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Arabidopsis *dth9* Mutation Identifies a Gene Involved in Regulating Disease Susceptibility without Affecting Salicylic Acid–Dependent Responses

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To determine which components of the plant defense response make important contributions to limiting pathogen attack, an M_2 mutagenized population of a transgenic Arabidopsis line was screened for mutants showing constitutive expression of β-glucuronidase activity driven by the promoter region of the *CEVI-1* gene. The *CEVI-1* gene originally was isolated from tomato plants and has been shown to be induced in susceptible varieties of tomato plants by virus infection in a salicylic acid–independent manner. We report here the characterization of a recessive mutant, *detachment9* (*dth9*). This mutant is more susceptible to both virulent and avirulent forms of the oomycete Peronospora and also exhibits increased susceptibility to the moderately virulent bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326. However, this mutant is not affected in salicylic acid metabolism and shows normal expression of pathogenesis-related (*PR*) genes after pathogen attack. Furthermore, after inoculation with avirulent pathogens, the *dth9* mutant shows a compromised systemic acquired resistance response that cannot be complemented by exogenous application of salicylic acid, although this molecule is able to promote normal activation of *PR* genes. Therefore, the *dth9* mutation defines a regulator of disease susceptibility that operates upstream or independently of salicylic acid. Pleiotropy is also evident in the *dth9* mutant in the sense that the shoots of *dth9* plants are insensitive to the exogenously applied auxin analog 2,4-dichlorophenoxyacetic acid.

INTRODUCTION

Plants have developed constitutive as well as inducible defense responses against pathogens. Systemic acquired resistance (SAR; Ross, 1961) is one such inducible defense response that is triggered in the plant by previous exposure to pathogens that cause cell death. SAR is long lasting and confers protection against a broad spectrum of pathogens (Ryals et al., 1996). A more rapid defense response that precedes the onset of SAR is the hypersensitive response (HR; Agrios, 1988), which is localized at the site of attempted pathogen entry. HR is characterized by programmed death of host cells and is a consequence of the interplay of the products of the pathogen avirulent genes (*Avr*) and the host disease resistance genes (*R*). Tightly correlated with the HR and the SAR is the production of antimicrobial compounds, the increased expression of a subset of the pathogenesis-related (PR) proteins, many of which possess antimicrobial activities, and the reinforcement of mechanical barriers such as cell walls (Sticher et al., 1997).

Salicylic acid (SA) is an important signal molecule in plant defense. SA accumulates in increased amounts during HR and SAR. Preventing the accumulation of SA compromises disease resistance and the accretion of PR proteins in infected plants (Gaffney et al., 1993; Delaney et al., 1994). Furthermore, exogenous application of SA induces disease resistance and the expression of *PR* genes (Ryals et al., 1996).

Studies on mutants altered in SAR responses suggest that plants have developed a complex network of interactions that ultimately orchestrate the activation of defense-related responses. In Arabidopsis, the *NPR1*/*NIM1*/*SAI1* locus operates downstream from SA and has been shown to be a key component of SA-regulated *PR* gene expression and disease resistance, because *npr1*/*nim1*/*sai1* mutants fail to express *PR* genes and display enhanced susceptibility to infection even after treatment with SA (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). Other mutants affected in plant resistance could be positioned upstream of the synthesis or action of SA. *lsd* (Dietrich et al., 1994) and *acd2* (Greenberg et al., 1994) mutants have high amounts of SA, express lesions similar to that of an HR, and show enhanced resistance to infection. The *cpr* mutants (Bowling et

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al., 1994) also show constitutive expression of *PR* genes as well as constitutively high amounts of SA, suggesting that they act upstream of SA. However, although *cpr1* is affected specifically in the SA pathway (Bowling et al., 1994), the *cpr5* mutant (Bowling et al., 1997) shows constitutive expression of the *PDF1.2* gene, which is independent of SA. Also, the *cpr6* and *ssi1* mutants are distinct in the sense that the expression of *PR* genes is independent of NPR1/NIM1/SAI1 (Clarke et al., 1998; Shah et al., 1999). In addition, *dnd1*, although exhibiting increased resistance and high constitutive expression of SA and *PR* genes, does not undergo an HR reaction when infected with avirulent pathogens (Yu et al., 1998).

In marked contrast, the *pad4* mutant (Zhou et al., 1998) is impaired in the accumulation of SA and the phytoalexin camalexin as well as in expression of the *PR1* gene after infection with a virulent pathogen. Likewise, the SA induction–deficient (*sid*) mutants (Nawrath and Métraux, 1999) are compromised in SAR and do not accumulate SA after pathogen infection, but only a subset of *PR* genes in these mutants is strongly reduced, and camalexin amounts are normal. The enhanced disease susceptibility (*eds*) mutants (Glazebrook et al., 1996; Parker et al., 1996; Rogers and Ausubel, 1997; Volko et al., 1998) represent another mutant type that is compromised in resistance, but the role of SA remains to be elucidated. Like *sid* mutants, the *eds5* mutant (allelic to *sid1*) is defective only in the expression of *PR1* but not in that of *PR2* or *PR5*, suggesting that there is an SA-independent compensation pathway activated in the plant once the pathogen has been detected (Nawrath and Métraux, 1999).

