DOI: 10.1534/genetics.104.032193

Fitness Costs of Mutations Affecting the Systemic Acquired Resistance Pathway in *Arabidopsis thaliana*

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Manuscript received June 9, 2004
Accepted for publication August 30, 2004

ABSTRACT

This study investigated the fitness effects of four mutations (*npr1, cpr1*, *cpr5*, and *cpr6*) and two transgenic genotypes (*NPR1-L* and *NPR1-H*) affecting different points of the systemic acquired resistance (SAR) signaling pathway associated with pathogen defense in *Arabidopsis thaliana*. The *npr1* mutation, which resulted in a failure to express SAR, had no effect on fitness under growth chamber conditions, but decreased fitness in the field. The expression of *NPR1* positively correlated with the fitness in the field. Constitutive activation of SAR by *cpr1*, *cpr5*, and *cpr6* generally decreased fitness in the field and under two nutrient levels in two growth chamber conditions. At low-nutrient levels, fitness differences between wild type and the constitutive mutants were unchanged or reduced (especially in *cpr5*). The reduced fitness of the constitutive mutants suggests that this pathway is costly, with the precise fitness consequences highly dependent on the environmental context.

ORGANISMS have evolved a variety of resistance mechanisms to defend themselves against their natural enemies, and there has been extensive discussion of the degree to which such mechanisms are likely to be costly to the organism when pests and pathogens are absent. From an evolutionary viewpoint it has been hypothesized that the magnitude of these fitness costs will determine whether such resistance mechanisms will evolve to be constitutive or inducible (Clark and Harvell 1992; Frank 1993). Resistance costs will also determine whether a stable polymorphism of resistant and susceptible genotypes can be maintained in a population (Jayakar 1970; Antonovics and Thrall 1994).

Despite the importance of fitness costs in determining the evolution of resistance mechanisms, consensus on when and how they occur is lacking. Thus while many studies have found costs of resistance in both plants and animals (Bryant and Julkunen-Tiitto 1995; Bergelson and Purrington 1996; Mauricio 1998; Elle *et al.* 1999; Raberg *et al.* 2000; Tian *et al.* 2003), other studies, even though carried out rigorously and with large sample sizes, have failed to find such costs (Simms and Rausher 1989; Agren and Schemske 1993). This may be because fitness costs are dependent on the particular environment in which they are measured as well as on whether the resistance is induced or not. Environmental conditions, especially nutrient availability, are thought to be important factors determining whether costs are seen. Several theories have been suggested to explain how nutrient levels affect resistance; for example, it has been argued that costs may be more evident under low-nutrient conditions (Purrington and Bergelson 1997). It is also particularly difficult to assess costs when the pathogen itself induces the resistance mechanism because then the costs of the mechanism are confounded with its benefits in warding off pathogen attack. As a result there has been considerable interest in examining fitness costs using chemical inducers of resistance pathways (Thaler *et al.* 1999; Heil *et al.* 2000).

Systemic acquired resistance (SAR) is an induced resistance mechanism that was first characterized by Ross (1961) in tobacco during response to infection by tobacco mosaic virus. This mechanism has been found to be present (with some variation) in all angiosperms examined to date (Heil 1999). Pathogens that produce necrotic lesions can induce SAR, and induction of this pathway leads to the expression of defense-related proteins and resistance against a wide spectrum of pathogens including viruses, bacteria, fungi, and oomycetes (Ryals *et al.* 1996). The proteins upregulated during SAR include the pathogenesis-related (*PR*) proteins; many of them are known to possess antimicrobial properties (Datta and Muthukrishnan 1999). Individual components of some of the SAR signaling pathway leading to resistance have been successfully identified. One set of responses is dependent on the production of salicylic acid (SA) and has been associated with the activity of NPR1, a protein that interacts with transcription factors to regulate the expression of defense-related

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PR genes (Zhang *et al.* 1999). The expression of the *PR-1* gene has been used, especially in tobacco and *Arabidopsis thaliana*, as a marker for these SA-mediated responses. Another set of responses is dependent on the production of either jasmonic acid (JA) or ethylene and involves the gene *PDF1.2*. These latter responses are effective against fungal and oomycete pathogens (Penninckx *et al.* 1996; Clarke *et al.* 2000). Evidence is accumulating that different defense pathways interact such that induction of one pathway may inactivate the other (Doares *et al.* 1995a,b; Fidantsef *et al.* 1999; Thaler *et al.* 1999; Traw *et al.* 2003), resulting in tradeoffs in resistance to different organisms.

Several mutants and transgenic variants in the SAR signaling pathway in *A. thaliana* were isolated as part of a study to characterize the SAR pathway (Bowling *et al.* 1994, 1997; Cao *et al.* 1994; Clarke *et al.* 1998). The present research was undertaken to assess the fitness of these genotypes under two nutrient treatments in growth chambers, as well as under natural conditions in a field experiment.

MATERIALS AND METHODS

Mutant and transgenic genotypes: Mutants were all isolated using an EMS-induced mutagenesis screen from the same wild-type strain of *A. thaliana* (Col-0 ecotype, Landsberg, Germany, containing the reporter construct *BGL2::GUS*) and then further backcrossed six times into the same wild-type background to remove possible undetected additional mutations that might have occurred during mutagenesis (Bowling *et al.* 1994, 1997; Cao *et al.* 1994; Clarke *et al.* 1998). The specific mutants used in these experiments were *npr1-1*, *cpr1-1*, *cpr5-1*, and *cpr6-1*; in this notation, the second number (*npr1-1*, *cpr1-1*, etc.) refers to the alleles of the gene in which the mutation occurred. However, since only one allelic variant of each gene was used, the allelic designation is henceforth omitted for simplicity.

