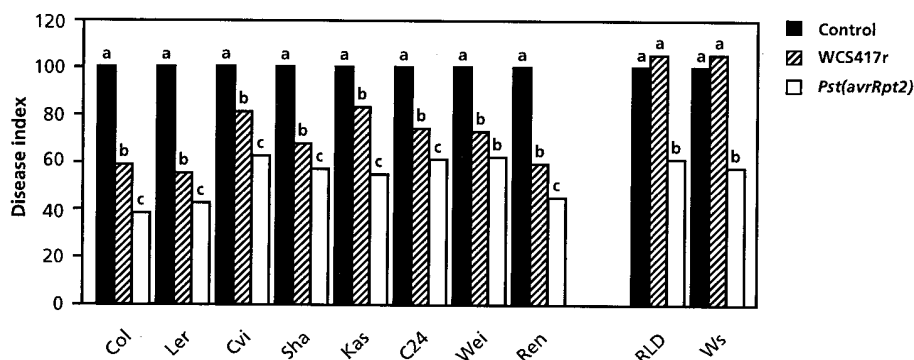
## RESULTS

### *Arabidopsis* ecotypes unable to express WCS417r-mediated ISR exhibit relatively low levels of basal resistance against *Pst*.

Ten *Arabidopsis* ecotypes were tested for their potential to express pathogen-induced SAR and WCS417r-mediated ISR against virulent *Pst*. All ecotypes developed a hypersensitive response in leaves that were injected with avirulent *Pst*(*avrRpt2*) (data not shown), and subsequently expressed SAR against virulent *Pst* in the remaining noninfiltrated leaves (Fig. 1). Ecotypes Col, *Ler*, Cvi, Sha, Kas, C24, Wei, and Ren developed ISR upon treatment of the roots with WCS417r bacteria, indicating that both types of biologically induced disease resistance can be triggered in these ecotypes (Fig. 1). ISR-expressing plants showed a reduction of disease symptoms ranging from 15 to 40%, compared with the control treatments, whereas SAR-expressing plants consistently showed a stronger reduction of disease symptoms (40 to 60%). Ecotypes RLD and Ws developed SAR to the same extent as the other ecotypes. However, they did not develop ISR after treatment of the roots with WCS417r. This nonresponsive phenotype was manifest irrespective of the level of disease incidence (data not shown), ruling out the possibility that ISR was masked by the high susceptibility of these ecotypes to *Pst* (see below).

To determine whether the nonresponsiveness to WCS417r in ecotypes RLD and Ws was due to poor colonization of the rhizosphere, the population density of WCS417r in the rhizosphere of all ecotypes was determined. Table 1 shows that at the end of the bioassays the number of rifampicin-resistant bacteria isolated from the rhizosphere of all ecotypes tested was well above 10<sup>6</sup> per gram of root fresh weight, indicating that the inability of RLD and Ws to express ISR was not the result of reduced root colonization by WCS417r.

As evident in non-induced plants, disease symptoms caused by *Pst* infection on the leaves of both RLD and Ws were characterized by many large necrotic or water-soaked spots, surrounded by extensive chlorosis, whereas disease symptoms on Col plants were much less severe (Fig. 2).



**Fig. 1.** Quantification of induced systemic resistance (ISR) and systemic acquired resistance (SAR) against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) in different *Arabidopsis* ecotypes. ISR was induced by growing plants in soil containing *P. fluorescens* WCS417r bacteria at  $5 \times 10^7$  CFU/g. SAR was induced by pressure-infiltrating a suspension of avirulent *Pst* (*Pst*(*avrRpt2*)) at  $10^7$  CFU/ml into the first two leaves, 4 days prior to challenge inoculation of the upper leaves. Challenge inoculation was performed with a bacterial suspension of virulent *Pst* at  $2.5 \times 10^7$  CFU/ml (ecotypes Col, *Ler*, Sha, Kas, Cvi, C24, Wei, Ren, Ws) or  $10^7$  CFU/ml (RLD). Disease symptoms were scored at 3 or 4 days after challenge. Disease index is proportion of leaves with symptoms relative to the control treatment, which was set at 100%. For each ecotype, different letters indicate statistically significant differences between treatments (Fisher's least significant difference test;  $\alpha = 0.05$ ,  $n = 20$  to 25). Data presented are from a representative experiment performed at least twice for each ecotype, yielding similar results.

Moreover, when inoculated with *Pst* at  $2.5 \times 10^7$  CFU per ml, RLD and Ws showed a higher proportion of leaves with symptoms than control-treated WCS417r-responsive ecotypes (within one representative experiment: RLD, 66.3% and Ws, 75.7%, versus Col, 51.8% and *Ler*, 49.3%). To quantify basal resistance against *Pst* infection, the proliferation of *Pst* in the leaves of non-induced plants was determined by 3 days after inoculation. In accordance with their more pronounced symptoms, the WCS417r-nonresponsive ecotypes RLD and Ws allowed significantly more growth of *Pst*, compared with the WCS417r-responsive ecotypes (Table 2). In the WCS417r-responsive ecotypes *Pst* multiplied up to 3.1 log units over the 3-day time interval, whereas proliferation in RLD and Ws was four- to fivefold higher. These results illustrate that the inability to express ISR is associated with a relatively low level of basal resistance against the challenging pathogen, suggesting that these two resistance mechanisms are related.

#### The potential to express ISR and the relatively high level of basal resistance against *Pst* are inherited as dominant traits.

