Isolation and Characterization of Broad-Spectrum Disease-Resistant Arabidopsis Mutants

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ABSTRACT

To identify Arabidopsis mutants that constitutively express systemic acquired resistance (SAR), we constructed reporter lines expressing the firefly luciferase gene under the control of the SAR-inducible *PR-1* promoter (*PR-1*/luc). After EMS mutagenesis of a well-characterized transgenic line, we screened 250,000 M_2 plants for constitutive expression of the reporter gene *in vivo*. From a mutant collection containing several hundred putative mutants, we concentrated on 16 mutants lacking spontaneous hypersensitive response (HR) cell death. We mapped 4 of these *c*onstitutive *im*munity (*cim*) mutants to chromosome arms. Constitutive expression of disease resistance was established by analyzing responses to virulent *Peronospora parasitica* and *Pseudomonas syringae* strains, by RNA blot analysis for endogenous marker genes, and by determination of salicylic acid levels in the mutants. The variety of the *cim* phenotypes allowed us to define distinct steps in both the canonical SAR signaling pathway and a separate pathway for resistance to *Erysiphe cichoracearum*, active in only a subset of the mutants.

PLANTS possess inducible disease defense systems. A major contribution to this innate defense response is systemic acquired resistance (SAR). SAR is induced in many species upon local infection by necrogenic pathogens and by hypersensitive response (HR; Ryals *et al.* 1996). During SAR, an increased ability to resist attacks of a wide array of pathogens is systemically induced, which lasts several weeks to several months after initiation. Induced expression of a subset of pathogenesis-related (*PR*) genes, called SAR genes, is highly correlated with the maintenance phase of SAR (Ward *et al.* 1991; Uknes *et al.* 1992). In Arabidopsis, the *PR-1* gene is the most reliable molecular marker for SAR.

Salicylic acid has been shown to be both necessary and sufficient for mediating systemic triggering of SAR in some plants (Vernooij *et al.* 1994). Transgenic tobacco and Arabidopsis plants expressing the bacterial salicylate hydroxylase gene, NahG, which significantly reduces accumulation of active salicylic acid (SA), are unable to establish SAR (Gaffney *et al.* 1993). The action of salicylic acid can be specifically mimicked by certain chemicals, such as 2,4 dichloroisonicotinic acid (INA) and benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH). The latter compound is used commercially for crop protection in various pathosystems (Friedrich *et al.* 1996; Goerlach *et al.* 1996). In the model system Arabidopsis, BTH induces resistance to many fungal and bacterial pathogens, such as the obligate biotrophic oomycete *Peronospora parasitica*, and virulent strains of *Pseudomonas syringae* (Lawton *et al.* 1996). Arabidopsis has been used to genetically dissect the signaling cascade leading to disease responses. Mutants in the SAR pathway that are either impaired in resistance responses or constitutively express SAR have been described (Dong 1998). This second group contains two classes: mutants exhibiting spontaneous, HR-like cell death, called *acd* (*a*ccelerated *c*ell *d*eath), *lsd* (*l*esion-*s*imulating *d*isease resistance), *cpr* (*c*onstitutive expression of *PR* genes; Greenberg and Ausubel 1993; Bowling *et al.* 1994; Dietrich *et al.* 1994), or *edr* (*e*nhanced *d*isease *r*esistance; Frye *et al.* 2001), and mutants that do not exhibit this cell death in absence of external trigger. They are less common, but may be crucial for the understanding of SAR signaling downstream or independent of cell death. Only a few mutants have been identified so far, including some *cpr* mutants (Dong 1998) and two *dnd* (*d*efense, *n*o *d*eath) mutants (Yu *et al.* 1998).

Mutations in the *NIM1/NPR1* (*n*oninducible *im*munity/*n*o *PR* gene expression) gene impair inducible disease resistance in Arabidopsis (Cao *et al.* 1994; Delaney *et al.* 1995). The cloning of this central member of the SAR signaling cascade revealed homologies to mammalian transcription factor regulators containing ankyrin domains (Cao *et al.* 1997; Ryals *et al.* 1997). By analogy to mammals, a protein kinase cascade may regulate the function of the NIM1/NPR1 protein. A MAP kinase

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that is activated by SA has recently been identified by biochemical means (Zhang and Klessig 1997; Romeis *et al.* 1999). One mutant, called *edr1*, has been identified that cannot be classified as a SAR mutant because it confers resistance to *Erysiphe cichoracearum* in the absence of increased *PR-1* gene expression and accumulation of elevated SA levels (Frye and Innes 1998). *edr1* supports wild-type infection upon inoculation with virulent *P. parasitica* isolates. *edr1*, which encodes a mitogen-activated protein kinase kinase (MAPKK) kinase in an SA-inducible defense response (Frye *et al.* 2001), may therefore define a different signal transduction pathway branch involved in plant-pathogen interactions. This signaling may be linked to SAR, which also provides resistance against Erysiphe infections.

Additional SA-independent disease resistance pathways have recently been described (reviewed by Maleck and Dietrich 1999; Glazebrook 2001). The specific roles and interdependences of these pathways are not yet well understood, in part due to a lack of distinctive marker genes.

To better understand cellular signaling leading to the establishment of SAR, we performed a near genome-saturating mutant screen in *Arabidopsis thaliana* on the basis of constitutive expression of a *PR-1*/luciferase reporter gene. We identified several hundred mutants with constitutive luciferase activity. We then focused on 16 mutants from this pool that lacked spontaneous cell death and still expressed constitutive *PR-1*/luciferase activity. All 16 mutants accumulated high levels of SA and expressed high constitutive levels of SAR-associated marker genes. On the basis of different resistance responses to several virulent pathogens, we classified the mutants and compared them to plants elicited by BTH.

MATERIALS AND METHODS

Construction and characterization of the *PR-1*/luciferase line: A *PR-1* genomic clone was identified by screening an Arabidopsis EMBL3 genomic library (CLONTECH, Palo Alto, CA) with the *PR-1* cDNA (Uknes *et al.* 1992). A 7-kb *Xho*I fragment of the *PR-1* genomic clone was subcloned into pBS+ (Stratagene, La Jolla, CA) using standard cloning techniques, and restriction mapping revealed the presence of a 4.2-kb promoter fragment 5′ of the *PR-1* coding region (GenBank accession no. AF0962949). This fragment was subcloned 5′ to a cDNA coding for luciferase (excised from pDO432, Ow *et al.* 1986), generating a translational fusion at the ATG that marks the start of translation for luciferase. The *PR-1*/luciferase construct was verified by sequencing and subcloned as a *Xho*I/*Sac*I fragment into pCIB200, a binary vector that contains the neomycinphosphotransferase II gene that confers resistance to kanamycin. The resulting construct was mobilized into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *A. thaliana* (ecotype Col-0) plants were transformed with this construct by Agrobacterium using the vacuum-infiltration method (Bechtold *et al.* 1993). A total of 32 independent transformants homozygous for the transgene were identified on the basis of resistance of the T3 progeny to kanamycin. *PR-1*/luciferase plants were characterized on the basis of inducibility of luciferase activity by 375 μM INA and one line, called 6E, that showed consistently a >100-fold inducibility was selected for further characterization by chemical and biological induction as described below and in the mutagenesis section.

