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The *Arabidopsis* gain-of-function mutant *dll1* spontaneously develops lesions mimicking cell death associated with disease

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Summary

We describe the characterization of a novel gain-of-function *Arabidopsis* mutant, *dll1* (*disease-like lesions1*), which spontaneously develops lesions mimicking bacterial speck disease and constitutively expresses biochemical and molecular markers associated with pathogen infection. Despite the constitutive expression of defense-related responses, *dll1* is unable to suppress the growth of virulent pathogens. However, *dll1* elicits normal hypersensitive response in response to avirulent pathogens, thus indicating that *dll1* is not defective in the induction of normal resistance responses. The lesion$^+$ leaves of *dll1* support the growth of *hrcC* mutant of *Pseudomonas syringae*, which is defective in the transfer of virulence factors into the plant cells, and therefore non-pathogenic to wild-type Col-0 plants. This suggests that *dll1* intrinsically expresses many of the cellular processes that are required for pathogen growth during disease. Epistasis analyses reveal that salicylic acid and NPR1 are required for lesion formation, while ethylene modulates lesion development in *dll1*, suggesting that significant overlap exist between the signalling pathways leading to resistance- and disease-associated cell death. Our results suggest that host cell death during compatible interactions, at least in part, is genetically controlled by the plant and DLL1 may positively regulate this process.

Keywords: compatible interactions, *PR* genes, disease, cell death, *dll1*, *Arabidopsis*.

Introduction

Since plants are sessile organisms, they have evolved elaborate defense mechanisms to resist pathogen infection. Most interactions between plant and pathogens can be classified as either compatible or incompatible. During an incompatible interaction, the plant recognises the pathogen and rapidly activates an extensive array of defense responses at the site of infection that limit pathogen ingress into neighbouring cells. In contrast, a compatible interaction is often characterised by a much delayed and attenuated defense response that fails to retard pathogen colonisation.

Both incompatible and compatible interactions are usually followed by the onset of systemic acquired resistance (SAR), a distinct plant defense response that results in a non-specific and long-lasting systemic resistance to a variety of pathogens (Dong, 2001; Ryals *et al.*, 1996). SAR establishment is preceded by the activation of pathogenesis-related (*PR*) genes and salicylic acid (SA) accumulation (Enyedi *et al.*, 1992; Uknes *et al.*, 1992). SA induces the expression of *PR* genes and disease resistance in several plant species, while SA depletion leads to enhanced susceptibility to multiple pathogens (Delaney *et al.*, 1994; Yang *et al.*, 1997). NPR1 (also known as NIM1 and SAI1) functions downstream of SA and is a critical component of the SA signalling pathway (Cao *et al.*, 1994; Ryals *et al.*, 1997; Shah *et al.*, 1997). The *npr1* mutant cannot activate SAR in response to SA application and displays enhanced susceptibility to virulent pathogens.

A nearly ubiquitous result of plant–pathogen interactions is host cell death. During incompatible inter-

actions, host cell death is generally manifested in the form of hypersensitive response (HR) and requires host participation (Greenberg, 1997; Richberg *et al.*, 1998). Mutants from several plant species, particularly maize and *Arabidopsis,* have been identified that spontaneously develop necrotic cell death resembling HR lesions. Because cell death in these mutants is accompanied by constitutive expression of several defense-related responses and enhanced resistance to virulent pathogens, these lesions have been suggested to mimic the plant's hypersensitive response to avirulent pathogens (Dangl *et al.*, 1996; Johal *et al.*, 1995).

How cell death is induced during susceptible interactions is not well understood. It is not known if cell death during disease involves active host participation or whether the cell death is a consequence of pathogen-derived toxic products that function to kill plant cells. It has been suggested that cell death during compatible interactions resulting in disease may be genetically programmed in plants (Dangl and Jones, 2001; Dangl *et al.*, 1996; Greenberg, 1997; Greenberg *et al.*, 2000). Recently, several studies indicate that the host may genetically condition pathogen susceptibility. For example, *Arabidopsis PMR* genes have been suggested to potentially encode host susceptibility factors. The *pmr* mutants are resistant to the normally virulent pathogen *Erysiphe cichoracearum*. These mutants neither develop lesions nor constitutively express elevated levels of *PR1* or *PDF1.2* (Vogel and Somerville, 2000). The *Arabidopsis dth9* mutant constitutively activates the promoter of *CEVI-1,* a gene from tomato that is induced only during compatible plant–virus interaction, suggesting that *DTH9* may function as a regulator of host disease susceptibility (Mayda *et al.*, 2000a, 2000b). Likewise, the *Arabidopsis COI1* gene is required for disease symptom development following virulent *Pseudomonas syringae* infection, suggesting that a host-encoded component plays an active role during compatible interactions (Kloek *et al.*, 2001). The plant hormone ethylene has been implicated in the development of disease symptoms (Bent *et al.*, 1992; Lund *et al.*, 1998). Thus, the possible involvement of hormones and other host factors in modulating the cell death response to pathogens argue for a plant genetic programme(s) that controls cell death during disease.

In this paper, we describe the characterisation of a novel gain-of-function *Arabidopsis* mutant designated *dll1* (*disease-like lesions1*) that spontaneously develops lesions mimicking many features of a compatible plant–pathogen interaction. We provide evidence that suggests that cell death during disease, at least in part, is host regulated and a significant overlap may exist between the signalling components involved in resistance and susceptible responses to pathogen infection.

