Functional Analysis of the Type III Effectors AvrRpt2 and AvrRpm1 of *Pseudomonas syringae* with the Use of a Single-Copy Genomic Integration System

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Gram-negative phytopathogenic bacteria require a type III secretion apparatus for pathogenesis, presumably to deliver Avr effector proteins directly into plant cells. To extend previous studies of Avr effectors that employed plasmids encoding Avr proteins, we developed a system that permits the integration of any gene into the *Pseudomonas syringae* genome in single copy. With this system, we confirmed earlier findings showing that *P. syringae* pv. *maculicola* strain PsmES4326 expressing the AvrRpt2 effector induces a resistance response in plants with the cognate *R* gene, *RPS2*. Chromosomally located *avrRpt2*, however, provoked a stronger resistance response than that observed with plasmid-expressed AvrRpt2 in *RPS2*⁺ plants. Additionally, chromosomal expression of AvrRpt2 conferred a fitness advantage on *P. syringae* grown in *rps2*⁻ plants, aiding in growth within leaves and escape to leaf surfaces that was difficult to detect with plasmid-borne *avrRpt2*. Finally, with the use of the genomic integration system, we found that a chimeric protein composed of the N terminus of the heterologous AvrRpm1 effector and the C-terminal effector region of AvrRpt2 was delivered to plant cells. Because the C terminus of AvrRpt2 cannot translocate into plant cells on its own, this indicates that the N-terminal region can direct secretion and translocation during an infection, which supports the view that Avr proteins have a modular design. This work establishes a readily manipulatable system to study type III effectors in a biologically realistic context.

Additional keywords: *hrp*–*hrc*, hypersensitive response, transmission, virulence.

Gram-negative plant pathogenic bacteria secrete a broad range of elicitors that can induce defenses in a host if it bears the appropriate cognate resistance (*R*) gene (Bonas and Van den Ackerveken 1999). This resistance response is characterized by a rapid programmed cell death called the hypersensi-

tive response (HR) (Greenberg 1997). A subset of bacterial elicitors are proteins called effectors that are secreted through a multisubunit complex called the type III secretion apparatus (Bonas and Van den Ackerveken 1999). This apparatus is encoded by the *hrp*–*hrc* pathogenicity island and shares substantial homology with the type III secretory apparatus bacterial pathogens of animals (Fenselau et al. 1992; Gough et al. 1992; Van Gijsegem et al. 1993; Van Gijsegem et al. 1994) and the flagellar motor (Van Gijsegem et al. 1995).

The best-studied type III effectors are produced by animal pathogenic bacteria of the genus *Yersinia* spp., which is closely related to many phytopathogens. The N termini of type III secreted effectors, called Yops, are sufficient to direct their secretion and translocation into the host cell (Sory and Cornelis 1994; Sory et al. 1995). In some cases, only a region encoded in the 5' mRNA is needed to direct Yops into the extracellular medium (Anderson and Schneewind 1997). Similar 5' mRNA signals are likely to be involved in *hrp*–*hrc*-dependent secretion of phytopathogenic Avr effector proteins into the extracellular medium (Anderson et al. 1999). In the case of YopE, additional amino acid sequences are needed for the translocation of the protein into host cells (Sory et al. 1995).

Whereas Avr proteins have been shown to secrete into the extracellular medium in vitro (Alfano and Collmer 1997; Mudgett and Staskawicz 1999; Pirhonen et al. 1996), translocation into plant cells has not been demonstrated directly. Nonetheless, it is likely that at least some Avr effectors are translocated into plant cells because their direct expression in plant cells confers *R*-gene-specific cell death (De Feyter et al. 1998; Gopalan et al. 1996; Leister et al. 1996; Rate et al. 1999; Scofield et al. 1996; Stevens et al. 1998; Tang et al. 1996; Van den Ackerveken et al. 1996). Currently, it is not known which nucleic acid and/or protein sequences are required for secretion and translocation of Avr effectors into plant cells. Mudgett and Staskawicz (1999) demonstrated that N-terminal deletions of the *Pseudomonas syringae* AvrRpt2 effector disrupt secretion into the medium and delivery to plant cells. Although these studies raise the possibility that Avr and Yop proteins reach the host cytoplasm by similar mechanisms, the demonstration of N-terminal sufficiency for secretion and translocation into plant cells or the demonstration that Avr proteins are translocated directly into host cells via the *hrp* secretory system is lacking.

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In addition to triggering a defense response in plants, some Avr proteins enhance virulence when expressed in bacteria infecting plants that lack the cognate *R* gene (Gabriel 1999). Enhanced growth of bacteria carrying effectors has been demonstrated in *P. syringae* (Jackson et al. 1999; Ritter and Dangel 1995) and *Xanthomonas campestris* (Kearney and Staskawicz 1990). Yang and colleagues (1994) took the analysis one step further by determining that the *X. campestris avrB6* gene enabled the bacterium to dramatically increase disease symptoms on its host (water soaking), thereby releasing more bacteria to the surface of the leaf, yet they were unable to show that the gene conferred a direct growth benefit.

The study of type III phytopathogenic virulence mechanisms is hampered partially by the use of plasmids and cosmids to express bacterial genes. The loss of extra-chromosomal elements during in planta growth is an especially difficult problem because it is usually impossible to select for the maintenance of the plasmid or cosmid vectors (Abebe et al. 1997). A recent example of the problems associated with plasmid loss was illustrated by Hendrickson and colleagues (2000). They found that an *rpoN* mutant of *P. syringae* pv. *maculicola* (PsmES4326) was unable to maintain a clone containing the *hrpL* gene during pathogen infection of *Arabidopsis* spp. Additionally, typical broad-host-range vectors are often large and difficult to manipulate experimentally. A further problem with the use of plasmids is the potential for nonphysiological levels of protein accumulation as a result of altered or variable gene dosage. To circumvent these problems, we developed a single-copy chromosomal integration system that greatly facilitates the rapid analysis of the function and structure of *P. syringae* Avr effectors in a biologically realistic manner. With this system, we report that two *avr* genes, *avrRpt2* and *avrRpm1*, can induce resistance responses in heterologous strains of *P. syringae* when present in single copy. These responses are stronger than those elicited by strains with plasmid-borne copies of these genes. We also find that the AvrRpt2 effector increases the fitness of *P. syringae* grown in host plants lacking the cognate resistance protein Rps2. Finally, we show that N-terminal deletions of AvrRpt2 defective for secretion and translocation can be delivered to plant cells by the N terminus of a heterologous Avr protein. This suggests that Avr proteins possess a modular structure and that the N terminus of Avr proteins shows the expected role in secretion and translocation in a biologically realistic assay. This work demonstrates that single-copy chromosomal integration offers a powerful and rapid way to dissect the functional regions of effectors and address their biological role in disease.