To elucidate the genetic basis underlying the relationship between ISR and basal resistance against *Pst*, the WCS417r-nonresponsive Arabidopsis ecotypes RLD and Ws were crossed with the WCS417r-responsive ecotypes Col and *Ler*.  $F_1$  progenies of crosses between RLD  $\times$  Col, Ws  $\times$  Col, and Ws  $\times$  *Ler* were tested for their ability to express ISR and their level of basal resistance against *Pst*. All WCS417r-treated  $F_1$  plants developed a level of ISR comparable to that of the WCS417r-responsive parent (Fig. 3), indicating that the potential to express ISR is inherited as a dominant trait. Also, the severity of disease symptoms caused by *Pst* infection (Fig. 3) and the extent of proliferation of *Pst* (Table 2) in control-treated  $F_1$  plants were always similar to those of their control-treated Col or *Ler* parent, indicating that the relatively high level of basal resistance against *Pst* is inherited likewise as a dominant trait.  $F_1$  plants derived from reciprocal crosses behaved similarly (data not shown). Thus, no maternal factors are involved in the potential to express ISR and in the relatively high level of basal resistance against *Pst*.

**Table 1.** Population densities of *Pseudomonas fluorescens* WCS417r bacteria on roots of different Arabidopsis ecotypes<sup>a</sup>

Ecotype	CFU/g of root fresh weight (log) <sup>b</sup>
WCS417r-responsive ecotypes	
Col	6.35 $\pm$ 0.39
<i>Ler</i>	6.10 $\pm$ 0.68
Cvi	6.28 $\pm$ 0.19
Sha	6.88 $\pm$ 0.08
Kas	6.65 $\pm$ 0.05
C24	6.38 $\pm$ 0.26
Wei	6.76 $\pm$ 0.05
Ren	6.54 $\pm$ 0.21
WCS417r-nonresponsive ecotypes	
RLD	6.32 $\pm$ 0.23
Ws	6.33 $\pm$ 0.25

<sup>a</sup> Values presented are means with standard deviations of the log of the average population densities from data of multiple bioassays.

<sup>b</sup> Roots were harvested at the end of the bioassays. In the rhizosphere of nontreated plants, no rifampicin-resistant bacteria were detected (detection limit =  $10^3$  CFU/g).

#### The potential to express ISR and the relatively high level of basal resistance against *Pst* are monogenically determined and genetically linked.

The inheritance of the potential to express ISR and basal resistance against *Pst* was further investigated by studying the segregation patterns of both traits in  $F_2$  plants and  $F_3$  families from the Col  $\times$  RLD cross. In two independent experiments, the percentage of leaves with disease symptoms in control- and WCS417r-treated Col, RLD, and  $F_2$  plants was determined at 4 days after challenge inoculation with *Pst*. Based on the percentage of diseased leaves, each plant was assigned to one of 10 disease severity classes. Control-treated Col plants showed a frequency distribution centering the >50 to 60% disease severity class (Fig. 4). Due to the expression of ISR, WCS417r-treated Col plants showed less symptoms, resulting in a frequency distribution around the >30 to 40% and >40 to 50% disease severity classes. WCS417r-nonresponsiveness and the relatively low level of basal resistance of RLD were reflected by a normal frequency distribution around the >70 to 80% disease severity class in the control- and WCS417r-treated plants. The  $F_2$  of the Col  $\times$  RLD cross showed a broad range of disease severities in control-treated plants. Treatment of the  $F_2$  plants with WCS417r resulted in a 3 : 1 segregation: Seventy  $F_2$  plants exhibited a normal frequency distribution over the same disease severity classes as their WCS417r-treated Col parents, suggesting that this fraction of the  $F_2$  population expressed ISR in association with a relatively high level of basal resistance. In contrast, 28  $F_2$  plants exhibited a normal frequency distribution over the same disease severity classes as their WCS417r-nonresponsive RLD parents, indicating that these plants were nonresponsive to WCS417r and possessed a relatively low level of basal resistance against *Pst* (Fig. 4). These 28 plants were clearly marked by typical RLD-like disease symptoms with many large necrotic or water-soaked spots surrounded by extensive chlorosis (Fig. 2). The cosegregation of responsiveness to WCS417r with a relatively high level of basal resistance on the one hand, and nonresponsiveness to WCS417r with a relatively low level of basal resistance on the other hand, suggests that the potential to express ISR and relatively high basal resistance are genetically linked. In the second experiment a similar cosegregation pattern was observed in the WCS417r-treated  $F_2$  population: 36 plants showed a Col-like phenotype, whereas 11 plants showed an RLD-like phenotype (data not shown). The segregation patterns in both experiments fit a statistically significant 3 : 1 segregation (Table 3), indicating that the potential to express WCS417r-mediated ISR and the relatively high level of basal resistance against *Pst* are dominant traits that are monogenically determined.