Plant cultivation and mapping strategy: Putative *c*onstitutive *im*munity (*cim*) mutants were isolated from an M_2 population comprising 250,000 plants of the homozygous 6E line, mutagenized by ethyl methanesulfonate. The 250,000 M_2 plants were derived from 168 independent M_1 seed pools containing 50 plants each. The coverage in the M_2 can be calculated with the formula $P = 1 - f^n$, with P, the probability to detect a recessive homozygous mutation in the M_2; n, the number of M_2 plants screened per M_1 plant = 250,000/168 × 50 = 29.7; and f, the theoretical fraction of M_2 plants that do not show a mutation present in one of the proposed two effective germ cells of a M_1 plant = $1 - \frac{1}{8}$. Therefore $P = 1 - 0.875^{29.7} = 98.1\%$ (Redei and Koncz 1992). Mutants were grown at 20°–24°, 60% relative humidity, 9-hr day/15-hr night cycle, 250 μE/m²/sec on Germination Mix Superfine (C. Farfard, Agawam, MA).

Crosses to the parental line (kan^r) and to other ecotypes were performed on half-closed buds of flowers from the female parent plant. Cross pollinations were confirmed by the presence of the luciferase gene or by selecting on plates containing 50 μg/ml kanamycin in cases of pollination from the parental line. In mixed ecotype crosses, race-specific microsatellites [single sequence length polymorphisms (SSLPs)] were used to confirm the cross. Crosses to NahG plants (hyg^r) were resistant to hygromycin and kanamycin.

Three- to 4-week-old progeny were screened for *in vivo* luciferase activity. Plants were evenly misted with a 7.5-mM luciferin solution (Biosynth International, Naperville, IL) containing 0.1% SilWet L77 (Union Carbide Chemicals), and after 10 min, photon emission was quantified during 10-min integration using a photon counter (Hamamatsu, Tokyo) at the most sensitive detection setting. F_1 plants of backcrosses to the *PR-1*/luciferase line showing luciferase activity were selected for selfing and further crosses. F_2 populations of single F_1 plants were analyzed if no F_1 progeny showed luciferase activity to identify recessive mutations and to determine the segregation ratios of progeny of F_1 plants with luciferase activity. Segregation ratios of crosses to other ecotypes and mutants were scored in the same way. Progeny that lost the luciferase marker gene due to segregation were eliminated on the basis of a luciferase gene-specific PCR (5′ primer, CTATGAAGAGAT ACGGCCTG; 3′ primer, ATGAGATGTGACGAACGTGT; 35 cycles of 30 sec 95°, 30 sec 60°, and 1 min 30 sec 72°). The selected F_2 progeny of mapping crosses were allowed to self-pollinate, and F_3 progeny were rescreened for luciferase activity, both on kanamycin-containing GM plates and on soil.

SSLP markers described by Bell and Ecker (1994) and cleaved amplified polymorphic sequences (CAPS) markers (Konieczny and Ausubel 1993; E. Drenkard and F. Ausubel, http://genome-www.stanford.edu/Arabidopsis/maps/CAPS.html) were used to identify genetic loci linked to the *cim* mutations and the luciferase transgene. Restriction fragment length polymorphism (RFLP) marker mi291a was converted into a CAPS marker (5′ primer, CCTTCTGCTGTTGTTAAAG; 3′ primer, CCAGTTCCTTTTGTTTGAC; 35 cycles of 30 sec 95°, 30 sec 51°, and 1 min 30 sec 72°, cleaved with *Xba*I; New England Biolabs, Boston). RFLP marker mi185 was converted into a CAPS marker (5′ primer, AGCCATCAGATTATGTT CCC; 3′ primer, TGTAGGAACTCGATCCTCC; 35 cycles of 30 sec 95°, 30 sec 56°, and 2 min 72°, cleaved with *Xmn*I; M. Hunt, unpublished data). Recombination frequencies were converted to genetic map distances using the Kosambi (1944) function as provided in the MapMaker 3.0b program (Lander *et al.* 1987; Lincoln *et al.* 1992).

Nucleic acid extractions and analysis: Plant DNA was extracted using a hexadecyltrimethyl-ammonium bromide method (Rogers and Milliman 1984) for single leaves. For polymerase chain reaction, DNA was resuspended in 200 μl 10 mm Tris, pH 8.5; 5 μl of this DNA solution was used per 25-μl reaction. For Southern blot analysis, 1–5 μg DNA was digested with several appropriate restriction endonucleases according to the manufacturer's instructions (New England Biolabs) and Southern blotting was performed as described by Ausubel *et al.* (1987). DNA was transferred onto GeneScreen Plus membranes (Du Pont-New England Nuclear, Boston) in 10× SSC and the membranes were hybridized and washed as described for Northern blot.

RNA was isolated by lithium chloride precipitation as described previously (Lagrimini *et al.* 1987) from 0.5 g frozen leaf tissue. For Northern blotting, RNA was photospectrometrically quantified and 10 μg total RNA was electrophoretically separated on formaldehyde-agarose gels and blotted onto a nylon membrane (GeneScreen Plus; Du Pont-New England Nuclear) as described (Ausubel *et al.* 1987). After UV-crosslinking (1200 μJ), RNA was hybridized to gene-specific probes that were radioactively labeled by random priming (GIBCO BRL, Gaithersburg, MD) to 200,000 counts/min/cm^2 membrane. Untreated wild-type Col-0, BTH-treated plants (treated 2 days prior to harvest with 300 μm BTH, 25% active ingredient; Lawton *et al.* 1996) and Peronospora-infected tissue, harvested 8 days after inoculation, served as controls. Blots were analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and standardized to α-tubulin. Each RNA blot was repeated at least twice with comparable results.