RESULTS

The *P. syringae* pv. *tomato avrRpt2* gene functions in single copy when expressed from the chromosome of a heterologous *P. syringae* strain.

avrRpt2 was originally cloned from a *P. syringae* pv. *tomato* (PtoJL1065) genomic cosmid library (Dong et al. 1991; Whalen et al. 1991) and is a genomic, single-copy locus (Innes et al. 1993). We wanted to introduce *avrRpt2* and derivative constructs stably into *P. syringae* strains that do not naturally possess the gene in single copy in order to study its function and structure in a biologically realistic manner, which

would alleviate problems associated with the use of plasmids. We created a "suicide" integration vector (Fig. 1) that contained a 527-bp internal fragment of the *P. syringae* pv. *maculicola* (strain PsmES4326) *recA* gene, which is dispensable for *P. syringae* virulence (Willis et al. 1988), as well as a kanamycin resistance marker, but no *P. syringae* origin of replication. After the integration vector was introduced into *P. syringae* by a single reciprocal homologous recombination event (Campbell integration, Fig. 1), the resulting strains were completely stable during in vitro and in planta growth and possessed a single copy of the vector integrated at the *recA* locus, as assayed by Southern blot analysis (data not shown). In contrast, when strains carrying the broad-host vector pLAFR3 were inoculated into plants, 20 to 45% of bacteria lost the vector after 4 days of growth in planta. PsmDG3 (PsmES4326 *recA*ΔpCKTR) grew as well as the parent strain PsmES4326 lacking the vector (Fig. 2) and showed the same level of disease symptoms (data not shown).

To test whether single-copy expression of an *avr* gene would yield the same results as found in earlier studies of plasmid-borne *avr* genes expressed in heterologous strains,

Fig. 1. Integration vector and Campbell integration strategy. At top is a schematic of the integration vector pCKTR. Bottom shows a schematic of the chromosomal structure of pCKTR integrated into the chromosomal locus of *recA*. The *recA* locus is numbered 1 to 8 arbitrarily to represent positions within the gene. Chloramphenicol resistance (*cmr*^r) is nonfunctional in pCKTR.

we cloned the *avrRpt2* gene into the integration vector and introduced it into PsmES4326. Host recognition was retained in the PsmDG6 (*recA*Δ*avrRpt2*) strain. We compared the growth of PsmES4326 carrying *avrRpt2*, either integrated into the genome or plasmid-borne, in *Arabidopsis* spp. expressing the RPS2 resistance gene product required for AvrRpt2 recognition. PsmES4326-(pLAFR::*avrRpt2*) and PsmDG6 (*recA*Δ*avrRpt2*) showed reduced growth relative to the congenic virulent *P. syringae* strains lacking *avrRpt2* (Fig. 2). Surprisingly, although PsmDG6 and PsmES4326-(pLAFR::*avrRpt2*) were avirulent on *RPS2*⁺ *Arabidopsis* spp., the integrated version induced a stronger HR than the plasmid-borne version, as evidenced by the enhanced tissue collapse of the PsmDG6-infected tissue (Fig. 3A; note the wrinkling of the leaf tissue infected with PsmDG6), enhanced ion leakage from PsmDG6-infected tissue (Fig. 4), and reduced growth of PsmDG6 relative to PsmES4326-(pLAFR::*avrRpt2*) (Fig. 2). Although the HR with PsmDG6 was stronger than that found with PsmES4326 carrying *avrRpt2* on a plasmid, the timing of HR initiation was not obviously different (data not shown). We detected a similar increase in the strength of the HR for integrated versus plasmid-borne *avrRpt2* when *avrRpt2* was introduced into random sites in the genome by a transposon (data not shown). The plant resistance response provoked by PsmES4326 harboring integrated *avrRpm1* also was stronger than that found with plasmid-borne *avrRpm1* (data not shown). These results show that recognition of avirulent *P. syringae* is retained when the *avr* genes are expressed in single copy in a heterologous strain. Furthermore, the effects of *avr* genes on bacterial recognition are enhanced when the genes are chromosomal compared with plasmid-borne. We have not explored the molecular basis for the observed differences. These experiments show that the use of plasmid-borne *avr* genes can underrepresent the effects they have on pathogen recognition.

AvrRpt2 is a virulence factor in *rps2*⁻ plants.

Some *avr* genes enhance the virulence of bacterial phytopathogens (Jackson et al. 1999; Kearney and Staskawicz 1990; Ritter and Dangl 1995; Yang et al. 1994). Having shown that *avrRpt2* provokes a resistance response in single copy, we tested for a possible virulence role in plants lacking the cognate *RPS2* gene. Previously, we found that growth of PsmES4326 carrying plasmid-borne *avrRpt2* showed some plasmid loss over the time course of the experiment (data not shown), making it difficult to definitively assess the potential virulence function of this gene. Additionally, the strain from which *avrRpt2* was isolated was recalcitrant to transformation (data not shown), precluding the functional analysis of AvrRpt2 by the creation of loss of a function mutant. To circumvent these problems, we used PsmDG6 (*recA*Δ*avrRpt2*) to assess the potential virulence function of AvrRpt2. Bacteria were inoculated at a low titer of optical density (OD) at 600 nm of 0.0002 into plants lacking the RPS2-mediated recognition of AvrRpt2 (*rps2-101c*). Leaves were washed extensively immediately after infection so that no *P. syringae* cells were detected on leaf surfaces of the majority of leaves at the beginning of the experiment. We monitored bacterial density in three ways over a period of 6 days. First we washed the leaves and titered the bacteria in the wash solution to assess the

