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The *avrRpm1* Gene of *Pseudomonas syringae* pv. *maculicola* Is Required for Virulence on Arabidopsis

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We demonstrate that the avirulence gene *avrRpm1*, isolated from *Pseudomonas syringae* pv. *maculicola* strain Psm M2 via interaction with the Arabidopsis resistance gene *RPM1*, is also required for maximal virulence on this host. Two *avrRpm1*::Tn3-Spice marker-exchange mutants do not elicit a hypersensitive reaction on *RPM1*-containing Arabidopsis accessions Col-0 and Oy-0. Surprisingly, these mutants neither generate disease symptoms, nor grow in planta, after inoculation onto susceptible accessions Nd-0, Fe-1, and Mt-0. These deficiencies can be corrected in a merodiploid containing a wild-type *avrRpm1* allele, and are not observed following gene-replacement with *avrRpm1*::Tn3-Spice alleles containing insertions just beyond the 3′ terminus of the avirulence gene open reading frame. *AvrRpm1* mRNA is expressed in low, but detectable amounts, in rich media. Induced accumulation of transcript is observed 3 h after shift to minimal media, and an *avrRpm1*::Tn3-Spice marker-exchanged reporter gene reaches maximal induction 30 min after shift. *AvrRpm1* transcription starts 5 base-pairs 3′ of the putative regulatory "*hrp*-box" cis-element found upstream of many *P. syringae avr* and *hrp* genes. Transcriptional induction of the marker-exchanged reporter gene in minimal media is enhanced by a carbon source. Induction in planta is the same in either resistant or susceptible Arabidopsis accessions, and is unaffected by the presence or absence of wild-type *avrRpm1*. As previously observed for many other *P. syringae avr* genes, transcriptional regulation of *avrRpm1* in minimal media is dependent on *hrpL* and *hrpS*.

Additional keywords: bacterial plant pathogen; environmental gene regulation; gene-for-gene resistance; plant-bacteria signalling; virulence mechanisms.

Avirulence (*avr*) genes from phytopathogenic Pseudomonads and Xanthomonads encode, either directly or indirectly, the necessary and su entire nt disease resistance, as predicited by Flor's "gene-for-gene hypothesis" (Flor 1971; Keen 1990). Since the first was cloned a decade ago (Staskawicz et al. 1984), a plethora of *avr* genes have been isolated using the same experimental regime: Conversion of virulence of a recipient strain to avirulence in a manner dependent on the presence of a corresponding resistance (*R*) gene in the test plant (reviewed by Keen and Staskawicz 1988; Long and Staskawicz 1993; Dangl 1994). Though initially thought to operate only within a given pathovar group to limit the effective host range within a plant species, seminal experiments proved that gene-for-gene interactions could govern pathogen host-range beyond the host species level (Whalen et al. 1988; Kobayashi et al. 1989). This notion has been strengthened by analogous findings in many pathosystems, and is the driving force behind the identification of functionally homologous bacterial disease resistance specificities in many plant species (e.g., Keen 1990; Debener et al. 1991; Whalen et al. 1991; Dangl et al. 1992; Innes et al. 1993b). Thus, bacterial *avr* genes restrict the number of colonizable plant genotypes within a particular host species, and the overall number of colonizable host species.

Several puzzling enigmas exist regarding bacterial avirulence genes. Since they have a negative effect on pathogen fitness on hosts capable of recognizing their activity, why do *avr* genes persist in bacterial populations? Though *avr* genes probably serve a positive function at some stage in the bacterial life strategy, clear roles as virulence or pathogenicity factors have been ascribed to only a miniority (Kearney and Staskawicz 1990; Swarup et al. 1991; Swarup et al. 1992; Lorang et al. 1994). Are they structural triggers of plant resistance, or are they enzymes whose products elicit plant resistance responses? This question has been satisfactorally addressed only for the *avrD* gene from *P. syringae* pv. *tomato*, whose expression leads to the production of a low molecular weight syringolide which acts as a specific trigger of resistance on appropriate plant genotypes (Keen et al. 1990; Midland et al. 1993). What is the mechanism of *avr* gene action, and is it fundamentally tied to pathogenesis in ways not yet recognized? In this regard, one could imagine that *avr*-gene function is required during epiphytic bacterial growth, or early in the shift to pathogenesis.

To date, delivery of all *avr*-gene encoded signals are dependent on functions encoded in *hrp* (hypersensitivity and pathogenicity) clusters found in phytopathogenic bacteria. Hrp clusters share some functional and structural homology, and several *hrp*-encoded proteins are similar to molecules required for secretion of virulence factors in mammalian pathosystems systems (reviewed by Willis et al. 1991; Van Gijsegem et al. 1993; Bonas 1994; Collmer and Bauer 1994). These findings led to the proposal that the *hrp* cluster encodes a similar system, used to deliver virulence, and potentially avirulence, functions to the plant cell. This idea is also sup-

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ported by findings in *P. syringae* pathovars regarding transcriptional regulation of *hrp* and *avr* genes. Two loci, *hrpRS* and *hrpL*, encode regulators of other *hrp* genes, and of all four *P. syringae avr* genes analyzed to date (Grimm and Panapoulos 1989; Xiao et al. 1994). Transcription of both putative regulators, the other *hrp* cluster genes, and *P. syringae avr* genes, is induced by environmental conditions thought to reflect in planta conditions (Lindgren et al. 1989; Fellay, et al. 1991; Rahme et al. 1991; 1992; Xiao et al. 1992; Miller et al. 1993 for *hrp* genes; Huynh et al. 1989; Innes et al. 1993a; Salmeron and Staskawicz 1993; Shen and Keen 1993 for *avr* genes). Thus far, the requirement for *hrp* regulators in activation of *P. syringae avr* genes is absolute, lending credence to the model that *avr* genes are part of a *hrp*-dependent regulon required for pathogenesis.

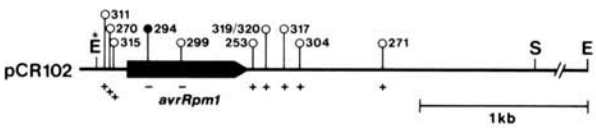

Fig. 1. Tn*3*-Spice insertions in and around the *avrRpm1* open reading frame (ORF). Mapped insertion sites are numbered. The filled symbol of insert 294 represents transcription of the *inaZ* gene in the same direction as the *avrRpm1* ORF. All other insertions were in the opposite orientation. Symbols below the line represent either loss (–) or retention (+) of avirulence activity when tested an accession Col-0. Experiments in all cases were performed on a minimum of 20 leaves.

