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Uncoupling PR Gene Expression from NPR1 and Bacterial Resistance: Characterization of the Dominant Arabidopsis *cpr6-1* Mutant

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In Arabidopsis, NPR1 mediates the salicylic acid (SA)–induced expression of pathogenesis-related (PR) genes and systemic acquired resistance (SAR). Here, we report the identification of another component, CPR6, that may function with NPR1 in regulating PR gene expression. The dominant *CPR6-1* mutant expresses the SA/NPR1–regulated PR genes (*PR-1*, *BGL2*, and *PR-5*) and displays enhanced resistance to *Pseudomonas syringae* pv *maculicola* ES4326 and *Peronospora parasitica* Noco2 in the absence of SAR induction. *cpr6-1*–induced PR gene expression is not suppressed in the *cpr6-1 npr1-1* double mutant but is suppressed when SA is removed by salicylate hydroxylase. Thus, constitutive PR gene expression in *cpr6-1* requires SA but not NPR1. In addition, resistance to *P. s. maculicola* ES4326 is suppressed in the *cpr6-1 npr1-1* double mutant, despite expression of *PR-1*, *BGL2*, and *PR-5*. Resistance to *P. s. maculicola* ES4326 must therefore be accomplished through unidentified antibacterial gene products that are regulated through *NPR1*. These results show that *CPR6* is an important regulator of multiple signal transduction pathways involved in plant defense.

INTRODUCTION

Pathogens annually cause billions of dollars in damage to crops worldwide (Baker et al., 1997). Consequently, an increasing amount of research has been dedicated to developing novel methods for controlling plant diseases. Such studies have centered on the plant's innate ability to resist pathogen invasion in an effort to buttress the plant's own defenses to counter pathogen attacks (Staskawicz et al., 1995; Baker et al., 1997). One such defense mechanism under study is known as systemic acquired resistance (SAR; reviewed in Ryals et al., 1996). SAR is defined as a generalized defense response, which is often induced by avirulent pathogens and provides enhanced resistance to a broad spectrum of virulent pathogens (Chester, 1933; Ross, 1961; Kuc, 1982; Ryals et al., 1994). Avirulent pathogens carry an avirulence (*avr*) gene whose product can be recognized by the product of a corresponding resistance (*R*) gene carried by plants. Such recognition triggers both a programmed cell death response, known as the hypersensitive response (HR), around the point of pathogen infection and release of a systemic SAR-inducing signal (Hammond-Kosack and Jones, 1996). After a rapid, localized HR, the elevated state of resistance associated with SAR is effective throughout the plant for a period of time ranging from several days to a few weeks (Van Loon and Van Kammen, 1970; Malamy et al., 1990; Ward et al., 1991; Yalpani et al., 1991; Uknes et al., 1992).

Salicylic acid (SA) is an integral signaling component of SAR (Gaffney et al., 1993). During an SAR response, endogenous levels of SA increase dramatically throughout the plant (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). Exogenous application of SA (White, 1979) or the SA analogs 2,6-dichloroisonicotinic acid (INA; Métraux et al., 1991) and benzo(1,2,3)thiadiazole-7-carbothioc acid *S*-methyl ester (Görlach et al., 1996) can induce SAR. Removal of SA results in plants that are unable to establish an SAR response and are supersusceptible to pathogen infection (Gaffney et al., 1993; Delaney et al., 1994). Coinciding with the onset of SAR is the transcriptional activation of the pathogenesis-related (PR) genes. These genes encode proteins that exhibit antimicrobial activities (Ward et al., 1991).

In Arabidopsis, expression of *PR-1*, β-1,3-glucanase (*BGL2*), and *PR-5* has been shown to be tightly correlated with resistance to virulent bacterial, fungal, and oomycete

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pathogens; therefore, these genes are used as molecular markers for SAR (Uknes et al., 1992). To identify genes that regulate SAR, we conducted a genetic screen in Arabidopsis for mutants that have an altered SAR response. We isolated the *npr1* mutant (for nonexpresser of PR genes; Cao et al., 1994; also called *nim1* and *sai1*; Delaney et al., 1995; Shah et al., 1997) that blocks the SA-induced expression of *PR-1*, *BGL2*, and *PR-5* and SA-induced resistance to virulent pathogens. *NPR1* was recently cloned using a map-based approach (Cao et al., 1997; Ryals et al., 1997). In addition to *npr1*, which is nonresponsive to SAR induction, we isolated several *cpr* mutants (for constitutive expresser of PR genes) that have elevated levels of PR gene expression and enhanced pathogen resistance in the absence of SAR induction (Bowling et al., 1994, 1997).

By using genetic epistasis analysis, we recently demonstrated that *cpr5*, which is a *cpr* mutant unique for its formation of spontaneous lesions and defective trichome development, confers not only NPR1-dependent resistance to the virulent bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326 but also NPR1-independent resistance to the virulent oomycete pathogen *Peronospora parasitica* Noco2 (Bowling et al., 1997). In the *cpr5 npr1-1* double mutant, expression of *PR-1*, *BGL2*, and *PR-5* is suppressed, whereas resistance to *P. parasitica* Noco2 is not, indicating that CPR5 regulates the expression of additional genes that contribute to oomycete resistance in an NPR1-independent manner.

Genetic and biochemical evidence has been accumulating recently in support of the existence of SA/NPR1–independent resistance pathways. Pieterse et al. (1996) demonstrated that resistance to *Fusarium oxysporum* induced by the root-colonizing biocontrol bacterium *Pseudomonas fluorescens* WCS417r is independent of SA and PR gene expression. More evidence has come from studies of induction pathways leading to the accumulation of defensin and thionin class peptides (Florack and Stiekema, 1994; Broekaert et al., 1995; Terras et al., 1995), especially defensin PDF1.2 (Penninckx et al., 1996) and thionin Thi2.1 (Epple et al., 1995), which have been shown to be pathogen inducible. In Arabidopsis, *PDF1.2* is induced by the avirulent pathogen *Alternaria brassicicola* (Penninckx et al., 1996), and *Thi2.1* is induced by the pathogen *F. o.* f sp *matthiolae* (Epple et al., 1995, 1997). Although both *PDF1.2* and *Thi2.1* seem to be regulated through the plant hormone jasmonic acid (JA), their expression was shown to be nonresponsive to SA or INA induction (Epple et al., 1995; Penninckx et al., 1996).

