The Plant Journal (2002) **30**(4), 467–480

The *Arabidopsis hrl1* mutation reveals novel overlapping roles for salicylic acid, jasmonic acid and ethylene signalling in cell death and defence against pathogens

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Received 30 November 2001; revised 19 February 2002; accepted 25 February 2002
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Summary

Defence against pathogens in *Arabidopsis* is orchestrated by at least three signalling molecules: salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). The *hrl1* (*hypersensitive response-like lesions 1*) mutant of *Arabidopsis* is characterized by spontaneous necrotic lesions, accumulation of reactive oxygen species, constitutive expression of SA- and ET/JA-responsive defence genes, and enhanced resistance to virulent bacterial and oomycete pathogens. Epistasis analyses of *hrl1* with *npr1*, *etr1*, *coi1* and SA-depleted *nahG* plants revealed novel interactions between SA and ET/JA signalling pathways in regulating defence gene expression and cell death. RNA gel-blot analysis of RNA isolated separately from the lesion$^+$ and the lesion$^-$ leaves of double mutants of *hrl1* revealed different signalling requirements for the expression of defence genes in these tissues. Expression of the ET/JA-responsive *PDF1.2* gene was markedly reduced in *hrl1 npr1* and in SA-depleted *hrl1 nahG* plants. In *hrl1 nahG* plants, expression of *PDF1.2* was regulated by benzathiadiazole in a concentration-dependent manner: induced at low concentration and suppressed at high concentration. The *hrl1 etr1* plants lacked systemic *PR-1* expression, and exhibited compromised resistance to virulent *Pseudomonas syringae* and *Peronospora parasitica*. Inhibiting JA responses in *hrl1 coi1* plants lead to exaggerated cell death and severe stunting of plants. Finally, the *hrl1* mutation lead to elevated expression of *AtrbohD*, which encodes a major subunit of the NADPH oxidase complex. Our results indicate that defence gene expression and resistance against pathogens in *hrl1* is regulated synergistically by SA and ET/JA defence pathways.

Keywords: *PR*, *PDF1.2*, cell death, *hrl1*, *Arabidopsis*, pathogen resistance.

Introduction

Plants induce a multi-faceted defence response against pathogens. One such resistance response, the hypersensitive response (HR), is usually associated with the appearance of necrotic flecks containing dead plant cells at the sites of pathogen infection. Hypersensitive cell death is often accompanied by the production of reactive oxygen species (ROS) and anti-microbial compounds (phytoalexins), rapid cross-linking of cell-wall proteins, activation of several defence-related genes, and ultimately enhanced resistance to pathogens (Goodman and Novacky, 1996; Hammond-Kosack and Jones, 1996). Several studies have shown that HR-associated cell death is controlled by a genetic programme(s) in the plant and requires active host participation (Dangl *et al.*, 1996; Dixon *et al.*, 1994; Greenberg, 1997). In many cases, HR is followed by the onset of systemic acquired resistance (SAR) (Hammond-Kosack and Jones, 1996; Ryals *et al.*, 1996; Yang *et al.*, 1997). SAR refers to a distinct plant defence response that results in a non-specific and long-lasting systemic resistance to a variety of pathogens. Several pathogenesis-related (*PR*) genes are induced during SAR and serve as molecular markers of plant defence. Salicylic acid (SA) is both essential and sufficient to induce SAR because transgenic expression of a bacterial salicylate hydroxylase

gene (*nahG*), which converts SA to an inactive catechol, blocks the onset of SAR and abolishes the expression of several *PR* genes (Delaney *et al.*, 1994).

In recent years, identification and analysis of several *Arabidopsis* mutants with altered response to pathogens and SAR-inducing chemicals have helped unravel the molecular basis of defence activation in plants. These mutants may be broadly divided into two classes. Mutants in the first class accumulate high levels of SA, constitutively express SAR and are resistant to variety of virulent pathogens. In addition to constitutive SAR expression, some of these mutants spontaneously develop HR-like lesions and are referred to as lesion-mimic mutants (Bowling *et al.*, 1997; Dietrich *et al.*, 1994; Greenberg *et al.*, 1994; Rate *et al.*, 1999; Shah *et al.*, 1999). Mutants in the second class exhibit enhanced susceptibility to a variety of pathogens (reviewed in Dangl *et al.*, 1996; Glazebrook, 2001; Ryals *et al.*, 1996; Shirasu and Schulze-Lefert, 2000). Of the mutants that exhibit enhanced susceptibility to virulent pathogens, only *npr1* and the independently isolated alleles *sai1* and *nim1* fail to respond to SA treatment (Cao *et al.*, 1994; Ryals *et al.*, 1997; Shah *et al.*, 1997).

Although SA is an important signal mediating defence against a variety of pathogens, the resistance response to some pathogens such as *Alternaria brassicicola* and *Botrytis cinerea* is independent of SA and NPR1 (Penninckx *et al.*, 1996, 1998). This SA/NPR1-independent pathway is characterized by the induction of *PDF1.2* and thionin genes that encode anti-microbial peptides, and requires functional ethylene (ET) and jasmonic acid (JA) signalling pathways.

Although SA- and ET/JA-mediated signalling appear to regulate distinct defence pathways, several studies indicate cross-talk between these pathways (Dong, 1998; Glazebrook, 2001; Maleck and Dietrich, 1999). Experiments with cDNA microarrays revealed that relatively large numbers of *Arabidopsis* genes are coordinately regulated by SA and methyl jasmonate (MJ) (Schenk *et al.*, 2000). Analysis of *Arabidopsis cpr* mutants revealed that components of the ET/JA-mediated resistance pathway are required for SA-mediated, NPR1-independent resistance (Clarke *et al.*, 2000), and SA is required for expression of the *PDF1.2* gene in the *ssi1* mutant (Shah *et al.*, 1999). Simultaneous activation of SA-dependent SAR and ET/JA-dependent induced systemic resistance (ISR) in *Arabidopsis* has an additive effect on induced resistance against *Pseudomonas syringae* (van Wees *et al.*, 2000). While these studies have demonstrated the synergistic effects of various defence signalling pathways, several studies have reported antagonistic effects between these pathways. For example, SA and its derivative acetyl SA suppress JA biosynthesis and downstream signalling in tomato (Doares *et al.*, 1995; Pena-Cortes *et al.*, 1993). While SA promotes HR-related cell death, JA suppresses superoxide-driven cell death resulting in lesion containment (Overmyer *et al.*, 2000; Rao *et al.*, 2000).

To identify the genetic components involved in regulating cell death and defence activation in response to pathogen infection in *Arabidopsis*, we isolated and characterized a mutant designated *hypersensitive response-like lesions 1* (*hrl1*). To analyse the signalling mechanisms that relay the oxidative stress to activate cell death and defence, and to evaluate the possible cross-talk between SA- and ET/JA-mediated defence pathways in *hrl1*, we constructed double mutants in combination with *npr1*, *etr1*, *coi1* and *nahG* expressing *hrl1* lines. Epistasis analysis revealed that defence genes are differentially regulated in lesion$^+$ and lesion$^-$ tissues of *hrl1*. We found that the components of SA and ET/JA signalling pathways function synergistically to regulate the expression of defence genes and resistance against pathogens in *hrl1*.

Results

Isolation and genetic analysis of the hrl1 *mutant*

We screened EMS-mutagenized populations of *Arabidopsis* ecotype Col-0 for mutants that developed reduced disease lesions (water-soaked lesions surrounded by chlorosis) in response to infection by virulent bacterial pathogens *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Whalen *et al.*, 1991). In one of these screens, we identified a mutant designated *hrl1* (*hypersensitive response-like lesions 1*) that spontaneously developed HR-like lesions (Figure 1a,g) and displayed reduced disease symptoms in response to *Pst* DC3000. Lesions in *hrl1* start as random necrotic patches of dead cells on the leaf blades of 2-week-old-plants and are confined to the rosette and cauline leaves. No lesions develop on the stem or on the inflorescence. Once formed, the lesions do not enlarge significantly. Lesions develop on aseptically grown plants, indicating that exposure to pathogens is not necessary for lesion formation. The rosettes of the mature *hrl1* plants are significantly smaller than those of the wild-type parent. All the experiments were performed with a mutant line that was back-crossed three times to the wild-type parent.