To further prove that the potential to express ISR is genetically linked to relatively high basal resistance against *Pst*, 74 individual  $F_2$  plants of the Col  $\times$  RLD cross were selfed, resulting in 74  $F_3$  families. Subsequently, sets of 16  $F_3$  plants of each family were challenge inoculated with *Pst*, after which disease symptoms were monitored at 3, 4, and 5 days after inoculation. Evaluation of the disease symptoms revealed that 17  $F_3$  families were homozygous for Col-like disease symptoms, 40  $F_3$  families were heterozygous and showed both Col- and RLD-like disease symptoms, and 17  $F_3$  families were homozygous for RLD-like disease symptoms. This segregation pattern statistically fits a 1 : 2 : 1 segregation ( $\chi^2 = 0.486$ ;  $P = 0.784$ ), confirming the monogenic inheritance of basal resis-

tance against *Pst*. Subsequently, five randomly selected RLD- and Col-like families were further tested for proliferation of *Pst* in the leaves and responsiveness to induction of ISR by WCS417r. The five F<sub>3</sub> families selected for Col-like disease symptoms were fully capable to express WCS417r-mediated ISR and allowed relatively low levels of *Pst* proliferation, comparable with ecotype Col (Table 4). In contrast, five F<sub>3</sub> families selected for RLD-like disease symptoms did not respond to ISR treatment with WCS417r, and exhibited relatively high levels of *Pst* proliferation, comparable with ecotype RLD (Table 4). The observed cosegregation in the F<sub>3</sub> families of responsiveness to WCS417r and relatively high basal resistance on the one hand, and nonresponsiveness to WCS417r and relatively low basal resistance on the other

hand, demonstrates that both defense mechanisms are genetically linked. We designate the corresponding locus *ISR1*.

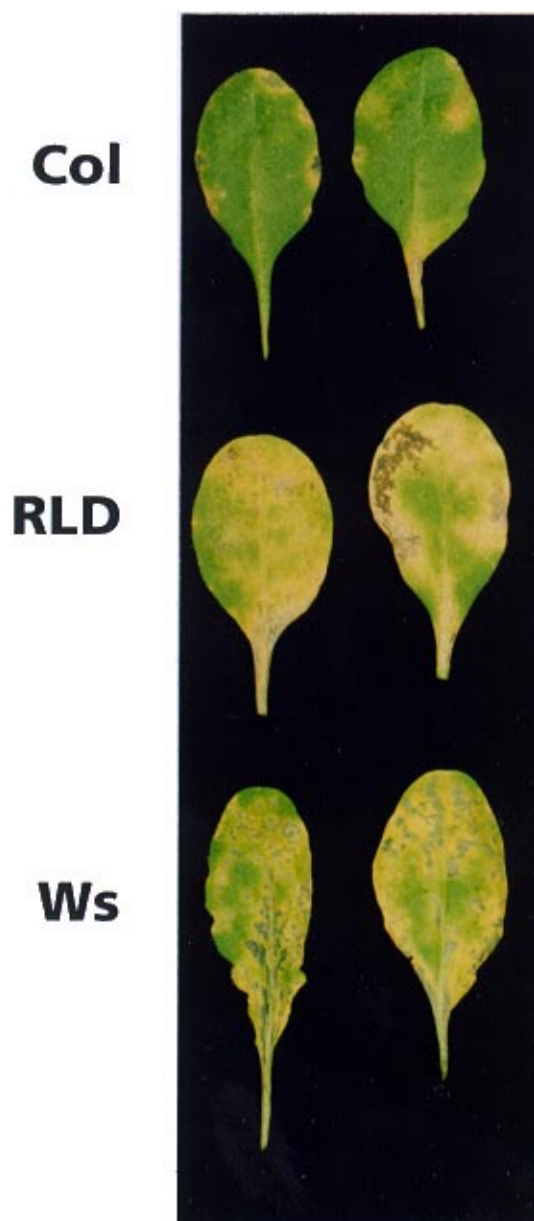
#### RLD and Ws are both affected in the *ISR1* locus.

To investigate whether the recessive alleles of RLD and Ws can complement each other for ISR and basal resistance

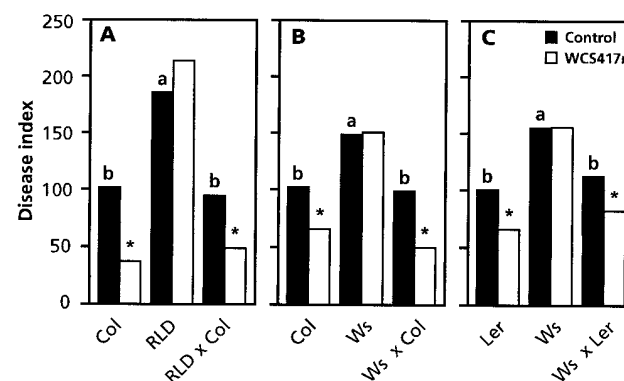
**Table 2.** Proliferation of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) in the leaves of different Arabidopsis ecotypes and F<sub>1</sub> plants over a 3-day time interval

Genotype	Proliferation of <i>Pst</i> (in log units) <sup>a</sup>
WCS417r-responsive genotypes	
Col	2.36 ± 0.18
RLD × Col	2.24 ± 0.15
Ws × Col	2.35 ± 0.07
Ler	2.83 ± 0.08
Ws × Ler	2.80 ± 0.08
Cvi	3.06 ± 0.17
Sha	2.71 ± 0.35
Kas	2.17 ± 0.23
C24	2.12 ± 0.09
Wei	2.96 ± 0.22
Ren	2.77 ± 0.18
WCS417r-nonresponsive genotypes	
RLD	3.61 ± 0.09
Ws	3.71 ± 0.21
RLD × Ws	3.51 ± 0.24

<sup>a</sup> Values presented are means with standard deviations of the log of the proliferation of *Pst* over a 3-day time interval. Plants were infected by pressure-infiltrating a suspension of virulent *Pst* at  $5 \times 10^3$  CFU/ml into the leaves. Immediately after pressure infiltration and 3 days later, the number of *Pst* bacteria per gram of leaf fresh weight was determined and proliferation over a 3-day time-interval was calculated. The data presented are from a representative experiment that was performed at least twice with similar results.

