Characterization of a New Arabidopsis Mutant Exhibiting Enhanced Disease Resistance

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In many plant-pathogen interactions, resistance is associated with the synthesis and accumulation of salicylic acid (SA) and pathogenesis-related (PR) proteins. At least two general classes of mutants with altered resistance to pathogen attack have been identified in *Arabidopsis*. One class exhibits increased susceptibility to pathogen infection; the other class exhibits enhanced resistance to pathogens. In an attempt to identify mutations in resistance-associated loci, we screened a population of T-DNA tagged *Arabidopsis thaliana* ecotype Wassilewskija (Ws) for mutants showing constitutive expression of the *PR-1* gene (*cep*). A mutant was isolated and shown to constitutively express *PR-1*, *PR-2*, and *PR-5* genes. This constitutive phenotype segregated as a single recessive trait in the Ws genetic background. The mutant also had elevated levels of SA, which are responsible for the *cep* phenotype. The *cep* mutant spontaneously formed hypersensitive response (HR)-like lesions on the leaves and cotyledons and also exhibited enhanced resistance to virulent bacterial and fungal pathogens. Genetic analyses of segregating progeny from outcrosses to other ecotypes unexpectedly revealed that alterations in more than one gene condition the constitutive expression of *PR* genes in the original mutant. One of the mutations, designated *cpr20*, maps to the lower arm of chromosome 4 and is required for the *cep* phenotype. Another mutation, which has been termed *cpr21*, maps to chromosome 1 and is often, but not always, associated with this phenotype. The recessive nature of the *cep* trait suggests that the *CPR20* and *CPR21* proteins may act as negative regulators in the disease resistance signal transduction pathway.

Additional keywords: *nahG*, pathogen resistance, systemic acquired resistance.

When a plant is infected by a pathogen, one of several possible outcomes can occur. The plant may be unable to control the growth and/or spread of the pathogen, which leads to disease. Alternatively, the plant may be able to resist pathogen invasion. In some cases, resistance is passive; the plant fails to provide the appropriate environment to support pathogen

colonization. In other cases, resistance is an active process in which the plant induces a cascade of defense responses. Genetic studies of plant-microbe interactions have shown that active resistance is usually conditioned by a plant resistance gene, whose product directly or indirectly recognizes a race-specific avirulence determinant produced by the pathogen (Keen 1990; Scofield et al. 1996; Tang et al. 1996; Yang et al. 1997). One of the most visible signs that a plant is resisting pathogen attack is the development of a hypersensitive response (HR) on the inoculated tissue. The HR is characterized by small, necrotic lesions that form around the infection site (Matthews 1991) and may help restrict the growth and spread of the pathogen (Slusarenko et al. 1991). In addition, antimicrobial compounds (phytoalexins) often are produced (Dixon 1986) and the cell wall is strengthened as a result of lignification and the cross-linking of cell wall proteins (Bowles 1990; Bradley et al. 1992; Brisson et al. 1994). In addition to the HR, the uninfected portion of the plant develops increased resistance to a secondary infection by even unrelated pathogens. This nonspecific resistance is known as systemic acquired resistance (SAR; Chester 1933; Ross 1961; Ryals et al. 1996) and it is associated with the systemic expression of SAR genes, which include several families of pathogenesis-related (*PR*) genes (Ward et al. 1991). In addition to serving as molecular markers for SAR, several of the *PR* proteins have been shown to have activity against pathogens in vitro and/or in vivo (Kombrink and Somssich 1997).

Still relatively little is known about the plant signal transduction pathways following pathogen infection that lead to the local and systemic activation of defense responses, such as *PR* gene expression. However, numerous studies have demonstrated that salicylic acid (SA) is an important component of one or more of these pathways. It has long been known that treating tobacco leaves with SA enhances resistance to tobacco mosaic virus (TMV; White 1979). In addition, SA induces the same or similar set of SAR genes as those expressed during a resistance response to pathogens (Ward et al. 1991). Furthermore, SA levels increase after infection with an avirulent pathogen in tobacco, cucumber, and *Arabidopsis* (Dempsey et al. 1997; Enyedi et al. 1992; Malamy et al. 1990, 1992; Métraux et al. 1990; Uknes et al. 1993; Mauch-Mani and Slusarenko 1996). In tobacco and *Arabidopsis*, this increase in SA precedes or coincides with *PR* gene expression. Some of the strongest evidence that SA plays a role in resistance comes from studies of transgenic tobacco and *Arabidopsis* plants expressing a salicylate hydroxylase-encoding

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(*nahG*) gene from *Pseudomonas putida*. Salicylate hydroxylase converts SA to catechol; thus, plants expressing the *nahG* gene are unable to accumulate SA at high levels. Strikingly, these NahG plants fail to develop SAR and exhibit enhanced susceptibility to pathogen infection (Delaney et al. 1994; Gaffney et al. 1993).

To further characterize the resistance signal transduction pathway, several laboratories have isolated *Arabidopsis* mutants that exhibit altered responses to pathogen infection. Some of these mutants are more susceptible to pathogen infection and fail to express defense genes after SA treatment. In contrast, other mutants are more resistant to pathogens. This latter group can be further divided into three classes. One class spontaneously forms HR-like lesions and concurrently exhibits increases in *PR* gene expression and endogenous SA levels. Included in this class are the *acd2* (accelerated cell death; Greenberg et al. 1994), *cpr5* (constitutive expressor of *PR* genes; Bowling et al. 1997), *ssi1* (suppressor of SA-insensitivity of *npr1-5*; Shah et al. 1999), and *lsd* (lesion simulating disease resistance; Dietrich et al. 1994; Weymann et al. 1995) mutants. The second class of mutants does not form spontaneous HR-like lesions but constitutively expresses *PR* genes and contains elevated levels of SA. Examples of this class are the *cpr1* (Bowling et al. 1994), *cim3* (constitutive immunity; Ryals et al. 1996), and *dnd1* (defense with no HR cell death; Yu et al. 1998) mutants. The last class of mutants confer pathogen resistance in the absence of constitutive *PR* gene expression, e.g., *edr1* (enhanced disease resistance; Frye and Innes 1998).