We are interested in the structure, function, and regulation of both bacterial and plant partners in interactions leading to specific disease resistance in Arabidopsis (reviewed in Dangl 1993). To that end, we defined and cloned the *avrRpm1* gene from *P. syringae* pv. *maculicola* isolate Psm M2 via its interaction with the *RPM1* resistance locus of Arabidopsis accession Col-0 (Debener et al. 1991). We subsequently demonstrated that resistance to *avrRpm1* exists in pea, bean, and soybean, and that in pea and bean this resistance specificity is defined by the *P. syringae* pv. *pisi* race 2 allele of *avrRpm1*, *avrPpiA1* (Dangl et al. 1992). Through the experiments presented here, we wished to address possible roles for *avrRpm1* in virulence, and we sought to define the environmental and genetic controls of *avrRpm1* expression.

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains	Genotypes, relevant features[a]		Reference, source
Escherichia coli			
HB101	F⁻ *hsdS20(hsdR hsdM) recA13 thi leu proA2 lacY1galk2 rpsL xyl-5 mtl-1 supE44*⁻	Sm^r	Boyer and Roulland-Dussoix 1969
DH5α	F⁻ *lacZ ΔM15 endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1* λ⁻	Nal^r; 10 μg/ml	Bethesda Research Labs
C2110	*polA*	Nal^r; 50 μg/ml	Stachel et al. 1985
S17-1	*Pro*, res-, mod+, RP4-2-Tc::Mu-Km:: Tn7 integrated into the genome	Sm^r	Simon et al. 1993
Pseudomonas syringae pv.:			
maculicola isolates:			
LMG5071	Psm M2	Rif^r	Debener et al. 1991
LMG5295	Psm M4	Rif^r	Debener et al. 1991
CR294	Psm M2 *avrRpm1* 294::Tn*3*-spice	Rif^r, Sp^r	This study
CR299	Psm M2 *avrRpm1* 299::Tn*3*-spice	Rif^r, Sp^r	This study
CR253	Psm M2 *avrRpm1* 3′ 253::Tn*3*-spice	Rif^r, Sp^r	This study
CR304	Psm M2 *avrRpm1* 3′ 304::Tn*3*-spice	Rif^r, Sp^r	This study
CR317	Psm M2 *avrRpm1* 3′ 317::Tn*3*-spice	Rif^r, Sp^r	This study
tomato isolates:			
DC3000	race 0	Rif^r	B. Staskawicz
CB191	DC3000 *hrpS*::Tn*3*-gus	Rif^r, Km^r	R. Innes
CB337	DC3000 *hrpL*::Tn*3*-gus	Rif^r, Km^r	R. Innes
CB196	DC3000 *hrpF*::Tn*3*-gus	Rif^r, Km^r	R. Innes
phaseolicola isolates:			
1448AR	derivative of race 6 1448A	Rif^r	Fillingham et al. 1992
1448AR	*hrpL*::Tn3-gus	Rif^r, Km^r	J. Mansfield
1448AR	*hrpF*::Tn3-gus	Rif^r, Km^r	J. Mansfield
Plasmids			
pRK2013	Tra⁺, Mob⁺; ColE1 replicon	Km^r	Figurski and Helinski 1979
pLAFR5	Tra⁺, Mob⁺; RK2 replicon	Tc^r	Staskawicz et al. 1987; Keen et al. 1988
pL6	Tra⁺, Mob⁺, RK2 replicon derived from pLAFR, but carrying transcription terminators flanking the multicloning site	Tc^r	D. Dahlbeck and B. Stakawicz, pers. comm.
pSPT19	pUC19 derivative	Ap^r	Boehringer-Mannheim
pSShe	*tnpA*⁺, pACYC184 replicon	Cm^r	Stachel et al. 1985
pTn*3*-spice	*inaZ*⁺	Ap^r, Sp^r, Sm^r	Lindgren et al. 1989
pCR102	A 6-kb *Eco*RI fragment from pCR100 cloned into pLAFR5, carries *avrRpm1*	Tc^r	Dangl et al. 1992
pCR104	Derived from pCR102 in two steps: A 2.5-kb *Eco*RI–*Sst*I fragment from pCR102 was subcloned in pSPT19, thus donating a *Bam*HI site. The insert was excised as an *Eco*RI–*Bam*HII fragment and cloned into pLAFR5	Tc^r	D angl et al. 1992
pCR106	Derived from pSPT19 subclone as an *Eco*RI–*Sac*I fragment ligated into pL6	Tc^r	This study
pCR253, 270, 271, 294, 299, 304, 311, 315, 317, 319, 320	Tn*3*-spice insertions in pCR102	Tc^r, Sp^r, Sm^r	This study

[a] Sm, streptomycin; Rif, rifampicin; Cm, chloramphenicol; Ap, ampicllin; Sp, spectinomycin; Km, kanamycin; ^{r}, resistant.

RESULTS

AvrRpm1 encodes a factor required for virulence on susceptible Arabidopsis accessions.

We peviously reported the generation of several Tn*3*-Spice insertions into a pLAFR5-based plasmid, pCR102 (Dangl et al. 1992). This construct contains the *avrRpm1* open reading frame (ORF), and 5.0 kb of 3′ flanking DNA. Several additional insertions, shown in Figure 1, were generated and mapped in the course of the experiments reported here (see Materials and Methods; Table 1). We limited our analysis to insertions in the *Eco*RI–*Sst*I fragment carried on pCR104, since we showed previously that pCR104 carries *avrRpm1* activity. New Tn3-Spice insertions were tested initially for insertion into the *avrRpm1* gene via transconjugation into a *P. syringae* pv. *maculicola* strain, Psm M4, which is virulent on *RPM1*-containing Arabidopsis accessions (strains described in Debener et al. 1991). Transconjugants incapable of triggering an HR on Col-0 carry Tn*3*-Spice insertions in sites necessary for *avrRpm1* activity. To assess the possible role of *avrRpm1* on bacterial fitness, marker-exchange mutants were constructed in Psm M2 using the *avrRpm1*::Tn3-Spice alleles carried on both pCR294 and pCR299. These contain Tn*3*-Spice insertions in either the correct or incorrect orientations, respectively, for generation of transcriptional fusions. Correct replacement of the wild-type *avrRpm1* allele with each mutant allele was confirmed by DNA blot analysis (not shown, see Materials and Methods).

