

Mutational Analysis of the *Arabidopsis RPS2* Disease Resistance Gene and the Corresponding *Pseudomonas syringae avrRpt2* Avirulence Gene

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Plants have evolved a large number of disease resistance genes that encode proteins containing conserved structural motifs that function to recognize pathogen signals and to initiate defense responses. The *Arabidopsis RPS2* gene encodes a protein representative of the nucleotide-binding site-leucine-rich repeat (NBS-LRR) class of plant resistance proteins. *RPS2* specifically recognizes *Pseudomonas syringae* pv. *tomato* strains expressing the *avrRpt2* gene and initiates defense responses to bacteria carrying *avrRpt2*, including a hypersensitive cell death response (HR). We present an in planta mutagenesis experiment that resulted in the isolation of a series of *rps2* and *avrRpt2* alleles that disrupt the *RPS2-avrRpt2* gene-for-gene interaction. Seven novel *avrRpt2* alleles incapable of eliciting an *RPS2*-dependent HR all encode proteins with lesions in the C-terminal portion of AvrRpt2 previously shown to be sufficient for *RPS2* recognition. Ten novel *rps2* alleles were characterized with mutations in the NBS and the LRR. Several of these alleles code for point mutations in motifs that are conserved among NBS-LRR resistance genes, including the third LRR, which suggests the importance of these motifs for resistance gene function.

Additional keywords: dexamethasone, genetic screen, hypersensitive response, inducible promoter, nucleotide sequence.

The *RPS2* gene of *Arabidopsis* conditions resistance against *Pseudomonas syringae* pv. *tomato* (*Pst*) expressing the avirulence (Avr) effector gene *avrRpt2* and encodes a typical resistance protein of the nucleotide-binding site-leucine-rich repeat (NBS-LRR) class (Bent et al. 1994; Mindrinos et al. 1994). Judging by studies of expressed sequence tags (ESTs) with homology to conserved NBS-LRR motifs (Botella et al. 1997), degenerate polymerase chain reaction (PCR) amplification of NBS-LRR homologs (Speelman et al. 1998) and the preliminary annotation of the complete sequence of *Arabidop-*

sis chromosomes 2 (Lin et al. 1999) and 4 (Mayer et al. 1999), the NBS-LRR class of resistance genes is abundant in the genome and likely represents a major mechanism of plant disease resistance. These proteins also can be classified by motifs present in the N terminus. Many NBS-LRR proteins have an N-terminal domain bearing either a region of similarity to the Toll protein of *Drosophila* and the mammalian interleukin-1 receptor (TIR) or a putative leucine zipper (LZ), whereas others have an N terminus with no recognizable protein motifs (Hammond-Kosack and Jones 1997). All NBS-LRR resistance genes encode a central NBS with a number of conserved motifs (Meyers et al. 1999) and a variable number of C-terminal LRRs. *RPS2* contains an N-terminal LZ, and is similar in primary structure to *Arabidopsis RPS5* (Warren et al. 1998) and *RPM1* (Grant et al. 1995). *RPS2*, *RPS5*, and *RPM1* also share a dependence upon the *NDR1* gene, which is predicted to encode a unique protein containing two membrane-integrated domains at the extreme N terminus and C terminus (Century et al. 1997). A loss-of-function mutation in *NDR1* eliminates disease resistance conditioned by these three genes (Century et al. 1995) but does not affect disease resistance mediated by several resistance proteins containing an N-terminal TIR domain (Aarts et al. 1998). This suggests that these LZ-NBS-LRR proteins act through a conserved mechanism that requires functional *NDR1*.

NBS-LRR resistance proteins often function to recognize extracellular pathogens, but are themselves predicted to be localized in the cytosol. Many gram-negative phytopathogens, including *Pseudomonas syringae*, contain a type III secretion system that most likely functions to deliver pathogen Avr proteins directly into the host cell (Galan and Collmer 1999). Consistent with this hypothesis are the observations that several Avr genes expressed within plant cells can elicit resistance gene-dependent defense responses (Kjemtrup et al. 2000). In addition, the recent detection of eukaryotic myristoylation of the avirulence proteins AvrB and AvrRpm1 in vivo (Nimchuk et al. 2000) suggests that these effectors must be translocated to the host for this posttranslational modification to occur. In the absence of resistance gene detection, it is thought that effector proteins are delivered into the plant cell where they act to promote virulence of the pathogen.

A major goal of current research is to assign function to the conserved protein motifs found in NBS-LRR resistance

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genes. It has been suggested that the LRR region of NBS-LRR proteins binds directly or indirectly to the Avr protein or an Avr-derived product. This model is developed on the basis of several lines of evidence, including the crystal structure of porcine ribonuclease inhibitor (a protein comprised entirely of LRRs) (Kobe and Deisenhofer 1993), the inference of diversifying selection acting upon LRR residues of R proteins (Noel et al. 1999; Parniske et al. 1997), and experimental evidence showing a direct role for LRRs in ligand binding in other plant proteins (Leckie et al. 1999). In this model, the NBS and N-terminal region function to initiate defense responses upon binding of the Avr ligand. Recently, evidence for direct or indirect interactions has been provided for the gene-for-gene systems *RPS2-AvrRpt2* (Leister and Katagiri 2000) and *Pi-ta-AVR-Pita* (Jia et al. 2000).