In order to identify other components in the resistance signal transduction pathway, we have screened a collection of T-DNA tagged lines from *Arabidopsis thaliana* (ecotype Wasilewskija [Ws]; Feldmann 1992) for mutants that constitutively express the *PR-1* gene. Here we describe the isolation and characterization of a new mutant, *cep*, that exhibits constitutive expression of the *PR-1*, *PR-2*, and *PR-5* genes, contains high endogenous levels of SA, spontaneously forms lesions, and shows enhanced resistance to both the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* and the fungal pathogen *Peronospora parasitica* isolate EMWA. Genetic analyses indicate that the *cep* phenotype in our mutant is due to at least two recessive, unlinked mutations that we have termed *cpr20* and *cpr21*, following the nomenclature of Bowling et al. (1994).

RESULTS

Identification of the *cep* mutant.

To date, the majority of mutants exhibiting enhanced disease resistance phenotypes (constitutive SAR mutants) were generated with the chemical mutagen ethylmethane sulfonate (Ryals et al. 1996). However, isolation of the resulting mutant genes requires map-based cloning. Because cloning with this approach is time-consuming and laborious we have screened a collection of T-DNA-containing mutants generated by Agrobacterium-mediated transformation of *Arabidopsis* seeds. Some of the resultant mutations are due to T-DNA insertion events (Feldmann et al. 1992) and, thus, the T-DNA can be used as a convenient tag for the rapid cloning of the mutated gene. Because *PR-1* gene induction is an excellent molecular marker for activation of defense responses in *Arabidopsis*, as

well as tobacco, we screened the T-DNA-containing collection of Ws-derived lines for constitutively high levels of *PR-1* mRNA. Titration analysis with RNA prepared from untreated and SA-treated *Arabidopsis* plants indicated that we should be able to use Northern (RNA) blot analysis to detect a single mutant plant constitutively accumulating high levels of *PR-1* transcript in a pool of 20 plants. A total of 3,000 lines were screened by repeated sowing of 60 seeds (representing 20 lines) in three pools of 20 seeds each. These plants were grown with a 14-h light cycle ($140 \mu\text{E s}^{-1} \text{m}^{-2}$) at 22°C . One leaf was excised from each of the 20 3-week-old plants in the pool, then combined and used for preparation of RNA and Northern analysis. Any pool that exhibited elevated levels of *PR-1* transcript, compared with the wild-type (wt; Ws) control, was further analyzed by separately extracting RNA from each plant in the pool. Through this screening process one mutant called *cep* was identified.

In addition to constitutively expressing the *PR-1* gene, *cep* plants displayed a variety of abnormal phenotypes. These included curly leaves, a slower growth rate, and stunted size at all stages of development, compared with wt plants. Interestingly, the cotyledons of *cep* plants prematurely senesced, and were completely necrotic by 18 days post planting (dpp). Close examination indicated that necrotic lesions began to appear by 10 dpp on the cotyledons. This time correlates with the beginning of constitutive expression of *PR* genes (data not shown).

PR genes are constitutively expressed in *cep* plants.

To analyze the expression of *PR* genes, Northern analysis was performed on individual 3-week-old *cep* and wt plants. As expected, the *cep* plants exhibited constitutive expression of the *PR-1* gene (Fig. 1). In addition, these plants constitutively accumulated transcripts for the *PR-2* and *PR-5* genes. By contrast, little or no *PR* gene expression was detected in any of the wt plants. The pattern of *PR* gene expression in *cep* plants resembles that observed in SA-treated plants, with *PR-1* transcript accumulating to high levels, and *PR-2* and *PR-5* transcripts accumulating to somewhat lower levels.

Endogenous levels of free SA and SAG are elevated in *cep* plants.

SA treatment is known to induce *PR* gene expression in a wide variety of plants and SA levels precede or parallel the

Fig. 1. Constitutive expression of *PR-1*, *PR-2*, and *PR-5* genes in *cep* (constitutive expression of the *PR-1* gene) plants. RNA was prepared from 3-week-old wild-type (wt) and *cep* mutant plants. As a positive control, RNA from a salicylic acid (SA)-treated wt plant was diluted 20-fold with RNA from untreated wt plants. Numbers correspond to RNA from individual wt or *cep* plants.

induction of *PR-I* genes in tobacco and *Arabidopsis* plants resisting infection by either TMV (Malamy et al. 1990, 1992) or turnip crinkle virus (Dempsey et al. 1997), respectively. Thus, the high level of *PR* gene expression in cep plants raised the possibility that this mutation might affect endogenous SA levels. To assess this possibility, endogenous levels of free SA and SA glucoside (SAG) were monitored in cep and wt plants. cep plants were found to contain an eightfold higher basal level of SA and a sevenfold higher level of SAG than those detected in wt plants in the absence of any inducer (Fig. 2).

The cep mutant develops spontaneous lesions.

Several of the *Arabidopsis* mutants that exhibit enhanced disease resistance also spontaneously form lesions (Ryals et al. 1996). These spontaneous lesions exhibit characteristics similar to a pathogen-induced HR, such as the accumulation of cell-wall-associated autofluorescent material (Lummerzhim et al. 1993; Yu et al. 1993). cep plants also were observed to form spontaneous lesions. Initially, these lesions appeared on the cotyledons approximately 10 dpp. Over time, the size of the lesions increased and by 18 dpp the cotyledons were completely necrotic. To determine whether the spontaneous lesions formed on cep cotyledons are associated with the accumulation of autofluorescent compounds, we performed microscopic analysis on 10-day-old cotyledons from cep and wt plants. On the cep cotyledons, the cells associated with the spontaneous lesions exhibited yellow autofluorescence around their perimeter (Fig. 3A). These cells also were irregularly shaped, suggesting some loss of internal structure. In contrast, no accumulation of cell-wall-associated fluorescent material was detected on the cotyledons of wt plants (Fig. 3B). Spontaneous lesions also formed on some of the true leaves of cep plants by 3 weeks post planting. They appeared as localized necrotic spots and exhibited limited expansion. Necrosis was also evident on some of the petioles of 4-week-old cep plants. Interestingly, lesions formed by cep plants were not suppressed by short day length, in contrast to those associated with several *lsd* mutants (Dietrich et al. 1994).