The resulting strains, CR294 and CR299, were inoculated into leaves of *RPM1*-containing Arabidopsis Col-0 plants at high titer, and observed for formation of either a typical HR, or formation of water-soaked disease lesions. The latter outcome is expected if mutation of *avrRpm1* removes the single function normally responsible for triggering resistance in Col-0 to *P. syringae* pv. *maculicola* Psm M2. Figure 2A shows that CR299 (and CR294, not shown) did not trigger a typical HR at 24 h postinoculation (h.p.i.), in contrast to the Psm M2 parent strain. Unexpectedly, however, CR299 (and CR294,

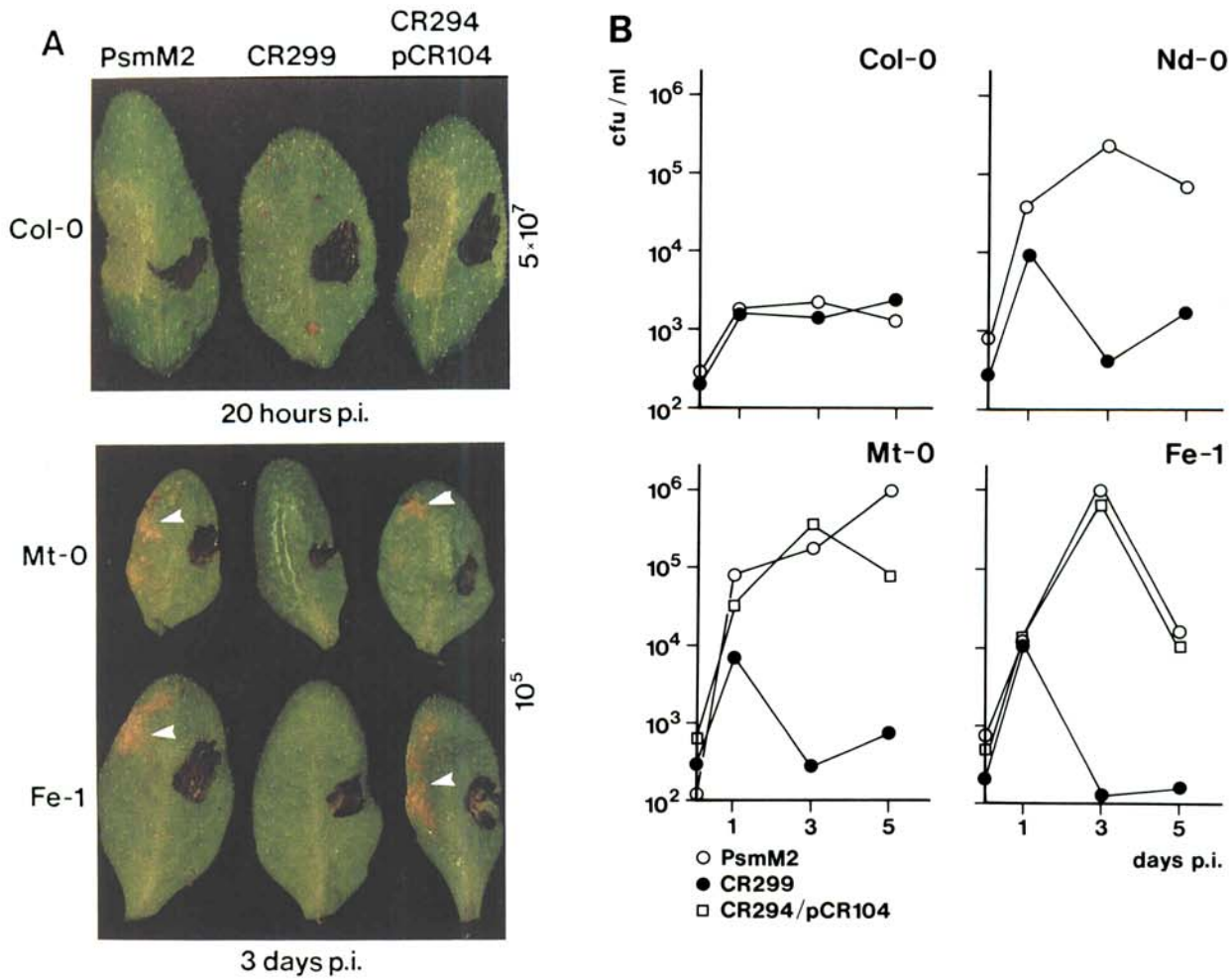

Fig. 2. *AvrRpm1* function is required for generation of hypersensitive response on resistant, and for virulence on susceptible, Arabidopsis. **A,** Leaves of resistant Col-0 were inoculated at high titer (right side of panel) with the strains denoted above each. Leaves were detached and photographed 20 h later (below panel). Alternatively, leaves of susceptible accessions Mt-0 and Fe-1 were inoculated with the same strains at low titer, and disease symptoms photographed 3 days later. More than 40 leaves were analyzed in each interaction. **B,** Growth of bacterial strains (lower left) on resistant (Col-0) or susceptible (Nd-0, Mt-0, Fe-1) accessions over 5 days. The beginning titer was 10^5 CFU/ml. Growth curves were performed two or three times with nearly identical results, according to Debener et al. (1991). Each data point represents a pool of four leaves. Absolute titers for data points on growth curves differ by not more than threefold.

not shown) was also unable to generate water soaking at this time point, as would have been expected if *avrRpm1* were a critical function restricting growth of Psm M2 on *RPM1*-containing Arabidopsis. Transconjugation of CR299 (or CR294, not shown) with pCR104, which carries a wild-type *avrRpm1* allele, restored the ability of this strain to trigger a normal HR on Col-0. As expected, this merodiploid strain did not grow in Col-0 leaves (not shown).

Surprisingly, typical disease symptoms failed to develop on several susceptible Arabidopsis accessions after inoculation with strains carrying mutant *avrRpm1* alleles, as depicted for accessions Mt-0 and Fe-1 in Figure 2A. In this assay, low titer inoculum of Psm M2 gives rise to typical chlorotic disease lesions 3 days postinoculation (d.p.i.) (Davis et al. 1991; Debener et al. 1991; Whalen et al. 1991). Again, merodiploids carrying a wild-type *avrRpm1* allele rescued the ability to cause disease lesions in both CR294 and CR299. As well, CR294 and CR299 were incapable of sustained in planta growth in either resistant or susceptible Arabidopsis accessions, as shown in Figure 2B for CR299. Strains with mutant *avrRpm1* alleles grew at the same initial rate as wild-type Psm M2 over the first 24 h.p.i., but numbers of recovered colonies fell sharply thereafter. As with both HR and disease lesion phenotypes, the presence of pCR104 carrying a wild-type *avrRpm1* allele restored normal in planta growth kinetics to the strains carrying mutant alleles as gene replacements.

The pCR104 construct, which complements all three defects derived from inactivation of *avrRpm1*, contains 1.8 kb of Psm M2 sequence 3′ of the *avrRpm1* ORF. It was therefore necessary to exclude the possibility that polar effects of the Tn*3*-Spice insertions on a function encoded downstream of the *avrRpm1* ORF were responsible for the lack of virulence described above. We thus constructed marker-exchange mutants with the *avrRpm1* 3′::Tn*3*-Spice insertions pCR253 and pCR304, shown in Figure 1, to generate the strains CR253 and CR304. The former carries a Tn*3*-Spice insertion less than 100 bp, and the latter around 300 bp, 3′ of the *avrRpm1* ORF. We performed the same sets of experiments described in Figure 2 to assay for HR on resistant Arabidopsis accessions Col-0 and Oy-0, and both disease symptoms and in planta bacterial growth on susceptible accessions Nd-0, Fe-1, and Mt-0. A subset of this data is displayed in Figure 3. Neither Tn*3*-Spice insertion downstream of the *avrRpm1* ORF affected any of the tested phenotypes. Thus, the *avrRpm1* gene encodes a function necessary for virulence of *P. syringae* pv. *maculicola* Psm M2 on an array of susceptible accessions, as well as being necessary and sufficient for induction of HR on *RPM1*-containing Arabidopsis.