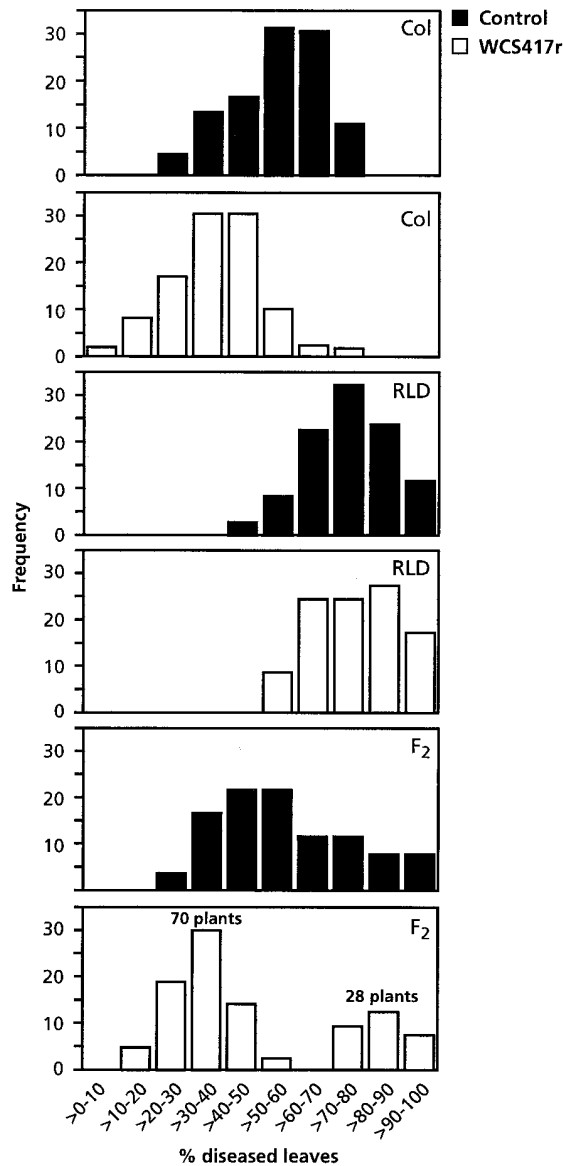


**Fig. 2.** Symptoms of bacterial speck disease caused by *Pseudomonas syringae* pv. *tomato* DC3000 on leaves of Arabidopsis ecotypes Col, RLD, and Ws, 4 days after inoculation. Plants were inoculated by dipping the leaves into a bacterial suspension at  $2.5 \times 10^7$  CFU/ml.



**Fig. 3.** Quantification of *Pseudomonas fluorescens* WCS417r-mediated ISR against *P. syringae* pv. *tomato* DC3000 (*Pst*) in F<sub>1</sub> plants of crosses between (A) RLD × Col, (B) Ws × Col, and (C) Ws × Ler; and their respective parents. Plants were grown in soil with (WCS417r) or without (control) bacteria. Challenge inoculation was performed with a bacterial suspension of virulent *Pst* at  $2.5 \times 10^7$  CFU/ml. Disease symptoms were scored 4 days after challenge inoculation. Disease index is the proportion of leaves with symptoms relative to the proportion of leaves with symptoms in (A and B) control-treated Col-parents or (C) control-treated Ler-parents. Proportion of leaves with symptoms in control treatments was set at 100%. A–C, Different letters above “control” bars indicate statistically significant differences in disease severity (Fisher’s least significant difference test;  $\alpha = 0.05$ ,  $n = 20$  to 25). Asterisks = statistically significant differences between control- and WCS417r-treated plants within each pair (Student’s *t* test;  $\alpha = 0.05$ ,  $n = 20$  to 25). This experiment was also performed with F<sub>1</sub> plants of the reciprocal cross, yielding similar results.

against *Pst*, the F<sub>1</sub> progeny were tested. F<sub>1</sub> plants of the RLD × Ws cross failed to express WCS417r-mediated ISR (Fig. 5). In addition, the percentage of leaves with disease symptoms (Fig. 5) and the extent of *Pst* proliferation (Table 2) in leaves of control-treated F<sub>1</sub> plants were similar to those observed in RLD and Ws. These observations demonstrate that ecotypes RLD and Ws are unable to complement each other for the potential to express ISR and the relatively high level of basal resistance against *Pst*. Therefore, they both must be affected in the *ISR1* locus controlling both resistance mechanisms.



**Fig. 4.** Frequency distributions of Col, RLD, and F<sub>1</sub> plants of the RLD × Col cross over 10 disease severity classes ( $n = 95$  to 100). Plants were grown in soil with or without *Pseudomonas fluorescens* WCS417r bacteria. Five-week-old plants were challenge inoculated with a suspension of virulent *P. syringae* pv. *tomato* DC3000 at  $2.5 \times 10^7$  CFU/ml. Four days later, plants were scored for disease severity; each plant was assigned to one of 10 disease severity classes, ranging from 0–10% to >90–100% of the leaves with symptoms. The experiment was performed twice, with similar results.