In an *npr1* mutant plant, pathogen- and SA-induced SAR is abolished (Cao *et al.* 1994). This is correlated with a loss of resistance and with a failure to induce the *PR* genes, *PR-1*, *PR-2*, and *PR-5* (Cao *et al.* 1994). The *NPR1* gene encodes a protein that is activated by SA and interacts with transcription factors to control *PR* gene expression (Cao *et al.* 1997; Zhang *et al.* 1999; Fan and Dong 2002; Mou *et al.* 2003). Microarray studies suggest that under SAR-inducing conditions hundreds of genes may be regulated by *NPR1* (Maleck *et al.* 2000; Glazebrook *et al.* 2003).

Transgenic plants were generated as part of a study investigating effects of overexpressing and underexpressing the wild-type *NPR1* gene (Cao *et al.* 1998). One genotype, designated *NPR1-H*, had high expression of the NPR1 protein (~3-fold higher than in wild type), while the other genotype, *NPR1-L*, had low expression of the protein (~20-fold lower than in wild type) due to cosuppression. Both transgenic genotypes retain an inducible SAR response and differ only in the intensity of that response (Cao *et al.* 1998).

The *cpr* mutants all express SAR constitutively and activate three well-characterized *PR* genes, *PR-1*, *PR-2*, and *PR-5* (Clarke *et al.* 2000). The *cpr5* and *cpr6* mutants, but not *cpr1*, also constitutively express *PDF1.2*, a gene associated with the JA pathway. All three *cpr* mutants have increased resistance to both the bacterial pathogen *Psuedomonas syringae* and the oomycete pathogen *Peronospora parasitica* (Bowling *et al.* 1994, 1997; Clarke *et al.* 1998). Putting *cpr5* and *cpr6* into an *npr1* background eliminates resistance to *P. syringae* but not to *P. parasitica*. This indicates that there is an oomycete resistance mechanism activated in *cpr5* and *cpr6* that is regulated independently of *NPR1* through the JA pathway (Bowling *et al.* 1997; Clarke *et al.* 1998, 2000). Mutant *cpr5* has stronger resistance than *cpr6* to *P. parasitica* and also has reduced trichome development (Bowling *et al.* 1997; Clarke *et al.* 2000). The *CPR5* gene encodes a putative transmembrane protein (Kirik *et al.* 2001) and is a negative regulator of programmed cell death (L. Anderson and X. Dong, unpublished data). Mutant *cpr1* has a dwarf phenotype that was partially removed during backcrossing (Bowling *et al.* 1994). Both *cpr1* and *cpr6* have been mapped to resistance (*R*) gene clusters (J. Clarke, M. Kesarwani and X. Dong; unpublished data), which encode structurally similar proteins mediating plant resistance against different pathogenic signals (Meyers *et al.* 2003). Since a phenotypically similar mutant, *snc1*, has been identified as a gain-of-function *R*-gene mutant (Zhang *et al.* 2003), it is likely that *cpr1* and *cpr6* are also mutants that constitutively activate either expression of an *R* gene or the activity of its product. Like the *snc1* mutant, constitutive disease resistance in *cpr1* and *cpr6* is blocked by the *R*-gene-signaling mutants *eds1* and *pad4* (Clarke *et al.* 2001; Jirage *et al.* 2001).

Growth chamber experiment 1: This experiment investigated the effects of two nutrient levels on the seed production of wild-type, *cpr1*, *cpr5*, *npr1*, and *NPR1-H* genotypes in a growth chamber at the Duke University Phytotron. The experiment was performed in eight flats, each consisting of an array of "cells," each 4.5 × 5.9 cm and 6.4 cm deep and filled with an autoclaved 1:1:1 mix of sand, gravel, and turface as the growth medium. The five genotypes were divided as equally as possible among the flats with random placement of genotypes within each flat. Half of the flats received the nutrient supplement described below and half did not. Three or four seeds of each genotype were planted in each cell after 10 days of cold treatment at 4° in water, and plants were randomly thinned to one per cell after 1 week of growth.

In the high-nutrient treatment, each cell received 5 ml of 1/2 strength Hoagland's solution weekly for 2 months. At the same time, in the low-nutrient treatment, each cell received 5 ml of distilled water instead of the fertilizer. All plants were watered as needed at least twice a day during the experiment except during the first 2.5 weeks when watering was not needed.

The plants in this study were simultaneously used as controls for a related pathogen treatment in the same growth chamber that demonstrated that the *cpr* mutants have higher resistance against *P. parasitica* regardless of nutrient conditions (Heidel 2002). As part of this control regime the following additional actions were taken. One week after germination, 2 μl of distilled water was placed on the cotyledons of each plant for 3 days. During the first 2 weeks, the flats had plastic domes over them to raise the humidity level to ~100%. One leaf was removed from each plant at day 45 for transcript analysis (Heidel 2002). Temperature inside the domes was kept at ~16° during the day and at 14° during the night. Two and a half weeks after planting, the plastic domes were removed and the temperature in the chamber was set to 22° day and 14° night with 60% relative humidity. Throughout the experiment, the light/dark cycle was 14 hr/10 hr with a light level of 450 mE/sec/m^2. To reduce the effect of plant placement on growth, the positions of flats in the growth chamber and the positions of groups of six cells between flats (within each nutrient treatment) were randomized every week, starting 1 month after planting.

