Natural Variation in the *Arabidopsis* Response to the Avirulence Gene *hopPsyA* Uncouples the Hypersensitive Response from Disease Resistance

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The plant hypersensitive response (HR) is tightly associated with gene-for-gene resistance and has been proposed to function in containing pathogens at the invasion site. This tight association has made it difficult to unequivocally evaluate the importance of HR for plant disease resistance. Here, *hopPsyA* from *Pseudomonas syringae* pv. *syringae* 61 is identified as a new avirulence gene for *Arabidopsis* that triggers resistance in the absence of macroscopic HR. Resistance to *P. syringae* pv. *tomato* DC3000 expressing *hopPsyA* was *EDS1*-dependent and *NDR1*-independent. Intriguingly, several *Arabidopsis* accessions were resistant to DC3000(*hopPsyA*) in the absence of HR. This is comparable to the *Arabidopsis* response to *avrRps4*, but it is shown that *hopPsyA* does not signal through *RPS4*. In a cross between two *hopPsyA*-resistant accessions that differ in their HR response, the HR segregated as a recessive phenotype regulated by a single locus. This locus, *HEDI* (HR regulator in *EDS1* pathway), is proposed to encode a protein whose activity can cause suppression of the *EDS1*-dependent HR signaling pathway. *HEDI*-regulated symptomless gene-for-gene resistance responses may explain some cases of *Arabidopsis* resistance to bacteria that are classified as nonhost resistance.

Additional keywords: pHIR11, *Pseudomonas fluorescens*, *P. syringae* pv. *glycinea*, *RPS6*

Plant disease resistance genes govern the plant response to pathogens expressing cognate avirulence genes according to the gene-for-gene hypothesis (Flor 1971). In the absence of either the resistance gene in the plant or the avirulence gene in the pathogen, disease ensues. If both are present, the plant mounts a rapid defense response that includes an oxidative burst, ionic fluxes at the membrane, increased salicylic acid content, and induction of defense genes (Dangl and Jones 2001; Dangl et al. 1996; Hammond-Kosack and Jones 1996; Martin et al. 2003). The avirulence gene-triggered plant defense response is often accompanied by the hypersensitive response (HR), a form of localized programmed cell death that is thought to delimit pathogens to the infection site (Goodman and Novacky 1994; Greenberg and Yao 2004). Although it is generally assumed that the HR contributes to or is essential for

resistance, it is also possible that the HR occurs as a consequence of overactive defense responses. Direct evidence for either hypothesis has been difficult to obtain, since the HR in general is tightly correlated with gene-for-gene disease resistance, and the path from pathogen recognition to cell collapse is poorly understood.

A few cases of disease resistance without HR have been described. Barley resistance to the necrotrophic leaf scald fungus *Rhynchosporium secalis* mediated by the *Rrs1* gene is characterized by an inhibition of hyphal growth and degradation of fungal structures in the absence of HR (Lehnackers and Knogge 1990; Rohe et al. 1995). Resistance to *Potato virus X* (PVX) governed by the *Rx1* and *Rx2* genes in potato is not accompanied by HR and is thought to be based on the extreme rapidity of the plant response. This prevents virus replication in single cells at a very early stage, thus preventing high accumulation of an HR-inducing signal (Bendahmane et al. 1999, 2000). Similar extreme resistance is observed in soybean resistance to *Soybean mosaic virus* (SMV), governed by the *Rsv1* resistance gene (Hajimorad and Hill 2001). Both PVX and SMV elicit HR on their respective hosts under certain experimental conditions, indicating that extreme resistance usually prevents macroscopic HR (Bendahmane et al. 1999; Hajimorad and Hill 2001). Interestingly, Cole and coworkers (2001) demonstrated that cell death and resistance are genetically separable in the interaction between *Cauliflower mosaic virus* (CaMV) and *Nicotiana* spp., where no extreme resistance is observed. In this system, plants that are not homozygous for the recessive cell death gene *ccd1* display resistance to CaMV in the absence of HR, leading to chlorotic local lesions (Cawly et al. 2005; Cole et al. 2001; Király et al. 1999). The inverse form of uncoupled HR and viral resistance was observed in the interaction between *Turnip crinkle virus* and *Arabidopsis* governed by the resistance gene *HRT* and a putative negative regulator of disease resistance called *RRT*. In this interaction, HR can occur in the absence of resistance (Cooley et al. 2000; Kachroo et al. 2000).

