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Analysis of Resistance Gene-Mediated Defense Responses in *Arabidopsis thaliana* Plants Carrying a Mutation in *CPR5*

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In resistant plants, pathogen attack often leads to rapid activation of defense responses that limit multiplication and spread of the pathogen. To investigate the signaling mechanisms underlying this process, we carried out a screen for mutants in the signaling pathway governing resistance in *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*. This involved screening for suppressor mutations that restored resistance to a susceptible line carrying a mutation in the *RPS2* resistance gene. A mutant that conferred resistance by activating defense responses in the absence of pathogens was isolated. This mutant, which carries a mutation at the *CPR5* locus and was thus designated *cpr5-2*, exhibited resistance to *P. syringae*, spontaneous development of necrotic lesions, elevated *PR* gene expression in the absence of pathogens, and abnormal trichomes. Resistance gene-mediated defenses, including the hypersensitive response, restriction of pathogen growth, and induction of defense-related gene expression, were functional in *cpr5-2* mutant plants. Additionally, in *cpr5-2* plants *RPS2*-mediated induction of *PR-1* expression was enhanced, whereas *RPM1*-mediated induction of *ELI3* was not. These findings suggest that *CPR5* encodes a negative regulator of the *RPS2* signal transduction pathway.

Additional keywords: disease lesion mimic, *gl3,* systemic acquired resistance (SAR).

Disease resistance in plants is often triggered by specific recognition of the invading pathogen. Pathogen recognition results in the rapid activation of a complex series of plant defense responses that limit multiplication and spread of the pathogen within the plant, thus leading to resistance. Common defense responses include the production of reactive oxygen species, the hypersensitive response (HR), which is characterized by rapid cell death and tissue necrosis at the site of infection, and the production of antimicrobial compounds (phytoalexins) and lytic enzymes (reviewed in Hammond-Kosack and Jones 1996; Baker et al. 1997; Greenberg 1997). Many of these induced responses are controlled, at least in part, by transcriptional activation of defense-related genes, including the pathogenesis-related (*PR*) genes (Lamb et al. 1989; Baker et al. 1997).

Pathogen recognition is governed by genes in both the plant and the pathogen (Keen 1990). Plant disease resistance genes confer on the plant the ability to recognize pathogens expressing specific recognition determinants. Production of these pathogen determinants is, in turn, controlled by specific pathogen avirulence (*avr)* genes. The prevailing hypothesis is that the recognition event mediated by matching resistance and *avr* genes triggers a signal transduction pathway that culminates in the activation of plant defense responses. The molecular isolation of several disease resistance genes has revealed that the majority cloned to date appear to encode components of signal transduction pathways (Bent 1996; Hammond-Kosack and Jones 1997). For example, the *RPS2* gene of *Arabidopsis thaliana*, which confers resistance to strains of the bacterial pathogen *Pseudomonas syringae* expressing *avrRpt2* (Kunkel et al. 1993; Yu et al. 1993), encodes a protein containing several motifs suggestive of a role in signaling, including a leucine-rich repeat (LRR) domain and a nucleotide-binding site (Bent et al. 1994; Mindrinos et al. 1994). However, the actual mechanisms by which *RPS2* and other resistance genes mediate resistance are not clear. A current area of intense research is focused on further elucidating the regulatory mechanisms governing resistance by identifying and characterizing additional components of disease resistance signal transduction pathways.

A number of genetic approaches have been used to further dissect disease resistance signaling pathways (reviewed in Dangl et al. 1996; Kunkel 1996; Ryals et al. 1996; Delaney 1997). These include screens for mutants that exhibit enhanced disease susceptibility (*eds* mutants; Glazebrook et al. 1996; Rogers and Ausubel 1997), mutants that have lost genotype-specific resistance to avirulent pathogens (*ndr1* and *eds1;* Century et al. 1995; Parker et al. 1996), mutants that either don't induce (*npr1, niml;* Cao et al. 1994; Delaney et al. 1995) or inappropriately regulate defense-related gene expression (*cpr, cim* mutants; Lawton et al. 1993; Bowling et al. 1994, 1997), and mutants that form HR-like necrotic lesions in the absence of pathogen infection (*acd2* and *lsd* mutants; Dietrich et al. 1994; Greenberg et al. 1994; Weyman et al. 1995). The *acd2*, *lsd*, and *cpr5* mutants are examples of a

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mutant class known as "disease lesion mimics" that behave as if they were under constant pathogen attack. These mutants often exhibit elevated levels of salicylic acid (SA), defense-related gene expression in the absence of pathogens or SA-inducing compounds, and enhanced resistance to a number of bacterial and fungal pathogens. It is likely that these mutants define genes encoding signaling components regulating the induction or containment of HR-related cell death in response to pathogen attack (Dangl et al. 1996; Ryals et al. 1996; Delaney 1997).

It is not yet clear whether resistance signaling pathways interact with other signal transduction processes in plants. The isolation of several *A. thaliana* mutants with pleiotropic phenotypes such as *ttg*, which affects both trichome development and anthocyanin production (Marks 1997), and several *cop* and *det* mutants that affect photomorphogenesis and the regulation of defense gene expression (Mayer et al. 1996), provides evidence for cross-talk between signaling pathways governing seemingly unrelated processes. As disease resistance signal transduction pathways become better understood, interactions between the signaling pathways governing pathogen defense and other processes, including development, may be revealed.

We have used a complementary genetic approach to dissect the signal transduction pathway coupling pathogen recognition to expression of disease resistance in *A. thaliana*. This involved screening for mutants that restore resistance to an *rps2* mutant line that is susceptible to *P. syringae* strains expressing *avrRpt2* (Kunkel et al. 1993). Our expectation was that this screen would identify mutants that restore *RPS2*-dependent resistance as well as mutants that bypass the requirement for pathogen recognition. One of the suppressors isolated in this screen carries a mutation at the *CPR5* locus (Bowling et al. 1997), and was thus designated *cpr5-2*. This mutant exhibits an intriguing combination of phenotypes, including enhanced resistance to *P. syringae,* spontaneous development of necrotic lesions in uninoculated plants, and abnormal trichomes. We show that disease resistance mediated by the *RPS2, RPS5,* and *RPM1* genes is functional in the *cpr5-2* mutant background and that *cpr5-2* specifically enhances the induction of *PR-1* gene expression triggered by *RPS2*-mediated pathogen recognition.

RESULTS

Isolation of mutations that suppress the disease-susceptible phenotype of *rps2* mutant plants.

We carried out a screen for *A. thaliana* mutants that suppress the disease-susceptible phenotype of an *rps2* mutant in order to identify additional genes important for resistance to the bacterial pathogen *P. syringae*. To facilitate isolation of components of the *RPS2*-mediated recognition pathway we screened for mutants with enhanced resistance after inoculation with a *P. syringae* strain expressing the avirulence gene *avrRpt2*.