We recently described the isolation of *dth* (*detachment*) mutants from Arabidopsis, which are defined as mutants displaying constitutive expression of the reporter β-glucuronidase (*GUS*) gene driven by the promoter of the tomato *CEVI-1* gene (Mayda et al., 2000). In tomato plants, *CEVI-1* expression was shown to be transcriptionally activated, along with other defense-related genes, during the course of compatible plant–virus interactions (e.g., tomato mosaic virus). Additionally, *CEVI-1* showed rapid activation if leaf segments were detached from the plant. However, the expression of *CEVI-1*, either during disease or on detachment, was controlled by way of an SA-independent pathway, and neither ethylene nor jasmonic acid and wounding were able to induce expression of *CEVI-1* in the plant (Mayda et al., 2000). These findings suggested that the expression of *CEVI-1* could be controlled by a novel signaling pathway activated during disease in susceptible tomato plants. Additionally, the observation that the constitutive expression of *CEVI-1*::*GUS* in the Arabidopsis *dth* mutants concurs with an auxin-resistant phenotype in the plant prompted us to suggest that the mechanism upregulating the expression of *CEVI-1* is related to an induced insensitivity to auxin that might be primed during disease (Mayda et al., 2000).

To study a causal link between the signal pathway mediating the activation of *CEVI-1* and that mediating disease susceptibility in plants, we report the characterization of the *dth9* mutant as a case study.

RESULTS

Identification of the *dth9* Mutant

The Arabidopsis *dth* mutants were identified in a screen for constitutive expressers of a *GUS* gene driven by the promoter region of the *CEVI-1* gene. The details of this screen have been described previously (Mayda et al., 2000). The *CEVI-1* gene (which encodes an anionic peroxidase) originally was isolated from tomato plants and shown to be induced in susceptible tomato plants, but not in resistant plants, by viral infection (e.g., tomato mosaic virus; Mayda et al., 2000).

Here, we report the characterization of *dth9*. Figures 1A and 1B show the constitutive expression of the *GUS* reporter gene in the Arabidopsis *dth9* mutant compared with the parental nonmutagenized transgenic *CEVI-1*::*GUS* line. The parental line showed no GUS activity except in a few cells located at the distal margin of the cotyledons, whereas the *dth9* mutant showed intense GUS staining throughout the plant body except in the roots. In full-grown plants (Figures 1C and 1D), GUS staining was present along the rosette leaves in the *dth9* plants, whereas expression of GUS activity in the parental line was again absent.

This tissue-specific distribution of GUS staining in *dth9* plants was similar to that observed for the previously reported nonallelic *dth2* and *dth23* mutants isolated in the same screen (Mayda et al., 2000). In addition to their constitutive expression of GUS driven by the *CEVI-1* promoter, *dth2* and *dth23* mutants also were characterized as auxin-insensitive mutants because they were resistant to the growth inhibition caused by (2,4-dichlorophenoxy)acetic acid (2,4-D; Mayda et al., 2000). To determine if the 2,4-D–resistant phenotype also concurred in the *dth9* mutant, we germinated *dth9* seed on agar plates containing 0.2 μM 2,4-D, and the extent of growth inhibition attributable to the perception of this hormone was compared with that observed in wild-type seed. Growth of wild-type seedlings was severely affected on this medium, whereas *dth9* seedlings appeared to resist the inhibitory effect of 2,4-D, at least in the aerial part of the plant (Figures 1E and 1F). At variance with *dth2* and *dth23* mutants, which show the auxin-resistance phenotype in both aerial and root tissues (Mayda et al., 2000), roots of *dth9* mutants did not elongate when grown in the presence of 2,4-D (Figure 1F).

Genetic Characterization of the *dth9* Mutation

To determine the genetic basis of the phenotype described above in *dth9* plants, we performed a backcross between *dth9*/*dth9* plants and wild-type *DTH9*/*DTH9* plants contain-

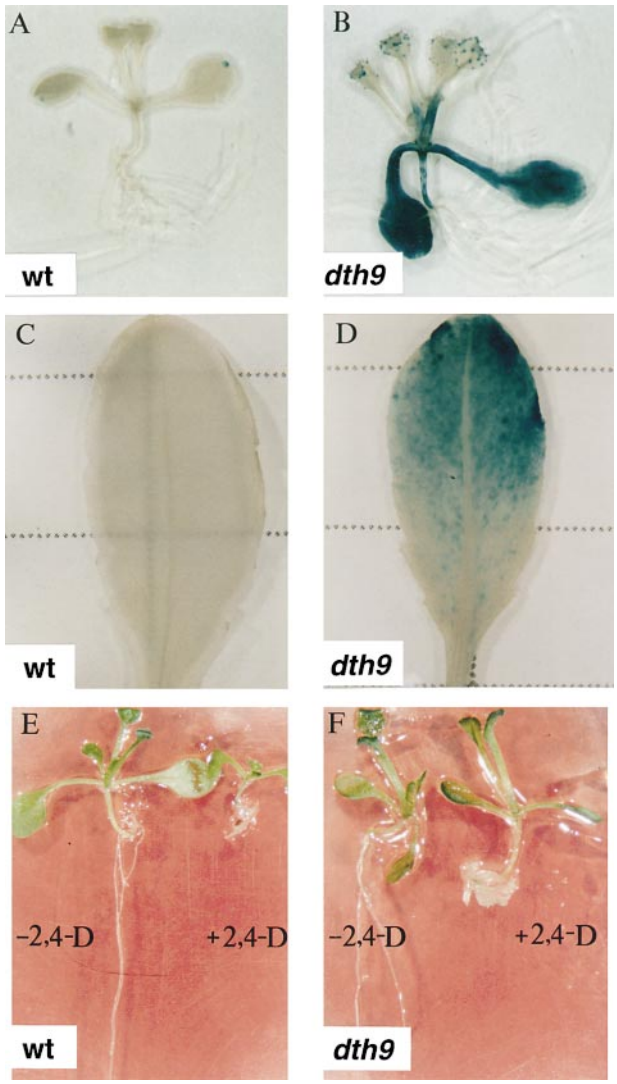

Figure 1. Characterization of *dth9* Plants and Comparison with Wild-Type Arabidopsis Plants Containing *CEVI-1*::*GUS*.