Pathogen treatments: We tested whether the *cim* mutants were resistant to the virulent oomycete parasite *P. parasitica* isolates *Noco2* (obtained from J. Parker, Norwich, England) and *Emco5* (obtained from E. Holub, Wellesbourne, England) by comparing disease symptoms to those of either BTH-treated (0.3 mm, 2 days prior to pathogen treatment) or water-treated wild-type Col-0 plants. *P. parasitica* isolate *Noco2* was sprayed as a conidial suspension (10^5 spores/ml) onto 4-week-old plants, and *P. parasitica* isolate *Emco5* was sprayed on 2-week-old seedlings. Following inoculation, plants were maintained in high humidity and symptoms were scored 8 days after inoculation for development of conidiospores, using the rating system proposed by Holub (Holub *et al.* 1994). For microscopic analysis of induced cell death and fungal development, Trypan Blue staining was performed on individual leaves (Keogh *et al.* 1980). Callose was detected using anilin blue staining on 5-μm-thick leaf sections (Hunt *et al.* 1997).

Resistance to *E. cichoracearum* strain UCSC (kindly provided by Dr. R. Innes, Indiana University) was tested by brushing sporulating Col-0 plants onto 4-week-old plants, as described by Frye and Innes (1998). To visualize the infection and fungal structures, a fluorescence dye staining was performed on infected leaves (Duckett and Read 1991). Leaves were incubated for 2 min in 50 μg/ml ($DiOC_6(3)$) stain (Sigma Chemicals, St. Louis), cleared for 30 sec in distilled water, and mounted in water under a coverslip. Fluorescent fungal hyphae were detected at 520 nm after blue light excitation (450–490 nm) with an epifluorescence microscope (Leitz, Wetzlar, Germany).

For the analysis of resistance to compatible phytopathogenic bacteria, the apoplast of leaves of 4-week-old *cim* plants and water-treated Col-0 and BTH-activated Col-0 (0.3 mg/ml) plants were injected with *P. syringae* pv *maculicola* ES 4326 (Schott *et al.* 1990). Samples were taken at 0, 1, 3, and 5 days after injection. For each time point, four leaf punches were pooled, ground in 10 mm $MgCl_2$, and plated in appropriate dilutions on Kings B medium supplemented with streptomycin (100 μg/ml). Standard deviations were calculated from four independent experiments. The significance of differences between mean values was evaluated by Student's *t*-test. Differences were considered to be significant at $P > 0.6$. For analysis of HR in incompatible interactions, *P. syringae* pv *tomato* (*avrRpt2*) was infected with 5×10^7 cfu/ml (Innes *et al.* 1993).

Measurements of salicylic acid levels: Free and total salicylic acid levels of triplicate samples were determined as previously described (Gaffney *et al.* 1993; Uknes *et al.* 1993). For comparison, we also measured SA in tissue of *E. cichoracearum*-infected plants harvested 3 days after inoculation.

RESULTS

Screening for disease-resistant mutants: To carry out a screen for constitutive expression of *PR-1*, a *PR-1*/luciferase reporter gene construct including a NPTII selection marker was transformed by Agrobacterium-mediated gene transfer into Arabidopsis and homozygous lines were generated. One transgenic line in the Arabidopsis ecotype Col-0 (referred to as 6E line) was chosen for further characterization on the basis of the ratio of *in vivo* luciferase background (noninduced) to induced (24 hr after 0.3 mm BTH treatment) activity. In an F_2 population of an outcross to untransformed Col-0 plants, 147 out of 203 plants survived on selection for kanamycin resistance ($\chi^2 = 0.724$, $P < 0.4$ for a 3:1 segregation ratio). Southern analysis using either the luciferase gene or the right border of the T-DNA as a probe showed that only one insert was integrated into the genome (data not shown). The extent and timing of expression (quantified as enzyme activity) from the *PR-1-LUC* transgene in the 6E line after chemical and biological induction matched the expression pattern of the endogenous *PR-1* gene (data not shown). Induction kinetics of luciferase activity following chemical treatment with different concentrations of BTH or INA paralleled *PR-1* gene expression, as confirmed by Northern blots. Similarly, luciferase activity over time matched *PR-1* gene expression kinetics in compatible and incompatible pathogen interactions (*P. parasitica Emwa* and *Noco*; data not shown). Luciferase activity was routinely induced >100-fold in these induction experiments. The 6E line was indistinguishable from wild type both morphologically and in terms of gene expression. No increased resistance to virulent pathogens or *PR*-gene expression was detected. On the basis of these observations, the 6E line was taken as a wild-type control in all further experiments.

A total of 8400 M_1 seeds of the 6E line were used for EMS mutagenesis (performed at Lehle Seeds) with an *M* value of 0.147 (Haughn and Somerville 1987; Mednik 1988). Out of 168 independent M_1 seed pools, screened with 98% coverage in the M_2 population (250,000 plants, see materials and methods for calculation of coverage), 160 pools contained at least one plant that constitutively expressed *PR-1*/luciferase, and there were 602 putative mutants in total. Sixteen of these mutants (Ta-

TABLE 1

Genetic analysis of *cim* mutants

16 *cims*[a]	M_1[b]	Luc in F_1[c]	F_2 (+:−)[d]	χ^2[e]	Chromosome
			Mutants described in this study		
cim5	36	Yes	57:25	1.32 ($P > 0.2$)	2
cim6	15	Yes	40:20	2.22 ($P > 0.1$)	1
cim7	2	Yes	ND[f]		
cim8	93	Yes	58:16	0.45 ($P > 0.5$)	
cim9	98	No	33:99	0 ($P > 0.95$)	
cim10	107	Yes	31:11	0.03 ($P > 0.95$)	5
cim11	115	Yes	43:12	0.30 ($P > 0.8$)	1
cim12	124	Yes	34:21	5.1 ($P > 0.01$)[g]	
779[h]	168	Yes	47:60	55.1[g]	
cim13	164	Yes	33:19	3.69 ($P > 0.05$)	
cim14	155	Yes	35:11	0.03 ($P > 0.95$)	
			Mutants not further analyzed		
2	17	No	ND		
11[i]	36	Yes	87:35	0.89 ($P > 0.4$)	
81	8	Yes	ND		
367	16	Yes	ND		
671	91	Yes	28:22	9.6 ($P > 0.01$)[g]	
714	121	Yes	15:5	0 ($P > 0.95$)	
741	132	Yes	22:18	8.53 ($P > 0.01$)[g]	

With 250,000 M_2 plants out of 168 M_1 pools (containing 50 M_1 plants), the coverage in the M_2 can be calculated with the formula $P = 1 - (f)^n$, with P, the probability to detect a recessive homozygous mutation in the M_2; *n*, the number M_2 plants screened per M_1 plant = 250,000/168 × 50 = 29.7; *f*, the theoretical fraction of M_2 plants that do not show a mutation present in one of the proposed effective two-germ cells of a M_1 plant = $1 - \frac{1}{8}$. *P* yield thus to $P = 1 - 0.875^{29.7} = 98.1\%$.