Genetic crosses were performed to determine the segregation of the *hrl1* locus, to test whether the *hrl1* mutation is allelic to other known lesion-mimic mutants, and to determine the map position of the *HRL1* locus. None of the 35 F_1 plants from a back-cross between *hrl1* and its wild-type parent (Col-0) developed lesions. The F_2 population segregated as 252 lesion$^-$ to 82 lesion$^+$ plants. This closely approximates a 3:1 ratio ($\chi^2 = 0.036$; $P > 0.95$), indicating that the phenotype is caused by a recessive nuclear mutation at a single locus. The reduced size of the plants, resistance to virulent *Pst* DC3000, and defence-related gene expression always co-segregated with the lesion

phenotype in a recessive manner. To facilitate genetic mapping, the *hrl1* mutant in the Col-0 background was crossed with Landsberg *erecta* (L*er*). Cleaved amplified polymorphic sequence marker (CAPS) (Konieczny and Ausubel, 1993) analysis of 240 Le*r* × *hrl1* F_2 progeny mapped the *hrl1* locus within a 6.88 cM interval, 6.25 cM from CAPS marker g8300 and 0.63 cM from the *RPS2* locus on chromosome IV. Of other lesion-mimic mutants, *lsd1* and *acd2* map close to *hrl1* (Dietrich *et al.*, 1997; Mach *et al.*, 2001). Therefore, to test whether *hrl1* is allelic to *lsd1* or *acd2*, complementation tests were performed. All these mutations are recessive and none of the F_1 progeny from the *hrl1* × *lsd1* (n = 25) or *hrl1* × *acd2* (n = 20) crosses developed lesions, suggesting that *hrl1* is not allelic to *lsd1* or *acd2*. In addition, a recently reported *Arabidopsis* lesion-mimic mutant, *agd2*, maps to the bottom arm of the chromosome IV (Rate and Greenberg, 2001). However, map positions of *HRL1* and *AGD2* suggest that they are more than 10 cM apart, and therefore represent different genetic loci. From these results, we conclude that *hrl1* defines a novel locus with a lesion-mimic phenotype.

hrl1-*associated lesions mimic several pathogen-induced responses*

To further analyse the nature of *hrl1* lesions and to test whether they phenocopy pathogen-induced HR, we analysed the presence of cellular and biochemical markers associated with the HR induced by plants in response to avirulent pathogens (Hammond-Kosack and Jones, 1996). Comparison of whole mounted leaves of *hrl1* with controls revealed substantial accumulation of autofluorescent material, callose, H_2O_2 and superoxide in and around the lesions in *hrl1*, and in the control tissue displaying HR (Figure 2). These results suggest that the *hrl1* mutant constitutively expresses cellular and biochemical markers associated with plant's hypersensitive response to avirulent pathogens.

To test whether spontaneous lesion formation in the *hrl1* mutant is accompanied by transcriptional activation of defence-related genes, total RNA was isolated from *hrl1* leaf tissue and RNA gel-blots were probed with cDNAs encoding *PR-1*, *PR-2* and *GST1* (markers of the SA-responsive defence pathway), and *PDF1.2* (a marker of the ET/JA-responsive defence pathway). To specifically distinguish the lesion-associated HR-like response from the SAR-like response, we analysed the expression of these genes in both the lesion$^+$ leaves and the lesion$^-$ leaves of 6-week-old *hrl1* plants. Leaf 1 and leaves 2 and 3 in Figure 1(g) represent lesion$^-$ and lesion$^+$ *hrl1* samples, respectively. No dead cells were found in lesion$^-$ leaves even after staining with trypan blue (data not shown). Results in Figure 3 (lanes 1 and 2) demonstrate that both lesion$^+$ and lesion$^-$ leaves of 6-week old *hrl1* plants show heightened defence gene activation.

Growth of virulent bacteria and oomycete pathogens is suppressed in hrl1

As *hrl1* plants constitutively express cellular and molecular markers associated with HR and SAR, we sought to determine whether they were resistant to virulent pathogens. The growth of the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Whalen *et al.*, 1991) was tested in the leaves of *hrl1* plants in which lesions had just initiated. The results presented in Figure 4(a) demonstrate that *hrl1* is more resistant to *Pst* DC3000 compared to wild-type Col-0: bacterial levels were more than 100-fold lower in *hrl1* plants 4 days post-infiltration. Growth of the virulent oomycete pathogen *Peronospora parasitica* Ahco2 (Holub *et al.*, 1994) was also tested on lesion$^+$ leaves of *hrl1* plants. As shown in Figure 4(b), compared to wild-type Col-0, growth of *P. parasitica* Ahco2 is strongly suppressed in *hrl1* plants. These results demonstrate that, compared to the wild-type parent, *hrl1* plants are more resistant to virulent bacteria and to at least one oomycete pathogen isolate.

hrl1 *accumulates elevated levels of SA*

SA is a key endogenous signal required for the expression of the SA-dependent defence signalling pathway. Furthermore, several lesion-mimic mutants accumulate elevated levels of SA (reviewed in Dangl *et al.*, 1996; Ryals *et al.*, 1996; Shirasu and Schulze-Lefert, 2000). We analysed the endogenous levels of free SA and salicylate glucoside (SAG) in the rosette leaves of 6-week-old soil-grown *hrl1* plants. As shown in Figure 5, levels of free SA and SAG in *hrl1* plants are two- and five-fold higher, respectively, than in the parental Col-0 plants. However, the increase in the levels of SA and SAG in *hrl1* is significantly lower compared to the increase in levels (up to 30-fold) observed in several other constitutive SAR mutants such as *cpr1*, *cpr5*, *cpr6* and *ssi1* (Bowling *et al.*, 1994; Bowling *et al.*, 1997; Clarke *et al.*, 1998; Shah *et al.*, 1999). Nonetheless, these results indicate that elevated levels of SA may, in part, activate SA-mediated defence pathways leading to constitutive defence gene expression and enhanced resistance to virulent pathogens in *hrl1* plants.

SA regulates expression of both SA- and ET/JA-responsive defence genes in hrl1

To determine the role of SA in regulating the cell death and defence expression in *hrl1*, we constructed *hrl1 nahG* plants by a genetic cross using a well-characterized *Arabidopsis* line harbouring the *nahG* gene. Plants express-

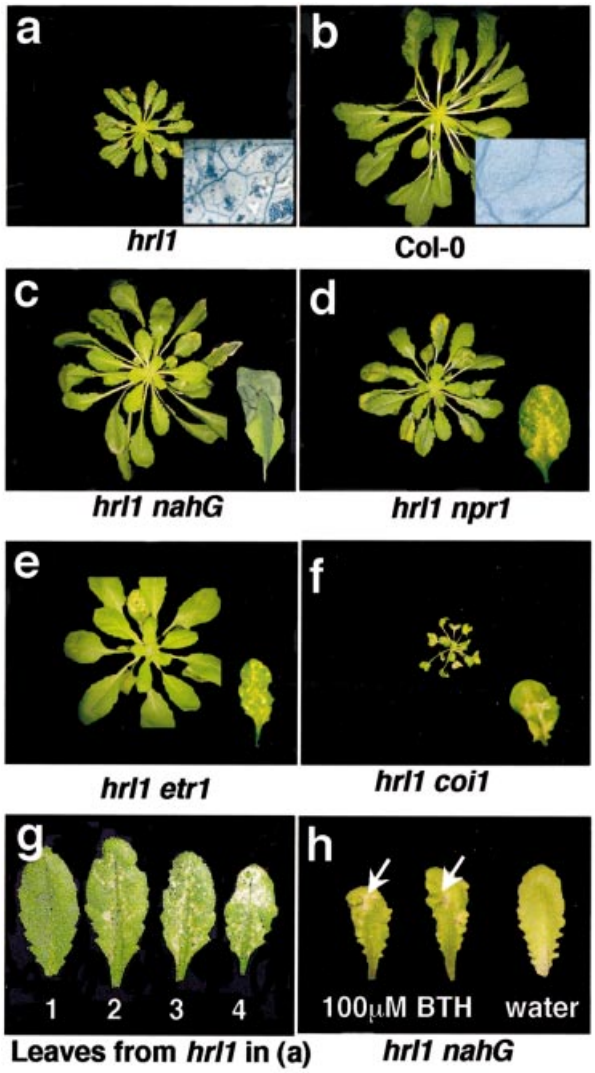

Figure 1. Phenotypes of 6-week-old *hrl1*, *hrl1 npr1*, *hrl1 nahG*, *hrl1 etr1* and *hrl1 coi1* plants.
Insets in (a) and (b) are trypan blue-stained leaves showing an intensely stained area of dead cells only in *hrl1*. Insets in (c)–(f) are close-up images of individual leaves showing characteristic lesions of the double mutants. (g) Lesion$^-$ leaf (1) and lesion$^+$ leaves (2–4) of *hrl1*. Leaves 1 and 4 represent the youngest and the oldest leaf, respectively. (h) Plants were treated as indicated and the leaves were photographed 6 days after treatment. Arrows show the BTH-induced *hrl1*-like lesions in *hrl1 nahG* leaves.

