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A Mutation in Arabidopsis That Leads to Constitutive Expression of Systemic Acquired Resistance

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Systemic acquired resistance (SAR) is a nonspecific defense response in plants that is associated with an increase in the endogenous level of salicylic acid (SA) and elevated expression of pathogenesis-related (PR) genes. To identify mutants involved in the regulation of PR genes and the onset of SAR, we transformed Arabidopsis with a reporter gene containing the promoter of a β-1,3-glucanase–encoding PR gene (*BGL2*) and the coding region of β-glucuronidase (*GUS*). The resulting transgenic line (*BGL2–GUS*) was mutagenized, and the M_2 progeny were scored for constitutive GUS activity. We report the characterization of one mutant, *cpr1* (constitutive expresser of PR genes), that was identified in this screen and shown by RNA gel blot analysis also to have elevated expression of the endogenous PR genes *BGL2*, PR-1, and PR-5. Genetic analyses indicated that the phenotype conferred by *cpr1* is caused by a single, recessive nuclear mutation and is suppressed in plants producing a bacterial salicylate hydroxylase, which inactivates SA. Furthermore, biochemical analysis showed that the endogenous level of SA is elevated in the mutant. Finally, the *cpr1* plants were found to be resistant to the fungal pathogen *Peronospora parasitica* NOCO2 and the bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326, which are virulent in wild-type *BGL2–GUS* plants. Because the *cpr1* mutation is recessive and associated with an elevated endogenous level of SA, we propose that the *CPR1* gene product acts upstream of SA as a negative regulator of SAR.

INTRODUCTION

Systemic acquired resistance (SAR) is a general defense response that, when activated, protects plants from infection by a wide variety of pathogens (reviewed in Kuc, 1982). The resistance response can be activated by both biological and chemical inducers; biological inducers include avirulent pathogens that usually cause necrotic lesions (Ross, 1961; Hecht and Bateman, 1964; Lovrekovich et al., 1968), whereas chemical inducers include salicylic acid (SA; White, 1979) and 2,6-dichloroisonicotinic acid (INA; Metraux et al., 1991). The appearance of SAR is accompanied by elevated levels of endogenous SA (Malamy et al., 1990; Metraux et al., 1991) and the expression of genes encoding pathogenesis-related (PR) proteins (Van Loon and Van Kammen, 1970; Ward et al., 1991).

A causal link between PR proteins and SAR has yet to be established, but the timing of PR gene expression correlates with the onset and duration of SAR (Ward et al., 1991; Uknes et al., 1992). Furthermore, some of the PR genes encode extracellular proteins with β-1,3-glucanase and chitinase activities. Purified chitinase and β-1,3-glucanase have been shown to inhibit the growth of several fungal pathogens (Schlumbaum et al., 1986; Mauch et al., 1988). β-1,3-Glucanase has also been suggested to be involved in releasing defense-activating elicitors (Keen and Yoshikawa, 1983; Mauch and Staehelin, 1989). Moreover, elevated levels of some of the PR proteins in plants have been shown to confer resistance to certain pathogens. For example, transgenic tobacco plants constitutively producing a chitinase are resistant to the fungal pathogen *Rhizoctonia solani* (Broglie et al., 1991), and plants constitutively expressing the PR-1a gene are resistant to the fungal pathogens *Phytophthora parasitica* and *Peronospora tabacina* (Alexander et al., 1993). Because of these associations with pathogen resistance, PR gene expression is used as a molecular marker for SAR.

Although little is known about the signaling pathway that leads to systemic resistance and the expression of PR genes, the importance of SA as a signal molecule for SAR has been well documented. Treatment of tobacco with exogenous SA induces resistance to pathogens (White, 1979) and expression of PR genes (Ward et al., 1991). Furthermore, increases in endogenous SA levels after infection with an avirulent pathogen have been demonstrated in tobacco, cucumber, and Arabidopsis (Malamy et al., 1990, 1992; Metraux et al., 1990; Rasmussen et al., 1991; Yalpani et al., 1991; Enyedi et al., 1992; Uknes et al., 1993). In addition, a soluble SA binding protein has been identified in tobacco whose binding affinity and specificity

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suggest its involvement in transducing the SA signal (Chen and Klessig, 1991); this SA binding protein has recently been shown to be a catalase whose activity is inhibited by SA (Chen et al., 1993). The most direct evidence that SA plays an essential role in SAR comes from studies of transgenic tobacco plants expressing the salicylate hydroxylase–encoding gene *nahG*, which prevents the accumulation of SA by converting it to catechol. These transgenic plants fail to develop SAR after pathogen infection (Gaffney et al., 1993; Vernooij et al., 1994). However, grafting experiments done between this transgenic line and a wild-type line indicate that, although SA is required in the distal tissues for the establishment of the resistant state, it is not the systemic signal for SAR (Vernooij et al., 1994).

Previous studies have verified that Arabidopsis exhibits the characteristics of SAR, including development of SAR after a hypersensitive response (HR) to an avirulent pathogen (Dempsey et al., 1993; Uknes et al., 1993; Cameron et al., 1994; Mauch-Mani and Slusarenko, 1994), responsiveness to SA and INA induction (Uknes et al., 1992), and expression of PR genes (Uknes et al., 1992; Dempsey et al., 1993). Studies of SAR in Arabidopsis have led to the identification of one class of mutants in which lesions resembling an HR form spontaneously, followed by induction of SAR; these include *acd2* (accelerated cell death; Greenberg et al., 1994), *lsd1*, *lsd2*, *lsd3*, *lsd4*, and *lsd5* (lesions simulating disease; Dietrich et al., 1994). However, mutations affecting signal transduction downstream of the HR have no known morphological phenotype and are thus difficult to identify. To aid in the isolation of such mutants, we constructed a resistance-related reporter gene and used it to transform Arabidopsis; this reporter gene contains the promoter of the *BGL2* gene (a PR gene encoding a β-1,3-glucanase) fused with the coding region of the β-glucuronidase (*GUS*) gene. We have previously reported the use of this reporter gene to isolate a mutant that is a nonexpresser of PR genes in the presence of SA or INA (*npr1*; Cao et al., 1994).

In this paper, we describe the isolation and characterization of an Arabidopsis mutant that does not spontaneously form HR-like lesions and yet constitutively expresses the *BGL2–GUS* reporter gene. Because other, endogenous PR genes are also constitutively expressed in this mutant, we have named it *cpr1* (constitutive expresser of PR genes 1). Significantly, *cpr1* plants are resistant to both the fungal pathogen *Peronospora parasitica* NOCO2 and the bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326 (Davis et al., 1991; Dong et al., 1991), which further indicates that we have identified a mutation leading to constitutive activation of SAR.

RESULTS

Identification of the *cpr1* Mutant Using the *BGL2–GUS* Reporter Gene

At the time we began our studies, there were no morphological phenotypes reported to be associated with mutants in SAR signaling. To circumvent this problem, we transformed Arabidopsis with a reporter gene responsive to resistance-inducing signals. This reporter gene contained the promoter of *BGL2* and the *GUS* coding region. Our screening rationale was that the reporter should reflect the expression of the endogenous PR genes and that in searching for mutants with aberrant expression of the reporter gene, we would identify mutations affecting the regulation of SAR.

There are three β-1,3-glucanase genes in Arabidopsis, *BGL1*, *BGL2*, and *BGL3*, which are in tandem array on a 12-kb segment of chromosomal DNA (Dong et al., 1991). A 2025-bp XbaI-SphI fragment spanning the entire region between *BGL1* and the start codon of *BGL2* was fused to the coding sequence of the *GUS* gene; an EcoRI-SalI fragment containing this chimeric gene was then placed in the transformation vector pBI101 with a selectable marker for kanamycin resistance, as shown in Figure 1. The construct was delivered into Arabidopsis through root transformation (Valvekens et al., 1988). The resulting transformants were tested for kanamycin resistance, and the insertion of *BGL2–GUS* into the Arabidopsis genome was confirmed by DNA gel blot analysis (data not shown).