We made use of an inducible promoter system designed to conditionally express the *Pst avrRpt2* gene inside transgenic *Arabidopsis* plants (McNellis et al. 1998). Seedlings of such transgenic plants are killed within 1 week after germination on media containing the inducing glucocorticoid hormone by a mechanism that may represent a systemic hypersensitive response (HR) triggered by an *RPS2*-dependent pathway. We used this transgenic line in a mutant screen to isolate plants that survive germination in the presence of the inducing hormone in order to further characterize the genetic requirements for the *avrRpt2-RPS2*-mediated HR. Here we describe the results of this screen, including the characterization of seven *avrRpt2* loss-of-function alleles and ten *RPS2* loss-of-function alleles.

Table 1. *avrRpt2* mutant alleles

Mutant	Mutagen	Nucleotide (nt) mutation	Amino acid (aa) mutation
E-123C-1 ^a	EMS ^b	nt 343 C to T	aa 115 Gln to Stop
E-288P-3 ^c	EMS	nt 365 G to A nt 392 G to A	aa 122 Cys to Tyr aa 131 Gly to Asp
E-122C-2	EMS	nt 422 G to A	aa 141 Gly to Arg
E-122E-1	EMS	nt 580 G to A	aa 194 Gly to Arg
E-116D-1	EMS	nt 581 G to A	aa 194 Gly to Glu
E-122I-1	EMS	nt 600 G to A	aa 200 Trp to Stop
E-115H-1	EMS	nt 682 C to T	aa 228 Arg to Stop

^a Mutants are described by an arbitrary code indicating the mutagenized pool from which they were derived.

^b Ethyl methanesulfonate.

^c Two independent *avrRpt2* mutations were found in line E-288P-3.

RESULTS

Isolation of mutants defective in *RPS2-avrRpt2* dependent cell death.

Transgenic *Arabidopsis* plants (ecotype Col-0) possessing a glucocorticoid-inducible *avrRpt2* transgene (pTA7001-*avrRpt2*) respond to hormone application with a systemic death of tissue that is presumed to model the events occurring during the *RPS2-avrRpt2*-mediated HR (McNellis et al.

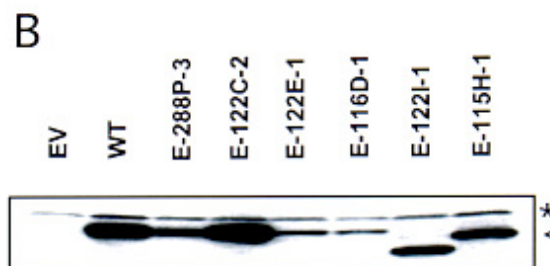
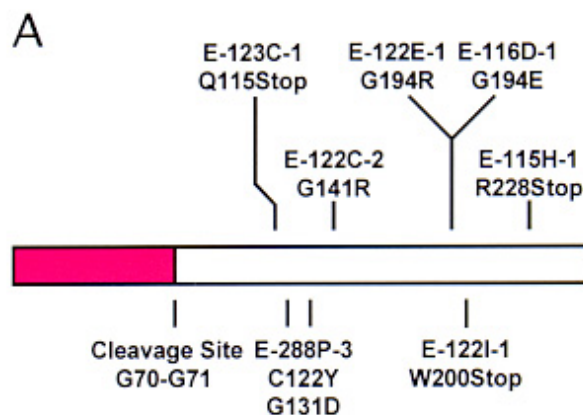


Fig. 1. **A**, Schematic of AvrRpt2 (255 amino acids) showing positions of mutations that cause loss of recognition by *RPS2*. The 70 amino acid portion of AvrRpt2 previously shown to be cleaved during infection is represented in red. Allele designation is in the top line and the predicted amino acid changes are on the bottom line. **B**, Immunoblot of mutant AvrRpt2 proteins extracted from *Pst* DC3000 bacteria expressing mutant *avrRpt2* alleles. Total protein was extracted from strains transformed with the indicated mutant alleles of *avrRpt2*, separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted, and probed with a polyclonal anti-AvrRpt2 sera. Allele E-123C-1 was not tested. * = position of nonspecific band; < = position of wild-type AvrRpt2. **C**, *Arabidopsis* Col-0 leaves 24 h after inoculation with 2×10^7 cells of *Pst* DC3000 per ml expressing the indicated allele of *avrRpt2* in pDSK519. Allele E-123C-1 was not tested. EV = empty vector control; WT = wild-type *avrRpt2*.

1998). We reasoned that this transgenic line could be mutagenized and used in a conditional lethal genetic screen for mutants defective in the *avrRpt2*-*RPS2*-mediated cell death response. M2 mutagenized pTA7001-*avrRpt2* seeds were sown on agar plates supplemented with 10 to 20 nM dexamethasone, a potent inducer of *avrRpt2* transgene expression. Seeds were allowed to germinate and grow for 10 days, after which most seedlings were white and had aborted growth subsequent to cotyledon emergence. Surviving mutants were detected easily by their green color, presence of true leaves,

and large size when compared with the majority of aborted seedlings. M2 mutants were transplanted from selection plates onto soil, where they were allowed to self-fertilize.