number of bacteria living on the surface, a measure of the transmission potential of the bacteria. Second, we surface sterilized the leaf and took a leaf punch from the most highly symptomatic area of the leaf. Finally, we assayed the remainder of the leaf for bacterial load. Leaves of similar sizes were sampled at each time point (Fig. 3B; unpaired *t* test of leaf area; *P* = 0.750). Figure 3B shows leaves sampled from *rps2-101c* plants infected with PsmDG3 (integration control) or PsmDG6 (integrated *avrRpt2*) after 6 days. A quantitative analysis of the percentage of healthy green tissue remaining after infection showed that PsmDG3 had 50.5% (standard error [SE] = 14%), whereas PsmDG6 had 11.6% (SE = 13.3%) green tissue. These values were significantly different (*P* < 0.0001; unpaired *t* test; *n* = 24). Thus, disease symptoms (overall yellowing) were stronger on *rps2* plants inoculated with the *avrRpt2*-containing strain than the control.

Growth experiments confirmed that the presence of *avrRpt2* (PsmDG6) allowed the bacteria to significantly increase their density by all three measures (Fig. 5). The presence of *avrRpt2* provided the greatest benefit during the first couple of days of infection, allowing the bacteria to more rapidly increase in density than those bacteria without the *avr* gene. This difference was maintained throughout the course of the infection. No significant growth difference was observed between these strains grown in either rich or minimal media in vitro (data not shown). Interestingly, bacteria carrying *avrRpt2* appeared on leaf surfaces in greater numbers than bacteria lacking *avrRpt2* (Fig. 5A), indicating a role for AvrRpt2 in aiding bacterial transmission. PsmDG6 did not grow better than PsmDG3 when inoculated directly on leaf surfaces (data not shown).

Fig. 2. Bacterial growth assay. Bacteria were inoculated into *Arabidopsis thaliana* with an *RPS2*⁺ background at an optical density of 600 nm at 0.002. PsmES4326 is the wild-type strain. PsmDG3 has a control vector integrated into the *recA* locus. PsmES4326 (pLAFR3) carries the broad-host-range cosmid pLAFR3. PsmDG6 has the *avrRpt2* containing vector integrated into the *recA* locus. PsmES4326 (pLH12) carries the *avrRpt2* gene on pLAFR3. Four replicate leaves were taken for each point. Error bars represent one standard error on each side of the mean. The experiment was replicated twice. Growth of PsmES4326 carrying pLH12 was significantly lower than PsmDG3 or PsmES4326 carrying pLAFR3 (*P* < 0.05 on days 2 and 3; unpaired *t* tests). Growth of PsmDG6 was significantly different from all other strains on days 2 and 3 (*P* < 0.02; unpaired *t* tests).

Avr proteins have modular regions required for their function in the host environment.

Previously, Mudgett and Staskawicz (1999) established that the effector region of AvrRpt2, required for RPS2-mediated recognition, resided between amino acids 120 to 255 in the C-terminal portion of the protein. We confirmed these findings independently with a transient expression system that relies on biolistic transformation of plant cells (data not shown). Deletion of sequences encoding the first 9 to 119 amino acids was previously shown to abolish secretion and translocation with-

out affecting the AvrRpt2 protein accumulation in *P. syringae* (Mudgett and Staskawicz 1999). This suggests that a secretion and translocation signal might reside in the N-terminal region of AvrRpt2. Recently, Anderson and colleagues (1999) showed that the 5' mRNA regions encoded by several phytopathogenic effector genes were sufficient for the secretion of a reporter protein from bacteria into the extracellular medium. Collectively, these experiments support the notion that Avr proteins are modular with respect to secretion, although it has not yet been addressed whether the 5' mRNAs or additional sequences are sufficient for the secretion and/or translocation of phytopathogenic effector proteins expressed during a host-pathogen interaction. This is an important consideration because in the *Yersinia* spp. YopE effector, different signals are required for in vitro secretion and in vivo translocation into animal cells (Cheng et al. 1997).

We tested our single-copy genomic expression system for its utility to further establish the modular structure of Avr effectors when they function in the context of host-pathogen interactions. This system allowed for the rapid construction of Avr chimeras because the integration vector was experimentally easier to manipulate than the available broad-host-range vectors as a result of its relatively small size. We made chimeras encoding the C terminus of AvrRpt2 and the N terminus of another Avr protein, AvrRpm1. Three chimeras were constructed: pDSG7 encodes a fusion of the first 43 amino acids of AvrRpm1 to the C-terminal 214 amino acid of AvrRpt2 expressed under the control of the AvrRpm1 promoter; pDSG8, which encodes a fusion of the first 43 amino acids of AvrRpm1 to the C-terminal 176 amino acids of AvrRpt2 under