In the pilot experiment for the mutant screen, 15-day-old wild-type *BGL2–GUS* seedlings were tested for GUS activity using a microtiter plate assay that scored for the conversion of the GUS substrate 4-methylumbelliferyl β-D-glucuronide (MUG) to the fluorescent product 4-methylumbelliferone (4-MU; Jefferson, 1987). As shown in Figure 2, seedlings that had been grown on agar plates with Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) showed little GUS activity without inducers, whereas seedlings of the same age grown on MS medium supplemented with either 0.5 mM SA or 0.1 mM INA expressed significant amounts of GUS activity. For this assay, leaves were placed into microtiter plate wells containing the substrate solution for GUS; we found that the amount of tissue used had little effect on the outcome of the assay and that tissue processing was not necessary.

Mutagenesis was performed in the *BGL2–GUS* transgenic line by exposing seed (~36,000 total) to ethyl methanesulfonate. The mutagenized seeds were sown onto 12 separate flats and allowed to grow, self-fertilize, and set seed. Using the microtiter plate GUS assay procedure, M_2 plants were tested for constitutive expression of *GUS*. Of 13,883 plants screened, 262 *GUS* expressers were identified, transplanted to soil, and allowed to set seed. GUS activity was assayed again in progeny of 147 of these putative mutants to determine whether the phenotype was heritable, and 28 lines again showed GUS activity. To begin characterization of these mutants, the first line that was found to be homozygous for *GUS* expression was chosen for further analysis. We named this mutant *cpr1* (Figure 2).

PR Genes Are Constitutively Expressed in *cpr1* Plants

A quantitative assay for GUS activity was performed to compare the expression of the reporter gene in the *cpr1* plants (*cpr1/cpr1*) with that of the wild-type *BGL2–GUS* transgenic

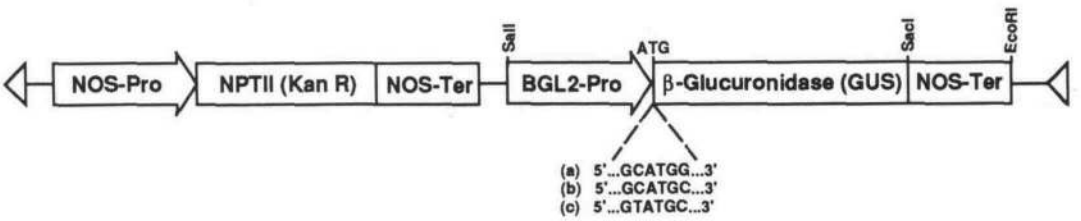

Figure 1. *BGL2–GUS* Reporter Gene Construct.

In (a) are sequences at the translation initiation site of the *BGL2–GUS* reporter gene, a chimera of SphI and NcoI sites; in (b) are sequences at the translation initiation site of *BGL2* after a T-to-C transition at the position −1 to the ATG codon, an SphI site; in (c) are sequences at the translation initiation site of *BGL2*. NOS, nopaline synthase gene; NPTII, neomycin phosphotransferase; Kan R, kanamycin resistance; Pro, promoter; Ter, transcription termination sequence.

line (*CPR1*/*CPR1*). As shown in Figure 3, the GUS activity observed in the uninduced *cpr1* plants was similar to the level observed in SA- or INA-induced wild-type plants, whereas the level of GUS activity in the uninduced wild-type plants was negligible. Although the addition of SA or INA to *cpr1* plants could further induce GUS activity, the induction was less than twofold. In contrast, the increase in GUS activity observed in the wild-type plants treated with 0.5 mM SA or 0.1 mM INA was 67- and 307-fold, respectively.

Histochemical staining was performed to investigate the pattern of constitutive reporter gene expression in *cpr1* plants and to compare it with that observed in the SA-induced wild type. A consistent pattern was observed in the aseptically grown *cpr1* plants. As shown in Figure 4A, in untreated wild-type seedlings, no GUS activity was observed (the sample on the left), whereas staining was detected in the shoots but not in the roots of untreated *cpr1* seedlings (the sample on the right). More specifically, the stain in *cpr1* plants was present at the tips of young leaves and throughout mature leaves and was concentrated in the veins of old leaves. A similar pattern was observed in the SA-treated wild-type leaves (Figure 4B). Stained tissues were then embedded and sectioned to examine the distribution of GUS activity further. As Figures 4C and 4E indicate, in *cpr1* plants, the reporter gene was expressed mainly in mesophyll cells and vascular tissues, including phloem and young xylem. Strong staining was not found in the epidermis except in stomatal guard cells. This tissue-specific distribution of staining in *cpr1* seedlings was similar to that of the wild type induced by the exogenous application of SA (Figures 4D and 4F).

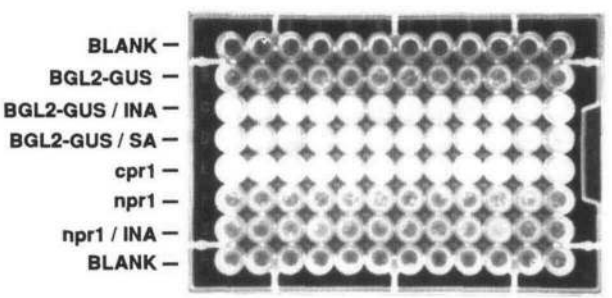

Figure 2. Fluorometric Assay of GUS Activity on a Microtiter Plate as Used for the Mutant Screen.

The plate was illuminated with long-wavelength UV light, which allows detection of the fluorescent product 4-MU formed by hydrolysis of MUG by the GUS enzyme. BLANK indicates that no plant tissue was added. BGL2–GUS, BGL2–GUS/INA, and BGL2–GUS/SA designate wild-type transgenic *BGL2–GUS* plants grown on MS medium, MS medium with 0.1 mM INA, and MS medium with 0.5 mM SA, respectively. cpr1 indicates constitutive expresser of PR genes mutant 1 grown on MS medium; npr1 and npr1/INA designate nonexpresser of PR genes mutant 1 grown on MS medium and MS medium with 0.1 mM INA, respectively.

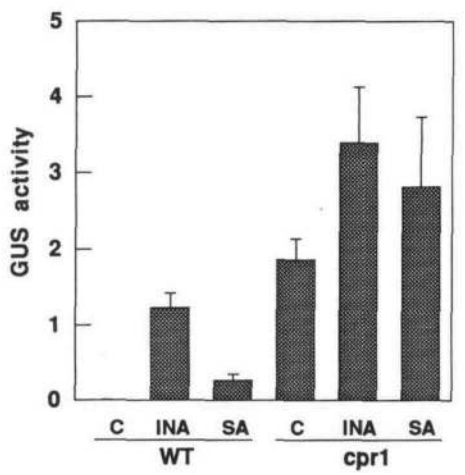

Figure 3. Quantitative Analysis of GUS Activity in Wild-Type and *cpr1* Plants.

Plant tissues assayed were from 15-day-old wild-type (WT) seedlings carrying the *BGL2–GUS* transgene and 15-day-old *cpr1* seedlings. The values represent the average of three replicates ± SE. GUS activity is given as absolute fluorescence units per minute per microgram of protein. C (control) indicates seedlings grown on MS medium; INA, seedlings grown on MS medium with 0.1 mM INA; SA, seedlings grown on MS medium with 0.5 mM SA.