(A) Histochemical staining of GUS activity in a 12-day-old wild-type transgenic seedling grown on Murashige and Skoog (MS; see Methods) agar medium.
(B) GUS expression of a *dth9* mutant plant grown as in **(A)**.
(C) Fully expanded rosette leaf from a wild-type transgenic plant stained for GUS activity.
(D) Fully expanded rosette leaf from a *dth9* plant stained for GUS activity.
(E) Twelve-day-old Arabidopsis wild-type seedlings grown on MS plates containing (right) or not containing (left) 0.2 μM 2,4-D for 12 days.
(F) Twelve-day-old Arabidopsis *dth9* mutant seedlings grown on MS plates containing (right) or not containing (left) 0.2 μM 2,4-D for 12 days.
wt, wild type.

ing the *CEVI-1*::*GUS* transgene and then analyzed the progeny. In the F_1 plants resulting from this cross, constitutive expression of GUS was absent in all 27 seedlings tested; in the F_2 plants, expression was present in 19 of 90 seedlings. The F_2 segregation ratio of the phenotype conferred by *dth9* was 1:3.7 (constitutive expressers:nonexpressers), and the χ^2 value calculated for goodness of fit to a single recessive nuclear mutation was 0.53 ($0.1 > P > 0.5$).

To define the chromosomal map position of *DTH9*, we crossed the *dth9* mutant, which is in a Columbia (Col-0) background, to wild-type Landsberg *erecta* (L*er*), and F_2 seedlings were phenotyped and examined using simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994). DNA was isolated from 31 *dth9* homozygous plants, and segregation of SSLP markers indicated that *dth9* showed linkage to the Nga1126 marker on chromosome 2. Of 62 chromosomes analyzed, 12 showed L*er* alleles (12 L*er*; 50 Col-0; data not shown).

dth9 Mutants Have Enhanced Susceptibility to Peronospora

The *CEVI-1* gene has been shown to be induced in susceptible tomato plants infected with viral pathogens, and its expression correlated spatiotemporally with that of other defense-related genes (e.g., *PR* genes) in diseased plants (Mayda et al., 2000). However, the expression of *CEVI-1* was not mediated by SA, ethylene, or jasmonate, thus favoring the interpretation that the signaling cascade controlling *CEVI-1* expression runs parallel to that of other defense cascades activated during disease (Mayda et al., 2000).

To determine whether the increased amounts of GUS expression driven by the *CEVI-1* promoter in *dth9* plants correlated with alteration of the disease resistance state of the plant, we tested the response of *dth9* plants to the obligate biotroph Peronospora (Figure 2).

We inoculated 25 *dth9* plants as well as 25 wild-type parental plants with the Peronospora isolate NOCO (10^5 conidiospores per milliliter), which is virulent on Arabidopsis Col-0, and the appearance of conidiospores was scored for as long as 7 days later (Figures 2A and 2B). Whereas sporulation occurred on only 40% of the leaves from wild-type plants, 90 to 100% of the leaves from *dth9* plants demonstrated its presence. Evaluation of the infection with a dissecting microscope revealed that the *dth9* mutants became so heavily colonized that they consisted almost only of oomycetes. Therefore, even the susceptible Col-0 wild-type plants become even more susceptible when the *dth9* mutation is present.

When the plants were similarly inoculated with the Peronospora isolate EMWA, which is avirulent on Arabidopsis Col-0, the wild-type plants were completely resistant, they reacted with an HR, and no sporulation was observed (Figure 2C). In contrast, the *dth9* mutants showed a strong shift toward susceptibility, as demonstrated by extensive colonization of the plants (Figures 2D and 2E). On the plant side,

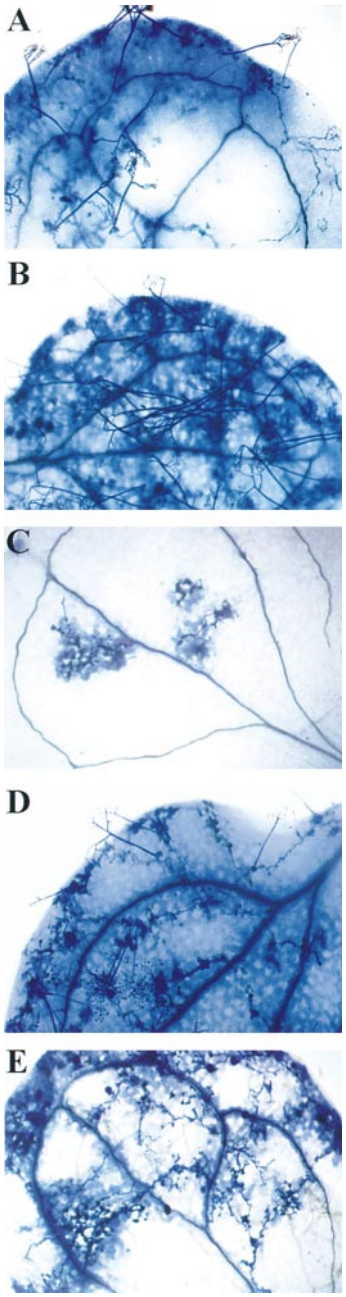

Figure 2. Resistance Response of Wild-Type and *dth9* Mutant Arabidopsis Plants to Peronospora Isolates.