These experiments show that the SA- and JA-induced resistance responses in Arabidopsis are regulated by distinct pathways. However, other experiments show that these separate defense-related responses may have synergistic or antagonistic effects toward each other. In rice, INA was shown to elevate JA and the expression of JA-regulated genes (Schweizer et al., 1997); in barley, INA was found to induce the expression of thionin (Wasternack et al., 1994). On the other hand, antagonistic mechanisms between signaling pathways may play a role in prioritizing a plant's response to different challenges. For example, in tomato, the accumulation of SA resulting from pathogen infection suppresses the JA-regulated expression of the herbivore-deterring proteinase inhibitor pathway (Doherty et al., 1988; Peña-Cortés et al., 1993; Doares et al., 1995). In addition, it has been shown in tobacco that the expression of defense-related genes induced by *Erwinia carotovora* is suppressed by the addition of SA (Vidal et al., 1997). Epistasis studies will be an important tool for distinguishing between different resistance responses and determining their points of interaction. By examining the phenotypes of double mutants that affect different pathways, the complex interplay between these pathways can be elucidated.

Here, we report the characterization of a dominant mutation, *cpr6-1*, of Arabidopsis. *cpr6-1* plants have elevated levels of SA, constitutive expression of the NPR1-dependent PR genes (*PR-1*, *BGL2*, and *PR-5*) and the NPR1-independent defense genes *PDF1.2* and *Thi2.1*, and enhanced resistance to *P. s. maculicola* ES4326 and *P. parasitica* Noco2. Epistasis analysis shows that PR gene expression in the *cpr6-1* mutant requires SA but is independent of NPR1. We believe that the CPR6 protein regulates the expression of multiple defense genes and functions with NPR1 in regulating PR gene expression.

RESULTS

Characterization of the *cpr6-1* Mutant

The Arabidopsis *cpr6-1* mutant was identified by using a screen for plants displaying constitutive expression of a reporter gene containing the *BGL2* promoter fused to the β-glucuronidase (*GUS*) coding region. This screen was designed to isolate mutants in the regulatory components of SAR. The details of this screen have been described previously (Bowling et al., 1994). All of the following experiments were conducted with a *cpr6-1* mutant line that was backcrossed twice. Crossing *cpr6-1* with a wild-type plant containing the *BGL2–GUS* reporter gene revealed reporter gene expression in the F_1 generation, implying that *cpr6-1* is a dominant mutation. In the F_2 population, reporter gene expression was present in 69 of 98 seedlings (three of four), verifying that the *cpr6-1* mutation is inherited as a single dominant locus ($\chi^2 = 1.1$; $0.05 < P < 0.1$). Because *cpr6-1* is in the Columbia (Col) ecotype, crosses into the Landsberg *erecta* (L*er*) ecotype were performed to determine the map position for the *cpr6-1* locus. By using codominant cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel, 1993) and simple sequence-length polymorphisms (SSLPs; Bell and Ecker, 1994), *cpr6-1* was mapped to chromosome 1 in the ~3-centimorgan (cM) interval defined by nga280 (1.9 cM) and g4026 (1.5 cM). *RPP7*, an *R* gene in the

L*er* ecotype that provides enhanced resistance to *P. parasitica*, also maps to this region (Crute et al., 1993).

Expression of the *BGL2–GUS* reporter gene in the *cpr6-1* mutant is influenced by the developmental stage of the plant. Figure 1A shows the expression pattern of the reporter gene in the *cpr6-1* mutant grown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Reporter gene expression was first detected 1 week after germination, primarily at the root–hypocotyl juncture. In the second week, expression was found throughout the hypocotyl, most prominently at the node between the hypocotyl and cotyledons. By the third and fourth weeks, expression spread from the cotyledons to the stem, petioles, and older leaves.

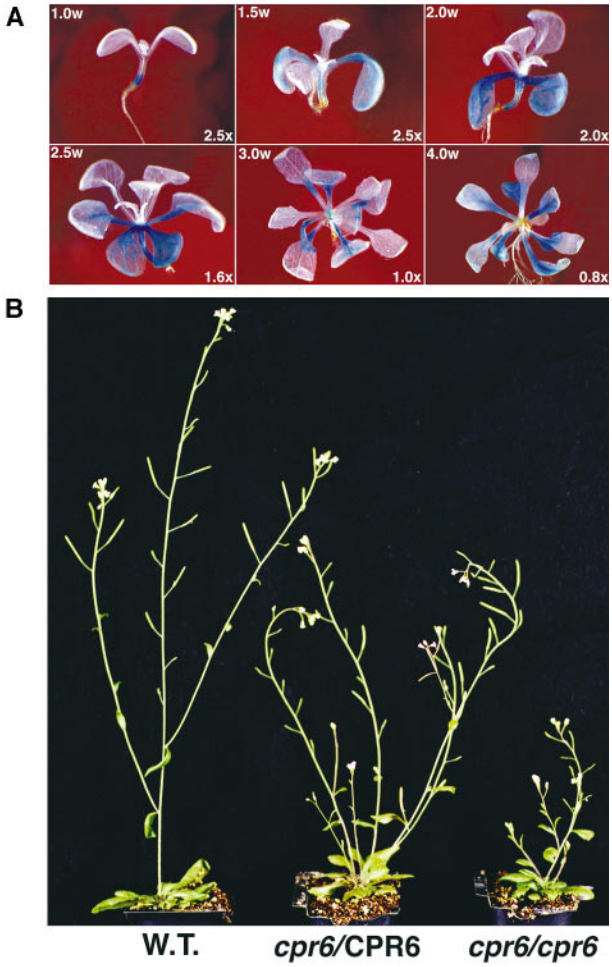


Figure 1. Developmental Expression of *BGL2–GUS* Reporter Gene and Morphological Phenotypes of *cpr6-1.*

(A) Expression of the *BGL2–GUS* reporter gene in *cpr6-1* plants is linked to the developmental stage of the plant. Plants from 1 to 4 weeks of age were infiltrated with X-gluc to stain for expression of the *BGL2–GUS* reporter gene, according to Jefferson et al. (1987). The age of the plant in weeks (w) is denoted at upper left, and the magnification of the image is denoted at lower right.

(B) A comparison between *cpr6-1* (*cpr6*) and *BGL2–GUS* plants (W.T.), showing the semidominant morphological phenotype associated with the *cpr6-1* mutation. The plants were photographed at 5 weeks of age.

