Suppressors of the Arabidopsis lsd5 Cell Death Mutation Identify Genes Involved in Regulating Disease Resistance Responses

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ABSTRACT

Cell death is associated with the development of the plant disease resistance hypersensitive reaction (HR). Arabidopsis lsd mutants that spontaneously exhibit cell death reminiscent of the HR were identified previously. To study further the regulatory context in which cell death acts during disease resistance, one of these mutants, lsd5, was used to isolate new mutations that suppress its cell death phenotype. Using a simple lethal screen, nine lsd5 cell death suppressors, designated phx (for the mythological bird Phoenix that rises from its ashes), were isolated. These mutants were characterized with respect to their response to a bacterial pathogen and oomycete parasite. The strongest suppressors—phx2, 3, 6, and 11-1—showed complex, differential patterns of disease resistance modifications. These suppressors attenuated disease resistance to avr isolates of the biotrophic Peronospora parasitica pathogen, but only phx2 and phx3 altered disease resistance to avr strains of Pseudomonas syringae pv tomato. Therefore, some of these phx mutants define common regulators of cell death and disease resistance. In addition, phx2 and phx3 exhibited enhanced disease susceptibility to different virulent pathogens, confirming probable links between the disease resistance and susceptibility pathways.

PLANTS are constantly challenged by infectious pathogens. However, because plants have developed sophisticated defense mechanisms, disease rarely occurs. One correlate of disease resistance, called the hypersensitive response (HR; Agrios 1988; Goodman and Novacky 1994), is manifested by the local triggering of a set of defense reactions and cell death. This localized plant cell death, around the infection site, may be responsible for halting pathogen growth. Alternatively, HR could be a cellular consequence of the mechanism that actually stops pathogen growth. HR is often governed by single genes in both the plant (resistance or R-gene) and the pathogen (avirulence or avr gene). Disease resistance is observed only when matching R and avr specificities are present (gene-for-gene relationship; Flor 1947).

The HR pathway can be separated into three steps: R-gene-mediated recognition of the pathogen, transduction of signals to the nucleus, and execution of the defense program. The cloning of several R-genes has led to the observation that, despite the diversity of pathogens recognized by these genes, common structural features are found among the proteins they encode (Bent 1996). Subsequent to recognition, signal transduction pathways are engaged. A number of different signals have been implicated in the triggering of the HR (Hammond-Kosack and Jones 1996). Generation of reactive oxygen intermediates (the oxidative burst; Baker and Orlando 1995) and changes in ion fluxes (Atkinson and Baker 1989) are observed during the early phases of many plant-pathogen interactions. Finally, defense genes such as the PR (pathogenesis-related; Linthorst 1991) are activated and defense products such as phytoalexins (Smith 1996) can be synthesized.

It is unclear what contributions cell death and defense gene activation make in halting pathogen growth. Cell death may be the result of the induction of defense products, many of which are toxic for the plant cell. However, HR cell death seems to be intrinsically controlled by the plant. Several lines of evidence support the idea that HR cell death is a form of programmed cell death (Mittler and Lam 1996; Morel and Dangl 1997). The best evidence derives from the existence of mutants, called lesion mimics, that spontaneously exhibit cell death reminiscent of the HR in the absence of pathogen. Such mutants have been found in maize (Waldorf et al. 1983), barley (Wolter et al. 1993), and other species (Dangl et al. 1996). In Arabidopsis, acc (Greenberg and Ausubel 1993; Greenberg et al. 1994) and lsd mutants (lesion simulating disease resistance; Dietrich et al. 1994) have been identified. In addition to the spontaneous cell death phenotype, these mutants exhibit hallmarks of the plant defense response, such as expression of defense genes and ectopic disease resistance.

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Involved in Regulating Disease Resistance Responses
On the basis of their phenotypes, two classes of lsd mutations were established. In the first class, lesions do not spread once initiated (initiation class). Most lesion mimic mutations that belong to this class (e.g., lsd5; Dietrich et al. 1994). It was hypothesized that these mutations represent defects in genes involved in the triggering of the HR (cell death pathway (Dangl et al. 1996; Mittler and Lam 1996). In the second class of mutations (propagation class), lesions spread once they have been initiated (e.g., lsd1; Dietrich et al. 1994). These mutations define genes necessary for the control of the extent of cell death (Dietrich et al. 1997). Recently, several of the genes responsible for cell death phenotypes have been cloned. The LDL gene encodes a novel class of zinc finger protein that could act as a negative regulator of signals involved in the propagation of cell death (Dietrich et al. 1997). The LLS1 gene from maize encodes a putative dioxygenase that could be responsible for the detoxification of signals generated during cell death (Gray et al. 1997). Finally, the MLO resistance gene from barley encodes a putative transmembrane protein (Büsches et al. 1997). The cloning of such genes and the study of their regulation will help define the regulatory components of cell death in plants.

Different approaches have been used to analyze genetically HR cell death. In the first, the phenotype used to define mutants consisted of loss of disease resistance to a particular avirulent pathogen. This led to the identification of loci required for disease resistance (RDR loci; Hammond-Kosack and Jones 1996), such as ndr1 (Century et al. 1995) and eds1 (Parker et al. 1996). Both of these mutations identify Arabidopsis genes required for resistance to multiple pathogens and triggered by multiple R-genes. Identification of these mutations suggests a possible convergence of the pathways triggered by distinct R-genes. Interestingly, both these mutants also exhibited enhanced growth of normally virulent pathogens, suggesting that the pathways leading to resistance and susceptibility share some components.

In other studies, Glazebrook and Ausubel (1994) and Glazebrook et al. (1997) directly addressed the role of one component of the defense response: They looked for mutants affected in the biosynthesis of the major Arabidopsis antimicrobial phytoalexin, camalexin. All pad mutants were impaired in resistance to virulent isolates of the oomycete Peronospora parasitica. However, those mutations had little impact on restricting growth of an avirulent strain of Pseudomonas syringae. Interestingly, those mutants are still able to mount an HR (Glazebrook and Ausubel 1994), suggesting that camalexin cannot be directly responsible for cell death.

Using a similar approach, we aimed at elucidating the role of the HR in the disease resistance pathway. We isolated suppressor mutations of the Arabidopsis lsd5 cell death control mutant. Some of these exhibited reduced resistance to avirulent pathogens and define new loci that modify disease resistance mechanisms. The results presented here support a model in which the genetic components regulating cell death are also required in plant disease resistance.