Seven days after spray inoculation of 2-week-old plants with 10^5 conidiospores per milliliter of Peronospora isolates, cotyledons were stained with lactophenol–trypan blue and viewed under a microscope.
(A) and **(B)** Plants inoculated with virulent isolate NOCO: wild-type plant with blue staining of hyphae and conidiospores **(A)** and *dth9* mutant with blue staining of hyphae, conidiospores, and oospores **(B)**.
(C) to **(E)** Plants inoculates with avirulent isolate EMWA: wild-type plant with blue staining of typical HR reactions **(C)** and *dth9* mutant with blue staining of hyphae with trailing necrosis, conidiospores, and oospores (**[D]** and **[E]**).

cell death occurred in the form of trailing necrosis that followed the growing hyphae, as revealed by intense retention of trypan blue in the vicinity of the hyphae. However, these plant defense reactions seem to be insufficient, because Peronospora was able to complete its cycle, forming sexual oospores and asexual conidia on conidiophores. Because the growth of Peronospora EMWA in the *dth9* plants always was associated with necrosis on the plant side, the resistance to EMWA was lost only in part.

The *dth9* Mutant Is More Susceptible to *Pseudomonas syringae*

Increased susceptibility of *dth9* plants to pathogens was investigated further by using the virulent bacterial pathogen *P. syringae* pv *maculicola* ES4326 (see Methods). After infection of wild-type and *dth9* plants with *P. s. maculicola* ES4326, the growth rate of these bacteria was monitored in extracts from infected leaves; the resulting growth curves are shown in Figure 3A. The number of *P. s. maculicola* ES4326 detected in *dth9* plants was more than 10-fold greater than the number in wild-type plants after 3 to 5 days of growth (cf. *wt*[L] and *dth9*[L] growth curves in Figure 3A).

To study this susceptibility of *dth9* plants to the virulent bacteria in more detail, we addressed whether or not the *dth9* mutant could be compromised in the SAR response. We inoculated wild-type and *dth9* plants with the avirulent bacteria *P. syringae* pv *tomato* DC3000 *AvrRpm1*, which elicit an HR response in the inoculated leaf. Then, 3 days after this first inoculation, we challenged other leaves of the same plants with the virulent *P. s. maculicola* ES4326 strain. The plants were examined visually for disease symptoms as well as for growth of the newly inoculated *P. s. maculicola* ES4326.

The bacterial titer of *P. s. maculicola* ES4326 in the wild-type plants expressing SAR after the first inoculation with the incompatible bacteria was observed to decrease with time (Figure 3A: cf. *wt*[L] with *wt*[SAR] growth curves). This suggested that SAR developed appropriately in the wild-type plants. Conversely, the number of *P. s. maculicola* ES4326 in the *dth9* mutants was 100-fold greater after 5 days of growth. Additionally, as shown for leaves photographed 5 days after the second inoculation with virulent bacteria (Figure 3B), severe chlorosis was observed in *dth9* plants, whereas symptoms were absent in wild-type plants. These results suggested an effect of the *dth9* mutation in interfering with the normal development of SAR as elicited by the avirulent *P. s. tomato* DC3000 *AvrRpm1* pathogen.

Endogenous Amounts of SA Are Not Affected in *dth9* Plants

Because SA is the master regulatory molecule necessary for the normal activation of defenses and SAR response, measurements were made to determine whether the amount of

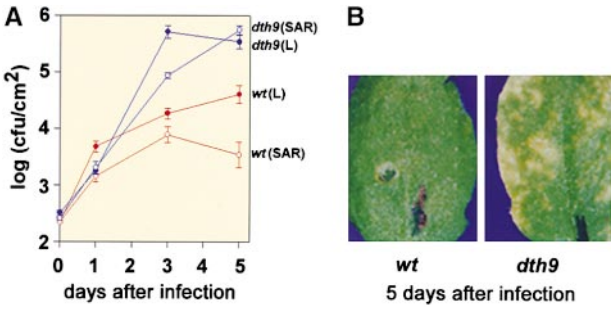

Figure 3. Growth of *P. s. maculicola* ES4326 in Wild-Type and *dth9* Plants during Local and SAR Responses.

(A) Bacterial growth curves. For local response studies (L), wild-type and *dth9* plants were infected by infiltrating leaves with a *P. s. maculicola* ES4326 suspension at a 500-fold dilution (10^5 cfu/mL) at OD_{600} = 0.1. Bacterial titer was determined at 0, 1, 3, and 5 days after infection. For SAR response studies (SAR), two leaves per plant first were infected by infiltration with a suspension of *P. s. tomato* DC3000 carrying *avrRpm1* (OD_{600} = 0.1); 3 days after this initial infection, the distal leaves were inoculated with a *P. s. maculicola* ES4326 suspension at a 500-fold dilution (10^5 cfu/mL) at OD_{600} = 0.1. Bacterial titer was determined at 0, 1, 3, and 5 days after the second inoculation. Error bars represent 95% confidence limits of log-transformed data. Eight samples were taken for each genotype at each time noted. The experiment was repeated three times with similar results. cfu, colony-forming unit.
(B) Symptoms of bacterial infection in local leaves from wild-type (left) and *dth9* (right) plants at 5 days after inoculation with *P. s. maculicola* ES4326.
wt, wild type.

endogenous SA was affected in *dth9* plants. Free SA and conjugated salicylate glucoside (SAG) concentrations were examined in leaf tissues from *dth9* and wild-type plants (Figure 4). In healthy noninoculated leaves of *dth9* plants, the basal amount of SA was 1.7-fold greater than that observed in wild-type plants, the same difference observed when SAG contents were analyzed. In infected leaf tissue at 4 days after inoculation with *P. s. maculicola* ES4326, the amounts of free SA increased twofold in wild-type plants and 1.5-fold in *dth9* plants (Figure 4). Similar increases in SAG were observed in both cases (Figure 4). This finding suggests that the increased susceptibility to pathogens observed in *dth9* plants was not the result of major defects in SA production.