At 97 days after planting, when all plants had senesced, seed

yield was determined for each plant. The total number of siliques was counted on the primary and secondary inflorescences. Then the number of seeds per silique was determined separately for the primary and secondary inflorescences by counting the number of seeds in every fifth intact silique in the high-nutrient treatment and in every intact silique in the low-nutrient treatment. The number of seeds was determined by multiplying the seeds per silique by the number of siliques. The number of seeds on the primary inflorescence and secondary inflorescences were then added together to give the total number of seeds per plant.

Growth chamber experiment 2: This experiment investigated the effects of two nutrient levels on the seed production of wild-type, *cpr1*, *cpr5*, *cpr6*, and *npr1* genotypes. The growth medium, watering, and nutrient treatments were the same as in experiment 1, except that 10 ml (rather than 5 ml) of 1/2 strength Hoagland's solution or 10 ml (rather than 5 ml) of distilled water was used weekly in the high- and low-nutrient treatments, respectively, and the cells were slightly larger (5.9×6.7 cm and 6.4 cm deep). Additionally, these treatments were applied not just for the first 2 months (as in experiment 1) but also through senescence.

Wild type, *cpr1, cpr5, cpr6*, and *npr1* were grown in six flats in a growth chamber at the Duke University Phytotron. Each flat was divided between the five genotypes as equally as possible. Three flats received the added nutrient treatment and the other three flats did not. A flat's location was randomized within the chamber every week. Starting at 2 weeks, cells were randomized between flats within nutrient treatments. The temperature in the chamber was 22° day and 14° night with a light/dark cycle of 14 hr/10 hr, a light level of 675 mE/sec/m^2, and 40% relative humidity. Three or four seeds of each genotype were planted in each cell after 4 days of cold treatment at 4° in water. Two weeks after planting, plants were thinned to 1 plant/cell, leaving 155 plants approximately equally divided between the five genotypes for two nutrient treatments. Two and a half months after planting, all plants had senesced, and seed yield was determined for each plant as described above.

Field experiment: This experiment investigated the performance of wild type, *npr1*, *cpr1*, *cpr5*, *cpr6*, *NPR1-L*, and *NPR1-H* under field conditions. The experiment was performed at the Central Crops Field Station in Clayton, North Carolina [United States Department of Agriculture (USDA) research station of North Carolina State University] located in a region of North Carolina where *A. thaliana* occurs naturally as a weed in cultivated fields. At the site where the experiment was established, naturally occurring *A. thaliana* plants were found. The field was divided into a grid pattern of 21 plots with ~4 m between plots.

Due to USDA regulations on transgenic plants it was not possible to measure seed yield directly because all plants were required to be harvested before seed set. Rosette diameter was therefore used as the proxy for fitness. Results from the first growth chamber experiment demonstrated that rosette diameter in *A. thaliana* was strongly correlated with seed yield. A rare winter thunderstorm and hail storm killed a large portion of the first planting seedlings, and a second planting of seedlings was added a month later. Both plantings were done within the span of the natural germination period of *A. thaliana* in North Carolina (our personal observation). As part of a related herbivore resistance study (Heidel 2002), half of the plots were sprayed with the insecticide Orthene and the other half of the plots were sprayed with water on February 23, 2001. The insecticide treatment was found to have no effect on rosette diameter so the insecticide treatment was not included in any further analyses.

First planting: Seeds of all seven genotypes were cold treated for 3 days at 4°. On November 22, 2000, 2016 seeds, equally divided among genotypes, were planted in 13 flats with soil from the field site. Each flat received approximately equal numbers of each genotype and seeds were planted within the flat in a completely randomized design. The plants were grown in the Duke University greenhouses. Prior to transplanting in the field, they were given two near-freezing treatments at night by moving to a cold room one night and an exposed enclosure a second night. On December 14, 2000, all plants were transported to the Central Crops Field Station in Clayton, North Carolina, and transplanted over the following 2 days. All plants were planted in a completely randomized plot design, with genotypes more or less equally represented in all 21 plots. Plants within plots were placed 10 cm apart in a grid.

Second planting: Seeds of all seven genotypes were cold treated for 6 days at 4°. From January 9 to 10, 2001, 1368 cold-treated seeds more or less equally divided between the seven genotypes were planted in 19 flats with field soil in the Duke University greenhouse. Each flat received a similar number of each genotype and seeds were planted within the flat in a completely randomized design. The seedlings were given eight near-freezing treatments at night by moving to an exposed enclosure. Plants were transported to the field site on February 8, 2001, and transplanted on February 8–9, 2001, to 19 of the 21 blocks already in use. To reduce transplantation time, all plants from a germinating flat were transplanted to one plot, unlike the first planting. As plants began to bolt, rosette diameter or mortality was determined on March 19, 2001, for plants from the first planting, and on March 22, 2001, for plants from the second planting.

RNA analysis: In the second planting of the field experiment one leaf was removed from each plant on March 20–23, 2001. Leaves of the same genotype within a plot were bulked for RNA analysis. RNA was extracted and probed as described by Clarke *et al.* (1998). Expression of *PR-1*, *PDF1.2*, *COR78*, as well as the control gene *UBQ5*, was examined. The *COR78* gene has been shown to increase cold tolerance probably by stabilizing membranes against freezing damage (Horvath *et al.* 1993; Tomashow 1999). *UBQ5*, a constitutively expressed ubquitin gene, was used as a control for the amounts of RNA loaded on the gel. Expression of mRNA was quantified using ImageQuant software and normalized by *UBQ5* expression and then divided by the mean wild-type expression for the blot. Division by wild-type expression reduces the blot effect and allows more accurate estimation of relative expression between genotypes.