Fig. 3. Hypersensitive response (HR) induction by bacteria harboring *avrRpt2*. **A**, HR assay of *Pseudomonas syringae* strains carrying the control vector (left), *avrRpt2* carried on the low-copy-number cosmid pLAFR3 (center), or single-copy integrated *avrRpt2* (right). Half leaves (inoculated side is indicated with a white dot) were inoculated at optical density (OD) of 600 nm at 0.01 and photographed after 24 h. Recognition of the AvrRpt2 protein by plants producing the resistance protein RPS2 resulted in an HR (cell death). Leaves infected by the control strain with the suicide vector integrated at the *recA* locus (PsmDG3) looked identical to the strain carrying the empty vector (no *avr* gene, data not shown). This experiment was repeated four times. **B**, Effect of AvrRpt2 on virulence in *rps2* plants. Congenic strains derived from PsmES4326, which differed only by the presence or absence of *avrRpt2* integrated in single copy into the genome, were inoculated at OD₆₀₀ at 0.0002 into *Arabidopsis* lacking the *RPS2* resistance gene. Plants were photographed after 6 days. The strain carrying the *avrRpt2* gene (PsmDG6) produced noticeably more disease symptoms than the strain lacking *avrRpt2* (PsmDG3). **C**, HR test of AvrRpt2 and AvrRpm1::AvrRpt2 chimeric protein. The chimera is the native promoter, and the first 43 amino acids of AvrRpm1 fused to AvrRpt2 harboring a 79 amino acid deletion of its N terminus. PsmES4326 strains carrying either a plasmid-borne copy of *avrRpt2* or an integrated single copy of the AvrRpm1::AvrRpt2 chimera were inoculated at OD₆₀₀ at 0.01 into one half of an *Arabidopsis* leaf (side inoculated is indicated with a white dot). In some cases, the leaf half that was inoculated showed such a severe HR that the whole side of the leaf was crushed against the uninoculated leaf half. The plant genotypes are presented above the leaves. Rps2 is the cognate resistance protein to AvrRpt2. Rpm1 is the cognate resistance protein to AvrRpm1. The same plant genotypes inoculated with control strains lacking the indicated *avr* genes or *avr* fusion genes showed no HR on the plants (data not shown). Additionally, PsmES4326 with full length *avrRpm1* integrated at the *recA* locus did not induce the HR on *RPS2*⁺*-rpm1*⁻ plants (data not shown). This experiment was repeated three times.

the control of the AvrRpm1 promoter; and pDSG9, which encodes a fusion of the first 89 amino acids of AvrRpm1 to the C-terminal 214 amino acids of AvrRpt2 under the control of the AvrRpm1 promoter. These constructs were integrated into PsmES4326 and used for infection experiments.

All three chimeras were capable of eliciting *RPS2*-dependent resistance responses, as evidenced by the decreased growth of the pathogens carrying these fusions (Fig. 6) and *RPS2*-dependent-*RPM1*-independent HR (Fig. 3C). The control strain bearing full-length *avrRpm1*, however, showed an HR only on *RPM1*⁺ plants (data not shown). No decrease in growth was found in infections of *RPM1*⁺*rps2*⁻ plants, except with the control strain (PsmDG34) bearing full-length integrated *avrRpm1* (Fig. 6). This indicates that the specificity of AvrRpt2 was retained in the chimeras. Thus, the *avrRpm1*-encoded N-terminal 45 amino acids fused to the C terminus of AvrRpt2 reversed the HR⁻ phenotype, previously reported for the truncated AvrRpt2 protein (Mudgett and Staskawicz 1999), which suggests that the N terminus of AvrRpm1 delivered the C terminus of AvrRpt2 to the cell surface and likely mediated its translocation into the plant cell where AvrRpt2 functions (Leister et al. 1996).

Mudgett and Staskawicz (1999) reported that N-terminal deletions of AvrRpt2 missing up to 119 amino acids were synthesized and stable in *P. syringae*. We could not detect AvrRpt2 protein by immunoblot analysis, however, in any of our *avrRpt2* N-terminal deletion constructs (even those deleting as few as three amino acids after the start methionine) in PsmES4326 (data not shown). Therefore, we could not independently verify that the HR⁻ and secretion-translocation deficiency phenotypes of *P. syringae* carrying similar deletion constructs of *avrRpt2* to those previously reported (Mudgett and Staskawicz 1999) were not simply a result of the lack of the protein. The deletion constructs reported by Mudgett and Staskawicz (1999) were driven by the heterologous *nptII* promoter, whereas our constructs retained the native *avrRpt2* promoter. Because truncated AvrRpt2 protein can accumulate in *P. syringae*, the lack of truncated AvrRpt2 accumulation in our strains with the native *avrRpt2* promoter was likely a result of the disruption of a 5' uncharacterized regulatory element, possibly encoded in mRNA (Anderson et al. 1999). This

element appears to require the native promoter in order to have an influence on AvrRpt2 production.

DISCUSSION

This study demonstrates the advantages of the use of single-copy chromosomal integrations at the *recA* locus for functional and structural studies of the AvrRpt2 and AvrRpm1 effectors of *P. syringae*, from which three major conclusions can be drawn. First, we found that *avrRpt2* and *avrRpm1* can function in single copy and induce resistance responses that were stronger than those evoked by the same strains bearing plasmid-borne *avr* genes. Second, we demonstrated that AvrRpt2 is involved in virulence (the ability to cause disease symptoms in the host), in planta growth, and transmission in the *P. syringae* infections of *rps2*⁻ plants. These functions were difficult to demonstrate with a plasmid-borne copy of the gene, probably as a result of variable gene doses and/or plasmid stability during the low-dose infections (*our unpublished observations*). Finally, we showed that C-terminal regions of AvrRpt2 could be delivered to plant cells when fused to the N terminus of AvrRpm1, suggesting modular structures for Avr proteins required for their function during host-pathogen interactions. The cloning and integration of the *avr* chimeras was greatly facilitated by the use of the genomic integration vector, which is smaller and easier to manipulate than most broad-host-range vectors currently in use.

The integration vector used in this study is broadly useful for expressing genes in *Pseudomonas* spp. We have successfully performed integrations in *P. syringae* pathovars *maculicola*, *phaseolicola*, and *tomato*. Additionally, the high conservation of the *recA* locus between *Pseudomonas* spp. allows the integration of our constructs into *P. aeruginosa* (L. Rahme, *personal communication*). While we could not detect any pathogen fitness cost for *P. syringae* pv. *maculicola* having constructs integrated at the *recA* locus, it is possible that such a cost would occur for bacteria growing under field conditions. This would have to be specifically tested by researchers interested in using this method to achieve heterologous expression of virulence genes. If *recA* integrants showed decreased fitness in the field, however, it should be possible to

Fig. 4. Ion leakage from *Pseudomonas syringae*-infected tissue. Indicated strains were inoculated into plants. Fifteen hours later, the tissue was assayed for ion leakage with conductivity measurements. Four samples were used for each measurement. Bars indicate standard error of the mean. Letters above each bar represent significance groups as determined by Fisher's protected least significant difference measure (a post-hoc multiple *t* test). Each letter group differs from the other letter groups at a level of *P* < 0.05 or better. Each test was performed only within an inoculation dose. This experiment was repeated twice with similar results.

modify the targeting locus and use double recombinants to achieve chromosomal insertions that are similarly useful to the ones documented in this study.

