Preexisting Systemic Acquired Resistance Suppresses Hypersensitive Response-Associated Cell Death in Arabidopsis *hrl1* Mutant[1]

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The hypersensitive response (HR) displayed by resistant plants against invading pathogens is a prominent feature of plant-pathogen interactions. The Arabidopsis *hypersensitive response like lesions1* (*hrl1*) mutant is characterized by heightened defense responses that make it more resistant to virulent pathogens. However, *hrl1* suppresses avirulent pathogen-induced HR cell death. Furthermore, the high *PR-1* expression observed in *hrl1* remains unaltered after avirulent and virulent pathogen infections. The suppressed HR phenotype in *hrl1* is observed even when an elicitor is expressed endogenously from an inducible promoter, suggesting that an impaired transfer of avirulent factors is not the reason. Interestingly, the lack of HR phenotype in *hrl1* is reversed if the constitutive defense responses are compromised either by a mutation in *NON EXPRESSOR OF PR-1* (*NPR1*) or by depleting salicylic acid due to the expression of the *nahG* gene. The rescue of HR cell death in *hrl1 npr1* and in *hrl1 nahG* depends on the extent to which the constitutive systemic acquired response (SAR) is compromised. Pretreating Arabidopsis wild-type plants with SAR-inducers, before pathogen infection resulted in a significant decrease in HR cell death. Together, these results demonstrate that the preexisting SAR may serve as one form of negative feedback loop to regulate HR-associated cell death in *hrl1* mutant and in the wild-type plants.

Successful host resistance against pathogen invasion requires expeditious recognition and activation of the necessary defense repertoire. One such robust response in plants involves resistance (*R*) gene-dependent recognition of pathogen-derived elicitors and initiation of localized cell necrosis at the site of pathogen infection (Goodman and Novacky, 1996). Undoubtedly, the most noticeable feature of this *R* gene-dependent resistance response is the rapid cell death that is well defined within the attempted infection site, a process known as hypersensitive response (HR). The HR cell death is often preceded by changes in ion fluxes, oxidative burst, and cross-linking of cell wall proteins. Most of the HR cell death processes are accompanied by an increase in salicylic acid (SA) biosynthesis, transcriptional activation of various pathogenesis-related (*PR*) genes, and the establishment of a long-lasting systemic response known as systemic acquired resistance (SAR; Hammond-Kosack and Jones, 1996; Ryals et al., 1996).

Several lines of evidence indicate that HR cell death is a form of programmed cell death that resembles apoptotic cell death in other organisms (Mittler and Lam, 1996; Morel and Dangl, 1997). Identification and analysis of several Arabidopsis mutants with spontaneous cell death that mimic pathogen-induced cell death support the idea that HR cell death may be controlled by plant's own genetic mechanisms (Dangl et al., 1996; Greenberg, 1997; Glazebrook, 1999). Genetic screens aimed at identifying the loss of HR to avirulent pathogens have resulted in the cloning of several *R* genes. These *R* genes, when mutated, fail to develop HR against one or more avirulence factors, and they represent genetic components that are required rather early in the signal transduction leading to HR cell death (Bent, 1996; Dangl and Jones, 2001). The relative scarcity in identifying new mutants that lack HR cell death may be due to the presence of functionally redundant genes, embryo lethality, or weaker HR^- phenotypes that were overlooked (Innes, 1998). Although HR cell death is intrinsically controlled by the plant, the relative importance of cell death in conferring resistance to pathogens is not well understood. For example, the Arabidopsis *non-race-specific disease resistance1* (*ndr1*) mutant is susceptible to several strains of *Pseudomonas* spp., although it elicits HR against some of these pathogens (Century et al., 1995, 1997). Conversely, in barley (*Hordeum vulgare*), *MLA*-conferred resistance against an obligate biotroph *Erysiphe graminis* f. sp. *hordei* is abolished when HR cell death is inhibited (Schiffer et al., 1997).

In recent years, some of the Arabidopsis mutants with elevated resistance response were shown to possess partial loss of HR phenotype against avirulent pathogens (Yu et al., 1998, 2000; Rate et al., 1999; Rate

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and Greenberg, 2001). The Arabidopsis *defense no death1* (*dnd1*) mutant was isolated based on its inability to develop HR against avirulent *Pseudomonas syringae* (Yu et al., 1998). The *DND1* gene encodes a cyclic nucleotide-gated ion channel, which may be required for eliciting HR (Clough et al., 2000). In *aberrant growth and death2* (*agd2*) mutant, the loss of HR phenotype was reversed in the *nonexpressor of PR1* (*npr1*) and in the SA-depleted (*nahG*) genetic backgrounds (Rate and Greenberg, 2001). In *accelerated cell death6* (*acd6*) mutant, upon delivery of the AvrRpt2 elicitor inside the plant cell, HR cell death was rescued (Rate et al., 1999). Many of these mutants have high SA levels, increased *PR*-gene expression, and enhanced resistance to virulent pathogens. Apart from these genetic studies, there are instances in a wide range of plant species where HR cell death was suppressed by treatments with a transcriptional inhibitor, an actin polymerization inhibitor, and incubation in low oxygen (Tomiyama et al., 1982; Mittler et al., 1996; Schiffer et al., 1997). Although the loss of HR phenotype has been observed in several Arabidopsis constitutive defense mutants, the mechanisms that lead to the suppressed HR cell death are not clear.

We have recently isolated an Arabidopsis mutant that displays enhanced disease resistance and constitutive expression of several defense-related genes (Devadas et al., 2002). The *hypersensitive response like lesions1* (*hrl1*) mutant was isolated from an ethyl methanesulfonate-mutagenized population of Arabidopsis ecotype Col-0 in a screen for plants that displayed reduced disease lesions in response to infection by virulent bacterial pathogens *P. syringae* pv *tomato* DC3000 (*Pst* DC3000). The *hrl1* mutant spontaneously develops necrotic patches of dead cells on the blades of older leaves and constitutively expresses several biochemical and molecular markers of defense. None of the 35 F_1 plants from a backcross between *hrl1* and its wild-type parent (Col-0) developed lesions. The F_2 population segregated 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 and the defense-related markers always cosegregated with the lesion phenotype. The *hrl1* locus maps within a 6.88-cM interval, 6.25 cM from CAPS markers g8300 and 0.63 cM from the *RPS2* locus on chromosome IV indicating that it is not allelic to *dnd1* or *agd2*, which map to other regions (Clough et al., 2000; Rate and Greenberg, 2001). Detailed characterization of *hrl1* will be described elsewhere (Devadas et al., 2002). In this report, we used *hrl1* as a tool to understand the role of preexisting SAR in regulating HR cell death. Here, we report that the elevated SAR response in *hrl1* plays an active role in suppressing HR cell death. This is further supported by our finding that pre-inducing SAR in wild-type plants also suppresses HR cell death in response to avirulent pathogens.