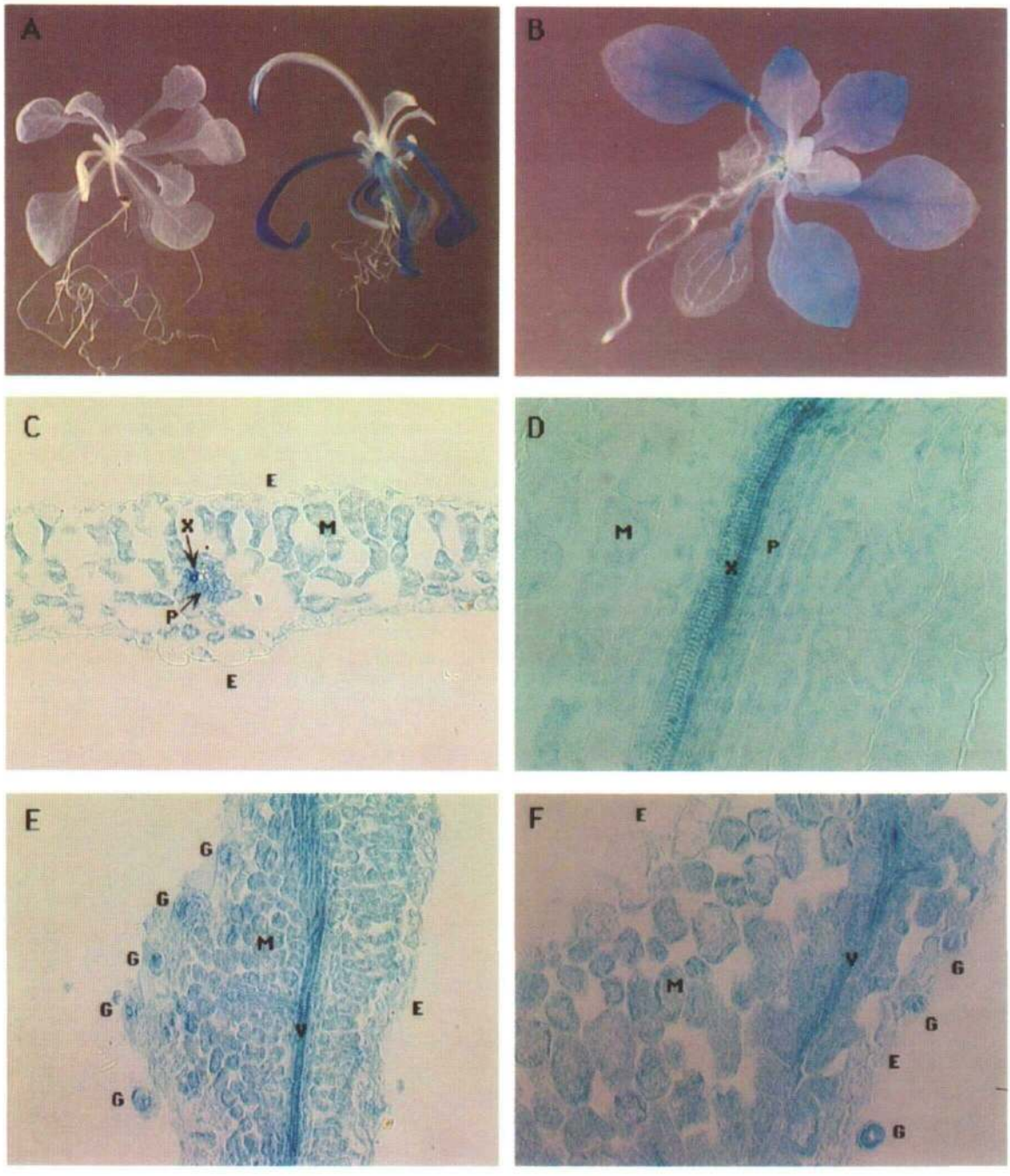

Figure 4. Pattern of *GUS* Expression in *cpr1* Plants Compared with SA-Induced Wild-Type Arabidopsis Plants Containing *BGL2–GUS*.

(A) Histochemical staining of GUS activity in a 15-day-old *cpr1* seedling grown aseptically in MS agar medium (right) and in a wild-type seedling carrying the *BGL2–GUS* transgene grown under the same conditions (left).
(B) An SA-induced *BGL2–GUS* wild-type seedling.
(C) A cross-section of a stained *cpr1* leaf.
(D) A longitudinal section of a stained, SA-induced *BGL2–GUS* wild-type leaf.
(E) A longitudinal section of a stained *cpr1* leaf.
(F) A longitudinal section of a stained, SA-induced *BGL2–GUS* wild-type leaf.
E, epidermis; G, guard cell; M, mesophyll; P, phloem; V, vascular bundle; X, xylem.

To examine the expression of SAR-responsive PR genes, RNA gel blot analyses were performed in both 2-week-old *cpr1* seedlings grown axenically on MS agar medium and 4-week-old mature *cpr1* plants grown on soil. The level of PR mRNA detected in each sample was normalized to that of a constitutively expressed β-ATPase gene and then compared with levels in wild-type plants grown under the same conditions. As shown in Figure 5, levels of the endogenous *BGL2*, PR-1, and PR-5 transcripts were low in wild-type plants, whereas expression of these genes was induced in the presence of 0.1 mM INA. In contrast, the basal level of *BGL2*, PR-1, and PR-5 gene expression was 3.2-, 11.7-, and 1.3-fold higher, respectively, in the *cpr1* seedlings and 4.8-, 12.1-, and 2.6-fold higher in the mature *cpr1* plants compared with the corresponding wild type. Although the amount of PR-5 transcript detected in *cpr1* seedlings was not much more than that in the untreated wild-type seedlings, the induction of PR-5 gene expression produced by INA treatment of the wild-type seedlings was also only 1.3-fold. The presence of 0.1 mM INA in the growth media also led to a further 4.0-fold increase of PR-1 expression in *cpr1* seedlings.

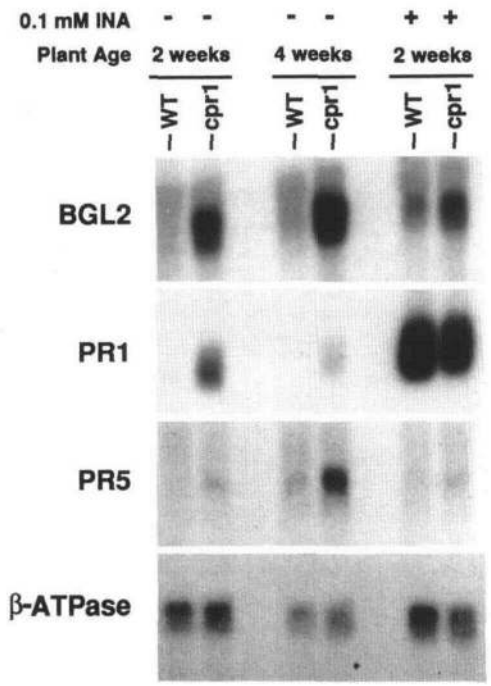

Figure 5. RNA Gel Blot Analysis of *BGL2*, PR-1, and PR-5 in Wild-Type and *cpr1* Plants.

RNA samples were extracted from 2-week-old seedlings grown on MS medium and MS medium with 0.1 mM INA and from 4-week-old plants grown on soil. Endogenous *BGL2*, PR-1, PR-5, and β-ATPase gene–specific probes were used. WT, wild-type *BGL2–GUS* transgenic line; (–) plants grown in the absence and (+) in the presence of INA.

Genetic Analyses Indicate That the *cpr1* Mutation Is Recessive and the *cpr1* Phenotype Is Suppressed by Salicylate Hydroxylase

For genetic analyses, the progeny were assayed for reporter gene expression using both the fluorometric GUS assay in microtiter plates to obtain the ratio of segregation and histochemical staining for GUS activity to verify the accuracy of the fluorometric assay. Plants scored as *cpr1* by the microtiter plate assay were stained and shown always to have the same pattern of staining as the parental *cpr1* plants. Likewise, plants scored as wild type by the microtiter plate assay showed no GUS staining.