As shown in Figure 1, wild-type *A. thaliana* ecotype Columbia (Col-0) plants were susceptible to the *P. syringae* pv. *tomato* (*Pst*) strain DC3000 and developed bacterial speck-like disease symptoms consisting of many small, individual gray lesions surrounded by a halo of chlorosis. In contrast, Col-0 plants (which possess a functional *RPS2* gene; Kunkel et al. 1993), were resistant to *Pst* DC3000 expressing the avirulence gene *avrRpt2* (*Pst* DC3000 [*avrRpt2*]) and thus exhibited no disease symptoms. *rps2* mutant plants were susceptible to both *Pst* DC3000 and *Pst* DC3000 (*avrRpt2*). To isolate mutations that suppress the disease-susceptible phenotype of *rps2* mutants, populations of M_2 plants derived from seed of *rps2* mutant plants that had been mutagenized with ethylmethane sulfonate (EMS) were inoculated with *Pst* DC3000 (*avrRpt2*). We screened approximately 17,500 M_2 plants and isolated 28 suppressor mutant lines that exhibited enhanced resistance upon re-testing in the M_3 generation. One of the most resistant of these suppressor mutants, *cpr5-2*, was chosen for further analysis. The remaining 27 suppressor lines are currently being characterized, and will be described in more detail elsewhere (G. Kalinowski, A. Kloek, M. Verbsky, and B. Kunkel, *unpublished results*).

cpr5-2 confers full resistance to *P. syringae.*

In contrast to the parental *rps2* line, the *cpr5-2 rps2* suppressor mutant line was resistant when inoculated with *Pst* DC3000 (*avrRpt2*) (Fig. 1). To determine whether resistance was restored specifically to *Pst* DC3000 (*avrRpt2*), we tested *cpr5-2 rps2* for resistance to several pathogenic *P. syringae* strains not expressing *avrRpt2,* including *Pst* DC3000, *Pst* 3455 (Whalen et al. 1991), and the *P. syringae* pv. *maculicola* (*Psm*) strain m4 (Debener et al. 1991). *cpr5-2 rps2* mutant plants were resistant to all three of these strains (Fig. 1; and data not shown). This indicates that enhanced resistance in *cpr5-2 rps2* plants is not specific to strains expressing *avrRpt2,* suggesting that resistance in this mutant line is independent of *avrRpt2*-mediated pathogen recognition. Furthermore, these observations suggest that resistance in the *cpr5-2 rps2* line is not due to gain of a novel capacity to specifically detect infection by *Pst* DC3000.

To determine if resistance in *cpr5-2 rps2* was associated with restricted growth of *P. syringae* within the plant, growth of the pathogen in mutant plants was monitored over the course of several days. As shown in Figure 2A, growth of *Pst* DC3000 (*avrRpt2*) was limited in *cpr5-2 rps2* mutant plants, obtaining a final concentration of only 10^4 to 10^5 CFU/cm^2. This was in marked contrast to the high levels of bacterial growth observed in *CPR5 rps2* plants, where the same strain reached a final concentration of 10^6 to 10^7 CFU/cm^2 (Fig. 2A). The 50- to 100-fold reduction of bacterial growth observed in *cpr5-2 rps2* plants was similar to the limitation of growth observed in wild-type Col-0 plants, where restriction of the pathogen was a result of resistance gene-mediated pathogen recognition (Fig. 2A). *Pst* DC3000, which grew to high levels in wild-type Col-0 plants, was similarly restricted in *cpr5-2 rps2* mutant plants (Fig. 2B).

cpr5-2 plants develop spontaneous lesions prior to infection.

The *cpr5-2 rps2* mutant was initially isolated based on its enhanced resistance to *Pst* DC3000. Upon closer inspection we observed that uninoculated *cpr5-2 rps2* plants developed macroscopic, localized, yellow or brown necrotic lesions, reminiscent of those observed during an HR. Lesion formation in *cpr5-2* mutant plants was, at least in part, developmentally regulated, as tissue necrosis initially appeared on cotyledons of 2- to 3-week-old seedlings, followed by chlorosis and grad-

ual necrosis of the entire cotyledon. As shown in Figure 3, as the mutant plants matured, chlorotic and necrotic lesions became apparent on the older leaves. Necrosis often started at the base of the midrib, and then extended into the leaf (Fig. 3B). Development of this lesion-mimic phenotype was independent of pathogen attack, as lesion development also occurred on seedlings grown in axenic culture (data not shown).

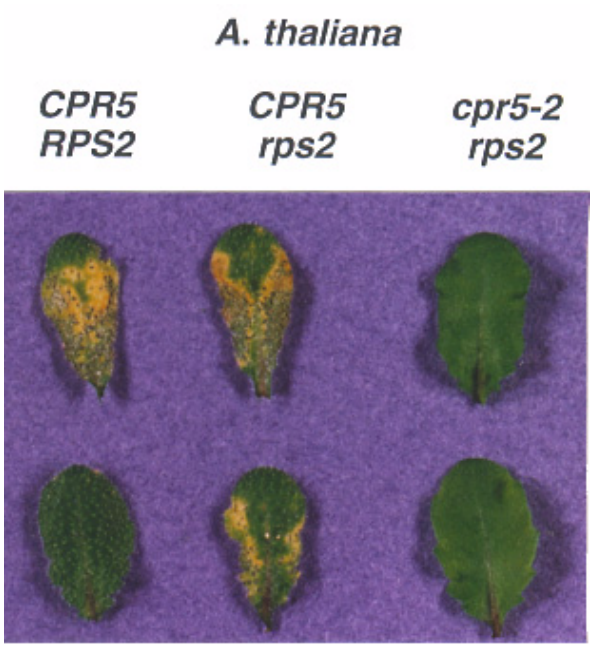

Fig. 1. Disease phenotypes of wild-type Col-0 (*CPR5 RPS2*), *CPR5 rps2*, and *cpr5-2 rps2* mutant *Arabidopsis thaliana* plants after inoculation with *Pseudomonas syringae* pv. *tomato (Pst)* strains. Leaves are shown 4 days after inoculation with *Pst* DC3000 (top) or *Pst* DC3000 (*avrRpt2*) (bottom). Plants were inoculated by dipping into bacterial suspensions containing the surfactant Silwet L-77.

We noted variation in lesion development from experiment to experiment, but this variability was not strongly dependent on day length or light intensity (data not shown). However, high humidity appeared to delay the formation of macroscopic lesions. Visible tissue necrosis was not evident until 3 to 4 weeks after germination in seedlings grown under the very humid conditions present in axenic culture (data not shown). *cpr5-2 rps2* mutant plants were also somewhat smaller than their *CPR5 rps2* progenitor or their wild-type siblings. The reduction in plant size, which was reflected both in overall stature of the plant (data not shown) and in leaf size (Fig. 3B), was evident both in seedlings grown under axenic conditions and in those grown in soil.

cpr5-2 mutants have abnormal trichomes.

A third phenotype of *cpr5-2 rps2* mutant plants was abnormal trichomes. As illustrated in Figure 4, trichomes on the leaves of *cpr5-2* mutants were much smaller than trichomes present on wild-type plants. In addition, the majority of *cpr5-2* trichomes had only two branches, while wild-type trichomes typically had three or four (Fig. 4; Hülskamp et al. 1994; Marks 1997). However, the spacing and number of trichomes per leaf appeared to be unaltered. The mutant trichomes also had a transparent, "glassy" appearance, and lacked the calcium-containing papillae found in the mature secondary cell wall of wild-type trichomes (data not shown; Marks 1997). Additional phenotypes often associated with trichome mutations, such as alterations in production of seed coat mucilage and root hair development, appeared to be normal in *cpr5-2* mutant plants (data not shown).

Genetic analysis of *cpr5-2*.