Two classes of mutations were expected to result from this screen: those that alter the capacity of the plant to recognize and respond to AvrRpt2 (including mutations in *RPS2* and other plant genes required for the HR) and those that eliminate the capacity of the *avrRpt2* transgene to trigger a HR upon induction (including mutations in *avrRpt2* and the pTA7001 expression cassette). To distinguish these two classes of mutants, we performed a secondary screen by inoculating mutant leaves with *Pseudomonas fluorescens* strain 55 (pHIR11) (pV288). This *P. fluorescens* strain is not pathogenic upon *Arabidopsis*, but is able to elicit an *avrRpt2*-dependent HR on *RPS2* plants (McNellis et al. 1998). Mutants defective in the HR in response to bacteria bearing *avrRpt2* and to dexamethasone treatment were considered most likely to be defective in plant genes required for the HR. Mutants capable of responding with a HR to bacteria bearing *avrRpt2* were considered most likely to carry mutations in the pTA7001-*avrRpt2* transgene. Out of 135 individual dexamethasone-resistant mutants tested by bacterial inoculation, 40 failed to give a HR in response to bacteria bearing *avrRpt2*, whereas 95 showed a normal HR to bacteria bearing *avrRpt2*. Detailed analyses performed on 17 mutants that failed to recognize *avrRpt2* expressing bacteria and seven mutants that retained the ability to recognize *avrRpt2* expressing bacteria are described below.

Analyses of novel *avrRpt2* alleles.

Dexamethasone-insensitive mutants capable of responding with a HR to bacteria bearing *avrRpt2* are not defective in *RPS2*-dependent cell death signal transduction. We thus suspected that some of these mutants contained lesions in the *avrRpt2* transgene. We sequenced 47 independent *avrRpt2* transgenes and identified eight different *avrRpt2* mutations in seven transgenes predicted to cause either nonsense or missense mutations in AvrRpt2 (Table 1 and Fig. 1A). We speculate that the remaining mutants that retain the ability to recognize *avrRpt2* expressing bacteria contain lesions in the two-component glucocorticoid-inducible system that led to loss of the ability to express *avrRpt2*. Interestingly, two different nucleotide changes were identified in codon 194 encoding glycine in *avrRpt2*. The mutations substituted two nonconservative amino acids, arginine and glutamate, for glycine. To verify that the mutations identified in *avrRpt2* truly affect AvrRpt2 protein specificity, we independently expressed the seven mutant *avrRpt2* alleles in *Pst* DC3000 (see below). With the exception of *avrRpt2* allele E-123C-1, all of the mutant AvrRpt2 proteins were detectable at levels comparable to wild-type AvrRpt2 (Fig. 1B). We did not attempt to detect E-123C-1 AvrRpt2 on the basis of earlier analyses, indicating that severely truncated forms of AvrRpt2 are unstable in *Pst* DC3000 (M.B. Mudgett and B.J. Staskawicz, unpublished results). Col-0 leaves infected with bacteria containing the mutant *avrRpt2* alleles did not exhibit an *avrRpt2*-dependent HR (Fig. 1C), indicating that the isolated *avrRpt2* mutants are indeed incapable of being perceived by *RPS2* plants. Thus, the majority of the *avrRpt2* mutations identified in this screen compromise *RPS2* recognition but do not appear to affect AvrRpt2 expression and protein stability in the pathogen.