To examine the genetic segregation of *cpr1*, a backcross was performed between *cpr1/cpr1* plants and wild-type *CPR1/CPR1* plants containing the *BGL2*–GUS transgene. In the F_1 generation, constitutive expression of *GUS* was absent in all 60 seedlings tested, whereas in the F_2 population, expression was present in 28 of 129 seedlings. The F_2 segregation ratio of the *cpr1* phenotype was 1:3.6, and the chi-square calculated for goodness of fit to a single recessive nuclear mutation was 0.744 ($P > 0.1$).

In addition to constitutive PR gene expression, morphological characteristics were also noted in *cpr1* plants. Compared with the wild type, *cpr1* plants growing on soil were found to have small, narrow, dark green leaves densely covered with trichomes on the adaxial surface and relatively long siliques. Segregation of these visible phenotypes was also studied in the backcross. After scoring for GUS activity, F_2 progeny with high GUS activity (*cpr1/cpr1*) and progeny with no GUS activity (*CPR1/cpr1* and *CPR1/CPR1*) were transplanted to soil, and the morphological phenotypes were examined later. All plants with GUS activity also exhibited the visible phenotypes, whereas all the F_2 progeny without GUS activity looked like the wild type. Therefore, the mutation resulting in the stunted phenotype either is the same mutation that causes the constitutive SAR phenotype or is tightly linked to it.

Further genetic analysis was performed to determine whether the *CPR1* gene product acts upstream or downstream of SA in the signal transduction pathway leading to PR gene expression. We constructed an Arabidopsis transgenic line carrying a bacterial salicylate hydroxylase gene (*nahG*; Yen and Gunsalus, 1982; Schell, 1986; You et al., 1991) controlled by the constitutive cauliflower mosaic virus 35S promoter (see Methods) using a strategy similar to that developed by Gaffney et al. (1993). The expression of the *nahG* gene was confirmed by RNA gel blot analysis, and the salicylate hydroxylase activity in the transgenic plants was shown by the accumulation of a brown oxidation product of salicylate in the roots of the transgenic plants when the growth medium was supplemented with 0.5 mM SA. Crosses were made between homozygous *cpr1* plants and a homozygous transgenic line expressing *nahG*. None of the F_1 progeny constitutively expressed the *GUS* reporter gene; this was expected because *cpr1* is a recessive mutation. To examine whether one copy of the *nahG* gene could produce sufficient levels of salicylate hydroxylase to inactivate SA induction of the reporter gene, F_1 heterozygous

nahG plants were grown in media supplemented with 0.5 mM SA. No reporter gene expression was detected in these plants, indicating that a single copy of the *nahG* gene is sufficient to suppress SA induction. Using this information, two expected ratios of *GUS* expressers to nonexpressers were calculated for the F_2 generation. Due to the involvement of three different loci and selection for kanamycin resistance in our assay, the derivation of these ratios is somewhat complex. The details of the calculation are described in Methods. A ratio of 1:4 would be expected if the *cpr1* phenotype is unaffected by salicylate hydroxylase, meaning *CPR1* acts downstream of SA, whereas a ratio of 1:19 would be expected if salicylate hydroxylase interferes with the *cpr1* phenotype, indicating that *CPR1* functions upstream of SA. In the population of F_2 plants, 28 of 506 plants were positive for GUS activity. This ratio (1:17) argues that salicylate hydroxylase suppresses the *cpr1* phenotype (χ^2 = 0.303; $P > 0.5$) and thus acts upstream of SA. Furthermore, the visible phenotypes associated with *cpr1* plants were mostly suppressed in the F_2 plants that were negative for GUS activity. This implies that these phenotypes are caused by the elevated SA level of *cpr1* plants.

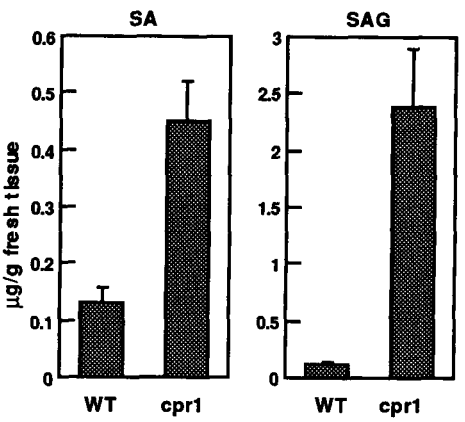

Figure 6. Endogenous Levels of SA and SAG in Wild-Type and *cpr1* Plants.

Leaves from 4-week-old plants grown on soil were harvested and analyzed by HPLC for free SA and SAG content. The values presented are the average of five replicates ± SE. WT, wild-type *BGL2-GUS* transgenic line.

The Endogenous Level of SA Is Elevated in *cpr1* Plants

Because genetic analysis indicated that the *CPR1* gene acts upstream of SA, measurements were made to determine whether the endogenous level of SA was affected in *cpr1* plants. Intracellular SA is found predominantly as free SA or its sugar conjugate, SA β-glucoside (SAG; Enyedi et al., 1992; Malamy et al., 1992). Leaf tissues from 4-week-old *cpr1* and wild-type plants were examined for both free SA and SAG concentrations; the results are presented in Figure 6. In the absence of any inducer, the basal level of SA in *cpr1* plants was 4.5-fold higher than that in wild-type plants, whereas the SAG level was 21-fold higher. The differences in both SA and SAG levels between the wild-type and *cpr1* plants were statistically significant ($P < 0.001$).

cpr1 Plants Are Resistant to the Pathogens *P. parasitica* NOCO2 and *P. s. maculicola* ES4326

To determine whether the elevated levels of SA and PR gene expression in *cpr1* plants lead to the constitutive activation of SAR, the growth of two normally virulent pathogens was examined in *cpr1* plants. To test for resistance to a fungal pathogen, 10-day-old plants were sprayed with a spore suspension of *P. parasitica* NOCO2, which causes downy mildew in Arabidopsis. As shown in Figures 7A and 7B, this pathogen caused disease in the wild-type plants, as indicated by the appearance of many conidiophores on leaves. In contrast, conidiophores were almost completely absent in *cpr1* plants, as shown in Figures 7C and 7D. In a random sample of plants scored 7 days after inoculation, conidiophores were present in 34 of 53 wild-type leaves examined, whereas they were found in only four of 74 *cpr1* leaves scored. To rule out the possibility that the resistance we observed was simply due to the presence of a dense covering of trichomes on *cpr1* leaves that prevented spores from reaching the leaf surface, we examined *cpr1* leaves at a ×4 magnification and confirmed that our method of applying spores using a fine mist thoroughly wets both the adaxial and abaxial leaf surfaces. Furthermore, conidiophores were generally absent even on the abaxial surface of *cpr1* leaves, which are not covered with trichomes. In the wild type, conidiophores were found on both surfaces of the leaves.

Further testing for resistance was performed by infecting 4-week-old wild-type and *cpr1* plants with a bacterial suspension of *P. s. maculicola* ES4326 (see Methods). Plants were examined visually for disease symptoms each day during the week following infection. As shown for plants photographed 4 days after infection (Figure 7E), severe chlorosis was observed in the wild-type leaves 3 days or more after infection, whereas symptoms were nearly absent in *cpr1* plants and in plants treated with INA 2 days prior to infection. The growth of *P. s. maculicola* ES4326 in the plants was also monitored by extracting bacteria from infected leaves at daily time points; the resulting growth curves are shown in Figure 8. The number of *P. s. maculicola* ES4326 detected in *cpr1* plants was more than fourfold lower than that in the wild type after 1 day of growth and nearly sevenfold lower after 2 days. Although the differences between the means of the bacterial concentrations in *cpr1* and wild-type plants were statistically significant at all time points taken after time zero, plants treated with INA had even less bacterial growth. Compared with untreated *cpr1* plants, the INA-treated mutants had fourfold fewer bacteria after 1 day of growth and sixfold fewer after 2 and 3 days.