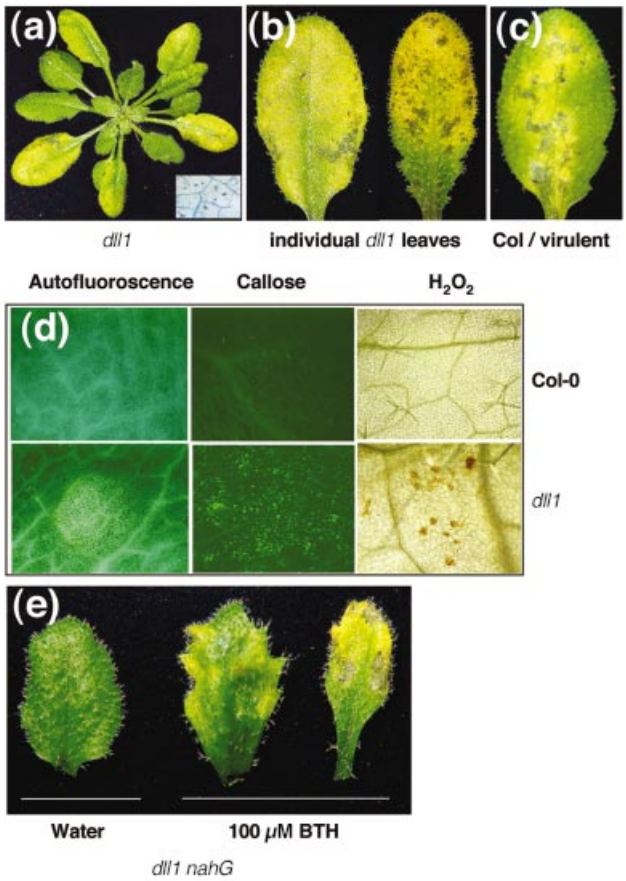

Figure 1. Phenotypes of *dll1* and *dll1 nahG* plants.
(a) Six-week-old *dll1* plant. Inset in a displays trypan blue-stained dead cells of *dll1*.
(b) Close-up of the leaves of plant in panel a.
(c) Leaf of a 6-week-old Col-0 inoculated with *Pst* DC3000 and photographed after 3 days. Compare the lesions on the leaves in the panel b with the lesions on the leaf in panel c.
(d) Accumulation of defense-related biochemical markers in uninfected Col-0 and *dll1* leaves. Biochemical markers were visualized as described in Materials and methods.
(e) Lesion development in *dll1 nahG* plants in response to BTH. Six-week-old *dll1 nahG* plants were sprayed either with water or with 100 μM BTH and photographed after 3 days.

Results

Identification of a gain-of-function mutant that mimics bacterial speck disease

The *Arabidopsis thaliana d*isease-*l*ike *l*esions *1* (*dll1*) mutant was identified as a spontaneous mutant in the wild-type Colombia (Col-0) background that developed water-soaked lesions surrounded by regions of chlorosis. These lesions remarkably resemble the bacterial speck disease caused by *Pseudomonas syringae* infection (Figure 1a, b, and compare with Figure 1c). Lesions initiate from the leaf margin of the oldest leaves of 4-week-old-plants and spread throughout the leaf blade, ultimately

engulfing the entire leaf. Frequently *dll1* plants die even before bolting. Trypan blue staining revealed the presence of dead cells in the lesion$^+$ leaves (Figure 1a). The *dll1* lesions form under all tested growth conditions; however, long-day growth conditions and high humidity exacerbate lesion severity. Since aseptically grown *dll1* plants develop lesions, cell death is not the result of enhanced susceptibility to soil-borne pathogens.

The *dll1* mutant was backcrossed to the wild-type parent Col-0. All of the F_1 progeny (n = 45) developed lesions. The F_2 progeny segregated 260 lesion$^+$ plants to 85 lesion$^-$ plants, thereby indicative of a monogenic dominant mutation (χ^2 = 0.015, 0.99 > P > 0.95). To determine whether the dominance of the *dll1* mutation is the result of haploinsufficiency or whether *dll1* constitutes a gain-of-function allele, we crossed the diploid *dll1* plants (*dd*) with a tetraploid Col-0 parent (*DDDD*) to generate triploid progeny (*dDD*). These triploid progeny contain two wild-type copies of *DLL1* and one mutant *dll1* allele. All of the F_1 triploid progeny (n = 30) developed *dll1*-associated lesions (data not shown) indicating that *dll1* is a true gain-of-function allele. In addition, similar to homozygous *dll1* (*dd*), these triploid (*dDD*) plants constitutively express molecular markers of defense (see below).

Analysis of 180 L*er* × *dll1* F_2 progeny with lesions mapped *DLL1* locus to the bottom arm of chromosome 1, within a 16-cM interval flanked by SSLP markers T23K23 and nga 280, 6 cM from T23K23 and 10 cM from g4026. No other mutant with a similar phenotype maps to this region of Chromosome 1 and therefore *dll1* defines a novel gain-of-function mutation.

