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Salicylate-Independent Lesion Formation in *Arabidopsis lsd* Mutants

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In many interactions of plants with pathogens, the primary host defense reaction is accompanied by plant cell death at the site of infection. The resulting lesions are correlated with the establishment of an inducible resistance in plants called systemic acquired resistance (SAR), for which salicylic acid (SA) accumulation is a critical signaling event in *Arabidopsis* and tobacco. In *Arabidopsis*, the lesions simulating disease (*lsd*) mutants spontaneously develop lesions in the absence of pathogen infection. Furthermore, *lsd* mutants express SAR marker genes when lesions are present and are resistant to the same spectrum of pathogens as plants activated for SAR by necrogenic pathogen infection. To assess the epistatic relationship between SA accumulation and cell death, transgenic *Arabidopsis* unable to accumulate SA due to the expression of the salicylate hydroxylase (*nahG*) gene were used in crosses with the dominant mutants *lsd2* or *lsd4*. Progeny from the crosses were inhibited for SAR gene expression and disease resistance. However, these progeny retained the spontaneous cell death phenotype similar to siblings not expressing *nahG*. Because lesions form in the absence of SA accumulation for *lsd2* and *lsd4*, a model is suggested in which lesion formation in these two mutants is determined prior to SA accumulation in SAR signal transduction. By contrast, the loss of SAR gene expression and disease resistance in *nahG*-expressing *lsd* mutants indicates that these traits are dependent upon SA accumulation in the SAR signal transduction pathway.

Additional keywords: autofluorescence, callose, feedback amplification loop, *Peronospora parasitica* isolate Noco, PR gene expression.

The correlation between rapid, hypersensitive cell death and plant disease resistance responses has been widely described (Klement 1982; Keen 1982; Keen and Staskawicz 1988). Pathogen-associated cell death is also known to activate an inducible, systemic, broad-spectrum resistance called systemic acquired resistance (SAR; for reviews see Ross 1961; Kuc 1982; Ryals et al. 1994; Chen et al. 1995; Neuenschwander et al. 1996; Hunt and Ryals 1996; Shirasu et al. 1996; Ryals et al. 1996). SAR is correlated with the expression of marker genes and an increase in salicylic acid (SA) levels in both infected and noninfected leaves (Uknes et al. 1992, 1993b). Expression of a bacterial salicylate hydroxylase (*nahG*) transgene in *Arabidopsis* and tobacco inhibits SA accumulation in response to pathogen infection and prevents not only the establishment of SAR (Gaffney et al. 1993; Lawton et al. 1995) but gene-for-gene resistance as well (Delaney et al. 1994).

Necrogenic pathogen infection is required for the biological activation of SAR. However, certain mutations can lead to pathogen-independent activation of the SAR pathway. For example, *Arabidopsis* lesions simulating disease (*lsd*) and accelerated cell death (*acd2*) mutants that form spontaneous lesions exhibit histochemical markers associated with defense-related cell death, show elevated SAR gene expression, and exhibit heightened disease resistance (Dietrich et al. 1994; Greenberg et al. 1994; Weymann et al. 1995). Similar lesioned phenotypes exist in other plant species, including maize (Walbot et al. 1983), barley (Wolter et al. 1993), and tomato (Langford 1948), with the latter two also displaying enhanced disease resistance. A correlation between spontaneous cell death and disease resistance is also evident in transgenic tobacco expressing a bacterial proton pump (Mittler et al. 1995), the A1 subunit of cholera toxin (Beffa et al. 1995), or a ubiquitin variant (Becker et al. 1993). Collectively, these data indicate that many types of cellular perturbation can trigger cell death, and some simultaneously trigger SAR. Because of the similarities between mutant and pathogen-activated SAR in plants, we have utilized *lsd* mutants as a model to explore the epistatic relationship between cell death, SA accumulation, and the induction of SAR genes and resistance. Here we describe results obtained from inhibiting SA accumulation in *lsd2* and *lsd4* plants by *nahG* expression and discuss the implications of these results regarding cell death and SAR signal transduction.

RESULTS

Lesion formation in *lsd* mutants expressing *nahG*.