These data piqued our curiousity regarding the role of *avrRpm1* in interactions of Psm M2 with other plant species, particulary since *avrRpm1* defines resistance specificities in pea, bean, and soybean when present as a transconjugant in *P. syringae* isolates virulent on those species (Dangl et al. 1992). We found no obvious effect of Tn*3*-Spice insertion alleles in, or downstream of, the *avrRpm1* ORF in Psm M2 on HR induction on either pea cultivars which either contain the R2 resistance gene corresponding to *avrRpm1* (cvs. Martus, Katrin, and Vinco), or a cultivar which does not (cv. Kelvedon

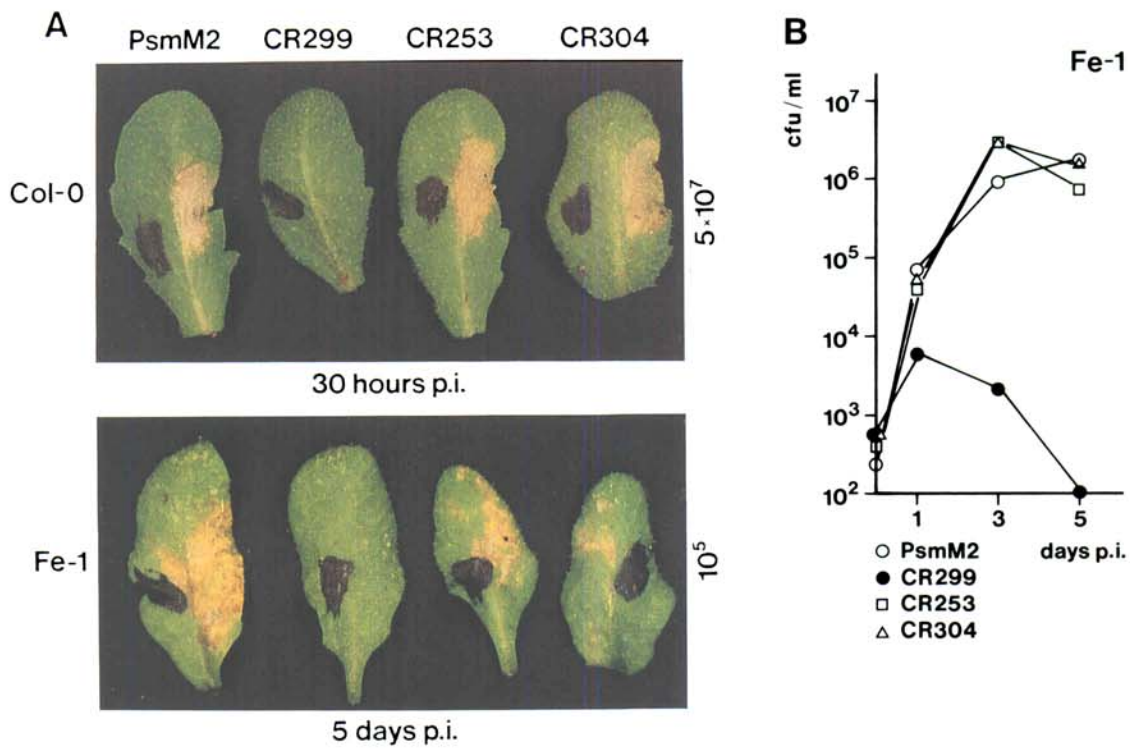

Fig. 3. Tn*3*-Spice insertions 3′ of the *avrRpm1* open reading frame (ORF) do not affect hypersensitive response (HR) on resistant, or virulence on susceptible, Arabidopsis. **A,** Phenotypes on resistant Col-0 leaves after inoculation of the various bacterial strains (top) at high titer (for HR) or low titer (for disease symptoms) were done as in Figure 2. Inocula titer is shown at the right, time to photograph below, each picture. **B,** Growth curves of several strains (lower left) on susceptible accession Fe-1. This experiment was performed three times. Each data point is the mean derived from the titer of two pools of four leaves from one experiment. Absolute titers for data points on growth curves differ by not more than threefold.

Wonder). Inoculation of CR299, CR253, or CR304 on bean (cvs. Canadian Wonder and Seafarer), radish, turnip (which is a host for Psm M2), white cabbage, and tobacco gave phenotypes identical to those following inoculation with Psm M2 (data not shown; see Materials and Methods for experimental details). Thus, *avrRpm1* does not function as a *hrp* gene. We thus conclude that Psm M2 contains avirulence/virulence functions other than *avrRpm1* which are responsible for limiting/expanding its host range, at least on the cultivars of each species tested. Since *avrRpm1* is present in only a minority of tested *P. syringae* pv. *maculicola* isolates, and since several of the isolates lacking it are pathogenic on Arabidopsis (e.g., Psm M4) (Dangl et al. 1992), it is apparent that *avrRpm1* does not encode a factor absolutely required for bacterial growth.

Transcriptional activation of *avrRpm1*.

All *P. syringae* avirulence genes analyzed to date are transcriptionally activated under culture conditions thought to be roughly analogous to those found in leaf intracellular spaces (Huynh et al. 1989; Innes et al. 1993a; Salmeron and Staskawicz 1993; Shen and Keen 1993). These conditions are thought to be mimicked in defined minimal media supplemented with either simple sugars such as fructose and sucrose, or sugar alcohols like mannitol. We tested accumulation of either wild-type *avrRpm1* mRNA in Psm M2, or reporter gene induction from the *avrRpm1*::Tn*3*-Spice transcription fusion allele carried on as a marker exchange in strain CR294.

RNA blot analysis presented in Figure 4 shows that *avrRpm1* mRNA levels in Psm M2 are low, but easily detectable, in rich media and accumulate to high levels after 3 h of growth in minimal media (M9, pH 5.5) supplemented with 10 mM fructose (the same results were obtained with 10 mM sucrose, not shown). We also used this RNA to determine the site of *avrRpm1* transcription initiation via primer extension. Figure 5 shows the single major product of reverse transcription from a primer complementary to the coding strand at amino acid positions 6–16 of the *avrRpm1* ORF (see Materials and Methods). The size of the *avrRpm1* mRNA observed in Figure 4 is around 700 bp, in agreement with the size of the *avrRpm1* ORF (663 bp; Dangl et al. 1992) plus a 28-bp 5′ untranslated leader defined by this primer extension analysis. The deduced transcription start site is 5 bp 3′ of the consensus "*hrp*-box" cis-element, as depicted schematically in Figure 5 (Innes et al. 1993a; Shen and Keen 1993). We also observed a much weaker transcription start 120 bp further upstream (not shown). This site is 20 bp upstream of an ATG which is in the same frame as the ATG of the *avrRpm1* ORF. However, no possible Shine-Delgarno sequence is present at this upstream site, and Tn3Spice insertion 315, which has no effect on *avrRpm1* activity, (Figure 1) is between it and the major start site shown in Figure 5.