Another developmental characteristic of the *cpr6-1* mutant, as shown in Figure 1B, is a loss of apical dominance and a reduction in overall plant size. *cpr6-1* plants typically have five to 10 bolts that emerge from the rosette soon after the primary bolt. All of the bolts tend to achieve the same height, which is approximately one-third the height of a wild-type plant, giving the *cpr6-1* mutant a bushy appearance. The cotyledons of *cpr6-1* senesce 5 to 7 days before the wild type does, and *cpr6-1* plants flower a week later than do wild-type plants. The morphological phenotypes of *cpr6-1* appear to be semidominant (Figure 1B). The *cpr6-1* heterozygote is larger than the *cpr6-1* homozygote but is still only one-half to three-quarters the height of wild-type plants. These morphological characteristics continue to cosegregate with the *cpr* phenotype after the sixth backcross.

The Arabidopsis *PR-1*, *BGL2*, and *PR-5* genes are used as molecular markers for SA/NPR1–dependent SAR (Uknes et al., 1992), whereas *PDF1.2* and *Thi2.1* represent SA/NPR1–independent pathogen-responsive genes (Epple et al., 1995; Penninckx et al., 1996). We performed RNA gel blot analysis to identify the defense-related genes expressed in *cpr6-1* plants. INA was used as an inducer of the PR genes, and rose bengal (RB) was used as an inducer of *PDF1.2* (Penninckx et al., 1996). We found that the *cpr6-1* mutant constitutively expresses these SA/NPR1–dependent and SA/NPR1–independent genes, as represented in Figure 2 by *PR-1* and *PDF1.2. PR-1* gene expression in *cpr6-1* can be slightly induced by the application of INA (Figure 2A) and SA (data not shown). Interestingly, the application of INA seems to suppress the constitutive expression of *PDF1.2* (Figure 2A) and *Thi2.1* (data not shown) in the *cpr6-1* mutant. The application of RB did not appear to increase *PDF1.2* or affect *PR-1* gene expression in *cpr6-1.* In the *cpr6-1* heterozygote, the expression levels of *PR-1* and *PDF1.2* (as well as *BGL2*, *PR-5*, and *Thi2.1*; data not shown) are the same as those of the *cpr6-1* homozygote, further confirming that *cpr6-1* is a dominant mutation (Figure 2A).

Constitutive expression of these defense-related genes suggests that *cpr6-1* plants may have enhanced resistance to pathogen infection. To determine the level of resistance bestowed by the *cpr6-1* mutation, we tested the growth of the virulent bacterial pathogen *P. s. maculicola* ES4326 in *cpr6-1* and wild-type plants. As shown in Figure 3A, *cpr6-1* plants exhibited enhanced resistance to *P. s. maculicola* ES4326 3 days after pathogen infection. The level of bacterial growth was 10 times less than that in wild-type plants and 12 times greater than that in INA-treated wild-type plants. Application of INA to *cpr6-1* further reduced the growth of *P. s. maculicola* ES4326 to the level found in INA-treated wild-type plants (data not shown). Resistance to the virulent

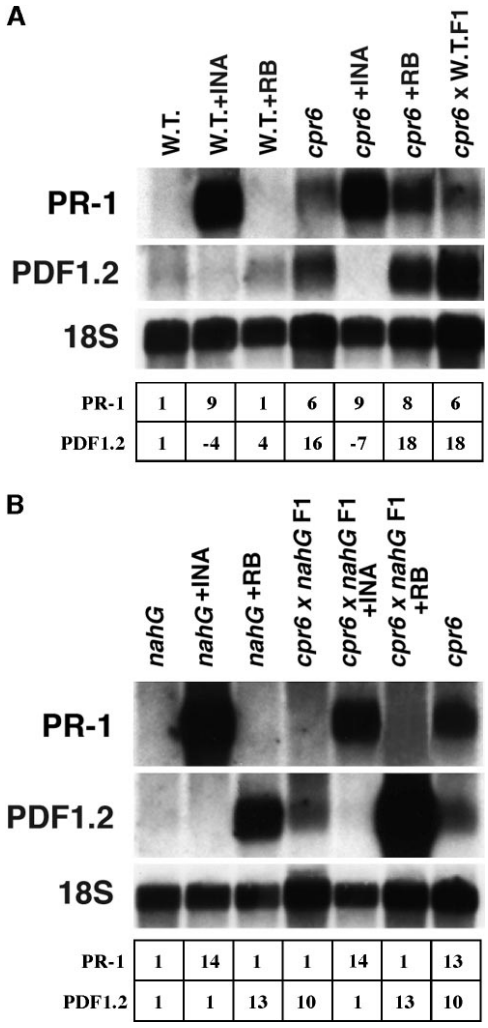


Figure 2. *PR-1* and *PDF1.2* Gene Expression in *cpr6-1*, *cpr6-1* × Wild Type F_1, and *cpr6-1* × *nahG* F_1 Plants.

(A) Expression of *PR-1* and *PDF1.2* genes in wild-type, *cpr6-1* heterozygous, and *cpr6-1* homozygous plants.

(B) Expression of *PR-1* and *PDF1.2* genes in *nahG*, *cpr6-1*, and *cpr6-1* × *nahG* F_1 plants.

The table below each RNA gel blot describes the fold induction of gene expression for each sample relative to that of the wild type. The values have been corrected based on the loading control and normalized to the wild type. *PR-1* and *PDF1.2* gene-specific probes were used for RNA gel blot analysis, and the 18S ribosomal subunit gene-specific probe was used as a loading control. RNA samples were extracted from 4-week-old plants grown on soil with or without treatment with 0.65 mM INA or 2 mM rose bengal (RB), as described by Penninckx et al. (1996), 3 days before tissue collection. W.T., *BGL2–GUS* transgenic line; *cpr6*, *cpr6-1* mutant line; *nahG*, transgenic line containing the *nahG* gene encoding salicylate hydroxylase.

oomycete pathogen *P. parasitica* Noco2 was also tested in the *cpr6-1* mutant. As shown in Figure 3B, *cpr6-1* plants have enhanced resistance to *P. parasitica* Noco2, displaying a reduced number of conidiosporangia 1 week after inoculation with oomycete spores. Application of INA to *cpr6-1* further increased the resistance to the level of INA-treated wild-type plants (data not shown). We also examined pathogen resistance in the *cpr6-1* heterozygote. The *cpr6-1* heterozygote exhibits the same degree of resistance to *P. s. maculicola* ES4326 and *P. parasitica* Noco2 as does the *cpr6-1* homozygote (Figures 3A and 3B). Therefore, we conclude that *cpr6-1* is a dominant mutation with respect to defense gene expression and pathogen resistance, despite the semidominant morphological phenotypes.