**MATERIALS AND METHODS**

**lsd5 seeds mutagenesis and suppressor screening:** Seeds of the lsd5 mutant (accession Ws0; Dietrich et al. 1994) were mutagenized with either fast-neutrons (0.031 Gy/s) or ethyl methanesulfonate (EMS 0.15% for 8 hr). M1 plants (600 and 100 for the fast neutron and EMS mutagenesis, respectively) were grown under permissive conditions for lsd5 [16-hr light; long-day (LD)] in 20 pools of 1 to 20 plants for each mutagenesis. Approximately 40 M1 seeds per M1 plant were then sown under nonpermissive conditions [8-hr light; short day (SD)], 60 μEinsteins total fluence] for suppressor screening. In vitro cultivation of lsd5 and M1 seeds from M2 candidate plants was performed on Murashige and Skoog medium (Gibco BRL, Gaithersburg, MD) solidified with 0.8% agar and supplemented with 50 μg/ml kanamycin to select for a T-DNA linked to lsd5 (J.-B. Morel and J. Dangl, unpublished results).

**Bacteria and Peronospora growth conditions:** P. syringae pv tomato DC3000 (Pst DC3000, Whalen et al. 1991) and P. syringae pv glycinea R4 (Psg; Kobayashi et al. 1989) were cultured overnight in modified King's B medium (20 g/liter peptone, 20 g/liter tryptone, 20 g/liter glycerol, 0.05% K2HPO4, 0.05% KH2PO4) supplemented with 100 μg/ml rifampicin. Kanamycin (30 μg/ml) was added in cultures of Pst DC3000 and Psg containing the avirulence geneavrRpm1 (Debener et al. 1991) or avrRps4 (Hinsch and Staskawicz 1996) cloned into the pVSP61 vector (Busgrove et al. 1994) and in cultures of Psg containing the empty vector pVSP61. Spores of P. parasitica isolates Ahco2, Noco2, and Emwa1 were prepared via propagation (Dangl et al. 1992), using the susceptible ecotypes Col-0 and Ws0, respectively. An rpp5 mutant (rpp5 P41; Parker et al. 1997) was used to ensure identity of the isolates Noco2 and Ahco2.

**Bacterial growth and HR assays:** Four-wk-old plants grown under short days were hand inoculated on half leaves with Pst DC3000 strains at a dose of 107 cfu/ml in 10 mM MgCl2 (OD600 = 0.0002), using a syringe (with no needle). At various time points, samples (consisting of four leaf disks of ~0.28 cm², each from separate infected plants) were ground in 10–20 ml water) were sprayed to runoff on 10- to 14-day-old seedlings using a sprayer (Preval, New York). To include lsd5 in these experiments, plants were grown under long-day and shifted to short-day conditions (8-hr light, 16±100% humidity) after inoculation to ensure appropriate Peronospora growth conditions. Samples were analyzed 1 day and 5 days after inoculation using trypan blue staining. In each independent experiment, from 25 to 150 interaction sites (5–30 cotyledons, ~5 interaction sites per cotyledon) were scored for each time point and for each genotype. For sporulation analysis with P. parasitica isolate Emwa1, 20 cotyledons were harvested, weighed, and washed in 100–200 μl water
(vortexed twice 15 sec). Spores were then counted using a hemocytometer (magnification ×100).

**Trypan blue staining:** Plant tissue was heated 3 min at 95° in trypan blue solution (Koch and Slusarenko 1990) and left to stain overnight. After destaining in chloral hydrate (2.5 g dissolved in 1 ml of water) for 2 days, samples were mounted in 70% glycerol for microscopy analysis.

**Statistical analysis:** For segregation analysis, the χ² test was used (1 d.f.). For pathogenic experiments, Student’s t-test was used to compare the means of the different mutant lines and Ws-0.

**Allelism tests and double mutant isolation:** Allelism between the phx/lsd5 mutants and the ndr1-1 (Col-0; Century et al. 1995), pad4-1 (Col-0; Glazebrook et al. 1997), and eds1-1 (Ws-0; Parker et al. 1996) was assayed as follows: The recessive phx/lsd5 mutants were crossed to the different mutants. Allelism in F₁ plants was determined using the loss-of-resistance phenotypes associated with P. parasitica isolate Ahco2 for eds1-1 and isolate Emw1 for pad4-1 and ndr1-1. The strain Pst DC3000 carrying the avrulence gene avrRpt2 (Whalen et al. 1991) was also used to test allelism to ndr1-1. For double mutant isolation, lsd5 was crossed to the eds1-1 and ndr1-1 mutants. Lesioned plants (homozygous for lsd5) were selected in the F₂ progeny and the genotype for the lsd5 mutation was then determined using a PCR marker specific for the lsd5-1 mutation (J. Parker, personal communication). Preselection for the ndr1-1 mutation among lesioned F₂ plants (homozygous for lsd5) was performed by PCR using CAPS marker GAPA, which is linked to this locus. Confirmation of the presence of the ndr1-1 mutation, which results in the absence of NDR1 mRNA (Century et al. 1997), was obtained by RNA blot analysis in the selected F₂ families.

**Separation of the phx mutations from the lsd5 mutation:** The lsd5 mutation is tightly linked to a 1.3-kb truncated T-DNA, as well as to a functional kanamycin resistance gene (see results). Segregation analysis showed that the genetic distance between the 1.3-kb T-DNA and the lsd5 phenotype was <0.36 cM. However, the lsd5 mutant is not tagged (J.-B. Morel and J. L. Dangl, unpublished results). We designed primers from the flanking genomic sequences of this T-DNA. Because both wild-type and lsd5 alleles could be amplified by PCR, this codominant marker (called TOC) was used to genotype plants at lsd5. In some cases, M₄ progeny from individual M₃’s had to be tested to obtain homzygous suppressor mutations that had been isolated as M₂ heterozygote individuals. On the basis of these criteria, a total of 11 mutants, all from independent M₁ lots, were isolated (Figure 1): 4 after fast neutron mutagenesis (0.6 = phx1, 4.2 = phx2, 8.12 = phx3, and 18.2 = phx4) and 7 after EMS mutagenesis (2.3 = phx9, 3.6 = phx10, 6.1 = phx11-1, 10.26 = phx12, 14.1 = phx11-2, 16.1 = phx8, and 17.10 = phx6; see below).

**RESULTS**

**Isolation of lsd5 suppressors by conditional lethal screening:** As described in Dietrich et al. (1994), the recessive lsd5 mutation is conditional. Grown under long days (16-hr light, LD), lsd5 plants are smaller than wild type (Ws-0) and rarely develop spontaneous foliar lesions. However, when transferred from long-day to short-day conditions (8-hr light, SD), adult lsd5 plants show macroscopic lesions 3–4 days later. More importantly, when grown under constant SD, the lsd5 mutation is lethal. We took advantage of this conditional lethality to isolate second site mutations that suppress lsd5. Mutagenized lsd5 M₁ seeds (fast neutron and EMS) were sown under SD, and plants that survived under these conditions were further analyzed. The lsd5 mutation is tightly linked to a kanamycin resistance gene (<0.39 cM; J.-B. Morel and J. L. Dangl, unpublished results) originating from the T-DNA used to obtain this mutant line (Dietrich et al. 1994). M₃ seeds from the putative mutants were analyzed for their resistance to kanamycin, and only mutants that were homozygous for this lsd5-linked marker were selected. M₄ progeny were sown under SD to confirm the suppressed phenotype. In some cases, M₄ progeny from individual M₃’s had to be tested to obtain homzygous suppressor mutations that had been isolated as M₂ heterozygote individuals.