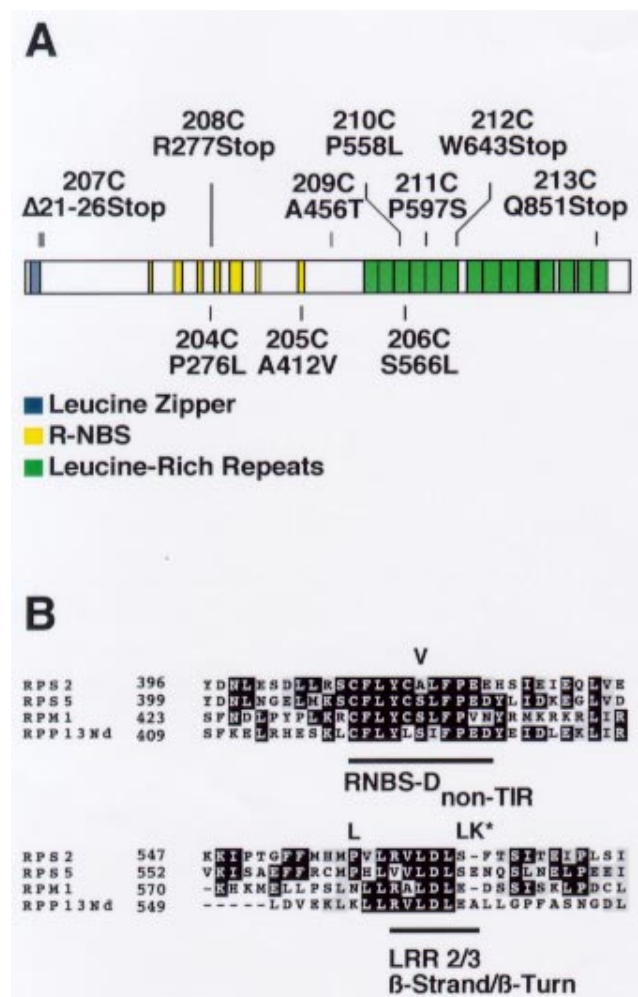


Fig. 2. A, Schematic of RPS2 showing positions of mutations that cause loss of the *avrRpt2* conditioned hypersensitive response. The 909 amino acid RPS2 protein is drawn with conserved motifs designated as Leucine Zipper (blue) (Bent et al. 1994; Mindrinos et al. 1994); R-NBS (nucleotide binding site; yellow) (Meyers et al. 1999); Leucine-Rich Repeats (green) (Bent et al. 1994; Mindrinos et al. 1994). Allele designation is on the top line and the predicted amino acid changes on the bottom line. **B**, Clustal W amino acid alignment of Col-0 RPS2, RPS5, RPM1, and Nd-1 RPP13 *Arabidopsis* disease resistance proteins showing portions where amino acid substitutions occur in the *rps2-205C* (A412V), *rps2-210C* (P558L), and *rps2-206C* (S566L) alleles. Black-shaded amino acids are identical to the consensus and shaded amino acids are similar to the consensus. K* = E572K amino acid substitution encoded by *rps5-1* that is able to suppress the function of multiple resistance genes (Warren et al. 1998). Conserved amino acid motifs are indicated below the alignment.

Analyses of novel RPS2 alleles.

Mutants that were resistant to induced expression of *avrRpt2* and failed to elicit a HR upon inoculation with *avrRpt2* expressing bacteria may represent lesions in genes required for AvrRpt2-triggered defense signaling. We performed complementation tests designed to determine whether the mutations were allelic to known mutants that abrogate the *avrRpt2*-RPS2-dependent HR. We crossed mutants to *rps2-101C-rps2-101C* plants and to *ndr1-1-ndr1-1* plants and analyzed the ability of the F₁ progeny to elicit a HR in response to *avrRpt2*-expressing bacteria. As shown in Table 2, all 17 F₁ heterozygotes failed to complement the *rps2-101C* lesion, suggesting that they were loss-of-function alleles of RPS2. Three of these mutants (*rps2-204C*, *rps2-205C*, and *rps2-206C*) also failed to complement the *ndr1-1* mutation in the initial allelism tests (data not shown). Backcrosses to the parental Col-0 were performed for a subset of the HR mutants. In the initial backcrosses, four of the HR mutants were complemented by Col-0 (*rps2-207C*, *rps2-208C*, *rps2-209C*, and *rps2-210C*), whereas three (*rps2-204C*, *rps2-205C*, and *rps2-206C*) were not complemented (Table 2).

The nucleotide sequence of RPS2 was determined for 10 of these mutant alleles. Each mutant possessed a lesion within the RPS2 open reading frame (ORF) predicted to lead to an amino acid substitution (Table 2 and Fig. 2A). Four of the novel RPS2 alleles are predicted to give rise to truncated protein products, either as a result of nonsense mutations or, in the case of *rps2-207C*, a two-base-pair deletion, leading to a frameshift and premature stop (Table 2 and Fig. 2A). Six of the novel *rps2* alleles characterized contained mutations predicted to give rise to amino acid substitutions at various positions within the RPS2 ORF (Table 2 and Fig. 2A). All of the amino acid substitutions that result in a nonfunctional RPS2 are located within the region bounded by the RNBS (Meyers et al. 1999) and the first five N-terminal LRRs. As shown in Figure 2B, three of these alleles, *rps2-205C*, *rps2-206C*, and

rps2-210C, encode amino acid substitutions within two motifs that are shared among members of the NBS-LRR disease resistance gene family. *rps2-205C* encodes an amino acid change within a conserved portion of the NBS, termed the RNBS_{non-TIR} (Fig. 2B) (Meyers et al. 1999). *rps2-206C* and *rps2-210C* cause mutations in the evolutionarily conserved third LRR (Bittner-Eddy et al. 2000; Warren et al. 1998; Warren et al. 1999).

The failure of the *rps2-204C*, *rps2-205C*, and *rps2-206C* alleles to complement *ndr1-1* mutants and their initial dominance to wild-type RPS2 suggests that they might have a novel dominant-negative phenotype. In the F₂ generation of the initial backcrosses, the *rps2-204C* phenotype segregated in a manner consistent only with a single recessive locus (17 HR+, 42 HR-; $\chi^2 = 0.458$; $P = 0.5$), whereas in the cases of *rps2-205C* (24 HR+, 24 HR-) and *rps2-206C* (115 HR+, 90 HR-), the ratios of HR+ to HR- plants were inconsistent with either a fully recessive or fully dominant locus controlling the phenotype. F₃ lines homozygous for the respective *rps2* alleles that lacked the pTA7001-*avrRpt2* transgene were selected from these backcrosses and crossed to Col-0. *rps2-204C* and *rps2-205C* appeared recessive in this backcross, whereas the *rps2-206C* phenotype was difficult to score. Different F₁ individuals were observed to respond with a strong, weak, or no HR (data not shown). After this backcross, all three alleles regained the ability to complement the *ndr1-1* mutation (data not shown), suggesting that an unlinked mutation was responsible for the dominant-negative effect initially observed.

