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Arabidopsis Mutants Simulating Disease Resistance Response

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Summary

We describe six Arabidopsis mutants, defining at least four loci, that spontaneously form necrotic lesions on leaves. Lesions resemble those resulting from disease, but occur in the absence of pathogen. In five mutants, lesion formation correlates with expression of histochemical and molecular markers of plant disease resistance responses and with expression of genes activated during development of broad disease resistance in plants (systemic acquired resistance [SAR]). We designate this novel mutant class *lsd* (for lesions simulating disease resistance response). Strikingly, four *lsd* mutants express substantial resistance to virulent fungal pathogen isolates. *lsd* mutants vary in cell type preferences for lesion onset and spread. Lesion formation can be conditional and can be induced specifically by biotic and chemical activators of SAR in *lsd1* mutants.

Introduction

Plants have sophisticated intra- and intercellular signaling mechanisms to generate both local and systemic responses to pathogen infection. Responses to pathogens are triggered by recognition of pathogen-encoded molecules, subsequent signal transduction, and biosynthesis or release of effector molecules acting to halt pathogen growth. These events occur at the site of pathogen ingress, are controlled by specific genes in both host and pathogen, and give rise to a continuum of host responses (Crute, 1985; Dangl, 1992; Dixon and Lamb, 1990; Hahlbrock and Scheel, 1989; Lamb et al., 1989). At one extreme is the rapid host cell collapse around the infection site observed in many plant disease resistance responses, termed the hypersensitive response (HR) (Keen, 1982; Keen, 1990; Keen and Staskawicz, 1988; Klement, 1982; Stakman, 1914). The HR may directly inhibit pathogen growth or may be a consequence of the primary resistance mechanism (Gabriel and Rolfe, 1990; Görg et al., 1993; Király et al., 1972). Larger necrotic regions that form more slowly are typical of disease lesions caused by virulent pathogens.

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In several plant species, mutants have been observed with a visible phenotype that resembles the lesions caused by pathogen attack. These have been identified in corn (disease lesion mimics; Emerson, 1923; Ullstrup and Troyer, 1967; Neuffer and Calvert, 1975; Walbot et al., 1983; Neuffer et al., 1984; Pryor, 1987) tomato (autogenous necrosis; Langford, 1948), barley, (*ml-o*; Wolter et al., 1993), and Arabidopsis (*acd1*; Greenberg and Ausubel, 1993). The phenotypes associated with these mutants hint that they may represent steps along normal response pathways triggered by pathogen infection, and the high frequency of nonallelic mutations in maize suggests that a large number of loci can exhibit these mutant phenotypes (Walbot et al., 1983). This mutant class suggests that host factors alone are sufficient to generate cell death potentially analogous to that associated with pathogen attack. However, disease response–specific markers were not analyzed in these mutants.

Biochemical, molecular, and phenomenological markers are associated with the necrosis induced by virulent or avirulent pathogens (Dixon and Lamb, 1990). Pathogen-induced necrosis also often induces systemic, long-lasting resistance to a broad range of pathogens; this is termed systemic acquired resistance (SAR) (Ross, 1961; Kuc, 1982; Uknes et al., 1993a, 1993b; Ryals et al., 1994). Biochemical markers of resistance responses include irreversible membrane leakage and formation of secondary metabolites and deposition of callose in and around dead cell foci. Each is thought to contribute to limiting pathogen growth (Hahlbrock and Scheel, 1987; Keogh et al., 1980; Koga et al., 1980; Koga et al., 1988; Mayama and Shishiyama, 1976; Woods et al., 1988). Several genes are known to be induced locally as well as systemically following pathogen attack. These include genes associated with SAR (Uknes et al., 1992; Ward et al., 1991). Some SAR gene products have direct antimicrobial activities in vitro and in vivo (Alexander et al., 1993; Broglie et al., 1991; Mauch et al., 1988). As well, several chemicals have been shown to induce SAR genes and resistance to pathogens (White, 1979; Métraux et al., 1991). One of these chemicals is salicylic acid (SA), which exists naturally in plants and is a probable endogenous signal molecule for SAR (Malamy et al., 1990; Métraux et al., 1990; Ward et al., 1991; Yalpani and Raskin, 1993). Recently, the role of SA in SAR has been demonstrated by showing that plants engineered to catabolize SA are unable to establish SAR (Gaffney et al., 1993).

No spontaneous lesion phenotype, in any plant species, has been shown to mimic plant responses to infection faithfully using the markers mentioned above. We describe a novel class of Arabidopsis mutants that develop disease response–like lesions and express markers of authentic plant defense responses, including cytological and biochemical indicators of plant defense, high levels of SAR gene products, and significant resistance to normally virulent plant pathogens. Since members of this mutant class