**DNA extraction, PCR conditions, and mapping techniques:** Small-scale genomic DNA preps were made from ~0.25 cm² leaf disks ground in 400-μl extraction buffer (200 mm Tris-HCl, pH 7.5, 250 mm NaCl, 25 mm EDTA, 0.5% SDS). Samples were centrifuged 3 min at 13,000 rpm, the supernatant was precipitated with 300 μl isopropanol, and the pellet DNA was resuspended in 20 μl TE. DNA (1 μl) was used in a 20-μl PCR reaction. For the TOC PCR, conditions were 94° 3 min, 40× (94° 30 sec, 50° 1 min, 72° 3 min), 72° 5 min. TOC lsd5-specific primers were 5′-CCAGTCAAAGGAAAAGAAG-3′ (TIC2) and 5′-ATGGTGTTCCATGGTTTAT-3′ (TAC2). Mapping was done using PCR-based CAPS (Konieczny and Ausubel 1993) and simple sequence length polymorphisms (Bell and Ecker 1994) markers. Other markers were obtained from the Arabidopsis database (http://genome-www.stanford.edu/Arabidopsis/).

**RNAS blot analysis:** Total RNA was purified using 1 ml of TRIZOL reagent (Gibco BRL) per ~0.5 ml of tissue ground in liquid nitrogen according to the manufacturer’s protocol. Samples (5 μg/lane) were separated on formaldehyde-agarose gels (Ausubel et al. 1995), transferred to Hybond-N hybridization membrane (Amer sham, Buckinghamshire, UK), hybridized in HYB- SOL solution (Yang et al. 1993), and washed 30 min in 2× SSC, 0.1% SDS at 65° and 30 min in 1× SSC, 0.1% SDS at 50°. Radiolabeled probes were generated using the random oligolabeling kit from Stratagene (La Jolla, CA). The probes used were cDNAs for PR1 (Uknes et al. 1992) and 18S rRNA (kindly provided by E. Lam, Rutgers, NJ).
Figure 1.—Reversion of the lsd5 cell death phenotype in the phx/lsd5 mutants. Each suppressor line and controls (wild type Ws-0 and lsd5) were grown under SD for 4 wk. (A) Macroscopic phenotypes. A plant is displayed for each line (left) and one typical leaf after staining for cell death with trypan blue (right). lsd5 pictures are magnified four times compared to the others. (B) Size measurements of the original phx/lsd5 lines (the numbers in parentheses refer to the original mutant designation; see Tables 1 and 2). Mean and standard deviation of the largest diameter of 12 plants. The percentage of plants exhibiting macroscopic lesions is indicated: *, 25%; **, 75%; and ***, 100%. (C) Reversion of the lsd5 PR1 gene expression phenotype. Three-week-old plants grown under long days (LD) were shifted to short days for induction of lsd5 lesions and RNA was extracted after 7 days (SD) or treated (under LD) with SA (0.5 mg/ml) and RNA extracted 2 days after treatment (SA). RNA samples (5 μg/lane) were hybridized with a PR1 probe and, after stripping, with an 18-S rRNA probe as a loading control. Experiments were repeated at least two times and gave similar results.

grown under SD (using trypan blue staining as a marker of cell death; see Figure 1A). Rosette size measurements also reflected the degree of lsd5 suppression (Figure 1B). The lines 4.2, 6.1, and 8.12, which are fully suppressed for lesion formation, reached ~75% of the size of wild-type plants, while the other lines were significantly smaller (Figure 1B). In addition to the macroscopic and microscopic examination of the suppression phenotypes, we assessed defense gene expression in the double mutants (Figure 1C). Constitutive PR1 expression has been shown to be associated with the HR-like lsd5 lesions under SD (Dietrich et al. 1994). PR1 is not inducible in the strong suppressor lines after shifting plants from LD to SD, as observed in lsd5, although the weak suppressor lines exhibited low levels of induction (Figure 1C). Consistent with the suppression of lsd5 lesions in plants continuously grown under SD, PR1 mRNA was undetectable in most of the suppressor lines, with the exception of several weak suppressors (lines 2.3, 14.1, 16.1, and 17.10; not shown). We also addressed whether PR-1 was still inducible in those lines by application of salicylic acid (SA), a natural inducer of systemic
TABLE 1
Genetic analysis of lsd5 suppressors

<table>
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<tr>
<th>Cross</th>
<th>F₂</th>
<th>F₂ segregation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Lsd5⁻</td>
<td>Lsd5⁺</td>
</tr>
<tr>
<td>0.6 × lsd5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Ws-0 × 0.6</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>4.2 × lsd5</td>
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<td>0</td>
</tr>
<tr>
<td>Ws-0 × 4.2</td>
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<td>0</td>
</tr>
<tr>
<td>8.12 × lsd5</td>
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<td>0</td>
</tr>
<tr>
<td>Ws-0 × 8.12</td>
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<td>0</td>
</tr>
<tr>
<td>18.2 × lsd5</td>
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<td>0</td>
</tr>
<tr>
<td>Ws-0 × 18.2</td>
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<td>0</td>
</tr>
<tr>
<td>2.3 × lsd5</td>
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</tr>
<tr>
<td>Ws-0 × 2.3</td>
<td>11</td>
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</tr>
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<td>3.6 × lsd5</td>
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</tr>
<tr>
<td>Ws-0 × 3.6</td>
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<td>9</td>
</tr>
<tr>
<td>6.1 × lsd5</td>
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<td>9</td>
</tr>
<tr>
<td>Ws-0 × 6.1</td>
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</tr>
<tr>
<td>10.26 × lsd5</td>
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<tr>
<td>Ws-0 × 10.26</td>
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<td>14.1 × lsd5</td>
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<tr>
<td>Ws-0 × 14.1</td>
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</tr>
<tr>
<td>16.1 × lsd5</td>
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<td>5</td>
</tr>
<tr>
<td>Ws-0 × 16.1</td>
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<td>3</td>
</tr>
<tr>
<td>17.10 × lsd5</td>
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<td>3</td>
</tr>
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</table>

a) Crosses are listed female × male. All F₂s with Ws-0 were segregating lsd5 genotype as assayed by segregation of resistance to kanamycin (not shown).
b) Plants were grown under LD and shifted to SD after 3 wk to induce lsd5 lesions.
c) Tests performed under SD.
d) Segregation data was evaluated with chi-square analysis using the null hypothesis (n.h.) indicated. Chi-square probabilities are indicated. Rejection of the null hypothesis is indicated (r).
e) Crosses were done in both directions and gave similar results.