We next assayed reporter gene induction from the *avrRpm1*::Tn3 spice transcription fusion carried in CR294 under various culture conditions (see Materials and Methods). We measured an approximately 17-fold induction from CR294 in minimal media plus either 10 mM fructose or sucrose at 30 min postshift. A low, but reproducible, transcriptional activation of *avrRpm1*::Tn3 spice activity was measured in the absence on any carbon source. Activation was apparently maximal at 30 min, since no further increases were observed over a 3-h time course (data not shown).

We further analyzed transcriptional activation of *avrRpm1* by monitoring reporter gene induction after inoculation of CR294 and control strains onto either resistant (accession Col-0) or susceptible (accessions Fe-1) Arabidopsis plants. Time course experiments presented in Figure 6 demonstrate three important points. First, reporter gene induction is rapid in planta, reaching a maximum 10^4- to 10^5-fold above initial

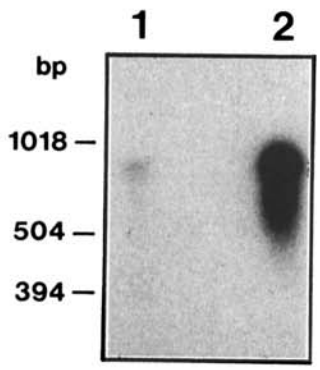

Fig. 4. Transcriptional activation of *avrRpm1*. Psm M2 was grown overnight in King's medium B (KB), diluted 20-fold in KB and grown to mid-log (OD_{600} = 0.5), washed in M9 salts, and diluted into either KB (lane 1) or M9 (lane 2), each supplemented with 10 mM fructose, for a further 3 h growth as described in Materials and Methods. RNA was prepared, blotted onto Hybond Plus and probed as described in Materials and Methods.

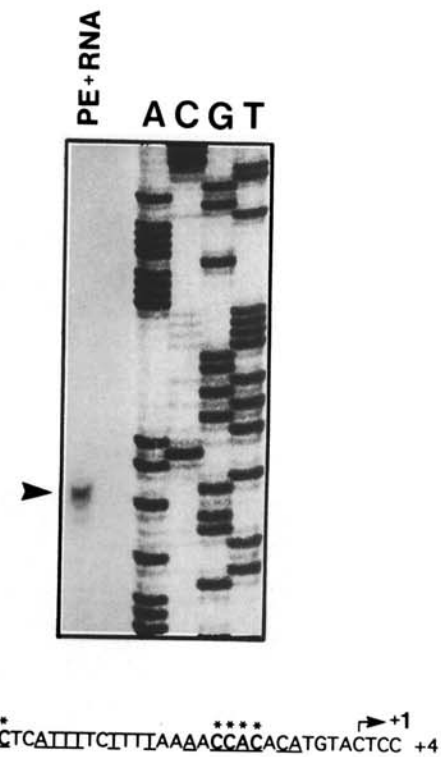

Fig. 5. The *avrRpm1* transcriptional start site. RNA from Figure 4, lane 2 was used for primer extension as described in Materials and Methods. Sequencing ladder was generated using the same primer and pCR102 subclone in pSPT19 (Table 2) as template. The start site is depicted with an arrow, in the context of homologies to other avr gene promoters (Innes et al. 1993). Highest homologies are denoted (*), others underlined.

levels by 2 to 3 h.p.i., but returning to lower levels by 20 h.p.i.. Second, the presence or absence of the *RPM1* resistance gene has little or no bearing on *avrRpm1* transcription induction, since both accessions assayed support essentially the same timing of ice nucleation reporter gene induction (compare open and closed squares). Third, there was only a minimal difference in timing of ice nucleation reporter gene induction accumulation when we compared the gene replacement strain, CR294, to the corresponding merodiploid version of *avrRpm1*::Tn*3*-Spice294, pCR294 (Figure 6, closed squares versus closed circles). We conclude that the presence or absence of a wild-type allele of *avrRpm1* has no effect on the perception of the signals necessary to activate *avrRpm1* promoter function.

To address whether *avrRpm1* transcriptional activation required the *hrp* regulatory functions, we conjugated pCR106 (see Table 1) into the *P. syringae* pv. *tomato* strain DC3000, and putative *hrpS, hrpL,* and nonregulatory *hrpF* mutants derived from it (CB191, CB337, CB196, respectively) and performed RNA blots. The DC3000 strain is pathogenic on Arabidopsis and tomato (Whalen et al. 1991), and delivers the *avrRpm1* derived signal to *RPM1*-containing Arabidopsis accessions (data not shown). The *hrp* mutants used no longer deliver the *avrRpm1* signal, and are also not pathogenic on Arabidopsis (data not shown). These and analogous *hrp*::Tn3gus alleles were previously used to demonstrate that transcriptional activation of both the *avrPto* and *avrRpt2* genes from *P. syringae* pv. *tomato* in minimal media are completely dependent on putative regulatory functions provided by *hrpS* and *hrpL*, but are not influenced by mutation in *hrpF* (Innes et al. 1993a; Salmeron and Staskawicz 1993).

Three hours after shift to minimal media plus 10 mM fructose, we prepared RNA and resulting blots were probed with an *avrRpm1*-internal PCR product (see Materials and Methods). Consistent with observations for other *P. syringae avr* genes analyzed to date, mutation of either hrp regulatory locus drastically reduces the accumulation of *avrRpm1* mRNA under normally inductive conditions. Mutation in the nonregulatory *hrpF* locus has no effect (Fig. 7).