Statistical analysis: The seed yield and rosette results in the growth chamber experiments were analyzed by ANOVA, with the different genotypes and nutrient treatments as fixed effects and the flat as a random effect nested within the nutrient treatments. Although the flats are not classical "blocks" since the cells were later randomized between flats, the original flat for each cell was found to have a significant effect. Therefore the original flat of each plant was included in each ANOVA. Plants that died before reproduction (16% in experiment 1 and 23% in experiment 2) were given a seed yield or a rosette diameter of 0.

Rosette diameter in the field was analyzed by ANOVA with genotype and planting date as fixed effects and block as a random effect. Plants that died after transplanting, but before the last rosette measurement (61%), were given a rosette diameter of 0. Survival in the field was analyzed by logistic regression with genotype and planting as fixed effects and block as a random effect. The analysis of differences in survival between wild type and all other genotypes was performed as planned comparisons. All other comparisons between genotypes used the Bonferroni correction.

Gene expression results were analyzed by ANOVA with ge-

TABLE 1

Effects on seed yield or rosette diameter in growth chamber experiments 1 and 2 and in field experiment

	Effect tests								
	Seed yield in growth chamber experiment 1			Rosette diameter in growth chamber experiment 1			Seed yield in growth chamber experiment 2		
Source	d.f.	SS	*F*	d.f.	SS	*F*	d.f.	SS	*F*
Genotype	4	1904	23.2***	4	276.8	20.61***	4	4.40×10^7	2.82*
Nutrient	1	715	34.9***	1	184.6	54.97***	1	5.51×10^8	141.30***
Genotype × nutrient	4	445	5.4***	4	175.7	13.08***	4	4.12×10^7	2.64*
Flat (nutrient)	6	651	5.3***	6	47.6	2.36*	4	3.71×10^7	2.38 ($P = 0.055$)

	Rosette diameter in field experiment		
Source	d.f.	SS	*F*
Genotype	6	1278	27.5***
Planting	1	7587	978.4***
Genotype × planting	6	286	6.1***
Block	20	80	0.5

Seed-yield data and rosette-diameter data from growth chamber experiment 1 were cube root and square root transformed, respectively. Seed-yield data from growth chamber experiment 2 were not transformed. Rosette-diameter data from the field experiment were box cox transformed. SS, sum of squares. *Significant *F*-values at $P < 0.05$; **significance at $P < 0.01$; ***significance at $P < 0.001$.

notypes as a fixed effect and the block and blot (nylon membrane used in the Northerns) as random effects. The blot was treated as an effect because even though the same probe was used on all blots, the probe may hybridize and be washed off at different levels between blots.

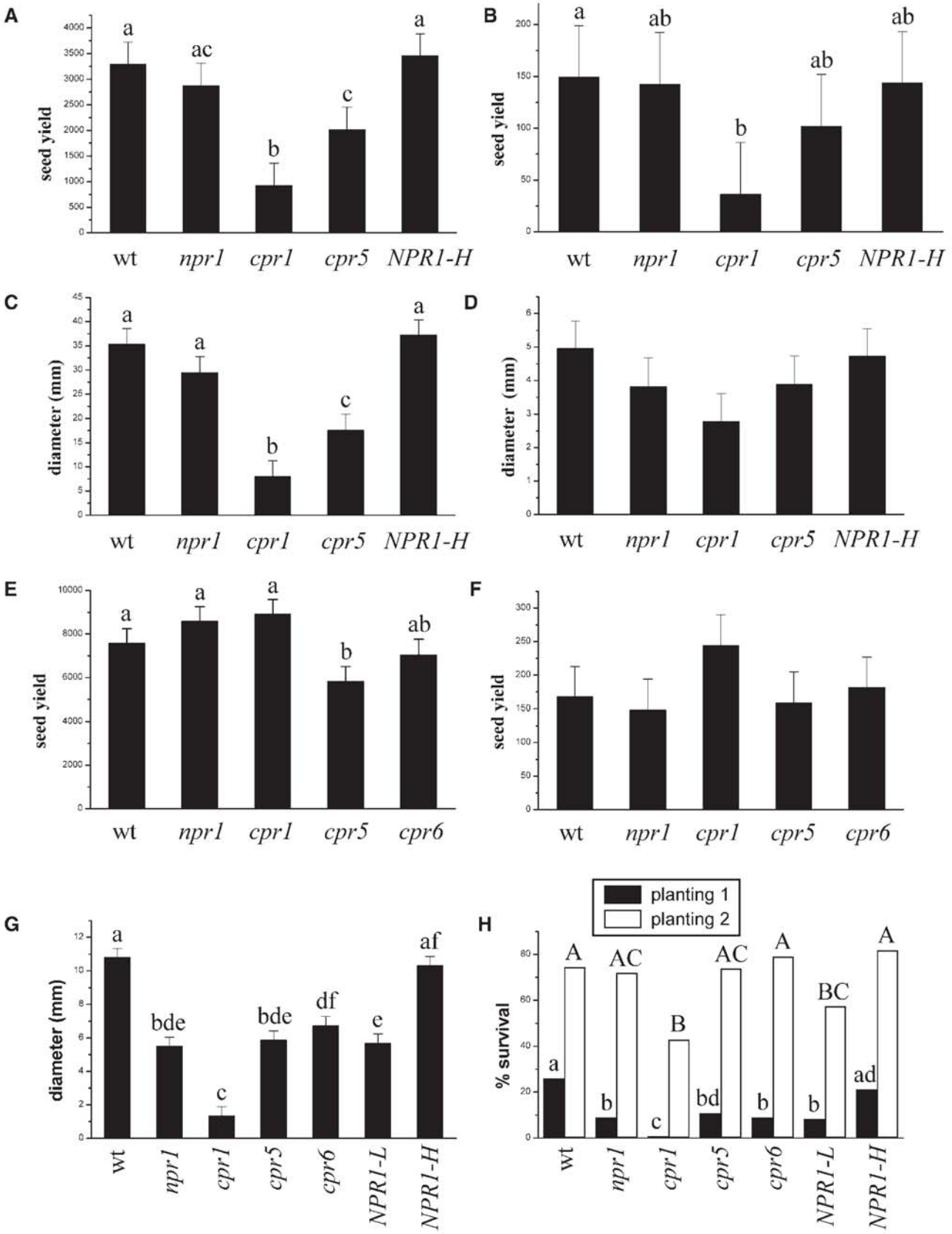
In the analyses, all interaction terms were tested and if they were not significant, they were removed from the analysis. All data were transformed as noted in the legends to Tables 1 and 2 to conform to normality requirements. After ANOVAs, contrast tests between wild type and all other genotypes were performed as orthogonal planned comparisons. All other contrasts were performed as Tukey-Kramer tests, which correct for multiple comparisons. All statistical analyses were performed using JMP 4 (SAS Institute).