Arabidopsis Cell Death Suppressors

acquired resistance (SAR) and defense-related genes (Ward et al. 1991). As shown in Figure 1C, PR-1 mRNA accumulation was inducible by SA under LD in all the suppressors to levels comparable to lsd5. Similar results were obtained for SA induction of PR1 in plants grown continuously under SD (not shown).

Genetic analysis of the lsd5 suppressor mutants: Because suppression of the lsd5 phenotype could result from reversion of the initial recessive lsd5 mutation, both intragenic and extragenic suppressors were expected. F₁ and F₂ analyses of backcrosses to lsd5 and crosses to Ws-0 were used to establish inheritance of the phx mutations. The following types of phx mutations were found (Table 1): recessive and linked to lsd5 (6.1, 14.1, and 16.1), dominant and linked (4.2), recessive and extragenic (0.6, 2.3, 3.6, 10.26, 17.10, and 18.2), and dominant extragenic (8.12). While F₁ and F₂ data from the cross 16.1 × lsd5 shows that the 16.1 locus is recessive, an unexpected ratio of ~15 wild type:1 lsd5 was found in F₂ from the cross Ws-0 × 16.1 (instead of the 13 wild type:3 lsd5 ratio expected if 16.1 is recessive and unlinked). This can be explained by genetic linkage between the lsd5 and 16.1 loci. In contrast, we were unable to separate, out of more than 3000 meioses, the suppressor mutation in the line 4.2 from the lsd5 mutation. It is likely that this suppressor corresponds to an intragenic mutation (distance to lsd5 <0.017 cM). Similarly, the 6.1 and 14.1 mutations are tightly linked to the lsd5 mutation and may represent intragenic revertants. Because the 4.2 mutant was dominant, allelism between this mutation and the recessive 6.1 and 14.1 mutations could not be addressed. Despite unexpected segregation ratios observed in the F₂ of the cross 8.12 × lsd5, further analysis and genetic separation of the suppressor mutation from lsd5 confirmed that the mutation in the line 8.12 is extragenic and dominant (see below).

Complementation testing was used to determine how many loci were defined by the eight extragenic recessive suppressors (0.6, 2.3, 3.6, 6.1, 10.26, 14.1, 16.1, and 17.10). phx/lsd5 double mutants were crossed pairwise, and the resulting F₁ seeds were sown under SD. The mutations in lines 6.1 and 14.1 failed to complement (Table 2) and therefore represent allelic mutations. It is noteworthy that the 6.1 line is a significantly stronger allele than 14.1 (see Figure 1). All other intercrosses still expressed the lsd5 early lethal phenotype in SD, indicat-
ing that these mutants represent mutations in different genes (Table 2). Based on this genetic analysis, at least seven complementation groups were obtained. These mutants were named phx after the mythological Phoenix that rises from its ashes as follows: phx1/lsd5 (0.6), phx2/lsd5 (4.2), phx3/lsd5 (8.12), phx6/lsd5 (17.10), phx8/lsd5 (16.1), phx9/lsd5 (3.6), phx10/lsd5 (2.3), phx11/lsd5 (6.1), phx11-2/lsd5 (14.1), and phx12/lsd5 (10.26). Due to its instability after backcross to lsd5, mutant 18.2 (phx4/lsd5) was not further analyzed. Similarly, phx5 is lethal in combination with lsd5. phx1 will be described elsewhere. All experiments described below were using progeny of lines backcrossed to lsd5 and reselected as not segregating for phenotypic suppression of lsd5 lesions.

**Bacterial resistance in the double phx1 lsd5 mutants:**
As a preliminary test that the lsd5 suppressors may impair disease resistance, we monitored the growth of the normally avirulent Pst DC3000 (avrRpm1) bacteria in the phx/lsd5 mutants. The presence of the RPM1 resistance gene in wild-type Ws-0 plants (the background for lsd5) reduces up to 1000-fold the growth of bacteria carrying the avrRpm1 avirulence gene as compared to the isogenic strain lacking avrRpm1 (Grant et al. 1995; Figure 2). As shown in Figure 2A, two suppressor lines, phx2/lsd5 and phx3/lsd5, showed a significant loss of resistance to this normally avirulent isolate 1 day postinoculation (dpi). No significant differences between the other suppressor lines and Ws-0 were detected in these experiments 1 dpi (Figure 2A) or 3 dpi (not shown). The phx2/lsd5 and phx3/lsd5 mutants were therefore further characterized. As shown in Figure 2B, 1 dpi, both suppressor lines allowed bacterial multiplication similar to that observed during the compatible interaction between Ws-0 and Pst DC3000. Bacterial titers remained slightly higher in the phx2/lsd5 and phx3/lsd5 than in Ws-0 at later time points. In addition, chlorotic symptoms associated with the compatible interaction between Ws-0 and Pst DC3000 were visible in these two lines by 5 dpi (Figure 3). It is noteworthy that under these light conditions lsd5 plants normally show enhanced levels of resistance to pathogens (Dietrich et al. 1994) and that despite the presence of the lsd5 mutation, the phx2/lsd5 and phx3/lsd5 mutants displayed reduced resistance. Plants heterozygous for either phx2 or phx3 also exhibited disease-like symptoms, suggesting that this phenotype, like the lsd5 suppression phenotype, is dominant (Figure 3A).

We also tested whether resistance triggered by a different combination of R-avr genes was modified in the phx/lsd5 lines. Plants were inoculated with the normally avirulent P. parasitica isolate Emwa1 (Hinsch and Staskawicz 1996) and bacterial growth measured. We did not measure any significant difference in bacterial growth or changes in symptoms between the phx/lsd5 lines and Ws-0 (1 and 3 dpi; data not shown).

**Reaction to the oomycete P. parasitica in the phx1 lsd5 mutants:**
Lsd5 plants exhibit enhanced resistance to the virulent P. parasitica isolate Emwa1 (Dietrich et al. 1994). We wanted to measure the possible effects of the suppressor mutations on this phenotype under conditions where lsd5 exhibits little or no cell death. Plants were therefore grown under LD and shifted to SD after inoculation with different P. parasitica isolates (SD; high humidity conditions are required for optimal Peronospora growth). Under these conditions, almost no spontaneous cell death was visible in lsd5 until day 3 after shift to SD, although background cell death could be measured microscopically (data not shown).