Defense-Related Genes Are Normally Induced in *dth9* Plants

Common to the SAR response is the activation of defense-related genes (e.g., *PR* genes). Because no gross differences were observed in SA contents, we thought it possible that the SAR defect observed in *dth9* plants was located downstream from SA, as observed in *npr1/nim1* plants, which are also unusually susceptible to pathogen infection and do not express *PR* genes (Cao et al., 1994, 1997). To study this possibility, we inoculated wild-type and *dth9* mutant plants with *P. s. tomato* DC3000 *AvrRpm1* and determined by RNA gel blot analysis the extent and duration of the activation of the *PR* genes in both pathogen-treated leaves and nontreated systemic leaves. As shown in Figure 5, expression of *PR1* and *PR2* was highly induced in both pathogen-inoculated leaves and noninoculated systemic leaves. In wild-type plants and *dth9* plants, the kinetics and intensity of *PR* induction were very similar. These results suggest that contrary to observations in other SAR-compromised mutants (e.g., *npr1/nim1*), *dth9* mutant plants are not blocked in the induction of *PR* genes.

Penninckx et al. (1998) reported an SA-independent resistance pathway in Arabidopsis that is characterized by the jasmonate-inducible *PDF1.2* gene. To determine whether dth9 had a defect on the induction of this jasmonate-dependent pathway, we hybridized the RNAs mentioned above with the PDF1.2 probe. Figure 5 shows that wild-type and dth9 plants expressed PDF1.2 transiently when inoculated with P. s. tomato DC3000 *AvrRpm1*. This suggests that dth9 plants are not compromised in the signal pathway that activates PDF1.2.

Exogenous SA Induces *PR* Gene Expression in *dth9* but Does Not Complement the Compromised SAR Response

The fact that normal *PR* gene expression took place in *dth9* plants after pathogen inoculation does not completely rule

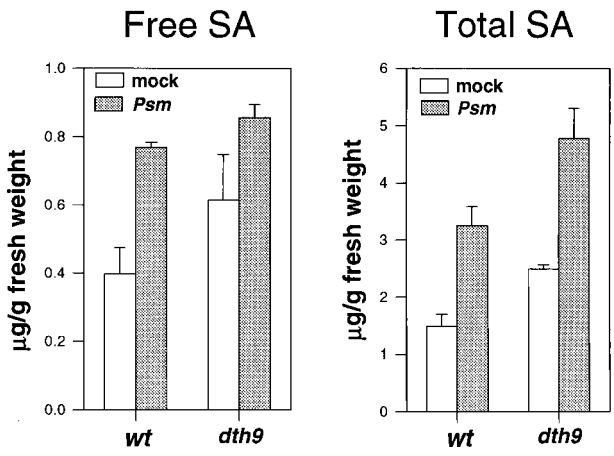

Figure 4. SA and SAG Contents in Wild-Type and *dth9* Plants.

Wild-type (*wt*) and *dth9* plants were inoculated with *P. s. maculicola* ES4326 (*Psm*) or 10 mM $MgCl_2$ (mock). Each bar represents the mean of five replicate samples. Error bars represent SD. SA and SAG were assayed in the same samples.

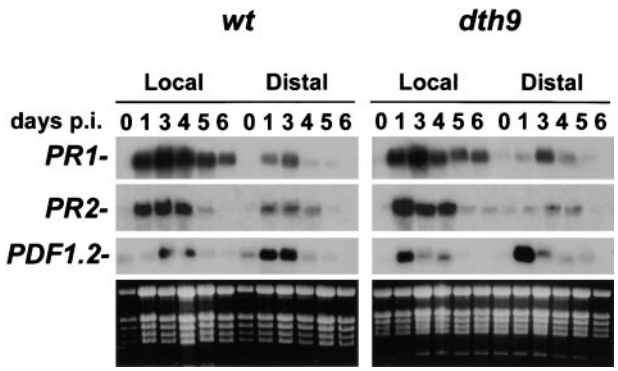

Figure 5. Defense Gene Expression in Local (Inoculated) and Distal (Noninoculated) Leaves of Wild-Type and *dth9* Plants at Different Times after Infection with *P. s. tomato* DC3000 Carrying *avrRpm1*.

Leaves were excised at 0, 1, 3, 4, 5, and 6 days after infection (p.i.), and the accumulation of *PR1*, *PR2*, and *PDF1.2* mRNAs was tested. The experiment was repeated twice with similar results. wt, wild type.

out the possibility that these genes could be activated in an SA-alternative or SA-independent pathway. To determine whether or not *dth9* mutant plants can perceive SA, and also whether SA could restore the compromised SAR response, we treated wild-type and *dth9* plants with 1 mM SA, and the induction of *PR* gene expression and the activation of SAR were studied. Figure 6A shows that after exposure of plants to SA, both wild-type and *dth9* plants had high and comparable expressions of *PR* genes. However, when the growth curves of *P. s. maculicola* ES4326 were compared (Figure 6B), we observed that although SAR was induced effectively by SA in wild-type plants, the *dth9* mutant still failed to develop the SAR phenotype. The growth of the bacteria in SA-treated *dth9* plants was comparable to that observed in plants before treatment with SA.

The fact that the exogenous application of SA could promote the expression of *PR* genes but not complement the defect in SAR in *dth9* plants suggests that the defective SAR response observed in this mutant was not caused by a lack of perception of SA.

The *dth9* Mutant Accumulates Camalexin Normally

The accumulation of the phytoalexin camalexin is important for a plant's defense system, and some mutants impaired in camalexin production show marked increases in susceptibility to pathogens (Glazebrook and Ausubel, 1994; Glazebrook et al., 1996; Thomma et al., 1999). Furthermore, because camalexin is an indole-type compound with some similarities to auxin, we tested whether or not camalexin production could be altered in *dth9* plants. Determination of camalexin content in mock-inoculated and *P. s. tomato* DC3000–inoculated plants revealed no marked differences between wild-type and *dth9* plants with regard to camalexin accumulation (Table 1). Wild-type plants responded to the bacterial infection with a 148-fold induction of camalexin content; *dth9* plants responded with a 113-fold induction. This finding suggests that the pathway controlling camalexin production is not affected in *dth9* plants.