One surprising outcome of this study is that AvrRpt2 induced a stronger host defense response, as evidenced by greater tissue collapse, greater ion leakage, and less pathogen

growth when the *avrRpt2* gene was integrated in single copy. We do not believe that the loss of *recA* as a result of the integration of the vector is a significant factor because we observed similar results when we introduced *avrRpt2* into random genomic locations with a transposon (our unpublished observations). It is possible that AvrRpt2 is overproduced from the multicopy cosmid and that the protein excess blocks the secretion apparatus and, as a result, inhibits translocation into the plant cell. It also is possible that the plasmid is lost at a low rate when the bacteria are grown in the plant, which would result only in a proportion of the infecting bacteria being able to induce the host defenses. In support of this possibility, we found that the extrachromosomal vector pLAFR3 has a loss rate of 20 to 45% from PsmES4326 after 4 days of

Fig. 5. Bacterial growth assay of the virulence advantage conferred by AvrRpt2. Bacteria harboring either an integrated *avrRpt2* (PsmDG6) or a control vector (PsmDG3) were inoculated into *rps2* plants. Three measures of bacterial growth were taken each day. **A**, Leaves were first washed to collect and measure the bacteria growing on the leaf surface. **B**, A fixed-diameter leaf punch was taken from the most highly infected region of the leaf. **C**, Remainder of the leaf was homogenized to determine total bacterial load. Twelve replicates were taken at each time point for each strain. The experiment was repeated three times. Error bars represent one standard error above and below the mean. Results of unpaired *t* tests are presented below each data point. $P = 0.05 > * > 0.01 > ** > 0.001$.

Fig. 6. Bacterial growth assays: AvrRpm1::AvrRpt2 chimeric protein function. Growth of bacteria containing either an integrated control vector (PsmDG3), integrated copy of *avrRpt2* (PsmDG6), integrated copy of *avrRpm1* (PsmDG34), or integrated copy of the *avrRpm1::avrRpt2* chimeric gene (PsmDG8, composed of the native *avrRpm1* promoter, and the first 43 amino acids of AvrRpm1 fused to a 79 amino acid N-terminal deletion of AvrRpt2) was measured on two plant genotypes. **A**, *RPS2*⁺–*RPM1*⁺: Growth of all the strains was significantly different from PsmDG3 on days 2 and 3 ($P < 0.02$; unpaired *t* tests). **B**, *rps2*[–]–*RPM1*⁺: Growth of strains PsmDG3, PsmDG6, and PsmDG8 was not significantly different, although of strain PsmDG3 was significantly different from all other strains on days 2 and 3, except for day 3 with strain PsmDG8, which had a *P* value of 0.06; unpaired *t* tests). *RPS2* is cognate resistance protein to AvrRpt2. *RPM1* is the cognate resistance protein of AvrRpm1. Bacteria were inoculated at optical density of 600 nm at 0.002. Eight replicates were taken at each time point, and the experiment was repeated three times. The error bars represent one standard error above and below the mean.

growth of the bacteria in planta, whereas the *recA* integrated vector is completely stable.

The virulence and growth function of AvrRpt2 in *rps2⁻* plants add to the growing number of type III effectors that increase bacterial fitness in plants lacking the cognate *R* genes (Gabriel 1999). Although the growth advantage conferred by AvrRpt2 was modest, even small differences in growth would confer a significant fitness advantage over pathogens lacking the gene. The leaf-wash experiments showed that bacteria carrying *avrRpt2* were more prevalent on leaf surfaces than the bacteria lacking the gene, indicating a role for AvrRpt2 in enhancing transmission. Because no bacteria were detected on the majority of leaf surfaces at the beginning of the experiment, any bacteria that were found there at subsequent times must have been released from the apoplast of the plant cells in the infected tissue. We envision at least two ways that AvrRpt2 might aid in allowing greater recovery of bacteria from plant surfaces after infection. First, bacteria carrying *avrRpt2* may be released earlier or in greater numbers onto leaf surfaces than bacteria lacking the gene. In a second scenario, bacteria harboring *avrRpt2* may be released at the same rate as bacteria lacking *avrRpt2*. Subsequent to release, AvrRpt2 might confer a fitness advantage, resulting in a greater growth rate of the bacteria on leaf surfaces. A combination of the two scenarios also may occur. Our data does not allow us to distinguish between a role for *avrRpt2*, specifically in release of bacteria, or in epiphytic growth once bacteria reach the leaf surface. Epiphytic-growth experiments in which bacteria were inoculated only on leaf surfaces, however, indicated that bacteria carrying *avrRpt2* grew the same or slightly worse than the control strain lacking the gene (*our unpublished observations*).

Similar evidence for increased in planta growth of *P. syringae* carrying *avrRpt2* comes from studies of *P. syringae* pv. *tomato* strain DC3000 carrying *avrRpt2* gene on a plasmid. *rps2⁻* plants in the relatively *P. syringae*-tolerant Nossen ecotype and mutants of the Col ecotype with constitutive disease resistance showed enhanced susceptibility to *P. syringae* carrying *avrRpt2* (Chen et al. 2000). A virulence effect was not discernible in the *P. syringae*-susceptible Col ecotype, however, unless *avrRpt2* was expressed directly inside the plant cells. This may be a result of variable gene doses of *avrRpt2* or plasmid loss from *P. syringae* during the growth studies. These authors did not examine a role for AvrRpt2 in transmission. The fact that *avrRpt2* conferred a fitness advantage in the context of two different *P. syringae* pathovars suggests that a virulence function for AvrRpt2 is biologically meaningful and not an artifact of expression in strain PsmES4326. The definitive assessment for its role in the strain in which it was discovered (PtoJL1065) (Dong et al. 1991; Whalen et al. 1991), however, would require a gene knockout in that strain. Unfortunately, our efforts to transform PtoJL1065 have not been successful.