Fig. 2. Endogenous levels of free salicylic acid (SA) and SA glucoside (SAG) in wild-type (wt) and cep (constitutive expression of the *PR-I* gene) plants. Leaves from 3-week-old wt and cep plants were harvested for SA and SAG analyses. Values presented for free SA and SAG (conjugated form) are the average for each of three different sets of samples with standard deviation. This experiment was done twice with similar results.

cep plants are resistant to the virulent pathogens *Pseudomonas syringae* pv. *maculicola* and *Peronospora parasitica* isolate EMWA.

To determine whether cep plants exhibit enhanced disease resistance, we analyzed the growth of two virulent strains of *Pseudomonas syringae* pv. *maculicola* (M4 and M5) in cep plants. Five-week-old plants were infected with either of these strains and bacterial growth and disease symptoms were monitored for several days post inoculation (dpi). Chlorosis was observed at 3 dpi in wt plants, whereas few, if any, symptoms were observed in cep plants (data not shown). While both strains of bacteria multiplied in the wt and cep plants, their growth in cep plants was consistently lower than in wt plants at all the times assayed. This difference was more evident between 3 and 5 dpi for both strains (Fig. 4).

To investigate whether cep plants exhibit enhanced resistance to other types of pathogens, we monitored the growth of *Peronospora parasitica* isolate EMWA, a fungal pathogen that is virulent on the Ws ecotype. Three-week-old plants were infected and sporulation and disease symptoms were monitored. At 6 dpi, conidiophores were observed on the leaves of all wt plants. In contrast, conidiophores were absent in almost all of the cep plants (Table 1). By 14 dpi, all the wt plants were chlorotic and wilted. By contrast, the cep plants did not show any of these symptoms.

The cep phenotype in Ws is conditioned by a single recessive mutation.

To analyze the genetic nature of cep, a backcross was performed between cep plants and Ws wt plants and the resultant progeny were monitored for constitutive *PR-I* gene expression. In the F_1 generation, none of the 14 plants tested exhibited constitutive *PR-I* gene expression, suggesting that the cep trait is recessive (Table 2). The F_2 plants were also scored for constitutive expression of *PR-I*. Out of 430 plants, 95 exhibited constitutive *PR* expression; this approximate ratio of 1 constitutive for *PR* expression : 3 wt ($X^2 = 1.79$; $0.5 > P > 0.1$) confirms that the cep phenotype in our original mutant is due to a recessive mutation in a single nuclear gene. Several cep-associated morphological abnormalities, including curly leaves, necrotic cotyledons, and stunted growth, were observed to segregate with the constitutive expression of the *PR-I* gene. Thus, these morphologies were either caused by the cep mutation or by another, tightly linked mutation.

The cep phenotype in Col-0 or Ler plants appears to require recessive mutations at more than one locus.

Surprisingly, when cep plants were crossed with those of a different ecotype (i.e., Columbia; Col-0), a deviation from the expected ratio of constitutive *PR* expressing to wt progeny was observed in the F_2 generation. None of 10 F_1 progeny plants tested exhibited constitutive expression of *PR-I*, confirming the recessive nature of the mutation (Table 2). However, in the F_2 generation, only 34 plants out of 648 total exhibited constitutive *PR* expression. This ratio (1:17; X^2 [monofactorial] = 134, $P < 0.001$; X^2 [bifactorial] = 2.01, $0.5 > P > 0.1$) could indicate that the cep phenotype is controlled by two independent nuclear loci. Alternatively, the cep phenotype may have a low penetrance in the Ws/Col-0 hybrid background, i.e., not just one but rather a host of modifiers could affect the expression of this phenotype. Similar results were observed in crosses between cep plants and those from

the Landsberg erecta (Ler) ecotype (data not shown). The fact that our initial analysis with Ws plants revealed only one locus suggests that, in this background, the allele(s) at the other locus (loci) does (do) not interfere with the expression of the cep phenotype. Thus, in order to fully characterize this phenotype, we continued to analyze it in plants from the Ws background.

To determine whether the newly arisen mutation is tagged by a T-DNA insert, 14 F₁ plants from a cross between cep and wt (Ws ecotype) were monitored for resistance to kanamycin (Table 3). All 14 plants were kanamycin resistant. These plants were allowed to self-pollinate and kanamycin resistance was tested in 136 F₂ progeny. A ratio of 3 kanamycin-resistant plants : 1 kanamycin-sensitive plant was observed, indicating the presence of a single locus containing one or more T-DNA inserts (Table 3). This was confirmed by Southern blot analysis with the left and right borders of the T-DNA inserts as probes (data not shown). An additional pool of F₂ plants (derived from the same F₁ parent) was screened for the curly leaves and necrotic cotyledons associated with the cep phenotype. A total of 88 putative cep mutants were identified and their cep phenotype was confirmed by analyzing *PR* gene expression. These 88 plants were allowed to self-pollinate and approximately 50 F₃ progeny from each line were tested for kanamycin resistance. If the cep phenotype were due to the T-

Fig. 3. Accumulation of autofluorescent cell wall material in (A) cep (constitutive expression of the *PR-I* gene) and (B) wild-type (wt) plants. Cotyledons from 10-day-old cep and wt plants were used for microscopic observation. Bars = 49 μ m.