[a] Identification number of *cim* mutant. A mutant was retained when the luciferase level was induced at least 10-fold over background level (which was at 200 cpm, integrated over 10 min). In cases where macroscopic injuries were visible, the plantlet was discarded.

[b] M_1 lot from which the mutant originated.

[c] Luciferase activity in F_1 of backcrosses to the 6E line at least five times above background; due to incomplete penetrance of the mutant phenotype, the percentage of plants in the F_1 populations expressing the PR1/luciferase gene varied between 10 and 100%.

[d] Segregation ratios of luciferase expressing to non-expressing plants in the F_2 generation of a backcross to the parental PR1/luc line.

[e] χ^2 and probability of the observed difference to the expected 3:1 (expressing to nonexpressing) segregation ratio for a single dominant mutation.

[f] ND, not determined.

[g] Hypothesis of a 3:1 segregation rejected.

[h] Lesion mimic.

[i] *cim* 11 originated from the same M_1 seed lot as *cim5*; they may be identical.

ble 1), isolated from different M_2 seed lots, did not exhibit spontaneous lesion formation under conditions used in our assays, as revealed by Trypan Blue staining of dead cell lesions and microscopy (Figure 1). As a control, a mutant with spontaneous cell death, designated mutant 779, was included in all the following experiments. Mutant 779 displayed patches of autofluorescence and callose that normally accompany HR-like cell death (data not shown).

Although free of lesions, other pleiotropic phenotypic alterations in the 16 mutants were not separated from the mutation that caused constitutive *PR* gene expression after three backcrosses. In general, *cim* mutants have a prolonged life cycle, a delayed flowering time (2 weeks later than in wild-type Col-0), and they set less seed (approximately one-third of Col-0). Some mutants also showed reduced germination. Leaf morphology varied from long, often curly leaves (*cim6*, *cim12*; Figure 1) to extremely small round leaves (*cim9*, *cim13*; Figure 1). Mutant *cim9* showed bright green leaf pigmentation. However, normal leaf morphology was also found, albeit mostly in the weaker mutants, *cim7* and *cim8* (weakness based on *PR-1* expression and SA content), as well as in the *cim11* that differed only in size to wild type (Figure 1). Ten *cim* mutants that were further characterized were denominated *cim5* through *cim14*. Mutants *cim1* through *cim4* were isolated in previous mutant screens (H. Steiner and J. Ryals, unpublished results).

Genetic analysis of the disease-resistant mutants: All

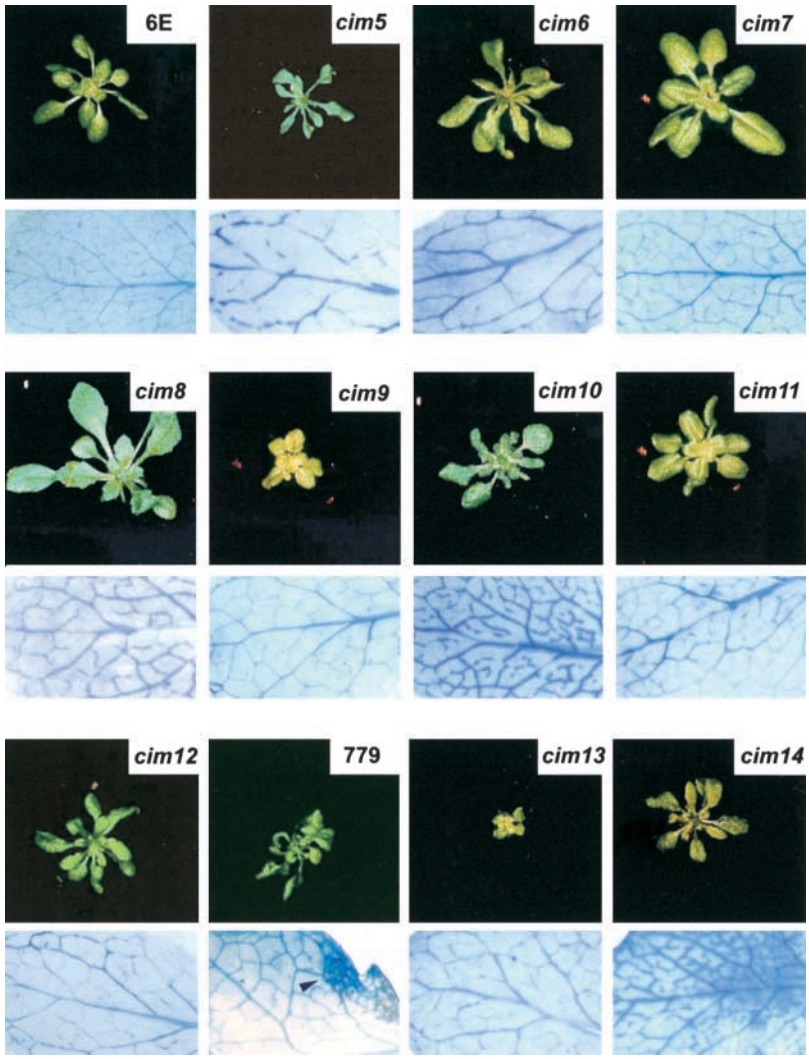

FIGURE 1.—The disease-resistant mutants do not exhibit spontaneous cell death, but morphological changes are common. Photography and Trypan Blue lesion staining of 10 *cim* mutants (*cim5–14*), wild-type *PR-1*/luc line (6E), and a lesion mimic mutant (779), which was included as a positive control in the staining, were performed as described in MATERIALS AND METHODS.

16 mutants originated from different seed pools and were therefore considered independent mutations. All mutants were backcrossed at least three times to the *PR-1*/luciferase parental line. Selfed progeny of all mutants stably expressed *PR-1*/luciferase.