ing the *nahG* gene do not accumulate SA and are more susceptible to virulent pathogens (Lawton *et al.*, 1996). Although the onset and the nature of lesions on the first true leaves of *hrl1 nahG* plants were very similar to those of the *hrl1* plants, their formation on the subsequent leaves was delayed by 2 weeks. The rosettes of *hrl1 nahG* plants were significantly larger than those of *hrl1* plants but somewhat smaller compared with the wild-type parent (Figure 1c).

We analysed the expression of SA- and ET/JA-responsive defence genes in lesion$^+$ and lesion$^-$ leaves of *hrl1 nahG* plants by RNA gel-blot analysis. The extent of cell death in

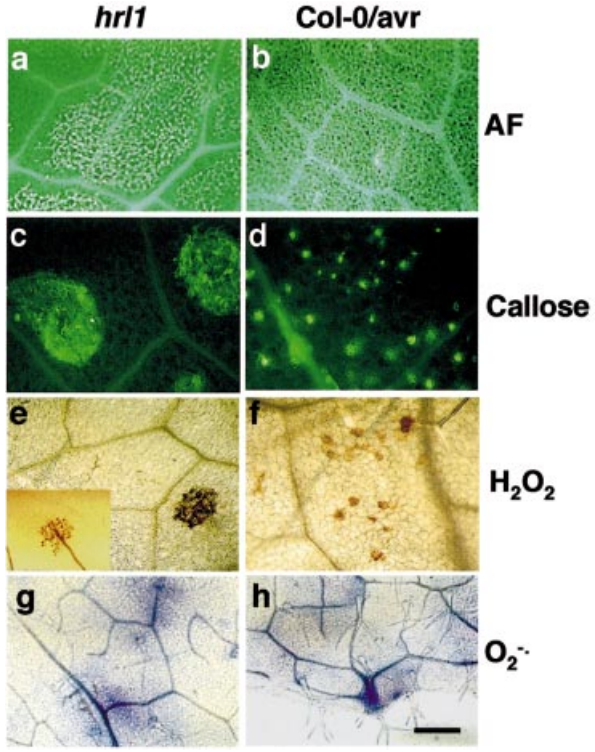

Figure 2. Accumulation of defence-related biochemical markers in *hrl1*.
Vertical columns represent the genotype of the plant/treatment used and the horizontal rows represent the biochemical marker tested. (a,b) Autofluorescent materials (AF) visualized by UV microscopy. (c,d) Callose deposition revealed by aniline blue staining. (e,f) H_2O_2 accumulation revealed by DAB staining. The inset in (e) shows frequently observed DAB staining around the vein endings in *hrl1*. (g,h) Superoxide ($O_2^{-\cdot}$) accumulation revealed by NBT staining. Col-0/avr, wild-type Col-0 leaves infiltrated with 10^7 cfu ml^{-1} of avirulent bacterial pathogen *Pst* DC3000 (*avrRpm1*). Leaves were harvested for analysis 24 h after infiltration. Bar in (h) = 1 mm (appiles to all images).

the lesion$^+$ leaves of *hrl1 nahG* and *hrl1* plants used in these experiments was similar. Results in Figure 3 (lanes 6 and 7) show that defence genes are differentially expressed in lesion$^+$ and lesion$^-$ leaves of *hrl1 nahG* plants. Expression of all the tested defence genes was suppressed in the lesion$^-$ leaves. However, in the lesion$^+$ leaves, while the expression of *PR-1* was completely suppressed, *PR-2* expression was significantly reduced (four-fold) and *GST1* expression remained unaltered. Furthermore, preventing SA accumulation in *hrl1 nahG* plants led to a significant reduction of *PDF1.2* expression (two-fold) in the lesion$^+$ leaves and to undetectable levels in the lesion$^-$ leaves. Thus, SA appears to positively regulate the expression of both SA- and ET/JA-responsive genes in *hrl1*.

PDF1.2 *expression in* hrl1 nahG *plants is dependent on BTH concentration*

The results described above and by others (Shah *et al.*, 1999) demonstrate that SA is required for the constitutive

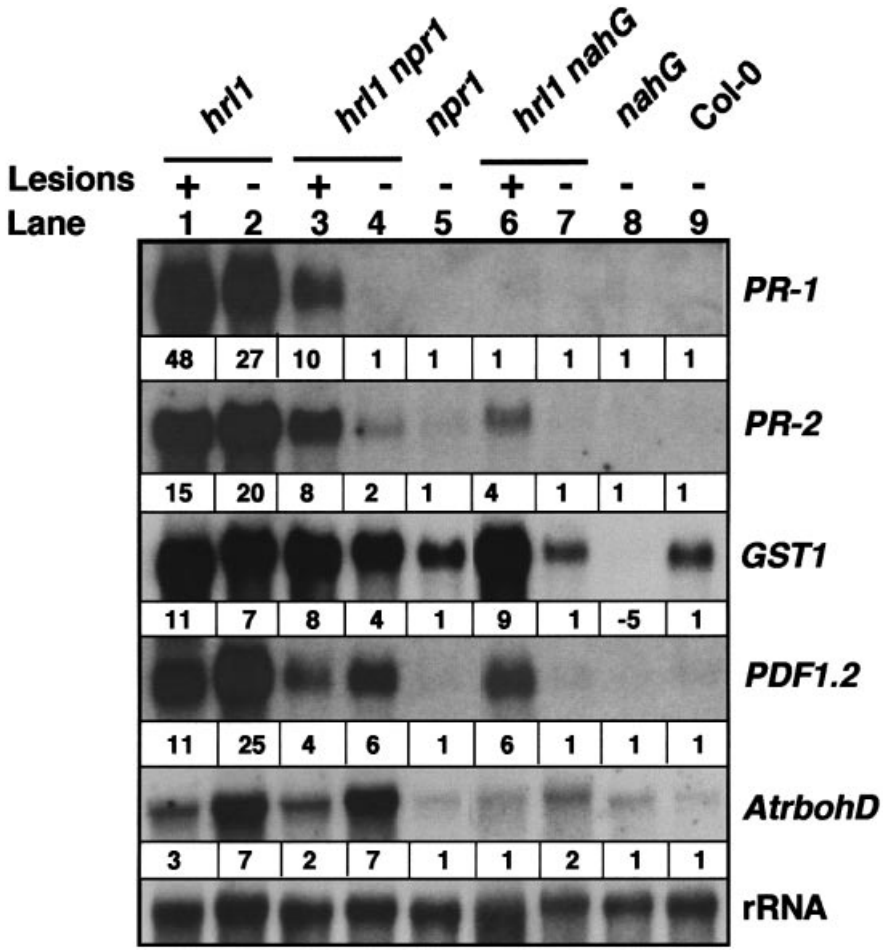

Figure 3. Defence gene expression in *hrl1*, *hrl1 npr1* and *hrl1 nahG* plants.
Transcript levels of *PR-1*, *PR-2*, *GST1*, *PDF1.2* and *AtrbohD* in the leaves of 6-week-old plants of the indicated genotypes were determined by RNA gel-blot analysis. Gene expression in *hrl1*, *hrl1 npr1* and *hrl1 nahG* plants was determined separately in the lesion$^+$ (+) and lesion$^-$ (–) leaves collected from the same set of plants. Signals were quantified using PhosphorImager and ImageQuant software (Molecular Dynamics) and were normalized relative to the loading control. The values under each row represent the fold induction of gene expression for each sample compared with the untreated control Col-0. This experiment was repeated twice with different sets of plants and similar results were obtained.

expression of *PDF1.2* in some *Arabidopsis* mutants. However, it has been shown that SA treatment suppresses the expression of the JA signalling pathway (Doares *et al.*, 1995). Therefore, to understand how SA and JA signalling pathways might interact in *hrl1*, we analysed the effects of SA and MJ treatments on the constitutive expression of *PR-1* and *PDF1.2* genes. Consistent with the idea of antagonistic effects, SA treatment suppressed the expression of *PDF1.2* and MJ treatment suppressed the expression of *PR-1* in *hrl1* plants (Figure 6a). These results and those described above demonstrate that addition as well as removal of SA from *hrl1* plants leads to suppression of *PDF1.2* expression.