To determine the genetic basis of the disease resistance, lesion-mimic, and abnormal trichome phenotypes in the *cpr5-2 rps2* mutant line, we crossed the mutant to both *CPR5 rps2* plants and wild-type Col-0, which carries a functional *RPS2* gene. As is summarized in Table 1, the F_1 progeny from both

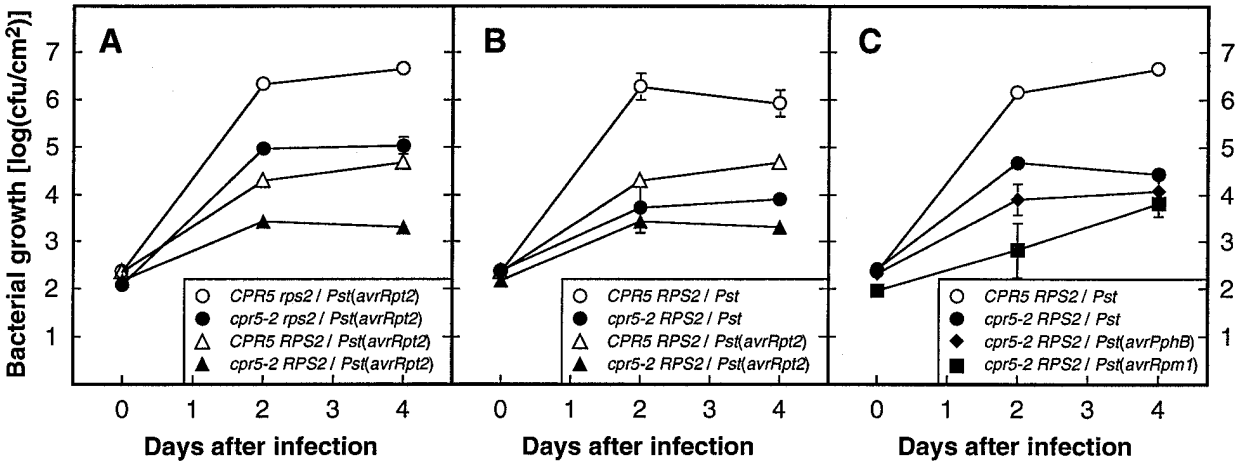

Fig. 2. Growth of *Pseudomonas syringae* pv. *tomato* (*Pst*) strains in suppressor mutant *cpr5-2*. Six-week-old *Arabidopsis thaliana* plants were inoculated by vacuum infiltration with the indicated *Pst* strains and the concentration of bacteria in the plant leaves assayed after 0, 2, and 4 days. **A,** Growth of *Pst* DC3000 (*avrRpt2*) in the indicated *A. thaliana* lines, **B,** Growth of *Pst* DC3000 and *Pst* DC3000 (*avrRpt2*) in Col-0 (*CPR5 RPS2*) and *cpr5-2 RPS2* plants. **C,** Growth of *Pst* DC3000, *Pst* DC3000 (*avrRpm1*), and *Pst* DC3000 (*avrPphB*) in wild-type Col-0 and *cpr5-2* plants. Data points represent means of three independent determinations ± SEM. Data presented in **A** and **B** are from the same experiment; thus, results for *Pst* DC3000 (*avrRpt2*) growth in Col-0 and *cpr5-2 RPS2* are presented in both panels for direct comparison. Experiments presented in **A** and **B** were repeated a minimum of three times with similar results.

crosses were susceptible to *Pst* DC3000, indicating that the *cpr5-2* mutation is recessive. The F_1 plants were allowed to self-pollinate and the resulting F_2 progeny were assayed to determine the segregation pattern of resistance conferred by the *cpr5-2* mutation. In the F_2 progeny from both crosses resistance segregated as a recessive, single-gene trait (Table 1). The lesion-mimic and abnormal trichome phenotypes cosegregated with resistance in these crosses (Table 1). Additionally, in 452 *cpr5-2* F_2 progeny from a cross between *cpr5-2* and Landsberg *erecta* (La-*er*), we did not detect any recombinant plants in which the lesion-mimic and trichome phenotypes were separated, indicating that these phenotypes are conferred by a defect at a single locus, or at two very tightly linked loci. The conclusion that all three phenotypes are due to mutation of a single locus is strengthened by the fact that an *A. thaliana* mutant exhibiting similar lesion-mimic and abnormal trichome development phenotypes was isolated in an independent screen. This mutant, *cpr5* (now referred to as *cpr5-1*), was isolated in a screen for mutants with elevated expression of the *BGL2* promoter, and was thus assigned the *cpr* (constitutive expressor of *PR* genes) designation (Bowling et al. 1997). Complementation tests between *cpr5-1* and *cpr5-2* indicated that the two mutants are allelic (data not shown; Bowling et al. 1997).

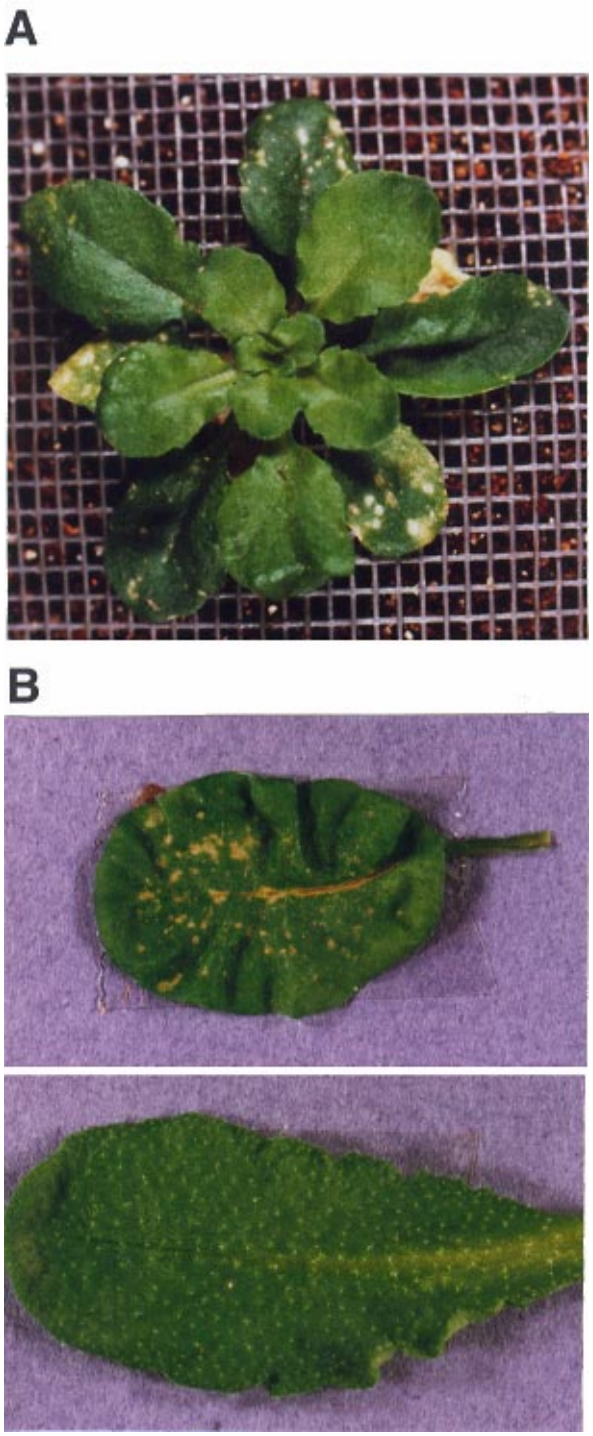

Fig. 3. Disease lesion-mimic phenotype of *cpr5-2* mutant plants. **A,** Uninfected *cpr5-2* plants exhibited macroscopic necrotic lesions on older rosette leaves. **B,** Lesion-positive leaf from uninfected *cpr5-2* plant (top) and leaf from uninfected wild-type *CPR5* plant (bottom). The leaves in **B** are developmentally matched and were taken from plants of the same age.