Interactions involving biotrophic oomycetes such as P. parasitica differ significantly from interactions between plants and necrotrophic bacteria. In particular, plant cell death may play a different role in interactions involving necrotrophic and biotrophic pathogens (Morel and Dangl 1997). The use of this pathosystem also allows for detailed characterization of the cellular events during infection of cotyledons (Koch and Slusarenko 1990; Holub et al. 1994; Holub and Beynon 1997). We

### TABLE 2

**Complementation testing of lsd5 extragenic recessive suppressors lines**

<table>
<thead>
<tr>
<th>Pollen phx recipient</th>
<th>Designation</th>
<th>Pollen donor (dpi)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>0.6</td>
<td>phx1</td>
<td>+</td>
</tr>
<tr>
<td>2.3</td>
<td>phx9</td>
<td>+</td>
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<tr>
<td>3.6</td>
<td>phx10</td>
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<td>phx11-1</td>
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</tr>
<tr>
<td>10.26</td>
<td>phx12</td>
<td>+</td>
</tr>
<tr>
<td>14.1</td>
<td>phx11-2</td>
<td>*</td>
</tr>
<tr>
<td>16.1</td>
<td>phx8</td>
<td>*</td>
</tr>
<tr>
<td>17.10</td>
<td>phx6</td>
<td>+</td>
</tr>
</tbody>
</table>

For each cross, more than five F1 seeds were tested for lsd5 phenotype under SD.
(*) Not tested but reciprocal cross tested.
(+) Complementation; (−) no complementation.

![Image of Table 2](https://example.com/table2_image.png)
Figure 2.—Modification of disease resistance to P. syringae in the phx/lsd5 mutants. Plants grown under SD were hand-inoculated with 10⁵ cfu/ml of Pst DC3000 (+/−avrRpm1). At the indicated times, samples were cut from infected leaves and bacterial titers were determined. (A) Pst DC3000 (avrRpm1) 1 day postinoculation (dpi) in the phx/lsd5 mutants and Ws-0 (gray bars). Pst DC3000 in Ws-0 (black bar) was also included as a growth-positive control. Stars indicate a statistically significant difference between Ws-0 and the corresponding lines (P > 99%) using a Student’s t-test. (B) Open triangles, Pst DC3000 in Ws-0; open squares, Pst DC3000 (avrRpm1) in Ws-0; solid circles, Pst DC3000 (avrRpm1) in phx2/lsd5; solid squares, Pst DC3000 (avrRpm1) in phx3/lsd5. The t values and confidence limits 1 dpi for Pst DC3000 (avrRpm1) are as follows: Ws-0-phx2/lsd5, 5.36 (>99%); Ws-0-phx3/lsd5, 2.53 (>95%). Each point represents the mean and standard deviation of four to five independent experiments. Because these experiments were done under SD, lsd5 could not be included.

used trypan blue staining to detect both fungal structures and plant cells showing either increased membrane permeability (light blue staining) or collapse (dark staining and absence of recognizable cell shape). Typically, six major types of reaction sites were observed during an incompatible interaction (Figure 4). After adhesion to the plant leaf surface, the spore had germinated and given rise to an infection structure called a haustorium (Figure 4A). From this point, two sets of events occurred. One was characterized by reaction of the infected plant cells (haustorium with reaction, Figure 4B) and later development of a typical HR (Figure 4C), as described by Koch and Slusarenko (1990), with intact neighboring mesophyll cells exhibiting light staining with trypan blue. In the other series of events, which only accounts for ~20% of the incompatible interaction sites in Ws-0 for either of the two isolates used, there was no apparent plant cell reaction and the parasite grew further (free hyphae, Figure 4D). The plant cells eventually detected those hyphae, leading to light trypan blue staining (hyphae with reaction, Figure 4E). Finally, HR occurred but behind hyphal growth, as suggested by the presence in some cases of hyphae emerging from HR (hyphae in HR, Figure 4F).

As a first characterization of the phx/lsd5 lines, we measured the frequency of HR (as in Figure 4C) after challenge with the avirulent isolates Ahco2 (recognized by an R gene near RPP12; Holub and Beynon 1997) and Noco2 (recognized in Ws-0 by the RPP1/10/14 complex; Reignault et al. 1996; Botella et al. 1998). For
Figure 5.—Differential modification of the HR mediated by separate R-genes in the phx/lsd5 mutants in response to P. parasitica isolates Ahco2 and Noco2. Two-week-old seedlings were spray-inoculated with a spore suspension of either isolate of P. parasitica. Tissues were stained using trypan blue as a marker of cell death 1 dpi, and the percentage of HR was determined (reaction types as defined in Figure 4C). (A) Isolate Ahco2; each point represents the mean and standard deviation of three to four independent experiments of 25–150 interaction sites on 5–30 cotyledons. Star denotes that a statistically significant difference was found using a t-test (95% confidence limit) between the phx/lsd5 line and Ws-0 or lsd5. (B) Isolate Noco2. One representative experiment from three experiments. All experiments were scored blind.

both isolates, the suppressor lines phx2/lsd5, phx3/lsd5, phx6/lsd5, and phx11-1/lsd5 displayed significantly fewer HR sites than either Ws-0 or lsd5 (Figure 5). The other suppressor lines were not significantly different from lsd5. Interestingly, the phx11-2 allele did not exhibit reduced HR as observed with the phx11-1 allele. This suggests that despite the absence of visible lsd5 lesions in growth conditions used in these experiments, the presence of lsd5 in the background renders weak suppressors (e.g., phx11-2; see Figure 1) slightly more resistant compared to Ws-0 (see also below). Little or no further growth of the pathogen was visible 5 dpi in Ws-0 and lsd5. However, in some of the lines where a reduced number of H Rs was observed 1 dpi, hyphal growth had occurred 5 dpi, along with extensive cell death (data not shown and see below). We never observed asexual sporulation in any of the suppressor lines or Ws-0 and lsd5 controls.

We then tested whether any of the suppressor mutations modified susceptibility to a normally virulent isolate of P. parasitica. Plants grown under LD were inoculated with the virulent isolate Emwa1 and shifted to SD. The putative intragenic suppressor mutant phx2/lsd5 exhibited enhanced susceptibility to the pathogen, as demonstrated by a threefold increase in sporulation in this line as compared to lsd5 (Figure 6). The other phx/lsd5 lines showed sporulation levels similar to those in lsd5 or Ws-0. lsd5 mutants were as susceptible as wild-type Ws-0, confirming that the conditions used in these experiments do not significantly trigger lsd5-mediated resistance as observed under SD (Dietrich et al. 1994).

The phx mutations define previously unidentified loci:
From the experiments described above, it appeared that several of the phx/lsd5 mutants affected resistance determined by multiple R-genes. Several recessive mutations required for R-gene function (collectively termed RDR, required for disease resistance; Hammond-Kosack and Jones 1996) have been identified in Arabidopsis, and different screening procedures have sometimes led to the identification of mutations at the same locus (e.g., Glazebrook et al. 1996). Therefore, some phx mutations could be allelic to known RDR mutations. Allelism between the phx mutations and three well-characterized RDR mutants, ndr1-1 (Century et al. 1995, 1997), pad4-1 (Glazebrook et al. 1997), and eds1-1 (Parker et al. 1996), was examined.