To explain this paradoxical result, we hypothesized that *PDF1.2* induction in *hrl1* requires an optimal concentration of SA and deviation from this concentration suppresses *PDF1.2* expression. To test this hypothesis, we used *hrl1 nahG* plants that are unable to accumulate SA, and analysed the expression of *PDF1.2* in response to increasing concentrations of benzathiadiazole (BTH), a biologically active analogue of SA (Lawton *et al.*, 1996). BTH was sprayed at three different concentrations (1, 10, 100 μM) on 6-week old *hrl1 nahG* and the control plants. We found that BTH treatment at 10 and 100 μM concentrations restored spontaneous punctate lesions within 6 days in the old and the newly emerging leaves of *hrl1 nahG* plants (Figure 1h) with no effect on Col-0 or *nahG* plants (data not shown). Furthermore, increasing amounts of BTH restored *PR-1* expression in *hrl1 nahG* plants, with significant induction occurring at 100 μM (Figure 6b). However, *PDF1.2* expression was highest at 1 μM BTH and then declined with increasing concentrations of BTH. Similar results were obtained with RNA isolated from 24 h lesion-free tissue samples, demonstrating that BTH-dependent *PDF1.2* induction in *hrl1 nahG* plants is not due to cell necrosis (Figure 6b). These results demonstrate that, depending on its concentration relative to other defence signals, BTH/SA can stimulate as well as suppress *PDF1.2* expression in *hrl1*.

Expression of PDF1.2 *in* hrl1 *is partially regulated through NPR1*

NPR1 functions downstream of SA and is required for some aspects of SA signal transduction in response to pathogen infection (Cao *et al.*, 1997). To understand the

role of NPR1 in regulating cell death and defence activation in *hrl1*, we constructed *hrl1 npr1* double mutants. Although the initial timing of appearance of lesions on the true leaves of *hrl1 npr1* double mutant is very similar to the lesions on *hrl1* plants, development of lesions on the subsequent leaves is delayed by at least 10 days. Furthermore, the rosettes of *hrl1 npr1* plants are larger in size than those of the *hrl1* plants but smaller than the wild-type parent Col-0 (Figure 1d). RNA gel-blot analysis revealed that, similar to *hrl1 nahG* plants, defence-related genes were differentially expressed in the lesion$^+$ and lesion$^-$ leaves of *hrl1 npr1* plants (Figure 3). It should be noted that the lesion$^+$ leaves of *hrl1 npr1* and *hrl1* plants used in this analysis had similar levels of cell death. In the lesion$^+$ leaves, the *npr1* mutation moderately suppressed constitutive expression of *PR-1*, but had little effect on the expression of *PR-2* and *GST1*. In the lesion$^-$ leaves, however, expression of *PR-1* and *PR-2* was markedly reduced but *GST1* expression was minimally affected. These results suggest that expression of *PR* genes in *hrl1* is regulated by NPR1-dependent and NPR1-independent pathways.

The expression of *PDF1.2* is reduced three- to four-fold in both lesion$^+$ and lesion$^-$ leaves of *hrl1 npr1* plants (Figure 3). These results suggest that *PDF1.2* expression in *hrl1* is partially regulated through NPR1. However, *npr1* mutants have been shown to accumulate elevated levels of SA compared to the wild-type parent (Clarke *et al.*, 2000;

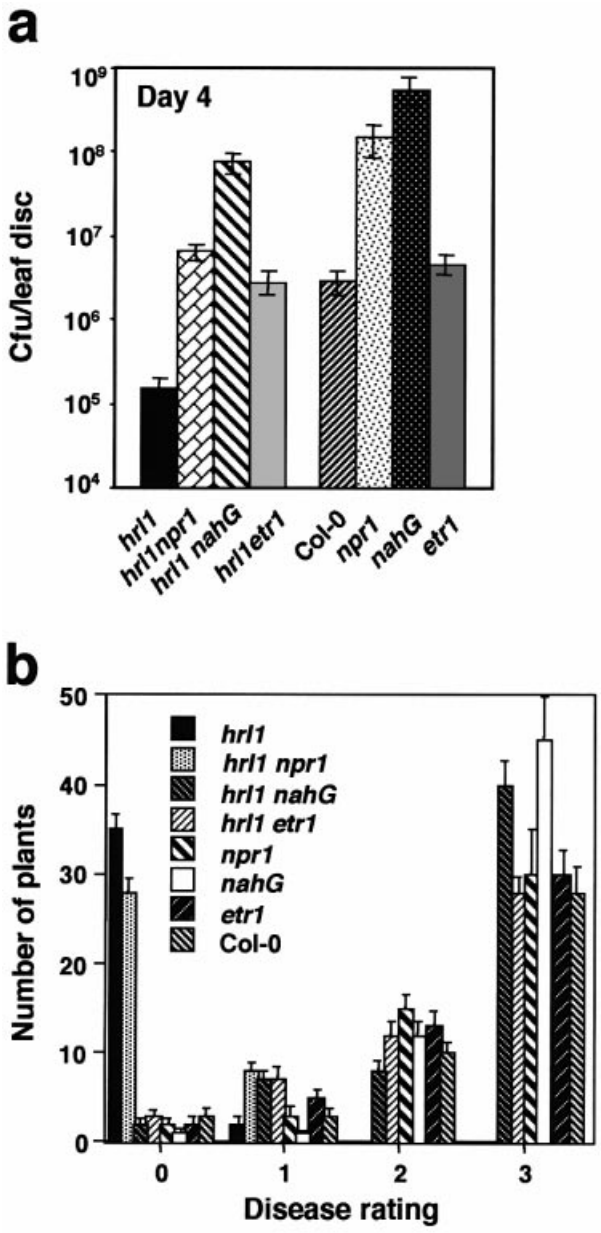

Figure 4. Effects of *npr1*, *nahG* and *etr1* on growth of *Pst* DC3000 and *P. parasitica* Ahco2 in *hrl1*.
(a) Leaves of 6-week-old plants of the indicated genotypes were infiltrated with a suspension of a virulent strain of bacterial pathogen *Pst* DC3000 in 10 mM $MgCl_2$ at a dose of 10^5 cfu ml^{-1}. Bacterial count was determined as described in Experimental procedures. The bacterial counts ± SD are presented as colony-forming units (cfu) per leaf disc and are means of three independent experiments. (b) Three-week-old seedlings of indicated genotypes were sprayed with a spore suspension of *P. parasitica* Ahco2 in water (2×10^4 spores ml^{-1}) and the number of conidiophores on each plant was counted 7 days after infection. Disease rating was determined as described by Bowling *et al.* (1994). The data are represented as means ± SD from three independent experiments.