The *rps2-204C*, *rps2-205C*, and *rps2-206C* mutations in the absence of the pTA7001-*avrRpt2* transgene were tested for any interference with the function of other *Arabidopsis* NBS-LRR resistance genes. As shown in Table 3, none of the three mutants interfered in either the HR or disease resistance conferred by *RPM1*, *RPS5*, or *RPS4* and were similar in all responses tested to the presumed null allele *rps2-101C*. Thus, these three alleles do not appear to retain any residual RPS2

Table 2. RPS2 mutant alleles

Mutant	Complements <i>rps2-101C</i> ^a	Complements <i>ndr1-1</i>	Recessive-dominant	Mutagen ^d	Nucleotide (nt) mutation	Amino acid (aa) mutation
<i>rps2-204C</i>	No	Yes ^b	Recessive ^b	EMS	nt 827 C to T	aa 276 Pro to Leu
<i>rps2-205C</i>	No	Yes ^b	Recessive ^b	EMS	nt 1,235 C to T	aa 412 Ala to Val
<i>rps2-206C</i>	No	Yes ^b	Semidominant ^c	EMS	nt 1,697 C to T	aa 566 Ser to Leu
<i>rps2-207C</i>	No	Yes	Recessive	FNe	nt 63-64 deleted	aa 21 frameshift aa 21 stop
<i>rps2-208C</i>	No	Yes	Recessive	EMS	nt 829 C to T	aa 277 Arg to Stop
<i>rps2-209C</i>	No	Yes	Recessive	EMS	nt 1,366 G to A	aa 456 Ala to Thr
<i>rps2-210C</i>	No	Yes	Recessive	EMS	nt 1,673 C to T	aa 558 Pro to Leu
<i>rps2-211C</i>	No	Not determined (ND)	ND	EMS	nt 1,789 C to T	aa 597 Pro to Ser
<i>rps2-212C</i>	No	ND	ND	EMS	nt 1,929 G to A	aa 643 Trp to Stop
<i>rps2-213C</i>	No	Yes	ND	EMS	nt 2,551 C to T	aa 851 Gln to Stop
E-117O-1 ^e	No	Yes	ND	EMS	ND	ND
E-137D-1	No	Yes	ND	EMS	ND	ND
E-121A-2	No	Yes	ND	EMS	ND	ND
E-135G-3	No	Yes	ND	EMS	ND	ND
E-136F-1	No	Yes	ND	EMS	ND	ND
E-136I-4	No	Yes	ND	EMS	ND	ND
E-137G-1	No	Yes	ND	EMS	ND	ND

^a Complementation and dominance relationships were ascertained by testing F₁ heterozygotes for the ability to mount an RPS2-dependent hypersensitive response (HR).

^b *rps2-204C*, *rps2-205C*, and *rps2-206C* appeared to be dominant and failed to complement the *ndr1-1* mutant with the pTA7001-*avrRpt2* transgene in the background.

^c *rps2-206C*-RPS2 heterozygotes give variable HRs.

^d EMS = ethyl methanesulfonate; FN = fast neutron bombardment.

^e Mutants whose RPS2 gene was not sequenced are described by an arbitrary code indicating the mutagenized pool from which they were derived.

function nor are they capable of interfering with the disease signaling caused by *RPM1*, *RPS5*, or *RPS4*.

DISCUSSION

We described a novel screen for loss of the *RPS2*–*avrRpt2* conditioned HR and the characterization of multiple loss of function alleles in both loci. By screening mutagenized, transgenic seedlings expressing *avrRpt2* in the *RPS2* Col-0 background for survivors, we were able to directly and rapidly identify mutants no longer capable of an *RPS2*-initiated HR.

Out of eight mutations within the *avrRpt2* transgene that cause loss of AvrRpt2 recognition by *RPS2*, five are amino acid substitutions and three are nonsense mutations, leading to a predicted truncated protein product. All amino acid substitutions leading to loss of the *RPS2*-dependent HR are located within the C-terminal portion of AvrRpt2 between amino acid 122 and 200, suggesting that this portion of the molecule is responsible for elicitation of the *RPS2*-dependent HR. This inference is consistent with the observation that AvrRpt2 is N-terminally processed during *Pst* infection of *Arabidopsis* and that the C-terminal fragment of AvrRpt2 comprising amino acids 120 to 255 is sufficient to give an *RPS2*-dependent HR in tobacco (Mudgett and Staskawicz 1999). The glycine at position 194 in AvrRpt2 may define an especially critical region for *RPS2* recognition, given that two independent mutations causing loss of *RPS2* recognition were found at this position. The molecular function of AvrRpt2 remains unknown, although it appears to act as a virulence factor promoting pathogen growth and symptom development in the absence of recognition by *RPS2* (Chen et al. 2000). Because the *avrRpt2* alleles we described are capable of producing stable protein products, at least in bacteria, future experiments will test whether these mutant AvrRpt2 proteins are capable of retaining their virulence function yet avoid recognition by *RPS2*.

