Molecular recognition of pathogen attack occurs inside of plant cells in plant disease resistance specified by the Arabidopsis genes RPS2 and RPM1

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ABSTRACT The Arabidopsis thaliana disease resistance genes RPS2 and RPM1 belong to a class of plant disease resistance genes that encode proteins that contain an N-terminal tripartite nucleotide binding site (NBS) and a C-terminal tandem array of leucine-rich repeats. RPS2 and RPM1 confer resistance to strains of the bacterial phytopathogen Pseudomonas syringae carrying the avirulence genes avrRpt2 and avrB, respectively. In these gene-for-gene relationships, it has been proposed that pathogen avirulence genes generate specific ligands that are recognized by cognate receptors encoded by the corresponding plant resistance genes. To test this hypothesis, it is crucial to know the site of the potential molecular recognition. Mutational analysis of RPS2 protein and in vitro translation/translocation studies indicated that RPS2 protein is localized in the plant cytoplasm. To determine whether avirulence gene products themselves are the ligands for resistance proteins, we expressed the avrRpt2 and avrB genes directly in plant cells using a novel quantitative transient expression assay, and found that expression of avrRpt2 and avrB elicited a resistance response in plants carrying the corresponding resistance genes. This observation indicates that no bacterial factors other than the avirulence gene products are required for the specific resistance response as long as the avirulence gene products are correctly localized. We propose that molecular recognition of P. syringae in RPS2- and RPM1-specified resistance occurs inside of plant cells.

In plants, robust defense responses to invading phytopathogens often conform to a gene-for-gene relationship: resistance to a pathogen is only observed when the pathogen carries a specific avirulence (avr) gene and the plant carries a corresponding resistance (R) gene (1–3). Because avr-R gene-for-gene relationships are observed in many plant-pathogen systems and are accompanied by a characteristic set of defense responses, a common molecular mechanism underlying avr-R gene mediated resistance has been postulated (4). One simple model which explains gene-for-gene relationships is that the pathogen avr genes directly or indirectly generate a specific molecular signal (ligand) that is recognized by cognate receptors encoded by plant R genes. Recent cloning of plant resistance genes and corresponding pathogen avirulence genes provided the tools for a direct test of this ligand-receptor model (5).

In the phytopathogenic interaction between the small flowering plant Arabidopsis thaliana and the bacterial phytopathogen Pseudomonas syringae, two R genes, RPS2 (6, 7) and RPM1 (8), and three corresponding avr genes, avrRpt2 (9), avrRpm1 (10), and avrB (11), have been isolated. RPS2 confers resistance to P. syringae strains expressing avrRpt2 (12, 13) and RPM1 confers resistance to P. syringae expressing avrRpm1 (14) or avrB (15). RPS2 and RPM1 belong to a major class of plant resistance genes which encode proteins containing nucleotide binding sites (NBS) and leucine-rich repeats (LRR) and which confer resistance to bacterial, fungal, or viral pathogens (5). The structural conservation among R genes is consistent with the presence of a common molecular mechanism underlying gene-for-gene mediated disease resistance.

The hypersensitive response (HR) is the most characteristic defense response associated with gene-for-gene interactions (1). The HR involves rapid plant cell death localized at the site of infection. Using the HR as a marker for disease resistance, a transient expression assay for RPS2 function was previously developed that involves biolistic introduction of an RPS2 cDNA clone into plant cells (7). In this assay, expression of a β-glucuronidase (GUS)-encoding reporter gene coinroduced with RPS2 is monitored as an indicator of the HR; when the HR causes plant cell death, low levels of GUS activity are observed.

Here we report that the RPS2 gene product is probably localized in the plant cell cytoplasm. We also report that when transiently expressed in plants, two P. syringae avr genes, avrRpt2 and avrB, can elicit an HR in a gene-for-gene specific manner. For these experiments, the transient expression assay was enhanced to make it quantitative. We propose that molecular recognition of the pathogen in RPS2- and RPM1-specified resistance occurs inside of plant cells.

MATERIALS AND METHODS

A. thaliana plants representing four different R gene genotypes were used; ecotype Columbia (Col-0) wild type (phenotype, RPS2 RPM1; genotype, RPS2/RPS2 RPM1/RPM1), rps2-101C (rps2 RPM1; genotype, RPS2/rps2 RPM1/rps2-101C RPM1/RPM1; Col-0 background) (7, 16), ecotype Niederzenz (Nd-0) wild type (RPS2 rpm1: RPS2/RPS2 Δrpm1/Δrpm1) (8, 14); and a hybrid line derived from a cross between rps2-101C and Nd-0 with an rps2-101C/rps2-101C Δrpm1/Δrpm1 genotype (phenotype rps2 rpm1) (7). Plants were grown at 22°C with ~80% relative humidity with a 12 hr light/12 hr dark cycle in environment-controlled growth chambers.