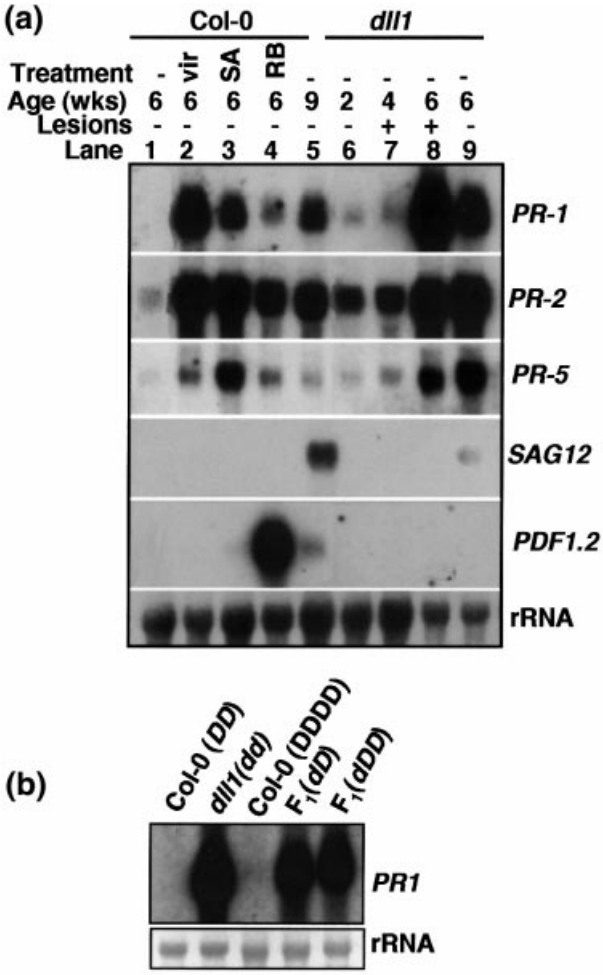

Figure 2. Defense-related genes are constitutively expressed in *dll1*.
(a) RNA in lanes 8 and 9 was isolated from lesion$^+$ and lesion$^-$ leaves of the same 6-week-old *dll1* plant. Lesion$^-$ leaves had no macroscopic or microscopic lesions. Six-week-old wild-type Col-0 plants were infiltrated with 10^7 cfu/ml *Pst* DC3000 (vir) or sprayed with 1 mM salicylic acid (SA) (control for SA-responsive defense genes) or sprayed with 20 μM Rose Bengal (RB) (control for *PDF1.2* gene). Pathogen-infiltrated and SA-treated leaves were harvested 24 h after treatment, and leaves treated with RB were harvested 48 h after treatment. RNA from senescing leaves of 9-week-old Col-0 plants in lane 5 was used as a control for *SAG12* gene.
(b) *PR-1* expression analysis of triploid *dll1* plants. All plants were 6-week-old and genotype of the plants is indicated. Blots were serially hybridized with the indicated probes. This experiment was replicated twice with different sets of plants and similar results were obtained.

dll1 *constitutively expresses multiple defense responses*

The expression of several biochemical and molecular markers is associated with the plant's response to pathogen infection. These markers are induced both by avirulent and virulent pathogens, but often in the later stages of infection by the virulent pathogen (Hammond-Kosack and Jones, 1996). Since *dll1* lesions resemble disease lesions, we sought to determine whether *dll1* expresses defense-related markers. Comparison of whole mounted *dll1* lesion$^+$ leaves with the controls reveals substantial deposition of autofluorescent material, callose, and accumulation of H_2O_2 in and around the lesions in *dll1*, but not in the control wild-type Col-0 tissue (Figure 1d). These results demonstrate that the *dll1* mutant constitutively expresses biochemical markers associated with pathogen infection.

We also examined the accumulation of defense-related gene transcripts in *dll1* plants and found that the spontaneous lesion formation in *dll1* is accompanied by the accumulation of elevated levels of transcripts of the pathogenesis-related genes associated with the SA-regulated defense pathway (*PR-1*, *PR-2*, *PR-5*) (Uknes *et al.*, 1992), but not the genes associated with the ethylene/jasmonic acid (ET/JA)-mediated defense pathway (*PDF1.2*) (Figure 2a) (Penninckx *et al.*, 1996). We also analysed the expression of these defense genes in the lesion$^-$ leaves of the 6-week-old *dll1* plant that had lesions on some leaves. We found that defense genes are expressed in the lesion$^-$ leaves at levels comparable with lesion$^+$ leaves, indicating the activation of SAR response (Figure 2a, lane 9). No dead cells were found in these lesion$^-$ leaves, even after staining with trypan blue. Since *dll1* lesion development begins

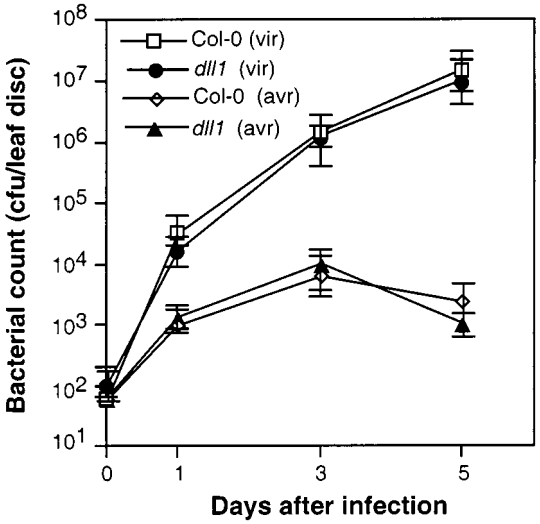

Figure 3. *dll1* does not suppress the growth of *Pst* DC3000.
Lesion$^+$ leaves of 6-week-old *dll1* and the leaves of Col-0 were infiltrated with bacterial suspension of *Pst* DC3000 or *Pst* DC3000 (*avrRpm1*) in 10 mM $MgCl_2$ at a dose of 5×10^5 cfu/ml (OD_{600} = 0.0005). Four leaf discs for each treatment were collected at 0, 1, 3 and 5 days after infiltration to determine the bacterial count as described in Experimental procedures. The bacterial counts ± SD are presented as colony-forming units (cfu) per leaf disc and represent the averages from three independent experiments. Col-0 (vir), Col-0 infiltrated with virulent pathogen *Pst* DC3000; Col-0 (avr), Col-0 infiltrated with avirulent pathogen *Pst* DC3000 (*avrRpm1*); *dll1* (vir), *dll1* infiltrated with virulent pathogen *Pst* DC3000; *dll1* (avr), *dll1* infiltrated with avirulent pathogen *Pst* DC3000 (*avrRpm1*).