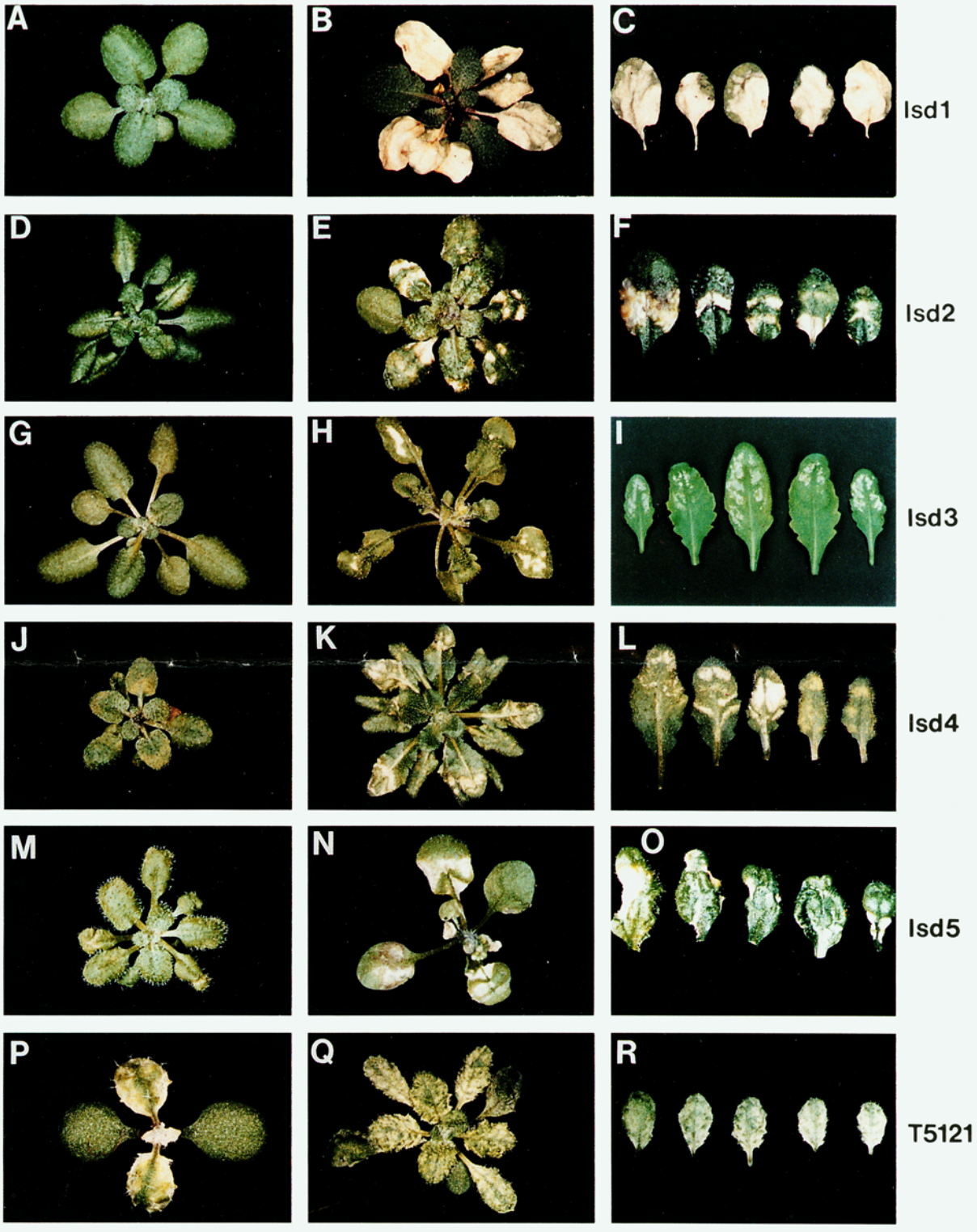

Figure 1. Lesion Phenotypes of Mutants

Mutant designation at right of each row. Left column (A, D, G, J, M, and P) shows plants grown under conditions suppressing lesion phenotype, if they exist. In the middle column (B, E, H, K, N, and Q) are plants grown under lesion-promoting conditions, and in the right column (C, F, I, L, O, and R) are detached leaves showing leaf phenotypes.

Table 1. Genetic Analyses of Lesion Mimic Mutants

Locus/Allele Designation	Genetic Background	F1 Progeny	F2 Segregation[a]			
			Phenotype$^+$	Phenotype$^-$	Phenotype$^+$	χ^2
lsd1^{T4855}	Ws-0	6	0	66	21	0.035; 0.9 > p > 0.5
lsd2^{CIM1}	Col-0	12	7[b]	19	28	5.96; 0.5 > p > 0.1[b]
lsd3^{T656}	Ws-0	6	0	52	17	0.005; 0.975 > p > 0.9
lsd4^{T6142}	Ws-0	18	18	26	87	0.237; 0.9 > p > 0.5
lsd5^{T6721}	Ws-0	11	0	178	50[c]	1.12; 0.5 > p > 0.1

[a] F2 data from backcross to Ws-0, except for *lsd2*^{CIM1} backcross to Col-0.
[b] Number of phenotype-positive class underrepresented, probably owing to incomplete penetrance; germination rate, 100%.
[c] Number of phenotype-positive class slightly underrepresented, owing to some seedling lethality.

exhibit many of the hallmarks of true disease responses, we term them *lsd* mutants, for their lesions simulating disease response phenotype.

Results

Mutant Phenotypes and Environmental Influences on Lesion Formation

Six mutants exhibiting spontaneous necrotic lesions were isolated as described in Experimental Procedures. Figure 1 shows representative phenotypes of each mutant, and a brief description of each follows. We noted an influence of developmental state and environment on the onset and extent of necrotic lesion formation in four of the lines. Similar lesions also formed under sterile culture conditions (data not shown), demonstrating that lesion formation in these mutants does not require an exogenous biotic trigger.

lsd1^{T4855}

Lesions formed on plants under long day (LD; see Experimental Procedures) growth conditions and were suppressed by growth under short day (SD; Figure 1A) growth conditions. When grown under SD and shifted to LD, lesions appeared within 2–3 days and involved the entire plant within 7–10 days. The lesions initiated as small foci of collapsed tissue appearing randomly on the leaf blade. These spread and coalesced into gray papery regions, eventually consuming the entire leaf (Figures 1B and 1C). Cotyledons were not affected, but newly emerging rosette leaves and other organs, including stems and flowers, were. This mutation is conditionally lethal, in that shift of young seedlings to LD conditions induced lesion formation that engulfed the entire plant before flowering.

lsd2^{cim1}

Lesions were suppressed under LD conditions (Figure 1D). Under SD, plants were smaller than wild type, and lesions first appeared on mature leaves of 2- to 3-week-old plants as pairs of chlorotic regions symmetrically oriented on leaf margins. These enlarged toward the midvein, coalesced into transversely oriented bands of necrosis that crossed the leaf midvein, and gave the leaves a striped appearance (Figures 1E and 1F).

lsd3^{T656}

Lesions formed under LD conditions and were suppressed under SD (Figure 1G). Lesions initiated 7–10 days after a shift to LD as dry gray foci on the rosette leaves, almost always in the apical two thirds of the blade (Figures 1H and 1I). Lesions occasionally coalesced to form a V-shaped area of lesioned tissue on the leaf. Lesions had a distinct margin and, after initial formation, did not spread significantly. Both rosette and cauline leaves could be affected.

lsd4^{T6142}

Lesions formed under all growth conditions tested and appeared on very young plants. In contrast with the other mutant phenotypes, these plants exhibited diffuse dispersed areas of chlorosis and bleached tissue without distinct margins, rather than necrosis (Figures 1J–1L). There was no apparent developmental pattern to formation of these chlorotic areas on the leaves, and most were affected. The plants were slightly smaller than wild type.

lsd5^{T6721}

The lesion phenotype is partially conditional (Figure 1M) and was more severe under conditions of low light intensity. Under SD, some seedling lethality occurred. Necrotic lesions developed on all leaves starting with the primary leaves and often formed on the leaf tips, but could initiate anywhere on the leaf (Figures 1N and 1O). Both rosette and cauline leaves were affected, and the plants were stunted.

T5121

The phenotype was seen under all growth conditions tested. The lesions had a mottled chlorosis and were found on all true leaves (Figures 1P–1R), but not on cotyledons (Figure 1P). Although selected using visual criteria as resembling maize lesion mimic mutants, this mutation is not in the *lsd* class, on the basis of criteria defined below.

Genetic Characterization

Genetic characterization of the mutants is shown in Table 1. The phenotypes in *lsd1*^{T4855}, *lsd3*^{T656}, and *lsd5*^{T6721} segregated in backcrosses to wild type as monogenic recessive

Table 2. Complementation Analyses of Lesion Mimic Mutants

Mutant Crosses[a]	Total F1 Plants	Phenotype^{+b}
lsd3^{T656}/*lsd3*^{T656} × *lsd1*^{T4855}/*lsd1*^{T4855}	8	0
lsd5^{T6721}/*lsd5*^{T6721} × *lsd1*^{T4855}/*lsd1*^{T4855}	5	0
lsd3^{T656}/*lsd3*^{T656} × *lsd5*^{T6721}/*lsd5*^{T6721}	6	0

[a] Crosses are listed as female × male.
[b] Phenotype-positive plants resembling each parent were recovered in the F2 progeny of these crosses.

mutations, while those of *lsd4*^{T6142} and *lsd2*^{cim1} segregated as dominant mutations. We defined three complementation groups via allelism tests between the three recessive mutants (Table 2) . The *lsd1*^{4855} mutation, which in some ways resembles the *acd* mutants (see Discussion; Greenberg and Ausubel, 1993), was nonallelic to both the *acd1* and *acd2* loci (R. A. D., unpublished data). Owing to phenotypic differences described below, we propose that *lsd2*^{cim1} and *lsd4*^{T6142} probably define distinct loci. Genetic and molecular mapping of these mutations is in progress.

lsd Mutants Express Histochemical and Molecular Markers Associated with Disease Resistance Responses