The abnormal *cpr5-2* trichome phenotype most closely resembles that observed for the *gl3* trichome mutant. The leaves of *gl3* mutant plants also have short, mostly unbranched trichomes (Marks 1997). Although *gl3* mutant plants do not exhibit the lesion-mimic or enhanced resistance phenotypes observed in *cpr5-2* (data not shown), it was possible that *cpr5-2* was an unusual allele of *gl3*. To investigate this possibility the *cpr5-2 rps2* mutant was crossed to *gl3* and the resulting F_1 and F_2 progeny scored for their resistance, lesion-mimic, and trichome phenotypes. The F_1 progeny exhibited wild-type phenotypes for all three traits, indicating that *gl3* and *cpr5-2* are not allelic. In addition, the random segregation of the resistance and lesion-mimic phenotypes from the *gl3* phenotype among the F_2 progeny indicated that the *GL3* and *CPR5* loci are unlinked (Table 1; and data not shown).

To determine the map position of the *CPR5* locus we took advantage of the fact that the *gl3* mutant used in the complementation test was in the La-*er* background (Table 1). F_2 progeny from this cross were used to map *cpr5-2* relative to selected molecular markers positioned at intervals of 20 to 40 centiMorgans (cM) on each of the five chromosomes (Konieczny and Ausubel 1993; Bell and Ecker 1994). As summarized in Table 2, *cpr5-2* mapped to chromosome 5, in an approximately 7 cM interval between the markers g2368 and m555. Although we mapped *cpr5-2* to the same region of chromosome 5 reported for *cpr5-1* by Bowling et al. (1997), we place *cpr5-2* between g2368 and m555.

Based on complementation tests and map position, *cpr5-2* defines a new locus affecting trichome development. Genetic analysis indicates that the *cpr5-2* mutation is not an allele of the *gl2, gl3, an, try, sti,* or *zwi* loci, which are known to affect trichome development (J. Larkin, *unpublished results;* Hülskamp et al. 1994; Oppenheimer et al. 1997).

Resistance gene-mediated induction of defense responses is functional in *cpr5-2* mutant plants.

We next wanted to address whether resistance gene-mediated resistance was altered, or possibly enhanced, by the presence of the *cpr5-2* mutation. Our finding that the *cpr5-2* resistance phenotype was expressed both in plants carrying the *rps2* mutation and in plants with a functional *RPS2* gene (Table 1) indicates that the *cpr5-2* mutation does not behave

as an allele-specific suppressor mutation. Further, the generation of homozygous *cpr5-2 RPS2* lines facilitated the investigation of any possible effects that the *cpr5-2* mutation may have on *RPS2*-mediated disease resistance.

We monitored the activities of *RPS2*, as well as two other resistance genes, by assaying for both expression of the HR and restriction of pathogen growth in *cpr5-2 RPS2* plants. Wild-type Col-0 plants carry functional *RPS2*, *RPM1*, and *RPS5* genes and exhibited visible macroscopic tissue collapse indicative of an HR within 20 h after inoculation with high doses of *Pst* DC3000 expressing *avrRpt2*, *avrRpm1*, or *avrPphB* (data not shown; Debener et al. 1991; Kunkel et al. 1993; Simonich and Innes 1995). *cpr5-2 RPS2* plants also exhibited clear HRs when inoculated with these strains (data not shown). The timing of the HRs observed in these plants was the same as that observed in wild-type Col-0. However, the degree of tissue collapse was reduced by varying degrees in all three interactions (data not shown). Neither *cpr5-2 RPS2* plants inoculated with *Pst* DC3000 nor *cpr5-2 rps2* plants inoculated with *Pst* DC3000 or *Pst* DC3000 (*avrRpt2*) exhibited an HR. These results indicate that, as in wild-type plants, the HR in *cpr5-2* plants is dependent on the presence of both the bacterial *avr* gene and the corresponding resistance gene. Further, the absence of an HR in *cpr5-2* plants inoculated with *Pst* DC3000 suggests that enhanced resistance conferred by *cpr5-2* is independent of the elicitation of an HR.

Additional evidence that resistance gene-mediated resistance is functional in the context of *cpr5-2* stems from the finding that in *cpr5-2 RPS2* plants growth of *Pst* DC3000 expressing *avrRpt2* is 50- to 100-fold lower than that observed in *cpr5-2 rps2* plants (Fig. 2A). These results indicate that in the *cpr5-2* mutant background *RPS2*-mediated resistance is functional and confers additional resistance to *Pst* DC3000 (*avrRpt2)*. However, given the recent finding that *avrRpt2* acts as a virulence factor by promoting pathogen growth in plants lacking a functional *RPS2* gene (A. P. Kloek, M. Lim, and B. N. Kunkel, *unpublished results*), we were concerned that the difference in growth of *Pst* DC3000 (*avrRpt2*) in *cpr5-2 rps2*

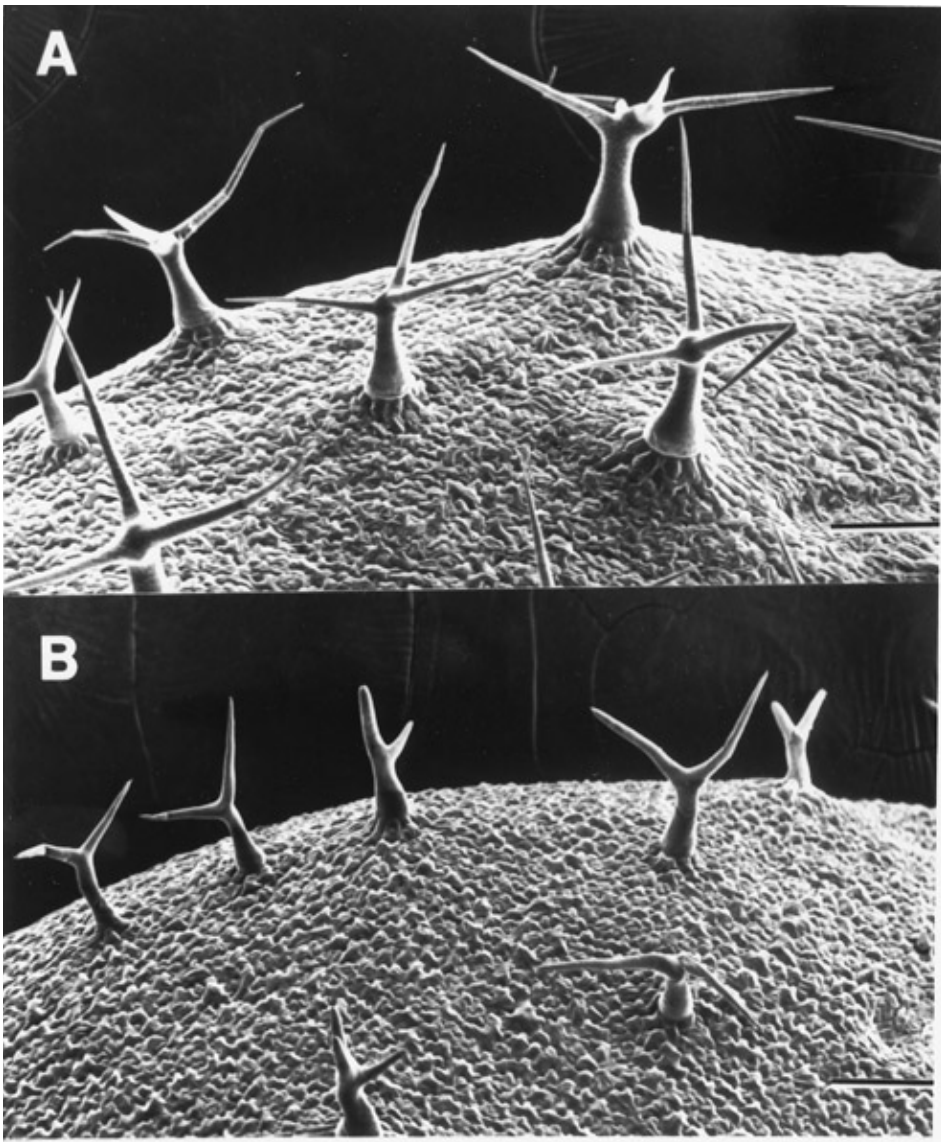

Fig. 4. Trichome phenotype of *cpr5-2* mutant plants. **A,** Wild-type *CPR5* (Col-0). **B,** *cpr5-2*. **A** and **B,** Printed at ×140. Bars = 100 µm.