with chlorosis of the leaves, a typical sign of senescence, we tested the expression of a senescence-associated gene *SAG12 (*induced during late stages of senescence) in these plants (Weaver *et al.*, 1998). No significant *SAG12* induction was detected in *dll1* plants (Figure 2a), suggesting that the onset of chlorosis in *dll1* is not the result of the accelerated senescence-like processes.

We also analysed the expression of *PR-1* gene in lesion$^+$ leaves of 6-week-old triploid (*dDD*) plants and found that it was induced to levels comparable with homozygous *dll1* (*dd*) (Figure 2b). This result further supports the contention that *dll1* is a gain-of-function mutation.

dll1 *mutant fails to resist the growth of virulent pathogens*

Since *dll1* plants constitutively express a vast repertoire of defense responses, we sought to determine whether these defense responses translate into increased resistance against virulent pathogens. We determined the growth of virulent *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) in *dll1* plants. Despite the constitutive expression of multiple defense responses, surprisingly, *dll1* plants did not suppress the growth of *Pst* DC3000 (Figure 3). Similarly, *dll1* failed to suppress the growth of another virulent bacterial pathogen (*P. syringae* pv. *maculicola* ES4326) and a virulent oomycete pathogen (*Peronospora parasitica* Ahco2) (data not shown). However, lesions were slightly more severe on *Pst* DC3000-infiltrated *dll1* plants compared to mock treated (buffer infiltrated) *dll1* plants (data not shown). To examine whether *dll1* is deficient in its ability to activate the normal defense response against pathogens, we tested the response of *dll1* to an avirulent strain of *P. syringae* (*Pst* DC3000 expressing *avrRpm1*). The *dll1* plants induced a normal visible HR (data not shown), and suppressed the growth of this pathogen, comparable to the wild-type Col-0 (Figure 3). Similar results were obtained with another avirulent pathogen, *Pst* DC3000 expressing *avrRpt2 (*data not shown). These results suggest that despite the abundance of defense-related responses, *dll1* does not resist the growth of virulent pathogens.

dll1 *supports the growth of a* hrp *mutant of* P. syringae

From the results described above, we demonstrate that despite the constitutive expression of biochemical and molecular markers associated with defense, *dll1* fails to suppress the growth of virulent pathogens. The presence of defense responses and yet the lack of resistance to pathogens is the hallmark of disease. Thus, *dll1* lesions mimic at least some aspects of the phenotypic and the molecular mechanisms associated with disease development. We tested if *dll1* lesions also mimic the physiological state of the disease by monitoring the growth of an *hrp* mutant of *Pst* DC3000 in *dll1* lesion$^+$ leaves. *P. syringae* employs a Type III secretion system, encoded by *hrp* genes, to transfer virulence factors from the pathogen into the plant cells (Collmer *et al.*, 2000). Thus, the *hrp* mutants of *P. syringae* do not cause disease on plants and are thus non-pathogenic. We reasoned that if *dll1* lesions truly mimic the physiological state of the disease, then *P. syringae* strains that are defective in the *hrp* genes should be able to grow in the lesion$^+$ leaves of *dll1*. To test this hypothesis, we compared the growth of the *hrcC* mutant of *Pst* DC3000 (Deng *et al.*, 1998) in the lesion$^+$ leaves of 6-week-old *dll1* mutant and in the wild-type parent Col-0. The *hrcC* mutant did not increase the severity of lesions in *dll1* plants (data not shown). However, the results in Figure 4 show that the growth of *hrcC* mutant is supported 100-fold more in *dll1* compared to Col-0 plants. The growth of *hrcC* mutant in the 3-week-old *dll1* plants (without lesions) was similar to its growth in the wild-type Col-0, suggesting that the growth of *hrcC* mutant requires *dll1*-associated cell death (data not shown). Together, these results suggest that *dll1* lesions mimic many of the critical aspects of both the molecular and the physiological state of disease.

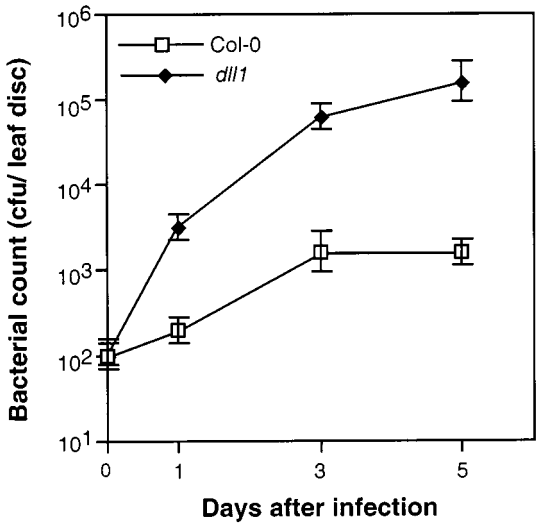

Figure 4. Lesion$^+$ leaves of *dll1* support the growth of *hrcC* mutant of *Pst* DC3000.
Lesion$^+$ leaves of 6-week-old *dll1* and the leaves of Col-0 were infiltrated with *hrcC* mutant of *Pst* DC3000 in 10 mM $MgCl_2$ at a dose of 10^5 cfu/ml (OD_{600} = 0.0001). Bacterial count was determined as described in Figure 3. The values represent averages of three independent experiments.

