The Plant Cell, Vol. 9, 1573–1584, September 1997 © 1997 American Society of Plant Physiologists

The *cpr5* Mutant of Arabidopsis Expresses Both NPR1-Dependent and NPR1-Independent Resistance

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The *cpr5* mutant was identified from a screen for constitutive expression of systemic acquired resistance (SAR). This single recessive mutation also leads to spontaneous expression of chlorotic lesions and reduced trichome development. The *cpr5* plants were found to be constitutively resistant to two virulent pathogens, *Pseudomonas syringae* pv *maculicola* ES4326 and *Peronospora parasitica* Noco2; to have endogenous expression of the pathogenesis-related gene 1 (*PR-1*); and to have an elevated level of salicylic acid (SA). Lines homozygous for *cpr5* and either the SA-degrading bacterial gene *nahG* or the SA-insensitive mutation *npr1* do not express *PR-1* or exhibit resistance to *P. s. maculicola* ES4326. Therefore, we conclude that *cpr5* acts upstream of SA in inducing SAR. However, the *cpr5 npr1* plants retained heightened resistance to *P. parasitica* Noco2 and elevated expression of the defensin gene *PDF1.2*, implying that NPR1-independent resistance signaling also occurs. We conclude that the *cpr5* mutation leads to constitutive expression of both an NPR1-dependent and an NPR1-independent SAR pathway. Identification of this mutation indicates that these pathways are connected in early signal transduction steps and that they have overlapping functions in providing resistance.

INTRODUCTION

Plants respond in a variety of ways to the presence of pathogens (Lamb, 1994). Recent studies of active plant disease resistance have centered around two major resistance responses, the hypersensitive response (HR) and systemic acquired resistance (SAR).

The HR occurs when a plant recognizes an invading pathogen. It involves specific avirulence (*avr*) genes expressed in the pathogen that are recognized by specific resistance (*R*) genes expressed by the host plant (reviewed in Staskawicz et al., 1995; Hammond-Kosack and Jones, 1996). When an avirulent pathogen expresses an *avr* gene, the plant is able to recognize it because of the presence of a corresponding *R* gene, whereas a virulent pathogen does not express an *avr* gene that the plant is able to recognize. This *avr–R* gene interaction initiates a signal cascade at the point of infection that results in rapid cell death, superoxide accumulation, callose deposition, local expression of the pathogenesis-related (PR) proteins, and release of the systemic signal that induces SAR (Chester, 1933; Enyedi et al., 1992; Ryals et al., 1994).

SAR is a plant defense response that occurs after infection by an avirulent or other necrotizing pathogen and results in a long-lasting, nonspecific, systemic resistance to subsequent pathogen infection (Ross, 1961; Kuc, 1982). SAR is characterized by enhanced resistance to virulent pathogens and transcriptional activation of the PR genes (Van Loon and Van Kammen, 1970; Ward et al., 1991; Yalpani et al., 1991; Uknes et al., 1992).

Several lines of evidence imply that SAR is dependent on salicylic acid (SA). SAR can be induced by the exogenous application of SA (White, 1979) or by chemicals, such as 2,6-dichloroisonicotinic acid (INA; Métraux et al., 1991) or benzo(1,2,3)thiadiazole-7-carbothioc acid *S*-methyl ester (Görlach et al., 1996), that are functional analogs of SA. Elevation of endogenous SA levels has been found near lesion sites during an HR and subsequently in systemic tissues, accompanying the onset of SAR (Malamy et al., 1990, 1992; Métraux et al., 1990; Rasmussen et al., 1991; Yalpani et al., 1991; Enyedi et al., 1992; Uknes et al., 1993). In addition, transgenic tobacco and Arabidopsis plants expressing *nahG*, a bacterial gene encoding salicylate hydroxylase (which converts SA to catechol; You et al., 1991), can neither accumulate significant levels of SA nor induce PR gene transcription or SAR in response to pathogen invasion (Gaffney et al., 1993; Delaney et al., 1994).

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To elucidate the SAR signaling cascade, two classes of mutants exhibiting an altered SAR response have been generated in Arabidopsis. The first mutant class is characterized by the gain of pathogen resistance. We originally reported the characterization of *cpr1* (constitutive expresser of PR genes), which constitutively expresses the hallmarks of SAR in the absence of induction (Bowling et al., 1994). Other mutants that lead to constitutive SAR include the *lsd* (lesions simulating disease resistance response; Dietrich et al., 1994, 1997; Weymann et al., 1995; reviewed in Dangl et al., 1996) and *acd* (accelerated cell death; Greenberg et al., 1994) mutants. Both exhibit spontaneous HR-like lesions as well as constitutive SAR expression. These mutants accumulate high levels of endogenous SA concurrently with *PR-1* gene expression and lose *PR-1* expression in the presence of *nahG* (and thus absence of SA). The *cim* (constitutive immunity) mutants are another *cpr1*-like class of mutants that do not express spontaneous HR-like lesions (Lawton et al., 1993).

The second class of mutants is characterized by the inability of the plant to mount an SAR response after biological or chemical induction. The characterization and cloning of *npr1* (nonexpresser of PR genes), which blocks expression of SAR and is super-susceptible to pathogen infection, have been reported (Cao et al., 1994, 1997). Several other mutant screens resulted in isolation of *npr1* alleles (Delaney et al., 1995; Glazebrook et al., 1996; Ryals et al., 1997; Shah et al., 1997), demonstrating the importance of NPR1 in the regulation of SAR. Other resistance-compromised mutants include the *eds* (enhanced disease symptoms; Glazebrook et al., 1996; Parker et al., 1996; Rogers and Ausubel, 1997) class, of which the lack of *PR-1* expression in *eds4* and *eds5* is rescued by SA treatment, and *ndr1-1* (non-race-specific disease resistance; Century et al., 1995), which fails to respond to several avirulent pathogens that normally elicit an HR.

By analyzing the mutants mentioned above, SAR induction has been defined as beginning with pathogen recognition, depending on SA accumulation, and resulting in PR gene expression and pathogen resistance. But what of the existence of alternative, SA- and NPR1-independent resistance pathways? Early evidence of the possibility of an alternative pathway was the demonstration that acidic (*PR-1*, *PR-2*, *PR-3*, *PR-4*, and *PR-5*) and basic (β-1,3-glucanase and basic chitinase) PR genes in tobacco are stimulated in different temporal and spatial patterns by different hormones (SA and ethylene, respectively; Brederode et al., 1991; Meins et al., 1991; Ward et al., 1991). Recently, Pieterse et al. (1996) described a systemic resistance pathway in Arabidopsis that is induced by plant growth–promoting rhizobacteria and that is independent of both SA accumulation and PR genes. Thus, it is apparent that SA-independent systemic resistance occurs and involves genes other than the PR genes that are markers of SA-dependent SAR.