and *cpr5-2 RPS2* plants might be enhanced by the *avrRpt2* virulence activity. Thus, we also assayed *RPS2* function by comparing the growth of *Pst* DC3000 and *Pst* DC3000 (*avrRpt2*) in *cpr5-2 RPS2* plants. As is shown in Figure 2B, growth of *Pst* DC3000 in *cpr5-2 RPS2* plants is 100-fold lower than in wild-type plants. The growth of *Pst* DC3000 (*avrRpt2*) in *cpr5-2 RPS2* plants is reduced an additional four- to 10-fold (Fig. 2B; and data not shown), confirming that *avrRpt2/RPS2*-mediated resistance is functional in *cpr5-2* plants. In contrast to the experiments summarized in Figure 2A, a cumulative effect of resistance mediated by *cpr5-2* and by *RPS2* was not reproducibly detectable in these experiments (Fig. 2B; and data not shown).

RPM- and *RPS5*-mediated resistances are also functional in the presence of the *cpr5-2* mutation. The growth of *Pst* DC3000 (*avrRpm1*), *Pst* DC3000 (*avrB*), and *Pst* DC3000 (*avrPphB*) was significantly lower than the growth observed for *Pst* DC3000 in *cpr5-2* plants (Fig. 2C; and data not shown). In these interactions resistance gene-mediated resistance was very effective in restricting pathogen growth (data not shown), and thus any additional resistance conferred by *cpr5-2* was not detected in these experiments.

Table 1. Genetic analysis of *cpr5-2*

Cross	Generation	Plants (no.) S[a]	Plants (no.) R[a]	$X^2_{3:1}$
cpr5-2 rps2 × *CPR5 rps2*	F_1	7[b,c]	0	
	F_2	105[b]	31[d]	0.35
cpr5-2 rps2 × *CPR5 RPS2* (Col-0)	F_1	6[c,e]	0	
	F_2	66[e]	16[d]	1.32
cpr5-2 rps2 × *CPR5 RPS2 gl3* (La-*er*)	F_1	2[c,e]	0	
	F_2	117[e]	44[d]	0.46
cpr5-2 rps2 × *CPR5 RPS2 nahG*	F_1	ND[f]	ND	2.66[g]
	F_2	91[e]	2[d]	

[a] Plants were inoculated by dipping them into bacterial suspensions containing the surfactant Silwet L-77, and were scored 4 to 5 days after inoculation. S = susceptible plants with disease symptoms; R = resistant plants exhibiting no disease symptoms.
[b] Plants were scored for resistance to the *Pseudomonas syringae* pv. *tomato* (*Pst)* strain DC3000 (*avrRpt2*).
[c] F_1 plants had wild-type trichomes and did not exhibit necrotic lesions.
[d] All resistant plants exhibited necrotic lesions and had abnormal trichomes.
[e] Plants were scored for resistance to *Pst* DC3000.
[f] Not determined.
[g] X^2 value for 15:1 segregation ratio.

Table 2. Linkage analysis of the *CPR5* locus

Marker[a]	Recombinant chromosomes[b]	Chromosomes scored (total no.)[b]	Recombination frequency (%)
DFR	17	58	29[c]
LFY3	29	904	3.2[c]
g2368	10	856	1.2[c]
m555	52	898	5.8[c]

[a] Other markers tested that showed no linkage to *cpr5-2: GapB*, nga111, nga128, *UFO*, and *PVV4* (chromosome 1); *Gl1* and nga172 (chromosome 3); PG11(chromosome 4); nga151 and *gl3* (chromosome 5).
[b] Data obtained from F_2 seedlings from the cross between *rps2 cpr5-2* (Col-0) and *gl3* (La-*er*)
[c] Three-factor analysis indicates that *cpr5-2* maps between g2368 and m555.

Defense-related *PR* genes are expressed in *cpr5-2* in the absence of pathogens.

The enhanced resistance and lesion-mimic phenotypes of *cpr5-2* are reminiscent of the *acd2* and *lsd* mutants described previously (Dietrich et al. 1994; Greenberg et al. 1994; Weyman et al. 1995). In these mutants enhanced resistance is correlated not only with the spontaneous appearance of necrotic lesions, but also with elevated defense-related gene expression. To determine whether this was also the case for *cpr5-2* we monitored *PR* gene expression in uninfected plants by RNA blot analysis. As is shown in Figure 5, the level of expression of two *PR* genes, *PR-1* and *BGL2*, was undetectable in leaves of mature, uninoculated wild-type Col-0 (*CPR5 RPS2*) and *CPR5 rps2* plants. However, both genes were expressed at elevated levels in leaves of mature, uninoculated *cpr5-2 rps2* plants grown under the same conditions (Fig. 5A). The level of *PR* gene expression was higher in leaves exhibiting lesions than in leaves without lesions harvested from the

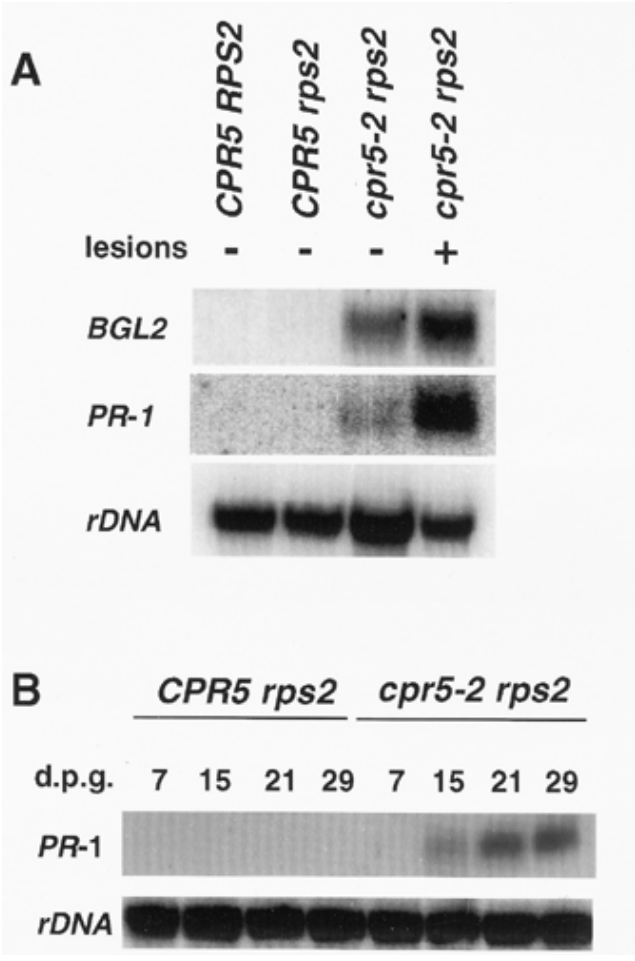

Fig. 5. Expression of *PR-1* and *BGL2* in uninoculated *cpr5-2* plants. **A,** Total RNA was extracted from mature plants grown in soil and subjected to RNA blot analysis. Lesion-positive (+) and lesion-negative (–) leaves were sampled from the same *cpr5-2* plants and RNA was prepared separately for each sample. **B,** Total RNA was extracted at indicated times (days post germination; dpg) from *CPR5 rps2* and *cpr5-2 rps2* seedlings grown axenically on Murashige and Skoog (MS) medium. Approximately 2 µg of RNA was loaded for each sample. Blots were first probed with *PR-1* and *BGL2*, followed by hybridization with an rDNA probe as a loading control.

same plant. Elevated *PR* gene expression was also detected in *cpr5-2* plants grown axenically on Murashige and Skoog (MS) agar plates (Fig. 5B).