Expression of a variety of biochemical and molecular markers correlates with a plant response to pathogen invasion (for reviews, see Dixon and Lamb, 1990; Hahlbrock and Scheel, 1989). We selected four classes of markers for analysis. First, we assayed accumulation of autofluorescent material in and around lesions. Autofluorescence derives from biosynthesis and deposition of secondary metabolites, including potentially antimicrobial phytoalexins, and is nearly always associated with plant cells undergoing pathogen-induced hypersensitive cell death (Koga et al., 1980; Koga et al., 1988; Mayama and Shishiyama, 1976). Second, we stained for the presence of callose at the sites of necrosis using aniline blue. Callose deposition at and around sites of hypersensitive cell death may be part of a complex cell wall–strengthening process meant to halt pathogen invasion (Stanghellini and Aragati, 1966; Aist and Israel, 1977; Görg et al., 1993). Third, we correlated presence of the first two histochemical markers with irreversible membrane damage (cell death) as measured by trypan blue uptake (data not shown; Keogh et al., 1980). Finally, we asked whether the formation of lesions might also induce the expression of SAR genes normally activated by pathogen-induced necrosis (Ward et al., 1991).

Figure 2 demonstrates that lesion formation in *lsd* mutants was accompanied by accumulation of cytological markers of plant disease resistance response (Figures 2A–2L). For comparison, we present a hypersensitive resistance response observed following inoculation of a wild-type plant with a bacterial pathogen (Figures 2Q–2T). These lesions contained both autofluorescent products and callose deposits. Histology of control uninfected wild-type plants is also presented (Figures 2U–2X). In each example, adjacent serial sections were visualized using differential interference microscopy (Figure 2, column 1, [A], [E], [I], [M], [Q], and [U]), ultraviolet light–stimulated autofluorescence (Figure 2, column 2, [B], [F], [J], [N], [R], and [V]), and secondary fluorescence following aniline blue staining for callose (Figure 2, column 3, [C], [G], [K], [O], [S], and [W]). Comparisons of autofluorescence and callose deposition revealed substantial differences among *lsd* mutants in cell types displaying these markers and in lesion morphology. First, both autofluorescence and callose markers were located in the mesophyll of *lsd1* and *lsd3* cross sections (Figures 2B, 2C, 2F, and 2G) as well as in *lsd2* and *lsd4* (data not shown). Analysis of whole-mounted leaves, however, demonstrated that callose was deposited in a discrete cellular border surrounding *lsd3* lesions, but was present in single cell foci that spread and coalesced in *lsd1* (compare [H] and [D]), giving rise to the distinctive lesions characteristic of *lsd1*. Second, lesion onset in *lsd5*, monitored by both autofluorescence and callose deposition, was localized to the epidermal cell layers ([J] and [K]), although the sharply bordered lesions of *lsd5* resembled those of *lsd3* in whole mounts (compare [H] and [I]). Third, and in contrast with all *lsd* mutants, *T5121* triggered no accumulation of autofluorescence or callose deposition (Figures 2M–2P). Instead, we noted a near absence of mesophyll cells in lesions. Absence of plant disease resistance response markers suggested that, although visually identified as a lesion mimic mutant, *T5121* does not satisfy the histochemical criteria for inclusion within the lesions simulating disease response class. Data presented in Figure 2, and the concordance of irreversible membrane damage with the presence of these markers in each mutant (data not shown), illustrate that lesion formation in *lsd* mutants can begin in a cell type–specific manner in each major leaf cell type, with the possible exception of vascular tissue. Irrespective of the cell type in which necrotic lesions initiate, they eventually coalesce throughout all cell types before lesion spread is arrested.

Does spontaneous lesion formation lead to expression of genes strongly correlated with onset of SAR? RNA was extracted from either lesion-negative or lesion-positive plants, and RNA blots were probed with three cDNAs encoding either PR-1 (unknown function), PR-2 (β-1,3 glucanase), or PR-5 (unknown function), whose accumulation is strongly correlated with onset of SAR in tobacco and Arabidopsis (Uknes et al., 1992; Ward et al., 1991). PR-1 is a particularly appropriate monitor for SAR, as its overexpression in transgenic tobacco plants was demonstrated to be sufficient to increase resistance to a range of oomycete fungal pathogens significantly (Alexander et al., 1993). Accumulation of *PR-1* mRNA was obvious in all *lsd* mutants, but not in *T5121* (Figure 3). PR-2 expression and PR-5 expression were similar, although the overall magnitude of mRNA accumulation was not as high as PR-1, and some background expression was apparent (data not shown). These levels of mRNA accumulation were comparable to or higher than those achieved using chemical inducers of SAR such as 2,6-dichloroisonicotinic acid (INA) (Ward et al., 1991; Figure 3). SAR gene mRNAs accumulated only when conditional *lsd1*, *lsd2*, *lsd3*, and *lsd5* mutants were in the lesion-positive state (Figure 3). In the absence of lesions, all lines, including *T5121*, showed high levels of INA-inducible gene expression (T. P. D., unpublished data). Moreover, for at least *lsd1*, onset of SAR mRNA accumulation paralleled onset of lesion formation after shifting plants to LD conditions (R. A. D., unpublished data). These observations strengthen the definition of the *lsd* class of mutants and further the criteria for exclusion of *T5121* from the *lsd* class.