The avrRpt2 and avrB genes were amplified by PCR using Pfu polymerase (Stratagene) from plasmids pLH12 (13) and pPSG0002 (17), respectively, using the primers, AVR1 and AVR2 for avrRpt2 and AVR1 and AVR2 for avrB: AVR1, 5'-CGCGGATCCACCATGATGAAAATTGCTC-3' and AVRB1, 5'-GGAGCGCGGCCGCTTGTCAT-3' for avrB, 5'-GAGGCCGGCGCGCTTTGATGCACGCACTTG-3' and AVRB2, 5'-CGCGATCCACCATGATGAAAATTGCTC-3' for avrRpt2. The primers

Abbreviations: avr gene, avirulence gene; R gene, resistance gene; HR, hypersensitive response; NBS, nucleotide binding site; LRR, leucine-rich repeats; GUS, β-glucuronidase; LUC, luciferase.

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were designed to change the sequence preceding the initiation codon into a eukaryote-type translation initiation site (18). Each PCR product was digested with BamHI and NotI and cloned into the BamHI–NotI site of the plant transient expression vector pKEX4tr (7) to obtain pEXavrRpt2 and pEXavrB, respectively. These plasmids were used for transient expression of theavr genes. A full-length RPS2 cDNA (clone 11) (7) in pKEX4tr was used for transient expression and for in vitro translation of RPS2. The RPS2 mutant I535K was created using PCR by changing the ATC codon of isoleucine 353 into the lysine codon AAG. Similarly, the rps2-101C nonsense mutation (7) was recreated by changing nucleotide G704 to A. These RPS2 wild-type and mutant genes in pKEX4tr were cut out with Pmel and SacI, and cloned into the Smal–SacI site of pBI11Rpro11 (a derivative of the plant transformation vector pBI2121) (Clonetech) in which the cauliflower mosaic virus 35S promoter was replaced with the 1.4-kb RPS2 promoter region; F.K. and F.M.A., unpublished) to obtain pR11-I535K, pR11-101C, and pR11-X11, respectively. These plasmids were used for generating transgenic plants. RPM1 cDNA clones were isolated from an A. thaliana cDNA library (7) in pKEX4tr by hybridization screening using a DNA probe based on the sequence of RPM1 (8). Three clones were purified, and one of the two full-length clones (clone 7), whose 5′ end starts at nucleotide number –86, was used for transient expression. pKEX4tr-G (7) was used for the GUS construct. The luciferase (LUC) construct p35S-LUC (19) was a gift from M. Bustos. Generation of transgenic plants and analysis of the plants for a macroscopic HR were performed as described (7).

For transient expression, gold biolistic particles (1 μm in diameter) were coated with DNA as described (7). Note that all genes for transient expression were linked to the cauliflower mosaic virus 35S promoter. pKEX4tr was cut out with NotI and SacI for baculovirus expression (20). Three clones were purified, and one of the two full-length clones (clone 7), whose 5′ end starts at nucleotide number –86, was used for transient expression. pKEX4tr-G (7) was used for the GUS construct. The luciferase (LUC) construct p35S-LUC (19) was a gift from M. Bustos. Generation of transgenic plants and analysis of the plants for a macroscopic HR were performed as described (7).

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utilizes rabbit reticulocyte lysate and dog pancreatic microsomes. In this procedure, if a protein, labeled with \(^{35}\text{S}\) by \textit{in vitro} translation, has a signal peptide for secretion, the protein is cotranslationally transported into microsomes. Proteinase K treatment and ultracentrifugation after \(\text{Na}_2\text{CO}_3\) treatment allow protein localization to be classified as cytoplasmic, membrane-integrated, or secreted (21). A cytoplasmic protein remains outside of microsomes, so that it is sensitive to proteinase K and is recovered in the supernatant after centrifugation. A secreted protein is transported into microsomes but remains soluble, so that it is protected from proteinase K and recovered in the supernatant after centrifugation. A membrane-integrated protein can be partially protected from proteinase K and is precipitated with the membrane after centrifugation. Fig. 3A shows the results of this analysis, including \(\beta\)-lactamase as a positive control for translocation. If \(\beta\)-lactamase, which is a secreted protein, was protected from proteinase K (lane 9) and detected in the soluble fraction after \(\text{Na}_2\text{CO}_3\) treatment (lane 11), demonstrating the high efficiency of the translocation system. \textit{In vitro}-translated RPS2 migrated with an apparent molecular weight consistent with that calculated from its deduced amino acid sequence (105 kDa; lane 2). RPS2 was not protected from proteinase K (lane 4). The major portion of RPS2 was detected in the soluble fraction after \(\text{Na}_2\text{CO}_3\) treatment (lane 6), but a significant amount was also detected in the precipitate fraction (lane 5). The amount of this precipitator remained the same in the absence of the translocation system (posttranslocation, Fig. 3B), indicating that the precipitate was an artifact. These results indicate a cytoplasmic localization for RPS2. Although the results must be interpreted with caution because of the use of a heterologous \textit{in vitro} system, taken together with the mutagenesis results described above, they suggest that RPS2 is a cytoplasmic protein.