RESULTS

Growth chamber experiment 1: There was a large effect of nutrients and genotype on seed yield (Table 1 and Figure 1, A and B). In general, wild-type, *npr1*, and *NPR1-H* genotypes had a higher seed yield than *cpr5*, which had a higher seed yield than *cpr1*. However, there was a significant genotype × nutrient treatment interaction caused largely by the increased relative seed yield of *cpr5* in low nutrients compared to that in high nutrients. Rosette diameter followed the same trends between genotypes as seed yield (Figure 1, C and D), although there are no significant differences between genotypes in the low-nutrient treatment. Genotype × nutrient treatment had strong effects on rosette diameter (Table 1), which was caused largely by the increased diameter of *cpr5* and *crp1* in low nutrients relative to that in high nutrients. There was a strong linear correlation between rosette diameter and seed yield across all genotypes and nutrient treatments ($R^2 = 0.889$, 384 plants, $P < 0.0001$).

Growth chamber experiment 2: In the second growth chamber experiment there were significant effects of genotype, nutrient treatment, genotype × nutrient treatment, and flat on seed yield (Table 1), but the relative differences among the genotypes were much less marked. The *cpr5* genotype had lower fitness than wild type only in the high-nutrient treatment (Figure 1E), and there were no significant differences among genotypes within

FIGURE 1.—Seed yield and rosette diameter in growth chamber and field experiments. Mean values (±SE) are shown for A–G. Letters above bars indicate significantly different values. (A) Seed yield in growth chamber experiment 1, high-nutrient treatment. (B) Seed yield in growth chamber experiment 1, low-nutrient treatment. (C) Rosette diameter in growth chamber experiment 1, high-nutrient treatment. (D) Rosette diameter in growth chamber experiment 1, low-nutrient treatment. (E) Seed yield in growth chamber experiment 2, high-nutrient treatment. (F) Seed yield in growth chamber experiment 2, low-nutrient treatment. (G) Rosette diameter in field experiment across both plantings. (H) Survival in field experiment for planting 1 (solid bars) and planting 2 (open bars). Lowercase letters indicate significant differences within planting 1; uppercase letters indicate significant differences within planting 2.


A
seed yield
a
ac
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npr1
cpr1
cpr5
NPR1-H
B
seed yield
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npr1
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cpr5
cpr6
NPR1-L
NPR1-H
H
planting 1
planting 2
% survival
a
A
b
AC
c
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bd
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A
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npr1
cpr1
cpr5
cpr6
NPR1-L
NPR1-H

TABLE 2

Effects on survival in field experiment

	Effect tests	
Source	d.f.	Chi square
Genotype	6	113.2***
Planting	1	695.3***
Block	20	31.0 ($P = 0.056$)
Genotype × planting	6	19.4**

Significance at $P < 0.05$; **significance at $P < 0.01$; ***significance at $P < 0.001$.

the low-nutrient treatment (Figure 1F). The genotype × nutrient interaction was significant largely due to the seed yield of *cpr5* relative to wild type in the two nutrient treatments. Unlike in the first growth chamber experiment, *cpr1* was not significantly different from wild type.

Field experiment: Rosette diameter (the measure of fitness in this experiment) was affected by genotype, block, planting, and genotype × planting (Table 1, Figure 1G). Wild type had a greater diameter than *npr1*, *NPR1-L* and all the *cpr* genotypes, with *cpr1* being the smallest. The first planting had larger plants than the second planting. Mortality significantly differed between genotypes in both plantings (Table 2, Figure 1H) with *cpr1* having the greatest mortality.

The two well-characterized oomycete pathogens of *A. thaliana*, *P. parasitica*, and *Albugo candida* have easily visible fruiting bodies, and these were the only two pathogens found at the field site in previous years (Heidel 2002). However, no visible symptoms caused by these pathogens were seen on any experimental plants or on native *A. thaliana* plants that grew adjacent to the experimental plots during the growing season.

Messenger RNA expression of *PR-1*, *PDF1.2*, and *COR78* was significantly affected by genotype (Table 3, Figure 2). The *cpr1* and *cpr5* mutants had higher expression of *PR-1* and lower expression of *PDF1.2*, compared to the other genotypes. Expression of *COR78* was significantly lower in *cpr1* than in the other genotypes.