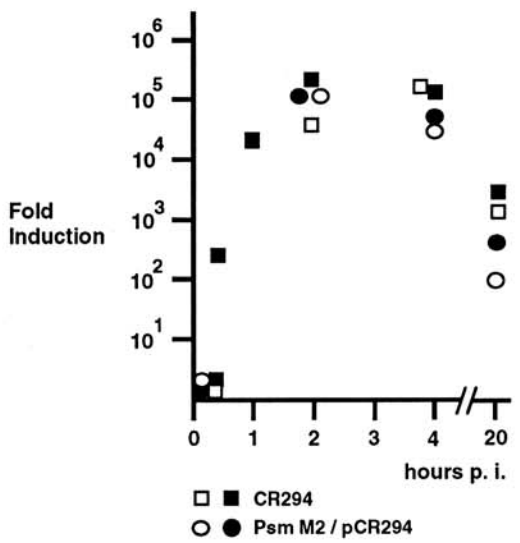

Fig. 6. Time course of *avrRpm1*::Tn*3*-Spice reporter gene induction in leaves of resistant or susceptible Arabidosis. Strains (lower left) were grown overnight in KB, washed and resuspended to 5×10^7 CFU/ml in 10 mM $MgCl_2$, and inoculated onto leaves as described for Figure 2. Experiments were performed on Col-0 or Oy-0 (resistant; filled symbols) and Fe-1, Mt-0, and Nd-0 (susceptible; open symbols). No difference was observed among accessions of either class, and the data for each class is pooled here. Fold inductions were calculated using the mean ice nucleation activity of CR294 from between 2 and 10 independent measurements per time point minus the mean activity of CR299 and Psm M2 (background control) at the same time point as numerator (each measurement in triplicate), and ice nucleation activity of CR294 in the initial inoculum (8×10^{-6} ice nuclei/CFU) as denominator (see Materials and Methods). It should be noted that pCR294 carries a *lacZ* promoter upstream of the *avrRpm1* promoter which may contribute to observed in planta *inaZ* activity. This amount must be negligible since the induction of the merodiploid strains carrying pCR294 is essentially the same as the marker exchanged CR294 strain. Standard deviations of the various measurements are less than the size of the symbols used.

DISCUSSION

Our results demonstrate two important characteristics of the *avrRpm1* gene, one of which illustrates an emerging modification to the prevailing paradigms for *avr* gene function and regulation in *P. syringae* pathovars (reviewed in Keen and Staskawicz 1988; Keen 1990; Dangl 1994). We define a clear role for *avrRpm1* in virulence of Psm M2 on three susceptible Arabidopsis accessions. We also provide both RNA blot and reporter gene induction data showing that transcriptional activation of *avrRpm1* is rapid, independent of gene-for-gene interactions, and dependent on the *hrpS* and *hrpL* regulatory functions, at least in minimal media supplemented with sugar.

There is a paucity of data ascribing pathogenicity or virulence functions to *avr* genes. To date, only the *avrBs2* gene from *X. campestris* pv. *vesicatoria* encodes a function required for pathogenicity (Kearney and Staskawicz 1990). *AvrBs2* is present in each (of over 500) *X. campestris* pv. *vesicatoria* isolates analyzed, as well as in isolates from other *X. campestris* pathovar groups, and mutations in this gene lower bacterial fitness on at least the two different hosts tested. Since *avrRpm1* is not widely distributed among *P. syringae* pv. *maculicola* isolates (Dangl et al. 1992), and since mutation of this gene did not affect pathogenicity on turnip, *avrRpm1* cannot be considered a basic pathogenicity factor.

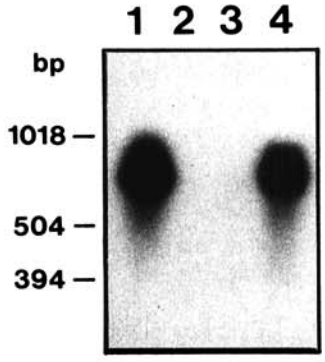

Fig. 7. Mutations in *hrp* regulatory genes abrogate *avrRpm1* mRNA accumulation in M9 containing 10 mM sucrose. Strains, all containing pCR106 (*avrRpm1* in pL6 with transcription terminators flanking the multi-cloning site) were grown as described as for Figure 4, and RNA prepared as described in Materials and Methods. Lanes: 1, DC3000; 2, CB191; 3, CB337; 4, CB196.

A more direct comparison to this aspect of our results was recently provided by Keen and colleagues, who showed that the *avrE* gene, and to a lesser extent *avrA*, from *P. syringae* pv. *tomato* also have virulence functions (Lorang et al. 1994). The particular strain from which *avrE* was cloned also contains *avrA* and *avrD* (Kobayashi et al. 1989; Kobayashi et al. 1990), and a functional allele of the *avrPto* gene, originally cloned from another *P. s.* pv. *tomato* strain (Ronald et al. 1992). Lorang et al. (1994) mutagenized all four of these genes in this strain, singly and in combination, and tested the resulting mutants for pathogenicity on tomato. They found that mutation of *avrE* mutation lowered the ability of low doses of bacteria to grow and cause disease symptoms on tomato, although very high titer inoculum was able to cause symptoms. Transconjugants carrying the cloned *avrE* gene in an *avrE* deletion mutant strain were complemented to full virulence at low-dose inoculum. These data argue strongly that *avrE* function is required to establish infection in tomato, but that it is not necessary for symptom production if large numbers of bacteria are inoculated into tomato tissue. Importantly, this virulence function is apparently strain dependent, since mutation of a functional *avrE* allele in another *P. s.* pv. *tomato* strain had no effect on virulence on tomato (Lorang and Keen 1994; Lorang et al. 1994). As with *avrE*, the requirement for *avrRpm1* function in virulence seems to be during establishment of infection, as high titer inoculation (5×10^7 CFU/ml) results in attenuated lesion formation on resistant Arabidopsis accessions, and water soaking on susceptible accessions (data not shown). Lorang et al. (1994) also show a similar, though less pronounced, role for *avrA* in virulence on tomato.

Two members of the *avrBs3* family from *Xanthomonas* have also been shown to encode virulence factors. The *pthA* gene from *X. citri* was first identified functionally via its ability to enhance the virulence of a weakly pathogenic *X. citremulo* strain on grapefruit (Swarup et al. 1991; Swarup et al. 1992). Mutation of *pthA* in *X. citri* results in loss of pathogenicity on grapefruit. The *pthA* gene also encodes cultivar-specific avirulence when conjugated into *X. campestris* pv. *malvacearum* and tested on cotton. Thus, *pthA* encodes a function that acts as an avirulence gene in *X. campestris* pv. *malvacearum,* a virulence factor in *X. citrumelo,* and is required for pathogenicity in at least one *X. citri* isolate. Another member of the *avrBs3* family, the *avrb6* gene from *X. campestris* pv. *malvacearum,* also apparently acts as a host-specific virulence factor in that it confers the ability to cause disease symptoms (water soaking) on cotton onto strains normally incapable of doing so, and it is required for maximal symptom formation (De Feyter et al. 1993). Oddly, presence of *avrb6* does not confer growth capability on the recipient strain tested.

These examples, and data presented here regarding *avrRpm1*, suggest that combinations of virulence factors, some probably redundant, operate in any particular strain on a particular host. This point is reinforced by the fact that, as discussed above, deletion of *avrE* in one strain affects that strain's fitness, but that a similar deletion in a second strain has no effect on virulence on the same host. We will also address how widely the virulence function ascribed here to *avrRpm1* operates. Emerging evidence suggests that gene-replacement mutation of the *avrRpm1* allele *avrPpiA1* in one race 2 strain of *P. syringae* pv. *pisi* has no effect on virulence on pea (M. Gibbon et al., submitted). It will be interesting to construct further gene replacement mutants of *avrRpm1* alleles in other isolates of both *P. syringae* pv. *maculicola* and *P. syringae* pv. *pisi* and test for reduction of virulence on the respective hosts. Since many *avr* genes, including *avrRpm1*, are borne on potentially transmissible plasmids, it is not surprising that combinations of *avr* functions (and any attendant fitness or virulence functions) are mixed and matched in the bacterial population. If most, or all, *avr* genes encode functions involved in additive or redundant mechanisms governing virulence, then it is also not surprising that some *avr* genes are apparently dispensible, or not broadly dispersed.