lsd Mutations Can Diminish Plant Susceptibility to Pathogens

We next assessed the effect of these mutations on the

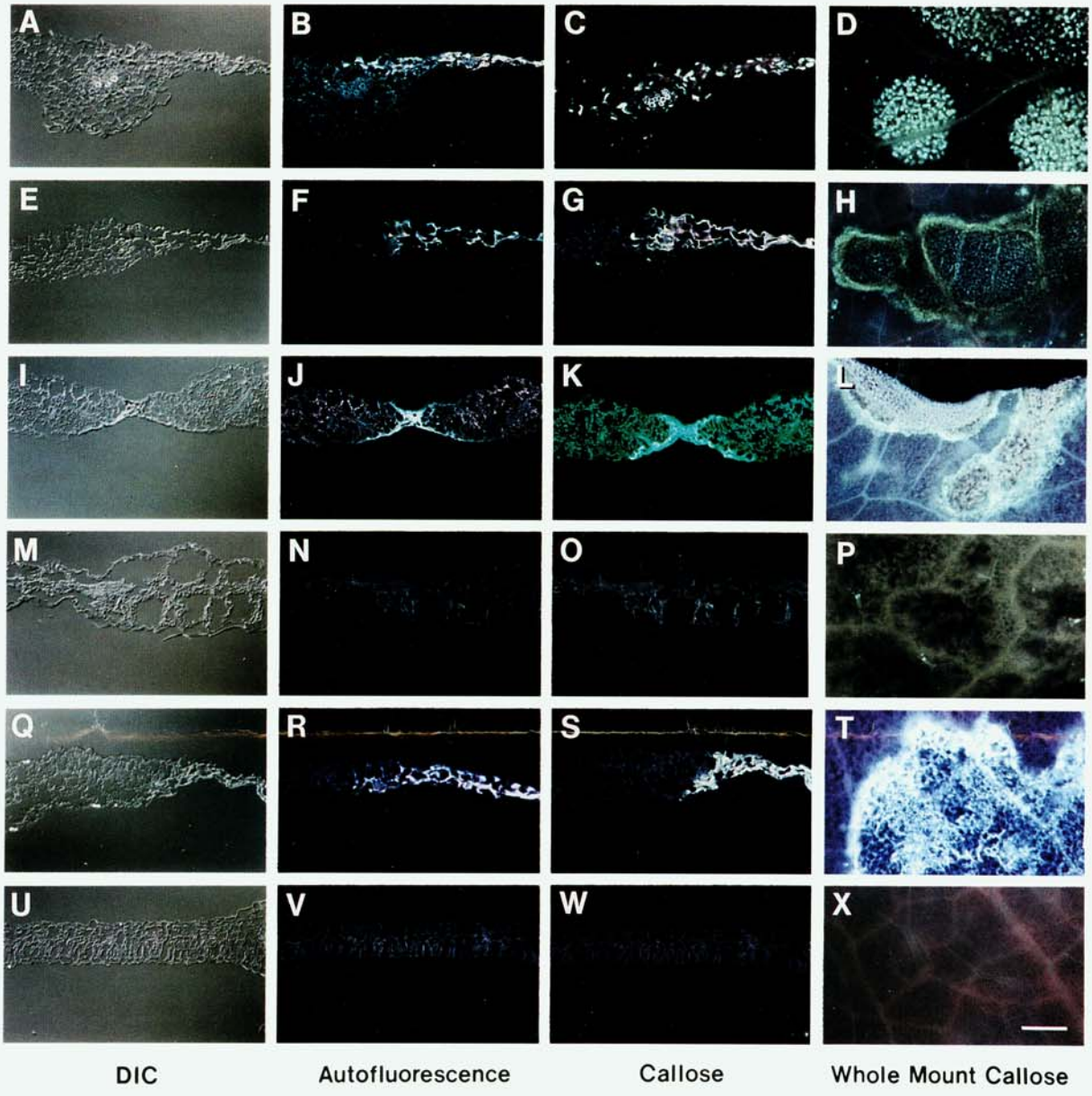

Figure 2. Lesion Histology

Horizontal rows display leaf morphology from lesions of *lsd1* (A–D), *lsd3* (E–H), *lsd5* (I–L), and *T5121* (M–P). Wild-type leaves exhibiting a P. syringae–induced HR (Q–T) or untreated (U–X) are shown as controls. Leaf cross sections through lesions are in columns 1–3. Within each row, these are adjacent serial sections, except in (R). Differential interference contrast images are in the far left column; UV-stimulated autofluorescence (Autofluorescence) is in the second column; and UV-stimulated fluorescence of aniline blue–stained tissue (Callose) is in the third column, and also in the far right column for leaf whole mounts (Whole Mount Callose). Scale bar in Panel X, 100 μM for cross section micrographs (columns 1–3) and 200 μM for the micrographs in column 4.

outcome of defined plant–pathogen interactions. We examined the interaction of mutant and wild-type plants with Peronospora parasitica, which causes downy mildew (Crute et al., 1993; Koch and Slusarenko, 1990). We evaluated infections macroscopically and microscopically to assess disease severity and the nature of host cell responses. Mutants were examined for conidiophore production relative to controls 6 days following inoculation with a fungal isolate virulent on wild-type parental plants. Table 3 shows that wild-type plants consistently exhibited substantial numbers of conidiophores (>20) on each inoculated leaf. In contrast, we observed significant levels of resistance on lesion-positive *lsd1*, *lsd2*, *lsd3*, *lsd4*, and *lsd5* mutants, and, exceptionally, on prelesion *lsd1* mutants (see below; see Experimental Procedures for definition of prelesion states). Wild-type controls, prelesion *lsd2* and *lsd3* mutants, and the *T5121* mutant were susceptible to fungal infection. Plants treated with the SAR-inducing

Table 3. Peronospora Parasitica Disease Ratings

Plants	Lesion Status[a]	Number of Plants Expressing Disease Levels[b]						
		0	+	++	+++	++++	+++++	Total Plants
Wild type[c]	–	0	0	0	0	0	20	20
lsd1	–	2	5	4	9	0	0	20
lsd1[d]	+	40	0	0	0	0	0	40
lsd2	–	0	0	0	0	2	8	10
lsd2	+	0	6	7	0	0	0	13
lsd3	–	0	0	0	0	0	20	20
lsd3	+	2	7	6	1	0	0	16
lsd4	+	6	4	3	5	2	0	20
lsd5[e]	+	0	6	4	8	2	0	20
T5121	+	0	0	0	0	0	20	20
Ws-0+INA	–	38	2	0	0	0	0	40

[a] Plus, obvious lesions on all plants prior to infection; minus, neither visible nor microscopic lesions observed prior to infection (see Experimental Procedures).

[b] This scale is defined as follows: 0, no conidiophores on plant; plus, at least one leaf with one to five conidiophores; double plus, at least one leaf with five to twenty conidiophores; triple plus, many leaves with five to twenty conidiophores; quadruple plus, all inoculated leaves with more than five conidiophores; quintuple plus, all inoculated leaves with more than 20 conidiophores.

[c] Wild type is Ws-0 for all except *lsd2*, which is Col-0.

[d] Since these plants exhibit spreading lesions at the time of infection, the time course of infection overlaps that of complete leaf collapse. Thus, lack of conidiophores may reflect either lack of living tissue or presence of defense functions.

[e] The definition of a prelesion state for this mutant is problematic, since phenotypic reversal from lesion-positive to lesion-negative following shift to LD conditions is never complete. Thus, only data for lesion-positive plants are shown.

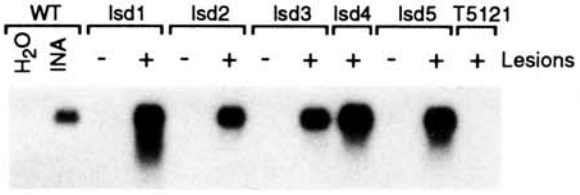

Figure 3. PR-1 Expression in Lesion Mutants

Total RNA was extracted from lesion-negative or lesion-positive plants (indicated over lanes), or from wild-type plants treated with water or INA.

chemical INA were completely resistant to P. parasitica. We observed a consistent correlation between lesion- or chemical-induced expression of SAR genes (Figure 3) and resistance to P. parasitica. Thus, according to a primary criterion of disease development, the *lsd* mutant class leads to significant levels of resistance to an otherwise virulent fungal pathogen.

We next examined pathogen growth and its interaction with the host plant by staining for callose deposition using aniline blue (Figure 4). To interpret plant cellular responses to fungal infection in the *lsd* mutants more succinctly, we first analyzed interactions of P. parasitica pathotype EMWA (Crute et al., 1993) with Arabidopsis accessions that are either susceptible or resistant to it (Figure 4). On susceptible Ws-0 plants, fungal spores germinated on the leaf surface, a germ tube penetrated between two epidermal cells, and a pair of haustoria formed in these neighboring epidermal cells (Figure 4A). Some callose was seen ringing the necks of the haustoria (Figure 4B). Hyphae invaded plant tissue and grew intercellularly (Figure 4C), from which feeding haustoria were elaborated inside leaf cells over the first 48 hr. Finally, either asexual sporulation structures (conidiophores, data not shown) or oosporangia containing sexual oospores formed (Figure 4D). In contrast, the resistance response of Arabidopsis accession La-*er* to the P. parasitica isolate EMWA was accompanied by callose deposition in the epidermal cells both at the site of attempted fungal penetration and in the mesophyll cells below that site (Figure 4H). Hyphae rarely penetrated into mesophyll cells beneath the primary inoculation site, and oospores were not produced. This type of resistance reaction is often mediated by single, dominantly acting resistance (*R*) genes (Koch and Slusarenko, 1990; Crute et al., 1993; Parker et al., 1993). Pretreatment of wild-type plants with INA also induces resistance to P. parasitica (Uknes et al., 1992), and cytological examination (Figure 4I) demonstrated that INA-triggered resistance is associated with accumulation of autofluorescent material in leaf mesophyll cells beneath attempted infection sites.