The dominant mutants *lsd2* and *lsd4* (Dietrich et al. 1994) were used as pollen donors in crosses to transgenic plants expressing salicylate hydroxylase (*nahG*; Gaffney et al. 1993; Lawton et al. 1995). Lesioned F_2 and F_3 progeny were evaluated for the expression of *nahG* by RNA blot analysis with a

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nahG probe. Figure 1 presents *lsd2* and *lsd4* progeny that either contain or lack *nahG*. Lesion morphology in *lsd2* is not altered by the expression of *nahG*. However, *nahG*-containing *lsd4* plants exhibit lesions that are more severe than the lesions exhibited by *lsd4* plants lacking the transgene.

Lesion histology in *lsd2* and *lsd4* mutants expressing *nahG*.

The accumulation of autofluorescent phenolic compounds and deposition of callose are markers associated with lesion formation in response to pathogen invasion (Koga et al. 1980) and lesions associated with *lsd2* and *lsd4* mutations (Dietrich et al. 1994). To assess the role of SA in lesion formation, we examined *lsd2* and *lsd4* progeny that expressed or lacked *nahG* for both the accumulation of autofluorescent material and the deposition of callose. Figure 2 shows micrographs of cross sections through leaf lesions examined by differential interference contrast (DIC) microscopy (column 1: A, D, G, J), ultraviolet light–stimulated autofluorescence (column 2: B, E, H, K), and callose deposition visualized by fluorescence following aniline blue staining (column 3: C, F, I, L). Callose deposition and autofluorescent material accumulation in lesions of *lsd2* plants lacking or expressing *nahG* are similar. However, *lsd4* plants expressing *nahG* contain more callose and autofluorescent material throughout the leaf mesophyll, whereas deposition of these materials is confined to the lower mesophyll in *lsd4* plants lacking *nahG*. Therefore, the more severely lesioned phenotype evident in *lsd4* plants expressing *nahG* is reflected at the histological level.

PR gene expression in *lsd2* and *lsd4* mutants expressing *nahG*.

Expression of the SAR-associated pathogenesis-related (PR) genes PR-1, PR-2, and PR-5 was assessed in *lsd2* and *lsd4* progeny segregating for *nahG*. Expression of these markers has previously been shown to correlate with the establishment of SAR in *Arabidopsis* in response to both biological and chemical SAR activators (Uknes et al. 1993a, 1993b; Lawton et al. 1996). A total of 57 F_3 *lsd2* progeny and 60 F_2 *lsd4* progeny were evaluated for *nahG* expression. Figure 3 shows PR gene expression in a representative subset of four *lsd2* or *lsd4* progeny that either contain (+) or lack (–) *nahG*. High-level PR-1 expression in *lsd2* and *lsd4* is substantially reduced by expression of *nahG*. While PR-2 and PR-5 expression is not as tightly correlated as that of PR-1 with SA accumulation, PR-2 and PR-5 also accumulate to substantially lower levels in *lsd* mutants expressing *nahG*.

Reduction of SA accumulation results in disease susceptibility.

Previously, Dietrich et al. (1994) demonstrated that lesion-positive *lsd2* and *lsd4* mutants were significantly resistant to isolates of *Peronospora parasitica* fully virulent on wild-type parental accessions. To assess the effect of inhibiting SA ac-