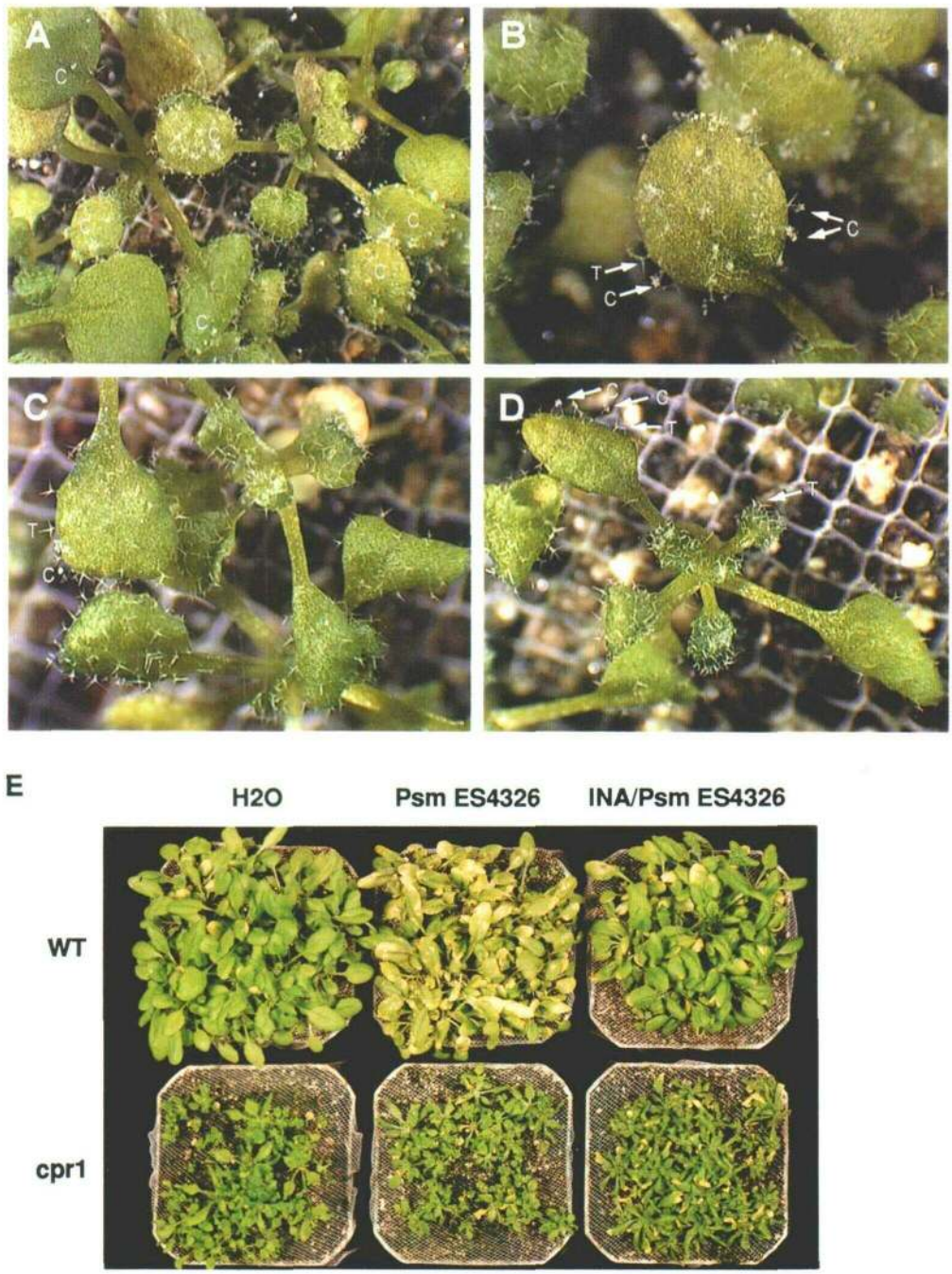

Figure 7. Symptoms Observed in Wild-Type and *cpr1* Plants after Infection with *P. parasitica* NOCO2 and *P. s. maculicola* ES4326.

The *P. parasitica* NOCO2 fungal conidiophore suspension used for infection contained 3×10^4 spores per milliliter; the *P. s. maculicola* ES4326 bacteria used for infection were suspended in 10 mM $MgCl_2$, 0.01% surfactant at an OD_{600} reading of 0.2.

(A) and **(B)** Wild-type plants 7 days after inoculation with *P. parasitica* NOCO2.

(C) and **(D)** *cpr1* plants 7 days after inoculation with *P. parasitica* NOCO2.

(E) Wild-type and *cpr1* plants 4 days after being dipped into solutions with or without *P. s. maculicola* ES4326.

C, conidiophore; T, trichome; WT, wild-type *BGL2–GUS* transgenic line; H2O, plants dipped in 10 mM $MgCl_2$, 0.01% surfactant without bacteria; Psm ES4326, plants dipped in the same solution containing 0.2 OD *P. s. maculicola* ES4326; INA/Psm ES4326, plants sprayed with INA 2 days before dipping in the solution containing bacteria.

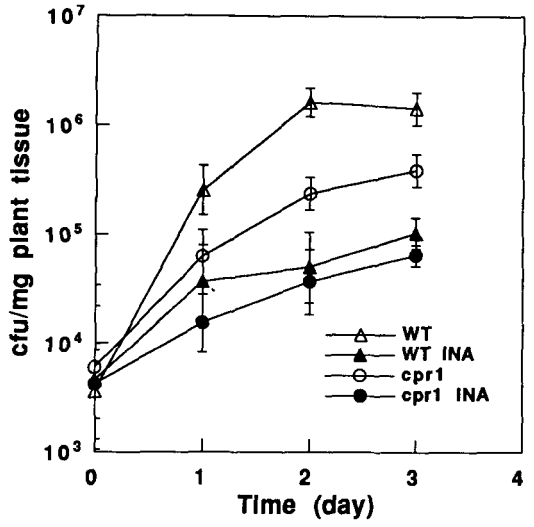

Figure 8. Growth of *P. s. maculicola* ES4326 in Wild-Type and *cpr1* Plants.

Plants were infected by dipping into a *P. s. maculicola* ES4326 bacterial suspension of 10 mM $MgCl_2$, 0.01% surfactant at an OD_{600} reading of 0.2. Samples were taken 0, 1, 2, and 3 days after infection. Error bars represent 95% confidence limits of log-transformed data (Sokal and Rohlf, 1981). Three samples were taken for each genotype at time zero, and thereafter seven samples were taken for each genotype at each time point. cfu, colony-forming units; WT, wild-type *BGL2–GUS* transgenic line; WT INA, wild-type *BGL2–GUS* transgenic line pretreated with INA; cpr1, *cpr1* mutants; cpr1 INA, *cpr1* mutants pretreated with INA.

DISCUSSION

To begin a systematic genetic analysis of the SAR signaling network, we transformed Arabidopsis with a reporter gene (*BGL2–GUS*) responsive to resistance inducers and screened for mutants with aberrant expression of this gene. The assay employed to screen for Arabidopsis mutants had to satisfy the following conditions: (1) the mutants had to be easily identifiable; (2) the assay had to be easily performed; and (3) the mutants had to be rescued to set seed. The GUS assay we derived satisfied these conditions. The combination of the use of microtiter plates and the omission of tissue processing enabled us to screen a large number of mutagenized seedlings and isolate a mutant, *cpr1*, that expressed the reporter gene without the application of exogenous inducers. Our strategy also allowed us to use the *BGL2–GUS* gene to study the levels and tissue-specific pattern of PR gene expression and to perform segregation analysis.

The constitutive expression of *BGL2–GUS* in *cpr1* plants is similar to that found in wild-type plants induced by the chemicals SA and INA. Fluorometric quantitation demonstrated that constitutive GUS activity in *cpr1* plants was at least as high as that in wild-type plants treated with INA and even higher than that in the wild type treated with SA (Figure 3). Also, histochemical staining revealed an expression pattern of the *BGL2–GUS* gene in *cpr1* matching the pattern seen in the chemically induced wild type (Figure 4). This similarity indicates that the constitutive expression of *GUS* in *cpr1* is probably due to a mutation in the same signaling pathway as the one involved in induction after the application of exogenous SA. These common patterns of *BGL2–GUS* expression also imply the presence of a tissue-specific factor(s) regulating PR gene expression.