We then examined interactions between virulent fungal isolates and plants carrying *lsd* or *T5121* mutations. Each *lsd* line in a lesion-positive state, and the exceptional *lsd1* mutation in a lesion-negative state, exhibited reduced or altered hyphal growth and reduced oospore production, consistent with reduced conidiophore elaboration (Table 3). In contrast, the other prelesion *lsd* mutants, the *T5121* line, and wild-type controls supported fungal growth. Prelesion *lsd1* mutants exhibited many cellular characteristics of plants resistant to P. parasitica, and Figures 4E, 4F, and 4G document these dramatic effects on normal fungal growth. First was a profound host response in both epidermal and mesophyll cells adjacent to elongating hyphae (compare Figures 4E and 4F with 4B and 4C), apparent within the first 24 hr after infection (data not shown), and obvious by 48 hr (Figure 4F). Interestingly, and in contrast with successful infection of wild-type plants, *lsd1* cell ultra-

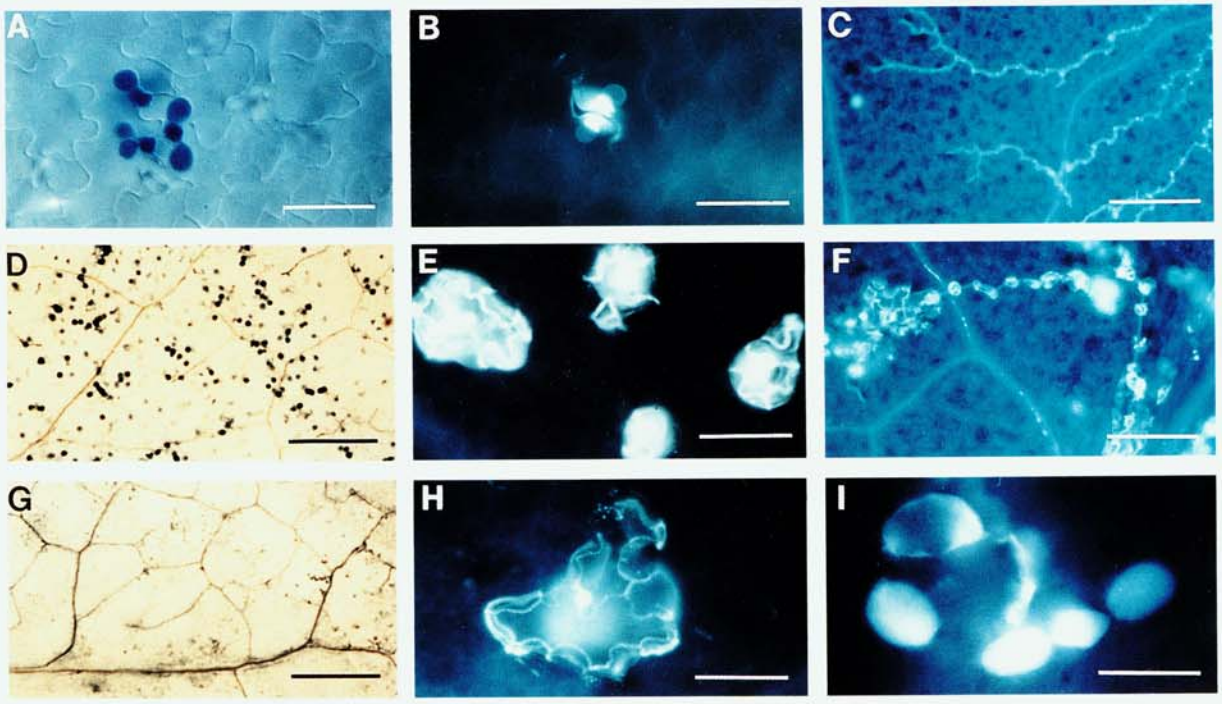

Figure 4. Responses of Resistant and Susceptible Wild-Type and *lsd1* Mutant Plants to Fungal Pathogen

P. parasitica isolate EMWA was used to infect susceptible wild-type Ws-0 plants (A–D), *lsd1* plants (E–G), and resistant wild-type La-*er* plants (E). (I) is from INA-treated wild-type Ws-0. (A), (B), (E), and (H) focus on infection sites at the leaf epidermis. In (A), three fungal penetration sites are visible, each flanked by two haustoria (dark blue under bright field). In (B), fluorescence is at the base of the fungal haustoria. In (E) and (H), intense fluorescence is in responding host cells surrounding the penetration site. (C), (D), (F), and (G) focus on fungal structures within leaf mesophyll. In susceptible Ws-0 plants, fungal hyphae spread without engendering appreciable callose deposition from the host (C) and later produce abundant oosporangia ([D], blue spheres). In *lsd1* mesophyll, hyphae trigger accumulation of callose in adjacent cells (F), and few oosporangia are produced (G). Wild-type plants treated with INA exhibit fluorescent mesophyll cells beneath sites of attempted fungal penetration (I). Scale bars: (A), (B), (E), and (H), 50 μM; (C) and (F), 100 μM; (D) and (G), 250 μM; (I), 80 μM.

structure also changed dramatically at this time, as chloroplasts in cells surrounding fungal hyphae disappeared (data not shown). Very few oosporangia formed in *lsd1* tissue (Figure 4G).

Fungal infection neither triggered lesion formation nor exacerbated the number or nature of existing lesions in the other *lsd* mutants, but variable and less dramatic host cell responses resulting in fungal growth inhibition were observed (data not shown). *Lsd2* mutants supported much less hyphal growth and conidiophore production than wild type, and reduced conidiophore production in *lsd4* and *lsd5* was often accompanied by a range of alterations in hyphal morphology, from the abnormally thick, highly branched, and often knobby to extremely thin with very few haustoria. Similarly altered fungal morphology was also observed in plants treated with small amounts of the SAR inducer INA prior to infection (Uknes et al., 1992). We conclude that each *lsd* mutation leads to heightened plant resistance to a virulent isolate of P. parasitica. Resistance was correlated with the presence of lesions and the expression of SAR genes. *Lsd1* is a unique case, because resistance was manifested on leaves that were outwardly normal, before any apparent sign of spontaneously forming, spreading necroses.

We next analyzed the response of *lsd* mutants to Pseudomonas syringae isolates capable of causing leaf-spotting disease on a variety of susceptible Crucifers, tomato, and Arabidopsis (Debener et al., 1991; Whalen et al., 1991; reviewed by Dangl, 1993). We chose P. syringae isolates to which the parental Arabidopsis accessions for the *lsd* mutants are either susceptible or resistant and performed two kinds of assays. First, we monitored in planta bacterial growth after low titer inoculation (10^5 cfu/ml). Susceptible plants support bacterial multiplication of 3–5 orders of magnitude over 5 days, resulting in formation of water-soaked lesions and chlorotic flecks. Second, we assayed for the rapid formation of macroscopic zones of collapsed plant cells (HR) exhibited by resistant Arabidopsis accessions after high titer inoculation (Dangl et al., 1992b; Debener et al., 1991; Dong et al., 1991; Whalen et al., 1991). These lesions have sharp borders and do not spread. At lower initial inocula, the HR occurs but is microscopic, because fewer plant cells are responding.