RESULTS

Delayed and Attenuated HR in *hrl1* Mutant

To characterize the HR of *hrl1* against avirulent bacterial pathogens, we infiltrated the leaves of 6-week-old *hrl1* plants with *P. syringae* pv *tomato* DC3000 expressing *avrRpm1* [*Pst* DC3000 (*avrRpm1*)] at a dosage of 10^8 colony-forming units (cfu)/mL (see "Materials and Methods"). Within 4 to 5 h, Col-0 plants usually show confluent collapse of tissue at the site of pathogen infiltration, a characteristic feature of HR-associated cell death. However, *hrl1* plants did not show any visible HR, neither in lesion$^+$ (older) nor in the lesion$^-$ (younger) leaves even after 8 h; a small percentage developed a very weak HR after 24 h (Fig. 1, A and B). The weak HR in the mutant plants was restricted to a small area surrounding the point of infiltration and was not confluent. The *hrl1* leaves are smaller than the Col-0 leaves because of the smaller stature of *hrl1* plants compared with the same aged Col-0 plants.

To test the possibility that the pathogen-infiltrated *hrl1* leaves might undergo membrane damage without visible HR, we measured the electrolyte leakage in *hrl1* and in the wild-type Col-0 after *Pst* DC3000 (*avrRpm1*) infection. Electrolyte leakage due to membrane damage is a characteristic feature and a quantitative measure of HR-associated cell death (Goodman and Novacky, 1996). The Col-0 plants infiltrated with 10^7 cfu/mL *Pst* DC3000 (*avrRpm1*) showed maximal conductivity within 8 h (see "Materials and Methods"). The *hrl1* mutant did not show any significant increase in ion leakage within 8 h, and the levels remained unchanged even after 24 h (Fig. 2). These results indicate that, unlike the wild-type parent Col-0, the *hrl1* mutant is significantly impaired in its ability to elicit HR cell death against avirulent bacterial pathogens.

Suppressed HR in *hrl1* Is Not Due to an Immediate Decrease in Viable Pathogen Concentration

Elicitation of HR requires live pathogens and the timing and the magnitude of the visible HR (macroscopic HR) in plants is often pathogen-dosage dependent. If the pathogen concentration falls below a certain threshold, then there is no visible HR (Turner and Novacky, 1974). Because the *hrl1* mutant constitutively expresses defense genes and perhaps accumulates antimicrobial compounds, it is possible that immediately upon infiltration, the number of live pathogens is reduced (below the threshold), leading to a suppressed HR. Another possibility is that not enough pathogen is infiltrated in the mutant leaves compared with the wild-type control. Therefore, we tested the growth of an avirulent pathogen, *Pst*

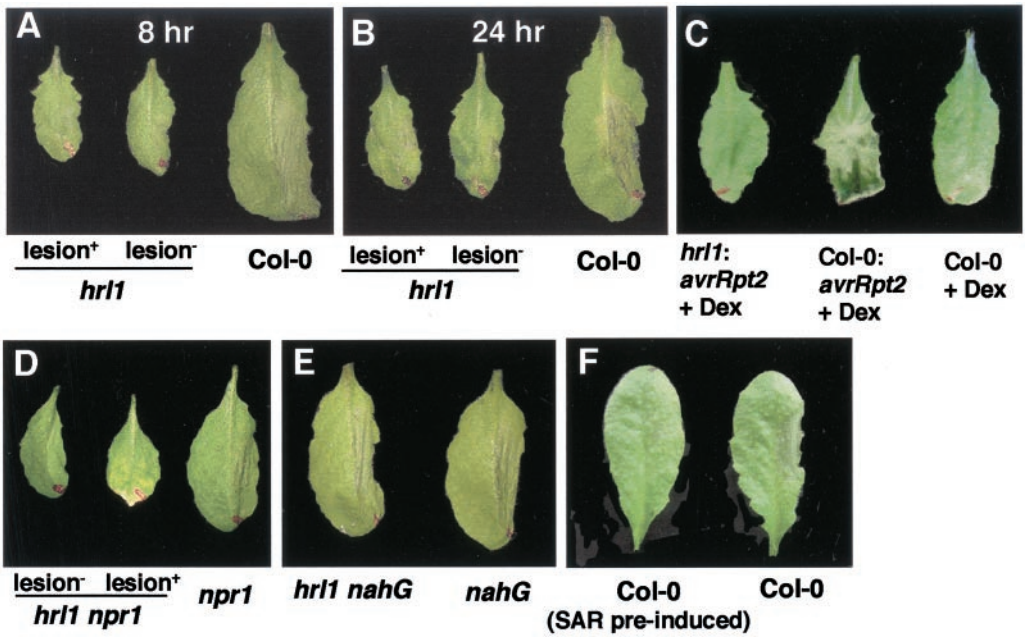

Figure 1. Suppressed HR of *hrl1* in response to an avirulent pathogen. A, Leaves of 6-week-old *hrl1* and Col-0 plants were infiltrated with 10^8 cfu/mL of *Pst* DC3000 (*avrRpm1*) and photographed after 8 h. B, Pathogen-infiltrated *hrl1* and Col-0 leaves as in A photographed 24 h after infiltration. C, Leaves of 6-week-old transgenic Col-0 and *hrl1* plants expressing *avrRpt2* and wild-type Col-0 infiltrated with 30 μM Dex. The picture was taken 24 h after Dex treatment. D, Leaves of 6-week-old *hrl1 npr1* and *npr1* plants were infiltrated with pathogen as described in A and photographed 12 h after infiltration. E, Leaves of 6-week-old *hrl1 nahG* and *nahG* plants were infiltrated with pathogen as described in A and photographed 12 h after infiltration. F, Col-0 plants were treated with 1.5 mM SA or water, and leaves were infiltrated 24 h later with pathogens as described in A and photographed 12 h after pathogen infiltration. All photographs are representative samples from a large experimental pool.