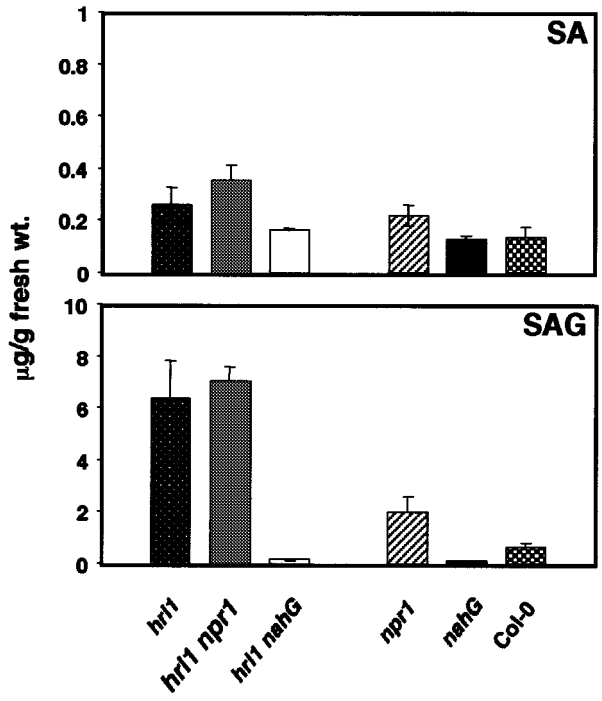

Figure 5. Comparison of free SA (SA) and sugar-conjugated SA (SAG) levels in *hrl1*, Col-0, *npr1*, *nahG*, *hrl1 npr1* and *hrl1 nahG* plants.
SA and SAG were extracted from the rosette leaves of 6-week-old soil-grown plants and analysed by HPLC as described in Experimental procedures. The values are presented as µg SA (± SD) g^{-1}fresh weight, and are averages from four sets of samples per genotype.

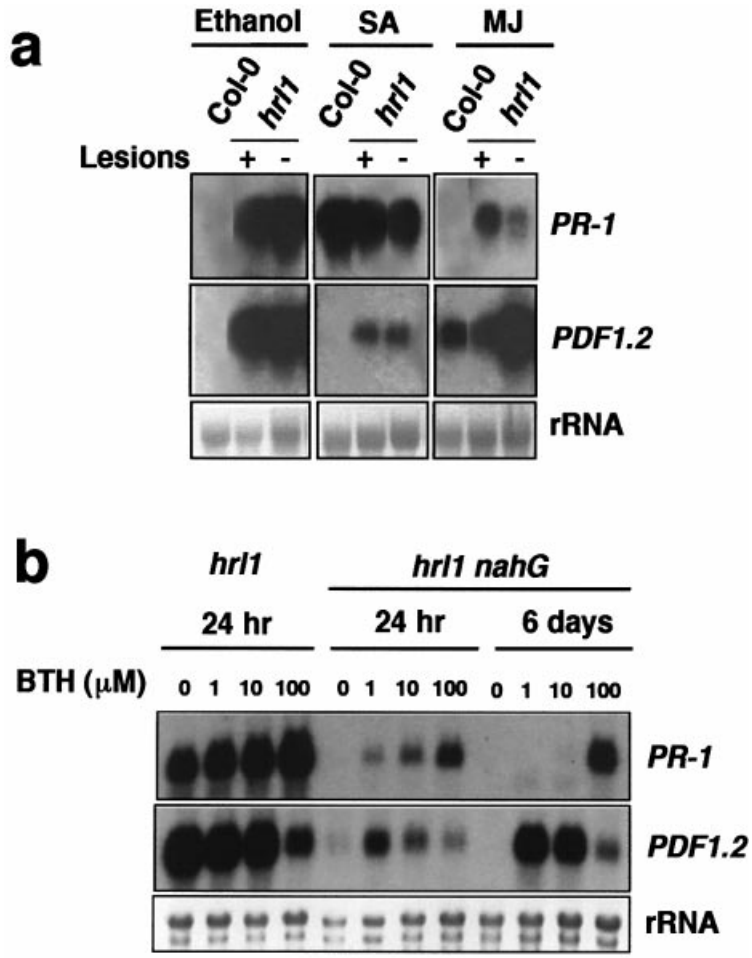

Figure 6. Expression of *PR-1* and *PDF1.2* genes in *hrl1* and *hrl1 nahG* plants in response to SA, MJ and BTH treatments.
(a) Six-week-old Col-0 and *hrl1* plants were sprayed with 1 mM SA, 50 μM MJ and 0.01% ethanol (solvent for MJ). Leaf samples were harvested 24 h after treatment. Expression in lesion^{+} (+) and lesion^{-} (–) leaves of *hrl1* was analysed separately. (b) Six-week-old *hrl1* and *hrl1 nahG* plants were sprayed with the indicated concentrations of BTH, and leaves were harvested at the indicated times after treatment. BTH treatment induced *hrl1*-like lesions on *hrl1 nahG* leaves 6 days after treatment (Figure 1h) but not within 24 h. This experiment was replicated twice with different sets of plants and similar results were obtained.

Delaney *et al.*, 1995). Hence the increase in SA levels due to the presence of *npr1* may lead to suppression of *PDF1.2* expression in *hrl1 npr1* plants. To test this possibility, we determined the levels of SA and SAG in *hrl1 npr1* and control plants. The levels of SA and SAG in *hrl1 npr1* plants are only slightly elevated compared to *hrl1* plants (Figure 5). This slight increase in the levels of SA in *hrl1 npr1* plants is unlikely to suppress the levels of *PDF1.2* by three- to four-fold.

hrl1 *constitutively activates the* AtrbohD *gene*

AtrbohD, an *Arabidopsis* homologue of mammalian gp91^{phox} gene, encodes a putative major subunit of the NADPH oxidase multi-enzyme complex and is induced during HR (Keller *et al.*, 1998; Torres *et al.*, 1998). As shown in Figure 3, *hrl1* plants express elevated levels of *AtrbohD* compared to the wild-type Col-0. While the induction of this gene remains unaffected in *hrl1 npr1* plants, it is reduced to background levels in *hrl1 nahG* plants. These results suggest that the induction of *AtrbohD* in *hrl1* is independent of NPR1 but requires SA accumulation. The two other homologues, *AtrbohB* and *AtrbohF*, were not significantly induced in *hrl1* (data not shown).

Ethylene signalling regulates cell death and systemic PR-1 *induction in* hrl1

The results described above demonstrate that the signalling components of the SA-mediated defence pathway (SA and NPR1) positively regulate the expression of both SA- and ET/JA-responsive genes. Therefore, we tested whether the converse held true for the signalling components of the ET/JA response pathways. First, we found that *hrl1* plants produced significantly more ethylene compared to the wild-type parent (Table 1). Next, we tested the effect of the *etr1* mutation on the expression of SA- and ET/JA-responsive genes in *hrl1*. *ETR1* encodes an ethylene receptor and *etr1* mutants are defective in ethylene perception (Chang *et al.*, 1993). The timing of appearance of lesions on the first true leaves of *hrl1 etr1* was similar to that of the *hrl1,* but lesion development in

Table 1 Ethylene production in *hrl1* and Col-0 plants

Genotype	Ethylene production (nmol h^{-1} mg^{-1} fresh weight)
hrl1	4.654 ± 1.19
Col-0	0.796 ± 0.17

Ethylene production by 6-week-old soil-grown *hrl1* and Col-0 plants was determined by gas chromatography as described in Experimental procedures. The values represent means ± SD for three independent samples involving at least 10 plants per measurement.

the subsequent leaves was delayed by 10 days. The rosettes of *hrl1 etr1* were significantly larger than those of *hrl1* plants but smaller compared with parent Col-0 (Figure 1e).

Expression of ET/JA- and SA-responsive genes was analysed in the lesion$^+$ and lesion$^-$ leaves of *hrl1 etr1* plants by RNA gel-blot analysis. As expected, constitutive expression of *PDF1.2* was markedly suppressed in both the lesion$^+$ and lesion$^-$ leaves of *hrl1 etr1* plants (Figure 7). Although the constitutive expression of SA-responsive genes was unaffected in the lesion$^+$ leaves of *hrl1 etr1* plants, in the lesion$^-$ leaves, expression of *PR-1* was markedly suppressed and that of *PR-2* and *GST1* moderately reduced. These results imply that, in addition to regulating the expression of ET/JA-responsive genes, ethylene signalling positively regulates the SAR expression of SA-responsive genes in *hrl1* plants.