Fig. 4. Growth of *Pseudomonas syringae* pv. *maculicola* (Psm) strains (A) M4 and (B) M5 in wild-type (wt) and cep (constitutive expression of the *PR-I* gene) plants. Bacterial strains M4 (10^5 CFU ml⁻¹) and M5 (10^4 CFU ml⁻¹) were suspended in 10 mM MgCl₂ and injected in the abaxial surface of 12 leaves per time point. CFU of M4 and M5 per leaf disk (0.28 cm²) were determined at various time points (days) after inoculation as described in Materials and Methods. Values represent average and standard deviation calculated from four samples containing three leaf disks each. This experiment was done three times with similar results.

Table 1. *Peronospora parasitica* isolate EMWA-induced disease

Plants ^b	Number of plants expressing disease levels ^a				Total plants
	0	+	++	+++	
Wild type	0	0	6	160	166
cep	158	4	4	0	166

^a Scale for level of disease: 0, no conidiophores on plant; +, at least one leaf with one to five conidiophores; ++, many leaves with five to twenty conidiophores; +++, all inoculated leaves with more than 20 conidiophores. All plants were scored at 6 days post inoculation.

^b All plants are from the Wassilewskija (Ws) ecotype. cep = constitutive expression of the *PR-I* gene.

DNA insertion, the progeny of all 88 families should be uniformly kanamycin resistant. However, only 11 were uniformly kanamycin resistant (Table 3), indicating that the new mutation is unlinked to the T-DNA.

The cep phenotype is suppressed in NahG plants.

The high endogenous levels of SA and SAG in cep plants suggested that the *CEP* gene product(s) might act upstream of SA in the pathway leading to *PR* gene expression. To confirm this, we crossed homozygous cep plants with a homozygous transgenic Ler line expressing the *nahG* gene (Bowling et al. 1994). Analysis of the resultant progeny showed that none of the F_1 plants expressed the *PR-1* gene constitutively, as expected of a recessive trait (Table 2). In the F_2 generation, different ratios of cep to wt plants could be predicted, depending on the number of factors that control the cep phenotype in the

cep $\times$ Ler intercross (see previous section). However, one prediction can be made with certainty. If the *nahG* gene product suppresses the cep phenotype, none of the mutant plants segregating in the F_2 should carry this gene. Alternatively, if expression of the cep phenotype is independent of the *nahG* product, 3/4 of the mutant plants should carry the *nahG* gene in either the homozygous or heterozygous condition. In the F_2 population, only 8 of 837 plants exhibited constitutive *PR-1* gene expression, stunted growth, and spontaneous lesion formation (Table 2). None of these 8 plants accumulated *nahG* transcript (data not shown), confirming that they lack the *nahG* transgene. The probability that, by chance alone, none of the 8 cep plants would have received at least one copy of the *nahG* transgene is $(1/4)^8$ or 1.5×10^{-5} . These observations support the notion that the *nahG* gene product suppresses the cep phenotype and that the only plants with a cep phenotype that can be recovered in the segregating population are those that lack the *nahG* transgene. We conclude from these data that CEP functions upstream of SA in the *PR* gene induction pathway.

The cep phenotype is not expressed in outcrosses to *cpr1*, *cpr5*, and *acd2*.

Like cep, mutants carrying either *cpr1*, *cpr5*, or *acd2* (Col-0 ecotype) constitutively express the *PR-1*, *PR-2*, and *PR-5* genes and exhibit enhanced disease resistance to various pathogens. In addition, the *cpr5* and *acd2* plants develop spontaneous lesions. To test for complementation of the constitutive phenotypes conditioned by these recessive mutations, cep plants were crossed to *cpr1*, *cpr5*, and *acd2* plants. For all three crosses, the F_1 progeny failed to constitutively express the *PR-1* gene or develop any of the morphological abnormalities associated with the different mutants (Table 2). The most likely interpretation of these results is that the cep phenotype is not due to a mutation in any of the above genes.

Mapping of mutation(s) producing the cep phenotype.

In order to map the mutation(s) responsible for the cep phenotype, we crossed Ws cep plants with Col-0 wt plants. As expected for a recessive trait, the F_1 progeny did not exhibit constitutive *PR-1* gene expression or the curly leaves and stunted growth morphology associated with cep (data not shown). These plants were allowed to self-pollinate and the F_2 progeny were screened for plants exhibiting curly leaves and stunted growth. Seventy-nine such plants were identified. To confirm the cep phenotype of these plants, F_3 progeny derived

Table 2. Genetic and complementation analyses

Cross ^a	Gen- era- tion	Total	PR-1 expression				<i>X</i> ²	<i>P</i>
			Consti- tutive		Not consti- tutive			
			O ^b	E ^b	O	E		
Ws cep × Ws wt	F ₁	14	0	0	14	14	1.79 ^c	>0.1
	F ₂	430	95	107	335	323		
Ws cep × Col-0 wt	F ₁	10	0	0	10	10	1.28 ^d	>0.1
	F ₂	648	34	41	614	607		
Ws cep × Ler <i>nahG/nahG</i>	F ₁	10	0	0	10	10		
	F ₂	837	8	— ^e	829	— ^e		
Ws cep × Col-0 <i>cpr1/cpr1</i>	F ₁	6	0	6	6	0		
	F ₂	137	26	137	111	0		
Ws cep × Col-0 <i>cpr5/cpr5</i>	F ₁	6	0	6	6	0		
	F ₂	41	15	41	26	0		
Ws cep × Col-0 <i>acd2-2/acd2-2</i>	F ₁	5	0	5	5	0		

^a Genotype of the pollen accepting plant is indicated first and that of the pollen donor second. Ws, Wassilewskija; Col-0, Columbia; Ler, Landsberg erecta; cep, constitutive expression of the *PR-1* gene.

^b O, observed; E, expected.

^c X^2 calculation based on a 1:3 ratio.

^d X^2 calculation based on a 1:15 ratio.

^e No expected value is provided as the number of factors controlling the cep phenotype in this cross is currently undetermined.