DC3000 (*avrRpm1*), in Col-0 and in *hrl1* plants at a dose of 10^7 cfu/mL (see "Materials and Methods"). We used a high dose of initial inoculum because we wanted to determine the viability of the pathogen at this dose, which failed to elicit an HR in *hrl1* plants. The zero hour time point demonstrated that a similar amount of pathogen was infiltrated in both the mutant and the wild type (Fig. 3). The extent of pathogen growth in *hrl1* and in Col-0 is very similar through 24 h, indicating that the avirulent bacterial population is reduced to similar levels in both the genotypes. A similar growth pattern was observed with a higher pathogen dosage (10^8 cfu/mL; data not shown). These results suggest that the delayed HR in *hrl1* is not because of a preferential decrease in viable bacterial population due to the preexisting defense responses. Furthermore, these results indicate that despite the lack of a visible HR, *hrl1* plants mount a similar level of resistance to an avirulent bacterial pathogen compared with Col-0 plants.

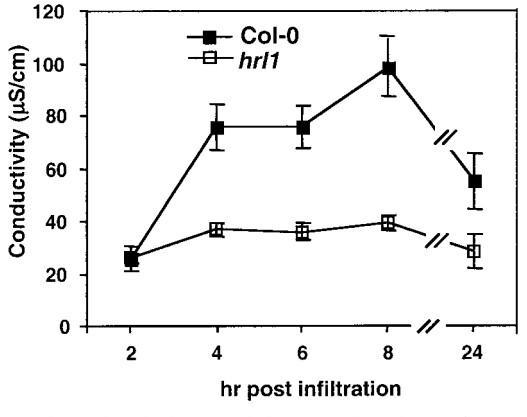

Figure 2. Electrolyte leakage in *hrl1* and Col-0 plants after pathogen infiltration. Plants were infiltrated with avirulent pathogens *Pst* DC3000 (*avrRpm1*) at a dose of 10^7 cfu/mL, and leaf discs were removed for conductivity measurements 24 h after pathogen treatment. The error bars represent ±SD from three independent measurements. μS, MicroSiemens.

Expression of a Bacterial Elicitor in *hrl1* Plants Fails to Elicit an HR

Because *P. syringae* pathogens depend on type III secretion for efficient delivery of avirulence factors into the plant cell (Alfano and Collmer, 1997), the suppressed HR in *hrl1* plants could be due to an impaired transfer of Avr proteins into the plant cell. Therefore, to circumvent the pathogen-based delivery of Avr protein into the plant cells, we constructed a transgenic *hrl1* line expressing *avrRpt2* from a glucocorticoid-inducible promoter by crossing to a wild-type Col-0 line containing this construct (McNellis et al., 1998; see "Materials and Methods").

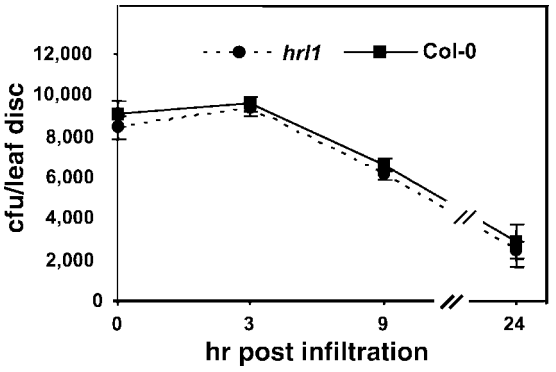

Figure 3. Growth of *Pst* DC3000 (*avrRpm1*) in *hrl1* and Col-0 plants. *hrl1* and Col-0 plants were infiltrated with *Pst* DC3000 (*avrRpm1*) at a dose of 10^7 cfu/mL. The bacterial titer in leaves was determined at the indicated time points. Each time point represents the average from three independent growth curve experiments. A minimum set of eight plants per genotype was included in each experiment. Values are represented as colony-forming units per leaf disc ± SD. Values on the *y* axis are on a linear scale rather than a log scale to increase the resolution of the graph because changes in the bacterial count are not in the orders of magnitude.

Plants harboring this construct express AvrRpt2 protein in response to dexamethasone (Dex) treatment and induce HR-associated cell death through the cognate RPS2-dependent signaling (McNellis et al., 1998). If the lack of HR in *hrl1* is primarily due to a block in the delivery of the Avr proteins, then the direct expression of an Avr protein within the plant cells should trigger normal HR. This strategy also overcomes the problems associated with pathogen viability. Such endogenous expression of bacterial elicitor proteins in plant cells has been shown to trigger HR cell death in a variety of experimental systems (Gopalan et al., 1996; Leister et al., 1996; McNellis et al., 1998). Dex treatment of transgenic Col-0 plants expressing the Dex-inducible *avrRpt2* elicited an HR within 24 h (Fig. 1C). The Dex-infiltrated region of the leaf showed HR within 8 h and the entire leaf collapsed within 24 h. However, the *hrl1* transgenic lines harboring the *avrRpt2* gene did not develop an HR even after 24 h following Dex treatment (Fig. 1C). The expression of the *avrRpt2* gene in the transgenic *hrl1* line after Dex treatment was confirmed by RNA gel-blot analysis using an *avrRpt2* gene-specific probe (data not shown). These results strongly suggest that the suppressed HR in *hrl1* plants is not because of a defect in the transfer of AvrRpt2 protein into the cells of *hrl1*.