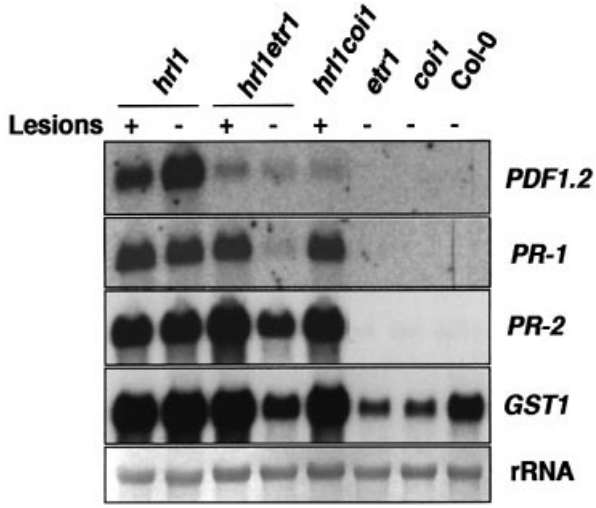

Figure 7. Effects of *etr1* and *coi1* on the expression of defence-related genes in the *hrl1* mutant.
Expression of *PR-1*, *PR-2*, *GST1* and *PDF1.2* in the leaves of 6-week-old plants of the indicated genotype was determined by RNA gel-blot analysis. Gene expression in *hrl1* and *hrl1 etr1* plants was determined separately in lesion$^+$ (+) and lesion$^-$ (–) leaves collected from the same set of plants. No lesion$^-$ leaves could be obtained from *hrl1 coi1* plants. This experiment was repeated twice with different sets of plants and similar results were obtained.

Mutation in COI1 *aggravates lesion formation in* hrl1

COI1 is required for sensitivity to coronatine and jasmonates in *Arabidopsis*. The *coi1* mutant is defective in jasmonate signalling and does not induce *PDF1.2* expression in response to chemical or biological inducers (Xie *et al.*, 1998). To assess the role of COI1 in regulating the expression of *PDF1.2* and SA-responsive genes in *hrl1*, we constructed a *hrl1 coi1* double mutant. Interestingly, unlike *hrl1 npr1*, *hrl1 nahG* or *hrl1 etr1* plants, in which lesion formation was attenuated and plants had larger rosettes, lesions in *hrl1 coi1* plants were exaggerated and the plant rosette was severely stunted (Figure 1f). Lesions in *hrl1 coi1* plants had severe bleaching and the entire leaf collapsed within a week following lesion initiation.

We analysed the expression of *PDF1.2* and SA-responsive defence genes in the lesion$^+$ leaves of *hrl1 coi1* plants by RNA gel-blot analysis. Consistent with its signalling requirements, *PDF1.2* gene expression was significantly reduced in these plants (Figure 7). Similar to *hrl1 etr1* plants, expression of *PR-1*, *PR-2* and *GST1* remained unaffected in the lesion$^+$ leaves. As *hrl1 coi1* plants were extremely dwarfed with severe lesions, we were unable to obtain lesion-free tissue samples from these plants for Northern analysis. These results show that, in addition to transducing JA-dependent defence signals, COI1 is necessary to limit the severity of cell death in *hrl1*.

Role of SA and ET/JA signalling in regulating resistance against pathogens in hrl1

To determine the role of SA and ET/JA signalling in the regulation of resistance against pathogens in *hrl1* plants, we determined the response of *hrl1 nahG*, *hrl1 npr1* and *hrl1 etr1* plants to virulent bacterial and oomycete pathogens. Due to the very small stature and severe lesions on *hrl1 coi1* plants, we could not reliably infect them to study their response to pathogens. Control plants and leaves of *hrl1 nahG*, *hrl1 npr1* and *hrl1 etr1* in which lesions had just initiated were inoculated with *Pst* DC3000 at a dose of 10^5 cfu ml^{-1}. Bacterial titre was determined 4 days after infection. Consistent with the previous reports, compared to Col-0, *npr1*- and *nahG*-expressing plants but not *etr1* plants were more susceptible to *Pst* DC3000. Resistance was compromised in *hrl1 npr1* and *hrl1 nahG* plants, although these double mutants were less susceptible than *npr1* and *nahG* plants, respectively (Figure 4a). Interestingly, *hrl1*-mediated resistance was also compromised in *hrl1 etr1* plants. These results suggest that resist-

ance to *Pst* DC3000 in *hrl1* is mediated by simultaneous expression of SA and ET signalling pathways.

We found that the resistance displayed by *hrl1* to *P. parasitica* Ahco2 was abolished in *hrl1 nahG* plants but was only slightly diminished in *hrl1 npr1* plants (Figure 4b). The *hrl1 etr1* double mutant had more conidiophores compared to *hrl1*, indicating that ethylene signalling is required for resistance to *P. parasitica* in *hrl1*. These results suggest that resistance to virulent oomycete in *hrl1* also requires the concurrent expression of SA and ET signalling pathways.

Discussion

We have isolated and characterized a novel *Arabidopsis* mutant, *hrl1*, which spontaneously develops HR-like necrotic lesions, constitutively expresses SA- and ET/JA-responsive defence genes, accumulates elevated levels of ROS, SA and ethylene, and displays enhanced resistance to virulent bacterial and oomycete pathogens. Analysis of defence gene expression separately in the lesion$^+$ and lesion$^-$ leaves of *hrl1* plants revealed that defence genes are induced in the lesion$^-$ leaves at levels comparable to the lesion$^+$ leaves, albeit with different signalling requirements. These results demonstrate that, similar to the response of wild-type plants to avirulent pathogens, signals originating from the necrotic tissue in the *hrl1* mutant spread systemically to induce the expression of defence-related genes in the healthy tissue. Alternatively, although the lesion$^-$ leaves do not have any dead tissue, some of the cells that are committed to die may activate defence-related gene expression. Compared to other lesion-mimic mutants, a unique feature of *hrl1* is that SA and ET/JA defence pathways function synergistically to regulate the expression of defence genes and resistance to virulent *P. syringae* and *P. parasitica* pathogens.

SA and NPR1 define a local versus systemic PR *gene expression pattern in* hrl1

Based on the expression analysis, we found that different signalling pathways regulate a subset of defence-related genes in the lesion$^+$ leaves (tissue mimicking HR) and in the lesion$^-$ leaves (tissue mimicking SAR) of *hrl1*. For example, expression of *PR-1* is partially suppressed in the lesion$^+$ leaves and abolished in the lesion$^-$ leaves of *hrl1 npr1*. However, removal of SA in *hrl1 nahG* plants blocked the expression of *PR-1* in both lesion$^+$ and lesion$^-$ tissue. These results indicate the participation of an additional signal generated only in the cells undergoing necrosis, which together with SA can activate *PR-1* gene expression independently of NPR1. *hrl1 npr1* plants retain partial and full resistance against bacterial and oomycete pathogens, respectively. However, resistance to both these pathogens is severely compromised in the SA-depleted *hrl1 nahG* plants. These results suggest that, while resistance to bacterial pathogen *Pst* DC3000 is partially regulated through NPR1, resistance to at least one isolate of the oomycete pathogen *P. parasitica* is independent of NPR1. Existence of a SA-dependent but NPR1-independent pathway for regulation of *PR-1* expression and resistance to bacterial pathogens has been suggested (Bowling *et al.*, 1997; Clarke *et al.*, 1998; Rate *et al.*, 1999).

Ethylene signalling modulates cell death, PR *gene expression and resistance in* hrl1

The role of ethylene in the transduction of SA-dependent defence responses against pathogens is not well understood. Analysis of *Arabidopsis* mutants impaired in ethylene signalling indicate that, although sensitivity to ethylene is required for development of disease symptoms in response to virulent bacterial pathogens, it is not essential for elicitation of the hypersensitive response against avirulent pathogens (Bent *et al.*, 1992; Lawton *et al.*, 1994a). These results suggest that, although ethylene may not be a global signal for establishing plant immunity, it can supplement some of the dominant resistance responses in a subset of host–pathogen interactions. The presence of *ein2* in *Arabidopsis* mutants *cpr5* and *cpr6* does not significantly alter the constitutive *PR-1* gene expression or resistance to *P. s. maculicola* ES4326 (Clarke *et al.*, 2000). However, in the *hrl1 etr1* double mutant, constitutive expression of *PR-1* is not affected in the tissue mimicking HR (lesion$^+$ leaves) but is blocked in the tissue mimicking SAR (lesion$^-$ leaves) (Figure 7). The down-regulation of *PR* gene expression in the systemic tissue of *hrl1 etr1* also correlates with the suppressed resistance against virulent bacterial and oomycete pathogens in these plants (Figure 4). Based on these results, we believe that ethylene plays an important role in relaying or amplifying the signal(s) that emanate from the necrotic tissue to potentiate SA-dependent SAR gene expression in the healthy tissue. This is consistent with a previous report that low concentrations of SA induce *PR-1* in Col-0 plants pre-treated with ethylene (Lawton *et al.*, 1994b).