Bacterial disease resistance and HR have also been genetically separated, first with the *Arabidopsis dnd1* mutant (Clough et al. 2000; Yu et al. 1998). The *dnd1* mutant does not mount an HR but still shows resistance to pathogens expressing the avirulence genes *avrRpt2*, *avrB*, *avrRpm1*, and *avrRps4*. This mutant also shows stunting, constitutively higher levels of salicylic acid, pathogenesis-related gene induction, and increased resistance to virulent pathogens. These characteristics are abolished in *dnd1* mutants transformed with the bacterial *nahG* gene, which degrades salicylic acid and is known to counteract constitutively active defenses (Clough et al. 2000). Interestingly, the *nahG* transgene only partially abolished the stunted phenotype and loss of HR in *dnd1* plants. *DND1* en-

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codes a cation channel of the cyclic nucleotide gated ion channel family (Clough et al. 2000; Leng et al. 1999). Recently, a closely related cyclic nucleotide gated ion channel mutant, *hlm1* (Balagué et al. 2003), and the allelic mutant *dnd2* (Jurkowski et al. 2004) were found to give similar phenotypes. The recessive *dnd1-1* and *dnd2-1* alleles and the *hlm1* knock-out allele indicate that wild-type DND1 and HLM1/DND2 function as positive regulators of HR. Although the phenotypes are somewhat pleiotropic, *dnd* mutants can be used to explore the general regulation of cell death activation in plants.

Among *Arabidopsis* accessions, variation exists in the HR response independent of the resistance response to *Pseudomonas syringae* pv. *tomato* DC3000 expressing *avrRps4*. Even though Col-0 is resistant to DC3000(*avrRps4*) as shown by reduced bacterial multiplication, it does not react with an HR. This is in contrast to Ws-0 or Po-1, which give a strong HR (Gassmann et al. 1999; Hinsch and Staskawicz 1996). Yet Col-0 is capable of giving a strong HR in response to bacteria expressing other avirulence genes. Resistance to bacteria expressing *avrRps4* in *Arabidopsis* is governed by *RPS4*, a member of the toll interleukin-1 receptor nucleotide-binding site leucine-rich repeat (TIR-NBS-LRR) class of resistance genes (Gassmann et al. 1999). Like most TIR-NBS-LRR genes, *RPS4* function is fully dependent on *EDS1* and not on *NDR1* (Aarts et al. 1998). *EDS1* (Falk et al. 1999; Parker et al. 1996) and *NDR1* (Century et al. 1995, 1997) are genetically downstream of resistance genes and are thought to define separate disease resistance signaling pathways (Glazebrook 2001; Hammond-Kosack and Parker 2003). Initially, it was hypothesized that the Col-0 response to *avrRps4* is a weak form of resistance dependent on the *RPS4-Col* allele. Consistent with this hypothesis, an *RPS4-Col* transgene in the susceptible accession RLD also gave resistance in the absence of HR (Gassmann et al. 1999).

In the present paper, it is shown that *hopPsyA* from *P. syringae* pv. *syringae* 61 is a new avirulence gene for *Arabidopsis*. Interestingly, Col-0 responded similarly to *hopPsyA* and *avrRps4*, in that Col-0 was resistant to DC3000 expressing *hopPsyA* in disease assays but gave no HR at high bacterial titer. Also, resistance to DC3000(*hopPsyA*) was dependent on *EDS1* and independent of *NDR1*. Importantly, resistance to *hopPsyA* was not mediated by *RPS4*, which would have explained the similar phenotypes. By crossing Col-0 and Ws-0, two *hopPsyA*-resistant accessions that differ in their HR response, it was found that the HR was a recessive phenotype regulated by a single locus. It is concluded that uncoupling of HR and disease resistance in Col-0 is governed by a new locus, *HED1* (HR regulator in *EDS1* pathway), on the bottom of chromosome 5, that is proposed to function as a negative regulator of HR in Col-0.

RESULTS

hopPsyA is a new avirulence gene for *Arabidopsis*.