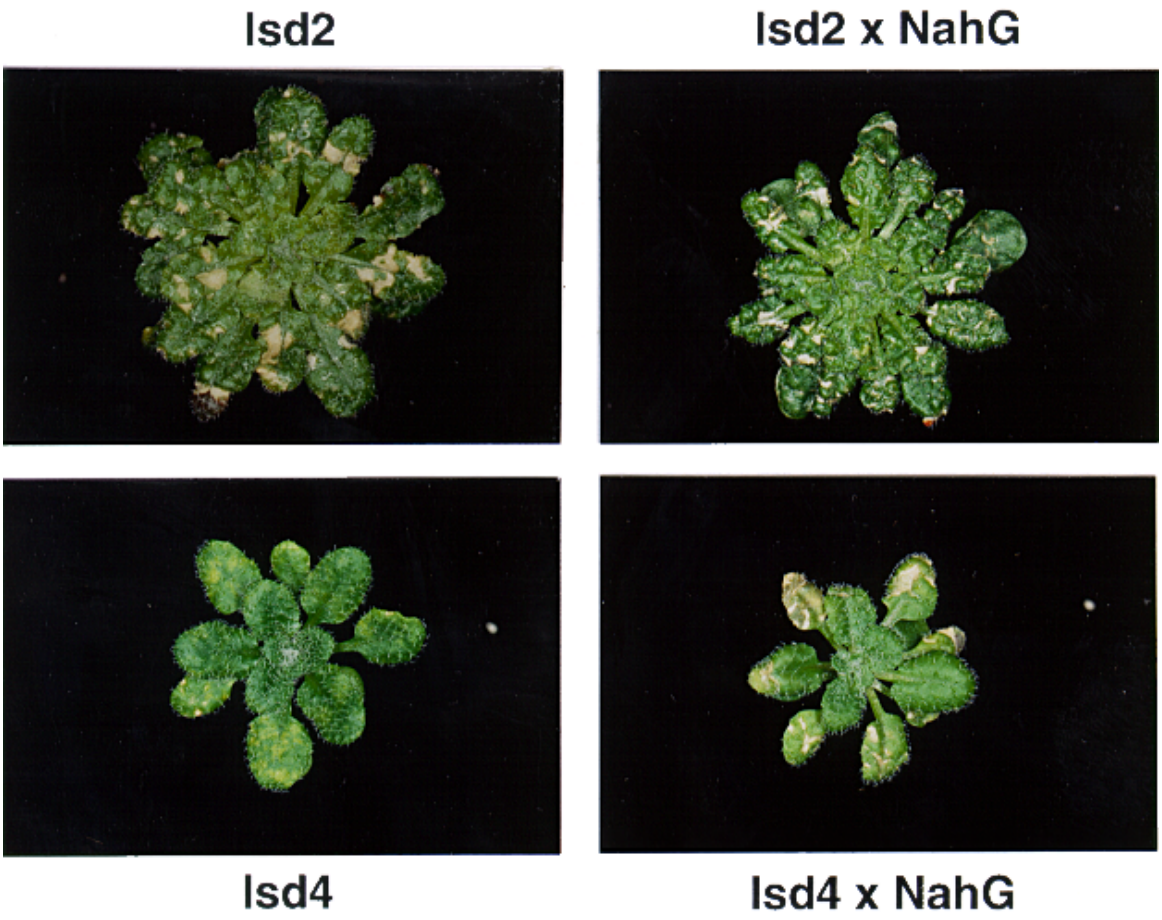

Fig. 1. Visible phenotype of *lsd*/NahG plants. Plant designations are located below and above each panel.

cumulation on the disease-resistant phenotype of *lsd* mutants, we tested *lsd2*/NahG plants by inoculation with the Noco isolate of *P. parasitica*. This fungal isolate is virulent on *Arabidopsis* ecotype Col-0, the parental accession for both *lsd2* and NahG lines. In F_3 populations from this cross, lesioned individuals were identified, a leaf sample was harvested for RNA blot analysis to identify *nahG*-expressing individuals (data not shown), and plants were inoculated with *P. parasitica*. Seven days after infection, plants were evaluated for severity of downy mildew disease. Table 1 shows that *lsd2* plants expressing *nahG* developed more severe disease symptoms than *lsd2* plants without *nahG*. A representative comparison between *lsd2* plants expressing or lacking *nahG* is presented in Figure 4. Because *lsd4* is in the Ws-0 ecotype while *nahG* was transformed into Col-0, similar tests of pathogen resistance in *lsd4*/NahG plants were not possible because fungal isolates virulent on both parental accessions were not available.

DISCUSSION

In this manuscript, we show that *lsd2* and *lsd4* mutants unable to accumulate SA due to *nahG* expression show reduced SAR gene expression and *lsd2* possesses a diminished disease resistance phenotype. However, development of lesions persists in the *nahG*-expressing *lsd2* and *lsd4* mutants. Interestingly, the lesioned phenotype of *lsd4*/NahG progeny is more severe than in siblings lacking *nahG*. The increased amount of callose and autofluorescent material in *lsd4*/NahG lesions is consistent with the more severe lesion morphology observed macroscopically. This correlates well with the observation that more severe lesions are evident in NahG tobacco and *Arabidopsis* plants infected with pathogens (Delaney et al. 1994; Ryals et al. 1995).