DISCUSSION

In the present study we investigated the fitness effects of two types of mutations and transgenics, namely those that cause constitutive expression of an inducible defense pathway (*cpr* mutants) and those that prevent the induction (*npr1*) or alter the level of expression (*NPR1-L* and *NPR1-H*) of this pathway. Inducible resistance pathways are expected to be costly, and their constitutive expression in the absence of a pathogen is expected to result in a decrease in fitness. On the other hand, the failure to induce such a pathway in the presence of pathogens is also expected to be detrimental to plant fitness. The fitness effects detected here were consistent with these expectations. The *cpr* mutants were "costly" in that they had reduced fitness relative to wild type, whereas the *npr1* mutant and *NPR1-L* resulted in reduced fitness in the field but not under the relatively more sterile growth chamber conditions.

Just as there is need for caution in interpreting the normal metabolic function of genes from "knockout" experiments, there is also need for caution in interpreting the fitness effects of mutants and transgenics in an evolutionary context. The fitness effects of mutants can throw light either on the "physiological costs" of various pathways or, more directly, on the direction of selection of those actual mutations under particular environmental conditions.

The latter type of interpretation is easier than the former, especially if there is supporting evidence that (a) the mutants being compared have a homogeneous background and (b) they reflect the type of mutations that are likely to occur "spontaneously." Chemical inducers of SAR can mimic the expressed phenotype, but they may not reflect the actual phenotypic effects of mutants and other genetic variants. In our studies, it is highly likely that the fitness effects were caused by the mutants themselves and not by differences in genetic background. The mutants were obtained using the same source strain, and six generations of backcrossing were used to homogenize their genetic background. Moreover, the mutations studied here are likely to occur in nature because the EMS mutagenesis used to generate

TABLE 3

Effects on *PR-1*, *PDF1.2*, and *COR78* expression in field experiment

	Effect tests								
	PR-1 expression			*PDF1.2* expression			*COR78* expression		
Source	d.f.	SS	*F*	d.f.	SS	*F*	d.f.	SS	*F*
Genotype	6	122	25.5***	6	15.7	16.2***	6	12.4	6.64***
Blot	6	20	6.28***	4	1.4	2.1	4	6.0	4.85**
Block	18	3	0.22	18	0.6	0.2	18	18.0	3.22***

PR-1, *PDF1.2*, and *COR78* data were box cox transformed. SS, sum of squares. *Significant *F*-values at $P < 0.05$; **significance at $P < 0.01$; ***significance at $P < 0.001$.