NPR1 and SA regulate PDF1.2 *expression in* hrl1

Induction of *PDF1.2* expression in response to *A. brassicicola* infection in *Arabidopsis* has been shown to be independent of both NPR1 and SA (Penninckx *et al.*, 1996). However, Bowling *et al.* (1997) observed elevated levels of *PDF1.2* expression in *npr1* plants grown on agar plates compared to the wild-type plants. In the *ssi1* mutant, constitutive expression of *PDF1.2* was found to be higher in the *npr1* background. Based on these studies, it has been suggested that NPR1 negatively regulates *PDF1.2*

expression (Shah *et al.*, 1999). In contrast to these reports, constitutive expression of *PDF1.2* in *hrl1* plants is reduced three- to four-fold in the absence of NPR1 function. As the presence of *npr1* in *hrl1 npr1* plants leads to only a slight increase in the levels of SA, this increase is unlikely to be the reason for the observed reduction in *PDF1.2* expression. In fact, the presence of *npr1* in other *Arabidopsis* mutants leads to a significant increase in SA levels, yet constitutive *PDF1.2* expression is not suppressed (Clarke *et al.*, 2000; Shah *et al.*, 1999). Involvement of NPR1 in regulating SA-independent defence pathways is not without precedent. For example, SA-independent but ET/JA-dependent ISR activated by *P. fluorescens* requires NPR1 function (Pieterse *et al.*, 1998). Together, these results demonstrate that NPR1 can transmit multiple signals from various pathogen defence pathways in *Arabidopsis*.

Synergistic and antagonistic effects of SA on PDF1.2 *expression*

Studies in several plant species have shown that SA and JA signalling can act both antagonistically and synergistically (Doares *et al.*, 1995; Pena-Cortes *et al.*, 1993; Schenk *et al.*, 2000). We speculate that synergism or antagonism between SA and JA signalling probably depends on the relative concentration of the signalling molecules. *hrl1* plants accumulate elevated levels of SA and ET (Figure 5 and Table 1) and possibly JA. Removal as well as addition of SA in *hrl1* plants suppressed *PDF1.2* expression (Figure 6), indicating that SA, depending on its concentration relative to ET and/or JA, can function both as a suppressor and an inducer of the ET/JA signalling pathway. How does SA function both as an inducer and a suppressor of ET/JA signalling in *hrl1 nahG* plants? One possible explanation is that, in *hrl1 nahG* plants, an inert signal is present that requires activation by SA. This SA-activated signal interacts with the components of the ET/JA pathway to induce *PDF1.2* expression. In wild-type plants, this signal is absent and hence SA or BTH alone cannot induce *PDF1.2* expression. Also, pathogens may overcome this *hrl1*-derived signal to induce *PDF1.2* independent of SA. A similar conjecture has been postulated to explain the SA-dependent induction of *PDF1.2* in *ssi1* (Shah *et al.*, 1999). However, when the endogenous SA concentration exceeds a certain critical threshold, it blocks JA/ET biosynthesis or their downstream signals, and thus suppresses constitutive expression of *PDF1.2*.

COI1 *limits lesion severity*

COI1 defines a crucial control element in transmitting JA-regulated responses against pests and pathogens in *Arabidopsis*. The *hrl1 coi1* double mutant, unlike other double mutants of *hrl1*, has exacerbated lesions and is extremely dwarfed compared to *hrl1*. *nahG*-expressing *hrl1*, *hrl1 npr1* and *hrl1 etr1* all had attenuated lesions and larger rosette compared to *hrl1*, presumably due to the reduced accumulation of toxic defence-related compounds and a reduced metabolic burden associated with their synthesis. Because COI1 is thought to play a role in targeting regulators of defence for modification by ubiquitination (Xie *et al.*, 1998), we speculate that the absence of COI1-mediated signalling may lead to further accumulation of toxic compounds constitutively produced in *hrl1*. Alternatively, unidentified signalling pathways that are repressed by JA/COI1 may be turned on in *hrl1 coi1* plants, leading to severe lesions. The fact that MJ pre-treatment of ozone-sensitive *Arabidopsis* ecotype Cvi-0 and *rcd1* mutant mitigate the propagation of cell death, and JA-insensitive *jar1* and JA-deficient *fad3/7/8* develop spreading lesions in response to ozone, suggest a protective role for jasmonates in containing cell death (Overmyer *et al.*, 2000; Rao *et al.*, 2000). The phenotype of *hrl1 coi1* indicates that COI1-assisted JA signalling may also serve to protect the cells against ROS-driven cell death.

Regulation of cell death and defence activation in hrl1

Although it is difficult to predict the function of the wild-type HRL1 protein, we speculate that wild-type HRL1 protein might be involved in regulating an early step leading to ROS production. Although the source of ROS during the oxidative burst is unclear, the NADPH oxidase multi-enzyme complex may participate in superoxide generation. *AtrbohD* encodes a major subunit of NADPH oxidase and its transcript level increases during HR (Keller *et al.*, 1998; Torres *et al.*, 1998). Because the *hrl1* mutant accumulates elevated levels of *AtrbohD* transcript, we speculate that wild-type HRL1 protein might negatively regulate the expression of *AtrbohD* and possibly other ROS-generating system(s). During incompatible plant–pathogen interactions, this suppression might be relieved by pathogen-derived signals. Accumulation of H_2O_2 can lead to synthesis of SA, and SA in turn can stabilize H_2O_2 by inhibiting catalase activity (Chen *et al.*, 1993; Leon *et al.*, 1995). Elevated levels of SA along with H_2O_2 can activate *PR* and *GST1* gene expression locally and serve as a systemic signal to activate SAR in the distal (lesion$^-$) leaves. In addition, superoxide generated due to the overexpression of *AtrbohD* can induce the *PDF1.2* gene. Chemicals such as paraquat or rose bengal that generate superoxide and singlet oxygen, respectively, induce *PDF1.2* gene expression (Penninckx *et al.*, 1996).

SA alone cannot induce cell death or accumulation of ROS, but it can potentiate elicitor-mediated generation of ROS and HR-associated cell death (Shirasu *et al.*, 1997). We found that the depletion of SA in *hrl1 nahG* plants significantly reduces *AtrbohD* expression (Figure 3). This

raises the possibility that SA or an SA-regulated signal may enhance the accumulation of ROS by positively regulating the transcription of *AtrbohD* and possibly other related genes. Recent evidence suggests that EDS1 and PAD4 may enhance resistance by processing ROS- and
SA-activated molecules (Rusterucci *et al.*, 2001).

While the results described in this paper demonstrate that *hrl1* induces all tested pathogen defence responses, there is some concern that some of the observed defence signalling in the lesion-mimic mutants might not necessarily represent cell death and defence induced by pathogens in wild-type plants but could be the result of disruption of cellular homeostasis. Considering the genetic complexity involved in maintaining cellular homeostasis, it is not surprising that many genetic and environmental insults are likely to induce cell death. Indeed, many genes known to be involved in regulating basic metabolism are differentially expressed in response to pathogen infection (Schenk *et al.*, 2000; A.M. Gómez-Buitrago and R. Raina, unpublished results). Many of these genetic components are likely to be part of overlapping signalling pathways, and might act as a 'funnel' to channel many different signals to regulate cell death in response to environmental and developmental signals. Such genetic components have been reported in animal systems (Anderson, 2000; Hatada *et al.*, 2000). However, in spite of significant overlap among different signalling pathways, it is important to note that, while cell necrosis can be induced by many stresses or genetic alteration, not all activate downstream SAR responses. For example, application of inorganic chemicals and the catalase inhibitor 3-aminotriazole can induce necrotic lesions that are cytologically similar to pathogen-induced lesions, but do not trigger SAR (Neuenschwander *et al.*, 1995; Tighe and Heath, 1982). Furthermore, while there are a large number of lesion-mimic mutants or transgenes whose overexpression induces lesions, only some trigger increased SA levels, *PR-1* expression and pathogen resistance, hallmarks of SAR (reviewed in Mittler and Rizhsky, 2000; Shirasu and Schulze-Lefert, 2000). Finally, recent epistasis analysis of constitutive SAR mutants (*cpr1*, *cpr6*, *dnd1* and *dnd2*) and lesion-mimic mutants (*crp5* and *lsd1*) with known defence regulators (*eds1*, *pad4* and *ndr1*) has demonstrated that defence expression in these mutants requires known defence regulators (Clarke *et al.*, 2001; Jirage *et al.*, 2001; Rusterucci *et al.*, 2001). Furthermore, such constitutive SAR and lesion-mimic mutants provide an opportunity to dissect the signalling pathways and cross-talk between multiple defence pathways that might not be ordinarily obvious by studying the response of wild-type plants to pathogens.