How might AvrRpt2 function to enhance virulence? One possibility is that it acts directly on a plant target to compromise the induction or execution of plant defenses. A second possibility is that plant defenses are not compromised, but AvrRpt2 stimulates a plant response that counteracts the effects of the other defenses. For example, AvrRpt2 might cause the release of needed nutrients from the plant into the apoplast by binding to and altering membrane permeability

or ion channels. Another possibility is that AvrRpt2 acts indirectly by allowing bacteria to increase the levels or activities of other virulence factors such as other effectors or toxins. A combination of these mechanisms also is possible. The fact that plants producing AvrRpt2 can enhance the virulence of *P. syringae* pv. *tomato* (Chen et al. 2000) suggests that its effects probably are exerted in the plant and not through enhanced activation of other virulence factors. An effect of AvrRpt2 on the levels of known virulence factors, however, has not yet been explored. Interestingly, some accessions of *Arabidopsis* spp. naturally lacking *RPS2* do not show enhanced disease when infected with PsmES4326 carrying *avrRpt2*, suggesting that there is genetic variability of the plant-host target(s) of AvrRpt2 that could be exploited to identify the molecular nature of the target(s) (B. A. Vinatzer and J. T. Greenberg, *unpublished observations*).

Structure–function analysis of phytopathogenic effectors shows that an mRNA sequence encoded in the 5' end of several genes is sufficient to mediate secretion into a heterologous protein medium, similar to effectors from animal pathogens (Anderson and Schneewind 1997; Anderson et al. 1999). Deletion experiments have localized the *R*-gene-mediated recognition of AvrRpt2 to the C-terminal portion of the protein (Mudgett and Staskawicz 1999). Our results show that the AvrRpm1 N terminus can complement a secretion–translocation-defective C-terminal region of AvrRpt2 and restore the ability of AvrRpt2 to activate an *R*-gene-specific resistance response. This finding further supports the notion that effector functions are encoded in contiguous stretches of primary sequence that comprise functional modules. Importantly, our results support the view that one of those separable modules functions to deliver effectors to the plant cell rather than only to the extracellular medium, as demonstrated in *in vitro* studies (Anderson et al. 1999). It has not been shown conclusively that phytopathogenic effectors are directly translocated into plant cells, although the fact that AvrRpt2 and AvrRpm1, when expressed inside plant cells, act in an *R*-gene-specific manner (Leister et al. 1996; Rate et al. 1999) strongly suggests that they are directly translocated. Thus, it is likely that the N terminus of AvrRpm1 encodes a sequence that is sufficient for translocation of the C-terminal effector module of AvrRpt2.

The N-terminal region of AvrRpm1 as well as several other effectors harbors myristoylation and palmitoylation sites (Nimchuk et al. 2000). Myristoylation is important for optimal function and localization of AvrRpm1 to the host plasma membrane (Nimchuk et al. 2000). The fact that the N terminus of AvrRpm1 could restore to the plant delivery of the secretion–translocation-deficient C-terminal region of AvrRpt2 suggests that AvrRpt2 may function when tethered to the membrane. It is unlikely, however, that AvrRpt2 is normally anchored to the membrane in this way. Although AvrRpt2 has a possible myristoylation site at amino acid 121, the protein is not processed in a manner likely to expose the myristoylation site for binding to the membrane (Mudgett and Staskawicz 1999). It is more likely, therefore, that if AvrRpt2 localizes to the membrane, it does so via interactions with a membrane-associated protein. Alternatively, AvrRpt2 might not normally localize to the membrane. Note that not all of the AvrRpm1 protein localizes to the membrane in planta and thus the chimeras between AvrRpm1 and AvrRpt2 may have membrane

and nonmembrane locations. In support of this possibility, we found that strains bearing any of several reciprocal chimeras in which the N terminus of AvrRpt2 was fused to the C terminus of AvrRpm1 failed to show any *RPM1*-dependent HR when inoculated into plants (*our unpublished observations*). This could indicate that the AvrRpt2–AvrRpm1 chimeras cannot localize to the appropriate location in planta as a result of the lack of a myristoylation site in the AvrRpt2–AvrRpm1 chimera. Further studies of the in planta locations of AvrRpt2 and the various chimeras will clarify the role of membrane localization in AvrRpt2 function.

In summary, the use of single-copy expression of *avrRpt2* has provided new insights into virulence and a transmission function for AvrRpt2. Additionally, it allowed for the rapid construction and analysis of altered versions of AvrRpt2 for the functional dissection of the protein modules. The quantitative difference in phenotypes between strains carrying *avr* genes on plasmids and those harboring single-copy integrations suggests that care should be taken when examining effector functions with the use of genes on multicopy plasmids.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 1. *P. syringae* strain PsmES4326 was obtained from F. M. Ausubel (Massachusetts General Hospital and Harvard University, Boston, MA, U.S.A.). *Escherichia coli* strains were grown in Luria-Bertani or SOC medium (Sambrook et al. 1989) in a tube roller at 37°C. *P. syringae* strains were grown in King's B (KB) medium (King et al. 1954) or SOC in a tube roller at 30°C. Where specified, kanamycin (Km), streptomycin (Str), tetracycline (Tet), ampicillin (Amp), and cycloheximide (CH) were added to the liquid media and plates in the following concentrations per ml: 50 µg of Km, 100 µg of Str, 25 µg of Tet, 100 µg of Amp, and 50 µg of CH. Strains harboring vector-borne *avrRpt2* or *avrRpm1* carried these genes on derivatives of pLAFR (plasmid pLH12) (Whalen et al. 1991) and pK48 (Dangl et al. 1992), respectively.

Plant lines and growth conditions.