To determine whether *PR* gene expression was constitutive, or alternatively whether it was induced during seedling development, we grew *cpr5-2 rps2* mutant plants in axenic culture, and harvested seedlings at 1, 2, 3, and 4 weeks after germination. The seedlings were examined through a dissecting scope to determine whether visible lesions were present, and then assayed for *PR-1* gene expression by RNA gel blot analysis. *PR-1* RNA was not detectable in 7-day-old *cpr5-2* seedlings but then was strongly induced by 15 days after germination (Fig. 5B). Macroscopic lesions did not appear until 21 days after germination. These results indicate that both induction of *PR-1* gene expression and lesion development in *cpr5-2 rps2* plants are developmentally regulated and occur in the absence of pathogens or other microorganisms.

We also investigated whether enhanced disease resistance and elevated levels of *PR* gene expression were dependent on SA by analyzing progeny from a cross between *cpr5-2 rps2* plants and an *A. thaliana* transgenic line carrying the bacterial salicylate hydroxylase gene (*nahG;* Bowling et al. 1994). We found that *nahG* is epistatic to *cpr5-2* with respect to both enhanced resistance and elevated *PR* gene expression (data not shown), indicating that *cpr5-2* activates the defense response signaling pathway at a point upstream of SA.

Pathogen recognition induces *PR-1* gene expression in *cpr5-2* mutant plants.

To determine whether the *cpr5-2* mutation also affects pathogen-induced defense gene expression we infected *cpr5-2 RPS2* plants with *Pst* DC3000 expressing either *avrRpt2, avrRpm1, avrB,* or *avrPphB* and assayed for *PR-1* gene expression at various time points after infection. As summarized in Figure 6A, *PR-1* expression was not detectable at early points in the time course in *CPR5 RPS2* plants, but then was strongly induced between 6 and 12 h after infection with *Pst* DC3000 (*avrRpt2*). This pattern of *PR-1* induction is typical of *RPS2*/*avrRpt2*-dependent defense responses (Reuber and Ausubel 1996). *PR-1* gene expression, which was slightly elevated in uninfected *cpr5-2* plants (Fig. 5A) and in infected *cpr5-2* plants at early time points, was also strongly induced between 6 and 12 h after infection, but reached significantly higher levels than was observed in wild-type plants (Fig. 6A). Interestingly, the elevated basal level of *PR-1* gene expression in *cpr5-2 RPS2* plants did not significantly alter the timing of *avrRpt2/RPS2*-mediated activation of *PR-1*. We observed this significant enhancement of *avrRpt2*-induced *PR-1* expression in four separate experiments. This finding is consistent with the observation that *cpr5-2 RPS2* plants exhibited an HR with normal kinetics when infected with *Pst* DC3000 (*avrRpt2*), and may account for the additional reduction of pathogen growth observed in *cpr5-2 RPS2* mutants infected with *Pst* DC3000 (*avrRpt2*) (Fig. 2A).

We did not detect significant induction of *PR-1* in either wild-type or *cpr5-2* plants infected with *Pst* DC3000 expressing *avrRpm1* or *avrB* (Fig. 6A). This is consistent with previous reports that *PR-1* is not induced upon infection with *P. syringae* expressing *avrRpm1* (Reuber and Ausubel 1996). Thus, to assess *RPM1*-dependent activation of gene expression, we monitored induction of *ELI3,* a gene that is rapidly and strongly induced during *RPM1*/*avrRpm1*-mediated resistance responses (Kiedrowski et al. 1992; Reuber and Ausubel 1996). *ELI3* was rapidly induced in wild-type plants after infection with *Pst* DC3000 (*avrRpt2*), *Pst* DC3000 (*avrRpm1*),

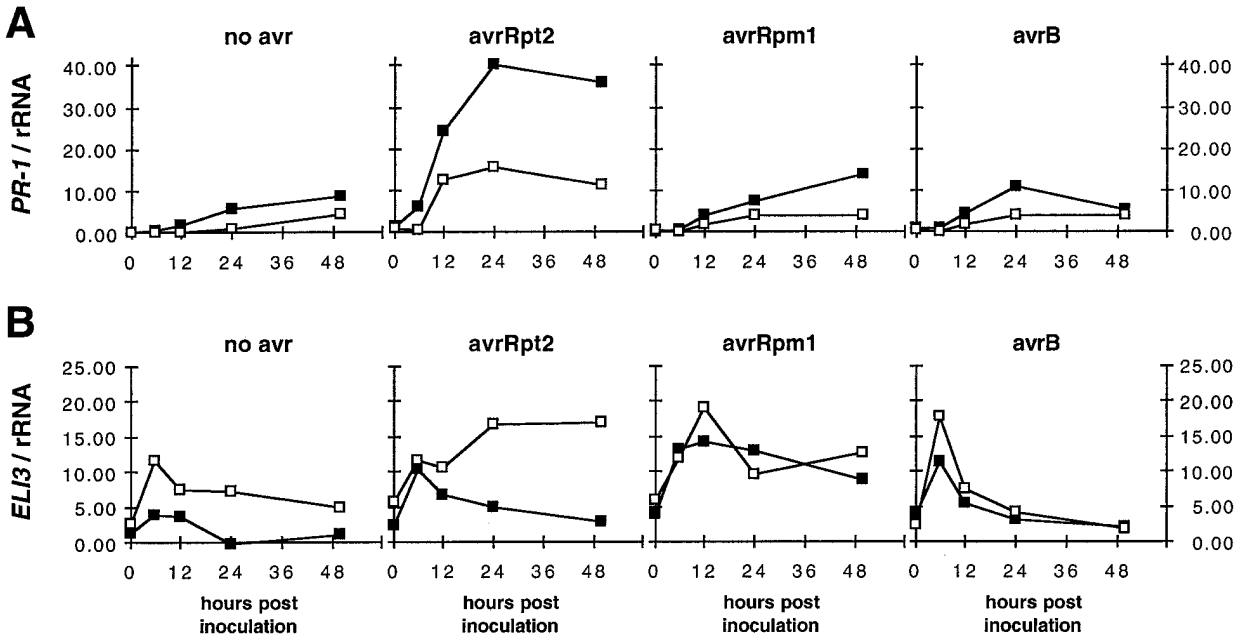

Fig. 6. Expression of *PR-1* and *ELI3* in *CPR5 RPS2* and *cpr5-2 RPS2* plants after inoculation with *Pseudomonas syringae* pv. *tomato* (*Pst*). **A,** *PR-1* expression. **B,** *ELI3* expression. Total RNA was extracted from wild-type Col-0 (*CPR5 RPS2*; open squares) and *cpr5-2 RPS2* (solid squares) leaves harvested at indicated times after vacuum infiltration with 5×10^5 CFU/ml of *Pst* DC3000, *Pst* DC3000 (*avrRpt2*), *Pst* DC3000 (*avrRpm1*), or *Pst* DC3000 (*avrB*). *PR-1* and *ELI3* hybridization signals were quantitated and normalized to signals obtained for the 28S rDNA. Similar results were obtained in a second, independent experiment.

and *Pst* DC3000 (*avrB*) (Fig. 6B). Interestingly, expression of *ELI3* in response to these strains was not enhanced in *cpr5-2* plants. The reduction of *ELI3* expression in response to *Pst* DC3000 and *Pst* DC3000 (*avrRpt2*) in *cpr5-2* plants was not consistently observed. We found the pattern of induction of *PR-1* and *ELI3* in response to *Pst* DC3000 (*avrPphB*) to be variable (data not shown), and thus we could not draw any meaningful conclusions from these experiments. The results from these experiments indicate that the *cpr5-2* mutation enhances the expression of *PR-1* in response to *RPS2*-mediated pathogen recognition, but does not effect *ELI3* expression activated by the *RPM1* signaling pathway.