Besides studies using the reporter gene, RNA gel blot analysis demonstrated that the endogenous PR genes *BGL2*, PR-1, and PR-5 were constitutively expressed in the *cpr1* plants compared with the wild type (Figure 5). This demonstrates that the *BGL2–GUS* reporter gene reflects the expression of endogenous PR genes and that *cpr1* contains a *trans*-acting mutation affecting transcription of these SAR-responsive genes. The presence of other factors that affect the regulation of each specific PR gene, along with such a general regulation modified by the *cpr1* mutation, cannot be ruled out. This is reflected in the RNA gel blot analysis in which differences in the levels of expression of individual PR genes were observed in plants of different ages. Such modifications in patterns of expression may reflect the specific role of each PR protein in SAR.

Because SA appears to be an endogenous signal molecule required for the induction of PR genes (Gaffney et al., 1993), the constitutive expression of PR genes in *cpr1* plants could result from an elevated level of endogenous SA or from the activation of a downstream regulatory factor that transduces the SA signal. The higher level of SA and its glucoside in *cpr1* plants suggests that the constitutive PR gene expression is a consequence of an elevated level of SA (Figure 6). This conclusion is further supported by epistasis studies in which expression of the *nahG* transgene for salicylate hydroxylase, which inactivates SA, suppressed reporter gene expression in *cpr1* plants. Whether the elevated endogenous level of SA in *cpr1* plants is as high as that induced systemically after an HR or as that supplied by exogenous application of SA is not known. Future experiments comparing the SA level in *cpr1* plants with that induced by treatment with avirulent pathogens will address this issue.

Although the molecular characteristics of an SAR response include an increase in the endogenous SA level and the induction of PR genes, the direct physiological evidence of SAR is resistance to a wide range of pathogens. *cpr1* plants that had not been treated with any resistance inducers were challenged with the fungal pathogen *P. parasitica* NOCO2 and the bacterial pathogen *P. s. maculicola* ES4326 and shown to be resistant to both (Figures 7 and 8). Because *cpr1* plants have an elevated level of endogenous SA, constitutively expressed PR genes, and enhanced resistance to very different pathogens, we concluded that *cpr1* is a mutation affecting the SAR signal transduction pathway.

The induction of SAR by the *cpr1* mutation is incomplete. This conclusion is supported by the higher GUS activity detected in the *cpr1* plants treated with either INA or SA (Figure 3), the further induction of the PR-1 gene in INA-treated *cpr1*

seedlings (Figure 5), and the more pronounced reduction of *P. s. maculicola* ES4326 growth in INA-treated *cpr1* plants (Figure 8). This leads us to speculate that the endogenous level of SA in *cpr1* may not be as high as the physiological level normally seen during SAR.

From the phenotype conferred by *cpr1*, we can begin to speculate on the function of the wild-type *CPR1* gene. *CPR1* could be a positively acting factor in the SAR signaling pathway, such as an enzyme involved in the synthesis of SA when activated. In the *cpr1* mutant, a positive factor could be constitutively activated and therefore lead to constitutive expression of SAR. Mutations leading to a constitutive positive signal would most likely be dominant. In contrast, *CPR1* could be a negative regulator in the SAR signaling pathway; it could act as a repressor of either the synthesis or the activity of enzymes involved in SA production. When this repression is inactivated, SAR would be induced. Such a negative factor could be permanently inactivated in the *cpr1* plants, resulting in constitutive SAR. The presence of a wild-type copy of such a gene should override the effect of the mutant copy in heterozygous *CPR1/cpr1* plants; therefore, a mutation in a negative factor would most likely be recessive. Indeed, genetic analysis of progeny from a *cpr1/cpr1* × *CPR1/CPR1* backcross demonstrated that *cpr1* is a recessive mutation. Thus, we propose that the *CPR1* gene functions as a negative regulator acting upstream of SA in the SAR signaling pathway.

SAR is a complex physiological response that can be induced by an HR to a range of viruses, fungi, and bacteria, and it results in enhanced resistance to an even greater number of microbial pathogens. However, the complex network involved in the perception of different pathogens appears to lead to a similar set of responses in the establishment of SAR; thus, a common signaling pathway(s) must exist. Endogenous production of SA appears to be part of such a common pathway (Gaffney et al., 1993). However, several studies suggest that SA is not the systemic signal that is produced at the primary site of infection and translocated throughout the plant to induce SAR (Rasmussen et al., 1991; Vernooij et al., 1994). Rather, SA appears to be required in the distal tissue for response to the unidentified systemic signal. At the end of the signaling pathway, a large number of SAR-related genes, including the PR genes, are induced. This physiological pathway can be overlaid with a genetic pathway, as shown in Figure 9. The previously identified mutations *acd2*, *lsd1*, *lsd2*, *lsd3*, *lsd4*, and *lsd5*, which lead to the spontaneous formation of HR-like lesions accompanied by the induction of SAR, all affect events prior to the establishment of the HR (Dietrich et al., 1994; Greenberg et al., 1994). Such spontaneous necrotic lesions have not been observed in *cpr1* plants; thus, the site of action for *CPR1* seems to be downstream of these HR-regulating genes. However, complementation tests between *cpr1* and *acd2* or *lsd* mutants must be performed to rule out definitively the possibility that *cpr1* is an allele of the known *ACD2* or *LSD* genes that has constitutive SAR in the absence of visible lesions. Furthermore, our studies argue that *CPR1* is a negative regulator acting prior to SA production. Finally, the pathway

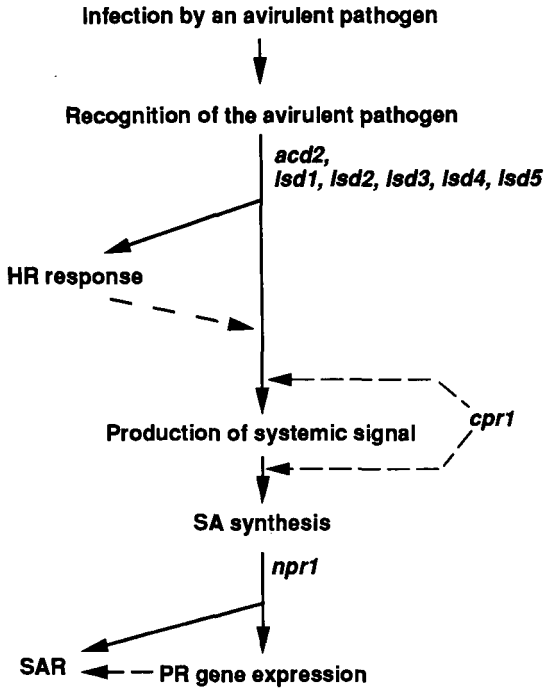

Figure 9. Proposed Placement of Mutants in the SAR Signaling Pathway.

The proposed signal transduction pathway is outlined by solid and dotted lines and arrows. The *cpr1* mutation is placed upstream of SA synthesis, either before or after production of the systemic signal(s).

transducing the SA signal has been marked by another Arabidopsis mutant, designated *npr1*, which is nonresponsive to SA and INA (Cao et al., 1994; Figure 2).

It is interesting that all mutations found thus far that lead to expression of SAR appear to have a detrimental effect on the plants, either by the formation of spontaneous lesions, in the case of *acd2* and *lsd* mutations, or by the appearance of a "stunted" phenotype with *cpr1*. These detrimental effects may explain why SAR is not normally constitutive. The negative effects may offset the advantage of enhanced resistance to pathogens afforded by constitutive SAR.