When transiently expressed in plants, \textit{avrRpt2} can elicit a specific resistance response. If RPS2 is indeed cytoplasmic and if it is the primary receptor for the \textit{avrRpt2}-generated ligand, the ligand must also be present in the plant cytoplasm. A possible ligand for RPS2 is the \textit{avrRpt2} gene product itself. If \textit{AvrRpt2} protein were expressed in plant cells, it would most likely be located in the plant cytoplasm because it is a hydrophilic protein and does not have an obvious signal peptide (9). Based on these considerations, we tested whether expression of \textit{avrRpt2} in plants can elicit a specific resistance response.

Transient expression by biolistic bombardment was used for this purpose. Based on the same principle as the transient expression assay for RPS2 (7), reduced expression of a coin- 

![Diagram](image-url)
the avrRpt2 construct was co-introduced with the GUS construct into either RPS2 wild-type (Right) or rps2-101C mutant (Left) plants, and plant cells that expressed GUS were visualized by histochemical staining (blue dots in the figure). The GUS expression in wild-type plants was reduced compared with that in the rps2 mutant plants, indicating the occurrence of the HR in an RPS2-dependent manner. Therefore, avrRpt2 can elicit a resistance response with gene-for-gene specificity when expressed in plants.

A Quantitative Transient Expression Assay for the Resistance Response. A problem associated with biolistic bombardment is that the efficiency of transient transformation varies to a great extent both for each bombardment and for different areas of the target in a single bombardment. To quantitate the assay, a reference for transformation efficiency must be included. This was achieved by introducing a second reporter gene, LUC (24), linked to the 35S promoter, into cells different from the cells into which the GUS and avrRpt2 genes were introduced. Gold particles were coated either with the LUC construct or with the GUS and avrRpt2 constructs, the particles were mixed and then bombarded together. Because a relatively small number of plant cells are transformed by the biolistics procedure, the cells transformed with LUC and the cells transformed with the GUS and avrRpt2 are statistically different and far apart. Because the HR is a local event (1), LUC expression is not expected to be affected very much by an HR occurring in other cells. Therefore, LUC activity represents the relative transformation efficiency in a particular bombardment and can be used as a reference to normalize the GUS activity.

Using this quantitative transient expression assay, avrRpt2 was examined in RPS2 and rps2 plants which had either an RPM1 or rpm1 phenotype (Fig. 4, second row of each panel). Irrespective of the RPM1 phenotype, expression of avrRpt2 caused ~50% reduction of the normalized GUS activity in RPS2 plants (A and C) compared with the activity in rps2 plants (B and D). Another P. syringae avirulence gene avrB, which corresponds to the RPM1 resistance gene, was also examined (Fig. 4, third row of each panel). Similar to the results obtained with avrRpt2, irrespective of the RPM2 phenotype, expression of avrB caused more than 85% reduction of the normalized GUS activity in RPM1 plants (A and B) compared with the activity in rpm1 plants (C and D). Thus the gene-for-gene relationship for avrRpt2 and avrB is strictly conserved in this assay, confirming that the assay indeed reflects a specific resistance response. Two different P. syringae avr genes with distinct specificities can elicit a specific resistance response when expressed in plants, and no other bacterial components are required for this response.

The reduction of GUS activity reflects both the percentage of dead cells and how quickly the cells die. The reduction of GUS activity caused by avrRpt2 was smaller than that caused by avrB (Fig. 4). This is consistent with the observation that the HR caused by avrB is faster than the one caused by avrRpt2 when the avr genes are carried by a P. syringae strain (25).

In the case of avrB expression in RPM1 wild-type plants, we often observed a statistically significant reduction of LUC reference gene expression (typically 50–70% reduction) compared with the LUC expression in controls in which no avirulence genes were expressed (not shown). Therefore, the actual reduction of relative GUS activity in the case of avrB-RPM1 interactions was even greater than that shown in Fig. 4. A similar reduction in LUC expression was not observed in any of the other assay conditions, including the case in which avrRpt2 was expressed in RPS2 wild-type plants. This difference in LUC gene expression between the avrRpt2-RPS2 and avrB-RPM1 interactions may be correlated with different gene induction patterns that are observed between these two interactions (25, 26).