Epistasis Analysis of *cpr6-1*

To place *cpr6-1* in the SAR signaling cascade and compare it with other mutants with constitutive SAR expression, such as the *cpr5* (Bowling et al., 1997), *lsd* (for lesion-simulating disease; Dietrich et al., 1994; Weymann et al., 1995; Hunt et al., 1997), and *acd2* (for accelerated cell death; Greenberg et al., 1994) mutants, we examined *cpr6-1* for HR-like lesions by using various approaches described previously (Bowling et al., 1997). We found no macroscopic or microscopic lesions in *cpr6-1* and therefore conclude that *cpr6-1* should probably be placed downstream of the HR (data not shown).

To determine the position of *cpr6-1* relative to SA, we measured the amount of endogenous SA and salicylate glucoside (SAG) in *cpr6-1.* As shown in Figure 4, levels of free SA and SAG in the *cpr6-1* mutant were seven and nine times higher, respectively, than those found in the uninduced wild-type plants. Interestingly, the amount of free SA accumulation in *cpr6-1* plants is similar to the amount of SA accumulation found in wild-type plants that have been challenged with an avirulent pathogen, and the level of SAG is threefold higher than that in an induced wild-type plant. These levels are significantly lower than is the increase (up to 30-fold) of SA and SAG accumulation found in *cpr1*, *cpr5*, *lsd6*, and *lsd7* (Uknes et al., 1993; Bowling et al., 1994, 1997; Weymann et al., 1995).

To define further the role of SA in *cpr6-1* plants, we performed RNA gel blot analysis with crosses made between *cpr6-1* and a transgenic plant containing the *nahG* gene of *Pseudomonas putida* (Bowling et al., 1994). *nahG* encodes a salicylate hydroxylase that converts SA to catechol (You et al., 1991). Gel blot analysis was used to determine whether constitutive PR gene expression in the *cpr6-1* mutation is dependent on SA. Because both *cpr6-1* and *nahG* are dominant, analysis was conducted in the F_1 generation. The results clearly show that *PR-1* gene expression is suppressed in the F_1 progeny of the cross between *cpr6-1* and *nahG* (Figure 2B). The same is also true for *BGL2* and *PR-5* (data not shown). We also found that *PDF1.2* (Figure 2B) and

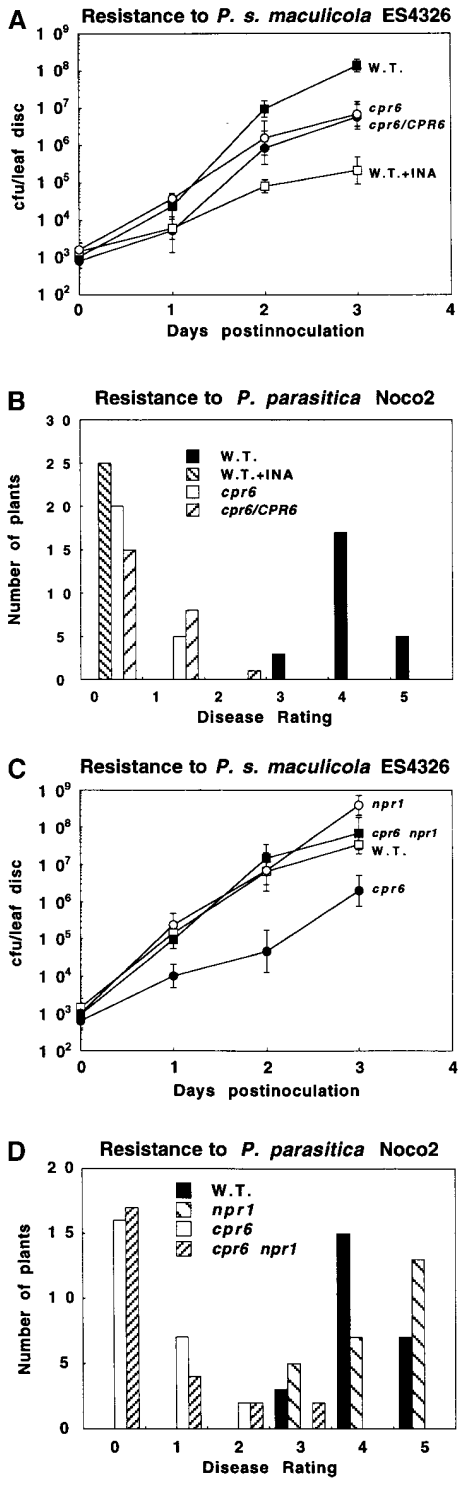


Figure 3. Effects of *cpr6-1* on Pathogen Growth in *cpr6-1* Homozygous, *cpr6-1* Heterozygous, and *cpr6-1 npr1-1* Double Mutant Plants.

(A) and **(C)** Growth of *P. s. maculicola* ES4326. Plants were infected by injecting a *P. s. maculicola* ES4326 bacterial suspension of 10 mM $MgCl_2$ at an OD_{600} reading of 0.001 into the abaxial surface of the leaf with a syringe. Samples were collected by using a hole punch to obtain a leaf disc at time points 0, 1, 2, and 3 days after infection. Eight samples were collected from each genotype at each time point, except on day 0, when only four samples were collected. Error bars represent 95% confidence limits of log-transformed data (Sokal and Rohlf, 1981). These experiments were replicated three times with similar results. **(A)** shows the level of *P. s. maculicola* ES4326 growth in wild-type, *cpr6-1* homozygous, and *cpr6-1* heterozygous plants. **(C)** shows the level of *P. s. maculicola* ES4326 growth in wild-type, *cpr6-1*, *npr1-1*, and *cpr6-1 npr1-1* plants.

(B) and **(D)** Disease rating of *P. parasitica* Noco2 infection. Plants were infected by spraying a conidiospore suspension (3×10^4 spores per mL) onto 3-week-old plants. Disease severity was scored 7 days after infection by counting the number of conidiophores on 25 plants for each genotype. Disease ratings are 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. These experiments were replicated three times with similar results. **(B)** provides disease ratings for wild-type, *cpr6-1* homozygous, and *cpr6-1* heterozygous plants. **(D)** provides disease ratings for wild-type, *cpr6-1*, *npr1-1*, and *cpr6-1 npr1-1* plants. The data were analyzed using Mann-Whitney U tests (Sokal and Rohlf, 1981). cfu, colony-forming unit; W.T., *BGL2–GUS* transgenic line; *cpr6*, *cpr6-1* mutant line; *npr1*, *npr1-1* mutant line; W.T.+INA denotes treatment of plants with 0.65 mM INA 72 hr before infection.