We also identified 17 novel alleles of *RPS2*, 10 of which harbor mutations within the *RPS2* ORF (the other seven were not sequenced). *rps2-213C* contains a nonsense mutation that causes a stop codon insertion at the extreme C terminus of the

protein, indicating that this region of the protein is critical for *RPS2* function or that the slightly truncated version of *RPS2* is unstable. Three other novel *RPS2* alleles (*rps2-207C*, *rps2-208C*, and *rps2-212C*) are predicted to result in truncated *RPS2* protein and do result in loss of *RPS2*-dependent HR. We also isolated 6 *RPS2* alleles whose loss of function is a result of single amino acid substitutions, although we cannot rule out the possibility that these mutations destabilize the resulting proteins. All of these amino acid substitutions are located between the R-NBS and the fifth LRR. The *rps2-205C* allele harbors an alanine to valine substitution at amino acid 412, which lies within a motif common to NBS–LRR resistance genes, termed the RNBS-D_{non-TIR} (Fig. 2B) (Meyers et al. 1999). The fact that a seemingly conservative amino acid change within this motif causes a complete loss of *RPS2* function argues for the functional significance of this motif in NBS–LRR proteins. The *rps2-206C* and *rps2-210C* alleles harbor single amino acid changes within the third LRR of *RPS2*. The serine-to-leucine substitution at amino acid 566 in *rps2-206C* is of particular interest because it occurs within the predicted β -strand– β -turn surface of the LRR (Bittner-Eddy et al. 2000; Kobe and Deisenhofer 1993; Parniske et al. 1997; Wang et al. 1998). A glutamate-to-lysine amino acid substitution near this position of the third LRR of *RPS5* causes loss of *RPS5* function and is able to interfere with the function of multiple R-genes (Fig. 2B) (Warren et al. 1998). We observed that *rps2-206C*–*RPS2* heterozygotes are partially impaired in their ability to give an *avrRpt2*-dependent HR. Unlike *rps5-1*, however, *rps2-206C* does not interfere with the function of other related NBS–LRR resistance genes. Nonetheless, the *rps2-206C* phenotype, together with the fact that the third LRR is highly conserved in several otherwise diverged NBS–LRR proteins (Bittner-Eddy et al. 2000; Warren et al. 2000), provides additional evidence that this region of NBS–LRR proteins has a conserved function in NBS–LRR resistance genes.

A striking result of this mutant screen was the failure to recover mutations in genes other than the resistance gene (*RPS2*) and the avirulence gene (*avrRpt2*), despite the fact that

Table 3. Characterization of *rps2-204C*, *rps2-205C*, and *rps2-206C*

Plant line	EV	<i>avrRpt2</i> ^b	<i>avrRpm1</i> ^c	<i>avrB</i> ^c	<i>avrPphB</i> ^d	<i>avrRps4</i> ^e
Hypersensitive response (HR) ^a						
Col-0	–	+	+	+	+	–
<i>rps2-101C</i> ^f	–	–	+	+	+	–
<i>rps2-204C</i> ^g	–	–	+	+	+	–
<i>rps2-205C</i>	–	–	+	+	+	–
<i>rps2-206C</i>	–	–	+	+	+	–
Disease susceptibility ^h						
Col-0	S	R	R	R	R	R
<i>rps2-101C</i> ^f	S	S	R	R	R	R
<i>rps2-204C</i> ^g	S	S	R	R	R	R
<i>rps2-205C</i>	S	S	R	R	R	R
<i>rps2-206C</i>	S	S	R	R	R	R

^a HRs induced by hand inoculation of 1×10^8 cells of appropriate *Pseudomonas fluorescens* strain 55 (pHIR11)(pVSP61) bacteria per ml.

^b *avrRpt2* is recognized by resistance gene *RPS2*.

^c *avrRpm1* and *avrB* are recognized by resistance gene *RPM1*.

^d *avrPphB* is recognized by resistance gene *RPS5*.

^e *avrRps4* is recognized by resistance gene *RPS4*. Col-0 does not respond with a strong HR to *avrRps4*.

^f *rps2-101C* harbors a nonsense mutation at Trp 235.

^g *rps2-204C*, *rps2-205C*, and *rps2-206C* plants are homozygous individuals without the pTA7001–*avrRpt2* transgene in the background.

^h Disease susceptibility tested by “dipping” plants in 4×10^8 cells suspension of appropriate strain of *Pst* DC3000 (pVSP61) per ml. S = susceptible; R = resistant.

several distinct loci are necessary for the *avrRpt2*–*RPS2* HR (Century et al. 1995; Yu et al. 1998; Yu et al. 2000). All plants that passed the secondary screen were allelic to *RPS2*, suggesting that the conditions used to isolate mutants would not allow recovery of mutants at other loci. We speculate that this may be a result of the different manner in which *AvrRpt2* is presented to the plant cell in a natural infection as compared with the inducible transgenic system, but this does not rule out other possibilities.

In the case of the *rps2-204C*, *rps2-205C*, and *rps2-206C* alleles, we observed a unique phenotype present only in the original dexamethasone-resistant lines. These mutants failed to complement *ndr1-1* plants and acted as dominant–negative alleles in the original backcross to Col-0. After one backcross and selection against the pTA7001–*avrRpt2* transgene, however, these alleles acted as recessive loss-of-function alleles and were able to complement *ndr1-1* plants, suggesting that a second-site mutation had caused the initial apparent dominant–negative effect. There are a number of possible explanations for this phenomenon. The chimeric transcription factor GVG is expressed at high levels in pTA7001–*avrRpt2* plants and has been associated with dexamethasone-induced expression of defense-related transcripts (Kang et al. 1999). It is therefore possible that the GVG is responsible for the prevention of the HR in the *rps2-204C*, *rps2-205C*, and *rps2-206C* backgrounds. Because most mutant lines examined did not show this effect, despite the fact that they were derived from the same pTA7001–*avrRpt2* parental transgenic line, this possibility seems unlikely. An alternative hypothesis is the presence of ethyl methanesulfonate (EMS)-induced mutations independent of either *RPS2* or the pTA7001–*avrRpt2* transgene capable of suppressing the HR in the original mutant lines. Regardless of the cause of this effect, it is clear that *rps2-204C*, *rps2-205C*, and *rps2-206C* are actually loss-of-function alleles that are not able to suppress the function of related R-genes.