Penninckx et al. (1996) reported an SA- and NPR1-independent resistance pathway in Arabidopsis after infection by the avirulent fungal pathogen *Alternaria brassicicola*. Activation of this pathway is characterized by induction of the *PDF1.2* gene. Expression of *PDF1.2* is not affected by *cpr1*, *npr1*, or *nahG*, indicating independence from SA and NPR1; however, the ethylene-insensitive mutant *ein2* and the jasmonic acid (JA)– and coronatine-insensitive mutant *coi1* block expression of *PDF1.2*, demonstrating possible dependence on JA and ethylene (Penninckx et al., 1996).

The *PDF1.2* gene encodes a defensin, a member of a class of small cysteine-rich peptides that are characterized by a cysteine-stabilized α-helical structure and potent antifungal properties (Broekaert et al., 1995; Terras et al., 1995). Defensins have been found in many other plants, including radish and potato (Terras et al., 1992; Moreno et al., 1994; Osborn et al., 1995), and have structural similarity to defensins that are integral to the immune response in mammals and insects (Broekaert et al., 1995). *PDF1.2* was first identified in a search for Arabidopsis genes with homology to a defensin from radish seed, *Rs-AFP1*, and the expression of *PDF1.2* corresponds with the production of an antifungal peptide in Arabidopsis (Penninckx et al., 1996). Thus, it is likely that PDF1.2 is involved in the resistance response, although a direct role has not been demonstrated.

Here, we report the characterization of a recessive mutant, *cpr5*, that constitutively expresses SAR and forms spontaneous HR-like lesions. This mutant maps to chromosome 5 and has an additional phenotype of reduced trichome development, which may indicate a connection with developmental pathways. We show strong genetic evidence for the confirmation of an NPR1-independent pathway by demonstrating that the *cpr5 npr1* double mutant loses PR gene expression and resistance to *Pseudomonas syringae* pv *maculicola* ES4326 but retains constitutive *PDF1.2* expression and resistance to *Peronospora parasitica* Noco2. We propose that *cpr5* be placed upstream of the HR on the signaling pathway for SAR and that the biological definition of SAR be expanded to include the NPR1-dependent and NPR1-independent pathways of systemic resistance.

RESULTS

Identification of the *cpr5* Mutant

The *cpr5* mutant was identified in a screen for constitutive expressers of a reporter gene containing the promoter region of the PR gene β-1,3-glucanase 2 (*BGL2*) and the β-glucuronidase (*GUS*) coding region; the details of this screen have been described previously (Bowling et al., 1994). Figure 1 shows the constitutive expression of the reporter gene in the *cpr5* mutant. Also evident in Figure 1 is that *cpr5* is significantly smaller than the wild type. This is a characteristic that has been observed in other *cpr* mutants. The *cpr5* mutation was also found to confer expression of the endogenous *PR-1* gene, as shown in Figure 2.

The expression of *PR-1* in *cpr5* plants varies and appears positively correlated with the number and size of lesions

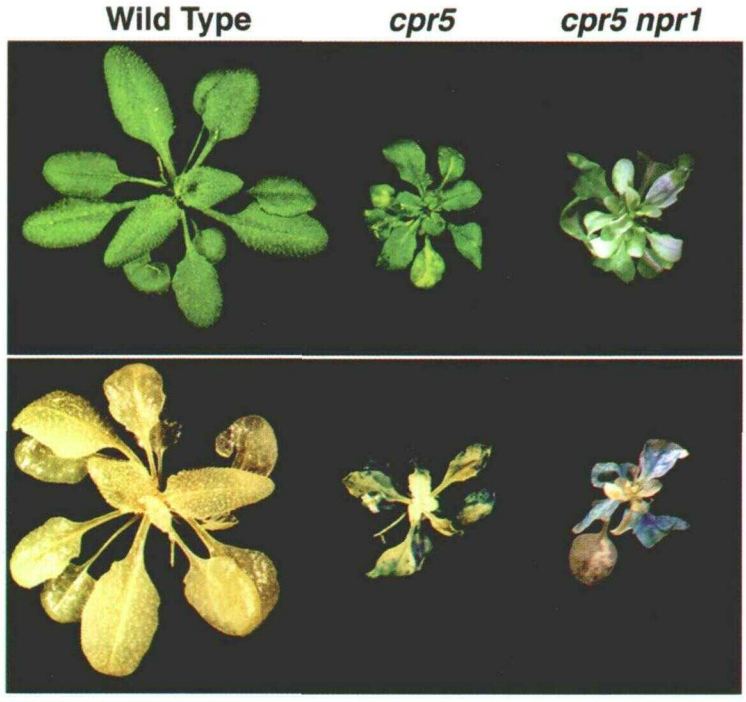

Figure 1. Phenotypes of *cpr5* and *cpr5 npr1* Plants.

Wild-type and *cpr5* plants were photographed when 5 weeks old; *cpr5 npr1* plants were photographed when 3 weeks old.
(Top) Wild-type *BGL2–GUS*, *cpr5*, and *cpr5 npr1* plants.
(Bottom) Wild-type *BGL2–GUS*, *cpr5*, and *cpr5 npr1* plants infiltrated with X-gluc to stain for expression of the *BGL2–GUS* reporter gene (Jefferson et al., 1987).

(S.A. Bowling, unpublished results); the plants used for the sample shown in Figure 2 had few lesions and resulted in one of the lower amounts of expression seen in six RNA gel blots performed that included *cpr5* plants. Quantification of the bands in this blot showed that the level present is significantly above background, at least fourfold higher than that found for either wild-type, *cpr5 npr1*, *npr1*, *cpr5 nahG*, or *nahG* samples.

In addition to the constitutive expression of *PR-1*, *cpr5* also exhibits the formation of spontaneous lesions, as shown in Figures 1, 3A, and 3B, and a reduction in both trichome number and development, as shown in Figure 3A. Lesion formation in *cpr5* plants was not found to be dependent on environmental conditions, including photoperiod or humidity. Rather, lesion formation on *cpr5* plants seems to follow a developmental pattern in which cotyledons develop lesions after true leaves have begun to emerge and in which true leaves do not show visible signs of lesions until several days after emergence.

To further investigate the nature of the spontaneous lesions in *cpr5* plants and to determine whether they phenocopy the lesions formed during the HR, *cpr5* plants were stained with trypan blue to assay for cell death (Dietrich et

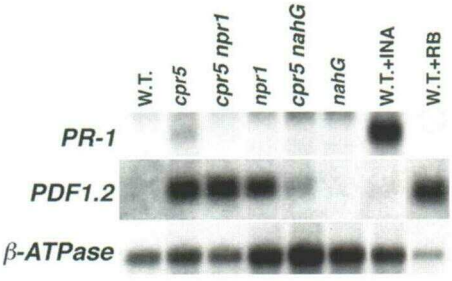

Figure 2. *PR-1* and *PDF1.2* Gene Expression in *cpr5* Plants and in the *cpr5 npr1* and *cpr5 nahG* Lines.