Thi2.1 (data not shown) expression in *cpr6-1* is not suppressed by the removal of endogenous SA. On the contrary, expression of *PDF1.2* and *Thi2.1* is suppressed by the exogenous addition of INA. From these data, it is apparent that *cpr6-1* requires SA for *PR-1*, *BGL2*, and *PR-5* gene expression but not for *PDF1.2* or *Thi2.1* gene expression. In fact, the SA analog, INA, seems to negatively affect the expression of both *PDF1.2* (Figures 2A and 2B) and *Thi2.1* (data not shown) in *cpr6-1.*

Because *cpr6-1* elevates PR gene expression, whereas *npr1-1* abolishes PR gene expression, we decided to generate the *cpr6-1 npr1-1* double mutant to determine their hierarchy in the signaling pathway. To interpret the data obtained from the epistasis analysis accurately, it is critical that the loss-of-function mutant has null phenotypes. We chose *npr1-1* because it shows no detectable PR gene expression and pathogen resistance in response to SA or INA treatment (Cao et al., 1994; Bowling et al., 1997). Crosses were performed between *cpr6-1* and *npr1-1*, and the resulting progeny were screened in the F_2 generation. Because of the ~20-cM linkage between *cpr6-1* and *npr1-1*, we expected to detect one *cpr6-1 npr1-1* recombinant in 98 F_2 progeny. *npr1-1* is recessive; therefore, we facilitated identification of the *cpr6-1 npr1-1* double mutant by exploiting *npr1*'s unique SA-intolerant phenotype. When grown on 0.5

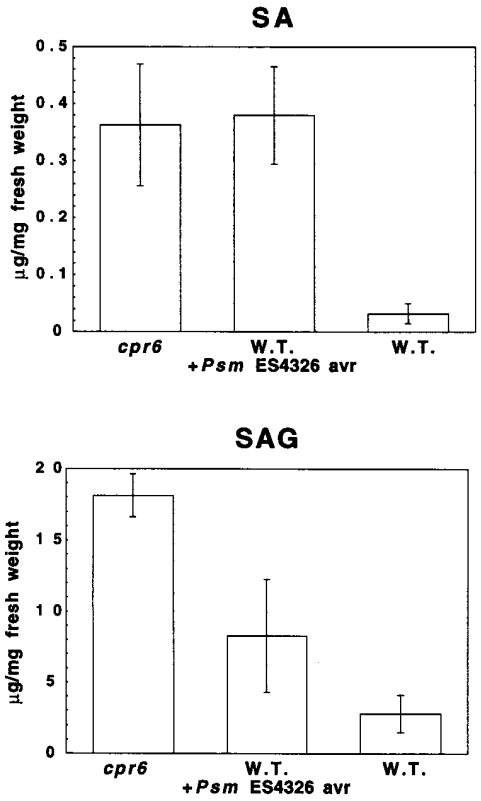


Figure 4. SA and SAG Levels in Wild-Type and *cpr6-1* Plants.

(A) Free SA.

(B) Sugar-conjugated SA (SAG).

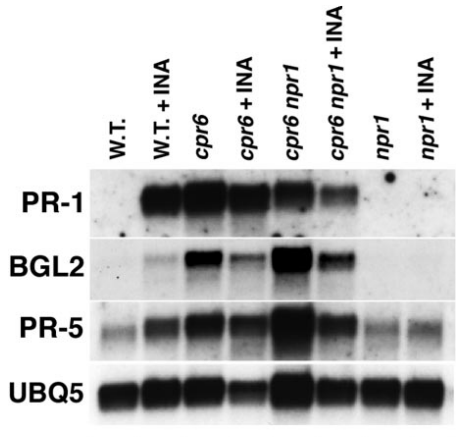
Leaves from 4-week-old plants grown on soil were collected and analyzed by HPLC for free SA and SAG content. The values presented are the average of five replicates ±SE (micrograms of SA per gram leaf fresh weight). W.T., *BGL2–GUS* transgenic line; *cpr6*, *cpr6-1* mutant line; W.T.+ *Psm* ES4326 avr, *BGL2–GUS* transgenic line infected with the *P. s. maculicola* ES4326 carrying the *avrRpt2* gene 2 days before sample collection.

mM SA, *npr1* plants bleach and do not develop beyond the cotyledon stage (Cao et al., 1994, 1997).

We began the screen for the *cpr6-1 npr1-1* double mutant by growing the F_2 progeny on SA plates. In the preliminary analysis of the F_2 progeny, one of four F_2 plants bleached, indicating that *cpr6-1* does not affect the expression of this *npr1* phenotype. All bleaching F_2 plants were rescued by transferring them to SA-free plates and then to soil, where F_3 seeds were collected from individual plants. Seeds from 178 of these F_3 lines, which were presumed homozygous for *npr1-1*, were grown on soil and scored for the *cpr6-1* phenotype. We found that two of the 178 lines were segregating for the *cpr6-1* phenotype, implying that the parental lines were heterozygous for the *cpr6-1* mutation. From these two F_3 populations, F_4 seeds were collected only from those plants that were phenotypically homozygous for *cpr6-1.* Because the *npr1-1* mutation abolishes an NlaIII restriction site (Cao et al., 1997), restriction digestion analysis of the *npr1-1* gene region amplified by polymerase chain reaction (PCR) was performed with the potential double mutants to confirm that these lines are homozygous for the *npr1-1* mutation. The homozygosity of *cpr6-1* in the double mutant was confirmed by backcrossing the *cpr6-1 npr1-1* double mutant to *BGL2–GUS* wild-type plants and scoring for the presence of *cpr6-1*–induced reporter gene expression in all F_1 progeny.

The *cpr6-1 npr1-1* double mutant phenotypically resembles *cpr6-1* in size and development but has shorter siliques and bleaches in the stems approaching the inflorescence (data not shown). Unlike the *cpr5 npr1-1* double mutant (Bowling et al., 1997), the *cpr6-1 npr1-1* double mutant does not bleach in the leaves. The pattern of reporter gene expression in the double mutant resembles the *cpr6-1* expression patterns in the hypocotyl, stem, and petioles but is fainter in the older leaves (data not shown).

RNA gel blot analysis was conducted with 3-week-old *cpr6-1 npr1-1* seedlings to determine whether *npr1-1* suppresses *cpr6-1*–induced expression of the PR genes. We were surprised to find that *cpr6-1* expresses *PR-1*, *BGL2*, and *PR-5* independently of the *npr1-1* mutation, as shown in Figure 5. This experiment was repeated two additional times



PR-1	1	21	22	25	19	16	1	1
BGL2	1	6	32	18	37	26	1	1
PR-5	1	10	12	12	13	12	1	1

Figure 5. Expression of PR Genes in the *cpr6-1 npr1-1* Double Mutant.