In summary, we have developed a useful strategy to identify and characterize SAR-related mutants, such as *cpr1*. With these mutants, we can begin genetic analyses to dissect the signaling pathway of SAR. Cloning and further characterization of the regulatory genes associated with these mutants will help to elucidate the molecular basis of SAR.

METHODS

Plant Growth Conditions

Arabidopsis thaliana ecotypes Columbia (Col-0) and Landsberg *erecta* were grown either in pots on Metro-Mix 200 soil (Grace-Sierra, Malpitas,

CA) or on plates with Murashige and Skoog (MS) media (Murashige and Skoog, 1962) containing 2% sucrose and 0.8% agar. Unless otherwise specified, those plants grown on soil were kept in a growth room at high humidity with a 14-hr photoperiod under fluorescent lamps; those grown on MS plates were kept in a growth chamber at 22°C and 70% humidity with a 14-hr photoperiod at a light intensity of 100 μE m^{-2} sec^{-1}. All seeds were vernalized at 4°C for 2 days before placement in a growth environment.

Construction of the Transgenic Line Containing the Reporter Gene

A 2185-bp XbaI-BglII fragment containing the 5′ region of the β-1,3-glucanase (*BGL2*) gene was cloned into the bacterial phage vector M13mp19. To generate an in-frame fusion, an oligonucleotide with the sequence 5′-CCCGTAGCATGCTCCGATTTG-3′ was used to create an SphI restriction site at the initiation codon of *BGL2* by making a T-to-C transition in the position −1 to the ATG codon (Figure 1). The XbaI-SphI fragment was then cloned into the vector pUC19GUS(NcoI) using XbaI-NcoI sites (Dewdney, 1993). The NcoI site in the vector pUC19GUS(NcoI) was at the ATG translation initiation codon of the β-glucuronidase (*GUS*) gene. The SphI-to-NcoI ligation was accomplished by removing the SphI 3′ overhang in the XbaI-SphI fragment by T4 polymerase and filling in the NcoI 5′ overhang of pUC19GUS by using the Klenow fragment of DNA polymerase I (Ausubel et al., 1994). The correct in-frame fusion of the *GUS* coding sequence to the *BGL2* transcription and translation control elements was verified by sequencing. The EcoRI-SalI fragment that contained the *BGL2–GUS* chimeric gene was transferred into the transformation vector pBI101, and the resulting plasmid was used to transform Arabidopsis ecotype Columbia (Valvekens et al., 1988). Transformants were identified by growth in MS medium containing 50 μg/mL kanamycin and by DNA gel blot analysis of the genomic DNA isolated from each transformant (Ausubel et al., 1994).

Mutant Isolation

An Arabidopsis transgenic line carrying the reporter gene *BGL2–GUS* was mutagenized by soaking ~36,000 seeds homozygous for *BGL2–GUS* in 100 mL of 0.3% ethyl methanesulfonate for 11 hr with gentle shaking. Seeds were then washed with water 15 times, resuspended in 0.1% agar, and sown at about 2 per cm^2 of soil. Seventy-five percent of the mutagenized seeds germinated and were allowed to grow, self-fertilize, and set seed. The resulting M_2 seeds were collected in 12 independent pools and germinated on MS agar with 2% sucrose, 50 μg/mL kanamycin, and 100 μg/mL ampicillin. Fifteen days after plating, seedlings were numbered and then a single leaf was removed from each and put into a corresponding microtiter plate well containing 100 μL of GUS substrate solution (50 mM Na_2HPO_4, pH 7.0, 10 mM Na_2EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 0.7 μL/mL β-mercaptoethanol, 0.7 mg/mL 4-methylumbelliferyl β-D-glucuronide [MUG]; Jefferson, 1987). Samples were infiltrated with substrate by placing the microtiter plates under vacuum, incubated at 37°C, and then examined for the fluorescent product of GUS activity (4-methylumbelliferone [4-MU]) under a long-wavelength UV light after 3, 6, 12, and 24 hr of incubation. Those M_2 seedlings constitutively expressing GUS activity were considered putative mutants and were transplanted to soil for seed setting. This procedure was repeated with the progeny of these putative mutants to ensure that the mutant phenotype was heritable and to identify the homozygous mutants. Controls included on each microtiter plate were wild-type *BGL2–GUS* plants grown on MS plates alone (for no GUS activity) and grown on MS plates supplemented with either 0.5 mM salicylic acid (SA) or 0.1 mM 2,6-dichloroisonicotinic acid (INA; obtained from Ciba-Geigy LTD, Basel, Switzerland) to induce *GUS* expression.

Quantitative GUS Assay

Fifteen-day-old wild-type and *cpr1* seedlings grown on MS medium, MS medium with 0.5 mM SA, and MS medium with 0.1 mM INA were collected and frozen in liquid nitrogen; three replicates were taken for each genotype and treatment. The samples were homogenized in extraction buffer (50 mM Na_2HPO_4, pH 7.0, 10 mM Na_2EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 0.7 μL/mL β-mercaptoethanol; Jefferson, 1987) and centrifuged for 5 min at 14,000 rpm in a microcentrifuge at 4°C. Supernatant aliquots were taken for determination of protein concentration and enzyme activity. The protein concentration was determined using the Bio-Rad dye reagent, and GUS activity was determined using a solution containing 5 μL of extract, 495 μL of extraction buffer, and 500 μL of 2 mM MUG in extraction buffer; the accumulation of the product (4-MU) was measured fluorometrically using a spectrofluorophotometer (RF5000U; Shimadzu Corporation, Kyoto, Japan).

Histochemical Localization of GUS Activity

GUS activity was localized histochemically using a staining solution containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc; first dissolved in dimethyl formamide at 25 mg/mL) in 0.1 M Na_2HPO_4, pH 7.0, 10 mM Na_2EDTA, 0.5 mM potassium ferricyanide/ferrocyanide, 0.06% Triton X-100 (Jefferson et al., 1987). Samples were infiltrated with substrate under vacuum and incubated at 26°C for 24 to 48 hr. The staining buffer was then removed, and the samples were cleared by sequential changes of 30, 75, and 95% ethanol. Sectioning and embedding were performed on some samples, from which slides were prepared for microphotography.

RNA Analysis

Tissue samples were collected from 2-week-old seedlings grown on MS plates or MS plates containing 0.1 mM INA or from 4-week-old plants grown on soil. Samples were frozen in liquid nitrogen after collection, and RNA was isolated from frozen samples by an 80°C phenol–chloroform extraction as previously described (Cao et al., 1994). The RNA concentrations were determined by UV absorbance, and 5-μg samples were separated by electrophoresis through formaldehyde–agarose gels and transferred to a hybridization membrane (GeneScreen; DuPont-New England Nuclear), as described by Ausubel et al. (1994). Ethidium bromide (40 μg/mL) was added to each sample to allow visualization of RNA under UV light for confirmation of equal sample loading. ^{32}P-labeled DNA probes for *BGL2*, pathogenesis-related protein-1 (PR-1), PR-5, and β-ATPase mRNAs were prepared as described by Cao et al. (1994). Hybridization and washing conditions were as previously described (Church and Gilbert, 1984; Cao et al., 1994). Relative radioactivity in each RNA band was determined using a PhosphorImager and ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA).