Plants (*Arabidopsis thaliana*) were grown in growth chambers under long-day conditions (cycle = 16 h of light, 8 h of dark), as described in Greenberg et al. (2000). All assays were performed with *Arabidopsis* spp. ecotype Columbia, unless indicated otherwise. *Arabidopsis* spp. *rps2-101c* (ecotype Columbia) was provided by F. M. Ausubel. Ecotype HS12 (*rpm1-RPS2*) was provided by R. Mauricio (University of Georgia, Athens, GA, U.S.A.).

Strain construction.

All polymerase chain reaction (PCR) amplifications were performed with Turbo PFU (Stratagene, La Jolla, CA, U.S.A.), following the manufacturer's recommendations. Table 2 provides primer sequences. PCR products and vectors were cleaned with QIAquick PCR purification columns (Qiagen, Chatsworth, CA, U.S.A.) prior to and following all enzymatic steps. Restriction and modification enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). All of the integration vectors were derived from pBluescript SK+ (Stratagene). A *trpA* terminator was constructed out of two oligonucleotides (*trpA*term+ and *trpA*term–) and cloned between the *NotI* and *XbaI* sites of pBluescript to create vector pBCT. Neomycin phosphotransferase (*nptII*, conferring resistance to Km and neomycin) was PCR amplified from pZERO2.1 (Invitrogen, San Diego, CA, U.S.A.) with *npt+* and *npt–* primers and cloned into the *AseI* site of pBCT to create vector pCKT. A 527-bp internal fragment of the *recA* gene was amplified from PsmES4326 with *recA*+271 and *recA*–797 and cloned into the *ApaI* site of pCKT to create vector pCKTR. *avrRpt2* was PCR amplified from pLH12. The promoter and full coding sequence were amplified with primers *avrRpt2*+4 and *avrRpt2*–1,146. Other primers used and notes regarding the constructs they were used for are presented in Table 2. All constructs are available upon request from D. S. Guttman or J. T. Greenberg. Southern blots were performed following Sambrook et al. (1989).

Avr chimeras were created by *SapI* seamless cloning. PCR primers extending from the ends of fragments to be cloned in

Table 1. Bacteria and plasmids used in this work

Strain/plasmid	Characteristics ^a	Reference/source
<i>Pseudomonas syringae</i> pv. <i>maculicola</i>		
PsmES4326	Str ^r	F. M. Ausubel
PsmDG3	Str ^r PsmES4326 <i>recA</i> integration of pCKTR	This study
PsmDG6	Str ^r PsmES4326 <i>recA</i> integration of pDSG6	This study
PsmDG7	Str ^r PsmES4326 <i>recA</i> integration of pDSG7	This study
PsmDG8	Str ^r PsmES4326 <i>recA</i> integration of pDSG8	This study
PsmDG9	Str ^r PsmES4326 <i>recA</i> integration of pDSG9	This study
PsmDG34	Str ^r PsmES4326 <i>recA</i> integration of pDSG34	This study
Plasmids		
pLAFR3	Tet ^r broad-host-range vector	Staskawicz et al. 1987
pLH12	Tet ^r pLAFR3 containing <i>avrRpt2</i>	Whalen et al. 1991
pK48	Tet ^r pLAFR5 containing <i>avrRpm1</i>	Debener et al. 1991
pBC	Cm ^r cloning phagemid	Stratagene, La Jolla, CA, U.S.A.
pCKTR	Km ^r pBluescript derivative contains <i>nptII</i> and a 527-bp internal <i>recA</i> fragment from PsmES4326	This study
pDSG6	pCKTR containing <i>avrRpt2</i> (including native promoter)	This study
pDSG7	pCKTR containing <i>avrRpm1</i> (pro + 43 aa):: <i>avrRpt2</i> (Δ41 aa)	This study
pDSG8	pCKTR containing <i>avrRpm1</i> (pro + 43 aa):: <i>avrRpt2</i> (Δ79 aa)	This study
pDSG9	pCKTR containing <i>avrRpm1</i> (pro + 89 aa):: <i>avrRpt2</i> (Δ41 aa)	This study
pDSG34	pCKTR containing <i>avrRpm1</i>	This study

^a pro = promoter of the respective gene; aa = amino acid.

the chimeric fusion junction had a 5' extension that included the recognition sequence for the restriction enzyme *SapI*, which cuts downstream from its recognition sequence and leaves cohesive ends that can be specified during primer design. PCR products were amplified with Turbo PFU, cut with *SapI*, and ligated, diluted, and reamplified with the two primers that would amplify the entire chimeric gene. These products were then cloned into pCKTR.

Some chimeric gene constructs and deletions were made by "round the vector" (RV) cloning. In this strategy, the locus or loci of interest were individually cloned into pCKTR. PCR primers were then designed to amplify away from each other (pointing out toward the vector), with their 5' ends corresponding to the desired fusion junctions. Turbo PFU PCR reactions were carried out to amplify the entire vector (approximately 4 to 7 kb in length, depending on the particular construct). This product was phosphorylated with polynucleotide kinase (PNK; New England Biolabs) and religated. Each construct was sequenced to ensure the integrity of the fusion junction and to control for mutations that could arise during the PCR reactions.

Vector integration and bacteria mating.

Vectors were electroporated into *P. syringae* at a capacitance of 3 µF, resistance of 500 ohms, and voltage of 2.5 kV. Approximately 10 ng of vector was used for each 40 µl of electrocompetent cells. Transformed bacteria were grown in 1 ml of SOC at 30°C for 1 h and pelleted at 5 K for 5 min. The entire pelleted culture was resuspended in approximately 100 µl of media, spread onto KB Km plates, and grown at 30°C. Positive colonies typically appeared within 3 days. Integration at *recA* was verified by Southern blot analysis and sensitivity to shortwave UV. *recA*⁻ *P. syringae* were three orders of magnitude more sensitive to UV irradiation than the parent strain.

HR, disease assay, and in vivo growth assay.