PR-1 and *PDF1.2* gene-specific probes were used for RNA gel blot analysis of the indicated plants, with the β-ATPase gene-specific probe used as a loading standard. W.T., wild-type *BGL2–GUS* transgenic line. RNA samples were extracted from 3-week-old seedlings grown on Murashige and Skoog (MS) medium or MS medium with 0.1 mM INA (W.T.+INA), except for the lane containing W.T.+RB, which was extracted from 4-week-old wild-type *BGL2–GUS* plants grown on soil and treated with rose bengal (Penninckx et al., 1996).

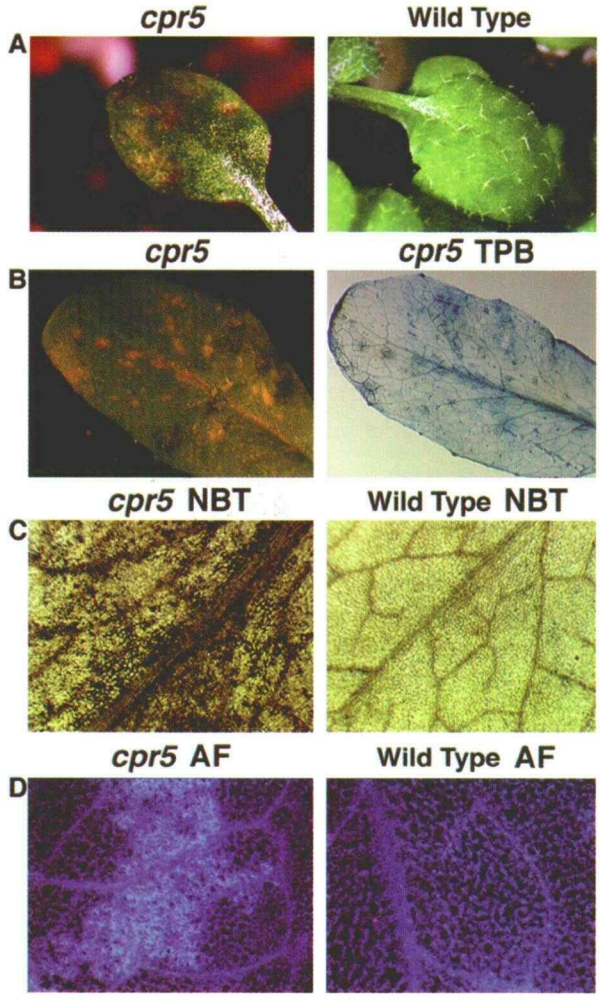

Figure 3. Trichome Development and Lesion Formation in *cpr5* Mutants.

(A) A typical *cpr5* leaf. Shown is a comparison of *cpr5* and wild-type leaves revealing spontaneous lesions and reduced trichome development on *cpr5* plants.

(B) Lesions of *cpr5* plants. Shown is the correspondence in a leaf of a 3-week-old *cpr5* plant of areas with lesions (left, *cpr5*) with areas of dead cells revealed by trypan blue staining (right, *cpr5* TPB).

(C) Superoxide accumulation in *cpr5* plants. Shown is a comparison of superoxide accumulation revealed by nitro blue tetrazolium staining in leaves with no visible lesions from 5-week-old *cpr5* (left, *cpr5* NBT) and wild-type plants (right, Wild Type NBT).

(D) Autofluorescence of *cpr5* plants. Shown is a comparison of autofluorescence revealed by UV microscopy between leaves of 4-week-old *cpr5* (left, *cpr5* AF) and wild-type plants (right, Wild Type AF).

al., 1994). As shown in Figure 3B, *cpr5* plants have areas of dead cells corresponding to sites at which there are macroscopic signs of lesions before staining. Such staining is not found in wild-type leaves, including those undergoing senescence, unless an HR has been induced on those leaves by an avirulent pathogen (data not shown). Further analysis of macroscopic lesion appearance with respect to trypan blue staining and *BGL2–GUS* reporter gene staining indicated that PR gene expression is most prominent on the margins of lesions, whereas dead cells are mostly on the interior. This pattern suggests that the signal inducing reporter gene expression emanates from the dead or dying cells found in the lesion. Figure 3C shows intense nitro blue tetrazolium staining (Jabs et al., 1996) of a representative *cpr5* leaf, confirming the presence of high amounts of reactive oxygen species in this leaf compared with the wild-type control, whereas Figure 3D presents UV microscopy revealing considerable autofluorescence corresponding to areas of lesion formation in *cpr5* plants. Thus, *cpr5* plants form lesions that phenocopy the HR. This is in contrast to two other *cpr* mutants that have been characterized in detail (*cpr1* and *cpr6*). *cpr1* and *cpr6* do not exhibit any macroscopic or microscopic signs of lesion formation and do not express the staining or autofluorescent markers of the HR (S.A. Bowling and J.D. Clarke, unpublished results).

Genetic Analyses of *cpr5*

Genetic crosses were conducted to define the Mendelian character of *cpr5*, to determine whether the *cpr5* mutation is allelic to other *cpr* or *cpr*-like mutations, and to allow mapping of the *CPR5* locus. In a backcross with wild-type plants containing the *BGL2–GUS* reporter gene, constitutive reporter gene expression was absent in F_1 plants and present in 44 of 175 F_2 seedlings. This segregation ratio of 1:3 indicates that *cpr5* is inherited as a single recessive nuclear mutation ($\chi^2 = 0.002$; $P > 0.95$). After the fourth backcross, lesion formation and reduced trichome phenotypes were still found to cosegregate with the constitutive expression of the reporter gene, suggesting that these traits are caused by the *cpr5* mutation. This conclusion is supported by the independent isolation of a mutant called *drt1* (disease resistant, abnormal trichomes) that also expresses these other phenotypes (M. Verbsky, T. Robertson, J. Boch, J. Larkin, and B. Kunkel, manuscript in preparation). The F_1 plants from crosses between *drt1*, which is also recessive, and *cpr5* clearly expressed lesions and the *BGL2–GUS* reporter gene, and had reduced trichome development, indicating that the same mutant gene is responsible for all of these phenotypes. Allelism tests between *cpr* mutants have revealed that *cpr1*, *cpr4*, *cpr5*, and *cpr11* each define unique complementation groups, whereas recombination studies have demonstrated that the dominant mutant *cpr6* is at a locus different from either *cpr1* or *cpr5* (S.A. Bowling and J.D. Clarke, unpublished results).