To analyze the segregation ratios of the mutations, F_2 populations of backcrosses, containing 20–100 plants, were screened for constitutive luciferase activity and the resulting data were subjected to χ^2 analysis (Table 1). The expression of the reporter gene in the F_1, confirmed in random samples by Northern blot analysis for endogenous *PR-1* expression, indicated that in all but two cases (mutant *2* and *cim9*) the mutant phenotype was dominant. However, the analysis of the F_2 segregation ratios suggested that many of these mutations were not fully penetrant (Table 1, segregation ratios in the F_2 populations). In addition, we cannot exclude the possibility that in some cases (*cim12*), two dominant genes are required to cause the observed phenotype (χ^2 for 9:7 = 0.69, $P < 0.4$). In the case of *cim11*, the morphological changes were inherited in a recessive manner, while the closely linked constitutive *PR-1*/luciferase expression was incompletely penetrant, with varying expression of the phenotype in the heterozygous plants. In cases where F_2 segregation ratios were normal, we mapped the mutations. *cim11* was placed on the genetic map of *A. thaliana* on chromosome 1 between the markers mi291a (5 recombinants in 120 meioses) and nga280 (2 recombinants in 124 meioses). *cim6* is also located on chromosome 1, between markers nga280 (20 recombinants in 116 analyzed meioses) and m185 (19 recombinants in 116 meioses). *cim5* is located on chromosome 2, between markers ve017 (16 recombinants in 148 meiosis) and nga168 (9 recombinants in 122 meioses). *cim10* lies on chromosome 5 between markers DFR (22 recombinants in 106 meioses) and LFY3 (17 recombinants in 110 meioses). The map positions of the mutations on chromosomes 1 and 2 do not match the map positions of known mutations in genes encoding functions in disease resistance and/or SAR. Mutant *cim10* is in a region of chromosome 5 termed MRC-J, which contains a number of *R* gene homologs (BOTELLA *et al.* 1997; HOLUB and BEYNON 1997). *cim11* and *cim6* map close to, but distinct from, *cpr6* (CLARKE *et al.* 1998).

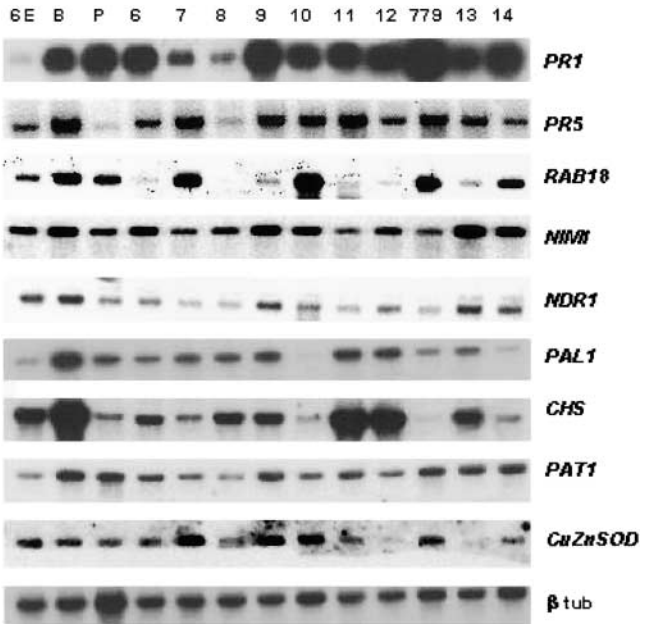

FIGURE 2.—Gene expression pattern of defense-related, *PR* genes and cell death-related genes in disease-resistant mutants. Columns are as follows: 6E, wild-type *PR-1*/luc line; B, wild type treated 2 days before harvest with 0.3 mM BTH; P, wild type treated 8 days before harvest with *P. parasitica* Noco2 (10^5 spores/ml); 6–14, 10 *cim* mutants; 779, a mutant that shows spontaneous cell death. Gene probes shown are as follows: *PR-1* and *PR-5*, pathogenesis-related proteins 1 and 5 (UKNES *et al.* 1992); *NIM*, *NIM1/NPR1* (RYALS *et al.* 1997); *NDR*, nonhost disease resistance (CENTURY *et al.* 1997); *PAL1*, phenylalanine ammonia lyase (WANNER *et al.* 1995); *CHS*, chalcone synthase (SHIRLEY *et al.* 1995); *PAT1*, phosphoribosyl anthranilate synthase (ROSE *et al.* 1992); *RAB18*, responsive to abscisic acid (GOSTI *et al.* 1995); and CuZnSOD, Cu-Zn superoxide dismutase (JABS *et al.* 1996). Genes whose expressions were quantified but not altered included lipid transfer protein (*LTP1*; THOMA 1994), lipoxygenase (*LOX1*; MELAN *et al.* 1993), an *At MLO* gene (BÜSCHGES *et al.* 1997), the Arabidopsis homolog of the defense against death (*DAD*) gene (SUGIMOTO *et al.* 1995), the lesion simulating disease resistance gene 1 (*LSD1*; DIETRICH *et al.* 1997), the Arabidopsis homolog of lethal leaf spot (*LLS1*; GRAY *et al.* 1997), superoxide dismutases (*MnSOD*, *FeSOD*), catalases 2 and 3 (*CAT2* and *CAT3*), peroxidase C (*PRX C*), glutathione-*S*-transferase type III (*GST*; all described in JABS *et al.* 1996), and the gene encoding for vascular storage protein (*AtVSP*; BERGER *et al.* 1995). Details about the probes used are available upon request. All blots were prepared with the same RNA; 5 μg RNA per sample was loaded.

SAR genes are overexpressed in the disease-resistant mutants: To confirm the identification of mutants affected in the SAR signaling cascade leading to *PR* gene expression, the expression of a variety of marker genes was analyzed in comparison to the parental line 6E (Figure 2), as well as to biologically and chemically induced tissue.

Several Arabidopsis SAR genes (*PR-1*, *-2*, and *-5*; UKNES *et al.* 1992) were induced in all mutants except *cim7* and *cim8* to levels comparable to a strong BTH induction or a pathogen treatment with a virulent race (Figure 2; data not shown). *PR-4* gene expression is inducible by ethylene. Its expression in *cim* mutants was ~20-fold weaker than that observed in an ethylene-treated control plant (data not shown).

TABLE 2

High levels of salicylic acid accumulation in *cim* mutants are correlated to *PR-1* gene expression levels

Mutant	Total SA (ng/mg FW)	*PR-1*[a]
6E (wild type)	296 ± 25	1.0
6E + Erysiphe	2030 ± 890	25[b]
cim5	1958 ± 835	11.4
cim6	1657 ± 436	17.5
cim7	899 ± 16	2.9
cim8	294 ± 21	1.7
cim9	4154 ± 211	38.2
cim10	2256 ± 223	11.8
cim11	1500 ± 78	12.6
cim12	1350 ± 267	10.5
cim13	3415 ± 331	7.3
cim14	2190 ± 491	24.7
6E + BTH	NA	11.0

PR-1 gene expression levels were quantified using a phosphorimager and normalizing for loading differences with α-tubulin. The 6E line showed consistently wild-type levels of *PR-1* gene expression. SA, salicylic acid; FW, fresh weight.