Table 3. Segregation analysis of the cep phenotype and kanamycin resistance

Kanamycin resistance										
Cross ^a	Generation	Total	Resistant		Segragating		Sensitive		Hypothesis	Fit ?
			O ^b	E ^b	O	E	O	E		
Ws cep kan ^r × Ws wt	F ₁	14	14	14 ^c			0	0 ^v		
	F ₂	136	102	102			34	34		
	F ₃	88 ^d	11	88 ^e	30	0 ^e	47	0 ^e	Link to T-DNA	NO

^a The pollen accepting plant is indicated first and the pollen donor second. Ws, Wassilewskija; cep, constitutive expression of the *PR-1* gene; kan^r, kanamycin resistance marker; wt, wild type.

^b O, observed; E, expected.

^c Expected for the dominant kan^r selectable marker.

^d Families of cep plants obtained from the F_2 families resistant to kanamycin with the morphological phenotype (curly leaves and stunted growth) associated with constitutive *PR-1* expression.

^e Expected for complete linkage of the mutation with the T-DNA.

by selfing the 79 individual F₂ plants were tested for constitutive *PR-1* gene expression. The chromosomal location of the mutation(s) responsible for the cep phenotype in this segregating population was then mapped with the 79 F₂ plants and a variety of CAPS (cleaved co-dominant amplified polymorphic sequences; Konieczny and Ausubel 1993) and SSLP (single sequence length polymorphism; Bell and Ecker 1994) markers (Table 4). Because these markers are co-dominant, the genotype for all 158 chromosomes could be monitored at every locus tested. Through this analysis it was determined that a mutation necessary for expression of the cep phenotype maps to the lower arm of chromosome 4, approximately 2.5 cM north of nga1107 and 4.5 cM south of PAP7.2. We have designated this mutation *cpr20*. This most likely corresponds to the new mutation identified in the T-DNA-mutagenized Ws population.

Among the 79 cep F₂ plants, the segment of chromosome 1 between nga280 and β -fructosidase gene was homozygous Ws in 62, heterozygous in 9, and homozygous Col-0 in 8. This chromosome segment segregated in the expected Mendelian

ratio within a random sample of wt F₂ segregants examined (data not shown), confirming that the strong deviation from expectation seen among the cep segregants was not due to abnormal transmission of the Col-0 markers. These data indicate that a second Ws-derived factor in chromosome 1 is often, but not always, associated with expression of the mutant phenotype in the F₂ segregants of a Ws cep $\times$ Col-0 wt cross. We have designated this factor *cpr21*. The simplest interpretation of the F₂ segregation data is that the normal *CPR20* and *CPR21* genes specify redundant functions.

DISCUSSION

In an effort to identify new components in the resistance signaling pathway, we screened T-DNA-mutagenized *Arabidopsis thaliana* plants for mutants exhibiting constitutive expression of defense responses. Through this process, a mutant that constitutively expresses the *PR-1*, *PR-2*, and *PR-5* genes was isolated (Fig. 1) and named cep. These plants also de-

Table 4. CAPS (cleaved co-dominant amplified polymorphic sequences) and SSLP (single sequence length polymorphism) analysis of cep mutant

Plant no. ^a	Chromosome 1						Chromosome 4			
	nga280	β -fructosidase	Nim	mi424	mi185	nga111	nga1139	AP2.3	PAP72.1	nga1107
18	H ^b	H	H	H	H	H	W	W	W	W
19	W ^c	W	H	H	H	H	W	W	W	W
52	W	W	W	H	H	C ^d	W	W	W	H
53	W	W	W	W	W	W	W	W	W	W
85	H	H	H	H	H	H	H	W	W	W
86	W	W	W	W	W	H	W	W	W	W
89	W	W	W	W	W	W	W	W	W	H
111	W	W	W	W	W	H	W	W	W	W
119	W	W	W	W	H	H	W	W	W	W
130	H	W	W	W	W	W	W	W	W	H
136	W	W	W	W	W	W	W	W	W	W
138	H	H	H	H	W	W	W	W	W	W
151	H	H	H	H	H	W	W	W	W	W
197	W	W	W	W	W	W	W	W	W	W
213	W	W	W	W	W	W	W	W	W	W
225	W	W	W	W	W	W	W	W	W	W
233	W	W	W	W	W	W	H	W	W	W
257	W	W	W	W	W	W	W	W	W	W
303	H	H	H	H	H	W	H	H	H	W
311	W	W	W	W	W	W	W	W	W	W
332	H	W	W	W	H	H	W	W	W	W
347	W	W	W	W	W	W	W	W	W	W
348	W	W	W	W	W	W	W	W	W	W
371	W	W	W	W	W	W	W	W	W	W
372	W	W	W	W	W	H	W	W	W	W
383	W	W	W	W	W	W	W	W	W	W
386	W	W	H	H	H	H	W	W	W	H
393	W	W	W	W	H	C	W	W	W	W
398	W	W	W	W	H	W	W	W	W	H
405	W	W	W	W	W	W	W	W	W	W
411	W	W	H	H	H	H	W	W	W	W
416	C	C	C	C	C	C	W	W	W	W
421-b	W	W	H	H	H	H	W	W	W	W
421-c	W	W	W	W	H	H	W	W	W	W
424-b	W	W	W	W	W	H	W	W	W	W
435-b	W	W	W	W	W	W	W	W	W	W

(continued on next page)

^a All 79 plants analyzed exhibited the cep phenotype including curly leaves and stunted growth morphology and constitutive expression of the *PR-1* gene.

^b Heterozygous.

^c Homozygous Ws; Wassilewskija.