The Specific Resistance Response Can Be Elicited by Transiently Expressed avr and R Genes. The results of the transient

![Fig. 4. avrRpt2 and avrB can elicit a specific resistance response when expressed in plants. RPS2 RPM1 (A), rps2 RPM1 (B), RPS2 rpm1 (C), and rps2 rpm1 (D) plants were bombarded with biolistics carrying the GUS and the indicated avr gene constructs. Each bar represents the mean value of four bombardment events. (Bars = SEM.)](image-url)

expression of the avirulence genes prompted us to test whether the assay works when both avr and R genes are transiently expressed. In the following experiments, rps2 rpm1 double mutant plants were used for biolistic bombardment. The avr gene, R gene, and GUS gene constructs were used to coat one set of gold particles and the LUC gene construct was used to coat another set. As shown in Fig. 5A, when avrRpt2 and RPS2 were transiently expressed together, reduction of GUS activity was observed, but was not observed when RPS2 was expressed together with avrB. Similarly, reduction of GUS activity was observed when RPM1 was transiently expressed together with avrB, but not when RPM1 was expressed together with avrRpt2 (Fig. 5B). This strict conservation of the gene-for-gene specificity indicates that the transient expression assay can be used for functional analysis of both avr and R genes.

As in the experiment described in Fig. 4, the avrB-RPM1 interaction resulted in greater reduction of the GUS activity than the avrRpt2-RPS2 interaction, when both the avr and R genes were transiently expressed (Fig. 5). A significant reduction of LUC reference gene expression specific to the avrB-RPM1 interaction was also observed in this experiment (not shown).

**DISCUSSION**

To determine where the molecular recognition occurs between avr-generated signals and R-gene mediated responses in gene-
genes used in this study, the bacterial hrp (hypersensitive response and pathogenicity) gene cluster is required to elicit the specific resistance response in plants (29, 30). Some of the genes in the hrp cluster encode components of a type III protein secretion system (31). Bacterial pathogens of mammals, such as Salmonella, Shigella, and Yersinia, apparently use the type III secretion pathway to transfer proteins important for pathogenicity directly into mammalian cells (32). By analogy, *P. syringae* could also use a type III secretion system to directly transfer *avr* gene products into plant cells. The *hrp* cluster is also required for pathogenicity (33), suggesting that the type III secretion system may also transfer virulence gene products into plant cells.

To demonstrate effects of avirulence gene expression in plants, we used transient expression using biolistic bombardment. The principle of the assay, in which reduction of a co-introduced reporter gene expression is used as an indicator of the HR, was initially developed by us to demonstrate complementation of an *rps2* mutant phenotype by an *RPS2* cDNA clone (7). In this study, we showed that the transient expression assay can also be used to monitor the activity of a cloned *avr* gene by delivering the *avr* gene constructs biolistically into plant cells. (Fig. 2B). While this work was in progress, Gopalan et al. also reported that *avrB* can elicit a specific resistance response when expressed in plants using a transient expression assay that is essentially the same as the assay used in Fig. 2B (29).

One shortcoming of the biolistic mediated transient expression assay is that the transformation efficiency varies to a large extent, both with respect to independent bombardments and to different areas of the target. To compensate for this variability, our original assay for *RPS2* function involved infection of only one half of each leaf with *P. syringae* carrying *avrRpt2*; the uninfected half of the leaf served as a reference for the transformation efficiency (7). However, the assay for *avr* genes used in Fig. 2B did not include an internal reference for transformation efficiency. We therefore modified the transient expression assay by including a second reporter gene (*LUC*) delivered on a separately coated set of biolistic particles in the same bombardment event (Fig. 4). Using this quantitative assay, we have unequivocally demonstrated that transient expression of *avrRpt2* or *avrB* in plants elicits an HR in a gene-for-gene specific manner (Fig. 4).

We further extended the application of the functional transient expression assay by simultaneously assaying for both *avr* and *R* gene functions (Fig. 5). This rapid assay will be applicable to studies of many other gene-for-gene plant-pathogen systems for three reasons. First, biolistic transient transformation is applicable to many plant species. Second, the HR is a characteristic resistance response in gene-for-gene resistance. Third, simultaneous bombardment of *avr* and *R* genes is not limited by pathogen type. This assay may also be used for a rapid determination of whether a particular *R* gene can function in a heterologous host.

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