[a] *PR-1* expression relative to wild type.

[b] *PR-1* expression in *Peronospora parasitica* pv Noco2-infected tissue (8 days after inoculation).

Hormone-inducible genes, such as *AtVSP* for monitoring jasmonic acid-induced gene expression (BERGER *et al.* 1995), were either weakly or not induced (data not shown). A possible exception may be the *RAB18* gene, an example of an ABA-inducible gene (MERLOT and GIRAUDAT 1997). Rab18 gene expression is induced in *cim7*, *cim10*, and mutant 779. The induction of SAR in *cim* mutants most likely does not activate or depend on other hormonally regulated pathways as monitored by marker gene expression.

Similarly, the expression of stress-inducible genes of secondary metabolism, such as *PAL* and *CHS* (WANNER *et al.* 1995), or of the SAR-inducible *NIM* gene and *NDR* gene in disease resistance pathways was not correlated to the particular phenotypes of the mutants. Induction of the shikimate pathway can lead to antimicrobial metabolites and to SA biosynthesis and hence to increased resistance. The induction of *NIM* has been shown to be sufficient to increase resistance in Arabidopsis (CAO *et al.* 1998; FRIEDRICH *et al.* 2001).

Expression of genes involved in regulating the cellular redox state or in the oxidative burst (LOX, GST, PRXC, MnSOD; not shown; JABS *et al.* 1996) was not induced in the lesion-free mutants with the possible exception of the *Cu-ZnSOD* gene (Figure 2). Cu-ZnSOD activity has previously been shown to be altered during oxidative stress and by pathogen infection (FODOR *et al.* 1997; KLIEBENSTEIN *et al.* 1999).

Most disease-resistant mutants accumulate high levels of salicylic acid: The direct dependence of the natural induction of SAR on SA (Willits and Ryals 1998) makes this compound a key metabolite to measure in mutants expressing altered SAR phenotypes. Although we expected to find some mutants downstream of SA, for example, possible gain-of-function *nim1/npr1* alleles, all mutants (with the exception of mutant *cim8*) accumulated 3–15 times more SA than untreated wild-type plants (Table 2). A control treatment with a virulent *E. cichoracearum* pathogen caused a sevenfold increase in total SA content after 3 days of infection. The levels of free and total SA were always correlated to each other, thus excluding from our collection mutations in the regulation of this equilibrium or in the degradation/conjugation of SA (data not shown). On the basis of SA content and *PR* gene expression, mutants can be classified into strong *cim* mutants (*e.g.*, *cim5*, *cim6*, *cim9*, *cim10*, *cim13*, *cim14*) and weak *cim* mutants (*e.g.*, *cim7*, *cim8*). Interestingly, no correlation between SA content and HR-like lesion formation has been found in the 90 lesion mimic mutants from this screen for which SA analysis has been performed (data not shown).

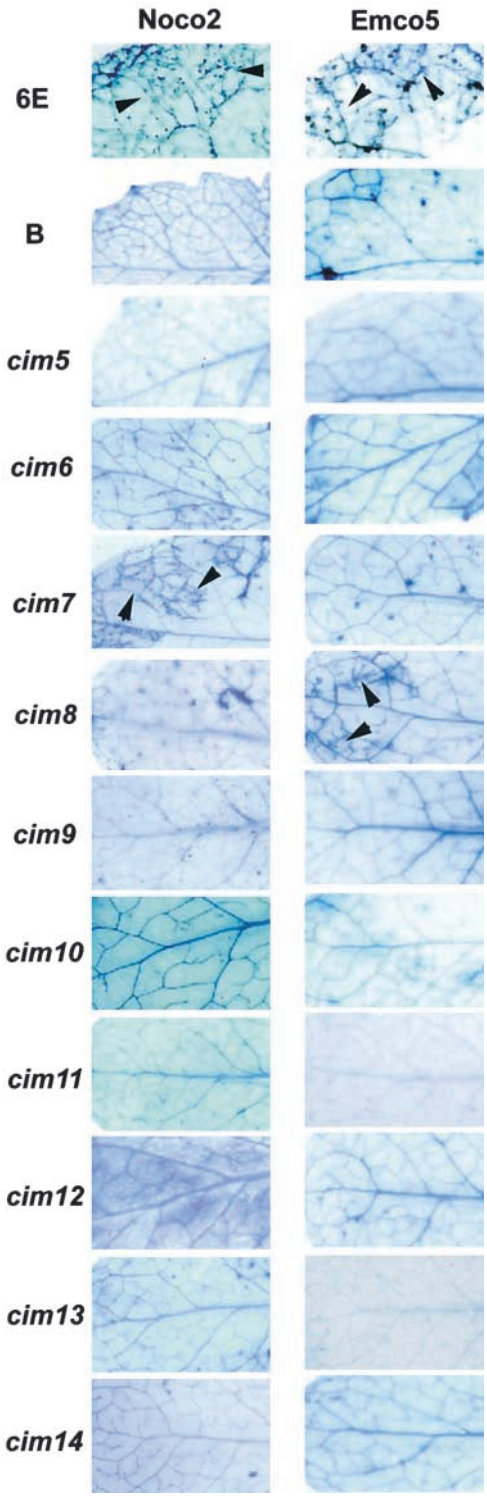

Figure 3.—*cim* mutants are resistant to two virulent *Peronospora parasitica* isolates. Trypan Blue staining of *cim* mutants 8 days after spray inoculation with *P. parasitica* spores of the isolates *Noco2* and *Emco5* is shown. With the exception of *cim7* and *cim8*, all mutants exhibit a complete protection against Peronospora. In mutants *cim10* and *cim12*, spore inoculation causes HR-like lesion formation. 6E, wild-type *PR-1*/luciferase line; B, wild-type *PR-1*/luciferase line pretreated with BTH (0.3 mm).