^d Homozygous Col-0; Columbia-0/

^e Not determined.

velop curly leaves and their growth is stunted, compared with wt plants. Interestingly, cep plants contained approximately eightfold higher levels of SA and SAG than those detected in wt plants (Fig. 2). Because SA appears to be a key component of the resistance signaling pathway, and endogenous SA levels increase in tobacco and Arabidopsis plants before or in parallel with the induction of *PR* gene expression during the HR and SAR (Ryals et al. 1996; Durner et al. 1997; Yang et al. 1997), these elevated SA levels are probably responsible for the constitutive *PR* gene expression observed in cep plants. This possibility was confirmed by the demonstration that both the morphological characteristics associated with the cep mutant (lesion formation, stunted growth, and curly leaves) and constitutive *PR* gene expression were suppressed in the F₂ progeny of a cross between cep and NahG plants (Table 2).

In addition to constitutively expressing several defense genes and containing elevated levels of SA, cep plants developed spontaneous lesions on their cotyledons and leaves. Microscopic analysis indicated that the deposition of auto-

fluorescent cell wall material was associated with these lesions (Fig. 3). The nature of this autofluorescent material is not known. However, it is thought to be composed of cross-linked phenolic compounds, which might strengthen the cell wall (Schröder et al. 1992). The deposition of autofluorescent cell wall material has previously been associated with the HR and resistance in several plant-pathogen interactions (Dempsey et al. 1997; Lummerzheim et al. 1993; Schröder et al. 1992; Yu et al. 1993). Accumulation of autofluorescent compounds has also been documented in the spontaneous lesions developed by some of the previously described enhanced disease resistance mutants of Arabidopsis (Greenberg et al. 1994; Dietrich et al. 1994; Weymann et al. 1995). However, unlike the lesions formed by the *acd2* and *lsd1* mutants, those developed by cep plants did not spread throughout the entire leaf. Furthermore, spontaneous lesion formation in cep plants was not environmentally regulated, whereas a specific day length was required to initiate lesion development in several of the *lsd* mutants (Dietrich et al.

Table 4. (continued from preceding page)

Plant no. ^a	Chromosome 1						Chromosome 4			
	nga280	β-fructosidase	Nim	mi424	mi185	nga111	nga1139	AP2.3	PAP72.1	nga1107
437-a	W	W	W	W	W	W	W	W	W	W
438	W	W	W	W	W	W	W	W	W	W
445	W	W	W	W	W	H	W	W	W	W
451	W	W	W	W	W	W	W	W	W	W
458	H	H	H	H	H	H	W	W	W	W
478-b	C	C	C	C	H	H	H	H	H	W
481	W	W	W	W	W	W	W	W	W	W
485	W	W	W	W	W	W	H	W	W	W
501	W	W	W	W	W	H	H	W	W	W
502	W	W	W	W	W	W	H	H	H	W
504	W	W	W	W	W	H	W	W	W	W
507	W	W	W	W	W	W	W	W	W	W
513	C	C	C	C	C	C	H	W	H	W
526a	C	C	C	C	C	C	H	W	W	W
526b	H	C	H	H	H	H	H	H	H	W
530	W	W	W	W	W	H	C	W	W	W
536	W	W	W	W	W	W	H	W	W	W
539a	W	W	W	W	W	W	W	W	W	W
539b	W	W	W	W	W	W	W	W	W	W
543	H	H	H	H	H	H	H	W	W	W
550	W	W	W	W	W	W	W	W	W	W
551	C	C	C	C	C	C	H	H	H	W
569	W	W	W	W	W	H	W	W	W	W
574	W	W	W	W	W	W	W	W	W	W
577	W	W	W	W	W	W	W	W	W	W
591a	W	W	W	W	W	H	W	W	W	W
591b	C	C	C	C	C	C	W	W	W	W
597b	W	W	W	W	H	H	W	W	W	W
601	C	C	C	C	C	H	H	H	H	W
606	W	H	H	H	H	H	W	W	W	W
607	W	W	W	W	W	W	W	W	W	W
610	H	H	H	H	W	H	H	H	H	W
622a	W	W	W	W	W	W	H	W	W	W
622b	W	W	H	ND ^c	W	W	W	W	W	W
625	W	W	W	W	W	W	W	W	W	W
627	H	W	W	W	W	W	W	W	W	W
541	W	W	W	W	W	H	W	W	W	W
549	W	W	W	W	W	W	W	W	W	W
579a	W	W	W	W	W	W	W	W	W	W
579b	H	W	W	W	W	W	W	W	W	W
587	W	W	W	W	W	H	W	W	W	W
597a	W	W	W	W	W	H	W	W	W	W
600	H	H	H	H	W	W	W	W	W	W

1994). Once visible lesions began to develop on cep plants, constitutive *PR* gene expression was activated. Interestingly, high levels of *PR* transcripts could be detected in the healthy leaves as well as those with visible lesions (data not shown). In contrast, high levels of *PR* expression were not found in the healthy leaves of *acd2* mutant plants until at least two leaves had become completely necrotic (Greenberg et al. 1994).

Because lesion formation, *PR* gene expression, and elevated SA levels are phenomena that correlate with disease resistance in many plant species, the response of mutant plants to infection with virulent pathogens was assessed. At every time point assayed, growth of either the M4 or M5 strain of *Pseudomonas syringae* pv. *maculicola* was lower in cep plants than in wt plants (Fig. 4). In addition, mutant plants were more resistant to infection by *Peronospora parasitica* isolate EMWA than wt plants. Almost none of the infected cep plants had conidiophores on their leaves, whereas all of the wt plants were efficiently colonized by *Peronospora parasitica* (Table 1). The reduction in pathogen growth and disease symptom severity demonstrates that cep plants exhibited enhanced disease resistance to both bacterial and fungal pathogens, presumably due to the constitutive activation of defense responses.