For plant inoculation studies, *P. syringae* were grown to saturation in KB, diluted, regrown to OD₆₀₀ at 0.5 to 1.0, centrifuged, and resuspended in 10 mM MgSO₄ to a concentration of OD₆₀₀ at 1.0 prior to dilution for plant inoculations. All

inoculations were performed with a blunt 1-ml syringe, as described previously (Greenberg et al. 2000). For the HR assay, bacteria were diluted in 10 mM MgSO₄ to a range of OD₆₀₀ at 0.01 to 0.1 and inoculated into leaves from unbolted long-day-grown plants. Plants were left open in the growth chambers and scored for HR symptoms or water soaking and yellowing (disease symptoms) after 18 to 24 h. For conductivity measurements to quantitate the strength of the HR, leaf punches (6 mm in diameter) were placed in 1 ml of ddH₂O with the abaxial side toward the solution for 1 h. Conductivity measurements of the solutions were measured with a model CDM2a conductivity meter (Radiometer Copenhagen, Brønshøj, Denmark). To assess the severity of disease symptoms, leaves were first photographed and scanned by a ScanMaker 4 (Microtek, San Francisco, CA, U.S.A.) with Photoshop LE software (Adobe, San Jose, CA, U.S.A.). Printouts of the scanned images of each leaf were cut to separate the fully green portion from the yellowed portion. Each of these portions was subsequently weighed to determine the percentage of green tissue.

For standard in vivo growth assays, bacteria were diluted to OD₆₀₀ at 0.002 and inoculated in young leaves approximately 1 cm in length. The leaves were allowed to dry for 2 h and then tightly covered with a plastic lid to maintain high relative humidity (Rate et al. 1999). Leaf punches (6 mm in diameter) were surface sterilized in 70% ethyl alcohol for 1 min, homogenized for 3 s in 200 µl of 10mM MgSO₄ with the use of a plastic disposable pestle and a 3/8-in. power drill (Skil, Chicago, IL, U.S.A.) set at low speed, diluted in 10 mM MgSO₄, and plated on KB-glycerol agar plates containing Km.

For the virulence assays, *rps2* plants were inoculated with *P. syringae* at OD₆₀₀ at 0.0002. The plants were then washed thoroughly with water to remove bacteria adhering to the surface, allowed to dry in the open for 2 h, and covered with a plastic dome. Whole leaves of similar age and approximately 2 to 3 cm in length were collected daily from day 0 through 6 (excluding day 5) in 500 µl of 10mM MgSO₄ and vortexed to remove surface bacteria. Dilutions of this surface wash were plated on KB-glycerol plates containing Km, Str, and CH. The washed leaves were assayed for bacteria, as described above.

Table 2. Polymerase chain reaction (PCR) primers used in the work

PCR primer	Primer sequence ^a	Notes ^b
npt ⁺	GGTCTGACGCTCAGTGAACGAAAAC	
npt ⁻	TCCAACTGGAACAACACTCAACCC	
recA + 271	TTCGTCGACGCCGAGCACGC	
recA - 797	CCCTTGCCGTAGAGGATCTGGAATC	
trpAterm ⁺	CAGCCCGCCTAATGAGCGGGCTTTTTTTGC	
trpAterm ⁻	GGCCGCAAAAAAAGCCCGCTCATTAGGCGGGCTGAGCA	
avrRpt2+4	GATTGATCTCTGGCTCAGTGCG	avrRpt2 5' primer
avrRpt2-1146	TGGCAGATGCTCATGGACAAGCG	avrRpt2 3' primer
avrRpm1 + 5p190	GAATTCGGCAAAAATCGTACGAG	avrRpm1 5' primer
avrRpm1-3p660	AAAGTCATCTTCTGAGTCAGACTGAACAGC	avrRpm1 3' primer
Primers used for <i>avrRpm1::avrRpt2</i> chimera cloning		
avrRpt2 + 296sap	(GATGCTCTTCAGGG)AAAAGTAGCGCTTCAAGCCCG	41 aa N-terminus deleted
avrRpt2 + 409sap	(GATGCTCTTCAGGG)AAGCACGAGACGGCGGTTCAAGTG	79 aa N-terminus deleted
avrRpm1 - 135sap	(GATGCTCTTCACCC)ATTGGAATGCTCGGAATCGGTTTC	N-terminus 45 aa
avrRpm1 - 268sap	(GATGCTCTTCACCC)CTCGGTTGCACCATCAGTTTTC	N-terminus 89 aa

^a Parenthetical segment used for *SapI* seamless cloning.

^b aa = amino acid.

Protein extraction and immunoblot assay.

P. syringae strains were grown in M9 minimal media (Sambrook et al. 1989) with sucrose to an OD₆₀₀ at 1.0. One milliliter of cells were pelleted, resuspended in 50 µl of alkaline lysis miniprep solution 1 (Sambrook et al. 1989), 50 µl of 2.22× denaturing sample buffer (125 mM Tris, pH 6.8; 20% glycerol; 4% sodium dodecyl sulfate [SDS]; 5 M urea; 0.01% bromophenol blue), and 10 µl of 1 M dithiothreitol, and heated to 95°C for 5 min. Protein samples were electrophoresed on SDS-polyacrylamide slab gel electrophoresis in Tris-glycine running buffer (1.9 M glycine; 250 mM Tris-base 0.1% SDS, pH 8.8) and transferred to a Millipore Immobilon-P membrane by semidry blotting in transfer buffer (1.9 M glycine, 250 mM Tris-base, 10% methanol). Membranes were blocked in BLOTTO (Sambrook et al. 1989) and incubated overnight at 4°C in 1:10,000 anti-AvrRpt2. Donkey anti-rabbit secondary antibody was used at 1:5,000 after four room-temperature washes in BLOTTO. Detection was carried out in Supersignal West Pico developing reagent (Pierce, Rockford, IL, U.S.A.) for 1 to 30 s.

Statistical analysis.

All statistical analyses were performed with StatView for Windows or Macintosh version 5.0.1 (SAS Institute, Cary NC, U.S.A.).

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