Pathogen Infection Does Not Superinduce *PR-1* Expression in *hrl1*

Active host resistance in plants is often accompanied by the induction of several *PR* genes (Ward et al., 1991). Proper recognition of the Avr factors by the cognate resistant gene products in plants leads to rapid induction of these defense-related genes during an incompatible interaction. The *PR* gene induction is slower and weaker during a compatible interaction. The *hrl1* plants constitutively express *PR-1* gene at elevated levels (Fig. 4A). We analyzed whether pathogen infection can further induce *PR-1* expression in *hrl1* plants. *PR-1* expression was monitored over a 3-d period in the *hrl1* and control Col-0 plants that were infiltrated with avirulent [*Pst* DC3000 (*avrRpm1*)] and virulent (*Pst* DC3000) pathogens at a dose of 10^7 cfu/mL. Col-0 plants showed a 11-fold increase in *PR-1* expression within 24 h and a 35-fold increase 3 d after infection with an avirulent pathogen However, compared with the uninfiltrated plants, pathogen-infiltrated *hrl1* plants did not show any significant enhancement of *PR-1* expression, with a maximal increase being only 1.3-fold 3 d post infection (Fig. 4A). Similarly, virulent pathogen was also unable to significantly super-induce *PR-1* expression in *hrl1*. However, treatment of *hrl1* plants with 100 μM benzothiadazole (BTH), a biologically active analog of SA, leads to more than 2-fold in-

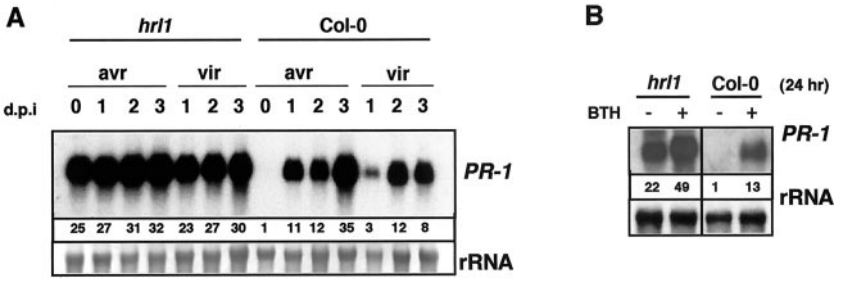

Figure 4. Analysis of *PR-1* expression in *hrl1* and Col-0 after pathogen infection and BTH treatment. A, Total RNA isolated from pathogen-infected *hrl1* and Col-0 plants. B, Total RNA isolated from BTH-treated *hrl1* and Col-0 plants. Blots contain 10 μg of total RNA of each sample. Pathogens were infiltrated at a dose of 10^7 cfu/mL, and tissue samples were collected at indicated time points. BTH (100 μM) was sprayed on the plants as an aqueous solution, and samples were collected 24 h later. Blots were serially hybridized with the indicated probes. The 18S ribosomal subunit gene-specific probe (rRNA) was used as a loading control. The numbers below the RNA gel blots indicate -fold induction of *PR-1* expression relative to untreated wild-type Col-0 after normalizing for loading variations. The quantifications were done using a phosphor imager. avr, *Pst*DC3000 (*avrRpm1*); vir, *Pst* DC3000; d.p.i., days post infection. This experiment was repeated twice with similar results.

crease in *PR-1* expression within 24 h after treatment (Fig. 4B). This suggests that the ability of *hrl1* to induce *PR* gene expression is not completely saturated, at least to an SAR-inducing chemical; but unlike wild-type Col-0, *hrl1* does not respond to pathogen stimulus. These results lead us to infer that the preexisting defense responses in *hrl1* restrain further *PR-1* induction and possibly other defense-related responses including HR cell death in response to avirulent pathogens.

The Reversal of HR$^-$ Phenotype in *hrl1* Depends on the Extent of SAR Induction

Because *hrl1* plants exhibited several defense responses associated with pathogen infection, we sought to determine whether the preexisting SAR has any role in desensitizing the HR induction in *hrl1* plants. To reduce SAR expression in the *hrl1* mutant, we crossed *hrl1* to an *npr1* mutant and to a *nahG* transgenic line. The *npr1* mutant was isolated based on its inability to transduce specific SA-mediated responses, whereas the *nahG* gene encodes a salicylate hydroxylase that converts SA into an inactive catechol (Gaffney et al., 1993; Cao et al., 1994, 1997). The *hrl1 npr1* double mutant and the transgenic *hrl1 nahG* plants displayed reduced *PR-1* gene expression (Fig. 5). The *PR-1* expression in the lesion$^+$ leaves of *hrl1 npr1* plants was reduced compared with the lesion$^+$ leaves of *hrl1* and was undetectable in the lesion$^-$ leaves of *hrl1 npr1* plants. However, *PR-1* expression in both the lesion$^+$ and lesion$^-$ leaves of *hrl1 nahG* was reduced to background levels. In addition, expression of other SAR-responsive genes that were induced in *hrl1* were also reduced to undetectable levels in *hrl1 nahG* plants (data not shown).

We monitored the HR of lesion$^+$ and lesion$^-$ leaves of *hrl1* plants in response to an avirulent bacterial pathogen [*Pst* DC3000 (*avrRpm1*), 10^8 cfu/mL] over a 24-h period. Within 6 h, more than 95% of the infiltrated wild-type Col-0 plants showed HR, whereas less than 10% of the lesion$^-$ leaves of *hrl1* showed an HR (Fig. 6A). This reduced HR phenotype was slightly more pronounced in the lesion$^+$ leaves of *hrl1*. Even after 24 h, less than 20% of the infiltrated leaves of *hrl1* developed a visible HR. The *hrl1* plants exhibited a delayed HR even when the pathogen concentration was increased by 10-fold to 10^9 cfu/mL or in response to another avirulent strain [*Pst* DC3000 (*avrRpt2*); data not shown].

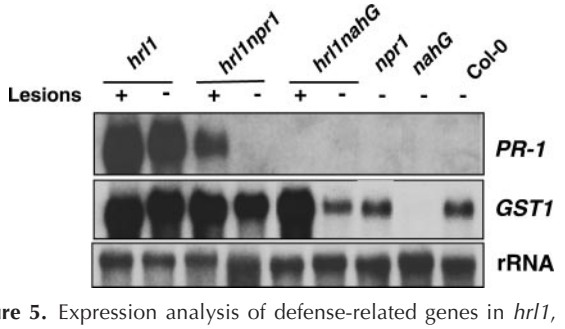

Figure 5. Expression analysis of defense-related genes in *hrl1*, *hrl1 npr1*, and *hrl1 nahG* plants. An RNA gel blot containing 10 μg of total RNA, isolated from the indicated genotypes, was hybridized with *PR-1* and *GST1* gene-specific probes. The 18S ribosomal subunit gene-specific probe (rRNA) was used as a loading control. Lesion$^+$ and lesion$^-$ indicate leaves with and without lesions, respectively, from the same set of 6-week-old plants that had leaves with lesions. Wild-type Col-0, *npr1*, or *nahG* plants had no lesions. This analysis was repeated three times with similar results.