**Table 3.** Genetic segregation of responsiveness to *Pseudomonas fluorescens* WCS417r and basal resistance against *P. syringae* pv. *tomato* DC3000 in WCS417r-treated F<sub>2</sub> plants of the RLD × Col cross

Experiment no. <sup>a</sup>	Total	Col-like <sup>b</sup>	RLD-like <sup>c</sup>	Expected ratio	$\chi^2$ value <sup>d</sup>	P
1	98	70	28	3 : 1	0.667	0.414
2	47	36	11	3 : 1	0.064	0.801

<sup>a</sup> In two independent experiments, cosegregation pattern of responsiveness to WCS417r and high basal resistance were determined in F<sub>2</sub> plants. Proportion of leaves with symptoms was determined per plant at 5 days after challenge inoculation with *P. syringae* pv. *tomato* DC3000 and each plant was assigned to one of 10 disease severity classes (see also Figure 4).

<sup>b</sup> Number of WCS417r-treated F<sub>2</sub> plants that exhibited a normal frequency distribution around the same disease severity classes as WCS417r-treated Col plants (Col-like).

<sup>c</sup> Number of WCS417r-treated F<sub>2</sub> plants that exhibited a normal frequency distribution around the same disease severity classes as control- and WCS417r-treated RLD plants (RLD-like).

<sup>d</sup> Numbers of Col- and RLD-like plants tested for the expected 3 : 1 ratio, with the Chi-square test.

**Table 4.** Genetic linkage between responsiveness to induction of induced systemic resistance (ISR) by *Pseudomonas fluorescens* WCS417r and basal resistance against *P. syringae* pv. *tomato* DC3000 (*Pst*)

Ecotype/ F <sub>3</sub> family	Test for ISR (% of induced protection) <sup>a</sup>		Test for basal resistance (proliferation of <i>Pst</i> in log units) <sup>b</sup>	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Col	46.3 (*)	27.1 (*)	2.67 ± 0.17	3.1 ± 0.15
Col-fam. #1 <sup>c</sup>	58.2 (*)		2.97 ± 0.12	
Col-fam. #2		26.5 (*)	2.57 ± 0.30	
Col-fam. #3	42.1 (*)		2.80 ± 0.32	
Col-fam. #4	49.4 (*)		2.71 ± 0.32	
Col-fam. #5		28.2 (*)	2.74 ± 0.20	
RLD	3.8	–4.3	3.54 ± 0.08	4.17 ± 0.09
RLD-fam. #1 <sup>d</sup>		–1.2		4.38 ± 0.10
RLD-fam. #2		–6.3		3.94 ± 0.42
RLD-fam. #3	1.9			4.49 ± 0.15
RLD-fam. #4	3.4			3.95 ± 0.30
RLD-fam. #5	–0.1			4.31 ± 0.19

<sup>a</sup> In two independent experiments, responsiveness to induction of ISR by *P. fluorescens* WCS417r was tested for 10 F<sub>3</sub> families of the RLD × Col cross and compared with RLD and Col. Five F<sub>3</sub> families showing Col-like symptoms and 5 F<sub>3</sub> families showing RLD-like symptoms after infection with *Pst* were used. Plants were grown in soil with or without WCS417r bacteria at  $5 \times 10^7$  CFU/g. Challenge inoculation was performed with a bacterial suspension of virulent *Pst* at  $2.5 \times 10^7$  CFU/ml. Disease symptoms were scored 3 days after challenge inoculation. Values presented are reductions in disease severity after WCS417r treatment relative to corresponding control treatment (% of induced protection). Asterisks = statistically significant differences between control- and WCS417r-treated plants within a plant genotype (Student's *t* test;  $\alpha = 0.05$ ,  $n = 20$ –25). The experiment was performed twice with similar results.

<sup>b</sup> Proliferation of *Pst* over a 3-day time interval in leaves of the different ecotypes and F<sub>3</sub> families. Plants were infected by pressure-infiltrating a suspension of virulent *Pst* at  $5 \times 10^5$  CFU/ml into the leaves. Immediately afterward and 3 days later, the number of *Pst* bacteria per gram of fresh weight was determined and proliferation calculated. Values presented are means and standard deviations ( $n = 5$ ) of the log of the proliferation of *Pst*.

<sup>c</sup> F<sub>3</sub> families homozygous for Col-like disease symptoms caused by *Pst* infection.

<sup>d</sup> F<sub>3</sub> families homozygous for RLD-like disease symptoms caused by *Pst* infection.

### Genetic mapping of the *ISR1* locus.

Thirty-two homozygous  $F_3$  families showing either Col- or RLD-like disease symptoms after infection with *Pst* were used to determine an approximate map position of the *ISR1* locus by cleaved amplified polymorphic sequence (CAPS) analysis. The Col- and RLD-like phenotypes of the  $F_3$  families showed cosegregation with markers *B4* ( $\chi^2 = 13.5$ ;  $P = 0.001$ ), *GL1* ( $\chi^2 = 18.0$ ;  $P < 0.001$ ), and *BGL1* ( $\chi^2 = 5.5$ ;  $P = 0.064$ ) on chromosome 3: Of the 64 chromosomes tested, 15 chromosomes were recombinant with marker *B4*, yielding a recombination frequency of 23%, 14 chromosomes were recombinant with marker *GL1*, yielding a recombination frequency of 22%, and 23 chromosomes were recombinant with marker *BGL1*, yielding a recombination frequency of 36%. These frequencies indicate that the map position of the *ISR1* locus is located between markers *B4* and *GL1* on chromosome 3.

### DISCUSSION

Pathogen-induced SAR and WCS417r-mediated ISR are two induced resistance responses that are controlled by different signaling pathways (Pieterse et al. 1996; Van Wees et al. 1997; Pieterse et al. 1998). Here we have shown that out of the 10 *Arabidopsis* ecotypes tested, eight have the ability to express both types of biologically induced disease resistance. The two other ecotypes, RLD and Ws, are able to express SAR but lack the ability to develop ISR upon treatment of the roots with WCS417r, confirming that ISR and SAR are distinct plant resistance responses.