In the course of studying the Col-0 response to high titers of bacteria, the nonpathogenic bacterium *Pseudomonas fluorescens* 55 containing the cosmid pHIR11 was used at 10^8 and 2×10^8 CFU/ml. Cosmid pHIR11 contains the type III secretion system genes of *P. syringae* pv. *syringae* 61 (Huang et al. 1988). Surprisingly, it was found that *P. fluorescens*(pHIR11) alone, without added avirulence genes, can cause a strong HR on some *Arabidopsis* accessions. As shown in Figure 1, Ws-0 gave a strong HR to *P. fluorescens*(pHIR11) inoculated at 10^8 CFU/ml, while no symptoms were seen on Col-0 after 24 h. Interestingly, the HR in Ws-0 was dependent on *EDS1*, since the *eds1-1* mutant line also showed no symptoms (Fig. 1). As expected, the *ndr1-1* line (in Col-0 background) gave no HR (Fig. 1). Since an *ndr1* mutant in the Ws-0 background was not available, it was not possible to determine whether the HR response is solely reliant on *EDS1* or on both *EDS1* and *NDR1*. Interestingly, a consistent chlorosis of Col-0 and *ndr1-1* leaves was observed 3 to 5 days after inoculation with *P. fluorescens*(pHIR11) (data not shown). This chlorosis was not observed with *eds1-1* or with pHIR11 derivatives that do not induce HR on Ws-0 (discussed below), indicating that perhaps Col-0 reacts with a form of delayed senescence rather than HR.

To determine which gene in *P. fluorescens*(pHIR11) triggers HR in Ws-0, a series of transposon-mutagenized and deletion constructs of pHIR11 (courtesy of J. Alfano, University of Nebraska-Lincoln, U.S.A.) were tested (Table 1). HR induction was abolished with pCPP2089 (data not shown), which carries a *TnPhoA* insertion in *hrcC* on pHIR11 (Huang et al.

Fig. 1. *EDS1*-dependent hypersensitive response (HR) induced by *Pseudomonas fluorescens*(pHIR11) in Ws-0. Leaves of indicated *Arabidopsis* lines were inoculated with *P. fluorescens*(pHIR11) at 10^8 CFU/ml. HR (leaf collapse) was recorded 24 h after inoculation.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Reference
Strains		
<i>Pseudomonas fluorescens</i> 55	Nal ^r	Huang et al. 1988
<i>P. syringae</i> pv. <i>tomato</i> DC3000	Wild type, Rif ^r	Cuppels 1986
<i>P. syringae</i> pv. <i>syringae</i> race 5	Wild type, Rif ^r	Shapiro 2000
Plasmids		
pHIR11	<i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrp</i> cluster cloned in pLAFR3, Tc ^r	Huang et al. 1988
pCPP2071	pHIR11 derivative with <i>TnPhoA</i> insertion in <i>hopPsyA</i> , Tc ^r Km ^r	Huang et al. 1991
pCPP2089	pHIR11 derivative with <i>TnPhoA</i> insertion in <i>hrcC</i> , Tc ^r Km ^r	Huang et al. 1991
pCPP2274	pHIR11 derivative with precise deletion of <i>hrpZ</i> ORF, Tc ^r	Gopalan et al. 1996
pLN18	pHIR11 derivative with precise deletion of <i>shcA-hopPsyA</i> operon, Tc ^r Km ^r	Jamir et al. 2004
pML123	Broad-host-range cloning vector, Gm ^r	Labes et al. 1990
pLN92	pML123 derivative containing <i>shcA</i> and <i>hopPsyA</i> , Gm ^r	van Dijk et al. 2002
pVSP61	Broad-host-range cloning vector, Km ^r	Hinsch and Staskawicz 1996
pV316-1A	pVSP61 derivative containing <i>avrRps4</i> , Km ^r	Hinsch and Staskawicz 1996

^a Gm = gentamycin, Km = kanamycin, Nal = nalidixic acid, Rif = rifampicin, and Tc = tetracycline.

1991). The *hrcC* gene encodes an outer membrane protein of the type III secretion machinery. The plasmid pCPP2274 has a precise deletion of the harpin *hrpZ* gene and also abolished HR induction (data not shown). However, this did not immediately implicate *hrpZ* as the avirulence gene, since *hrpZ* was also found to be necessary for AvrB and AvrRpt2 delivery from *P. fluorescens*(pHIR11) (Gopalan et al. 1996). Interestingly, a TnPhoA-insertion in *hopPsyA* on plasmid pCPP2071 abolished HR (Table 2). This strain still secretes harpin (Alfano et al. 1996), showing that *hrpZ* is not the avirulence gene.

HopPsyA (formerly called HrmA) is a pHIR11-encoded effector protein that triggers HR in certain *Nicotiana* species (Alfano et al. 1997). The *hopPsyA* gene is physically linked in an operon with *shcA*, which encodes a cochaperone that is required for HopPsyA secretion (van Dijk et al. 2002). A precise deletion of *shcA* and *hopPsyA* in plasmid pLN18 (Jamir et al. 2004) abolished HR, further suggesting that *hopPsyA* is the avirulence gene (Table 3). For unknown reasons, pHIR11 deletion derivatives were not complementable by the plasmid pLN92 carrying *shcA-hopPsyA* (Table 3).