We analyzed HR induction in lesion$^+$ and lesion$^-$ leaves of *hrl1 npr1* and *hrl1 nahG* plants. Similar to *hrl1*, lesion$^+$ leaves of *hrl1 npr1* elicited delayed and attenuated HR in response to *Pst* DC3000 (*avrRpm1*) (Figs. 1D and 6B). However, the lesion$^-$ leaves of *hrl1 npr1* exhibited HR similar to wild-type Col-0, in terms of both timing and magnitude (Figs. 1D and 6B). In addition, the suppressed HR phenotype was completely reversed in the lesion$^+$ and in the lesion$^-$ leaves of *hrl1 nahG* (Figs. 1E and 6C). The positive HR in the lesion$^-$ leaves of *hrl1 npr1* and in the lesion$^+$ and lesion$^-$ leaves of *hrl1 nahG* plants correlated well with the loss of *PR-1* expression in these tissues (Fig. 5). These results suggest that the preexisting defense responses (SAR) negatively regulate HR-associated cell death in *hrl1* plants.

Pre-Inducing SAR in Wild-Type Col-0 Suppresses HR Cell Death against an Avirulent Pathogen

The results described above demonstrate that constitutive SAR expression suppresses HR-associated cell death in *hrl1* plants. We hypothesized if the constitutive SAR in *hrl1* plants down-regulates HR cell death, then pre-inducing SAR in wild-type Col-0 plants should suppress HR cell death as well. To test this hypothesis, SAR was induced in Col-0 plants either by application of 1.5 mM SA or by infiltration with a low dose (10^5 cfu/mL) of an avirulent pathogen, *Psm* ES4326 (*avrRpm1*). At this low dose, *Psm* ES4326 (*avrRpm1*) does not elicit macroscopic HR, but induces several SAR-associated genes (A.M. Gómez-Buitrago and R. Raina, unpublished data). The *npr1* and *nahG* plants, incapable of activating SAR in response to SA, were included as controls. Twenty-four hours after SA or pathogen treatment, these plants were infiltrated with an avirulent pathogen, *Psm* ES4326 (*avrRpm1*), at a dose of 10^7 cfu/mL to assess the effects of the preexisting SAR on HR elicitation. SA- or pathogen-treated Col-0 plants experienced significantly reduced HR-associated cell death compared with the water-treated Col-0 plants, as judged by the reduced electrolyte leakage (Fig. 7). However, SA-treated *npr1* and *nahG* plants, in which SAR signaling is blocked, did not show any reduction in the levels of electrolyte leakage. Similar results were obtained when 300 μM BTH (biologically active analog of SA) was used for inducing SAR in Col-0 plants (data not shown). These results clearly demonstrate

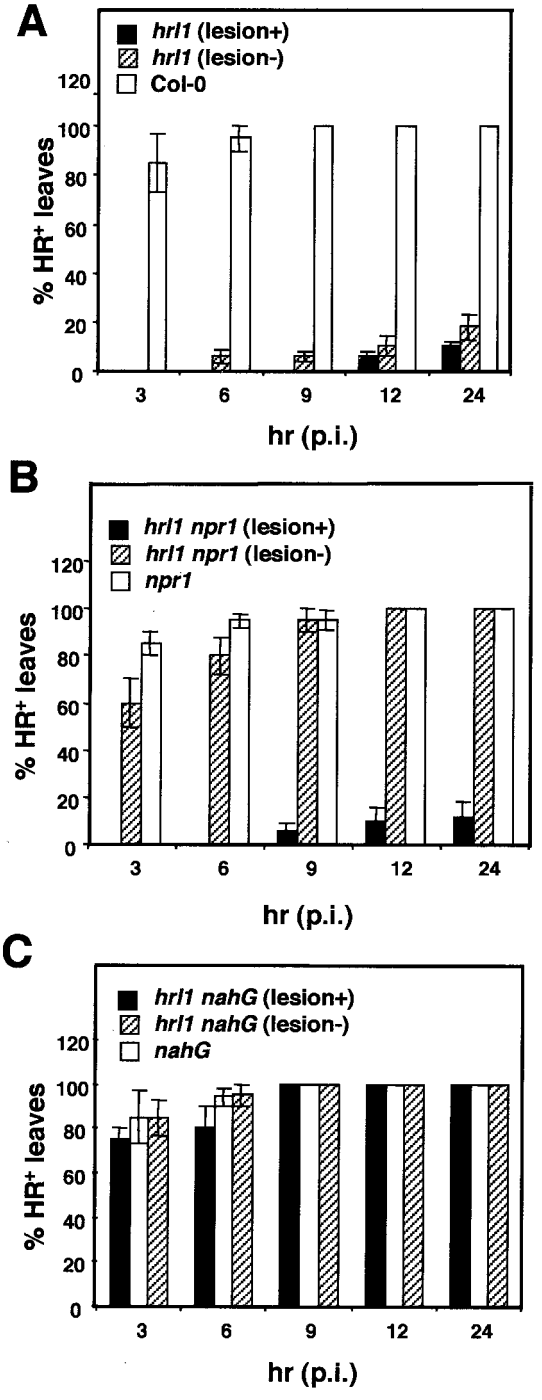

Figure 6. Quantitative representation of the HR in *hrl1*, *hrl1 npr1*, and *hrl1 nahG* plants after avirulent pathogen infection. One-half of the leaves of the 6-week-old plants were infiltrated with *Pst* DC3000 (*avrRpm1*) at a dose of 10^8 cfu/mL. Infiltrated leaves were scored for the characteristic HR cell death at the indicated time points. The leaves were scored as HR^+ if more than one-half of the infiltrated area developed confluent HR cell death. About 30 leaves from six plants per genotype were infiltrated. The graphs represent the percentage of infiltrated leaves that developed HR. A, Leaves of *hrl1* and Col-0 plants. B, Leaves of *hrl1 npr1* and *npr1* plants. C, Leaves of *hrl1 nahG* and *nahG* plants. The mean values ± SD from three independent experiments are plotted.