For the phx2, phx11-1, phx11-2, and phx3 mutants, allelism was ruled out by mapping. In the case of phx2, phx11-1, and phx11-2 mutants, strong genetic linkage was found with the lsd5 locus (Table 1). The lsd5 mutant (Ws-0 background) was crossed to the polymorphic wild-type ecotypes L a er- or Col-0. lsd5 phenotype F1 plants were used for mapping using CAPS and SSLP PCR markers (Konieczny and Ausubel 1993; Bell and Ecker 1994). Linkage to lsd5 was found on the bottom of chromosome 2, 1.2 cM telomeric to AthBIO2. As none of ndr1-1, pad4-1, or eds1-1 maps to chromosome 2 (Holub 1997 and references therein), we concluded that phx2, phx11-1, and phx11-2 are not allelic to them. The dominant phx3 mutation was mapped via the wild-type PH X 3 recessive allele. The double mutant phx3/lsd5 was crossed to Col-0, and F2 plants that showed lsd5 lesions (double homozygote lsd5/PH X 3) were used for mapping. Linkage to chromosome 5 was found between markers nga76 and spl2. As expected, linkage to chromosome 2 corresponding to the lsd5 locus (for which the mapping population was selected) was also found.

The recessive phx/lsd5 mutants were each crossed to ndr1-1, pad4-1, and eds1-1 recessive mutants. For complementation in the F2 progeny, in which the recessive lsd5 mutation has no impact, we tested for recovery of phenotypes associated with the test RDR-type mutations (see materials and methods). None of the tested phx/lsd5 lines were allelic to any of these RDR mutations in F2 plants, and recovery of lsd5 lesions in the F2 progeny further confirmed this conclusion (not shown). These results are consistent with the fact that lsd5 lesions are not suppressed in eds1-1/lsd5 or ndr1-1/lsd5 double mutants (data not shown). We conclude that the phx mutants define new loci that can differentially modify R-gene function.

Genetic separation of the phx mutations from the lsd5 mutation: Because the lsd5 mutation confers heightened levels of disease resistance under SD (Dietrich et al. 1994), there was a possibility that the phenotypes observed in the phx/lsd5 lines were combinatorial. Therefore, it was critical to isolate the phx mutations from the lsd5 mutation. Because the phx2, phx8, phx11-1, and phx11-2 mutations are linked to lsd5 (and potentially intragenic; Table 1), we did not attempt to separate them from the lsd5 mutation. Lines carrying four suppressor mutations (phx3, phx6, phx9, and phx12) exhibiting various degrees of suppression (Figure 1) were isolated.

The principle of isolation of the phx mutations is described in materials and methods. A PCR marker that is linked to the lsd5 mutation was used to assist the isolation of the phx mutations, on the assumption that this marker was reliably reflecting the lsd5 genotype (see materials and methods). Putative isolated phx lines were then backcrossed to lsd5 and Ws-0 to check for the absence of the lsd5 mutation and for homozygosity at the phx locus. For phx3, progeny from 18 independent (Ws-0 × phx3) F2 backcross families segregated no lsd5 plants, proving that the phx3 line was homozygous LSD5. This was confirmed in that all nine independent F2 progenies from the phx3 × lsd5 cross segregated 15 wild type: 1 lsd5, indicating that the line isolated was homozygous for the phx3 mutation and that this mutation was extragenic and dominant. Similar analysis was done with the recessive phx6, phx9, and phx12 mutations (data not shown). None of the isolated phx lines showed any visible phenotype.

Bacterial resistance in the phx3 and phx6 mutants: We first assessed bacterial growth in phx3 and phx6 using Pst DC3000 (with or without avrRpm1). As shown in Figure 7A, the phx3 line allowed significantly more growth of the normally avirulent Pst DC3000 (avrRpm1) than Ws-0. In addition, disease symptoms were visible in phx3 3 dpi (Figure 3A). We conclude from this experiment that the observed reduced disease resistance in...
Figure 7.—Bacterial growth in the phx3 and phx6 single mutants. Plants grown under SD were hand-inoculated with 10^6 cfu/ml of Pst DC3000 (+/- avrRpm1 or avrRps4). At the indicated times, samples were cut from infected leaves, and bacterial titers were determined. Each point represents the mean and standard deviation of three to four independent experiments. (A) Bacterial growth in the phx3 mutant. Squares, Pst DC3000; circles, Pst DC3000 (avrRpm1). Open symbols, Ws-0; solid symbols, phx3. The t values and confidence limit for Pst DC3000 (avrRpm1) between phx3 and Ws-0 are 4.5 (>99%, 1 dpi) and 3.88 (>98%, 5 dpi). (B) Growth of Pst DC3000 (avrRps4) in phx3 (solid squares), phx6 (solid circles), and Ws-0 (open squares). The star denotes a significant difference between phx3 and Ws-0 (t-test value 5.65, >99%).

the phx3/lsd5 line is caused by the phx3 mutation itself. When challenged with the virulent Pst DC3000, the phx3 lines showed enhanced susceptibility compared to Ws-0 (Figure 7A) and this was also correlated with increased chlorosis (Figure 3B). This enhanced susceptibility could be reverted by application of benzothiadiazole (125 mg/ml, not shown), an inducer of SAR (Görlich et al. 1996). When inoculated with the normally avirulent Pst DC3000 (avrRps4), phx3, but not phx6, showed slightly reduced resistance compared with Ws-0 (Figure 7B). This was not accompanied by appearance of symptoms as observed with the interaction between phx3 and Pst DC3000 (avrRpm1). This suggests that phx3 alters resistance triggered by RPM1 more than resistance triggered by RPS4. In contrast, no significant difference was detected in the phx6, phx9, and phx12 lines with either Pst DC3000 or Pst DC3000 (avrRpm1, not shown).

We also used a bacterial isolate of P. syringae pv. glycinea, which is nonpathogenic on Arabidopsis, to assess delivery of avirulence to the phx mutants. Typical necrosis was visible 7 hr after inoculation with Psg (avrRpm1) in the phx3, phx6, phx9, and phx12 mutants (not shown). Thus we concluded that HR following high-dose inoculation is not impaired in these mutants.

Peronospora resistance in the phx mutants: The isolated phx mutants were also tested for their reaction to both incompatible and compatible isolates of P. parasitica. In contrast to simply measuring changes in HR frequency, as shown in Figure 5 for phx/lsd5 lines, we performed detailed histology experiments, as defined in Figure 4.