Based on genetic analyses of plants from the Ws ecotype, the cep phenotype is inherited as a recessive mutation at a single locus that, unfortunately, is not tagged with T-DNA. Unexpectedly, analyses of the progeny derived from crosses between Ws cep and normal plants from other ecotypes revealed that at least two unlinked mutations from the Ws cep parent play a major role in determining the cep phenotype. One mutation was mapped to the lower arm of chromosome 4, between the markers PAP7.2 and nga1107. Since this mutation is necessary for the appearance of the cep phenotype, it most likely corresponds to the mutation originally identified in the Ws ecotype. We have designated this mutation *cpr20*. The second mutation maps to chromosome 1, between the β -fluctosidase gene and nga 280. We have designated this mutation *cpr21*. In contrast to *cpr20*, *cpr21* is not absolutely required for expression of the mutant phenotype, suggesting the involvement of possibly a third locus in determining the cep phenotype in the F_2 segregants.

In addition to *cpr20*, three other recessive mutations (*lsd1*, *acd2*, and *cpr1*), which cause constitutive *PR* gene expression, elevated SA levels, and enhanced disease resistance, have been mapped to chromosome 4 (Dietrich et al. 1997; Greenberg et al. 1994; Bowling et al. 1997). To determine whether the mutations carried by the *acd2* and *cpr1* mutants are allelic to *cpr20*, crosses between these mutants and cep plants were performed. The F_1 progeny from all of these crosses neither constitutively expressed *PR* genes nor displayed any of the cep-associated morphologies; thus, none of these mutations appear to be allelic (Table 2). The observed complementation between *cpr20* and *cpr1* is consistent with their different patterns of *PR* gene expression. In contrast to cep plants, whose constitutive pattern of *PR-1*, *PR-2*, and *PR-5* expression resembles that of SA-treated wt plants, *cpr1* only exhibits high constitutive expression of *PR-1* and *PR-2*, with *PR-2* transcripts accumulating to substantially higher levels than those of *PR-1*. Complementation analysis between *lsd1* and the cep mutant was not per-

formed because the *lsd1* mutation has been shown to be tightly linked to the *AG* locus (Dietrich et al. 1997), which our data indicate is approximately 17.6 cM away from the *CPR20* locus. Another mutant of *Arabidopsis*, called *cpr5*, which constitutively expresses *PR* genes, contains elevated SA levels, develops spontaneous lesions, and shows enhanced resistance, has been identified (Bowling et al. 1997). Complementation analysis suggested that *cpr5* and *cpr20* are not allelic (Table 2). This is consistent with recent mapping studies that have placed *cpr5* on chromosome 5 (Bowling et al. 1997). Several other *lsd* mutants besides *lsd1* have been shown to exhibit phenotypes similar to that of cep; however, complementation analyses with these mutants were not performed for several reasons. The dominant *lsd6* mutation maps to chromosome 1 (Weymann et al. 1995). The *lsd2*, *lsd4*, and *lsd7* mutants are also dominant (Dietrich et al. 1994; Weymann et al. 1995), which makes complementation analyses difficult. *lsd3* and *lsd5* have not yet been mapped, but, unlike cep, spontaneous lesion formation in these mutants is environmentally regulated (Dietrich et al. 1994), which suggests that they are not allelic to *cpr20*. Furthermore, while cep, *lsd3*, and *lsd5* are in the same background (Ws), when crossed to Col-0, *lsd3* and *lsd5* show a pattern of inheritance consistent with a recessive mutation at a single locus (J. Dangl, *personal communication*).

Based on these results, the *cpr20* and *cpr21* mutations responsible for the cep phenotype appear to affect a novel component of a disease resistance signaling pathway upstream of SA. Moreover, the constitutive phenotype of the cep mutant suggests that the *CPR20* and *CPR21* products act as early negative regulators in this defense pathway. Based on the genetic analysis of F_2 segregants from a Ws cep $\times$ Col-0 wt cross, the *CPR20* and *CPR21* genes would appear to encode proteins with redundant functions. However, though 62 of the 79 cep segregants from the cross were *cpr20 cpr21* double mutants, the remaining 17 were mutant only for *cpr20*. These data suggest that the *CPR20* and *CPR21* products are not fully redundant and that the *CPR21* product may need to be modified by the action of another gene to perform the equivalent negative function of *CPR20*.

Alternatively, one or more of these mutations may be in a component of another pathway that impinges on the SA-mediated defense pathway at or upstream of SA. The discovery that four non-allelic mutations causing enhanced disease resistance map to a region on the lower arm of chromosome 4 is intriguing. Whether this loose clustering of resistance-associated genes to a portion of chromosome 4 is significant from a biological or evolutionary point of view remains to be determined. The fine mapping and the cloning of the *CPR20* gene located in this region on chromosome 4 and further analysis of this region should help provide a better understanding of these aspects.

MATERIALS AND METHODS

Bacterial and fungal maintenance.

Pseudomonas syringae pv. *maculicola* strains M4 and M5 were grown at 30°C in King's B medium (King et al. 1954) supplemented with rifampicin (50 mg/liter). *Peronospora parasitica* isolate EMWA was propagated on Ws plants as described by Dangl et al. (1992) and Dietrich et al. (1994).

Plant maintenance.

Arabidopsis thaliana (Ws ecotype) wt and 3,000 lines of T-DNA-mutagenized transgenic plants obtained from ABRC (Ohio State University) were grown in Pro-Mix (Premier Horticulture, Red Hill, PA) in a growth chamber with a 14-h light cycle ($140 \mu\text{E s}^{-1} \text{m}^{-2}$) at 22°C . Dependence of the cep phenotype on light conditions was assessed under the same conditions described by Dietrich et al. (1994). To test kanamycin resistance, seeds were surface sterilized in 0.1% Triton X-100 for 30 min, 0.1% Triton X-100 plus 70% ethanol for 5 min, and 0.1% Triton X-100 plus 2% commercial bleach for 10 min, washed five times in sterile, distilled water, and sown on Murashige-Skoog (MS) media containing 0.8% agar, 1% sucrose, and 50 mg of kanamycin per liter. Growth conditions were the same as described above.

Bacterial infections.