Col-0 is resistant to DC3000(*hopPsyA*) in disease assays.

To prove that *hopPsyA* is the avirulence gene on pHIR11 recognized in *Arabidopsis*, pLN92 carrying *shcA-hopPsyA* (van Dijk et al. 2002) was introduced into DC3000. For HR assays, DC3000 strains were infiltrated into leaves at a density of 2×10^7 CFU/ml. Consistent with *hopPsyA* being the avirulence gene, DC3000(*hopPsyA*) caused an HR on Ws-0 within 24 h but not on Col-0, similar to DC3000(*avrRps4*) (Fig. 2). Transfer of *hopPsyA* to DC3000 allowed the analysis of disease resistance independent of an HR phenotype. Col-0 is resistant to DC3000(*avrRps4*) in disease assays with low inoculum of bacteria, despite the absence of an HR phenotype at high inoculum (Gassmann et al. 1999; Hinsch and Staskawicz 1996). Similar to DC3000(*avrRps4*), both Ws-0 and Col-0 gave a resistant phenotype to DC3000(*hopPsyA*), based on the absence of chlorosis 5 days after inoculation with bacteria at 10^6 CFU/ml (Fig. 3A).

The finding of resistance in Col-0 to DC3000(*hopPsyA*) allowed the testing of resistance in the *ndr1-1* mutant line. Unlike *eds1-1*, *ndr1-1* plants were resistant to DC3000(*hopPsyA*) (Fig. 3A). These results were confirmed by measuring in planta bacterial growth. As shown in Figure 3B, the resistant plants Ws-0, Col-0, and *ndr1-1* showed approximately 100 to 1,000-fold less bacterial growth with DC3000(*hopPsyA*) than *eds1-1* plants, while virulent DC3000 grew to high levels indica-

tive of a susceptible interaction on all plant lines (Fig. 3B). Similar to its response to DC3000(*avrRps4*), Col-0 is therefore resistant to DC3000(*hopPsyA*) without displaying a macroscopic HR phenotype at high bacterial inoculum. Experiments to detect single-cell death using Evan's blue staining or autofluorescence did not provide evidence for an increase in single-cell death in Col-0 infiltrated with *P. fluorescens*(pHIR11) compared with the background observed with *P. fluorescens*(pLN18) (data not shown).

Two *Arabidopsis* loci control the response to bacteria expressing *hopPsyA*.

A simple explanation for the identical HR and resistance phenotypes observed with *avrRps4* and *hopPsyA* would be if *hopPsyA* also signals through *RPS4*. This situation would be comparable to *avrRpm1* and *avrB*, both of which interact with the *RPM1* resistance gene (Grant et al. 1995). To exclude this possibility, *hopPsyA* was tested on RLD, which has a nonfunctional allele of *RPS4* (Gassmann et al. 1999; Hinsch and Staskawicz 1996), and an *RPS4* knock-out line from the SAIL knock-out collection (line 519-B09) (Sessions et al. 2002), which was named *rps4-1* (Kwon et al. 2004). The *rps4-1* allele has a T-DNA insertion in exon 2 encoding the NBS domain and is presumed to be functionally null. As shown in Figure 3C, both RLD and *rps4-1* were resistant to DC3000(*hopPsyA*) in disease assays. This demonstrated that *hopPsyA* does not signal through *RPS4*. It is therefore proposed that the name *RPS6* be reserved for the hypothesized resistance gene responsible for *hopPsyA* recognition.

Thus, two unrelated avirulence genes that signal through the *EDS1* pathway via different resistance genes give identical HR and disease resistance phenotypes in Col-0 and Ws-0. In addition, the resistant accessions Po-1 (strong HR), Ler (intermediate HR), and RLD (no HR) gave identical phenotypes to *hopPsyA* and *avrRps4* (data not shown; in the case of *avrRps4* and RLD, a transgenic line containing the *RPS4-Col* allele was used). Consequently, it was hypothesized that a common mechanism downstream of the resistance genes regulates the HR phenotype in response to *hopPsyA* and *avrRps4*. To under-

Table 2. Hypersensitive response (HR) assays with *Pseudomonas fluorescens* containing pHIR11 or pCPP2071^a

Plants	pHIR11	pCPP2071
Ws-0	17/21 ^b	0/21
Col-0	2/20	0/20
<i>eds1-1</i> (Ws)	0/21	0/21
<i>ndr1-1</i> (Col)	0/12	0/12

^a HR was scored 24 h after inoculation with the indicated strain at a density of 10^8 CFU/ml.