Our data indicate that the cell death triggered in *lsd2* and *lsd4* mutants is determined prior to the requirement for SA-dependent processes during the onset of SAR signaling. Additional support for this proposal comes from two previous observations. First, induction of SAR with physiologically effective concentrations of SA or with the synthetic activating chemicals 2,6-dichloroisonicotinic acid (INA; Vernooij et al. 1995) or benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; Friedrich et al. 1996; Lawton et al. 1996; Görlach et al. 1996) does not cause lesions to form in wild-type plants. Second, *Arabidopsis* mutants exist that accumulate SA and display con-

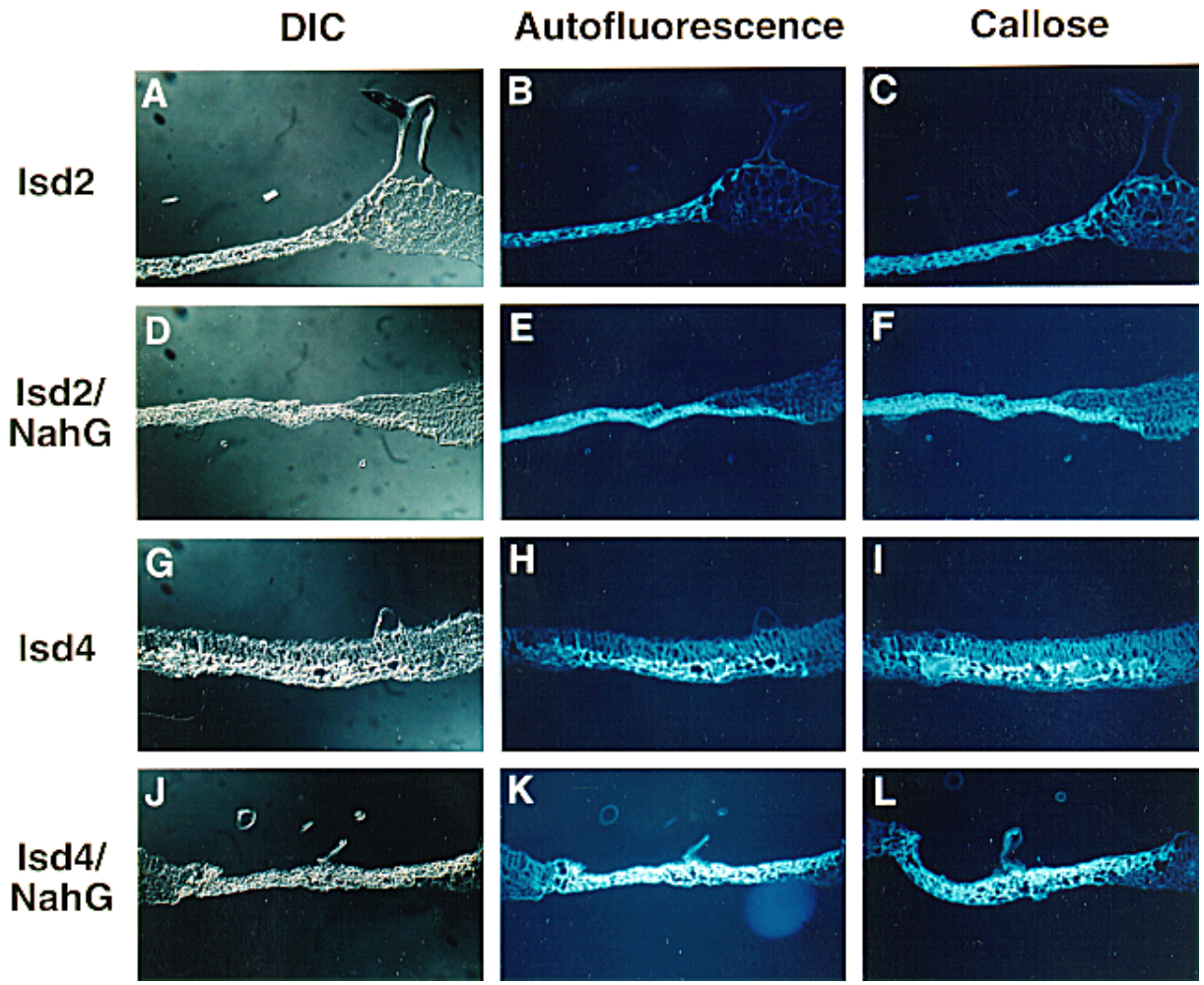

Fig. 2. Histology of lesions. Horizontal rows display leaf morphology from lesions of *lsd2* (**A–C**), *lsd2* /NahG (**D–F**), *lsd4* (**G–I**) , and *lsd4* /NahG (**J–L**). Differential interference contrast images are shown in column one (**A, D, G, J**), ultraviolet light–stimulated autofluorescence in column two (**B, E, H, K**), and secondary fluorescence of callose following aniline blue staining in column three (**C, F, I, L**).