PR-1, *BGL2*, and *PR-5* gene-specific probes were used for RNA gel blot analysis of the indicated plants, with the *UBQ5* gene-specific probe being used as a loading control. The table below the RNA gel blot describes the fold induction for each sample. The values have been corrected based on the loading control and normalized to that of the wild type. RNA samples were extracted from 2-week-old plants grown on MS media or MS media with 0.1 mM INA (+INA). W.T., *BGL2–GUS* transgenic line; *cpr6*, *cpr6-1* mutant line; *npr1*, *npr1-1* mutant line.

with plants at different developmental stages. Although slight variations in expression levels were observed between experiments, significant levels of PR gene expression were consistently detected in the *cpr6-1 npr1-1* double mutant. In addition, the NPR1-independent defense-related genes *PDF1.2* and *Thi2.1* are also expressed in the double mutant (data not shown).

Because *cpr6-1 npr1-1* mutant plants express *PR-1*, *BGL2*, *PR-5*, *PDF1.2*, and *Thi2.1*, we speculated that the double mutant would be resistant to *P. s. maculicola* ES4326 and *P. parasitica* Noco2. We first tested the double mutant for resistance to the oomycete pathogen *P. parasitica* Noco2 and found that it was indeed resistant to this virulent pathogen (Figure 3D). We then examined the double mutant for resistance to the bacterial pathogen *P. s. maculicola* ES4326 and found that the resistance to *P. s. maculicola* ES4326 conferred by *cpr6-1* was abolished by the presence of *npr1-1* (Figure 3C). In both cases, the addition of INA did not enhance resistance to pathogen infection (data not shown).

DISCUSSION

cpr6-1 Is a Dominant Mutant That Induces Multiple Defense Pathways

We have identified and characterized *cpr6-1* as a dominant mutant that constitutively expresses the defense-related genes *PR-1*, *BGL2*, *PR-5*, *PDF1.2*, and *Thi2.1*, that has elevated levels of SA and SAG, and that exhibits enhanced resistance to the virulent bacterial pathogen *P. s. maculicola* ES4326 and the virulent oomycete pathogen *P. parasitica* Noco2. The levels of defense-related gene expression and pathogen resistance are equivalent in *cpr6-1* heterozygous and homozygous plants (Figures 2A, 3A, and 3B). Therefore, we speculate that CPR6 is probably a positive regulator of SAR that activates defense gene expression in response to an internal signal. Without a loss-of-function allele in the gene, however, we cannot rule out the possibility that the *cpr6-1* phenotype results from a dominant loss-of-function mutation in a negative SAR regulator. Although one copy of *cpr6-1* is sufficient to activate SAR (dominance), two copies of *cpr6-1* amplify the plant's mutant phenotypic appearance (semidominance). This implies that *CPR6* may have a dual role in SAR and development. This is not entirely unexpected, because we have previously reported that the *cpr5* mutant is defective in trichome development (Bowling et al., 1997). The morphological phenotypes are unlikely to result from a second, tightly linked mutation because they cosegregate with the *cpr* phenotypes through six rounds of backcrosses.

Like *cpr5*, *cpr6-1* expresses the defense genes *PR-1*, *BGL2*, *PR-5*, *PDF1.2*, and *Thi2.1*, but without detectable lesions. Also similar to the *cpr5 npr1-1* double mutant, *cpr6-1 npr1-1* plants are resistant to *P. parasitica* Noco2. Therefore, the NPR1-independent resistance to *P. parasitica* Noco2 initially characterized in the *cpr5 npr1-1* double mutant is genetically reproducible in the absence of lesions in the *cpr6-1 npr1-1* double mutant. These data also suggest that the NPR1-dependent and NPR1-independent pathways share common regulatory elements and that the activation of the pathways are not dependent on the formation of lesions. *CPR6* may therefore be a prominent regulator of multiple defense pathways in Arabidopsis.

PR Gene Expression in *cpr6-1* Plants Requires SA but Is Independent of *NPR1*

To define where CPR6 acts in the SAR signaling cascade, we analyzed the F_1 cross between *cpr6-1* and *nahG* and constructed the *cpr6-1 npr1-1* double mutant. We found that expression of *PR-1*, *BGL2*, and *PR-5* in *cpr6-1* plants requires SA but does not require a functional NPR1 protein. These data argue against placing CPR6 upstream of NPR1 in the SAR signal cascade, as shown in Figure 6A, because PR gene expression, although requiring SA, occurs independently of NPR1. CPR6 cannot be directly downstream of NPR1, as shown in Figure 6B, because the addition of SA to *npr1-1 CPR6* plants does not rescue the *npr1-1* mutant phenotypes, namely, the lack of SA-induced PR gene expression and resistance. The simplest interpretation of the data we have accumulated is that CPR6 requires activation by SA and interaction with NPR1 to induce PR gene expression, as shown in Figure 6C. This suggests a role for NPR1 as a modifier of CPR6. Because the *cpr6-1* mutation results in activation of multiple defense signaling pathways, we speculate that CPR6 is directed toward transcription of the PR genes by SA and NPR1, whereas other factors may direct CPR6 toward transcriptional activation of the SA/NPR1–independent *PDF1.2* and *Thi2.1* genes. Evidence in favor of this interpretation of the data is the finding that addition of INA to *cpr6-1* suppresses expression of *PDF1.2* (Figures 2A and 2B) and *Thi2.1* (data not shown). INA may sequester CPR6 (the mutant protein) from activating the SA/NPR1–independent pathway. Supporting this argument is the repeated observation that in the *cpr6-1* background, the addition of INA also reduces the expression levels of *BGL2* (Figure 5), which has been shown also to be induced by pathways other than the NPR1 pathway (Bowling et al., 1997).