^b Numbers denote plants that were HR⁺/total number tested.

Table 3. Hypersensitive response (HR) assays with *Pseudomonas fluorescens* containing pHIR11, pLN18, or pLN18 and pLN92^a

Plants	pHIR11	pLN18	pLN18 + pLN92
Ws-0	11/11 ^b	0/11	1/11
Col-0	1/11	0/11	0/11

^a HR was scored 24 h after inoculation with the indicated strain at a density of 10^8 CFU/ml.

^b Numbers denote plants that were HR⁺/total number tested.

Fig. 2. *hopPsyA* is the avirulence gene on pHIR11 and gives phenotypes in *Arabidopsis* similar to *avrRps4*. The hypersensitive response (HR) phenotype in Ws-0 (top panels) and Col-0 (bottom panels) inoculated with DC3000 at 2×10^7 CFU/ml was recorded 24 h after inoculation. In the left panels, DC3000 contained either the empty vector pML123 (-), or vector pLN92 (+). In the right panels, DC3000 contained either the empty vector pVSP61 (-) or vector pV316-1A (+).

stand the genetic basis for resistance in the absence of HR, Col-0 (resistant, HR⁻) was crossed with Ws-0 (resistant, HR⁺). F1 plants from this cross showed no HR when challenged with *P. fluorescens*(pHIR11), whereas HR segregated as a recessive phenotype conditioned by a single locus in the F2 generation (Table 4). The postulated gene at this locus was named *HED1* (for HR regulator in *EDS1* pathway). The recessive nature of the HR⁺ phenotype indicated that, genetically, *HED1* acts as a negative regulator of HR.

A rough map position for *HED1* was determined by genotyping a subset of 14 HR⁺ lines that were verified in the F3 generation as being homozygous HR⁺ and, therefore, homozygous Ws-0 at the *HED1* locus. Based on linkage analysis, using a collection of cleaved amplified polymorphic DNA sequence (CAPS) (Konieczny and Ausubel 1993) and microsatellite (Bell and Ecker 1994) markers (Table 5), and examination of individual chromosome breakpoints, *HED1* mapped to a 22-cM region on the bottom of chromosome 5 between the markers RPS4-NT and LFY3. Consistent with *HED1* acting downstream of resistance genes, the three F3 lines with breakpoints between RPS4-NT and LFY3 that were tested were also homozygous HR⁺ when inoculated with DC3000(*avrRps4*) (data not shown).

The results with *P. fluorescens*(pHIR11) prompted an investigation of *P. syringae* pv. *glycinea* race 5, since this strain was reported to be a useful nonpathogenic strain that allows observation of *avrRps4*-induced HR on Col-0 at high inoculum (Shapiro 2000). Interestingly, a strong HR was observed on Ws-0 with the negative control strain containing an empty vector (Figure 4). This HR was absent in *eds1-1* plants (Figure 4). Although some loss of turgor was observed in Col-0 leaves in a few cases, the reported HR with *P. syringae* pv. *glycinea* race 5 (*avrRps4*) in Col-0 could not be replicated under these conditions (Fig. 4).

DISCUSSION

HED1 as a negative regulator of HR in Col-0.

Here, *hopPsyA* on cosmid pHIR11 from *P. syringae* pv. *syringae* 61 is identified as a new avirulence gene for *Arabidopsis*. Cosmid pHIR11 contains the type III secretion system genes of *P. syringae* pv. *syringae* 61 and allows delivery of diverse bacterial avirulence proteins from nonpathogenic bacteria such as *P. fluorescens* and *Escherichia coli* to plant cells for HR assays (Gopalan et al. 1996; Huang et al. 1988; Pirhonen et al. 1996). Surprisingly, it was found that *P. fluorescens*(pHIR11) alone induced a strong HR in Ws-0 while Col-0 showed no HR. Precise deletions of *shcA-hopPsyA* on pHIR11 abolished the HR in Ws-0. For unknown reasons, pHIR11 deletion derivatives were not complemented by a plasmid carrying *shcA-hopPsyA*.