One-week-old wt and cep seedlings were transferred to a growth chamber set for an 8-h light cycle ($350 \mu\text{E s}^{-1} \text{m}^{-2}$), 60% relative humidity (RH), and $24/20^\circ\text{C}$ (day/night). Three weeks later, four leaves per plant were infiltrated with either 10 mM MgCl_2 (mock) or suspensions of *Pseudomonas syringae* pv. *maculicola* strains M4 or M5. Bacteria were grown overnight at 30°C in King's B medium containing rifampicin and resuspended to $\text{OD}_{600} = 0.2$ (10^8 CFU ml^{-1}) in 10 mM MgCl_2 . Plants were infected with the M4 strain at a dose of 10^5 CFU ml^{-1} ; the M5 strain was used at 10^4 CFU ml^{-1} . Infiltration and analysis of bacterial growth were performed as described by Shah et al. (1997), except that rifampicin (50 mg/liter) was the only antibiotic used. Three independent experiments were done.

Fungal infections.

cep and wt seeds were sown on soil and placed in a growth chamber with a 10-h light cycle ($150 \mu\text{E s}^{-1} \text{m}^{-2}$) at 18°C and 90% RH. At the time of the infection, growth conditions were changed to 17°C , 10-h light cycle ($75 \mu\text{E s}^{-1} \text{m}^{-2}$), and 99% RH (accomplished by placing the plants inside plastic bags). Infection and analysis of *Peronospora parasitica* isolate EMWA growth were performed as described in Dangel et al. (1992) and Dietrich et al. (1994) except that 5-week-old plants were used. This experiment was done twice.

Free SA and SAG analyses.

Determination of free SA and SAG levels in wt and cep plants was performed with 0.5 g fresh weight of leaf tissue as described by Bowling et al. (1994). The data shown have been adjusted for the approximately 50% recovery of SA during extraction.

RNA and DNA analyses.

Total RNA was extracted from leaves of pooled or individual plants with the reagent TRIzol (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions. Genomic DNA was extracted according to Das et al. (1990).

Southern analyses were performed according to Shah et al. (1997), except $5 \mu\text{g}$ of total genomic DNA was used. Northern analyses were performed as described by Conrath et al. (1997). Probes specific for the *Arabidopsis PR-1*, *BGL2*, (*PR-2*), and *PR-5* genes, and the right and left borders of the T-DNA insert, were generated by random-primer labeling the inserts of various cDNA clones.

Mutant isolation.

A titration was initially performed to determine the sensitivity of our screen for mutants with constitutive expression of the *PR-1* gene. RNA extracted from plants treated (sub-irrigation) with $500 \mu\text{M}$ SA for 24 h was diluted with differing amounts of RNA extracted from untreated plants. With $20 \mu\text{g}$ of a 1:20 dilution, *PR-1* mRNA was still readily detected on a Northern blot after the autoradiogram was exposed overnight.

Sixty seeds representing 20 lines were sown in pools of 20 seeds each. From 3-week-old plants, single leaves from each plant in the pool were detached, combined, and used for total RNA extraction. Twenty micrograms of RNA from each pool, along with $20 \mu\text{g}$ of a positive control (consisting of a 1:20 dilution of RNA from SA-treated versus untreated plants) was subjected to Northern blot analysis. Any pool containing elevated levels of *PR-1* mRNA was then analyzed in greater detail by extracting RNA from the individual plants within the pool and subjecting $10 \mu\text{g}$ of each to Northern blot analysis to identify the putative mutant plant. One line exhibiting constitutive expression of *PR-1* (cep) was isolated from 3,000 T-DNA-mutagenized lines (Feldmann 1992). This putative mutant was allowed to self-pollinate and the cep phenotype was confirmed in the next generation.

Genetic analysis of cep.

For all crosses, cep plants derived from the ecotype Ws were used as the pollen-accepting plants. To determine the basis of the cep phenotype, cep homozygous plants were crossed to wt Ws plants. The F_1 progeny were scored for Kan^r and constitutive expression of *PR-1* at 3 weeks. To determine the chromosomal location of the mutation responsible for the cep phenotype, cep plants were crossed to Columbia ecotype (Col-0). The F_1 progeny were allowed to self-pollinate and the F_2 progeny were scored for the curly leaf morphology and necrotic cotyledons associated with the cep phenotype. Analysis of *PR-1* expression was performed on F_3 progeny to confirm the cep phenotype. These cep plants were used for CAPS and SSLP marker analysis (Bell and Ecker 1994; Konieczny and Ausubel 1993). DNA was isolated from individual plants as described by Edwards et al. (1991) and the polymerase chain reactions (PCRs) for CAPS markers were performed as described by Konieczny and Ausubel (1993) except that $0.04 \mu\text{g}$ of primers was used instead of $0.2 \mu\text{g}$ and the PCR products were separated in a 2% agarose gel. SSLP markers were used to fine map mutations on chromosome 4 and chromosome 1. PCR conditions for SSLP markers were as described by Bell and Ecker (1994) except that KCl, Tris-HCl, gelatin, and Triton X-100 were excluded from the reactions and the PCR products were fractionated in a 4% agarose gel.

All primers were purchased from Research Genetics (Huntsville, AL). Linkage analysis and genetic map distances were calculated with the MapMaker Macintosh V2.0 program with a maximum theta equal to 0.40 and a minimum LOD score of 3.00 (Landers et al. 1987). The Kosambi map function (Kosambi 1944) was used to convert the recombination frequencies to map distances in centimorgans (Koornneef and Stam 1992). Crosses between cep and the different mutants were analyzed for curly leaf morphology and necrotic cotyledons as well as constitutive expression of the *PR-1* gene. These analyses were performed on 3-week-old plants.

Microscopic analysis of cell-wall-associated autofluorescent materials.

cep and wt cotyledons were analyzed at 6, 8, 10, and 14 dpp, while leaves were monitored for lesion formation from 1 to 5 weeks. Cotyledons and leaves were analyzed as described by Dempsey et al. (1997).

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