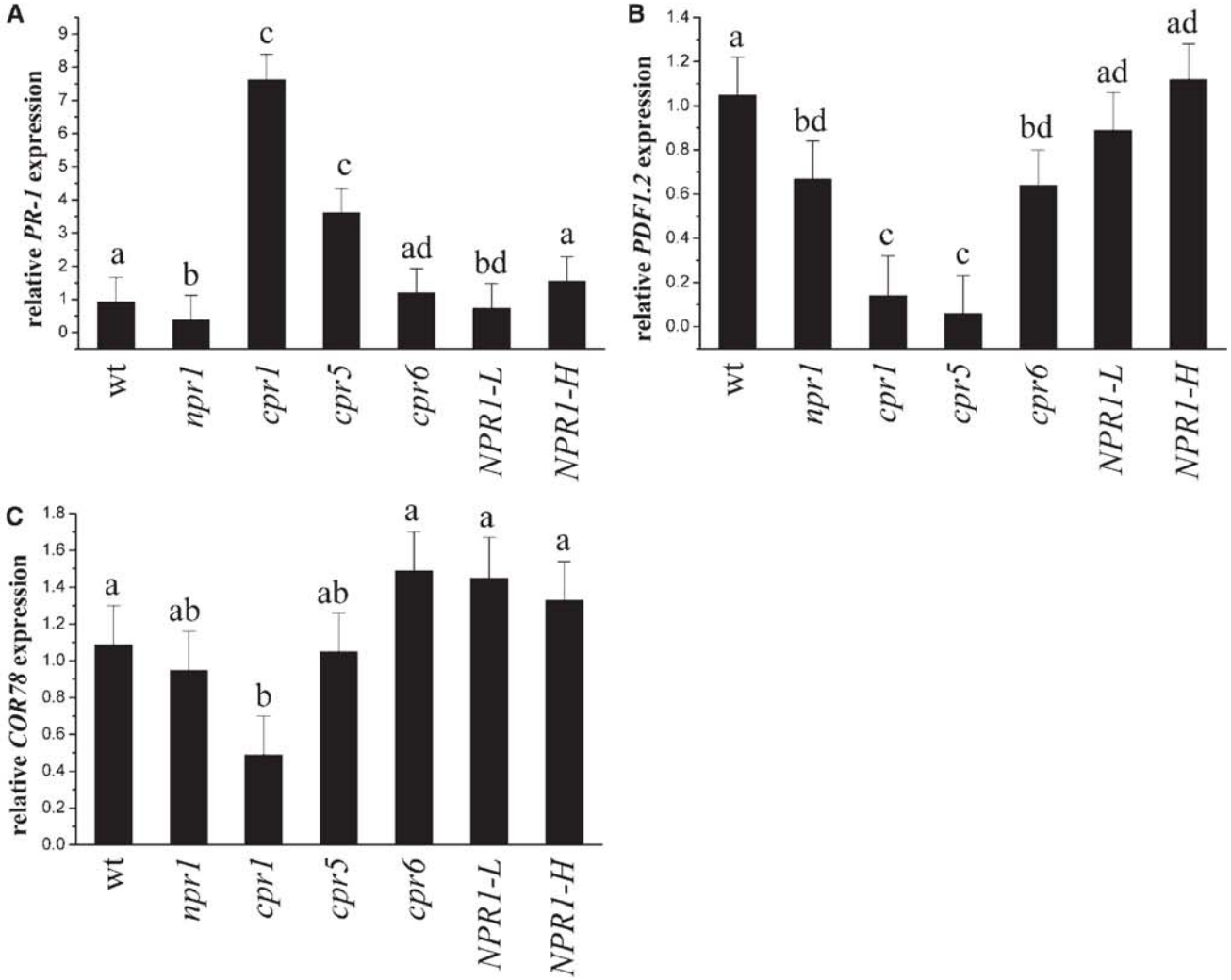

FIGURE 2.—Relative *PR-1, PDF1.2,* and *COR78* expression by genotype in field experiment. Mean values (±SE) are shown of gene expression in the field experiment. Letters above bars indicate significantly different values. (A) *PR-1* relative expression. (B) *PDF1.2* relative expression. (C) *COR78* relative expression.

them is characterized by single-nucleotide changes that can also be envisaged to occur under natural conditions. Obviously, the transgenic "mutations" used to create the *NPR1-L* and *NPR1-H* genotypes are unlikely to happen in nature, but mutations in the promoter of *NPR1* could easily produce the same effect. Our results clearly show that in the absence of high pathogen pressure, mutations causing constitutive expression of the resistance pathway are very unlikely to become established, whereas mutations affecting the expression of *NPR1* would be under much weaker selection.

Interpreting our experiments in terms of the "physiological costs" of the pathways affected by the various mutations is more difficult because the mutations may have pleiotropic effects. This may be because the affected protein itself has multiple "functions" (*i.e.*, is involved in reactions in other pathways) or may result from less direct effects such as the buildup of upstream metabolites or the accumulation of the defective products of the mutated gene itself. Studies suggest that *cpr1* and *cpr6* are mapped to *R*-gene clusters (J. CLARKE, M. KESARWANI and X. DONG, unpublished data) and their phenotypes, including reduced plant size and heightened disease resistance, can be suppressed in the loss-of-resistance *eds1* and *pad4* mutants (CLARKE *et al.* 2001; JIRAGE *et al.* 2001). This argues that the loss of fitness in *cpr1* and *cpr6* is not from pleiotropy but rather from the constitutive activation of the SAR pathway itself. The *cpr5* mutant could also have pleiotropic effects; a different allele of *cpr5* was shown to function in cell death of trichomes (KIRIK *et al.* 2001). However, the relevance to the allele used here is hard to estimate.

In spite of the difficulty of excluding the possibility of pleiotropic effects, our results showing that the *cpr* mutants have a lower fitness than wild type are consistent with many other lines of evidence that these pathways are costly. For example, SAR has also been found to be costly when induced with chemical inducers. HEIL *et al.* (2000) showed that when SAR was induced in wheat by a chemical analog of salicylic acid, BTH, there was a

5–75% reduction in fitness. Redman *et al.* (2001) induced resistance in tomato by JA and showed that it caused a 25% reduction in seed yield. Similarly, a high dose of SA caused a reduction in seed yield in *A. thaliana* (Cipollini 2002). Moreover, the significant cost of SAR is consistent with the large number of proteins that are induced by SAR (Ward *et al.* 1991). In laboratory studies, the *cpr* mutants have been shown to induce expression of the pathogenesis-related marker gene *PR-1* (Clarke *et al.* 2000), and in the field experiments we confirmed elevated expression of *PR-1* in these mutants (for the first time in field-grown *A. thaliana).* In tobacco, two PR proteins can reach 3% of soluble protein (Vogeli-Lange *et al.* 1988). In a survey of 25–30% of the Arabidopsis genes, SAR affected the expression of 413 genes, with the majority having their expression increased (Maleck *et al.* 2000). Many additional genes regulated by the SAR pathway are now being identified (Chen *et al.* 2002; Glazebrook *et al.* 2003).

Finally, any negative pleiotropic effects observed in the *cpr* mutants are unlikely to be allele- or gene-specific because *cpr1*, *cpr5*, and *cpr6* are three independent mutations in three different genes. Nevertheless, all of them exhibit costs under some conditions. This supports the idea that constitutive SAR expression, no matter how it is brought about, has costs.

The role of the JA pathway in these costs is less clear. Expression of the marker gene *PDF1.2* is associated with JA signaling and Clarke *et al.* (2000) showed elevated *PDF1.2* expression in *cpr5* and *cpr6*, but not in *cpr1*. However, in the field experiment described here, both *cpr5* and *cpr6* showed significantly lowered expression of *PDF1.2* compared to wild type (Figure 2B). The reasons for the conflict with the results of Clarke *et al.* (2000) are unknown, but expression of *PDF1.2* in the mutants may be environmentally dependent. Our results indicate that the fitness cost we observed correlates well to *PR* expression following induction of SAR, but the role of the JA pathway remains to be clarified.

Even though a large array of genes may be induced in SAR, parallel transcriptional reduction in other genes could potentially limit the immediate cost of the SAR. Exactly how these genes contribute to overall fitness is unclear. Indeed, reductions in photosynthesis transcripts/proteins are common in defensive responses (Somssich and Hahlbrock 1998; Roitsch 1999) and while these reductions may "save" on protein synthesis, they themselves may be symptoms of how costs are expressed (*e.g.*, reduced photosynthetic rate). Similarly, in the present study we found a lower expression of *COR78* in *cpr1* in the field experiment (Figure 2C). Because *COR78* has been implicated in cold tolerance, a lower expression level in *cpr1* might lead to a reduced cold tolerance and lowered overwinter survival rate. As shown in Figure 1H, this was indeed the case for the *cpr1* mutant. Whether there is a direct causal connection between reduced *COR78* expression and reduced overwinter survival rate for *cpr1* remains unknown.