Crosses between *cpr5* (which are in the Columbia [Col]

ecotype) and Landsberg *erecta* (L*er*) were performed to determine the chromosomal position of *cpr5* by using codominant cleaved amplified polymorphic sequences (Konieczny and Ausubel, 1993). Among chromosomes scored in F_2 lines that are homozygous for *cpr5*, 34 of 978 have L*er* alleles at the *LFY3* locus. In addition, only two of 748 tested chromosomes have L*er* alleles at the *g2368* locus, and these recombinant plants are homozygous for the Col allele at the *LFY3* locus. This places *cpr5* between these markers, ~3.5 centimorgans from *LFY3* and 0.3 centimorgans from *g2368* on chromosome 5. This map position confirms that *cpr5* is not allelic to either *cpr1* or *cpr6*, which map to chromosome 4 and chromosome 1, respectively (S.A. Bowling and J.D. Clarke, unpublished results).

Epistasis between *cpr5* and *npr1*

Because *cpr5* forms spontaneous lesions that phenocopy the HR, it was assumed that *npr1* would be downstream of *cpr5* in SAR signaling and therefore suppress PR gene expression and SAR in *cpr5* plants. To test this assumption, crosses were performed to establish double mutants of *cpr5* and *npr1*. Because *cpr5* is recessive, analysis of the effect of *npr1* in these double mutants was performed with plants from the F_2 generation and later.

To establish a *cpr5 npr1* double mutant line, we used the lesion-forming phenotype caused by *cpr5* to identify those F_2 plants homozygous for *cpr5*. This phenotype is likely to be independent of *NPR1*, given that *NPR1* functions downstream of the HR (Cao et al., 1994). Plants forming lesions identified in the F_2 generation were allowed to self-fertilize, and the F_3 plants were tested for expression of the *BGL2–GUS* reporter gene by histochemical staining for GUS activity (Jefferson et al., 1987). If *npr1* suppresses induction of PR gene expression by *cpr5*, then the F_3 families of *cpr5 npr1* double mutants would be expected to have reduced reporter gene activity compared with the *cpr5* plants. Such plants were found and were confirmed to be *cpr5 npr1* double mutants by a backcross with *npr1* (pollen donor), which produced F_1 plants that all had *npr1* phenotypes (little or no reporter gene induction when grown on SA or INA; bleaching when grown on 0.5 mM SA). RNA gel blot analysis showed that *npr1* completely suppresses expression of *PR-1* in the double mutant (Figure 2); quantification of the bands as described earlier shows that expression level in the double mutant is reduced to the level found in the untreated wild type. These results demonstrate that *cpr5* is upstream of *npr1* in inducing PR gene expression.

The *cpr5 npr1* double mutant has a unique phenotype characterized by a partial bleaching of the leaves and stems (Figure 1), which phenocopies the *npr1* plants grown on SA (Cao et al., 1994, 1997) and is probably a consequence of the high production of SA in these plants. Although the double mutant retains some residual expression of the *BGL2–GUS* reporter gene (Figure 1), it is considerably reduced from that in the *cpr5* mutant alone. A quantitative assay for GUS activity using the substrate 4-methylumbelliferyl β-D-glucuronide, presented in Figure 4, shows that the level of GUS activity in *cpr5 npr1* plants is approximately one-third of that in *cpr5* plants. Because INA induction of *npr1* plants does not lead to significant GUS activity, the residual GUS reporter gene activity in the *cpr5 npr1* mutant is not due to "leakiness" of the *npr1* mutation; instead, this activity may be attributed to an NPR1-independent induction of the *BGL2* promoter. This is not unexpected because the *BGL2* promoter is a complex promoter that is responsive to factors other than NPR1.

Relationship of *cpr5* to SA

With *cpr5* placed upstream of *npr1* in the SAR pathway, we then used both genetic and biochemical approaches to determine the relationship of *cpr5* to SA. Crosses between *cpr5* plants and plants containing the *nahG* gene were used for genetic suppression of SA production. The line homozygous for both *cpr5* and *nahG* was identified by taking advantage of the appearance of brown deposits in the roots of *nahG*-expressing plants when grown on SA (Bowling et al., 1994). These deposits are phenolic by-products from the breakdown of catechol, which is the compound formed

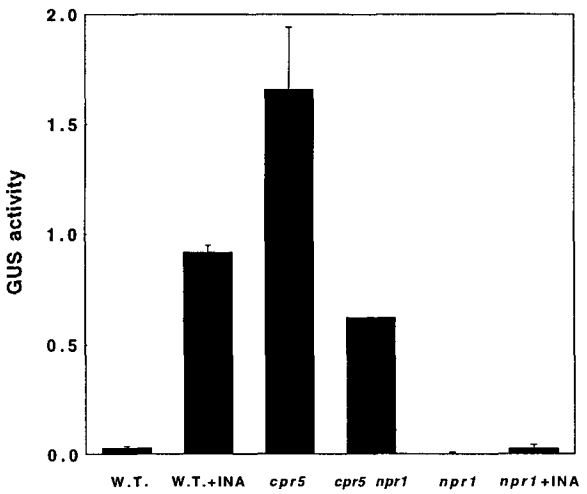

Figure 4. Quantitative Analysis of GUS Activity in Wild-Type, *cpr5*, *npr1*, and *cpr5 npr1* Plants.

Plant tissues assayed were from 10-day-old wild-type, *cpr5*, *npr1*, and *cpr5 npr1* seedlings grown on MS medium or MS medium with 0.1 mM INA. The values represent the average of three replicates ±SE. GUS activity is given as fluorescence units per minute per microgram of protein. W.T., wild-type *BGL2–GUS* transgenic line; W.T. + INA, wild-type *BGL2–GUS* seedlings grown on MS medium with 0.1 mM INA; *npr1* + INA, *npr1* seedlings grown on MS medium with 0.1 mM INA.

when salicylate hydroxylase oxidizes SA. Thus, seed were collected from individual F_2 plants with spontaneous lesions and reduced trichome development to select for homozygous *cpr5* lines, and among them, the F_3 families were scored for the uniform presence of brown roots to select for a line that was also homozygous for *nahG*.

Endogenous PR gene expression and free SA and salicylate glucoside (SAG) concentrations were examined in leaf tissues from *cpr5*, wild-type, and *cpr5 nahG* plants; the results are presented in Figures 2, 5A, and 5B, respectively. The *cpr5* plants express *PR-1* and exhibit a high level of endogenous SA and SAG, indicating that *cpr5* acts before SA. The SA and SAG levels in *cpr5* plants were each ~30-fold higher than in the wild type, similar to those reported for *cpr1*, *lsd6*, and *lsd7* (Bowling et al., 1994; Weymann et al., 1995) and to those found in Arabidopsis leaves after infection with a necrogenic pathogen (Uknes et al., 1993). The *cpr5 nahG* plants had dramatically reduced SA and SAG levels compared with *cpr5* plants. Although the SA level in the *cpr5 nahG* line was still above that found for the wild type (Figure 5A), this was not sufficient to activate *PR-1* expression in these plants (Figure 2). The levels of SA and SAG and the effects on *PR-1* expression support the assertion that *cpr5* acts before and via SA to induce SAR.