stitutive activation of SAR but are distinguished from the *lsd* and *acd2* mutants by their absence of lesions (Bowling et al. 1994; H.-Y. Steiner, S. Uknes, K. Weymann, D. Chandler, S. Potter, E. Ward, and J. Ryals, *unpublished data*). Together, these data indicate that SA accumulation is not sufficient to cause lesion formation, consistent with a view that lesion formation can be determined prior to SA-dependent processes.

In contrast to the SA-independent lesion development exhibited by *lsd2* and *lsd4*, SA accumulation is required for lesion formation in two additional mutants, *lsd6* and *lsd7* (Weymann et al. 1995). This contradiction indicates that cell death can cause or require SA accumulation, depending upon the nature of the mutation analyzed. These observations are consistent with the existence of a feedback amplification loop that is activated by SA or SA-dependent processes and can modify the initiation of cell death (Jones and Dangl 1996; Hunt and Ryals 1996; Ryals et al. 1996; Dangl et al. 1996). It appears that lesion formation occurs by at least two broadly defined mechanisms: those that are SA-independent and can stimulate SA-requiring processes (*lsd2* and *lsd4*) and those that may require feedback amplification or SA-dependent potentiation of signaling events leading to cell death (*lsd6*/*lsd7* [Weymann et al. 1995] and *lsd1* [U. Neuenschwander, R. A. Dietrich, J. L. Dangl, and J. A. Ryals, *unpublished results*]).

Further support for a feedback loop in the SAR pathway can be found from recent results in tobacco (Mur et al. 1996) or cucumber (Fauth et al. 1996). In tobacco, treatment with SA prior to wounding or pathogen infection enhances or "potentiates" the expression of defense gene promoter fusions. Similarly, wounded cucumber seedlings treated with either SA or INA showed an enhanced rate of H_2O_2 production in response to fungal elicitors. Thus, in both cases early resistance or defense-related responses were conditioned by the activation of downstream components consistent with the existence of a feedback loop in the SAR pathway.

Pathogen-induced lesion formation has often been associated with the presence of defined histological markers (Koga et al. 1988). The deposition of autofluorescent phenolic compounds is one such marker (Mayama and Shishiyama 1976; Koga et al. 1980), as is callose deposition around sites of pathogen-induced lesion formation (Aist and Israel 1977; Görg et al. 1993). Lesion formation in *lsd* mutants leading to disease resistance was also accompanied by accumulation of these histological markers (Dietrich et al. 1994). However, deposition of autofluorescent compounds and callose may not be specific for pathogen-associated cell death leading to SAR. Callose deposition has been documented to result from mechanical injury (Eschrich 1975), bruising (Dekayos 1972), injection of inorganic chemicals (Tighe and Heath 1982), thigmomorphogenesis (Jaffe et al. 1985), and the application of exogenous elements such as copper (Prokipcak and Ormrod 1986). Activation of SAR in response to these treatments has not been fully assessed, but SAR is not activated by mechanical wounding or stress (K. Lawton and S. Potter, *unpublished results*; Bozarth 1962). Lesion formation in response to the application of certain herbicides is also accompanied by the deposition of callose and autofluorescent material. However, there is not a consistent correlation with SAR gene expression and resistance (M. D. Hunt and J. A. Ryals, *unpublished data*; A. Molina, E. Ward, and J. A. Ryals, *unpublished data*). Taken together, these observations indicate that SAR signal transduction is not associated with all cell death events, even those that histochemically resemble pathogen-associated lesions, further illustrating the significance of the loci defined by the *lsd* mutants in dissecting these processes.