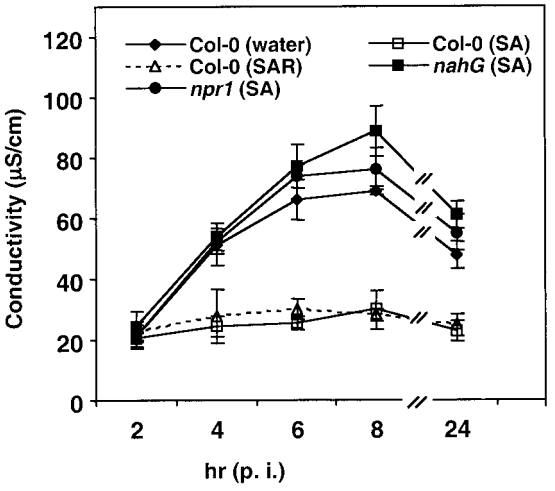

Figure 7. Electrolyte leakage in Col-0, *npr1*, and *nahG* plants pretreated with SAR-inducing agents followed by avirulent pathogen infection. All plants were 6-week-old and HR was induced by infiltrating 10^7 cfu/mL of *Psm* ES4326 (*avrRpm1*) 24 h after indicated treatments. Col-0 (water), Water-treated Col-0 plants; Col-0 (SAR), Col-0 plants infiltrated with 10^5 cfu/mL of *Psm* ES4326 (*avrRpm1*); *npr1* (SA), *npr1* plants treated with 1.5 mM SA; Col-0 (SA), Col-0 plants treated with 1.5 mM SA; *nahG* (SA), *nahG* plants treated with 1.5 mM SA. Values represent the average of three independent experiments ± SD.

that pre-inducing SAR in wild-type plants suppresses HR-associated cell death.

DISCUSSION

In an attempt to understand the regulation of HR against pathogens in plants, we employed an Arabidopsis constitutive SAR mutant *hrl1* and showed that the preexisting defense responses antagonize HR cell death. The HR was compromised even when the AvrRpt2 elicitor was expressed within the *hrl1* plants, suggesting that the repressed HR cell death was not because of the defective transfer of Avr proteins into the plant cells. Our results suggest that constitutive SAR expression suppresses the HR-associated cell death in *hrl1* plants. In support of this finding, we showed that the HR^- phenotype could be reversed in the double mutants of *hrl1* in which the elevated defense-responses were compromised. Finally, we demonstrated that pretreating wild-type Col-0 plants with SAR-inducing agents suppressed HR-associated cell death.

The induction of HR cell death in resistant plants upon pathogen attack is probably the most well-recognized active resistance response. Although the exact role of cell death during HR is unclear, the controlled initiation and execution of HR cell death are thought to limit the spread of pathogens and other unwanted toxic products into healthy cells (Morel and Dangl, 1997). Hence, it is necessary for the host cellular machinery to precisely control the untoward spread of HR cell death. The fact that the

resistance gene product RPM1 undergoes rapid degradation soon after HR initiation strongly suggests the existence of a negative feedback loop modulating the extent of cell death at the site of infection (Boyes et al., 1998). The best line of evidence for the genetic control of HR-like cell death stems from the analyses of several lesion-mimic mutants that may be perturbed in regulating certain aspects of pathogen-induced cell death. The Arabidopsis *dnd1* mutant, originally identified in a screen for reduced HR against *P. syringae* pv *glycinea* (*avrRpt2*), was later found to be a rare/conditional lesion mimic mutant (Clough et al., 2000). However, why such a mutation that suppresses HR leads to systemic resistance is not clear. Alternatively, the systemic resistance itself may be responsible for the reduced HR in the *dnd1* mutant.

The constitutive defense responses in *hrl1* may lead to desensitization of the HR activating machinery after pathogen attack. The suppression of HR in *hrl1* may not be a direct effect of the *hrl1* mutation but may be the consequence of a sustained SAR response exhibited by *hrl1* plants. This SAR-induced suppression of HR is supported by our results with the *hrl1 npr1* double mutant and transgenic *hrl1 nahG* plants. In the presence of *npr1* allele, *PR-1* expression is partially reduced in the lesion$^+$ leaves but is completely absent in the lesion$^-$ leaves of *hrl1 npr1* plants. Accordingly, in *hrl1 npr1* plants, the lesion$^+$ leaves showed suppressed HR but the lesion$^-$ leaves developed normal HR in response to avirulent pathogens. In addition, both the lesion$^+$ and the lesion$^-$ leaves of *hrl1 nahG* plants exhibited normal HR to *Pst* DC3000 (*avrRpm1*). The full HR recovery in *hrl1 nahG* plants correlates well with the loss of *PR-1* expression in the lesion$^+$ and lesion$^-$ leaves. Expression of several other *PR* genes (*PR-2* and *PR-5*) was also significantly reduced in *hrl1 nahG* (data not shown), suggesting that SAR induction was severely compromised in *hrl1 nahG*. The role of systemic resistance in suppressing HR cell death is further demonstrated by the reduced electrolyte leakage in Col-0 plants that were pretreated with inducers of SAR. However, pretreating *npr1* and *nahG* plants with SA did not alter the electrolyte leakage levels, demonstrating that SAR signaling is critical for suppressing HR cell death.

It is not clear whether elevated defense responses present in *agd2*, *acd6*, or the *dnd* class of mutants play any role in altering HR-associated cell death (Rate et al., 1999; Clough et al., 2000; Yu et al., 2000; Rate and Greenberg, 2001). Arabidopsis *agd2* mutant recently was shown to suppress avirulent *P. syringae*-mediated HR cell death. The suppressed HR cell death was reversed in the presence of *npr1*- and *nahG*-expressing plants. Whereas these phenotypes of *agd2* are similar to *hrl1*, *AGD2* and *HRL1* define different genetic loci. The *AGD2* locus maps to an interval of 0.23 cM flanked by markers L23H3 and nga1139 (Rate and Greenberg, 2001), whereas *HRL1* maps 0.63 cM from *RPS2* and 12.14 cM from nga1139 marker (S.K. Devadas and R. Raina, unpublished data). Furthermore, unlike in *hrl1*, presence of the *nahG* gene in *agd2* does not suppress cell death and the plant size is not rescued. Our results with *hrl1 npr1* and *hrl1 nahG* suggest that the suppressed constitutive SAR in these plants make them more responsive to eliciting HR during pathogen attack. Such elicitation competency might have been suppressed in *hrl1* plants because of the sustained high level of SAR expression. The lack of enhancement of *PR-1* expression in *hrl1* plants after pathogen inoculation can also be explained by the reduced elicitation competency in response to pathogen infection. However, it should be noted that the magnitude and the nature of SAR induction in *hrl1* might not reflect the physiology of other constitutive defense mutants, and, hence, other mutants might respond differently to HR-inducing pathogens. For example, a prelesion *lsd1* mutant is hyper-responsive and exhibits faster HR-like symptoms to both virulent and avirulent pathogens at a very low dosage (10^5cfu/mL) (Dietrich et al., 1994). However, *lsd1* mutants do not express SAR before pathogen inoculation when grown in the permissive environment (Dietrich et al., 1994). On the other hand, the *acd6* mutant is impaired in its ability to perceive the elicitor and, therefore, does not develop an HR against avirulent pathogens (Rate et al., 1999). However, *acd6* plant tissue exhibited normal HR cell death when the elicitor was delivered inside the plant cell through biolistic transformation. Interestingly, *acd6 nahG* plants developed HR in response to *Pst* DC3000 (*avrRpm1*) (Rate et al., 1999). Tobacco (*Nicotiana tabacum*) plants pretreated with resistance-inducing heat killed *Ralstonia solanacearum* cells develop reduced HR, presumably because of the activation of some of the defense responses (Lozano and Sequeira, 1970).