Most mutants are resistant to fungal pathogens: We tested the response of the mutants to various pathogens to which BTH confers significant protection in wild type. We tested resistance to two isolates of the oomycete parasite *P. parasitica*, which are virulent on wild-type Col-0 (Holub *et al.* 1994). We scored resistance to *P. parasitica* isolate *Noco2* in adult leaves 8 days after infection. Neither chlorosis nor spontaneous macroscopic necrosis were observed in the "strong" *cim* mutants, defined as those with high levels of SA and *PR* gene expression. Only two "weak" mutants, *cim7* and *cim8*, allowed some hyphal growth and slight sporulation (Figure 3). Trypan Blue staining for hyphal growth and cell death revealed, however, that in some mutants (*cim10*, *cim12*, and to a lesser extent in mutants *cim6*, *cim9*, and *cim13*) very occasional trailing necrosis (Holub *et al.* 1994) occurred around the hyphal penetration sites (data not shown). This phenomenon was not correlated to relative SA content or *PR* gene expression. A second compatible *P. parasitica* isolate *Emco5* (Holub and Beynon 1997, no. 2253; McDowell *et al.* 1998) was applied to younger plants in a cotyledon assay, because infection of wild type is more effective at this earlier stage (data not shown). Each *cim* line expressed a similar phenotype when infected with either *P. parasitica* isolate *Emco5* or isolate *Noco2* (Figure 3). These results suggest that the observed resistance is not an age-dependent or an isolate-specific reaction.

We also tested resistance of the *cim* mutants to a fungal pathogen, *E. cichoracearum*, which is virulent on most *A. thaliana* ecotypes (Adam and Somerville 1996), including Col-0. Col-0, however, can be completely protected

TABLE 3

Some *cim* mutants exhibit resistance to *Erysiphe cichoracearum*

Mutant	Experiment 1	Experiment 2	Experiment 3
Wildtype	3.00 ± 0.00[a]	3.00 ± 0.00	3.00 ± 0.00
Wild type + BTH	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
cim5	2.08 ± 0.76	2.92 ± 0.28	NT[b]
cim6	1.13 ± 0.33	1.56 ± 0.68	1.67 ± 0.67
cim7	NT	1.00 ± 0.00	1.00 ± 0.00
cim9	1.40 ± 0.66	1.27 ± 0.45	1.60 ± 0.66
cim10	2.50 ± 0.61	2.62 ± 0.74	2.54 ± 0.78
cim11	1.56 ± 0.50	1.92 ± 0.79	1.90 ± 0.94
cim12	2.30 ± 0.64	2.50 ± 0.81	2.71 ± 0.45
779	2.50 ± 0.71	3.00 ± 0.00	1.90 ± 0.83
cim13	1.09 ± 0.29	1.36 ± 0.48	1.09 ± 0.29
cim14	1.50 ± 0.50	2.40 ± 0.66	2.63 ± 0.48

[a] Disease was scored on at least 10 plants per mutant, per experiment 10 days after inoculation, according to the following rating: rating 1, zero to one leaf per plant showed hyphal growth; rating 2, two to four leaves per plant infected; rating 3, more than four leaves per plant infected. Mean and standard deviation were calculated for one experiment. The experiment was repeated three times.

[b] NT, not tested.

from Erysiphe infection by BTH pretreatment (0.3 mM; K. Maleck, unpublished observation). We utilized a disease rating system between 1 (resistant) and 3 (susceptible) to quantify macroscopic symptoms. Interestingly, this assay revealed a differential response among the *cim* mutants. Some *cim* mutants (*e.g.*, *cim7*, *cim13*) are completely resistant to *E. cichoracearum*, and others are completely susceptible (Table 3). The resistance did not correlate with the strength of *PR-1* gene expression or SA content. The two strongest mutants *cim9* and *cim13* were resistant, but *cim7*, with low *PR-1* gene expression and SA accumulation, also displayed an almost complete resistance (rating 1.01).

Many *cim*s are resistant to bacterial pathogens: To check for resistance to prokaryotic pathogens, we inoculated the *cim* mutants with several different virulent *P. syringae* strains. Significance of these experiments was often hampered by the non-wild-type leaf morphology and developmental stage of the *cim* mutants. It became clear, however, that resistance to *P. syringae* isolates was, in many mutants, not as good as resistance to Peronospora and Erysiphe. Differences in resistance to the aggressive pathogen *P. syringae* pv *syringae* DC 3000 were small among the mutants. We therefore chose the less virulent strain *P. syringae* pv *maculicola* ES4326 to better illustrate the spectrum of resistance to *P. syringae* among these mutants. Mutants *cim9*, *cim10*, *cim11*, and *cim13* exhibited a bacterial proliferation reduced >10-fold compared to wild type at 5 days after inoculation (Figure 4). For mutants *cim6* and *cim12*, the bacterial titer 5 days after inoculation was 2-fold lower than that in wild type. While mutants *cim5* and *cim14* are both in the class of strong mutants, they were at least as susceptible to this *P. syringae* isolate as wild type (Figure 4).

DISCUSSION

We isolated and characterized new disease-resistance mutants by screening for plants that constitutively express the *PR-1* gene. *PR-1* gene expression is the most reliable marker for monitoring the onset of SAR in Arabidopsis (Uknes *et al.* 1992), although its function remains unclear. To saturate the genome with this mutant class, we used a reporter gene that is readily detectable *in vivo*. Out of 250,000 M_2 plants, we isolated 16 mutants that constitutively expressed the *PR-1* gene without spontaneous microscopic cell death. These were called *cim* mutants. Interestingly, our screen for *cim* mutants yielded mainly dominant or semidominant mutations, thus rendering genetic analysis, complementation tests, and pathway classification by epistasis studies more difficult. We did, however, map four of the *cim* mutants, demonstrating that several independent loci have been identified. In spite of the theoretically near-saturating screen, only 16 *cim* mutants were identified, maybe because many lesion mimic mutants were allelic to *cim* mutants or because of lethality, poor growth, and penetrance of *cim* phenotypes. Hence, given our near-saturating screen, neo- or hypermorphic mutations in the SAR signaling pathway are extremely rare.