An alternative interpretation to the epistasis data is that PR gene induction occurs via two parallel pathways represented by CPR6 and NPR1. If true, cpr6 would require SA and an additional signal to induce PR gene expression, as shown in Figure 6D. The absence of this unidentified signal in *npr1-1 CPR6* plants prevents expression of the PR genes after SA treatment. Evidence in favor of this interpretation of the data is that in the *npr1* mutants, even though SA-induced PR gene expression is completely abolished, significant amounts of PR gene expression were still detected

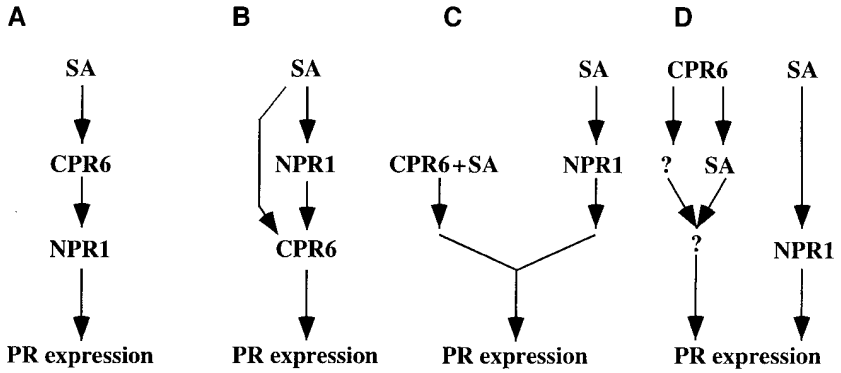


Figure 6. Possible Epistatic Relations between SA, *CPR6*, and *NPR1.*

(A) *CPR6* could be downstream of SA and upstream of *NPR1*; however, this possibility does not account for the *NPR1*-independent PR gene expression in *cpr6-1.*

(B) *CPR6* could be downstream of *NPR1* and require activation by SA; however, the lack of PR gene expression in *npr1-1* mutants after SA treatment precludes this possibility.

(C) *CPR6* could require activation by SA and interaction with *NPR1* in PR gene regulation.

(D) *CPR6* could stimulate both SA accumulation and an unknown, independent signal. Both would then act together in some way to induce PR gene expression that is independent of *NPR1.*

when *P. s. maculicola* ES4326 was used as an inducer (Glazebrook et al., 1996). Perhaps this unidentified second signal is produced as a result of *P. s. maculicola* ES4326 infection. However, it must be noted that the expression of these PR genes is not sufficient to establish resistance to *P. s. maculicola* ES4326 in *npr1* plants, even in the presence of SA (Cao et al., 1994; Glazebrook et al., 1996).

The Mechanism by Which *cpr6-1* Accumulates SA

The *cpr6-1* mutant has elevated levels of endogenous SA and requires SA for PR gene expression. We propose two possible mechanisms to explain the observed elevation of SA in *cpr6-1* that can be reconciled with NPR1-independent expression of the PR genes. The first mechanism suggests that the *cpr6-1* mutation affects a function or a component upstream of SA synthesis that stimulates both SA accumulation and an independent signal, which then work together to induce PR gene expression (Figure 6D). Although this hypothesis adequately explains elevated SA levels in *cpr6-1*, an unidentified signal has to be introduced to account for the fact that SA alone does not induce PR gene expression in *npr1-1 CPR6* plants. This model also assumes the existence of an SA-responsive pathway leading to PR gene expression that is completely independent of *NPR1.* However, such a pathway has not yet been reported.

The second mechanism is a simpler interpretation of the data and suggests that the *cpr6-1* mutation has dual effects downstream of SA synthesis. One effect, triggered by SA, is PR gene expression, whereas the other effect is continuation of SA synthesis, as shown in Figure 7. This suggests that PR gene expression is a consequence of SA-activated *cpr6-1* rather than a consequence of *cpr6-1*–activated SA accumulation. This also implies that there are downstream components of the *cpr6-1*–induced defense responses that regulate subsequent SA accumulation. A feedback loop was previously proposed to explain the SA dependence of lesions in the *lsd6* and *lsd7* mutants (Weymann et al., 1995), but the way in which a plant maintains systemically elevated SA has not yet been defined. Our data suggest that maintenance of elevated SA may occur via *CPR6* after the initial burst of SA accumulation induced by the HR. Mechanistically, the systemic signal released after avirulent pathogen infection could stimulate SA accumulation throughout the plant and then rely on activated CPR6 to maintain elevated SA levels after production of the systemic signal has ceased.

Expression of *PR-1*, *BGL2*, and *PR-5* Is Not Sufficient to Confer Resistance to *P. s. maculicola* ES4326

Expression of *PR-1*, *BGL2*, *PR-5*, *PDF1.2*, and *Thi2.1* in the *cpr6-1 npr1-1* double mutant led us to believe that *cpr6-1* would be resistant to infections by virulent oomycete and bacterial pathogens. With respect to the virulent oomycete pathogen *P. parasitica* Noco2, we were correct. However, the *cpr6-1 npr1-1* double mutant is as susceptible as *npr1-1* plants to the virulent bacterial pathogen *P. s. maculicola* ES4326, despite expression of *PR-1*, *BGL2*, and *PR-5*, which are the molecular markers for SAR used in Arabidopsis. In the *cpr6-1 npr1-1* double mutant, we have genetically

uncoupled SAR-induced resistance to *P. s. maculicola* ES4326 from expression of *PR-1*, *BGL2*, and *PR-5.* These data demonstrate that *NPR1* regulates a set of unidentified antibacterial genes and support previous observations that PR-1, BGL2, and PR-5 may be primarily antifungal peptides (Kauffmann et al., 1987; Woloshuk et al., 1991; Vigers et al., 1992; Neiderman et al., 1995; Hu and Reddy, 1997). The bacterial resistance observed in the *cpr6-1* mutant could be stimulated by *cpr6-1* itself with a functional NPR1 protein or result from *cpr6-1*–induced SA accumulation, which then activates the NPR1-regulated antibacterial genes. It is unclear at this point exactly how *cpr6-1* induces bacterial resistance. It is clear, however, that *cpr6-1* is an inducer of multiple defense pathways because it displays NPR1-independent resistance to *P. parasitica* Noco2 and constitutively expresses the antifungal defense genes *PDF1.2* and *Thi2.1* as well as the PR genes (*PR-1*, *BGL2*, and *PR-5*).

The Implications of Uncoupling PR Gene Expression from NPR1 and Bacterial Resistance

Based on our data, we present a model showing that CPR6 is a regulator of multiple defense pathways. Through CPR6, expression of the PR genes occurs either by interaction with NPR1 or via a parallel pathway. In addition, we show that induction of *PR-1*, *BGL2*, and *PR-5* is not sufficient to confer resistance to the virulent bacterial pathogen *P. s. maculicola* ES4326 and that *NPR1* must therefore regulate a set of unidentified antibacterial genes (Figure 7).

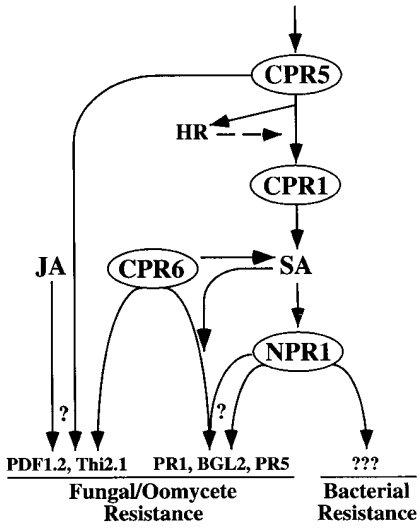


Figure 7. Proposed Model for Functions of *CPR6* and *NPR1* in the Induction of Defense-Related Genes.