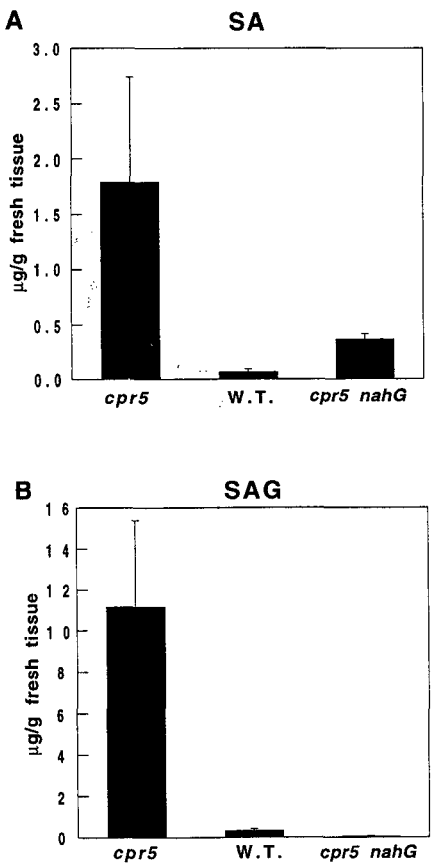

Figure 5. SA and SAG Levels in *cpr5* and *cpr5 nahG* Plants.

(A) Free SA.

(B) Sugar-conjugated SA (SAG).

Leaves from 4-week-old plants grown on soil were collected and analyzed by HPLC for free SA and SAG content. The values presented are an average of four replicates ±SE (micrograms of SA per gram leaf fresh weight). W.T., wild-type *BGL2–GUS* transgenic line.

The *cpr5 nahG* line also reveals unique phenotypes. The *cpr5 nahG* plants are severely limited in size even when compared with *cpr5* plants, and they often have brown to black lesions, as opposed to the yellow lesions found on *cpr5* plants. These extra effects could result from the buildup of products from the degradation of SA. These plants also retain expression of the *BGL2–GUS* reporter gene, which may be explained by the still-elevated level of SA (Figure 5A) remaining in *cpr5 nahG* plants or by the presence of an SA-independent pathway activating the reporter gene in *cpr5* plants. The latter explanation is supported by the lack of *PR-1* expression in these plants (Figure 2) and the low level of reporter expression also found in *cpr5 npr1* plants.

cpr5 Resistance

The biological function of SAR is to render plants resistant to normally virulent pathogens. The growth of the virulent bacterial pathogen *P. s. maculicola* ES4326 was tested for *cpr5*, *npr1*, and *cpr5 npr1* plants to determine the functional status of SAR in these mutant lines. As shown in Figure 6A, the *cpr5* plants exhibited constitutive resistance to *P. s. maculicola* ES4326 3 days after infection, with bacteria titer statistically equivalent to INA-treated wild-type plants and 10-fold less than untreated wild-type plants, whereas both *npr1* and *cpr5 npr1* plants displayed bacteria titers as high as or higher than those in the untreated wild-type plants. Similar results were obtained when *cpr5 nahG* plants were tested (data not shown). Thus, both *npr1* and *nahG* suppress the resistance to *P. s. maculicola* ES4326 bestowed by *cpr5*, further supporting the conclusion that *cpr5* acts before SA and NPR1 in SAR signaling.

Resistance to the virulent oomycete pathogen *P. parasitica* Noco2 was also tested for *cpr5* and the *cpr5 npr1* mutants. This pathogen was not used for the *cpr5 nahG* line because the *nahG* transgenic line is in the L*er* ecotype, which carries resistance genes to *P. parasitica* Noco2. As shown in Figure 6B, *cpr5* plants are constitutively resistant to *P. parasitica* Noco2, as are wild-type plants treated with INA, with no production of conidiosporangia 1 week after exposure to conidiospores. In contrast, wild-type, *npr1*, and *npr1* plants treated with INA had considerable levels of infection. However, the *cpr5 npr1* plants also showed considerable resistance to *P. parasitica* Noco2, with only a few conidiosporangia found on some plants and none on most plants. Statistical analysis using Mann–Whitney U tests (Sokal and Rohlf, 1981) showed *cpr5* plants, wild-type

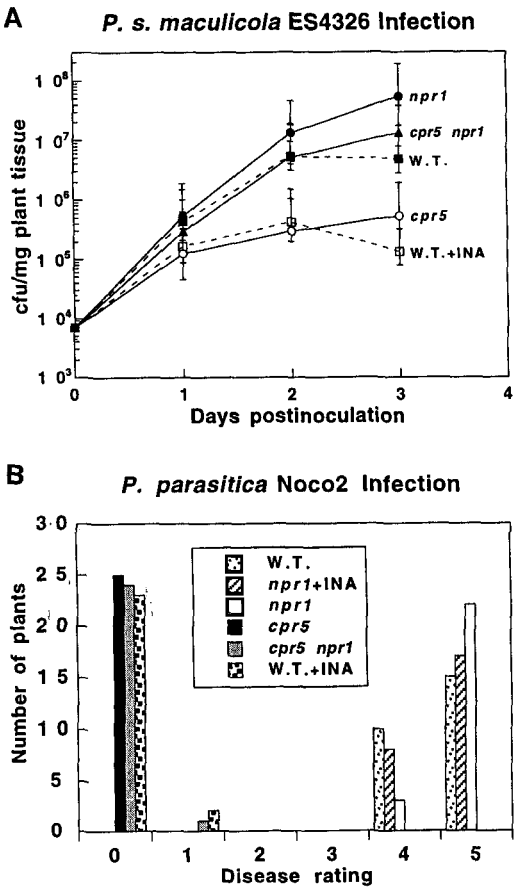

Figure 6. Effect of *cpr5* on Pathogen Growth in Both *cpr5* and *cpr5 npr1* Plants.

(A) Growth of *P. s. maculicola* ES4326. Plants were infected by dipping them into a *P. s. maculicola* ES4326 bacterial suspension of 10 mM $MgCl_2$ and 0.01% surfactant at an OD_{600} reading of 0.2. Samples were collected at time points 0, 1, 2, and 3 days after infection. On day 0, four samples were collected from each genotype; at each time point thereafter, eight samples were collected from each genotype. Error bars represent 95% confidence limits of log-transformed data (Sokal and Rohlf, 1981). cfu, colony-forming unit; W.T., wild-type *BGL2–GUS* transgenic line; W.T. + INA, wild-type *BGL2-GUS* treated with INA (0.65 mM) 72 hr before infection.