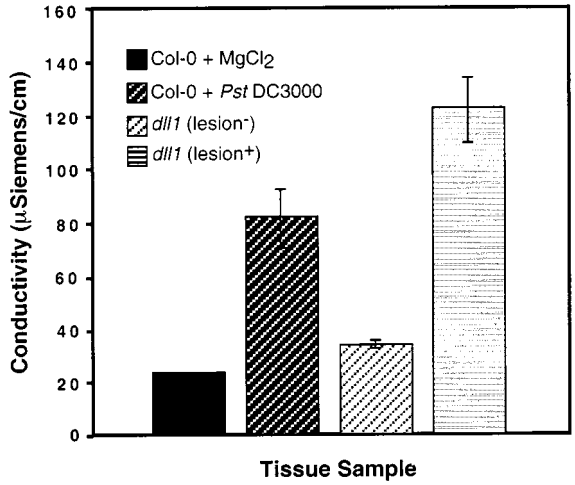

Figure 5. Cell death in *dll1* plants is accompanied by electrolyte leakage. Electrolyte leakage of lesion$^+$ and lesion$^-$ leaves of 6-week-old *dll1*, and the control leaves was determined as described in Experimental procedures. Values (μsiemens/cm) are average of 4 replicates ± SD from 4 different sets of plants. Buffer and *Pst* DC3000 infiltrated (10^5 cfu/ml) leaves were harvested 3 days after infiltration.

Cell death in dll1 *is accompanied by membrane damage*

Necrotrophic pathogens like *P. syringae* grow in the intercellular spaces of mesophyll tissue by using the nutrients that are released into the intercellular space due to pathogen-induced membrane damage. The fact that lesion$^+$ leaves of *dll1* support the growth of the *hrcC* mutant of *P. syringae* indicates that *dll1*-associated cell death may be accompanied by membrane damage, which leads to the release of nutrients into the intercellular space. To test this hypothesis, we quantified electrolyte leakage in *dll1* and in the control plants. Compared with the mock-treated Col-0 leaves, significant increase in electrolyte leakage was observed in virulent pathogen *Pst* DC3000-infected Col-0 leaves. Furthermore, similar to *Pst* DC3000-infected Col-0 leaves, the lesion$^+$ leaves of *dll1*, but not the lesion$^-$ leaves, exhibited high electrolyte leakage (Figure 5). These results suggest that in the absence of pathogen infection, *dll1* induces a cell death programme that is similar to pathogen-induced cell death leading to membrane damage.

Salicylic acid signalling regulates dll1*-associated phenotype*

Many *Arabidopsis* lesion-mimic mutants that spontaneously develop HR-like lesions and display enhanced resistance to virulent pathogens accumulate elevated levels of SA, and in many cases SA is required for lesion formation and constitutive defense expression (Dangl *et al.*, 1996; Ryals *et al.*, 1996). We tested what role, if any, SA might play in regulating *dll1*-associated phenotypes. We determined the endogenous levels of free SA and salicylate glucoside (SAG) in the rosette leaves of 6-week-old soil-grown *dll1* and control plants. As shown in Figure 6, levels of free SA and SAG in *dll1* plants are elevated. While free SA levels in *dll1* lesion$^+$ leaves are about 3-fold higher compared to the levels in the Col-0 plants in which disease lesions were induced by infection with the virulent bacterial pathogen *Pst* DC3000, the levels of SAG in these plants are similar.

We constructed transgenic *dll1* plants that are unable to accumulate SA due to the expression of the *nahG* gene from *P. putida* (You *et al.*, 1991). Expression of the *nahG* gene in *dll1* abolished lesion formation in *dll1 nahG* plants and these plants were phenotypically indistinguishable from their wild-type parents. Furthermore, expression of the *nahG* gene abolished constitutive *PR* gene expression in *dll1 nahG* plants (Figure 7). To further address whether the *dll1* lesion phenotype is dependent on salicylic acid, *dll1 nahG* plants were treated with 100 μM benzothiadiazole (BTH), a biologically active analogue of SA (Lawton *et al.*, 1996). BTH treatment restored lesion formation in *dll1 nahG* plants (Figure 1e), but did not induce lesions in wild-type or *nahG* plants. Furthermore, BTH restored *PR* gene expression in *dll1 nahG* plants within 24 h (before lesions development), which further increased at 72 h (after lesion development) (Figure 7). Together these results suggest that SA is required for *dll1*-associated phenotypes.