In summary, we provide new evidence that the signalling components of SA- and ET/JA-regulated defence pathways may function synergistically to regulate expression of both the SA- and ET/JA-responsive genes and the resistance against virulent strains of bacterial and oomycete pathogens. Furthermore, we provide support for an emerging paradigm that both the presence and relative concentrations of various endogenous signals enable plants to fine tune their transcriptional read-out against a wide variety of stress responses through synergistic or antagonistic regulation (Feys and Parker, 2000; Reymond and Farmer, 1998). These studies further highlight the complexity involved in regulation of the defence response in plants against pathogens. In the future, microarray analysis of the various double mutants of *hrl1* constructed in this study should help us to identify common and unique genes that define distinct signalling pathways for cell death and defence against pathogens in plants.

Experimental procedures

Plant growth conditions

Arabidopsis thaliana were grown in soil (Metro-Mix 360; Scotts Company, Marysville, Ohio, USA) or on plates containing Murashige and Skoog (MS) medium (Life Technologies, Grand Island, New York, USA) supplemented with 1% sucrose and 0.8% agar. Plants were grown in growth chambers at 25/23°C (day/night), 60–70% relative humidity, and a photosynthetic photon flux density (PPFD) of 100–150 μmol m^{-2} sec^{-1} with a 10 h photoperiod unless otherwise specified.

Genetic analysis

For back-crosses with the parental *HRL1* line, *hrl1* was used as the pollen donor. To facilitate mapping, pollen from *hrl1* was used to fertilize the flowers of the Landsberg *erecta* ecotype (L*er*). The resulting F_2 progenies were scored for the *hrl1* phenotype and then used for recombination analysis. Recombinant plants were scored by the method of co-dominant cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993). CAPS primer sequences were obtained from The Arabidopsis Information Resource (TAIR) website (http://www.*Arabidopsis*.org).

Histochemistry and microscopy

The leaves for autofluorescense and callose deposition were prepared as described previously (Dietrich *et al.*, 1994). Trypan blue staining of dead cells was performed as described by Vogel and Somerville (2000). Staining for the presence of H_2O_2 via the DAB uptake method was performed as described by Thordal-Christensen *et al.* (1997). NBT staining for the presence of superoxide was performed as described previously (Doke, 1983).

Chemical treatment and pathogen infection of plants

Mutant and wild-type plants were sprayed with an aqueous solution of SA (1 mM), BTH (1, 10, 100 μM), or MJ (50 μM in 0.01%

ethanol) until run-off. Control plants were sprayed with water or 0.01% ethanol.

Bacterial pathogen infiltration and growth estimation were performed as described previously (Clarke *et al.*, 2000; Greenberg *et al.*, 2000). Infection with *Peronospora parasitica* Ahco2 on 3-week-old soil-grown plants was performed as described by Bowling *et al.* (1997).

Construction of double mutants

The *hrl1 npr1* double mutant was generated using pollen from the *npr1-1* mutant (Cao *et al.*, 1994) to fertilize the flowers of *hrl1*. The homozygous *hrl1 npr1* double mutant was identified in the F_2 population by performing CAPS analysis for the *npr1-1* mutation with plants showing *hrl1*-like phenotype as described by Cao *et al.* (1997).

The *nahG* gene was introduced into *hrl1* plants by a genetic cross, using the pollen of *nahG* to fertilize the *hrl1* flowers. The transgenic *nahG* line in the Col-0 ecotype (line B15) was obtained from Syngenta Biotechnology Inc (Research Triangle, North Carolina, USA). Kanamycin-resistant F_2 seedlings were transferred to soil and scored for *hrl1*-like phenotype. Lines homozygous for *hrl1* and *nahG* loci were identified by screening F_3 populations of individual F_2 lines.

To construct the *hrl1 etr1* double mutant, pollen from *etr1-1* (Chang *et al.*, 1993) was used to fertilize the *hrl1* flowers. To identify the *hrl1 etr1* double mutants, F2 seeds were plated on 0.8% agar plates containing 50 μM 1-amino-cyclopropoane-1-carboxylic acid (ACC; Sigma, St Louis, Missouri, USA), incubated in the dark for 5 days and screened for lack of the ET-mediated triple response and *hrl1*-like phenotype.

To construct the *hrl1 coi1* double mutant, pollen from *hrl1* was used to fertilize the male sterile *coi1-1* flowers (Xie *et al.*, 1998). The homozygous *hrl1 coi1* plants were identified in the F2 progeny by screening for insensitivity to MJ and through CAPS analysis (Xie *et al.*, 1998).

RNA analysis

Tissue samples were collected from plants grown on soil at the indicated time points. Samples were flash-frozen in liquid nitrogen and the total RNA was isolated using TRIzol reagent according to manufacturer's instructions (Gibco BRL, Gaithersburg, Maryland, USA). For RNA gel-blot analysis, 10 μg of the total RNA was fractionated by electrophoresis through denaturing formaldehyde–agarose gels and transferred to Hybond N+ hybridization membrane (Amersham-Pharmacia, Piscataway, NJ, USA) as described by Ausubel *et al.* (1994). Gene-specific probes were synthesized by random-primed ^{32}P-labelling of gel-purified DNA fragments using the RediPrime kit according to the manufacturer's instructions (Amersham-Pharmacia). Gene expression was quantified using PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, California, USA).

Salicylic acid and ethylene measurements

SA was extracted from leaf tissue (500 mg) and quantified by spectrofluorescence HPLC as described previously (Enyedi and Raskin, 1993). To determine the concentration of salicylic acid glucoside (β-glucosylsalicylic acid; SAG), the methanolic leaf extract was dried and resuspended in 1.25 ml of hydrolysis buffer (100 mmol l^{-1} sodium acetate buffer, pH 5.5) containing 20 units of β-glucosidase (EC 3.2.1.21; almond). After 1.5 h incubation at 37°C, extracts were acidified to pH 1.0 with 10% w/v trichloroacetic acid and subjected to SA extraction and quantification.

Leaves for ethylene measurements were collected and placed immediately on MS medium in airtight vials sealed with silicone septum. After 12 h, 1 ml of gas sample was withdrawn with a syringe and analysed by gas chromatography (GC) on a Hewlett Packard 6890 instrument equipped with an alumina column and a flame ionization detector.

Acknowledgements

We thank Dr Jeff Dangl for *lsd1* seeds and *P. parasitica* strains, Dr Xinnian Dong for *npr1-1* seeds, Dr Jean Greenberg for *acd2* seeds, Dr John Turner for *coi1-1* seeds, Dr Fred Ausubel for *GST1* and *PDF1.2* cDNAs, Dr Nina Fedoroff for *AtrbohB* and *AtrbohF* cDNAs, Dr Jill Deikman for 18S rDNA, Dr Barbara Kunkel for *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*) strains, and Arabidopsis Biological Resource Center (ABRC) for *etr1-1* seeds. We gratefully acknowledge Syngenta Biotechnology Inc. for their gift of BTH and *nahG* transgenic seeds. We thank Dr Kathy Brown for assistance with ethylene measurements. We thank Drs Bob Dietrich, Nina Fedoroff and Hong Ma for useful comments on the manuscript. This work was supported by the Department of Biology at Penn State University. S.K.D. was supported in part by the Department of Biology and the Intercollege Graduate Program in Plant Physiology at Penn State University.

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