(B) Disease rating of *P. parasitica* Noco2 infection. Infection was accomplished by spraying a conidiospore suspension (3×10^4 spores per mL) onto 2-week-old plants, and disease symptoms were scored 7 days after infection with respect to the number of conidiophores observed on each plant (25 plants examined for each genotype), with disease ratings as follows: 0, no conidiophores on the plant; 1, no more than five conidiophores per infected leaf; 2, six to 20 conidiophores on a few infected leaves; 3, six to 20 conidiophores on most infected leaves; 4, more than five conidiophores on all infected leaves; 5, ≥20 conidiophores on all infected leaves. The data were analyzed using the Mann–Whitney U test (Sokal and Rohlf, 1981). W.T., wild-type *BGL2–GUS* transgenic plants; W.T. + INA, wild-type *BGL2–GUS* plants treated with 0.65 mM INA 3 days before infection; *npr1* + INA, *npr1* plants treated with 0.65 mM INA 3 days before infection.

plants treated with INA, and *cpr5 npr1* plants to have no significant difference in their low disease ratings, whereas wild-type plants, *npr1* plants, and *npr1* plants treated with INA all had significantly higher disease ratings and were not substantially different from each other. This indicates that there are components of resistance in the *cpr5 npr1* double mutant that are not affected by the presence of *npr1*. This resistance is clearly not due to leakiness in the *npr1* mutation, because *npr1* plants treated with INA do not exhibit resistance to *P. parasitica* Noco2.

Expression of *PDF1.2* in Plants with the *cpr5* Mutation

Because an NPR1-independent resistance pathway is induced in *cpr5* plants, we examined the expression of the defensin *PDF1.2* in *cpr5*, *cpr5 npr1*, and *cpr5 nahG* plants. *PDF1.2* was shown to be a molecular marker for an SA- and NPR1-independent resistance pathway by Penninckx et al. (1996). An RNA gel blot probed with *PDF1.2* showed that this gene is constitutively expressed in *cpr5* plants as well as in *cpr5 npr1* and *cpr5 nahG* plants (Figure 2), although its level of expression is reduced in the *cpr5 nahG* line. This gene is not expressed in the wild-type or INA-induced wild-type lines, but it is expressed to a high level in plants that have been treated with rose bengal, which causes the formation of reactive oxygen species and has been shown to induce *PDF1.2* expression (Penninckx et al., 1996). Because *PDF1.2* expression is extremely high under rose bengal treatment, we underloaded the RNA sample for this treatment, which is reflected by the relatively lower intensity of the band for the loading control (β-ATPase).

The reason for reduced expression of the *PDF1.2* gene in the *cpr5 nahG* line is not known. This result is unexpected, because Penninckx et al. (1996) found that *nahG* does not affect the induction of *PDF1.2* expression. One possibility is that the stressed nature of the *cpr5 nahG* plants, indicated by their small size, low seed yield, and black lesions as described earlier, may result in some downregulation of *PDF1.2* expression or degradation of *PDF1.2* mRNA.

Although the *PDF1.2* gene product may or may not play a role in resistance to *P. parasitica* Noco2, its expression in *cpr5* plants demonstrates that *cpr5* induces gene expression in a manner that is independent of NPR1. Thus, the NPR1-dependent resistance pathway is clearly not the only manifestation of systemic resistance expressed in *cpr5* plants.

One intriguing result is the expression of *PDF1.2* in *npr1* plants (Figure 2). This was demonstrated repeatedly for *npr1* plants grown on plates but was not found by others (Penninckx et al., 1996) or for *npr1* plants grown on soil (S.A. Bowling and H. Cao, unpublished results). This is in contrast to expression in *cpr5* plants, which occurs whether the plants are grown on plates or soil (S.A. Bowling, unpublished results). The growing conditions on sealed Petri plates may lead to an accumulation of a product(s), such as ethylene, that in turn may induce *PDF1.2* expression in *npr1* plants. If this is

the case, then the *npr1* plants must either produce more of or be more sensitive to the activating product(s), because *nahG* and wild-type plants do not express *PDF1.2* when grown on plates. Further investigation of *PDF1.2* expression in the double mutant under different growth conditions may provide insight into the interaction between these pathways. Regardless of the reason for expression of *PDF1.2* in *npr1* plants, this result does not conflict with the interpretation that *cpr5* induces *PDF1.2* expression in a manner that is independent of NPR1.

DISCUSSION

Among the mutants identified in the screen for constitutive expression of the *BGL2–GUS* reporter gene, *cpr5* is unique because it also has the additional phenotypes of the formation of spontaneous chlorotic lesions and reduced trichome development. This indicates that the *cpr5* gene affects multiple signaling pathways and implies that it may activate SAR through mimicking the recognition of an avirulent pathogen. The *cpr5* mutant was shown to fit the criteria for a mutant leading to constitutive activation of SAR, with constitutive expression of *PR-1* (Figure 2) and constitutive resistance to two normally virulent pathogens, *P. s. maculicola* ES4326 and *P. parasitica* Noco2 (Figures 6A and 6B).

The spontaneous chlorotic lesions produced in *cpr5* plants were examined in more detail because lesions are intimately associated with specific plant resistance responses. The lesion sites were found to contain dead plant cells, as indicated by trypan blue staining (Figure 3B); to have reactive oxygen species present in high amounts, as indicated by intense nitro blue tetrazolium staining (Figure 3C); and to exhibit autofluorescence (Figure 3D). These characteristics are similar to those exhibited by plants undergoing an HR to an avirulent pathogen and to the spontaneous lesions found on the *lsd* and *acd2* mutants (Dietrich et al., 1994; Greenberg et al., 1994); however, the *CPR5* gene defines a new locus on chromosome 5 between *LFY3* and *g2368.*

Because *cpr5* constitutively activates the SAR signaling pathway, epistasis studies were done with the *cpr5* and *npr1* mutant lines. Our results show that in the *cpr5 npr1* line, resistance to *P. s. maculicola* ES4326 is lost, whereas resistance to *P. parasitica* Noco2 is constitutively present. It is unlikely that this is due to *npr1* being "leaky" enough to allow NPR1-dependent *P. parasitica* Noco2 resistance signaling to occur, because the expression of *PR-1* is eliminated in the *cpr5 npr1* double mutant and neither SA nor INA treatment of *npr1* renders resistance to *P. parasitica* Noco2 (Figure 6B; Cao et al., 1997). In addition, *cpr5* constitutively activates *PDF1.2*, and the antifungal activity of defensins indicates a likely role of the PDF1.2 protein in resistance (Penninckx et al., 1996). Thus, we conclude that an NPR1-independent resistance pathway is constitutively induced by *cpr5*, leading to resistance to *P. parasitica* Noco2 in the *npr1* background.