NPR1 functions downstream of salicylic acid in the SA-dependent signalling pathway and is required for the

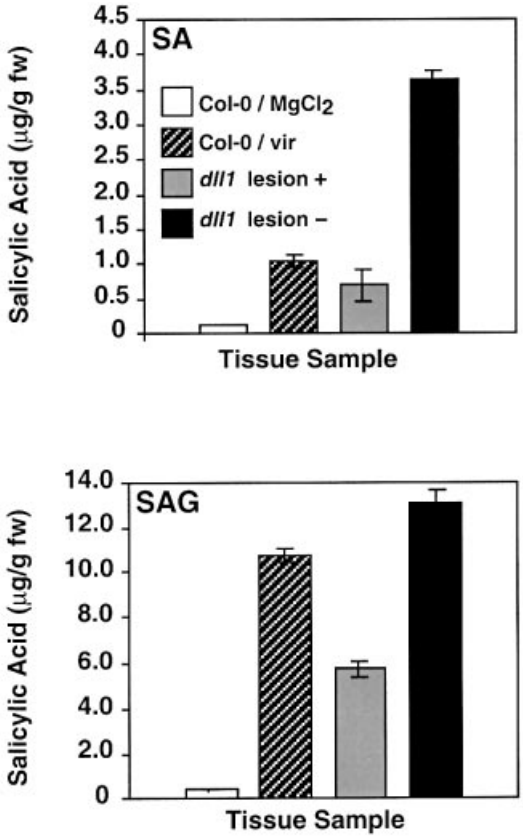

Figure 6. *dll1* accumulates elevated levels of SA.
Free SA (SA) and sugar-conjugated SA (SAG) were extracted from 6-week-old soil-grown plants. The values are averages from 4 different sets of plants per genotype and error bars represent SD. Mock (10 mM $MgCl_2$) and pathogen treated (vir; 10^5 cfu/ml, *Pst* DC3000) leaves were harvested 3 days after treatment.

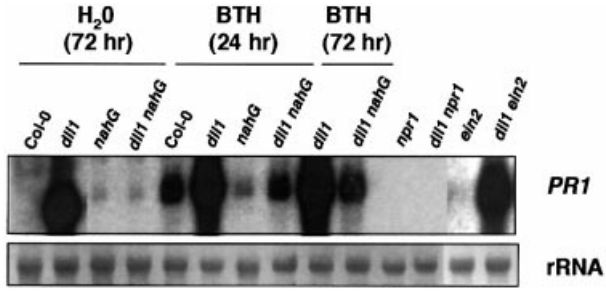

Figure 7. Expression of *PR-1* is suppressed in *dll1 nahG* and *dll1 npr1* plants.
Total RNA was isolated from 6-week-old soil-grown plants of indicated genotype and treatment. Plants were sprayed with water or 100 μM BTH and the leaves were harvested at the indicated times. The 72-h BTH-treated *dll1 nahG* plants had lesions.

Table 1. Ethylene evolved by *dll1* plants

Genotype	Number of samples	p.p.m. g^{-1} h^{-1} ± SE
Col-0	6	0.07 ± 0.00
dll1	6	0.25 ± 0.02

This experiment was repeated twice with similar results.
Six-week-old lesion$^+$ leaves were used for this experiment.
SE, standard error.

transduction of some of the SA dependent defense responses (Cao *et al.*, 1994). Since SA is required for *dll1*-associated phenotypes, we considered the role of NPR1 in regulating *dll1*-associated phenotypes. We generated *dll1 npr1* double mutants by a genetic cross with *npr1–1* line. Similar to the *dll1 nahG* plants, *dll1 npr1* plants did not develop lesions and were phenotypically indistinguishable from Col-0 or *npr1* plants (data not shown). Furthermore, constitutive *PR-1* expression was also suppressed in *dll1 npr1* plants (Figure 7). These results suggest that cell death and defense gene expression in *dll1* are regulated through the SA/NPR1 signalling pathway.

Ethylene signalling plays a critical role in lesion formation in dll1

Ethylene sensitivity is not required for HR-associated cell death induced by avirulent pathogens, but is required for disease-associated cell death following virulent pathogen infection (Bent *et al.*, 1992; Lund *et al.*, 1998). Since *dll1* lesions resemble disease lesions induced by virulent pathogen infection, we sought to evaluate the role of ethylene in regulating lesions development in *dll1*. As illustrated in Table 1, 6-week-old *dll1* plants evolved 8-fold more ethylene compared to the wild type parent Col-0. To further evaluate the role of ethylene signalling in regulating *dll1*-associated phenotypes, we constructed *dll1 etr1* and *dll1 ein2* double mutants. ETR1 is an ethylene receptor and EIN2 functions downstream of ETR1 in the ethylene signal transduction pathway (Alonso *et al.*, 1999; Chang *et al.*, 1993). *dll1 etr1* and *dll1 ein2* double mutants were created by genetic crosses with *etr1–1* and *ein2–1* mutants. We observed that perturbing ethylene signalling significantly delayed lesion initiation (by 3 weeks) as well as the severity of lesions in both *dll1 ein2* and *dll1 etr1* plants (data not shown). *PR-1* gene expression was not affected in the *dll1 ein2* (Figure 7) or *dll1 etr1* plants (data not shown).

Discussion

We identified a novel gain-of-function mutant of *Arabidopsis* designated *dll1*. At the age of 4 weeks, *dll1* spontaneously develops disease-like lesions, constitutively expresses several defense-related markers, and yet fails to suppress the growth of virulent pathogens. Several features of *dll1* suggest that *dll1* lesions mimic cell death caused by the necrotrophic pathogens. First, spontaneous