In summary, we performed a rapid, in planta mutational analysis of the *avrRpt2*–*RPS2* gene-for-gene interaction. Future genetic and biochemical experiments should benefit from the diversity of alleles generated in this study.

MATERIALS AND METHODS

Mutant screen.

Col-0 *Arabidopsis* seed homozygous for the pTA7001–*avrRpt2* transgene (McNellis et al. 1998) was mutagenized with either 50 mM EMS or fast neutron bombardment of seed at nine gray units (International Atomic Energy Agency, Vienna, Austria). Resultant M₁ plants were allowed to self-fertilize and M₂ seed was used for all screens. M₂ seed was surface sterilized with 0.1% Triton X-100 and 1.5% sodium hypochlorite for 5 min, followed by five successive washes with distilled water. Seed was allowed to stratify overnight at 4°C, followed by suspension in 0.1% agarose. Seed suspensions were sown on petri plates containing GM medium (McNellis et al. 1998) supplemented with 10 or 20 nM dexamethasone. Seeds were allowed to germinate and grow for 10 days at 22°C with 16 h of light–day, after which surviving M₂ plants were removed from plates and transferred to soil. M₂ mutants were allowed to self-fertilize and the resultant M₃ seed was used for secondary screens and test crosses. M₃ mu-

tant plants were grown under short-day conditions (9 h light–day) for 4 to 5 weeks and tested for the ability to give a HR in response to *P. fluorescens* strain 55 (pHIR11)(pV288) expressing *avrRpt2*, as described below.

Crosses.

M₃ mutant plants (see above) were crossed to wild-type Col-0, Col-0 *rps2-101C*–*rps2-101C* (Yu et al. 1993) and to Col-0 *ndr1-1*–*ndr1-1* (Century et al. 1995) plants. M₃ mutants were used as pollen donors for the wild-type Col-0 test crosses to enable confirmation by PCR amplification of the *avrRpt2* transgene (forward primer 5′-CCGCTCGAGATGAAAATTGCTCCAGTTGCC-3′; reverse primer 5′-GACTAGTTTAGCGGTAGAGCATTGCGTG-3′) from genomic DNA isolated from F₁ plants. Test crosses to *rps2-101C* plants were confirmed by PCR amplification of the central region of the *RPS2* ORF (forward primer 5′-CACGATGATGGAACAG-3′; reverse primer 5′-GGTGTAAACGCCATG-3′) from genomic DNA isolated from F₁ plants. The *rps2-101C* allele harbors a G to A nucleotide substitution at position 704 (Mindrinos et al. 1994) that creates a cleaved amplified polymorphic sequence marker via the introduction of a *DdeI* restriction site. PCR fragments were therefore digested with *DdeI* to detect the presence of the *rps2-101C* allele. M₃ mutant plants were used as pollen donors to enable confirmation by PCR amplification (forward primer 5′-AATCTACTACGACGATGTCCAC-3′; reverse primer 5′-GTAACCGATGGCAACTTTCC-3′) of the wild-type allele of *NDRI* from F₁ heterozygotes. The annealing site for the forward primer lies within the deleted 1.2-kb region of the *ndr1-1* allele (Century et al. 1997) so that an 850-bp PCR product results only if at least one copy of *NDRI* is present. Confirmed F₁ heterozygotes were tested for the ability to display an *RPS2*-dependent HR by inoculation with 1 × 10⁸ cells of *P. fluorescens* 55 (pHIR11)(pV288) per ml, as described below. Homozygous mutant lines *rps2-204C*, *rps2-205C*, and *rps2-206C*, free of the pTA7001–*avrRpt2* transgene, were isolated in the following manner: F₂ individuals, resulting from the initial backcross to Col-0 that failed to give a HR, were screened via PCR (forward primer 5′-CCGCTCGAGATGAAAATTGCTCCAGTTGCC-3′; reverse primer 5′-GACTAGTTTAGCGGTAGAGCATTGCGTG-3′) for the presence of the pTA7001–*avrRpt2* transgene. Plants that failed to give a PCR product were allowed to self-fertilize and the resultant F₃ progeny were tested for the HR, as described above. F₃ families that were 100% HR negative were pooled and used for extraction of genomic DNA to confirm the absence of *avrRpt2* by DNA blot analysis (Ausubel et al. 1994).

Sequence analysis and alignment.

Genomic DNA was isolated from mutant plants by a method discussed by Shure et al. (1983). The *avrRpt2* transgene or *RPS2* ORF was PCR amplified from total genomic DNA with primers developed on the basis of published sequences (Bent et al. 1994; Innes et al. 1993; Mindrinos et al. 1994). PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Valencia, CA, U.S.A.), according to the manufacturer's instructions, prior to sequencing by an ABI 377 DNA sequencer in conjunction with dye-terminator cycle sequencing (Applied Biotechnology Inc., Foster City, CA, U.S.A.), according to the manufacturer's instructions. Se-

quencing primers were designed so that annealing sites were spaced approximately every 300 bases. All sequences were confirmed with reverse and forward primers, and mutations were confirmed by sequencing genomic DNA from mutant siblings or progeny. Multiple sequence alignment was performed by CLUSTAL W 1.8 analysis with the blosum matrix (Thompson et al. 1994).