CPR6 is shown to induce the expression of the PR genes in conjunction with or independent of *NPR1* to confer resistance to pathogens. *NPR1* is shown to regulate the expression of antibacterial genes that have not yet been identified. *CPR6* is hypothesized to regulate the accumulation of SA through an autoregulatory mechanism, and its role in PR gene regulation is shown to require SA. It is not known at this time whether *cpr6-1*–induced expression of *PDF1.2* and *Thi2.1* is accomplished through the same pathway as *cpr5* and/or JA.

Our model suggests a possible mechanism in which the plant uses a modifier (NPR1) to direct a positive regulator of multiple resistance pathways (CPR6) toward transcriptional activation of a specific set of genes (PR genes). Alternatively, the activation of particular resistance pathways may be redundant and rely on a specific *avr–R* gene interaction (reviewed in Bent, 1996). Other studies have shown that different *avr–R* gene interactions can induce the expression of different genes while competing antagonistically to induce the expression of the same genes (Reuber and Ausubel, 1996; Ritter and Dangl, 1996). Pathogen signals may be able to specify the induction of CPR6- or NPR1-regulated PR and other defense-related genes.

In conclusion, the regulatory activity of CPR6 has significant implications for understanding the mechanisms of acquired resistance. By biochemically and genetically analyzing *cpr6-1* plants, we further dissected the SAR signaling pathways and demonstrated how the SA/NPR1–dependent pathway may relate to other pathways leading to acquired resistance. In Arabidopsis, SAR has been defined as an SA-dependent response that uses NPR1 to induce resistance to the pathogens *P. parasitica*, *P. s. maculicola* ES4326, *P. s.* pv *tomato* DC3000, and turnip crinkle virus along with the concurrent expression of *PR-1*, *BGL2*, and *PR-5* (Lawton et al., 1996; Ryals et al., 1996). We have shown that resistance to *P. parasitica* Noco2 and PR gene expression can occur independently of NPR1, that expression of *PR-1*, *BGL2*, and *PR-5* in the *cpr6-1 npr1-1* double mutant is not sufficient to confer resistance against *P. s. maculicola* ES4326, and that expression of the PR genes and the *PDF1.2* and *Thi2.1* genes is regulated by common genetic elements. Future studies will focus on more epistatic analyses between different acquired resistance mutants, the identification of NPR1-regulated antibacterial genes, and the cloning of *CPR6* in Arabidopsis.

METHODS

Plant Growth Conditions and Isolation of *cpr6-1*

Plants (*Arabidopsis thaliana*) 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 *cpr6-1* mutant was isolated in a screen for constitutive expression of the β-1,3-glucanase promoter–to–β-glucuronidase (*BGL2–GUS*) reporter gene fusion in transgenic Columbia (Col) plants mutagenized with ethyl methanesulfonate, as described by 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 with or without 0.1 mM 2,6-dichloroisonicotinic acid (INA) or 0.3 mM salicylic acid (SA) and from 4-week-old plants grown in soil with or without 0.65 mM INA or 2 mM rose bengal (RB), as described by Bowling et al. (1997). 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 absorbence, and 5- or 10-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 pathogenesis-related (PR) *PR-1* mRNA and 18S rRNA were labeled as described by Cao et al. (1994); probes for *BGL2*, *PR-5*, ubiquitin (*UBQ5*), *PDF1.2*, and *Thi2.1* were generated using a strand-biased polymerase chain reaction (PCR) protocol (Schowalter and Sommer, 1989; Bowling et al., 1997). The templates for *BGL2*, *PDF1.2*, and *Thi2.1* were generated by PCR from the plasmid containing the cDNA clone for each gene, using primers described by Rogers and Ausubel (1997), Penninckx et al. (1996), and Epple et al. (1995), respectively. The template for *UBQ5* was generated by PCR from Col genomic DNA, using primers described by Rogers and Ausubel (1997). PCR amplification of the template included one cycle of 94°C for 2 min; 30 cycles of 94°C for 15 sec, 55°C for 15 sec, and 72°C for 45 sec; and finally one cycle of 72°C for 10 min. PCR cycles were the same for strand-biased probe production, as described by Bowling et al. (1997). Hybridization and washing conditions were as previously described (Church and Gilbert, 1984; Cao et al., 1994). Bands were quantified using ImageQuant and NIH 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 and grown on MS plates or soil. Staining was with trypan blue for dead cells, nitro blue tetrazolium for the presence of superoxides, and autofluorescence fixing solution for UV epifluorescence microscopy. Trypan blue staining was performed in accordance with previous descriptions (Keogh et al., 1980; Dietrich et al., 1994; Bowling et al., 1997). Nitro blue tetrazolium staining was performed as described by Jabs et al. (1996). Samples for autofluorescence examination were prepared as described by Bowling et al. (1997). Autofluorescence was examined as described by Dietrich 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 *cpr6-1* plants as the pollen donor. The *cpr6-1 npr1-1* double mutant was generated using pollen from homozygous *cpr6-1* plants to fertilize homozygous *npr1-1* plants. The mapping population was generated by using the pollen from *cpr6-1* to fertilize a transgenic line of the Landsberg *erecta* (L*er*) ecotype transformed with the *BGL2–GUS* reporter gene. Successful crosses were scored based on loss of the L*er* phenotype in the F_1 plants. Homozygous *cpr6-1* mutants were isolated in the F_2 generation based on the *cpr6-1* homozygous phenotype and were later tested for expression of the reporter gene in all of the F_3 progeny.

PCR-Based Mapping

Mapping was performed by the codominant cleaved amplified polymorphic sequences (CAPS) protocol described by Konieczny and Ausubel (1993) and the single sequence-length polymorphisms (SSLPs) protocol described by Bell and Ecker (1994). The primers and restriction enzyme for converting the g4026 restriction fragment length polymorphism marker to a CAPS marker are reported in the Arabidopsis database web site (http://genome-www.stanford.edu).

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 were grown for 18 days on soil with a 12-hr photoperiod. Seven days after inoculation, the plants were scored for the presence of conidiophores by using a dissection microscope. The plants were given a disease rating of one to five, as previously described by Cao et al. (1997), and the data were analyzed using Mann-Whitney U tests (Sokal and Rohlf, 1981).

Measurement of SA

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

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