Intriguingly, Col-0 was as resistant as Ws-0 to DC3000(*hopPsyA*) in disease assays. Similar to *avrRps4*, resistance to DC3000(*hopPsyA*) was *EDS1*-dependent and *NDR1*-independent. However, resistance to DC3000(*hopPsyA*) required a resistance gene other than *RPS4*. The absence of HR in Col-0 to *hopPsyA* and *avrRps4* stands in contrast to the avirulence genes *avrRpt2*, *avrB*, *avrRpm1*, and *avrPphB*, which all depend on *NDR1* and signal via coiled coil-NBS-LRR resistance proteins (Aarts et al. 1998) and are known to give robust HR phenotypes in Col-0. The locus controlling the HR response to *hopPsyA* and *avrRps4* was therefore named *HED1*.

Using a small number of F3 families that were homozygous for the HR⁺ phenotype, *HED1* was mapped to the bottom of chromosome 5 between the markers RPS4-NT and LFY3. Although known disease resistance-associated genes fall within this interval, none are consistent with the phenotypes described here. *RARI* (Muskett et al. 2002; Tornero et al. 2002) and *HSP90* (Hubert et al. 2003; Liu et al. 2004; Lu et al. 2003;

Takahashi et al. 2003) are positive regulators of disease resistance and do not have an HR-specific phenotype. The *DND*-type *HLM1/DND2* gene is HR-specific but acts as a dominant HR-activating gene (Balagué et al. 2003; Jurkowski et al. 2004). The gene encoding AtBII1, an *Arabidopsis* homolog of the mammalian inhibitor of the cell death-promoting protein Bax, also is found in this interval (Sanchez et al. 2000). However, the *AtBII1* gene is upregulated to the same extent in both incompatible and compatible interactions by nonpathogens such as *E. coli* and wounding and in both Col-0 and Ws-0. This gene induction is not dependent on either *EDS1* or *NDR1*, and overexpression of *AtBII1* does not compromise HR (Sanchez et al. 2000).

Fig. 3. Col-0 is resistant to DC3000(*hopPsyA*) in the absence of hypersensitive response, and resistance is independent of *RPS4*. **A**, Disease assay with DC3000 at 10⁶ CFU/ml containing either pML123 (-) or pLN92 (+) in the indicated *Arabidopsis* lines. Only the lower half of leaves were infiltrated, and disease (chlorosis) was scored 5 days after inoculation. **B**, In planta bacterial growth of DC3000(pML123) (left) and DC3000(*hopPsyA*) (right) in Ws-0 (Ws), *eds1-1* (*eds*), Col-0 (Col), and *ndr1-1* (*ndr*) plants. Bacterial numbers at days 0 (open bars) and 3 (closed bars) after inoculation are shown. Error bars denote standard deviation. This experiment was repeated once with similar results. **C**, Disease assay with DC3000 at 10⁶ CFU/ml containing either pML123 (-), pLN92 (*hop*), or pV316-1A (*avr*). Only the left half of leaves were infiltrated, and disease (chlorosis) was scored 5 days after inoculation.

Resistance of the *avrRps4*-susceptible lines RLD and *rps4-1*, which both carry mutations in *RPS4* (Gassmann et al. 1999; Kwon et al. 2004), showed that resistance in *Arabidopsis* to *hopPsyA* required a resistance gene other than *RPS4*. This gene was provisionally called *RPS6*. A total of 32 accessions of *Arabidopsis* in addition to Col-0, Ws-0, Po-1, Ler, and RLD were surveyed with DC3000(*hopPsyA*) for resistance, to possibly identify a *hopPsyA*-susceptible line for map-based cloning of *RPS6*. However, all accessions tested were resistant to DC3000(*hopPsyA*). Interestingly, with these resistant accessions, there was a continuum in the HR response to *P. fluorescens*(pHIR11), from extremely rapid tissue collapse (within 6 to 7 h after inoculation in accession Est) to strong chlorosis in Aa-0 and very weak chlorosis in Gre-0 (data not shown). Possibly, this continuum reflects the degree to which the cell death response to *hopPsyA* is down-regulated by different *HED1* alleles.

Uncoupling of HR and disease resistance by *HED1*.

The natural variation in *Arabidopsis* HR responses to *hopPsyA* developed here is an ideal genetic system that will allow important open questions regarding the function and mechanism of HR in disease resistance to be addressed. Mapping of *HED1* was dependent on the use of *P. fluorescens*(pHIR11) to induce HR because *P. fluorescens* causes no disease symptoms, an approach first exemplified by the use of *Arabidopsis* and the bean pathogen *P. syringae* pv. *phaseolicola* NPS3121 (Yu et al. 1993). In contrast, HR assays with DC3000 provide only a small window of time between HR leaf collapse and collapse of susceptible leaves caused by a high titer of pathogenic bacteria. This would lead to an intolerable number of false-positives for mapping purposes.