We found an elevated SA level in *cpr5* plants similar to that detected in wild-type plants after treatment with a necrogenic pathogen or in other constitutive resistance mutants of Arabidopsis (Uknes et al., 1993; Bowling et al., 1994; Weymann et al., 1995). To further explore the role of SA in *cpr5* plants, a line homozygous for both *cpr5* and *nahG* was created. Similar experiments with the *lsd* mutants have revealed different relationships, with the lesion formation and SAR induction phenotypes being suppressed by *nahG* for *lsd6* and *lsd7* but not for *lsd2* and *lsd4* (Weymann et al., 1995; Hunt et al., 1997). We found that lesion formation in *cpr5* plants is not suppressed by *nahG* but that *PR-1* expression and resistance to the bacterial pathogen *P. s. maculicola* ES4326 is. Therefore, lesion formation is not the direct cause of resistance to *P. s. maculicola* ES4326 in *cpr5* plants; instead, there is an SA-dependent component to the resistance response induced by *cpr5.*

Because the mutation leading to SAR is present in all cells of the plant, whether *cpr5* affects SAR signaling before or after production of the signal that generates systemic resistance after recognition of an avirulent pathogen cannot be directly addressed. However, because *cpr5* plants form spontaneous chlorotic lesions similar to those that are found at sites of avirulent pathogen recognition, it is likely that the *cpr5* mutation affects events in the early part of the SAR signaling pathway, before production of a systemic signal.

The resistance to *P. parasitica* Noco2 found in *cpr5 npr1* plants (Figure 6B) demonstrates the existence of an alternative NPR1-independent resistance response that is connected to the NPR1-dependent pathway in early signaling steps. Thus, we propose a model of SAR signaling, presented in Figure 7, that is similar to the one proposed by Penninckx et al. (1996). The biological definition of SAR is expanded in our model to include both NPR1-dependent and NPR1-independent resistance responses. Whereas the NPR1-dependent and NPR1-independent pathways of Arabidopsis overlap in generating resistance to *P. parasitica* Noco2, only the NPR1-dependent pathway leads to resistance to *P. s. maculicola* ES4326.

Examples of overlapping resistance pathways can be found in other model systems. In Drosophila, the pathogen-responsive Toll receptor induces the production of an antifungal peptide and several antibacterial peptides through a specific Rel-related transactivator (possibly Dif; Ip et al., 1993). Another pathogen-responsive pathway acts through Imd to produce the same antibacterial peptides as the Toll receptor as well as two additional antibacterial peptides, but it does not produce the Toll-responsive antifungal peptide (Ip et al., 1993; Lemaitre et al., 1996). Although the Drosophila pathways differ from Arabidopsis by having overlapping antibacterial rather than antifungal components, the overall model of separate but overlapping resistance responses is the same. "Cross-talk" between these redundant and overlapping resistance pathways would be expected.

One obvious area for exploration is the signals involved in the NPR1-independent pathway that lead to resistance to *P.*

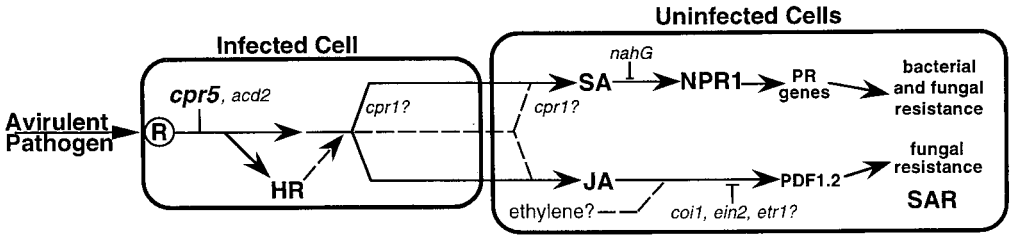

Figure 7. Proposed Placement of *cpr5* and the NPR1-Dependent and NPR1-Independent Branch Point in the SAR Signal Transduction Pathway.

The proposed signal transduction pathway is denoted by solid and dashed lines and arrows. The *cpr5* mutation is placed upstream of the branch point of the NPR1-dependent and NPR1-independent pathways and upstream of the HR. It is not known whether the branch point between the pathways of SAR occurs in the primary infected tissue or in distal, uninfected tissues. See also Penninckx et al. (1996).

parasitica Noco2. Because mutants defective in ethylene (*ein2* and *etr1*) or JA (*coi1*) perception are defective in defensin induction (Penninckx et al., 1996), the effect of these mutations on *cpr5* should help to determine the roles of ethylene and JA in this response. In addition, triple mutants with *cpr5*, *npr1*, and the ethylene or JA mutants should provide some interesting clues as to the nature of the interaction between the NPR1-dependent and NPR1-independent resistance pathways. Exploration of the genes still induced in *cpr5 npr1* that are blocked in the triple mutants will help to define the NPR1-independent pathway and to determine whether there is more signaling in *cpr5* that is independent of ethylene and JA as well as *NPR1*.

An interesting aspect of the *cpr5* mutation is its effect on trichome development. This is an area we have not explored in much detail. This suggests another similarity to the Drosophila system, in which Toll and Pelle are involved in regulation of transcription of developmental genes as well as defense genes (Morisato and Anderson, 1995). Although the comparison between the Arabidopsis and Drosophila pathways is tenuous, it nonetheless suggests at least by analogy that *cpr5* may provide clues about how defense signaling is integrated with the overall signaling network in plants. It is noteworthy that such a connection may be conserved from an ancient evolutionary origin common to both plants and animals.

The identification of *cpr5* has provided a genetic integration of two different resistance pathways, which shows that these pathways are connected in some early signal transduction steps and that they have separate but overlapping roles in resistance. The similarities to the Drosophila immune response reveal that pathogen-responsive signaling may be part of a surprisingly ancient pathway conserved in most eukaryotes. Further study of *cpr5* will provide a deeper understanding of the components of these pathways and their relationships to the defense responses and development.

METHODS

Plant Growth Conditions and Isolation of *cpr5*

Arabidopsis thaliana plants were grown on soil (Metro-Mix 200; Grace-Sierra, Malpitas, CA) or on plates containing Murashige and Skoog (MS) media, as described previously (Murashige and Skoog, 1962; Bowling et al., 1994). The *cpr5* mutant was isolated in a screen for constitutive expressers of the *BGL2–GUS* reporter gene in transgenic Columbia (Col) plants mutagenized with ethyl methanesulfonate, as described in Bowling et al. (1994). Assays for *BGL2–GUS* reporter gene activity were performed as described previously (Jefferson et al., 1987; Cao et al., 1994).