When challenged with the incompatible isolates Ahco2 and Noco2, we observed a reduction in the number of HR present 1 dpi in both phx3 and phx6 (Figure 8), similar to our observations with the corresponding phx/lsd5 lines (Figure 5). Therefore, the phx3 and phx6
mutations are responsible for the reduced resistance previously detected. The reduction of the number of HRs in reaction to Ahco2 (Figure 8A) was not as pronounced as the one observed in the case of Noco2 (Figure 8B), and this parallels the results obtained with these isolates when tested on the phx/ lsd5 lines (Figure 5). In the case of Noco2, the increase in interaction sites containing haustoria, or hyphae, accompanied by plant cell reaction was more striking than that in Ws-0. This suggests that R-gene action is delayed in phx3 and phx6, as these types of reactions precede the development of HR (Figure 4). Five dpi, the Peronospora life cycle was complete in Col-0, as demonstrated by profuse hyphal growth (Figure 9A) and oosporangia production. The resistant ecotype Ws-0 supported little hyphal growth and most of the reactions observed were complete HRs (Figure 9B). As a consequence of delayed triggering of the HR, pathogen growth occurred in phx3 (Figure 9C), followed by development of massive cell death along hyphal tracks. We did not observe further growth of the pathogen in phx6, suggesting that this mutant was only affected in early stages of the interaction with P. parasitica (Figure 9D). However, neither pathogen isolate completed its life cycle as measured by lack of sporulation. The phx9 and phx12 lines did not show any significant difference from Ws-0 when challenged with isolate Ahco2 or Noco2 (not shown). Also, we did not observe modified susceptibility of the phx lines isolated when challenged with the virulent isolate Emwa1 (not shown), consistent with results presented in Figure 6.

DISCUSSION

We devised a screening procedure to genetically decipher the pathway(s) regulating cell death in Arabidopsis. This screening was also designed to address the role of a single component, cell death, in the multifaceted HR phenomenon. Our screening did not rely on phenotypes such as loss of resistance, as in the case of the screenings used to identify the ndr1 (Century et al. 1995) or eds (Glazebrook et al. 1996; Parker et al. 1996) mutants. We used the spontaneous cell death phenotype of the lsd5 mutant as a way to isolate suppressors of cell death and then assayed the effects of such mutations on disease resistance. In a similar manner, Glazebrook and Ausubel (1994) and Glazebrook et al. (1997) addressed the role of camalexin in resistance with the phytoalexin-deficient (pad) mutants. Our screening led to the identification of 10 phx genes, ashyphal growth (Figure 9A) and oosporangia production. The resistant ecotype Ws-0 supported little hyphal growth and most of the reactions observed were complete HRs (Figure 9B). As a consequence of delayed triggering of the HR, pathogen growth occurred in phx3 (Figure 9C), followed by development of massive cell death along hyphal tracks. We did not observe further growth of the pathogen in phx6, suggesting that this mutant was only affected in early stages of the interaction with P. parasitica (Figure 9D). However, neither pathogen isolate completed its life cycle as measured by lack of sporulation. The phx9 and phx12 lines did not show any significant difference from Ws-0 when challenged with isolate Ahco2 or Noco2 (not shown). Also, we did not observe modified susceptibility of the phx lines isolated when challenged with the virulent isolate Emwa1 (not shown), consistent with results presented in Figure 6.

Figure 9.—Trailing necrosis in the phx3 mutant. Plants were inoculated with P. parasitica Ahco2 as in Figure 8 and tissue was stained 5 dpi for microscopical examination. (A) Col-0, absence of cell death, profuse hyphal growth and elaboration of oosporangia. (B) Ws-0, HR, and no hyphal growth. (C) phx3, HR accompanied by free hyphal growth that eventually triggers trailing necrosis. (D) phx6, HR and no hyphal growth, hyphae; o, oosporangia; tn, trailing necrosis. Bar, 20 μm.
that programs specific to each developmental process regulate cell death and that the phx mutations described here may be specific to disease resistance.

A range of suppressed phenotypes was found. We found no uncoupling of cell death suppression and suppression of PR1 expression. Instead, there was a correlation between the extent of residual lsd5-dependent cell death and PR1 expression (Figure 1). Genetic analysis of the disease resistance pathway led to several models in which cell death was placed upstream of SA-dependent defense gene activation (Dangl et al. 1996; Ryal's et al. 1996). For example, the dominant lsd2 and lsd4 mutants (Hunt et al. 1997) and the recessive cpri5 mutant (Bowling et al. 1997) still showed spontaneous lesions when crossed to nahG transgenic plants that accumulate very low levels of SA. In contrast, SA-dependent gene induction was suppressed in these plants (Bowling et al. 1997; Hunt et al. 1997). These results showed that in the case of these mutations, suppressing defense gene expression (by removing SA) did not suppress the appearance of cell death. Thus, cell death can be upstream of the point of action of SA. Our results indicate that suppression of cell death also results in suppression of defense gene expression, suggesting that at least lsd5 cell death acts upstream of defense gene activation. This suggests that the phx mutations may represent defects in common regulators of cell death and defense gene expression.

The strongest suppressor mutations (phx2, phx3, phx6, and phx11-1) significantly reduced resistance against several normally avirulent pathogens compared with lsd5 or Ws-0 (Figures 2 and 5). In these cases, suppression of the lsd5 phenotype did not result in a simple reversion to a wild-type phenotype with respect to pathogen response. Furthermore, the slightly increased resistance of lsd5 (Figure 5 and Dietrich et al. 1994) was converted to a decreased disease resistance in the phx/lsd5 lines. This was also true when the phx3 and phx6 mutations were isolated from the lsd5 mutation (Figures 7 and 8). Therefore, we concluded that the phenotypes observed were due to the phx mutations and not to their interaction with lsd5. Moreover, because isolation of the phx mutations from lsd5 was based on the lsd5 suppression phenotype, it is likely that the mutation suppressing lsd5 lesions is the same as that impairing disease resistance. Supporting this hypothesis is the observation that the phx2 and phx3 mutations impair both lsd5 lesion formation and disease resistance in a dominant manner (Figure 3A) and that the phenotypes described were observed in independent phx3 and phx6 isolation lines.

The phx2 and phx3 mutations affected resistance to both necrotrophic and biotrophic pathogens. This is similar to other known RDR mutants. For example, the ndr1-1 and pad4-1 mutants are impaired in their resistance to both necrotrophic bacteria and biotrophic pathogens (Century et al. 1995; Glazebrook et al. 1997). Altogether the existence of such mutants indicates possible convergence of the pathways controlling resistance to various types of pathogens. However, the phx6 mutation showed impaired resistance to the isolates Ahco2 and Noco2 of the biotrophic oomycete P. parasitica (Figure 9B) but not to the different strains of Pst DC3000 tested (Figure 7B). This finding suggests a possible divergence of the pathways leading to resistance to necrotrophic and biotrophic pathogens. Alternatively, the signaling events triggered by RPS4 may overcome the requirement for a fully functional PHX6, whereas the signals triggered by the RPP1/10/14 complex require a fully functional PHX6 for appropriate triggering of downstream events. Like other RDR mutations, such as the pad mutations (Glazebrook and Ausubel 1994; Glazebrook et al. 1997), the phx mutations show only partial loss of resistance and, although delayed, resistance was always the outcome of the interaction in the most affected phx lines. This probably reflects that the HR phenomenon is multifaceted and that modifying one element of the pathway cannot abolish it.