Bacterial growth in leaves of *lsd1* versus wild type over a 5 day time course is shown in Figure 5. The *lsd1* mutation consistently rendered plants more able to restrict growth of all tested bacterial phytopathogens. The plateau in planta bacterial titers were uniformly reduced by approximately 10-fold in this mutant compared with wild type, although the initial rate of growth was roughly the same as in wild-

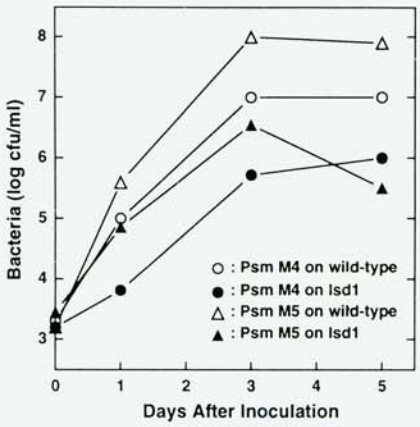

Figure 5. In Planta Bacterial Growth

Two P. syringae pv. maculicola strains (Psm M4 and Psm M5) were inoculated into Ws-0 and *lsd1* leaves at 10^5 cfu/ml, and in planta growth monitored over 5 days as described in Experimental Procedures. Ws-0 is susceptible to both strains. Data points represent the mean titer of three individual leaves per point from one experiment, and similar results were obtained in two other independent experiments.

type leaves. The *lsd2* mutation also led to reduced bacterial growth in some experiments, but there was no consistent alteration of growth in the other three mutants (data not shown). The decrease in bacterial growth observed in the *lsd1* mutant was similar to that reported for wild-type plants protected from infection by INA induction of SAR (Uknes et al., 1992). Except for *lsd1* (see below), the mutants responded to high titer inoculation with avirulent bacteria similarly to wild-type plants (data not shown). These data show that at least the *lsd1* mutant also expresses heightened resistance to normally virulent bacterial pathogens.

The *lsd1* Mutation Causes Hair-Trigger Perception of Signals Leading to the Inability to Control Cell Death

Inoculation of prelesion *lsd1* plants caused formation of lesions that spread throughout the leaf into noninoculated tissue over 2–3 days, independent of initial inoculum titer, as summarized in Figure 6. Following high density inoculation (10^7 cfu/ml) with the avirulent P. syringae pv. maculicola strain M2, a sharply defined HR formed over a short time course identical to that of wild-type Ws-0 (figure 6A, left). After a 1–2 day lag phase, the lesions began to spread, eventually crossing the leaf midrib. No bacteria were found in these spreading lesions beyond the inoculation site. Strikingly, the onset of spreading necrosis in *lsd1* was triggered by low inoculum levels (10^5 cfu/ml) that have no visible effect on wild-type Ws-0 (Figure 6A, right). Spreading lesions were induced by P. syringae isolates that cause disease (Figure 6A, strains M4 and M5) and by a P. syringae pv. phaseolicola strain that is nonpathogenic on wild-type Ws-0 (Figure 6A, strain 110). Spreading lesions were also induced by inoculation of Escherichia coli and a P. syringae pv. phaseolicola mutant lacking the ability to trigger an HR or to be pathogenic on a nominal host (an *hrpF* mutant; data not shown). Intercellular spread

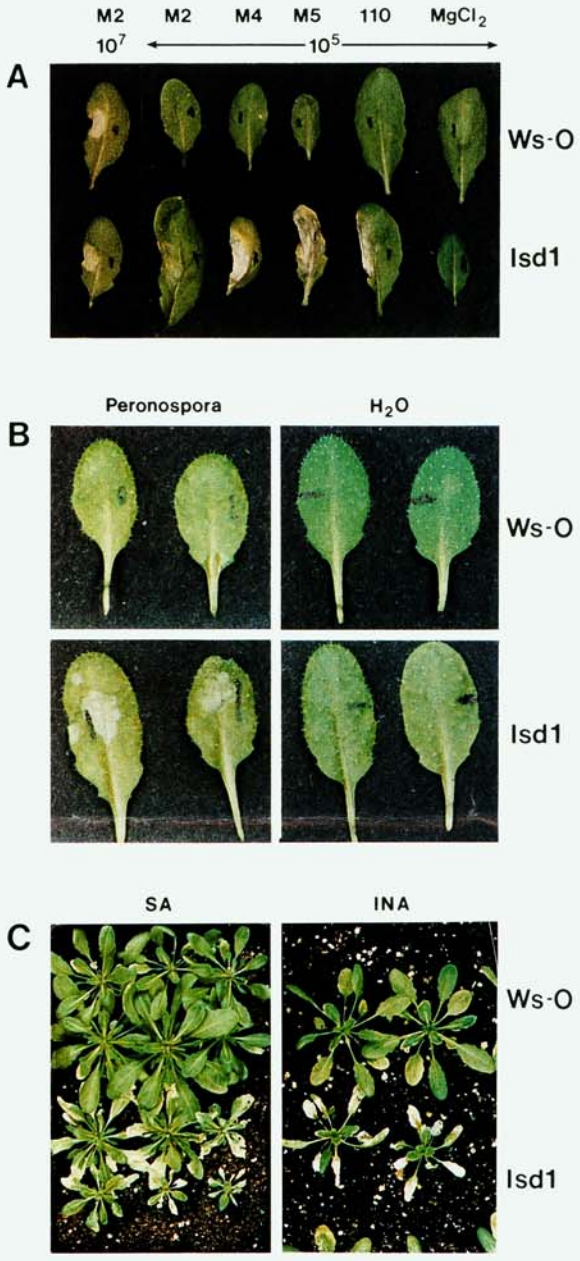

Figure 6. Induction of Spreading *lsd1* Lesions by Biotic and Abiotic Effectors

(A) P. syringae isolates (strain designations listed at top; buffer control is $MgCl_2$) were inoculated at either high (10^7 cfu/ml) or low (10^5 cfu/ml) titer into Ws-0 and *lsd1* leaves at sites opposite black ink marks. Leaves were detached and photographed 24 hr (10^7 cfu/ml) or 5 days (10^5 cfu/ml) after inoculation.

(B) Droplet inoculations (2 μl of spore suspension) of P. parasitica isolate EMWA (left) and water control (right) onto leaf surfaces of Ws-0 and *lsd1*. Leaves were detached and photographed 4 days after inoculation.

(C) Chemical inducers of SAR SA or INA were sprayed onto Ws-0 and *lsd1* plants under SD conditions. Plants were photographed 5 days after application.

of necrosis was not observed after mock inoculation with $MgCl_2$ (Figure 6A, right), heat killed bacteria, or by mechanical wounding (R. A. D., unpublished data).

We next tested whether *lsd1* lesion formation might also be triggered by localized infection of prelesion plants with a low density of P. parasitica infection. Droplets (2 μl containing 50–100 spores) were applied to *lsd1* and wild-type leaves. Figure 6B demonstrates that even low levels of P. parasitica infection caused spreading lesions. From these observations, we reason that lesion initiation in *lsd1* might reflect a hair-trigger phenotype, in which normally subthreshold levels of pathogen recognition aberrantly lead to a hypersensitive plant cell death response.