RNA Analysis

Tissue samples were collected from 3-week-old seedlings grown on MS plates or from MS plates containing 0.1 mM 2,6-dichloroisonicotinic acid (INA) or from 4-week-old plants grown on soil. Samples were frozen in liquid nitrogen after collection, and RNA was isolated by a phenol–chloroform extraction, as previously described (Cao et al., 1994). The RNA concentration was determined by UV absorbance, and 5-μg samples were separated by electrophoresis through formaldehyde–agarose gels and transferred to a hybridization membrane (GeneScreen; Du Pont–New England Nuclear), as described by Ausubel et al. (1994). ^{32}P-labeled DNA probes for *PR-1* and β-ATPase mRNAs were prepared as described by Cao et al. (1994); the probe for *PDF1.2* was generated using a strand-biased polymerase chain reaction (PCR) in a protocol modified from Schowalter and Sommer (1989). The template for *PDF1.2* was generated by PCR from the plasmid containing the cDNA clone for the *PDF1.2* gene, using primers described by Penninckx et al. (1996). PCR for amplification of the template included one cycle of 94°C for 2 min; 30 cycles of 15 sec at 94°C, 15 sec at 55°C, and 45 sec at 72°C; and finally, one cycle at 72°C for 20 min. PCR cycles were the same for strand-biased probe production, with 10 pmol of the antisense strand primer and 200 fmol of the sense strand primer. The dATP, dTTP, and dGTP amounts were 1.3 nmol each, and both unlabeled and ^{32}P-labeled dCTP were added in nearly equal amounts of 33 pmol.

Hybridization and washing conditions were as previously described (Church and Gilbert, 1984; Cao et al., 1994). Bands were quantified using Image 1.56 software (National Institutes of Health, Bethesda, MD).

Histochemistry and Microscopy

Leaf samples were taken from plants ranging in age from 1 to 4 weeks that were grown on MS plates or soil for trypan blue staining for dead cells, nitro blue tetrazolium staining for the presence of superoxides, or UV epifluorescence microscopy for detection of autofluorescence. Trypan blue staining was performed similar to previous descriptions (Keogh et al., 1980; Dietrich et al., 1994). Samples were submerged in a 70°C lactic acid–phenol–trypan blue solution (LPTB; 2.5 mg/mL trypan blue, 25% [w/v] lactic acid, 23% water-saturated phenol, 25% glycerol, and H_2O), slow-release vacuum infiltrated for 5 min, and then reinfiltrated. Samples were then heated over boiling water for 2 min and cooled for 1 hr before replacement of the LPTB solution with a chloral hydrate solution (25 g in 10 mL of H_2O) for destaining. After multiple exchanges of chloral hydrate solution, samples were equilibrated for several hours in 70% glycerol and mounted. Nitro blue tetrazolium staining was performed as described by Jabs et al. (1996). Samples for autofluorescence examination were placed in autofluorescence fixing solution (10% formaldehyde, 5% acetic acid, 45% ethanol, and H_2O) for 15 min. The autofluorescence fixing solution was removed, and the samples were soaked in 50% ethanol for 20 min and then in 95% ethanol overnight. The 95% ethanol was removed, and the samples were equilibrated in 70% glycerol before mounting. Autofluorescence was examined as described by Dietrich et al. (1994).

Quantitative GUS Assay

Plant tissues were assayed from 10-day-old plants grown on MS medium or MS medium with 0.1 mM INA, as described by Bowling et al. (1994).

Genetic Analysis

Crosses were performed as described previously (Bowling et al., 1994). Backcrosses with the parental *BGL2–GUS* transgenic line were performed using *BGL2–GUS* plants as the pollen donor. The *cpr5 npr1* double mutant was generated using pollen from the homozygous *npr1* plants to fertilize homozygous *cpr5* plants. Double mutants were detected in the F_2 generation based on the appearance of a new phenotype combination, including lesion formation, reduced trichomes, reduced induction of reporter gene expression, and bleached areas on leaves and stems of approximately one-sixteenth of the plants. Double mutant confirmation was accomplished by crossing potential double mutants into parental mutant lines. The *cpr5 nahG* line was generated using pollen from homozygous *cpr5* plants to fertilize the transgenic line expressing salicylate hydroxylase in the Landsberg *erecta* (L*er*) ecotype (*nahG/nahG er/er*). Successful crosses were scored based on a loss of the *er* phenotype in the F_1 plants. The double homozygous (*cpr5/cpr5 nahG/nahG*) line was isolated by allowing the approximately three-sixteenths of the F_2 plants that had both the *cpr5* phenotype (spontaneous lesions and reduced trichome development) and the *nahG* phenotype (brown roots when grown on MS plates containing 0.5 mM salicylic acid [SA]) to self-pollinate. The F_3 plants were then scored for uniform brown roots on MS plates with 0.5 mM SA (indicating that the plants were homozygous for *nahG*; Bowling et al., 1994) and tested for uniform reporter gene expression by the addition of INA (which induces reporter gene expression in the presence of *nahG*). The mapping population was generated by using the pollen from a homozygous *cpr5* plant to fertilize plants of the L*er* ecotype. Successful crosses were scored based on loss of the *er* phenotype in the F_1 plants. Homozygous *cpr5* mutants were isolated in the F_2 generation based on *cpr5* phenotypes.

PCR-Based Mapping

Mapping was performed by the codominant cleaved amplified polymorphic sequences protocol described by Konieczny and Ausubel (1993), with new markers as reported on the Arabidopsis database web site (http://genome-www.stanford.edu). The marker for *g2368* was improved by performing a double digest with both HindIII and PvuII, producing detectable products of ~780 and 620 bp for ecotype Col and 730 and 620 bp for L*er*. This was necessary because heterozygous individuals were hard to distinguish from homozygous Col with just HindIII digestion, which produces no digest of the 1400-bp product for Col and removal of only 50 bp from the L*er* product.

Pathogen Infections

Infections with *Pseudomonas syringae* pv *maculicola* ES4326 and *Peronospora parasitica* Noco2 were performed as described previously (Bowling et al., 1994). Plants used for the *P. s. maculicola* ES4326 infection were grown on soil for 4 weeks and were assayed as described by Bowling et al. (1994). Statistical analyses were performed by Student's *t* tests of the differences between two means of log-transformed data (Sokal and Rohlf, 1981). Plants infected with *P. parasitica* Noco2 had grown for 14 days on soil with a 12-hr photoperiod. Seven days after inoculation, the plants were scored for the presence of conidiophores by using a dissecting microscope. The plants were given a disease rating of one to five, as previously described by Cao et al. (1997), and were analyzed using Mann–Whitney U tests (Sokal and Rohlf, 1981).

Measurement of SA

SA and SA glucoside measurements were performed on leaf tissue from 4-week-old plants, as described previously (Bowling et al., 1994).

ACKNOWLEDGMENTS

We thank Hui Cao, Mark Kinkema, and Xin Li for useful discussions and comments on this manuscript; Susan Gordon for her work on the initial screen that identified the *cpr5* mutant; Tranese Green for performing the complementation experiments with *drt1*; and Barbara Kunkel for providing the *drt1* mutant. We also thank Novartis (Basel, Switzerland) for the gift of INA. S.A.B. is supported by a Howard Hughes Medical Institute Predoctoral Fellowship. This work was supported by National Science Foundation Grant No. MCB 9310371 to D.F.K. and by U.S. Department of Agriculture Grant No. 93-37301-8925 to X.D.

Received April 29, 1997; accepted July 17, 1997.

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