DISCUSSION

The constitutive expression of *CEVI-1*::*GUS* in the Arabidopsis *dth9* mutant is similar to that observed in virally infected *CEVI-1*::*GUS* transgenic tomato plants (Mayda et al., 2000). This similarity indicates that the constitutive expression of GUS activity in intact *dth9* plants probably results from a mutation in the same signaling pathway that controls its induction during disease in tomato. Furthermore, the absence of constitutive expression of *PR* genes in this mutant supports the notion that the *dth9* mutation is distinct from mutations resulting in constitutive upregulation of SA-inducible genes (e.g., *cpr* [Bowling et al., 1994]).

To determine the effect of the *dth9* mutation on suscepti-

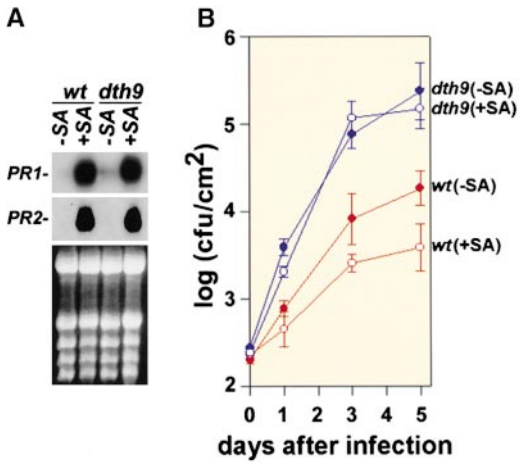

Figure 6. Exogenous SA Induced *PR* Gene Expression in Wild-Type and *dth9* Plants but Enhanced Resistance to *P. s. maculicola* ES4326 in Wild-Type Plants Only.

Plants were sprayed with 1 mM SA or with buffer alone until uniformly wet.

(A) *PR1* and *PR2* gene expression in response to SA. Samples were taken 2 days after spraying with SA (+SA) or with buffer (−SA).

(B) Effect of spraying plants with SA or buffer alone on *P. s. maculicola* ES4326 growth. Two days after treatment, plants were infected with *P. s. maculicola* ES4326 at a 500-fold dilution (10^5 cfu/mL) at $OD_{600} = 0.1$. Bacterial titer was determined at 0, 1, 3, and 5 days after infection. Each symbol represents the mean and SD of eight replicates. The experiments were repeated three times with similar results. cfu, colony-forming unit. −SA, plants not treated with SA before infection; +SA, plants treated with SA before infection; wt, wild type.

Table 1. Accumulation of Camalexin in Leaves of Wild-Type and *dth9* Plants[a]

Treatment	Camalexin[b]	Induction
dth9 + $MgCl_2$	40.7 ng/g FW	
dth9 + *P.s.* DC3000	4587.7 ng/g FW	113-fold
wt + $MgCl_2$	24.6 ng/g FW	
wt + *P.s.* DC3000	3640.6 ng/g FW	148-fold

[a] Four weeks after sowing, plants were vacuum-infiltrated (30 sec) with a suspension (in 10 mM $MgCl_2$) of *P. syringae* pv *tomato* DC3000 at a concentration of 10^5 cfu/mL. Camalexin levels were determined 2 days after infection according to Nawrath and Métraux (1999). The experiment was repeated twice with similar results.
[b] The camalexin content in each sample is expressed as nanograms per gram of fresh tissue. Each value is calculated from the peak height of each HPLC chromatogram and compared with o-anisic acid and SA standards.
FW, fresh weight; wt, wild type.

bility to pathogens, we examined the growth of two normally virulent pathogens. *dth9* plants that had not been treated with any resistance inducers were challenged with either the fungal pathogen Peronospora NOCO or the bacterial pathogen *P. s. maculicola* ES4326. For both pathogens, *dth9* plants showed greater susceptibility than did the parental wild-type plants, allowing the pathogens to grow massively (Figures 2A, 2B, and 3A).

To extend these studies in more detail, we determined whether or not activation of SAR also was affected in this mutant. After inoculating *dth9* plants with the avirulent pathogen *P. s. tomato* DC3000 *AvrRpm1* to elicit HR, we determined the extent of SAR responses by inoculating other leaves from the same plants with the virulent pathogen *P. s. maculicola* ES4326. These studies revealed that SAR was compromised in *dth9* plants, which, unlike the wild-type plants, were unable to stop the growth of the bacteria (Figure 3). The inoculated leaves of *dth9* plants, but not those of wild-type plants, very early developed the characteristic symptoms (e.g., pronounced chlorosis) of massive growth of the bacteria, further reinforcing the notion that the *dth9* mutants failed to mount a proper SAR response.

SA is required for the induction of *PR* genes and for the activation of SAR responses (Gaffney et al., 1993). The pathway transducing the SA signal has been marked by different Arabidopsis mutants that represent only one genetic locus, designated *NPR1/NIM1/SAI1*. This type of mutant is nonresponsive to induction of SAR by avirulent pathogens, does not respond to SA, and is a nonexpresser of *PR* genes (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). Thus, the inability of *dth9* plants to mount SAR could result initially from a defect in the downstream regulatory factor (e.g., *NPR1/NIM1/SAI1*) that transduces the SA signal to activate *PR* genes. However, our observation that in *dth9* plants, the spatiotemporal expression of *PR* genes after infection with an avirulent pathogen (Figure 5) took place the same as in wild-type plants excludes this possibility. Furthermore, the exogenous application of SA also induced the expression of *PR1* and *PR2* in *dth9* plants but was not able to correct the defective SAR response in this mutant (Figure 6). This finding favors the interpretation that SA functions normally for the activation of *PR* genes in *dth9* plants and is consistent with the proposal that *DTH9* operates upstream of SA or independently of *NPR1/NIM1/SAI1* and *PR* gene expression or possibly both. Alternately, the inability of *dth9* plants to mount SAR could reflect a defect in the synthesis and accumulation of SA without affecting the *NPR1/NIM1/SAI1* locus. However, the observation that SA metabolism is not affected in *dth9* plants (Figure 4) excludes the possibility that the observed compromised SAR response in *dth9* plants is the result of a defect in SA metabolism.