Because hypersensitive cell death seems linked to pathway(s) regulating SAR, we asked whether treatment with the inducing chemicals SA or INA might also cause lesion formation on *lsd1* plants. Figure 6C illustrates that very low levels of SA and INA induced the formation of spreading lesions in *lsd1*. We also tested structural analogs of SA known to be active (2,6-dihydroxybenzoic acid) or inactive (4-hydroxybenzoic acid and 2,5-dihydroxybenzoic acid) at inducing SAR (Métraux et al., 1991). Only compounds that induce SAR triggered *lsd1* lesions (data not shown). Lesions induced by these compounds were indistinguishable histochemically from those occurring after shift from SD to LD conditions (R. A. D., unpublished data). We conclude that spreading lesions in *lsd1* can also be induced not only through biotic agents, but also by natural (SA) or synthetic (INA or hydroxybenzoic acids) chemical agents that effect plant disease responses. None of the other *lsd* mutants were affected by treatment with these compounds.

Discussion

Plant mutants with necrotic lesion phenotypes have long been documented, especially in the rich literature of maize genetics (summarized by Walbot et al., 1983). Yet, while the resemblance to pathogen-caused lesions has been noted, these mutants have never been shown to mimic the cellular and molecular processes involved in plant disease responses. We sought necrotic lesion mutants in Arabidopsis and determined whether they faithfully mimic disease response or just superficially resemble diseased plants. Our criteria for this assessment were expression of cytological (autofluorescence and callose deposition) and molecular (SAR gene expression) markers of disease responses in plants. We also examined interactions with fungal and bacterial pathogens to ask whether these mutants expressed altered resistance functions. We identified six mutant lines with leaf phenotypes resembling disease resistance response lesions. Using the criteria described above, we found that five of those lines exhibited characteristics highly correlated to plant disease responses. In contrast, one line (*T5121*) had a visible phenotype resembling disease response, yet showed none of the detailed characteristics of infected plants. Based on these observations, we define a new phenotypic class that exhibits necrotic lesions simulating disease resistance response (hence, *lsd*) in the absence of pathogen. These mutants identify genes that may be important in signal transduction pathways involved in pathogen recognition, programmed cell death, or SAR.

The distinction between *lsd* mutants and other leaf necrosis mutants provides a useful context within which to evaluate additional lesion mutants in Arabidopsis and other plant species. Interestingly, disease-resistant germplasm sources can have associated leaf lesions (Langford, 1948; Wolter et al., 1993) or "undesirable leaf spotting characters" (Wernsman and Rufty, 1987). Because these germplasm sources may exhibit both lesion and disease resistance phenotypes, they could conceivably be additional examples of *lsd* mutants. The powdery mildew–resistant barley *mlo* mutants are also candidates for additional *lsd*-like mutations, since they exhibit spontaneous lesion formation and subcellularly localized callose deposition (Wolter et al., 1993).

We distinguish two classes of *lsd* mutations. The first, which we refer to as initiation mutants (sensu Walbot et al., 1983), form spontaneous lesions whose growth is determinate (*lsd2*, *lsd3*, *lsd4*, and *lsd5*). Lesion formation is not dramatically influenced by either pathogen infection or treatment with compounds known to trigger the onset of SAR. Since both recessive and dominant mutations give rise to the initiation class, we suggest that both the removal of negative regulatory control and the constitutive activation of signaling components can give rise to cell death in plants. These pathways may normally function in regulating the molecular responses culminating in the rapid cell death often associated with resistance (the HR). Alternatively, some *lsd* mutations may cause an unbalanced biochemical state misinterpreted by the cell as resulting from pathogen infection, thus triggering a host response. Such metabolic perturbation could be analogous to inactivation of specific host enzymes by pathogen-derived toxins (Johal and Briggs, 1992). Recently, Becker et al. (1993) also reported that perturbation of the ubiquitin-dependent protein degradation pathway could also induce heightened resistance to a viral pathogen. Whether the *lsd* mutations also impinge upon normal metabolic systems to effect activation of resistance responses awaits further investigation.

The second class, defined by *lsd1*, we call feedback or propagation mutants. This class forms lesions that can spread indeterminately, eventually consuming the leaf or plant. Lesion initiation in *lsd1* can be triggered by fungal or bacterial pathogens, and by chemical inducers of SAR, but not by wounding. Lesions radiate beyond the site of initiation, and lesion formation is accompanied by SAR gene expression. These results suggest that the pathway leading to establishment and maintenance of SAR can also modulate development of pathogen-derived lesions. The recessive nature of the *lsd1* mutation and its phenotype suggest that this mutant has lost a control required to set the local boundaries of cell death during lesion formation: once lesions are triggered, they spread unchecked beyond the site of initiation. The mechanism disrupted in *lsd1* may lead to either continuous generation, or lack of perception, of intercellular signals controlling cell death at the front of the advancing lesion. We have repeatedly attempted to construct double mutants be-

tween *lsd1* and one of the initiation class *lsd* mutants to ask whether the latter can induce lesion formation in the former. However, since *lsd1* lesions rapidly spread and kill affected leaves in LD conditions, unambiguous identification of double mutants relies on a synergisitc phenotype such that initiation class lesions are found under normally permissive SD conditions. We have not observed this.

Signals for cell death in other species also operate via removal of control steps that normally suppress apoptosis, as shown for the interactions of mammalian c-*myc* and *bcl-2* gene products, and for the function of the *ced-9* gene from Caenorhabditis elegans (Bissonnette et al., 1992; Hengartner et al., 1992). We speculate that the heightened resistance to fungal and bacterial pathogens exhibited by prelesion *lsd1* may operate in advance of spreading lesion formation. This speculation is fueled by the observation of host cell response to fungal or bacterial infection as rapidly as 24 hr post infection, with no spread of lesions occurring until approximately 2 days later.

Interestingly, the *sl* (Sekiguchi lesion) mutation in rice leads to spreading lesions that can engulf the rice leaf blade (Kiyosawa, 1970; Marchetti et al., 1983). The *sl* lesion phenotype is induced by isolates of two fungal pathogens (Bipolaris oryzae and Pyricularia oryzae) to which the parent plant is normally resistant and by several abiotic compounds, but not by virulent fungal isolates. Because histochemical analysis of *sl* lesions revealed no growing fungi, Marchetti et al. (1983) concluded that this mutation results in an unchecked hypersensitive response normally induced by avirulent fungal isolates. The Arabidopsis *acd1* mutant also displays spreading lesions (Greenberg and Ausubel, 1993). However, *acd1* differs from *lsd1*, since lesions in the former can be induced by nonspecific stresses, including mechanical wounding and inoculation with $MgSO_4$, and since *acd1* exhibits relaxed growth control for several avirulent bacteria. Developmental control of lesion formation is also different, since newly emerging secondary rosette leaves of *acd1* are not lesioned, while those of *lsd1* are. Further analysis will determine whether *sl* and *acd1* belong to the *lsd* phenotypic class defined here.

Independent, developmentally specified sets of signal pathways leading to programmed cell death are the norm in other eukaryotic systems (reviewed by Raff, 1992; Raff et al., 1993; Williams and Smith, 1993). Programmed cell death in plants is apparenly involved in anther, megagametophyte, and vascular tissue development, as well as in at least one mechanism of sex determination (DeLong et al., 1993). However, no clear biochemical or molecular markers of apoptosis have been characterized in plants. *Lsd* mutants vary with respect to leaf cell types involved during onset of lesion formation as well as the pattern of necrosis on the leaf. This variability may reveal an underlying pattern modifying expression of the lesion phenotype through cell- and tissue-specific cues. Whether these cues also participate in pathways functioning during pathogenesis is unknown. Genetic analysis of plant disease and mutants affected in these processes, such as the *lsd* mutants, will help establish relative roles of plant and pathogen activities during pathogenesis. Such mutants may also point to cellular processes that are shared in disease resistance and plant development.