DISCUSSION

Identification of a mutant with enhanced resistance to *P. syringae.*

We have isolated a new mutant allele of *CPR5* in a screen for second-site mutations that restore resistance of an *rps2* mutant line to *Pst* DC3000 expressing *avrRpt2*. The finding that *cpr5-2* plants were also resistant to *Pst* DC3000 not expressing *avrRpt2* (Figs. 1 and 2) indicates that resistance in this mutant is independent of *RPS2*-mediated pathogen recognition. Rather, resistance in *cpr5-2* appears to be due to the activation of defense responses in the absence of pathogen recognition.

cpr5-2 mutants express defense responses in the absence of pathogen infection.

cpr5-2 mutant plants exhibited two additional phenotypes indicative of "de-regulated" expression of defense responses: spontaneous development of necrotic lesions and expression of *PR* genes in the absence of pathogens. Defense-related genes, such as the *PR* genes, are not expressed at high levels in uninfected wild-type *A. thaliana* plants. Rather, expression of these genes is induced after pathogen infection (Fig. 6; Ryals et al. 1996). In contrast, *cpr5-2* mutant plants expressed elevated levels of the defense-related genes *PR-1* and *BGL2* in the absence of pathogen infection (Fig. 5A). Expression of these genes was highest in leaves exhibiting visible necrotic lesions, but was also detectable at lower levels in leaves without macroscopic lesions. These results are consistent with the findings of Bowling et al. (1997) for *cpr5-1*, and suggest that lesion formation in *cpr5* plants results in activation of both localized and systemic induction of systemic acquired resistance (SAR) gene expression, and thus disease resistance. This hypothesis is supported by the fact that *cpr5-1* mutant plants also exhibit elevated levels of SA (Bowling et al. 1997).

The *cpr5-2* phenotypes are reminiscent of the phenotypes of *acd2* and *lsd* mutants, where lesion formation leads to induction of *PR* gene expression and SAR (Dietrich et al. 1994; Greenberg et al. 1994; Weyman et al. 1995). Surprisingly, we found that in axenically grown *cpr5-2* mutant seedlings, *PR-1* gene expression preceded the appearance of visible lesions (Fig. 5). One possible explanation for this observation is that microscopic lesions were present prior to induction of *PR-1* expression in *cpr5-2* seedlings grown under these conditions. Alternatively, a signal that induces both lesion formation and *PR-1* gene expression may have been present at between 1 and 2 weeks of age in these seedlings, but the environmental conditions present in axenic plant culture caused a delay in lesion formation.

CPR5 is a novel locus involved in disease resistance and trichome development.

One of the most intriguing aspects of the *cpr5-2* mutation is that it also affects trichome development. *cpr5-2* trichomes are shorter and have fewer branches than those present on wild-type plants (Fig. 4). Our genetic analysis of *cpr5-2* suggests that enhanced disease resistance, lesion development, and the unique trichome defect are conferred by a single, recessive mutation that maps to the lower arm of chromosome 5. The fact that the *cpr5-1* mutant also has a similar trichome defect (Bowling et al. 1997) lends support to our conclusion that the trichome phenotype can be attributed to a mutation at the *CPR5* locus. The *CPR5* locus is novel in that it is both important in disease resistance signal transduction and required for normal trichome development.

RPS2, RPM1 and *RPS5*-mediated defense responses are functional in *cpr5-2* plants.

Resistance gene-mediated pathogen recognition is functional in the *cpr5-2* mutant background. This conclusion is supported by results from three different assays for resistance gene function. First, *cpr5-2* plants exhibited HRs when inoculated with high levels of *Pst* DC3000 expressing *avrRpt2, avrRpm1,* or *avrPphB.* Second, *avr* gene-mediated restriction of pathogen growth of *Pst* DC3000 expressing *avrRpt2, avrRpm1, avrB,* or *avrPphB* was observed in *cpr5-2* mutant plants (Fig. 2). Third, resistance gene-mediated induction of defense gene expression was induced in *cpr5-2* plants after infection with *Pst* DC3000 expressing these *avr* genes (Fig. 6). These results indicate that the *cpr5-2* mutation does not interfere with resistance gene-mediated signal transduction.

In fact, the *cpr5-2* mutation enhances *RPS2*-mediated signaling. In *cpr5-2* plants infected with *Pst* DC3000 (*avrRpt2*), induction of *PR-1* expression was significantly higher than in wild-type plants (Fig. 6). Interestingly, this stimulatory effect of *cpr5-2* appears to be specific for the *RPS2* signaling pathway, as *RPS5* and *RPM1*-mediated induction of defense genes was not enhanced in the mutant. This result is not surprising given the finding that *RPS2* and *RPM1* activate different signal transduction pathways leading to defense-related gene expression and resistance (Reuber and Ausubel 1996; Ritter and Dangl 1996). It is entirely possible that different resistance signaling pathways are regulated in different ways, and that the *cpr5-2* mutation affects only a subset of these.

Although *RPS2*- and *cpr5-2*-mediated activation of defense responses is cumulative at the level of *PR-1* expression, it is difficult to detect enhanced resistance at the level of restriction of pathogen growth. This is presumably due to the fact that resistance gene-mediated defense responses are very effective at limiting pathogen growth, thus masking any additional resistance conferred by *cpr5-2*. However, we were able to measure a significant cumulative resistance effect in the context of plants carrying an *rps2* mutation, in which growth of *Pst* DC3000 is significantly enhanced by the presence of the *avrRpt2* gene (Fig. 2A). In this case, the cumulative effect of *RPS2* and *cpr5-2*-mediated defense responses can be monitored in pathogen growth assays.

Possible roles for *CPR5* in disease resistance signaling pathways.

The results of our analysis of *cpr5-2* agree with those reported for *cpr5-1* by Bowling et al. (1997). Consistent with their model for the role of *CPR5* in defense, we propose that *cpr5-2* affects an early step in the defense response signaling pathway, upstream of SA, and presumably downstream of pathogen recognition. However, our analysis of the interactions between *cpr5-2* and several different resistance gene signaling pathways provides additional information that can be incorporated into a model for how *CPR5* may be regulating disease resistance.

The *cpr5-2* mutant belongs to the "initiation class" of cell death mutants, which Dangl et al. (1996) define as mutants that "stochastically form lesions of determinate size at inappropriate locations." In this class of mutants it is proposed that the HR signaling pathway and, presumably, the defense response pathway are inappropriately regulated. Based on this hypothesis we envision that the wild-type *CPR5* gene product normally acts as a negative regulator of one or more branches of the pathogen response signaling cascade, preventing inappropriate flux through the pathway. Mutations at *CPR5* would either partially or entirely remove this level of control, resulting in sensitization or inappropriate activation of the pathway. The phenotype of the *cpr5-2* mutant is consistent with this model, as lesion development and the expression of *PR* genes are uncoupled from the normal induction by pathogen recognition.

In the case of the *cpr5-2* mutant, however, the resistance signaling pathways are still capable of responding to positive inputs such as pathogen recognition mediated by the resistance genes *RPS2, RPM1,* and *RPS5*. Interestingly, *cpr5-2* appears to specifically potentiate signaling through the *RPS2* pathway, as *PR-1* expression in response to *Pst* DC3000 (*avrRpt2*) is significantly enhanced in the mutant. This finding suggests that *CPR5* impinges on *RPS2* signaling, but does not directly modulate the *RPM1* pathway.