It is important to point out that uncoupling of HR and resistance by *HED1* is qualitatively different from previously described systems. The Col-0 response to *hopPsyA* is distinct from rapid extreme resistance, as evidenced by chlorosis of the whole inoculated leaf area 3 to 5 days after inoculation with *P. fluorescens*(pHIR11). This indicates that the response of Col-0 challenged with bacteria expressing *hopPsyA* or *avrRps4* is actually a strongly attenuated HR that occurs in the presence of high amounts of elicitor. It is opposite from *HRT/RRT* (HR in absence of resistance) in that Col-0 displays resistance in the absence of HR when challenged with *hopPsyA* or *avrRps4*. Finally, unlike in *dnd1* and *hlm1/dnd2* mutants, the HR/resistance⁺ phenotype of Col-0 and other wild-type accessions is not accompanied by stunted growth, increased basal resistance,

or constitutive expression of pathogenesis-related genes. Also, *DND1* and *HLM1/DND2* genetically act as positive regulators of HR (Balagué et al. 2003; Clough et al. 2000; Yu et al. 1998), whereas *HED1* acts as a negative regulator. *HED1*-dependent uncoupling of HR and disease resistance most closely resembles the CaMV-*Nicotiana* spp. interaction regulated by *CCD1* (Cawly et al. 2005; Cole et al. 2001; Király et al. 1999).

Implications of *HED1* for molecular plant-pathogen interactions.

The discovery of *HED1*-controlled symptomless gene-for-gene resistance possibly has wider implications for studies in molecular plant-pathogen interactions. Efforts to identify the complete suite of effector proteins that a bacterial pathogen expresses most commonly include tests for avirulence function of each effector in Col-0, and many new avirulence genes may be missed. In fact, *P. fluorescens*(pHIR11) was previously described as failing to elicit the HR in *Arabidopsis*, even though *hopPsyA* was known to be an avirulence gene for *Nicotiana* spp. (Alfano et al. 1997). Based on observations with *P. fluorescens*(pHIR11) and *P. syringae* pv. *glycinia* race 5, the use of other bacterial strains considered nonpathogenic on *Arabidopsis* may also be problematic.

The term nonhost resistance is often used to describe plant species-level resistance to all races of a given pathogen species or pathovar. This resistance appears to be the result of overlapping disease resistance mechanisms and is therefore poorly understood mechanistically (Hammond-Kosack and Parker 2003; Mysore and Ryu 2004). For bacterial pathogens and a given plant species, nonhost resistance is usually manifested by an HR at high inoculum and an absence of disease symptoms on those individuals that do not give an HR, perhaps because of missing virulence factors in the pathogen or missing susceptibility factors in the host. However, the data presented here show that absence of both HR and disease symptoms in Col-0, the reference accession for *Arabidopsis*, does not necessarily signify that *Arabidopsis* is a nonhost for a given pathogen, since the dominant avirulence genes in this pathogen may

Table 4. Segregation of the HR phenotype in response to *hopPsyA*

Cross ^a	F1		F2		χ^2
	HR ⁺	HR ⁻	HR ⁺	HR ⁻	
Col-0 × Ws-0	0	10	46	156	0.59 ($P > 0.3$) ^b

^a Col-0 was recipient. Data were pooled from two independent crosses. Cleaved amplified polymorphic sequence marker analysis established that F1 progeny were true.

^b χ^2 values are given for the expected ratio 1:3 HR⁺/HR⁻.

Table 5. Mapping of *HED1*

Marker ^a	Position (cM)	Recombinant chromosomes	Total number of chromosomes	Recombination frequency (%)	χ^2
nga151	30	15	28	54	0.14 ($P > 0.7$) ^b
nga139	50	11	28	39	1.29 ($P > 0.2$)
RPS4-NT	95	3	28	11	17.29 ($P < 0.001$)
LFY3	117	4	20	20	7.20 ($P < 0.01$)
M555	133	7	26	27	5.54 ($P > 0.02$)

^a Only markers on chromosome 5 are shown. No linkage was observed with the following markers: nga63, nga248, nga280, and ATPase on chromosome 1; GPA1.2, ER, and nga168 on chromosome 2; nga162, GAPA, and nga6 on chromosome 3; and nga8, AG, and nga1107 on chromosome 4.

^b χ^2 values are given for the expected 1:1 ratio for random segregation.