Experimental Procedures

Mutant Isolation, Plant Maintenance, and Genetic Analyses

Five lines exhibiting spontaneous leaf necroses (*lsd1*, *lsd3*, *lsd4*, *lsd5*, and *T5121*) were isolated from 9000 T-DNA-mutagenized lines (accession Ws-0) as described in Feldmann (1991). Phenotype-positive T3 plants were backcrossed using wild-type Ws-0 as the female. Flowers were hand emasculated, and pollen was applied by hand under a dissecting microscope. Phenotype-positive F2 segregants were used for all experiments. These expressed phenotypes identical to those of the original T3 plants. Only the *lsd5* mutation is linked to a T-DNA insertion and may be tagged (J.-B. Morel, R. A. D., and J. L. D., unpublished data).

The *lsd2*^{cim1} allele was identified in a screen of EMS-mutagenized Arabidopsis thaliana ecotype Col-0 seed (provided by Dr. J. Ecker) consisting of RNA blot analysis of M2 plants to identify mutants constitutively expressing SAR genes (S. Potter and D. Chandler, unpublished data). This mutant was initially identified as such and was later shown to manifest necrotic lesions also.

Phytochamber growth conditions include an 8 hr photoperiod, 24°C day and 20°C night temperatures, 60% relative humidity, and a light intensity of 250 μeinsteins/m^2/s. Greenhouse conditions included a 16 hr photoperiod (sunlight supplemented to 16 hr with artificial lamps). Conditions of temperature and relative humidity in the greenhouse were variable. Day length conditionality could also be affected by other parameters, particularly light quality, that vary between SD (phytochamber) and LD (greenhouse) environments.

Seeds for sterile growth were surface sterilized in 70% ethanol, soaked for 30 min in 5% bleach, 0.5% SDS, washed five times in sterile distilled water, and sown in either sterile soil in glass jars or in Murashige–Skoog (MS) media containing 0.8% agar and 1% sucrose. Conditions during sterile growth were either 8 hr days in the phytochamber or in the greenhouse as described above. Prelesion states under permissive conditions for all mutants (except *lsd4* and *lsd5*) were defined as follows: no visible leaf morphology alterations compared with wild type; no visible lesions of any kind; and, most importantly, no microscopic lesions observed via autofluorescence or callose deposition in a minimum of 20 leaves from independent experiments.

Histochemistry and Microscopy

Leaves for autofluorescence and callose examination were boiled for 2 min in alcoholic lactophenol (95% ethanol:lactophenol, 2:1), rinsed in 50% ethanol, and then rinsed in water. Cleared leaves were mounted on slides in 70% glycerin in water and autofluorescence observed using ultraviolet epifluorescence (excitation filter, 365 nm; dichroic mirror, 395 nm; and barrier filter, 420 nm). For callose, cleared and rinsed leaves were stained for 1 hr at room temperature in a 0.01% (w/v) solution of aniline blue in 0.15 M K_2HPO_4 (Eschrich and Currier, 1964). Stained leaves were examined under ultraviolet epifluorescence using the same filter set.

Leaves for microscopic examination of P. parasitica–infected tissue were first cleared and then stained to highlight either the fungal hyphae (Bruzzese and Hasan, 1983; with slight modifications) or callose. Leaves were harvested and vacuum infiltrated with lactophenol for 10 min and then incubated at room temperature in lactophenol overnight or until the leaves were clear, using at least three changes of lactophenol. To stain the fungal tissue, cleared leaves were incubated for 1 hr at room temperature in lactophenol plus 0.06% (w/v) aniline blue. Stained tissue was examined using differential interference contrast microscopy. For callose staining of infected tissue, cleared leaves were rinsed in 50% ethanol and then water, then incubated for 1 hr at room temperature in 0.01% aniline blue in 0.15 M K_2HPO_4. Stained material was mounted in 70% glycerol, 30% stain and examined using ultraviolet epifluorescence as above. Leaf material for sectioning was fixed in FAE (10% formaldehyde:5% acetic acid:45% ethanol) overnight, dehydrated, and embedded in paraffin. Embedded leaves were sectioned on a rotary microtome at a thickness of 8 μm. Leaf sections were mounted on microscope slides, de-waxed, rehydrated, and then examined for autofluorescence or stained for callose as above.

Pathogen Infections

Bacterial strains used in these experiments were as follows: Pseudomonas syringae pv. maculicola isolates M2 (avirulent on wild-type Ws-0), M4, and M5 (virulent on Ws-0), P. syringae pv. phaseolicola isolate 110 (pathogen of bean, nonpathogenic on Arabidopsis), an *hrpF* mutant derived from P. syringae pv. phaseolicola race 6 (gift of J. Mansfield) or E. coli HB101. Bacterial injections and growth curves were done on 5-week-old plants grown in the phytochamber using inocula of 10^5, 10^6, 10^7, and 10^8 cfu/ml as described in Debener et al. (1991). For bacterial growth curves, an entire leaf half was infiltrated with a suspension of 10^5 cfu/ml. In one experiment, four leaf discs were pooled prior to homogenization; in a second experiment, two pools consisting of two discs each were analyzed; and in a third experiment, three single leaves for each treatment were titered. No significant difference was observed between these experiments.

Inoculation with P. parasitica was done on 4-week-old plants with fresh conidiospores suspended in water. Spore suspensions were prepared as described (Dangl et al., 1992a). Plants used for histological examination of the infection process were inoculated by placing a 2 µl droplet of spore suspension (approximately 10^5 spores/ml) on the leaf surface. To score for disease rating, whole plants were sprayed with a spore suspension (approx 10^6 spores/ml). Inoculated plants were kept covered to maintain high humidity throughout the course of the experiment, and fungal growth was evaluated on day 6.

Test of Chemical Inducers of SAR

Phytochamber-grown plants (5 weeks old) were sprayed to imminent runoff with 1 mM solutions of either SA, 4-hydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, or INA at a concentration of 0.4 mM as a suspension of 25% active ingredient, 75% wettable powder in water. After applications, plants were returned to the phytochamber and covered with a clear plastic cover for 24 hr to maintain high humidity and prevent localized increases in concentration of the applied compound caused by evaporation. Concentrations used induce moderate levels of SAR gene expression, but are at least 10-fold lower than those causing phytotoxicity.

RNA Isolation and Analysis

Leaves were harvested and frozen in liquid nitrogen. After grinding to powder, RNA was extracted as described by Verwoerd et al. (1989). Electrophoresis of 5 µg of RNA through agarose–formaldehyde gels, transfer to nylon membranes (Gene Screen Plus, New England Nuclear), probes (PR-1, PR-2, and PR-5 cDNAs), and washing conditions have been described (Uknes et al., 1992).

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Note Added in Proof

Another locus of the cross discussed in this paper has been identified by Greenberg et al.: Greenberg, J. T., Guo, A., Klessig, D. F., and Ausubel, F. M. (1994). Programmed cell death in plants: a pathogen-triggered response activated coordinately with multiple defense functions. Cell *77*, this issue.