An alternative hypothesis is that the *cpr5-2* mutation gives rise to an alteration in cellular metabolism that indirectly results in abnormal trichome development, lesion formation, elevated SA levels, expression of *PR* genes, and the resulting resistance. In this scenario, elevated levels of SA could potentiate enhanced signaling through the *RPS2* pathway (Shirasu et al. 1997). Thus, *cpr5-2* could be a mutation in a gene not normally involved in disease resistance or trichome development. A third possibility is that *CPR5* may be required for the normal function of a cellular component, such as the plant cell wall, that plays an important role in both disease resistance signaling and trichome development.

The molecular isolation and further characterization of *CPR5* should contribute to our understanding of both pathogen resistance and trichome development, and is likely to lead to important insights into how two seemingly separate processes may be governed by a shared regulatory component.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The bacterial pathogen strains *Pseudomonas syringae* pv. *tomato (Pst)* DC3000, *Pst* 3435, *P. syringae* pv. *maculicola* m4, and the *avrRpt2, avrRpm1, avrB,* and *avrPphB* avirulence genes, have been described previously (Staskawicz et al. 1987; Debener et al. 1991; Jenner et al. 1991; Whalen et al. 1991; Innes et al. 1993; Kunkel et al. 1993; Ritter and Dangl 1995). *P. syringae* strains were cultured at 28°C in King's B medium (King et al. 1954) containing 50 µg of rifampicin per ml plus appropriate antibiotics required for plasmid maintenance. The *avrRpt2* gene was introduced into these *P. syringae* strains on plasmids pABL18, pLH12, or pV288 (Whalen et al. 1991; Kunkel et al. 1993), and the *avrRpm1, avrB,* and *avrPphB* genes were introduced on plasmids K48 (Debener et al. 1991), pPSG0002 (Staskawicz et al. 1987), or pPPY424 (Fillingham et al. 1992), respectively. *P. syringae* strains not expressing *avr* genes carried control plasmids pLAFR3 or pVSP61 (vectors without insert).

Plant material, growth conditions, and inoculation procedures.

The susceptible *rps2-201C* mutant used in this work was described previously (Kunkel et al. 1993). The *nahG* transgenic line (Bowling et al. 1994) and the *cpr5-1* mutant (Bowling et al. 1997) were obtained from Scott Bowling and Xinnian Dong (Duke University). *A. thaliana* plants were grown from seed in growth chambers under an 8-h photoperiod at 24°C. Mass inoculation of plants was carried out by dipping entire leaf rosettes of 3- to 5-week-old plants into bacterial suspensions of 2 to 4×10^8 CFU/ml containing the surfactant Silwet L-77 as described in Kunkel et al. (1993). Pipette infiltrations to assay for the HR were carried out with *Pst* strains suspended in 10 mM $MgCl_2$ to a density of approximately 2×10^7 CFU/ml (Kunkel et al. 1993). Leaves were scored for tissue collapse approximately 20 h after inoculation. Bacterial growth within leaf tissue was monitored as described by Whalen et al. (1991).

Mutagenesis and isolation of mutants.

A genetically marked line of *rps2-201C* carrying a tightly linked molecular marker, PG11, was chosen for the mutant screen to facilitate verification of true suppressor mutants. PG11, which maps within 0.5 cM of *RPS2* (Bent et al. 1994), was used both to confirm that the suppressor mutants were derived from the originally marked *rps2-201C* mutant line and to follow inheritance of the *rps2-201C* allele in subsequent crosses. The *rps2-201C* marked line was derived from an F_2 individual from a cross between the *rps2-201C* mutant (Col-0 background) and the Nossen (No-0) ecotype that carried a crossover between the mutant *rps2* locus and PG11.

Seeds from the marked *rps2-201C* line were mutagenized by soaking them for 8 h at room temperature in 30 mM EMS. Lots of approximately 30 M_1 seeds were planted and harvested separately to obtain independent populations of M_2 seed. Approximately 2,000 M_2 seeds from each of 10 lots were screened by the L-77 dip-inoculation procedure (Kunkel et al. 1993).

The *cpr5-2 RPS2* plants used in our experiments were from homozygous F_3 *cpr5-2 RPS2* families derived from a second backcross of *cpr5-2 rps2* to *CPR5 RPS2* (wild-type Col-0). Families homozygous for the wild-type *RPS2* allele were identified by polymerase chain reaction screening with the closely linked PG11 marker.

Genetic analysis and mapping.

The *cpr5-2 rps2* mutant was crossed to both *CPR5 rps2-201C* and wild-type Col-0 to determine the genetic basis of

resistance. Allelism tests were carried out by crossing to *gl3* (La-*er* background; Koornneef et al. 1982) and *cpr5-1* (Bowling et al. 1997). The F_1 progeny from the cross to *cpr5-1* retained the trichome, disease lesion mimic, and enhanced resistance phenotypes characteristic of the mutants, indicating that *cpr5-2* and *cpr5-1* are allelic. *cpr5-2* was also crossed to a transgenic La-*er* line carrying the *nahG* gene (Bowling et al. 1994). As the dominant *nahG* gene renders plants very susceptible to *P. syringae* (Delaney et al. 1994), the F_1 progeny from this cross were not assayed for their resistance phenotype. The F_1 plants were allowed to self-pollinate, and the resulting F_2 progeny were scored for their disease lesion mimic and trichome phenotypes. Tissue was then harvested from individuals with *cpr5-2* lesion-mimic and trichome phenotypes for RNA isolation and gel blot analysis to monitor expression of *nahG*, *PR-1*, and *BGL2*. F_2 progeny from both *cpr5-2* and wild-type phenotypic classes were then inoculated with *Pst* DC3000 to assay their resistance phenotypes.

Restriction fragment length polymorphism (RFLP) linkage analysis with codominant cleaved amplified polymorphic DNA sequences (CAPS; Konieczny and Ausubel 1993) and simple sequence length polymorphism (SSLP; Bell and Ecker 1994) markers was performed with progeny from the cross between *cpr5-2* (in a Col-0/No-0 hybrid background) and *gl3* (La-*er* background). Plant genomic DNA was isolated from leaf tissue of F_2 plants according to the procedure of Tai and Tanksley (1990) with modifications as described in Kunkel et al. (1993).

Scanning electron microscopy.

Samples fixed in FAA (3.7% formaldehyde, 50% ethanol, 5% acetic acid) were prepared for scanning electron microscopy by standard methods (Irish and Sussex 1990).

RNA isolation and analysis.

Total RNA was isolated from *A. thaliana* leaf tissue with the RNeasy Plant RNA isolation kit (Qiagen, Valencia, CA). RNA gel blot analysis was carried out according to Sambrook et al. (1989). Hybridization probes were prepared by random-primer DNA synthesis (Sambrook et al. 1989). The following DNA probes were used: *A. thaliana PR-1* cDNA obtained from Ciba-Geigy (Uknes et al. 1992); *A. thaliana BGL2* cDNA (Dong et al. 1991); *A. thaliana ELI3* cDNA (Kiedrowski et al. 1992); and *nahG* (Bowling et al. 1994). As a loading standard we used a 3.7-kb *Eco*RI fragment from the 10-kb genomic region carrying *A. thaliana* rRNA genes (Vongs et al. 1993). This probe was kindly provided by Eric Richards (Washington University).

The RNA blots were analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The data summarized in Figure 6 were obtained by volume integration of the hybridization signals obtained with the *PR-1* or *ELI3* probes followed by normalization to the hybridization signals obtained with the rDNA probe.

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