Mutational analyses of the disease resistance pathway have often led to the recovery of allelic mutations, independent of the mode of screening used. For example, in a screen for mutants showing enhanced disease susceptibility (eds mutants), Glazebrook et al. (1996) identified mutations in the PAD2 and NPR1 genes that had been recovered using different screening procedures (Cao et al. 1994). We asked if any of the phx mutations were allelic to the known RDR mutations ndr1-1, pad4-1, and eds1-1 (Holub 1997 and references therein). Mapping and allelism tests showed that none of the tested phx mutations were allelic to these mutations. This was expected because neither the ndr1-1 nor the eds1-1 mutations suppress lsd5 lesions.

As more R-genes are being cloned, at least two distinct classes have been established based on sequence similarities (Bent 1996). The first class (LZ-NBS-LRR class) is represented by genes showing a leucine-zipper (LZ), a nucleotide-binding site (NBS), and leucine-rich repeats (LRR). The genes from the second class (TIR-NBS-LRR) show similarities with the toll and interleukin-1 receptors (TIR) and also possess a NBS and LRRs. Recent reports suggest that these two classes reflect the existence of at least two different disease resistance signaling pathways. In this model (Aarts et al. 1998), one pathway, dependent on NDR1, would trigger resistance governed by genes of the LZ-NBS-LRR class, whereas another pathway, dependent on EDS1, would govern resistance triggered by genes of the TIR-NBS-LRR class. We analyzed disease resistance governed by R-genes from both classes in this study: RPM1 belongs to the LZ-NBS-LRR class (Grant et al. 1995) while RPS4 (B. Staskawicz, personal communication) and RPP1/10/14 (Botella et al. 1998) belong to the TIR-NBS-LRR class. The phx2 and phx3 mutations modify resistance triggered by both subclasses of NBS-LRR genes. There-
Therefore, they must act downstream (or independently) of the pathways dependent on NDR1 and EDS1.

All suppressor lines, and the phx3 and phx6 lines (not shown), retained the ability to express PR1 after treatment with SA (Figure 1C). Therefore, these mutations must act upstream or independently of the point of action of SA. To our knowledge, the phx2 and phx3 mutations are the first mutations of this type described: They impair disease resistance downstream (or independently) of the NDR1- and EDS1-dependent pathways and before the point of action of SA. When tested, the eds mutants alter susceptibility to virulent pathogens but not resistance to avirulent ones (Rogers and Ausubel 1997). Other eds mutants have not been tested with both virulent and avirulent pathogens. However, the finding that some of the eds mutants represent mutations in the NPR1 gene (Glazebrook et al. 1996) suggests that these mutations are likely to be found downstream of SA action. The pad4-1 mutation is most likely to be similar to some of the phx mutations, because pad4-1 mutants showed reduced resistance to both avirulent strains of P. syringae pv. maculicola and avirulent isolates of P. parasitica (Glazebrook et al. 1997). In addition, SA can still induce defense gene expression in pad4-1 mutants, suggesting that this mutation acts upstream of SA (Zhou et al. 1998).

Interestingly, phx3 in isolation showed some level of enhanced susceptibility when challenged with Pst DC3000 (Figure 7A), and the phx2/lsd5 double mutant exhibited enhanced susceptibility to the virulent isolate of P. parasitica Emwa1 (Figure 6) but no eds phenotype with Pst DC3000 (not shown). This suggests that the pathways leading to R-gene-mediated disease resistance share some components with basic resistance mechanisms that act to limit pathogen growth during compatible interactions. Similar observations have been made with the eds-1 and pad4-1 mutants where both a decrease in resistance and an increase in susceptibility were measured (Parker et al. 1996; Glazebrook et al. 1997).

The lines phx8/lsd5, phx9/lsd5, phx10/lsd5, phx11-2/lsd5, and phx12/lsd5 did not show any altered disease resistance to the different pathogens tested. Therefore, these suppressor mutations may not be in the disease resistance pathway. They may be affecting the initial perturbation caused by the lsd5 mutation. For example, if the lsd5 mutation leads to the accumulation of a toxic compound, subsequent cell death, and expression of defense-related markers, detoxification of this compound could revert the lsd5 phenotype without affecting disease resistance. In addition, the lsd5 mutation has been shown to more specifically affect the cells of the epidermis layer, and suppressors specific to this tissue are unlikely to alter disease resistance to pathogens developing in the mesophyl. However, it is possible that the presence of lsd5 in these weak suppressor lines counterbalanced their weak effects on triggering resistance. Accordingly, we did not observe enhanced susceptibility to Pst DC3000 in the phx3/lsd5 line while we did in the phx3 mutant (Figure 7A). Likewise in the weakly suppressed phx/lsd5 lines, it is possible that some residual resistance was effective due to the presence of the lsd5 mutation in the background.

Whether in isolation or in the presence of the lsd5 mutation, only the strongest suppressors showed altered disease resistance. This suggests that a certain suppression threshold must be reached in order to significantly perturbate disease resistance. We hypothesize that only the mutations strongly suppressing the lsd5 phenotype have an impact on disease resistance. The comparison of the effects of the phx11-1 and phx11-2 alleles supports this hypothesis. While the strong lsd5 suppressor allele phx11-1/lsd5 shows impaired resistance to avirulent isolates of P. parasitica, the weak suppressor allele phx11-2 does not (Figure 5). Accordingly, when isolated from the lsd5 mutation, the weak suppressor mutations phx9 and phx12 still did not exhibit altered resistance to avirulent pathogens (not shown).

None of the phx suppressors abolished the HR cell death triggered by avirulent pathogens. In particular, biotrophic pathogens can still induce HR in the phx mutants. Because these pathogens do not primarily kill their host, but instead need living cells, it is unlikely that the cell death observed in the phx mutants is a result of the pathogen alone. Similarly, HR is retained in several RDR mutants (Glazebrook and Ausubel 1994; Cen tury et al. 1995; Parker et al. 1996). Several models can explain the development of HR cell death in the presence of lsd5 cell death suppressors. In the first, the overall amount of pathogen-triggered signals overrides the suppression threshold of the phx mutations. This model supposes that the amount of lsd5-triggered signal is below the one generated by pathogen attack. Alternatively, several independent signaling pathways could trigger cell death during the HR and the lsd5 suppressors could affect only one of them. Several signals are generated during the HR, such as reactive oxygen intermediates (Baker and Orlando 1995) and ion flux changes (Atkinson and Baker 1989). Bifurcation of intracellular resistance responses has also been described. The lsd5 suppressors may be able to block some of these signals but not others. In either model, the strength of the suppressor mutations is expected to influence the extent of alteration in disease resistance. Epistasis analysis between the phx mutants and other lsd mutants, as well as study of the physiological defects in these mutants, should allow refinement of models of disease resistance pathways.

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Peronospora parasitica


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