Cloning of *avrRpt2* alleles.

Mutant *avrRpt2* genes were amplified directly from plant genomic DNA and TOPO-TA subcloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, U.S.A.). The inserts were sequenced and subcloned into the *Nde*I–restriction endonuclease designation (*Eco*RI) of p519ngfp (Matthysse et al. 1996) to produce vectors expressing the *avrRpt2* alleles from the *nptII* promoter (Mudgett and Staskawicz 1999).

Immunoblot analysis.

Proteins were extracted from *Pst* DC3000 strains and separated on sodium dodecyl sulfate-polyacrylamide gels, as described by Mudgett and Staskawicz (1999). AvrRpt2 was detected with rabbit polyclonal anti-N-his6-AvrRpt2 sera at a dilution of 1/2000. Goat anti-rabbit immunoglobulin G secondary antibodies coupled to horseradish peroxidase at a dilution of 1/5000 were used to detect the primary antibodies via chemiluminescence (Amersham Pharmacia, Piscataway, NJ, U.S.A.).

Disease assays and HR inoculations.

To test for the HR with the *P. fluorescens* strain, we followed the technique of McNellis et al. (1998), inoculating leaves at a density of 1×10^8 cells per ml. Presence or absence of the HR was scored 24 h after infiltration. To test for the HR with *Pst* DC3000, we followed the method of Kunkel et al. (1993), inoculating at a density of 2×10^7 cells per ml, and

scored plants 24 h after infiltration. Disease assays with varying strains of *Pst*DC3000 (Table 4) were performed essentially as described by Whalen et al. (1991), except an inoculum level of 4×10^8 cells per ml was used. Resistant reactions were scored after 5 days as a complete lack of lesion formation and chlorosis. Susceptible reactions were scored as multiple leaves with large chlorotic areas containing water-soaked lesions.

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Table 4. Bacterial strains and vectors

Strain	Characteristics	Reference–Source
<i>Escherichia coli</i>	Strain DH5alpha; F ⁻ , <i>lacZ M15 endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1 I- Nal^r</i>	Life Technologies, Rockville, MD, U.S.A.
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	Strain DC3000; virulent on <i>Arabidopsis</i> spp.; Rif ^r	Cuppels et al. 1986
<i>P. fluorescens</i>	Strain 55; nonpathogenic on <i>Arabidopsis</i> spp.; Nal ^r	Huang et al. 1988
Plasmids		
pCR2.1-TOPO	T overhang vector for cloning and sequencing PCR products; Col E1 replicon; Km ^r , Ap ^r	Invitrogen, Carlsbad, CA, U.S.A.
pTA7001- <i>avrRpt2</i>	T-DNA vector carrying GVG system for inducible expression of <i>avrRpt2</i> in transformed plants; RK2 replicon; Kmr	McNellis et al. 1998
pVSP61	Stable plasmid in <i>Pseudomonas</i> spp.; pVS1 replicon; Km ^r	DNA Plant Technology, Oakland, CA, U.S.A.
pV288	pVSP61 carrying <i>avrRpt2</i> ; Km ^r	Kunkel et al. 1993
pVARM	pVSP61 carrying <i>avrRpm1</i> ; Km ^r	Bisgrove et al. 1994
pVB01	pVSP61 carrying <i>avrB</i> ; Km ^r	Kunkel et al. 1993
pVSP61(<i>avrPphB</i>)	pVSP61 carrying <i>avrPphB</i> ; Km ^r	Simonich and Innes 1995
pV316-1a	pVSP61 carrying <i>avrRps4</i> ; Km ^r	Hinsch and Staskawicz 1996
p519ngfp	<i>nptII</i> promoter in front of <i>gfp</i> ; IncQ replicon; Km ^r	Matthysse et al. 1996
pDSK519(<i>navrRpt2</i>)	p519ngfp carrying wild-type <i>avrRpt2</i> ; Km ^r	Mudgett and Staskawicz 1999
pDSK519(<i>navrRpt2</i> -M20)	p519ngfp carrying <i>avrRpt2</i> allele E-288P-3; Km ^r	This study
pDSK519(<i>navrRpt2</i> -M13)	p519ngfp carrying <i>avrRpt2</i> allele E-122C-2; Km ^r	This study
pDSK519(<i>navrRpt2</i> -M17)	p519ngfp carrying <i>avrRpt2</i> allele E-122E-1; Km ^r	This study
pDSK519(<i>navrRpt2</i> -M22)	p519ngfp carrying <i>avrRpt2</i> allele E-116D-1; Km ^r	This study
pDSK519(<i>navrRpt2</i> -M40)	p519ngfp carrying <i>avrRpt2</i> allele E-122I-1; Km ^r	This study
pDSK519(<i>navrRpt2</i> -M45)	p519ngfp carrying <i>avrRpt2</i> allele E-115H-1; Km ^r	This study
Cosmid		
pHIR11	Cosmid vector containing cloned <i>hrp</i> cluster from <i>P. syringae</i> ; RK2 replicon; Tet ^r	Huang et al. 1988

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