All mutants exhibited increased resistance to at least two virulent pathogens, thus validating the marker-gene-based approach. *cim* mutants define a diverse group of loci with different disease-resistance spectrums. It is tempting to speculate about the mechanistic nature of broad-spectrum disease resistance. Since all *cim* mutants are resistant to at least two virulent pathogens, the resistance appears *R*-gene independent and does generally not require an HR. Most *cim*s are able to develop an HR in response to an avirulent pathogen (data not

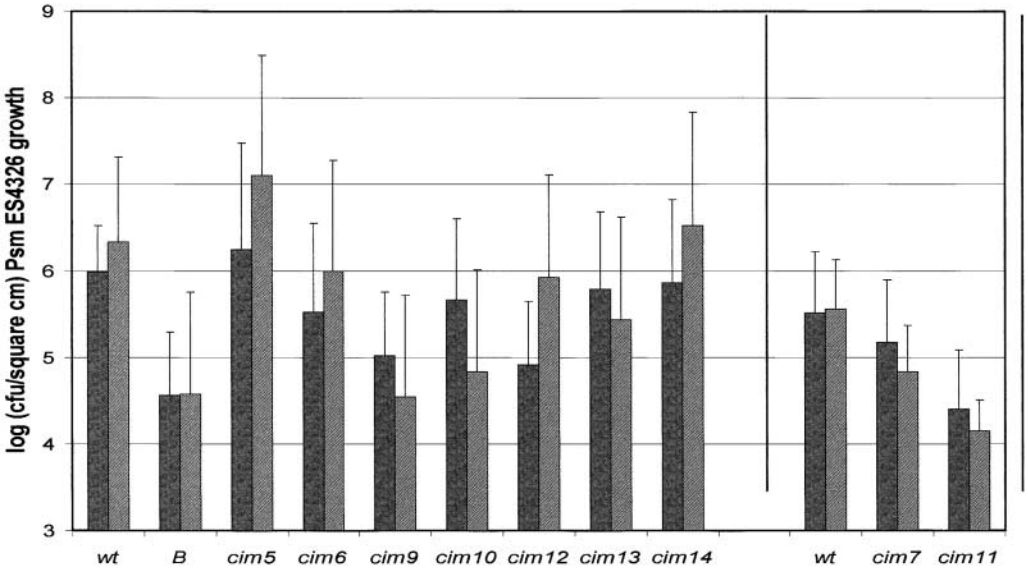

FIGURE 4.—Resistance of *cim* mutants to *Pseudomonas syringae* pv *maculicola* (Psm) ES4326. Three and 5 days after inoculation with Psm, infected leaves were assayed for bacterial density. Bacterial viable counts, expressed as colony forming units (cfu) per 1 cm^2 corresponding to four-leaf discs, calculated from four independent repetitions, are indicated with standard deviations. Lightly shaded bars, 3 days after inoculation (dpi); darkly shaded bars, 5 dpi. The *t* values and confidence limits are as follows, for each mutant compared with the 6E line: 3 dpi, BTH-induced plants (*B*), 5.82($P > 0.995$); *cim5*, 0.53 ($P > 0.65$);*cim6*, 2.53 ($P > 0.975$); *cim9*, 5.40 ($P > 0.995$); *cim10*, 1.33 ($P > 0.85$); *cim12*, 5.47 ($P > 0.995$); *cim13*, 1.13 ($P > 0.85$); *cim14*, 0.56 ($P > 0.7$); *cim7*, 0.94 ($P > 0.8$); *cim11*, 1.74 ($P > 0.9$); 5 dpi, BTH-induced plants, 1.74 ($P > 0.9$); *cim5*, 3.37 ($P > 0.99$); *cim6*, 0.60 ($P > 0.7$); *cim9*, 1.74 ($P > 0.9$); *cim10*, 1.71 ($P > 0.9$); *cim12*, 1.04 ($P > 0.8$); *cim13*, 1.5 ($P > 0.9$); *cim14*, 0.58 ($P > 0.7$); *cim7*, 3.23 ($P > 0.99$); *cim11*, 4.66 ($P > 0.995$).

shown) but some appear to simply bypass HR and thus resemble *dnd* mutants (YU *et al.* 1998). As in the barley *mlo* mutants (PETERHANSEL *et al.* 1997), a compatible interaction is converted into an incompatible interaction. Because of the very different lifestyles of the pathogens used (Erysiphe, Peronospora spp, Pseudomonas), it is unlikely that simple host morphological changes, for example, in the cuticle, are responsible for this resistance. In principle, a mutation in an *R* gene could activate the cascade leading to an activation of SAR, but it has previously been shown that such mutations can also cause a lesion mimic phenotype (*Rp1* in maize; HU *et al.* 1996). The similar nature of the *dnd* mutants, which were identified because of an altered gene-for-gene interaction, when compared to some of the *cim* mutants, reveals a link between SAR and *AVR/R*-gene mediated resistance. The *DND1* gene was recently cloned and encodes a probable ion channel (CLOUGH *et al.* 2000). A truncation in this protein leads to high levels of SA and *PR*-gene expression and stunted growth, thus mimicking constitutive SAR. Yet another constitutive SAR mutant is caused by a mutation in a MAP kinase (PETERSEN *et al.* 2000). A direct interaction between SA and the MAPK4 protein is unlikely, and the entire pathway is clearly far from being understood. However, the characterization of the MAPK4 mutant confirmed that SAR induction might be negatively regulated (PETERSEN *et al.* 2000).

We can assemble a first-order classification of these *cim* mutants, using differential resistance against pathogens. Several mutants were highly resistant to all tested pathogens (*e.g.*, *cim9*, *cim13*). Others were resistant only to fungal pathogens (*e.g.*, *cim6*). Mutant *cim10* was resistant to Pseudomonas and *P. parasitica* spp., but not to Erysiphe. Mutant *cim7* was strongly resistant only to Erysiphe. The identification of an *edr*-like mutant such as *cim7* with weak accumulation of *PR* gene transcripts was possible because the luciferase reporter gene assay is very sensitive. Together with the different map positions obtained for some of the mutants, these quantitative differences confirm our identification of several novel disease-resistance mutants and reveal a complex regulation pattern for the different branches of resistance signaling in Arabidopsis. Thus, monitoring the expression of one marker gene provided us with an array of mutant phenotypes. Using all our *cim* mutants (that might contain the majority of all possible mutants in this category) we conceivably could dissect a variety of branch points and possibly discern divergences in the plant's innate immune system (CLARKE *et al.* 2000, 2001; JIRAGE *et al.* 2001). These mutants may also provide good starting points for dissection of transcriptional responses (MALECK *et al.* 2000).

Molecular cloning of the underlying *cim* genes and epistasis studies of known recessive regulatory mutants, such as *ndr1* (CENTURY *et al.* 1995), *eds1* (PARKER *et al.* 1996), and *pad4* (GLAZEBROOK *et al.* 1997), together with broadening the spectrum of diseases tested will give further insight into the relative relationships among the loci identified by this collection of *cim* mutants and others like it (CLARKE *et al.* 2000, 2001; JIRAGE *et al.* 2001) and by similar mutants such as *dnd1*.

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