Construction of the Transgenic Line Expressing the Salicylate Hydroxylase Gene

A 1.8-kb HindIII-HpaI fragment containing the *nahG* gene encoding salicylate hydroxylase (Yen and Gunsalus, 1982; Schell, 1986; You et al., 1991) was purified from the plasmid pSR20 (M.A. Schell, unpublished data) and cloned into the pBluescript SK+ (Stratagene, La Jolla, CA) HindIII and SmaI sites. Single-stranded DNA was made from this pSKnahG clone using the helper phage R408. A 24-nucleotide oligonucleotide, 5′-ACGAGTACACCATGGAAAACAATA-3′, was used to direct the synthesis of the complementary strand that created a NcoI site at the ATG translation initiation codon of the *nahG* gene. The A-to-G transition in the +4 position of *nahG* also led to a lysine-to-glutamic acid change in the second amino acid of salicylate hydroxylase. The activity of the modified enzyme was tested by plating the bacteria containing this mutant clone on Luria-Bertani medium supplemented with 0.1% SA. Bacteria that produced active salicylate hydroxylase formed brown colonies due to the oxidation of SA by the enzyme. A NcoI-BamHI fragment that contained the entire *nahG* coding sequence was generated by a partial NcoI digestion at the ATG start codon of *nahG* and a complete BamHI digestion in the pBluescript SK+ vector BamHI site. This fragment was then ligated into NcoI and BamHI sites of the vector pRTL2 (Restrepo et al., 1990), which generated an in-frame fusion of the *nahG* gene under the regulation of the following: the cauliflower mosaic virus 35S promoter with a duplicated enhancer; the 5′ untranslated sequence of tobacco etch potyvirus, which enhances translation initiation (Carrington and Freed, 1990; Carrington et al., 1990); and the 35S poly(A) signal. The fusion gene was moved into a Ti plasmid vector pBin19 (Bevan, 1984) through HindIII digestion. This 35S–*nahG* fusion construct was then transformed into Arabidopsis ecotype Landsberg *erecta* (*erler*) using the transformation procedure previously described by Valvekens et al. (1988).

Genetic Analysis

Crosses were performed by dissecting and emasculating unopened buds and then using the pistils as recipients for pollen from three to four opened flowers. Pollen from homozygous *cpr1* plants was used to fertilize both the wild-type *BGL2–GUS* plants and the transgenic line expressing salicylate hydroxylase (*nahG*/*nahG*, *erler*). The reciprocal crosses were also performed. F_1 and F_2 plants were grown on MS plates and tested for GUS activity by the microtiter plate assay and histochemical staining, as previously described. The absence of the recessive *er* phenotype in F_1 plants proved that the crosses with *nahG* plants were successful. GUS activity was scored for F_2 plants grown on MS plates supplemented with 50 μg/mL kanamycin. For the F_2 generation of the *BGL2–GUS* backcross, three-fourths of the plants were expected to have GUS activity if *cpr1* were dominant and one-fourth if *cpr1* were recessive. For analysis of the F_2 generation of the cross with *nahG*, it was known that (1) the *cpr1* mutation is recessive; (2) only one copy of the *BGL2–GUS* gene is required for positive detection of GUS; and (3) a single copy of the *nahG* gene produces a sufficient amount of salicylate hydroxylase to suppress the SA-induced *BGL2–GUS* expression. Thus, the predicted F_2 segregation ratios were calculated as follows: three-fourths of the plants should have at least one copy of the *BGL2–GUS* gene, and one-fourth should have two copies of the recessive *cpr1* mutation; thus, three-sixteenths would be expected to express GUS activity constitutively if *nahG* has no effect on *cpr1*. However, plants were required to have at least one copy of the kanamycin resistance gene carried in either the *BGL2–GUS* or the *nahG* construct to survive the selection. Therefore, one-sixteenth of the plants died, and the expected fraction of *GUS* expressers in the F_2 progeny was modified to three-fifteenths (one-fifth), or a ratio of expressers to nonexpressers of 1:4. The alternative hypothesis that *nahG* suppresses the expression of *BGL2–GUS* in *cpr1*/*cpr1* plants would predict that among these potential *GUS* expressers, only the one-fourth of them lacking *nahG* should express GUS activity. This brings the predicted fraction of *GUS* expressers in the F_2 generation to one-twentieth (a ratio of 1:19) if the presence of a single *nahG* gene will suppress the *cpr1* phenotype. Segregation in the F_2 generations was analyzed with chi-square tests for goodness of fit (Sokal and Rohlf, 1981).

Determination of Endogenous Levels of SA and SA β-Glucoside

Leaf tissue was collected from 4-week-old wild-type and *cpr1* plants grown on soil, weighed, and frozen in liquid nitrogen. For each sample, ~1 g of the frozen tissue was extracted and quantitated for free SA and SA β-glucoside (SAG) essentially as described previously (Malamy et al., 1992; Hennig et al., 1993). Briefly, the tissue was homogenized in 3 mL of 90% methanol. After centrifugation, the pellet was reextracted with 100% methanol. The combined supernatants were dried in a speed vacuum with heat (~40°C). The residue was resuspended in 2.5 mL of 5% trichloroacetic acid and sonicated for 10 min. The free SA was then separated from conjugated SA through organic extraction with 2 volumes of ethylacetate–cyclopentane–isopropanol (50:50:1). The organic phase containing the free SA was then dried under nitrogen. The dried extract was suspended in 0.5 mL of 0.01 N H_2SO_4, filtered, and analyzed by HPLC as previously described (Hennig et al., 1993). HPLC was performed on an ARH-602 aromatic acids column (0.95 × 10 cm; Interaction Chemical Inc., Mountain View, CA) run at 45°C in 0.01 N H_2SO_4 with a flow rate of 0.6 mL/min.

The amount of SA conjugate was quantitated as follows. After the two-phase separation previously described, the aqueous phase (which contains the conjugated SA) was acidified with HCl to pH 1 and boiled for 30 min to release SA from any acid-labile conjugated forms. The released free SA was then extracted with the organic mixture and analyzed as previously described. To confirm that the SA released from conjugated SA by acid hydrolysis was derived mainly from SAG, we compared SA levels released after either acid hydrolysis or β-glucosidase digestion. Greater than 80% of the acid-releasable SA from Arabidopsis extracts could also be recovered after β-glucosidase treatment. We were unable to use area integration to quantify very low levels of SA reliably (e.g., some of the wild-type samples) because Arabidopsis samples contained large amounts of a fluorescent compound that eluted off the column immediately preceding the SA peak. Despite changing HPLC columns (aromatic acid versus Zorbax phenyl and Zorbax ODS columns; BTR Separations, Wilmington, DE), flow rates, sample volume, and solvent conditions, we have been unable to remove or separate further this unknown contaminant from the SA peak. Therefore, free SA levels, which were often lower than the amounts of the fluorescent contaminant, were determined based on peak height using a standard curve constructed with known amounts of SA. Because this contaminant was extracted into the first organic phase, it did not interfere with determination of the SAG levels, which were quantitated by area integration of the HPLC peaks.

Infection with *Peronospora parasitica* NOCO2

Both wild-type and *cpr1* plants grown on soil with a 12-hr photoperiod were infected with a conidial suspension of *P. parasitica* NOCO2 (3 × 10^4 spores per mL) by spraying and were then kept in a growth chamber at 80% or greater humidity and 18 to 19°C. Seven days after inoculation, plants were scored for the presence of conidiophores using a magnifying glass (×4) and photographed.

Infection with *Pseudomonas syringae* pv *maculicola* ES4326

P. s. maculicola ES4326 was grown at 28°C on King's B agar plates or in King's B liquid medium (King et al., 1954) supplemented with 100 μg/mL streptomycin for selection. Bacteria were then collected by centrifugation and resuspended at OD_{600} = 0.2 in a solution of 10 mM $MgCl_2$ plus the surfactant Silwet L-77 (0.01%; Union Carbide, Danbury, CT). Both wild-type and *cpr1* plants were grown on soil as previously described in 2.5-in square pots covered with fine mesh veil. Four-week-old plants were thoroughly wetted by dipping into the solution described above with or without bacteria. Two days prior to dipping, some plants were sprayed with a 0.65 mM solution of INA. Tissue samples of about 100 mg were collected and weighed at time points of 0, 1, 2, and 3 days after inoculation with bacteria; these samples each contained leaf tissue from 10 to 15 plants growing on one pot. The leaf tissue was homogenized to extract the bacteria as described by Cao et al. (1994). Dilutions were plated on King's B plates with streptomycin for determination of colony-forming units. Statistical analyses were performed by Student's *t* tests of the differences between two means of log-transformed data (Sokal and Rohlf, 1981).

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