In addition to its role as a virulence factor, we demonstrated that *avrRpm1* transcription is rapidly activated following shift to minimal media containing either sucrose or fructose as a carbon source. This rapid activation also occurs in planta independent of the presence of either functional *avrRpm1* in the pathogen, or *RPM1* in the host, albeit with a slightly different time course. This modes of transcriptional control are typical of *P. syringae avr* genes analyzed to date (Lindgren et al. 1989; Fellay et al. 1991; Rahme et al. 1991; Rahme et al. 1992; Xiao et al. 1992; Miller et al. 1993, see Bonas 1994 for review).

Environmental and *hrp*-dependent aspects of transcriptional control have been published for five *P. syringae avr* genes. In the first example, Huynh et al. (1989) showed that sugars and sugar alcohols optimally induced *lacZ* activity from the *avrB* promoter from *P. syringae* pv. *glycinea*. They were unable to detect *lacZ* activity in the absence of a utilizable carbon source, or in the same rich media used in our experiments. These authors also provided the first evidence for *hrp*-dependence of *avr* gene transcription, both in minimal media suplemented with sugar and in planta. In the former, mutations physically corresponding to *hrpS* and *hrpL* reduced *lacZ* activity at least 100-fold. The same mutations also had drastic effects on ice nucleation activity measured from the same promoter in planta, reducing them to background levels measured in rich media. Thus, *avrB* transcription is apparently completely controlled by regulatory *hrp* functions, and requires sugars or sugar alcohols for maximal induction. Similar conclusions were reached by Shen et al. (1993) for the *avrD* gene from *P. syringae* pv. *tomato*. This group used promoter fusions to the GUS reporter to measure transcriptional activation in both minimal media supplemented with fructose and in soybean leaves. In both cases, reporter activity was decreased 30- to 50-fold from maximum, and reduced to levels barely above background in *hrp* regulatory mutants. The *avrPto* gene from *P. syringae* pv. *tomato* provides the third example (Salmeron and Staskawicz 1993). Here, a promoter-GUS fusion was detectably active at extremely low levels in rich media, but induced some 800-fold in fructose suplemented minimal media. The effect of *hrp* regulatory mutants on *avrPto* accumulation, measured via RNA blots, is apparently absolute. Expression of the *avrRpt2* gene from *P. syringae* pv. *tomato* was also analyzed in media using RNA blots (Innes et al. 1993b). There, no expression was seen in rich media at pH 7.0 (our conditions also) and expression was maximal in a minimal media supplemented with both fructose and citrate. As with the *avrPto* analysis, no *avrRpt2* mRNA was observed in *hrp* regulatory mutants. No in planta experi-

ments were reported for *avrRpt2*. Together, these data support the idea of a strict *hrp* regulatory dependence, and a requirement for sugars or sugar alcohols, for transcriptional activation of these *avr* genes. In agreement with our findings, a rapid in planta transcriptional induction was observed for *avrB, avrPto,* and *avrD,* independent of both the resistance gene status of the inoculated plant, and the *avr* gene status of the test bacterial strain.

To identify genetically other potential *avrRpm1* regulatory components, we have mutagenized Psm M2 with Tn*phoA* (Manoil and Beckwith 1985) (on full media to repress *hrp* activity) and have isolated mutants impaired in the delivery of *avrRpm1* function to Arabidopsis. At least one mutant isolated is defective in accumulation of *avrRpm1* mRNA after shift to minimal media (our unpublished results). It is thus a candidate for another environnmental sensor directly or indirectly influencing *avrRpm1* transcriptional induction. Its effects on *avrRpm1* expression in planta, and its molecular characterization are under way.

MATERIALS AND METHODS

Maintenance of bacteria.

Pseudomonas syringae strains (Table 1) were grown on King's B media (King et al. 1954) shaken at 25 to 28°C. *Escherichia coli* strains (Table 1) were grown in Luria-Bertani (LB) broth, or LB agar plates at 37°C (Sambrook et al. 1989). Antibiotics (Sigma) were used as follows (mg/liter): for *Pseudomonas* strains: rifampicin, 100; tetracycline, 15; nalidixic acid, 10; spectinomycin 100; for *E. coli*: ampicillin 100; tetracycline 5 to 15; nalidixic acid 10 or 50 (see Table 1); kanamycin, 30 to 50; spectinomycin 10 to 20; streptomycin 50 to 100.

Triparental matings.

Conjugations from *E. coli* strains DH5α, HB101, or S17-λpir were performed via a modification (Debener et al. 1991) of standard protocols using pRK2013 as a helper plasmid when necessary (Ditta et al. 1980). Details are found in Dangl et al. (1992).

Transposon mutagenesis.

Mutagenesis with Tn*3*-Spice giving rise to the mutants in this study has been described in detail (Dangl et al. 1992).

Gene replacements.

Conjugations into Psm M2 were performed by first tranforming the plasmid into S17-λpir (Simon et al. 1993), and subsequent conjugation into Psm M2. We found this to be more efficient than triparental mating for this strain. For gene replacement, individual transconjugants were grown in the absence of tetracycline for 15 or more growth cycles in rich media. Colonies were then tested for retention of Tn*3*-Spice markers, and loss of tetracycline resistance. The orientation of gene replacements in insertions 253, 294, 299, and 304 were confirmed by Southern hybridizations. Insertions 253, 299, and 304 have the same orientation, in the opposite direction of the *avrRpm1* gene promoter, whereas 294 is in the same orientation. Southern blots were prepared, hybridized, and washed under high stringency conditions according to standard procedures (Ausubel et al. 1987).

DNA manipulations.

Total DNA or plasmid DNA was prepared as described (Dangl et al. 1992). DNA fragments used in DNA and RNA blot analysis were either a 681-bp *Eco*RI–*Pst*I fragment from pCR102 which covers the 5′ portion of the *avrRpm1* ORF, and 191-bp upstream, or a 432-bp PCR fragment derived from positions 305 to 737 of the published *avrRpm1* sequence (Dangl et al. 1992). The primers used were: Forward primer 5′-AAC CGA TTC CGA GCA TTC CAA T-3′ and Reverse primer 5′-CGG CTG GCT GCT CTT GCT TC-3′ (complementary strand beginning at nucleotide 737).