Fig. 4. *EDS1*-dependent hypersensitive response (HR) induced by *Pseudomonas syringae* pv. *glycinia* race 5 in Ws-0. The indicated *Arabidopsis* lines were inoculated at 1×10^9 CFU/ml with *P. syringae* pv. *glycinia* race 5 containing the empty vector pVSP61 (–) or pV316-1A (+). Symptoms were recorded 24 h after inoculation.

be in the same class as *hopPsyA* and *avrRps4*. Work on *HED1* may thus suggest that, even in the absence of HR, some non-host interactions are governed by gene-for-gene interactions, as was proposed for nonhost interactions involving HR (Mysore and Ryu 2004; Whalen et al. 1988).

In the future, the cloning of *HED1* will enable the generation of isogenic plant lines that only differ in the ability to mount an HR. Among other things, these lines will allow a quantitative analysis of the contribution of HR to bacterial disease resistance, if any. Further, it will be possible to challenge these lines with different kinds of pathogens to determine if cell death plays a role in resistance against pathogens with different life-styles.

MATERIALS AND METHODS

Plasmids and bacterial strains.

Plasmids and bacterial strains used in this study are listed in Table 1. *P. fluorescens* and *P. syringae* pv. *glycinea* race 5 strains were grown on Difco pseudomonas agar (Becton, Dickinson and Company, Sparks, MD, U.S.A.), and DC3000 strains were grown on nutrient yeast growth medium (Daniels et al. 1984) or pseudomonas agar. Plasmids pML123 and pLN92 were mobilized into recipient DC3000, and plasmids pVSP61 and pV316-1A were mobilized into recipient *P. fluorescens*(pHIR11) from *E. coli* by triparental mating using the helper plasmid pRK2013 (Ditta et al. 1980). All other *P. fluorescens* strains were provided by J. Alfano, University of Nebraska-Lincoln.

Plant growth and inoculations.

Arabidopsis plants were grown in soil (Pro-Mix BX; Premier Horticulture, Inc., Quakertown, PA, U.S.A.) in a TC-30 environmental growth chamber (Conviron, Winnipeg, Canada). Temperature was set at 24°C and humidity at 70%, with an 8-h light and 16-h dark cycle and a photosynthetic flux of 90 to 130 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Plants were used for experiments 4.5 to 6 weeks after planting.

Unless otherwise noted, HR assays with *P. fluorescens* strains were performed with a bacterial suspension in 10 mM MgCl_2 with an optical density at 600 nm (OD_{600}) of 0.1 (equivalent to 1×10^8 CFU/ml) and with DC3000 strains at an OD_{600} of 0.02 (equivalent to 2×10^7 CFU/ml). *P. syringae* pv. *glycinea* race 5 strains were used at an OD_{600} of 1.0 (equivalent to 1×10^9 CFU/ml). Individual leaves were inoculated with bacterial suspensions with a needleless syringe placed over a needle hole. Inoculated plants were returned to the growth chamber. HR symptoms were recorded 24 h after inoculation.

For disease assays with DC3000, bacterial suspensions with an OD_{600} of 0.001 (equivalent to 1×10^6 CFU/ml) were infiltrated into individual leaves as described above. Plants were returned to the growth chamber, and disease symptoms were recorded 5 days after inoculation. For in planta bacterial growth assays, leaves were infiltrated with DC3000 suspensions equivalent to 5×10^4 CFU/ml. At the indicated time-points, leaf tissue was harvested with a cork borer in triplicate, was ground in 10 mM MgCl_2 , and was plated on selective medium as described previously (Whalen et al. 1991).

Arabidopsis crosses and mapping of *HED1*.

For mapping, the Col-0 and Ws-0 accessions of *Arabidopsis* were crossed, with Col-0 as the female. Crosses were performed by hand-emasculating flowers before anther dehiscence and brushing donor pollen over the stigmas. Testing of F1 progeny with CAPS markers (Konieczny and Ausubel 1993) verified that they were progeny of a true cross. F1 and F2 plants were scored for HR⁺ phenotype after syringe-inoculating with *P. fluorescens*(pHIR11). F2 plants that showed HR were selected

for generation of F3 families to confirm their F2 phenotypes. DNA for analysis with microsatellite (Bell and Ecker 1994) and CAPS markers was isolated from two or three inner rosette leaves of HR⁺ F2 plants as described previously (Gassmann et al. 1999). To identify linkage, the percentage of chromosomes derived from the Col-0 parent at each marker was calculated. Deviation from random segregation was determined statistically, using the χ^2 test with one degree of freedom.

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