Like *dth9*, the *eds* mutants (Glazebrook et al., 1996; Parker et al., 1996; Rogers and Ausubel, 1997; Volko et al., 1998) also show an altered susceptibility to virulent pathogens. However, at variance with *dth9*, some *eds* mutants are still capable of mounting a SAR response if they have been inoculated with an avirulent pathogen (Rogers and Ausubel, 1997). This finding suggests that *dth9* and *eds* mutants represent different genes.

Another category of mutants with increased susceptibility to pathogens is represented by the phytoalexin-deficient (*pad*) mutants (Glazebrook and Ausubel, 1994). In particular, the *pad4* mutant resembles the *dth9* mutant in that SA can still induce defense gene expression (Glazebrook et al., 1996; Zhou et al., 1998). However, although *dth9* plants accumulate normal amounts of SA (Figure 4) and camalexin (Table 1), the *pad4* mutant is impaired in the synthesis of these two compounds (Zhou et al., 1998), suggesting that *dth9* and *pad4* mutants are different.

The *sid* mutants also have been described as more susceptible to pathogens (Nawrath and Métraux, 1999), but unlike *dth9*, the *sid* mutants do not accumulate SA and the expression of *PR1* is strongly reduced.

Studies of the response of *dth9* plants to the incompatible Peronospora isolate EMWA yielded an interesting observation. For this pathogen, *dth9* plants show a reduction in resistance. However, this effect is only partial because the pathogen growth is followed by the development of massive cell death along hyphal tracks (trailing necrosis), suggesting that the *R* gene action in the mutant is only delayed. This phenotype is similar to that observed in *nahG* plants (Nawrath and Métraux, 1999) and also in the dominant *phx3* mutant, which functions as a suppressor of the *lsd5* cell death mutation (Morel and Dangl, 1999). However, *dth9* plants allow the incompatible pathogen to complete its life cycle and sporulate, whereas the *phx3* mutant does not. Interestingly, and although not as evident as for *dth9*, the *phx3* mutant also shows some enhanced susceptibility to virulent *P. syringae*. Furthermore, because *dth9* and *phx3* retain the ability to express *PR* genes after treatment with SA, both mutants probably act upstream or independently

of the point of action of SA. These observations indicate that the dominant *phx3* mutant and the recessive *dth9* mutant may participate in a similar signal transduction pathway evolved to limit pathogen growth.

From the phenotype conferred by the *dth9* mutation, we can speculate on the function of the wild-type *DTH9* gene. DTH9 could function as a regulator of disease susceptibility operating upstream or independently of SA (Figure 7A). When this regulator is inactivated, the basic mechanism of resistance is blocked and, consequently, an increase in susceptibility to pathogens is primed. Such a factor could be inactivated permanently in *dth9* plants, resulting in enhanced susceptibility to pathogens and the inability to mount an effective SAR response. A mutation of this type most likely would be recessive. Indeed, genetic analysis of progeny from a *dth9*/*dth9* × *DTH9*/*DTH9* backcross demonstrated that *dth9* is a recessive mutation. Interestingly, pleiotropy is evident in the *dth9* mutant, which is also insensitive to the auxin analog 2,4-D. Thus, the same DTH9 could be required for proper auxin sensitivity (Figure 7B). When this regulator is inactivated, a discrete auxin insensitivity character would be primed, leading to the expression of the *CEVI-1* gene. Thus, we speculate that *DTH9* could function as a regulator in both the pathogenic pathway and certain aspects of the auxin signaling pathway. This might indicate the existence of common components in certain aspects of the pathway that transduces auxin signals and that activates an effective resistance response, as suggested previously (Mayda et al., 2000).

In conclusion, the identification of the *dth9* mutant provides new information on the complex mechanism or mechanisms that control the susceptibility of plants to pathogens and adds support to the notion that a complex, interrelated network of signaling pathways has evolved in plants. Characterization of the *DTH9* gene should provide further information on the elements involved in controlling the susceptibility to disease in plants.

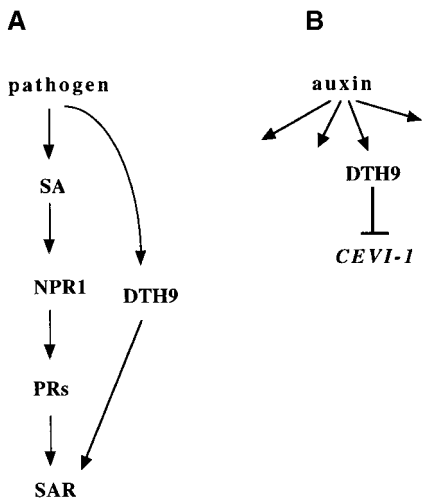

Figure 7. Model for Putative Roles of DTH9.

(A) DTH9 contributes in an SA-independent manner to promote an effective defense response when a pathogen is perceived.
(B) DTH9 mediates certain aspects of auxin perception.
One model does not necessarily exclude the other, and both pathways can operate in the same plant.