A first prerequisite for the establishment of rhizobacteria-mediated ISR is an efficient colonization of the rhizosphere by the resistance-inducing strain, enabling the rhizobacteria to reach a threshold population density necessary for the initiation of ISR (Raaijmakers et al. 1995). Insufficient rhizosphere colonization by WCS417r could not explain the WCS417r-nonresponsive phenotype of ecotypes RLD and Ws, as WCS417r colonized WCS417r-responsive and WCS417r-nonresponsive ecotypes equally well (Table 1). Thus, the lack of WCS417r responsiveness in RLD and Ws is not caused by poor root colonization, but must result from the absence of (a) genetic determinant(s) essential for induction and expression

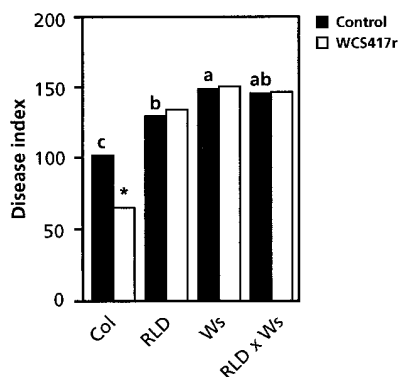
of ISR. As the recessive alleles of RLD and Ws failed to complement each other in their  $F_1$  progeny for responsiveness to WCS417r (Fig. 5), it is likely that both ecotypes lack the same genetic determinant(s) controlling WCS417r-mediated ISR.

In *Arabidopsis*, ecotype specificity of WCS417r-mediated ISR against *Pst* was clearly associated with ecotype-specific basal resistance against *Pst*. The observed phenomenon that *Arabidopsis* ecotypes capable of expressing ISR exhibit a substantially higher level of basal resistance to *Pst* than ecotypes impaired in their ISR response strongly suggests that WCS417r-mediated ISR in *Arabidopsis* utilizes components of the basal resistance pathway. Our results are consistent with previously obtained results of van Peer et al. (1991), who demonstrated that WCS417r-mediated ISR in carnation was less consistently expressed in a susceptible cultivar than in a resistant cultivar, and support the hypothesis that induced resistance constitutes an enhancement of basal resistance responses (Van Loon 1997). In this study, we further showed that the plant's potential to express ISR against *Pst* and its potential to express basal resistance against *Pst* are both dominant traits that are monogenically inherited and genetically linked. The corresponding *ISR1* locus was mapped at chromosome 3 between the markers *GL1* and *B4*. We postulate that the *ISR1* locus controls a basal resistance response against *Pst* that is enhanced under conditions leading to WCS417r-mediated ISR.

Previously, Glazebrook et al. (1996) reported the isolation of *Arabidopsis* mutants that exhibit enhanced disease susceptibility to the moderately virulent *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*). These *eds* mutants showed significantly enhanced growth of *Psm* in the leaves, which is proportional to the differences in growth of *Pst* observed in the leaves of the WCS417r-responsive ecotypes and ecotypes RLD and Ws. The 12 *eds* mutants isolated included two alleles of the recently identified defense regulator gene *NPRI*, two genes encoding proteins that are involved in the phytoalexin biosynthesis, and seven so far unidentified genes. An affected *NPRI* gene seems an unlikely explanation for the WCS417r-nonresponsive and highly susceptible phenotype of RLD and Ws, as both ecotypes are capable of expressing pathogen-induced SAR (Fig. 1), unlike the *npr1-1* mutant (Cao et al. 1994). Also, a disturbance in phytoalexin biosynthesis seems unlikely, because three phytoalexin deficient mutants, *pad1*, *pad2*, and *pad3*, were found to be responsive to ISR treatment with WCS417r (C. M. J. Pieterse, unpublished results).

Rogers and Ausubel (1997) reported that four of the previously isolated *eds* mutants, with so far unknown gene function, showed enhanced susceptibility to a subset of taxonomically related bacterial phytopathogens, including *Pst*. In addition, the four *eds* mutants were found to be unaffected in their hypersensitive reaction to *Psm*, carrying the avirulence gene *avrRpt2*, and were still capable of expressing SAR. This phenotype resembles the phenotype of ecotypes RLD and Ws, with respect to the loss of basal resistance against *Pst* on the one hand (Table 2), and the unaffected hypersensitive reaction to *Pst*(*avrRpt2*) and the SAR response (Fig. 1) on the other hand. Whether the *eds* mutations affect the WCS417r-induced ISR response as well needs to be elucidated.