Pretreatment of tobacco plants with high oxygen pressure before pathogen infection resulted in a delayed HR (Mittler et al., 1999). It was suggested that the anti-oxidant responses that were activated during the oxidative stress might scavenge the reactive oxygen intermediates (ROI) generated during pathogen infection leading to the suppression of HR. However, the expression of SAR genes was not analyzed in those plants. In *hrl1* plants, we found high *GST1* expression, and its expression was not suppressed in the *hrl1 npr1* plants (Fig. 5). Nevertheless, the lesion$^-$ leaves of *hrl1 npr1* developed normal HR, suggesting that the induction of anti-oxidant responses alone is not sufficient to suppress HR. Although the anti-oxidant responses induced in *hrl1* as a consequence of cell death might play a role in suppressing the HR cell death, the constitutive SAR induction appears to have a greater effect on the compromised HR.

What are the possible mechanisms that suppress HR in *hrl1* plants? There are numerous signaling

steps in the HR cascade, which, when affected, can influence HR. For example, down-regulation of K^+ and Cl^- efflux channel activities in *hrl1* plants could lead to a severe reduction in HR. Ca^{2+} channel blockers have been shown to inhibit HR in tobacco and soybean (*Glycine max*) cells (Atkinson et al., 1990, 1996). Continuous generation of ROI and other antimicrobial compounds such as phytoalexins may render *hrl1* mutant refractory to changes in membrane permeability that is crucial for HR cell death (Hahlbrock et al., 1995). Rapid turnover of various *R* gene products might prevent HR induction (Boyes et al., 1998). Although we cannot accurately predict which step is perturbed in *hrl1* that leads to suppression of HR, our results indicate that an induced SAR response down-regulates further HR cell death (Fig. 8).

In addition to the defense-related processes discussed above, there are instances where interfering with normal cellular homeostasis leads to the suppression of HR. For example, treatments of potato (*Solanum tuberosum*) cells with inhibitors of actin polymerization such as cytochalasin B and colchicine blocked HR cell death triggered by *Phytophthora infestans* (Tomiyama et al., 1982). The inhibition of cytoplasmic aggregation in elicitor-treated potato cells delayed some of the resistance reactions that are involved in HR cell death (Furuse et al., 1999). However, at present, it is not known whether perturbation of any of these normal cellular functions in *hrl1* affects HR induction.

Desensitization provides a way for cells to adapt to permanent changes in levels of certain signaling compounds. Receptor down-regulation as a tool to achieve desensitization and tolerance is a common cellular adaptation in many hormonal and neuronal responses in animal systems (Pawson, 1995). In plants, suspension-cultured tomato cells undergo desensitization to alkalanization of the growth medium in response to repeated stimuli with chitin elicitors (Felix et al., 1998). In addition, elicitor-induced oxidative burst in cultured soybean cells render the cells insensitive to further induction of ROI generation by the same or a different stimulus (Chandra et al., 2000). These observations demonstrate that desensitization can serve as a general mechanism to tightly regulate cellular processes that have significant overlap. In *hrl1*, the constitutive SAR response may alter the cellular physiology at various nodes that lead to insensitivity to subsequent pathogen attack. Down-regulation of HR cell death in the presence of an already existing systemic resistance response may be a way for plants to prevent excessive cell death and further defense induction (Fig. 8). Identification of host factors and the mechanisms that lead to desensitization without compromising the resistance response will be valuable in developing plants with enhanced defense in the absence of unwanted cell death.

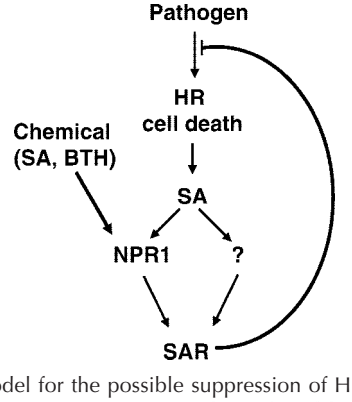

Figure 8. A model for the possible suppression of HR cell death by preexisting SAR responses. SAR can be induced by application of chemicals (such as SA or BTH), or infection by an avirulent pathogen (HR cell death). Once SAR is established through any one of these methods, it suppresses further HR cell death induced by avirulent pathogens. Avirulent pathogen-mediated SAR can be induced by NPR1-dependent or -independent pathways (Rate et al., 1999; Shah et al., 1999). This model also explains the suppression of HR-associated cell death in lesion$^+$ leaves of *hrl1 npr1* plants. At this time, it is not clear whether overall resistance responses or a particular component of SAR is required to suppress HR cell death, therefore the arrow emanating from the SAR is shown to suppress HR-associated cell death in response to avirulent pathogens.

MATERIALS AND METHODS

Plant Growth Conditions

Plants (Arabidopsis) were grown in soil (Metro-Mix 360, Scotts-Sierra, Maysville, OH) or on plates containing Murashige and Skoog media (Invitrogen, Carlsbad, CA) supplemented with 1% (w/v) Suc and 0.8% (w/v) agar. The plant growth chambers were set at 25°C/23°C (day/night), 60% to 70% relative humidity, and a photosynthetic photon flux density of 100 to 150 μmol m^{-2} s^{-1} with a 10-h photoperiod, unless otherwise specified.