lesions of *dll1* (water soaked lesions surrounded by chlorosis) are very similar to the lesions of bacterial speck disease caused by *P. syringae*. Second, despite the constitutive expression of all tested biochemical and molecular markers of defense, *dll1* is unable to resist the growth of virulent pathogens, which is the hallmark of compatible plant–pathogen interactions. Third, the lesion$^+$ tissue of *dll1* supports the growth of *hrcC* mutant of *P. syringae* that is non-pathogenic to wild-type Col-0. The ability of the lesion$^+$ tissue of *dll1* to support the growth of *hrcC* mutant implies that the *dll1* mutant mimics the physiological state of disease, thus allowing the bacterium to grow even in the absence of any transferred virulence factors. Similar to disease lesions, cell death associated with *dll1* lesions induces membrane damage leading to electrolyte leakage in the intracellular space, thereby providing a source of nutrients for the growth of the *hrcC* mutant. The ability of the *hrcC* mutant to grow in *dll1* is not the result of enhanced susceptibility to pathogens because 3-week-old *dll1* plants, which do not have lesions, cannot support the growth of *hrcC* mutant. Cell death is required to support the growth of *hrcC* mutant in *dll1*, probably to provide nutrients that are released as a result of cell death. Fourth, the lesion$^+$ tissue of *dll1* produces significantly more ethylene compared with the wild-type Col-0 and blocking ethylene signalling in *dll1 etr1* and *dll1 ein2* plants delays the initiation and severity of the *dll1* lesions. These findings are consistent with previous studies that virulent pathogens stimulate the host to produce ethylene (Boller, 1991) and the application of ethylene inhibitors attenuates cell death in susceptible plants (Moussatos *et al.*, 1994). Additionally, ethylene signalling positively modulates disease- but not HR-associated cell death (Bent *et al.*, 1992; Lund *et al.*, 1998; O'Donnell *et al.* 2001). Taken together, these results indicate that *dll1*-associated cell death mimics many aspects of cell death induced during compatible interactions. No lesion-mimic mutant with features similar to *dll1* has been reported yet. This resemblance at both the molecular and physiological levels provides compelling evidence that the host might, at least in part, actively regulate cell death during disease.

While the features of *dll1* discussed above are consistent with our hypothesis that *dll1* lesions mimic disease-associated cell death, the absence of lesions in *dll1 nahG* and *dll1 npr1* plants suggests that the formation of lesions in *dll1* is dependent on SA signalling. Because lesion development in *Arabidopsis* in response to virulent pathogens such as *P. syringae* or *P. parasitica* is not dependent on SA signalling, and is in fact enhanced in *nahG* and *npr1* plants, there appears to be a difference in the signalling requirements between *dll1*-associated and disease-associated lesions. One explanation to account for this discrepancy is that disease-associated cell death may be regulated by both SA-dependent and -independent pathways. Indeed, examples of SA-dependent disease-associated cell death have been reported. Disease symptoms induced by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) in tomato is dependent on SA (O'Donnell *et al.*, 2001). The inhibition of SA accumulation in tomato plants prevented spreading chlorosis and necrosis in response to *Xcv* infection. Similarly SA also promotes symptom formation during the interaction between tomato and *Cladosporium fulvum* (Bradling *et al.*, 2000). This is analogous to SA/NPR1-dependent and -independent pathways regulating HR-associated cell death. For example, HR-associated cell death mediated by *RPM1* resistance gene is SA-independent, while *RPS2* mediated cell death is SA-dependent (Rate *et al.*, 1999). Similarly, while HR-mimic mutants, *lsd1*, *lsd6*, *lsd7*, and *ssi1* require accumulation of SA for lesion formation, *lsd2*, *lsd4*, and *cpr5* do not (Bowling *et al.*, 1997; Dangl *et al.*, 1996; Ryals *et al.*, 1996; Shah *et al.*, 1999). Thus, similar to HR-associated cell death, it appears that different host–pathogen interactions may involve different signalling pathways to regulate disease-associated host cell death. The *dll1*-induced symptoms may represent a novel SA-dependent signalling pathway for pathogen-induced disease development that has not yet been studied with *Arabidopsis*.

Since *dll1* is a gain-of-function mutation, it is difficult to predict the normal function of the wild-type *dll1* gene. However, recently, bacterial virulence effectors have been suggested to enter the host cell, interact with host targets, and modify their function to promote cell death to nourish the extracellular bacteria (Dangl and Jones, 2001). We speculate that the DLL1 protein may represent one such cellular target for the bacterial virulence factors. Since *dll1* accumulates elevated levels of SA and cell death is blocked in *dll1 nahG* and *dll1 npr1* plants, the DLL1 protein probably functions upstream of SA and NPR1. SA treatment does not induce cell death in wild-type plants but is required for cell death in *dll1* plants. This suggests that in the wild-type plants, a pathogen-derived signal (possibly a virulence factor) may modify the DLL1 protein to activate cell death that is SA-dependent. However, in the mutant plants, *dll1* allele bypasses the requirement of the pathogen-derived signal, but still requires SA-signalling to cause cell death.

An intriguing feature of the *dll1* mutant is that constitutive expression of prototypical *Arabidopsis* defense responses is not sufficient for resistance against virulent pathogens. However, *dll1* plants induce normal defense and HR against avirulent pathogens. This observation strongly suggests that unidentified host gene(s) induced by avirulent pathogens are critical for resistance. Analogously, it is foreseeable that virulent pathogens induce host gene(s) to promote disease. Indeed host genes that might be involved in disease development have been identified (Mayda *et al.*, 2000a; Vogel and

Somerville, 2000). In addition, we have identified several *Arabidopsis* genes that are induced specifically or at least preferentially during bacterial compatible interactions (Gómez-Buitrago and Raina, in preparation). Thus, although some overlap exists between the signalling components (such as SA and NPR1) and downstream genes (such as *PR* genes analysed in this study) that regulate incompatible and compatible interactions, it appears that each response is represented by a set of unique genes that may play a crucial role in determining the outcome of the interaction. Global gene expression analysis of *dll1* using DNA microarrays should help us identify additional factors that regulate host cell death during disease.