PR-1 gene expression in *lsd2* and *lsd4* expressing *nahG* was inhibited; however, PR-2 and PR-5 displayed variable expression in different progeny, exhibiting some degree of SA-

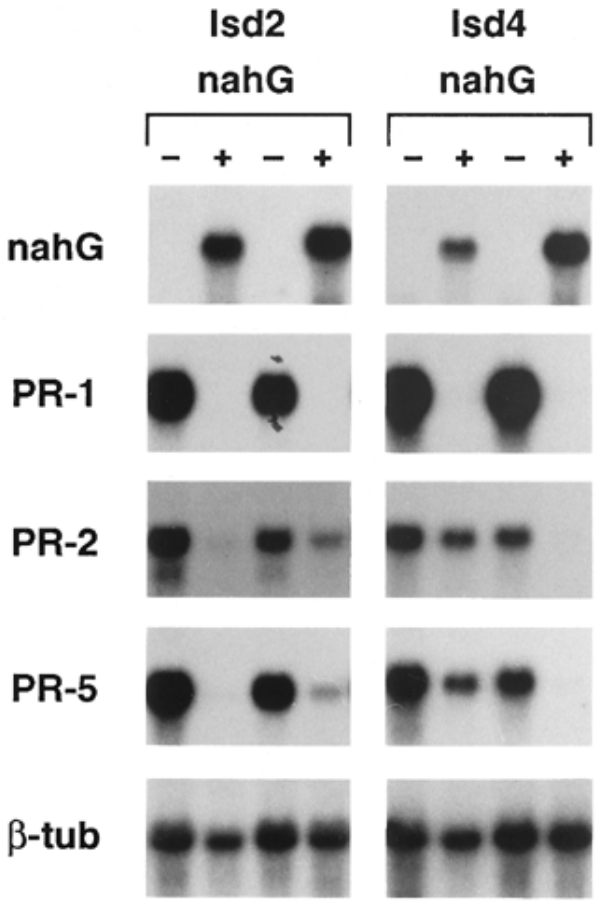

Fig. 3. Systemic acquired resistance (SAR) gene expression in *lsd*/NahG plants. Total RNA was extracted from *lsd2* and *lsd4* lesion-positive progeny that either contained (+) or lacked (–) *nahG* expression. Markers for SAR (PR-1, PR-2, and PR-5) and *nahG* status were used as probes. A β-tubulin probe was used to evaluate RNA loading.

Table 1. Disease rating of *lsd2* and *lsd2*/NahG progeny following infection with *Peronospora parasitica*[a]

	Plants expressing disease levels (no.)[b]						
Plants	**0**	**+**	**++**	**+++**	**++++**	**+++++**	**Total plants**
lsd2	12	2	2	1	0	0	17
lsd2/NahG	0	6	12	9	15	41	83

[a] *lsd2* and *lsd2*/NahG progeny were inoculated with a suspension of *P. parasitica* isolate Noco conidiospores (1×10^5 spores per ml) and rated for disease in individual plants after 10 days.

[b] Scale: 0 = no conidiophores on plant; + = one leaf with 1 to 15 conidiophores; ++ = two leaves with 1 to 15 conidiophores; +++ = one leaf with 15 or more conidiophores; ++++ = two leaves with 15 or more conidiophores; +++++ = more than two leaves with 15 or more conidiophores.

independence. Variable basal and stress-induced expression of PR-2 and PR-5 has been previously documented in *Arabidopsis* (Uknes et al. 1992; Mauch-Mani and Slusarenko 1994). In tobacco, PR-5 expression is elevated in response to methyl jasmonate and ethylene treatment (Xu et al. 1994). Such variability may indicate that PR-2 and PR-5 expression is also induced through signal transduction events effected by various stimuli but not necessarily leading to SAR. Therefore, we conclude that PR-2 and PR-5 mRNA accumulation in *Arabidopsis* is not tightly correlated with SAR activation.

The results presented in this study demonstrate that the cell death controls perturbed in *lsd2* and *lsd4* mutants can trigger SAR but do not depend upon SA for the formation of lesions. By contrast, SAR gene expression and resistance in these mutants depend upon SA accumulation. Identification of the genes affected in these mutants and further genetic dissection of the SAR signal transduction pathway will allow more refined understanding of pathways that control cell death and activate SAR.

MATERIALS AND METHODS

Crosses with NahG *Arabidopsis*.