Fragments were isolated from agarose gels via binding to and elution from DE81 paper. Briefly, paper is prepared by immersion in 2 M NaCl in TE for several minutes, washed repeatedly with TE buffer, and stored dry. After the fragment is electrophoresed onto the membrane, the paper is placed in a 0.5-ml Eppendorf tube with a hole in the bottom, and this is placed into a larger 1.5-ml tube for elution with three washes of 10 ml of 1 M NaCl in TE, each followed by brief centrifugation. The eluate is desalted over a Sephadex G50 column in a yellow pipetteman tip which is prepared by loading and is spun dry before application of the eluate. Probe for blots was labeled using the PRIME-IT kit from Strategene (La Jolla, Calif.). All other enzymes were from either Gibco-BRL or Boehringer Mannheim, and were used according to the supplier's recommendations.

Care of plants, inoculations of plants.

Arabidopsis plants were maintained as described (Dangl et al. 1992; Debener et al. 1991). All other plants used were greenhouse grown. Fully expanded leaves of all species were inoculated with bacteria in exactly the manner described by Dangl et al. (1992). Bacteria were prepared as described by Debener et al. (1991) except that overnight cultures of OD_{600} = 1.0 to 1.5 were diluted to OD_{600} = 0.1 and allowed to grow for 1 to 2 h before washing and adjustment to the desired density for inoculation. Alternatively, overnight cultures were washed and adjusted to the desired OD_{600}. An OD_{600} = 0.2 was taken as roughly 10^8 CFU/ml. Typically, dose-response assays were made at several bacterial titers, especially for non-Arabidopsis species. Phenotypic data presented here is from inocula of 5 $\times 10^7$ CFU/ml for rapid HR, and 10^5 CFU/ml for disease symptoms and growth curves, as previously described (Debener et al. 1991; Dangl et al. 1992).

RNA analysis.

RNA from bacteria was prepared with a modification of the hot phenol method (von Gabain et al. 1983) as described (Innes et al. 1993a). Ten milliliters of mid-log phase cultures (OD_{600} = 0.5), in rich media, was harvested and washed twice in M9 minimal media (33.7 mM Na_2HPO_4; 22 mM KH_2PO_4; 8.5 mM NaCl; 18.7 mM NH_4Cl; 6 mM $MgSO_4$; 5.7 mM K_2HPO_4; 0.1 mM $CaCl_2$; pH 5.5; as in Sambrook et al. 1989). These bacteria were then resuspended in the test media, supplemented with various sugars at 10 mM, and grown for the desired time with no antibiotics. The pH of the media was monitored before and after bacterial growth. No change was observed. Cells were harvested by centrifugation at 4°C, resuspended in 100 μl of distilled water, transferred to a 1.5-ml Eppendorf tube and lysed by addition of 400 μl of 90°C buffer (50 mM Tris-HCl, pH 9.0; 50 mM EDTA; 300 mM

sodium acetate; and 0.625% SDS). Following quick vortexing, this was incubated 30 s in a boiling water bath. Debris was sedimented in a microfuge for 5 min at room temperature. Hot phenol (400 µl, 68°C) was added, and emulsified via vortexing for 1 min. The aqueous layer was further extracted one time with hot phenol, and once with chloroform before precipitation of nucleic acids with an equal volume of isopropanol. Genomic DNA was removed by spooling, and RNA collected by centrifugation. The pellet was resuspended in 200 µl of water, and precipitated again via addition of an equal volume of 4 M lithium acetate for 1 h on ice. RNA was collected by centrifugation for 10 min at 13,000 × *g*, 4°C, was resuspended in 100 µl of water, and again reprecipitated with 1/10 volume 3 M sodium acetate and 2.5 volume ethanol. The final pellet was resuspended in 100 µl of water and quantified by optical density. From 10 ml of M9 culture, approximately 20 mg of RNA was typically isolated. Ten milligrams was used for RNA blot analysis after electrophoresis through standard agarose formaldehyde gels (Ausubel et al. 1987).

This RNA was also used for primer extension analysis, using the protocol provided in unit 4.8 of Ausubel et al. (1987). The primer for these experiments was: 5′-CCG GAA TAA TAT CCA GTA CTT CTT GAA GTG C-3′. This primer is the complement of positions 238 to 208 of the sequence of *avrRpm1* shown in Dangl et al. (1992).

Ice nucleation reporter gene induction measurements.

Assay conditions were first established as described in (Lindow 1990). We first found that high expression from pCR294 was observed in ice nucleation experiments at –5°C, and that Psm M2 and DC3000 had essentially no activity at this temperature. At lower temperatures, however, the endogenous ice nucleation activity of these strains was expressed. Thus, all assays were performed at –5°C. For measurements from bacteria inoculated onto plants, a titer of 5×10^7 CFU/ml was used, and cells prepared as for bacterial growth curves, in 10 mM Mg_2Cl. This buffer had no effect on ice nucleation activity levels, even after prolonged storage. Dilutions were made into 10 mM potassium phosphate, buffered to pH 7.0, and we followed the protocol given (Lindow 1990) exactly, except that repel-silane was used to coat the aluminum foil boats, and that our ethanol bath was contained in a pyrex glass dish on a cooling plate set to –22°C (surface ethanol temperature –5°C). All ice nucleation activity measurements were independently calculated from 3 × 40 droplets, and means were calculated. The experiments were repeated as described in the Figure 6 caption. Parallel samples were plated on KB plates with the appropriate antibiotics to determine cell number. Activity calculations were made using the formula

$$NT = \ln(N_0/N_0 - N_f)/V \qquad /CFU$$

N_0 = total number of droplets (always 40 in our experiments); N_f = number of droplets of that suspension that has frozen; V = volume of individual droplets (always 10 µl); /CFU = corrected for number of colony forming units in the same dilution from which ice activity was measured.

Since ice nucleation activity may not be linear with INAZ protein amount, all activity measurements are displayed as fold-induction where the denominator for each calculation is the same strain under control environmental conditions (time zero control), as discussed by Lindgren et al. (1989). For experiments comparing rich and minimal medias, the denominator was ice nucleation activity of CR294 in rich media at the time of shift (8×10^{-6} ice nuceli/CFU; time zero control). Absolute values for Psm M2 and CR299 (background control) in minimal media containing sucrose were 1.2×10^{-6} and 1.6×10^{-6} ice nuceli/CFU. For in planta experiments, fold inductions were calculated using the mean ice nucleation activity of CR294 from between 2 and 10 independent measurements per time point minus the mean activity of CR299 and Psm M2 (background control) at the same time point as numerator (each measurement in triplicate), and ice nucleation activity of CR294 in the initial inoculum (8×10^{-6} ice nuclei/CFU, time zero control) as denominator. The absolute values for CR299 and Psm M2 ranged from 8×10^{-5} to 1.3×10^{-3} ice nuceli/CFU in planta. They did not change over the time course in any obvious manner, and were not different between tested accessions.

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