The *BGL2::GUS* reporter gene used for the initial mutant screen was present in all of the genotypes used in this study. Nevertheless, the expression of the single *BGL2::GUS* gene probably had an extremely small effect on fitness compared to the expression of hundreds of genes activated during SAR. Furthermore, a similar construct in *A. thaliana* has been shown to have no detectable fitness cost (Purrington and Bergelson 1997).

The *npr1* mutant and the *NPR1-L* transgenic (with inactivated NPR1 protein and reduced NPR1 expression, respectively) did not differ in fitness from wild type in the growth chamber experiments (Figure 1, A, B, E, and F), indicating that in the absence of a pathogen attack, the inability to mount a defense should not be costly. Our results also indicate that there were no pleiotropic effects of *npr1* that affected fitness. Vanacker *et al.* (2001) found that *npr1-1* plants had fewer leaf cells and higher DNA content than wild type, but if these differences were expressed in the conditions of our experiments, they did not cause any fitness reduction. However, the *npr1* and *NPR1-L* genotypes did show a significant fitness reduction relative to wild type in the field experiment. This result is consistent with the fact that the environment in the field experiment was less sterile, as compared to the growth chambers with filtered air and sterilized nonorganic media. Even though the highly visible pathogens *P. peronospora* and *A. candida* were not present, natural microbes in the soil could have negative effects on plants with impaired resistance such as *npr1* and *NPR1-L*. Some rhizobacteria have been shown to induce an *NPR1*-dependent defense mechanism (Pieterse *et al.* 1998; van Wees *et al.* 1999; Iavicoli *et al.* 2003).

There was no significant difference in fitness between wild type and *NPR1-H* either in the growth chamber or in the field (Figure 1, A, B, E, F, and G). In the growth chamber, SAR induction is not expected. In the field, however, it is possible for SAR to be induced by pathogens or soil microbes, especially given the fitness reduction observed in *npr1* and *NPR1-L*. The increased intensity of the SAR induction in *NPR1-H* might be expected to be more costly than that in wild type. Cao *et al.* (1998) found that *PR-1* expression in this transgenic was not constitutively elevated, and our analyses of field-collected samples found that while *NPR1-H* showed somewhat greater expression of *PR-1* than wild type did, the difference was not significant. Therefore, it appears that *NPR1-H* did not show a substantially different reaction compared to wild type and correspondingly the fitness of these two genotypes was similar.

There has been substantial discussion on the role of resource availability in the expression of induced defenses in plants (Purrington and Bergelson 1997; Heil *et al.* 2000). One expectation is that metabolic costs will be enhanced under resource-limited conditions. On

the other hand, it can also be argued that if a resistance is costly, then it will be downregulated under nutrient stress to minimize the cost. In this study, there was a significant genotype by nutrient-treatment effect in both growth chamber experiments, indicating that nutrients may change the relative fitness cost (Table 1). In the first growth chamber experiment, the cost in low nutrients is greater for *cpr1*, but less for *cpr5*. In the second growth chamber experiment, *cpr5* also has a reduced relative cost under low nutrients, while *cpr1* has no cost under either nutrient treatment. Therefore, our results were inconclusive about the effect of resources on resistance costs. Heil *et al.* (2000) used a SA analog, BTH, to studying the effects of nutrients on the cost of the SAR pathway in wheat. They found that under sterile conditions low nutrients caused a greater, rather than smaller, relative cost. However, under field conditions they found the reverse situation: the relative cost of induction was less under low nutrients.

Our experiments also suggest that conditions other than resource availability may have a large effect on the expression of resistance pathways. The first and second growth chamber experiments were run under very similar conditions, except that in the first experiment the humidity levels were 100% for the first 2.5 weeks and 60% for the remainder compared to the second experiment where they were 40% throughout. It is interesting that fitness costs were far less in the second experiment: there was no cost seen for *cpr1* in the high-nutrient treatment and no cost seen for *cpr1*, *cpr5*, or *cpr6* in low nutrients. It is therefore interesting to note that at a humidity level of 40% (as in the second experiment) two natural pathogens of *A. thaliana*, *P. parasitica*, and *A. candida* cannot cause successful infection because they require much higher humidity levels (Holub *et al.* 1994, 1995). Therefore, at 40% humidity there might be no need for defenses against these and perhaps other fungal or oomycete pathogens in the evolutionary history of *A. thaliana*. It is possible that the lack of fitness costs is an adaptive response whereby resistances against fungi are inactivated at low humidity.

In conclusion, the results of this study show that the SAR resistance pathway in *A. thaliana* is costly in the absence of pathogens whereas its expression may provide protection against pathogens under field conditions. Our results also suggest that the expression of this pathway may be greatly influenced by environmental conditions and that further studies are needed to pinpoint the degree to which this environmental dependence is itself adaptive. Moreover, the expression of SAR results in a broad suite of changes in the expression of various genes. We have just begun to understand the consequences of these changes for plant fitness and the environmental dependence of these changes.

We thank David Shew for use of the field site and Noland Martin, Joel Kniskern, and members of the Dong laboratory for assistance in the field work. A. Heidel was supported in part by a Cell and Molecular Biology program fellowship from Duke University and by the Duke University Arts and Sciences Research Council.

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Communicating editor: D. Weigel