Another set of mutations shown to affect pathogen resistance are mutations that cause insensitivity to the plant stress



**Fig. 5.** Quantification of *Pseudomonas fluorescens* WCS417r-mediated induced systemic resistance (ISR) against *P. syringae* pv. *tomato* DC3000 in Col, RLD, Ws, and  $F_1$  plants of a cross between RLD  $\times$  Ws. For details see caption to Figure 3. The experiment was performed three times, with similar results.

hormones ethylene and jasmonic acid. In tobacco the *etr1-1* mutation, conferring ethylene insensitivity, was demonstrated to cause susceptibility to a normally nonpathogenic soilborne fungus (Knoester et al. 1998), indicating that ethylene controls defense mechanisms essential for nonhost resistance. Likewise, the jasmonic acid response mutant *jar1* of Arabidopsis was demonstrated to be highly susceptible to the soilborne fungus *Pythium irregulare*, indicating that jasmonate signaling plays an important role in the resistance response to soil microorganisms (Staswick et al. 1998). Moreover, growth of *Pst* in the leaves of the Arabidopsis mutants *etr1-1* and *jar1-1* has been shown to be increased 10-fold, compared with wild-type plants (Pieterse et al. 1998). This indicates that insensitivity to ethylene and jasmonic acid causes at least a partial loss of basal resistance against *Pst*. Development of SAR against *Pst* appears normal in *etr1-1* and *jar1-1* mutants (Pieterse et al. 1998). In contrast, both mutants are impaired in their WCS417r-mediated ISR response against *Pst*. Apparently, insensitivity to ethylene and jasmonic acid in Arabidopsis causes a phenotype that resembles the phenotype of ecotypes RLD and Ws with respect to the lack of WCS417r responsiveness and the relatively low level of basal resistance against *Pst*. To our knowledge, the area on chromosome 3 in which the *ISR1* locus is located does not contain mapped genes with functions related to ethylene and jasmonate signaling, indicating that *ISR1* represents a novel locus involved in disease resistance.

## MATERIALS AND METHODS

### Cultivation of rhizobacteria, pathogens, and plants.

For treatment of the roots with ISR-inducing rhizobacteria, the rifampicin-resistant *P. fluorescens* strain WCS417r (Pieterse et al. 1996) was grown on King's medium B agar plates (King et al. 1954) for 24 h at 28°C. Bacterial cells were collected by centrifugation and resuspended in 10 mM MgSO<sub>4</sub> to a final density of 10<sup>9</sup> CFU per ml. For induction of SAR, the rifampicin-resistant *Pst* carrying the avirulence gene *avrRpt2* (*Pst*(*avrRpt2*); Whalen et al. 1991) was cultured overnight in liquid King's medium B at 28°C, collected by centrifugation, and resuspended in 10 mM MgSO<sub>4</sub> to a final density of 10<sup>7</sup> CFU/ml. The virulent pathogen *Pst* (Whalen et al. 1991), used for challenge inoculations, was cultured in a similar manner.

In this study, the Arabidopsis ecotypes Columbia (Col), Landsberg *erecta* (*Ler*), Shahdara (Sha), Kashmir (Kas), Cape Verdi islands (Cvi), Weiningen (Wei), C24, Wassilewskija (Ws), RLD, and Renkum (Ren) were used. Ecotype Ren was selected from a natural population in Renkum, The Netherlands. Seedlings were grown in quartz sand for 2 weeks. Subsequently, the seedlings were transferred to 60-ml pots, containing a sand/potting soil mixture that had been autoclaved twice for 1 h. Plants were cultivated in a growth chamber with a 9-h day (200 µE s<sup>-1</sup> m<sup>-2</sup> at 24°C) and 15-h night (20°C) cycle and 65% relative humidity. For the duration of the experiments, all ecotypes remained vegetative and developed at least 10 to 15 mature leaves. Plants were watered on alternate days and once a week supplied with modified half-strength Hoagland solution: 2 mM KNO<sub>3</sub>, 5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, and trace elements, pH 7 (Hoagland and Arnon 1938), containing 10 µM sequestren

(Fe-ethylenediamide-di[*o*-hydroxyphenylacetic acid]; Novartis, Basel, Switzerland).

### Induced resistance bioassays.

Prior to transfer of the Arabidopsis seedlings to the pots, a suspension of ISR-inducing WCS417r bacteria (10<sup>9</sup> CFU/ml) was mixed thoroughly through the sand/potting soil mixture, to a final density of 5 × 10<sup>7</sup> CFU/g. Control soil was supplemented with an equal volume of 10 mM MgSO<sub>4</sub>. Induction of SAR was performed 4 days before challenge inoculation by pressure-infiltrating two lower leaves with a suspension of *Pst*(*avrRpt2*) at 10<sup>7</sup> CFU/ml in 10 mM MgSO<sub>4</sub>. One day before challenge inoculation, the plants were placed in 100% relative humidity. Plants were challenged when 5 weeks old by dipping the leaves in a suspension of virulent *Pst* bacteria, containing 10<sup>7</sup> or 2.5 × 10<sup>7</sup> CFU/ml in 10 mM MgSO<sub>4</sub>, 0.015% (vol/vol) Silwet L-77 (Van Meeuwen Chemicals, Weesp, The Netherlands). Three, 4, or 5 days after challenge inoculation, the percentage of leaves with symptoms was determined per plant (*n* = 20 to 25). Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased.

### Basal resistance bioassay.

Leaves of 5-week-old plants were inoculated by pressure infiltration with a suspension of *Pst* at 5 × 10<sup>5</sup> CFU/ml in 10 mM MgSO<sub>4</sub>. Immediately afterward and 3 days later, replicate leaf samples from five plants per ecotype were collected, weighed, and homogenized in 10 mM MgSO<sub>4</sub>. Serial dilutions were plated on selective King's medium B agar supplemented with 100 mg of cycloheximide per liter and 50 mg of rifampicin per liter. After incubation at 28°C for 2 days, the number of rifampicin-resistant CFU per gram of infected leaf tissue was determined and bacterial proliferation over the 3-day time interval was calculated.