Experimental procedures

Plant growth conditions

Arabidopsis thaliana plants were grown in soil (Metro-Mix 360; The Scotts Company, Marysville, OH, USA) or on plates containing Murashige and Skoog (MS) media (Life Technologies, Grand Island, New York, NY, 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 light intensity of 100–150 μmol m^{-2} sec^{-1} with a 10-h photoperiod.

Genetic analysis

Backcrosses with the parental *DLL1* line was performed using *dll1* as the pollen donor. To facilitate mapping, pollen from *dll1* was used to fertilize the flowers of Landsberg *erecta* ecotype (L*er*). The resulting F_2 progenies displaying *dll1* phenotype were used for recombination analysis by the methods of codominant cleaved amplified polymorphic sequences (CAPS; Konieczny and Ausubel, 1993) and simple sequence length polymorphisms (SSLP; Bell and Ecker, 1994). DNA isolation and PCR analysis were performed as described (Konieczny and Ausubel, 1993). To construct triploid plants, *dll1* pollen was used to fertilize the flowers of a tetraploid Col-0 plant (Stock number CS3432, Arabidopsis Stock Center).

Histochemistry and microscopy

Leaves for autofluorescense and callose deposition were prepared as described (Dietrich *et al.*, 1994), trypan blue staining of dead cells was performed as described (Vogel and Somerville, 2000), and staining for the presence of H_2O_2 was performed as described (Thordal-Christensen *et al.*, 1997).

Chemical treatment and pathogen infection of plants

The plants were sprayed with an aqueous solution of SA (1 mM), BTH (100 μM), rose bengal (RB; 20 μM) or water until run off and covered overnight to maintain high humidity. Leaves were harvested at the indicated times after treatment. Bacterial pathogen infiltration and disease assays were performed as described (Whalen *et al.*, 1991).

Construction of double mutants

To construct *dll1 nahG* line, the *nahG* gene was introduced into *dll1* plants by a genetic cross, using pollen of the *nahG* plants to fertilize *dll1* flowers. Transgenic *nahG* line in the Col-0 ecotype (line B15) was obtained from Syngenta Biotechnology Inc. F_2 seedlings were sprayed with 100 μM BTH to score for *dll1*-like phenotype. Homozygous *dll1 nahG* lines were identified as F_2 lines in which 100% of the F_3 progeny (n = 55) were kanamycin-resistant (marker linked to *nahG*) and developed *dll1* lesions upon BTH treatment.

To construct *dll1 npr1* plants, *dll1* pollen was used to fertilize the *npr1–1* flowers (Cao *et al.*, 1994). F_2 progeny was screened for lines homozygous for *npr1* mutation by CAPS analysis as described (Clarke *et al.*, 1998). Lines homozygous for *npr1* were backcrossed to parental Col-0 to identify the lines carrying *dll1* allele, as evidenced by the gain of *dll1* lesions. F_2 lines whose 100% progeny of backcross to Col-0 (n = 40) displayed *dll1* lesions were considered as homozygous *dll1 npr1* lines.

To construct *dll1 etr1–1* plants, *etr1–1* pollen was used to fertilize *dll1* flowers (Chang *et al.*, 1993). F_1 seeds were screened for insensitivity to ethylene as described (Greenberg *et al.* 2000). Homozygous *dll1 etr1* lines were identified as those F_2 lines whose 100% F_3 populations (n = 65) were ethylene-resistant and displayed delayed and attenuated *dll1* lesions.

To construct *dll1 ein2* plants, *dll1* pollen was used to fertilize *ein2–1* flowers (Alonso *et al.*, 1999). F_1 progeny displaying *dll1* phenotype were selfed and F_2 progeny was screened as described above. Homozygous *dll1 ein2* lines were identified as described above.

RNA analysis

Total RNA was isolated from soil-grown plants using TRIzol reagent according to manufacturer's instructions (Gibco BRL, Gaithersburg, MD, USA). For RNA gel-blot analysis, 10 μg of total RNA was fractionated by electrophoresis through denaturing formaldehyde-agarose gels and transferred to a Hybond N + hybridization membrane (Amersham-Pharmacia, IL, USA) as described (Ausubel *et al.*, 1994). Gene-specific probes were synthesized by random primed ^{32}P-labelling of gel-purified DNA fragments using RediPrime kit according to the manufacture's instructions (Amersham-Pharmacia, IL, USA). Hybridizations and washes were performed as described (Ausubel *et al.*, 1994).

Salicylic acid and ethylene measurements

Salicylic acid (SA) was extracted from leaf tissue (0.5 g) and quantified by spectrofluorescence HPLC as described (Enyedi and Raskin, 1993). Leaves for ethylene measurements were collected and immediately transferred to airtight vials containing MS media and 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.

Electrolyte leakage measurements

The electrolyte leakage measurements were done as described previously (McNellis *et al.*, 1998; Mittler *et al.*, 1996; Rate and Greenberg, 2001). For each treatment, four leaf discs (0.5 cm in diameter) from four different plants were suspended in 2 ml of double distilled water. Leaf suspensions were shaken at 28°C for 8 min and the 2 ml liquid sample was immediately transferred

into the electrode chamber of a conductivity meter. Conductivity measurements were performed using a VWR brand portable conductivity meter (VWR Scientific Products Corporation, USA).

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