METHODS

Plants and Growth Conditions

Arabidopsis thaliana plants were grown on soil or on plates containing Murashige and Skoog (1962) (MS) medium, as described previously (Mayda et al., 2000). The *dth9* mutant was isolated in a screen for constitutive expressers of the *CEVI-1*::*GUS* reporter gene in transgenic Columbia (Col-0) plants mutagenized with ethyl methanesulfonate, as described by Mayda et al. (2000). The *dth9* mutant line used in these experiments has been backcrossed twice to the wild-type parental line. Plants were grown in a growth chamber (20 to 22°C, 85% relative humidity (RH), 100 $\mu E/m^{-2}$ sec^{-1} fluorescent illumination) on a 14-hr-light/10-hr-dark cycle. Unless indicated otherwise, fully expanded leaves of 4-week-old plants were used for all experiments.

To determine the response of *dth9* and Col-0 parental line seedlings to 2,4-dichlorophenoxy acetic acid (2,4-D), surface-sterilized seed were germinated on MS plates supplemented with 0.2 μM 2,4-D, and the effects were monitored as described (Maher and Martindale, 1980).

Bacteria Inoculations and Quantification of Infection

Pseudomonas syringae pv *maculicola* ES4326 was grown at 28°C on King's B agar plates or in King's B liquid medium supplemented with 100 μg/mL streptomycin for selection. Bacteria then were collected by centrifugation, washed twice, and resuspended at $OD_{600} = 0.1$ in a solution of 10 mM $MgCl_2$. Wild-type and *dth9* plants were grown on soil for 4 weeks and inoculated with the bacterial suspension at a 1:500 dilution by infiltration on the abaxial surface of leaves, using a 1-mL syringe without a needle. *P. syringae* pv *tomato* DC3000 carrying the avirulence gene *AvrRpm1* was grown similarly, and selection was performed on medium containing 50 μg/mL kanamycin and 50 μg/mL rifampicin. Three leaf discs (~100 mg) from infiltrated leaves and from 10 independent plants were excised from independent leaves at the indicated times after inoculation. The discs were divided randomly into sets of three and macerated in 10 mM $MgCl_2$. The density of the bacterial populations was determined by plating serial dilutions on King's B medium supplemented with streptomycin (50 μg/mL) at 28°C and counting the colony-forming units (cfu). Data are reported as means and SD of the log (cfu/cm^2) of at least six replicates.

For chemical treatments, plants were sprayed with 1 mM salicylic acid (SA) in phosphate buffer, pH 7.1, or with buffer alone.

Inoculation with *Peronospora parasitica*

Peronospora isolate NOCO was transferred every week on 2- to 3-week-old Arabidopsis Col-0 plants by spray inoculation with a spore

suspension. Peronospora isolate EMWA was cultivated every week on Arabidopsis Wassilewskija plants. Plants inoculated with Peronospora were kept in a 12-hr-light/12-h-dark cycle with a night temperature of 19°C for the first day and the last day of the growth cycle; plants were kept in 100% RH to ensure infection and sporulation, respectively.

For resistance experiments, wild-type parental plants and *dth9* mutant plants were sprayed with a suspension of 10^5 conidiospores per milliliter of Peronospora isolate NOCO or EMWA and incubated as described above. Plants were examined for sporulation, or leaf samples were stained with lactophenol–trypan blue at different intervals (days) after inoculation and examined under a microscope (Koch and Slusarenko, 1990).

RNA Gel Blot Analysis

Tissue samples of four to five leaves were collected, frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted, and 5 μg of total RNA per sample was separated on a formaldehyde–agarose gel (Mayda et al., 2000). Ethidium bromide was added to each sample to allow visualization of RNA under UV light for confirmation of equal sample loading. ^{32}P-labeled DNA probes for *PR1*, *PR2*, and *PDF1.2* were prepared, and filters were hybridized as described (Mayda et al., 2000).

Genetic Analysis

Crosses were performed by dissecting and emasculating unopened buds and then using the pistils as recipients for pollen from five opened flowers. Backcrosses with the parental *CEVI-1*::*GUS* transgenic line were performed by using *CEVI-1*::*GUS* plants as the pollen donor. The reciprocal crosses also were performed. F_1 and F_2 plants were grown on MS plates and tested for β-glucuronidase (*GUS*) activity as described previously (Mayda et al., 2000). Segregation of constitutive *GUS* activity in the F_2 generation was analyzed with the χ^2 test for goodness of fit.

Polymerase Chain Reaction–Based Mapping

A *dth9* plant (in the Col-0 background) was crossed with a Landsberg *erecta* (L*er*) plant, and the progeny that segregated *dth9* homozygous mutants when selfed were used for mapping. Thirty-five seedlings in the F_2 population were selected for DNA extraction, and recombinant seedlings were identified by using simple sequence length polymorphism (SSLP) markers according to the protocol described by Bell and Ecker (1994) as well as new markers reported on the Arabidopsis database World Wide Web site (http://genome-www.stanford.edu).

Determination of Endogenous Amounts of SA and Camalexin

To determine how much free and conjugated SA was present, we infected leaves of 4-week-old wild-type plants and *dth9* plants with *P. s. maculicola* ES4326 or mock-inoculated them with 10 mM $MgCl_2$ as described above. Four days after inoculation, samples were collected (1 g of tissue per sample from six plants) and frozen in liquid nitrogen. SA and salicylate glucoside (SAG) were determined as described previously (Mayda et al., 1999).

For analysis of camalexin, leaves of 4-week-old wild-type plants and *dth9* plants were vacuum-infiltrated for 30 sec with a suspension (in 10 mM $MgCl_2$) of *P. s. tomato* DC3000 at a concentration of 10^5 cfu/mL. To achieve better penetration, we added 10 μL/100 mL Silwet L-77. The control plants were infiltrated only with $MgCl_2$ containing Silwet L-77. Camalexin amounts were determined 2 days after infection according to Nawrath and Métraux (1999).

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