Bacterial Inoculations

Bacterial pathogens *Pst* DC3000 with an empty vector and *Pst* DC3000 carrying a plasmid borne *avrRpm1* gene [*Pst* DC3000 (*avrRpm1*)] or *avrRpt2* gene [*Pst* DC3000 (*avrRpt2*); Debener et al., 1991; Whalen et al., 1991] were grown at 28°C on King's B agar plates or in liquid medium (King et al., 1954) supplemented with 100 μg mL^{-1} rifampicin and 25 μg mL^{-1} kanamycin. Bacterial culture was prepared by resuspending the overnight grown cells in 10 mM $MgCl_2$ to the required optical density (OD_{600}; 1 OD_{600} = 10^9 cfu/mL). A titer of 10^8 cfu/mL was used to score HR phenotype. For conductivity measurements, a titer of 10^7 cfu/mL was used, because higher bacterial titer lead to rapid collapse of infiltrated tissue, making it difficult to take leaf punches. The bacterial suspension was pressure-infiltrated on the abaxial side of the leaves using a 1-mL syringe. For growth curves, eight leaf discs (0.5 cm in diameter) from eight different plants were collected for each time point at indicated times and ground in 10 mM $MgCl_2$ using a pestle. Serial dilutions were plated on King's B agar plates supplemented with appropriate antibiotics.

Plates were incubated at 28°C for 2 d to determine the number of colony-forming units.

Chemical Treatments

Plants were treated with aqueous solution of 1.5 mM SA or 300 μM BTH by spraying until runoff. These concentrations are significantly below the phytotoxic levels but induce SAR (Dietrich et al., 1994; Lawton et al., 1996). Treated plants were covered with a dome for 4 h to prevent rapid localized increase in concentration of the applied chemicals.

Dex (Sigma, St. Louis) stock solution (30 mM) was made in 100% (v/v) ethanol and was stored at −20°C in a dark vial. For treatments, the stock solution was diluted to a final concentration of 30 μM in water and was pressure-infiltrated on the abaxial side of the leaves using a 1-mL syringe. Control infiltrations were performed with 0.1% (v/v) ethanol solution. Plant responses were recorded at indicated times.

Electrolyte Leakage Measurements

Electrolyte leakage measurements were performed as described previously (Mittler et al., 1996; McNellis et al., 1998; Rate and Greenberg, 2001). In brief, four leaf punches (0.5 cm diameter) were taken at indicated time points and were shaken for 10 min at 28°C in 2 mL of distilled water with the abaxial side toward the solution. The solution was transferred to a portable VWR brand conductivity meter (VWR Scientific Products, Pittsburgh) for conductivity measurements.

Construction of Double Mutants

The *hrl1 npr1* double mutant was generated using pollen from *npr1-1* mutant (Cao et al., 1994, 1997) to fertilize flowers of *hrl1*. Success of the cross was judged by loss of *hrl1* phenotype in F_1 plants. Homozygous *hrl1 npr1* double mutant was identified in the F_2 population by performing cleaved amplified polymorphic sequence analysis for *npr1-1* mutation on plants showing *hrl1*-like phenotype as described by Cao et al. (1997).

To construct *hrl1 nahG* line, the *nahG* gene was introduced into *hrl1* plants by a genetic cross, using pollen from *nahG* to fertilize *hrl1* flowers. Transgenic *nahG* line in the Col-0 ecotype (line B15) was obtained from Syngenta Biotechnology (Research Triangle Park, NC). Success of the cross was judged by the loss of *hrl1* phenotype in F_1 plants. F_2 seeds were plated on Murashige and Skoog media supplemented with 50 μg/mL kanamycin (marker linked to the *nahG* gene). Kanamycin-resistant seedlings were transferred to soil and scored for *hrl1*-like phenotype. Homozygous *hrl1* lines expressing *nahG* gene were identified as kanamycin-resistant plants showing *hrl1*-like phenotype. Lines homozygous for *hrl1* and *nahG* loci were identified by screening F_3 populations derived from individual F_2 lines. The F_2 lines that showed 100% resistance to kanamycin in the F_3 population and displayed *hrl1*-like phenotype were considered homozygous for *hrl1* and *nahG* loci.

To construct a transgenic *hrl1* line expressing inducible *avrRpt2*, pollen from a transgenic Col-0 line containing a glucocorticoid-inducible *avrRpt2* cassette was used to fertilize *hrl1* flowers (McNellis et al., 1998). The resulting F_1 seedlings were selected on Murashige and Skoog media containing 20 mg L^{-1} hygromycin B (Sigma, St. Louis) and were allowed to set seeds. The F_2 seedlings were again selected on 20 mg L^{-1} hygromycin B and the resistant ones were scored for *hrl1* phenotype. Genomic DNA was isolated from the hygromycin-resistant *hrl1* plants using a DNeasy isolation kit following the manufacturer's protocol (Qiagen USA, Valencia, CA). A 400-bp *avrRpt2* fragment was PCR-amplified using primers 5′-GCTCCAGTTGCCATAAATCACA-3′ (sense) and 5′-CAGGCATACCAACATCCCATT-3′ (antisense) to confirm the presence of the transgene.

RNA Isolation and RNA Gel-Blot Analysis

Tissue samples were collected from plants grown on soil at indicated time points. Samples were flash frozen in liquid nitrogen, and total RNA was isolated using TRIzol reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA concentration was determined by UV A_{260}. For RNA gel-blot analysis, 10 μg of total RNA was fractionated by electrophoresis through denaturing formaldehyde-agarose gels and transferred to the Hybond N+ hybridization membrane (Amersham-Pharmacia, Piscataway, NJ) according to Ausubel et al. (1994). Gene-specific probes were synthesized by random primed ^{32}P-labeling of gel-purified DNA fragments using Redi-Prime kit according to the manufacturer's instructions (Amersham-Pharmacia, Piscataway, NJ). A cDNA clone for *GST1* gene was obtained from Dr. Fredrick Ausubel (Harvard Medical School, MA), and 18s rRNA from Dr. Jill Deikman (Monsanto, MO). *PR-1* gene-specific probe was obtained by PCR from Col-0 genomic DNA. Primers used for *PR-1* were 5′-CCACAAGATTATCTAAGGGTTC-3′ (sense) and 5′-GGCTTCTCGTTCACATAATTCC-3′ (antisense). Hybridizations and washes were performed following the methods described by Ausubel et al. (1994). Gene expression was quantified using PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

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