### Root colonization.

Root colonization by WCS417r was determined at the end of the bioassays. Roots were harvested, weighed, and shaken vigorously in 5 ml of 10 mM MgSO<sub>4</sub> containing 0.5 g of 0.17-mm glass beads. Appropriate dilutions were plated on King's medium B agar, supplemented with 100 mg of cycloheximide per liter and 150 mg of rifampicin per liter. After incubation at 28°C for 2 days, the number of rifampicin-resistant CFU per gram of root fresh weight was determined.

### Genetic analysis.

F<sub>1</sub> plants were obtained by reciprocally crossing ecotypes RLD and Col, Ws and Col, Ws and *Ler*, and RLD and Ws. The resulting F<sub>1</sub> progenies were tested for ISR and basal resistance against *Pst* with the induced resistance and basal resistance bioassays, respectively. F<sub>2</sub> plants, obtained by selfing F<sub>1</sub> plants of the RLD × Col cross, were tested for ISR with the induced resistance bioassay with approximately 50 or 100 plants per treatment. F<sub>3</sub> families were collected by selfing individual F<sub>2</sub> plants of the RLD × Col cross. Sets of 16 plants per F<sub>3</sub> family were tested for symptom development by dipping control-treated plants in a suspension of *Pst* at 2.5 × 10<sup>7</sup> CFU/ml, after which disease symptoms were monitored at 3, 4, and 5 days after challenge. For testing genetic linkage between ISR and basal resistance, 10 randomly selected F<sub>3</sub>

families displaying either Col- or RLD-like disease symptoms were tested for ISR and basal resistance against *Pst*. Mapping the *ISR1* locus was performed with 16 homozygous F<sub>3</sub> families showing Col-like disease symptoms and 16 homozygous F<sub>3</sub> families showing RLD-like disease symptoms, with cleaved amplified polymorphic sequence (CAPS) analysis as described by Konieczny and Ausubel (1993).

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## LITERATURE CITED

- Cao, H., Bowling, S. A., Gordon, A. S., and Dong, X. 1994. Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* 6:1583-1592.
- Gaffney, T. P., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261:754-756.
- Glazebrook, J., Rogers, E. E., and Ausubel, F. M. 1996. Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. *Genetics* 143:973-982.
- Hammerschmidt, R., and Kuć, J. 1995. Induced Resistance to Diseases in Plants. Kluwer, Dordrecht, The Netherlands.
- Hoagland, D. R., and Arnon, D. I. 1938. The water culture method for growing plants without soil. *Calif. Agric. Exp. Stn. Bull.* 347:36-39.
- King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of phycocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
- Knoester, M., Van Loon, L. C., Van den Heuvel, J., Hennig, J., Bol, J. F., and Linthorst, H. J. M. 1998. Ethylene-insensitive tobacco lacks non-host resistance against soil-borne fungi. *Proc. Natl. Acad. Sci. USA* 95:1933-1937.
- Konieczny, A., and Ausubel, F. M. 1993. A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. *Plant J.* 4:403-410.
- Leeman, M., Van Pelt, J. A., Den Ouden, F. M., Heinsbroek, M., Bakker, P. A. H. M., and Schippers, B. 1995. Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. *Eur. J. Plant Pathol.* 101:655-664.
- Liu, L., Kloepper, J. W., and Tuzun, S. 1995. Induction of systemic resistance in cucumber against Fusarium wilt by plant growth-promoting rhizobacteria. *Phytopathology* 85:695-698.
- Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. 1990. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250:1002-1004.
- Métraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M. M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. 1990. Increase of salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250:1004-1006.
- Pieterse, C. M. J., Van Wees, S. C. M., Hoffland, E., Van Pelt, J. A., and Van Loon, L. C. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* 8:1225-1237.
- Pieterse, C. M. J., Van Wees, S. C. M., Van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P. J., and Van Loon, L. C. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* 10:1571-1580.
- Raaijmakers, J. M., Leeman, M., van Oorschot, M. M. P., van der Sluis, I., Schippers, B., and Bakker, P. A. H. M. 1995. Dose-response relationships in biological control of Fusarium wilt of radish by *Pseudomonas* spp. *Phytopathology* 85:1075-1081.
- Rogers, E. E., and Ausubel, F. M. 1997. *Arabidopsis* enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in *PR-1* gene expression. *Plant Cell* 9:305-316.
- Ross, A. F. 1961. Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14:340-358.
- Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H., and Hunt, M. D. 1996. Systemic acquired resistance. *Plant Cell* 8:1809-1819.
- Staswick, P. E., Yuen, G. Y., and Lehman, C. C. 1998. Jasmonate signaling mutants of *Arabidopsis* are susceptible to the soil fungus *Pythium irregulare*. *Plant J.* 15:747-754.
- Van Loon, L. C. 1985. Pathogenesis-related proteins. *Plant Mol. Biol.* 4:111-116.
- Van Loon, L. C. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *Eur. J. Plant Pathol.* 103:753-765.
- Van Loon, L. C., Bakker, P. A. H. M., and Pieterse, C. M. J. 1998. Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36:453-485.
- Van Peer, R., Niemann, G. J., and Schippers, B. 1991. Induced resistance and phytoalexin accumulation in biological control of Fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. *Phytopathology* 81:728-734.
- Van Wees, S. C. M., Pieterse, C. M. J., Trijssenaar, A., Van 't Westende, Y. A. M., Hartog, F., and Van Loon, L. C. 1997. Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. *Mol. Plant-Microbe Interact.* 10:716-724.
- Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Métraux, J. P., and Ryals, J. A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3:1085-1094.
- Whalen, M. C., Innes, R. W., Bent, A. F., and Staskawicz, B. J. 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* 3:49-59.