NahG *Arabidopsis* was generated as described in Delaney et al. (1994). *lsd2* and *lsd4* were isolated as described in Dietrich et al. (1994). Dominant *lsd2* and *lsd4* mutants were used as pollen donors in crosses to a homozygous NahG line. Successful F_1 crosses were verified by the presence of lesions. F_2 seeds from the *lsd2* × NahG F_1 plants were plated on germination medium as described in Weymann et al. (1995) supplemented with 50 μg of kanamycin per ml to identify *nahG*-expressing progeny. Lesion-positive plants were transferred to soil and F_3 seed was collected and used for the reported experiments. F_2 seed harvested from *lsd4* × NahG F_1 plants was used for all experiments.

RNA isolation and analysis.

Whole plants or leaf samples were harvested and frozen in liquid nitrogen. After plants and leaf samples were ground to powder, RNA was extracted as described by Verwoerd et al. (1989). Electrophoresis of RNA through agarose-formaldehyde gels, transfer to Gene Screen Plus nylon membranes (New England Nuclear, Boston), PR gene probes (PR-1, PR-2, and PR-5 cDNAs), and washing conditions have been described (Uknes et al. 1992). The *nahG* gene was described in Gaffney et al. (1993) and the β-tubulin gene probe was described in Delaney et al. (1995).

Histochemistry and microscopy.

Leaf material for sectioning was fixed and sectioned as described in Dietrich et al. (1994). Leaf sections were mounted on microscope slides and de-waxed by two successive 5-min washes in xylene. Samples were rehydrated by 5-min incubations in 100, 100, 75, and 40% ethanol, and water, respectively. For callose staining, samples were treated for 5 min in 0.15 M K_2HPO_4 plus 0.01% aniline blue. Samples were observed by ultraviolet epifluorescence as described in Dietrich et al. (1994).

Peronospora parasitica assays.

Peronospora parasitica isolate Noco was maintained and inoculated as described previously (Uknes et al. 1993b). *lsd2* × NahG progeny were 4 weeks old prior to inoculation with a suspension of 1×10^5 conidiospores ml^{-1} of water.

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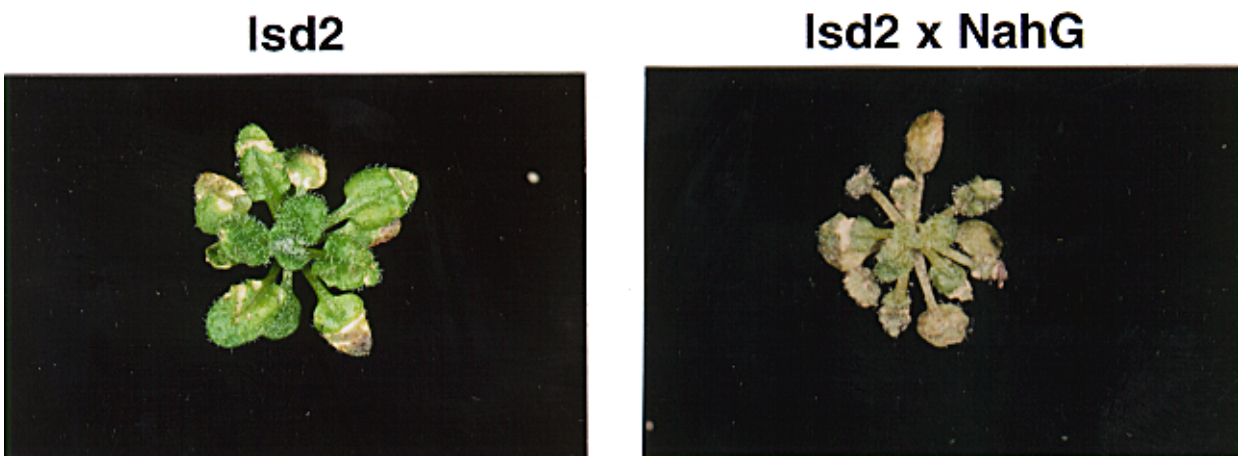

Fig. 4. *Peronospora parasitica* susceptibility in *lsd2*/NahG plants. Inoculation of *lsd2* progeny that either lack (left) or contain (right) *nahG* shows that fungal resistance in *lsd2* is lost by expression of salicylate hydroxylase. Photograph of plants was taken 7 days after inoculation.

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