The Disease Resistance Signaling Components *EDS1* and *PAD4* Are Essential Regulators of the Cell Death Pathway Controlled by *LSD1* in Arabidopsis

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Specific recognition of pathogens is mediated by plant disease resistance (*R*) genes and translated into a successful defense response. The extent of associated hypersensitive cell death varies from none to an area encompassing cells surrounding an infection site, depending on the *R* gene activated. We constructed double mutants in Arabidopsis between positive regulators of *R* function and a negative regulator of cell death, *LSD1*, to address whether genes required for normal *R* function also regulate the runaway cell death observed in *lsd1* mutants. We report here that *EDS1* and *PAD4*, two signaling genes that mediate some but not all *R* responses, also are required for runaway cell death in the *lsd1* mutant. Importantly, this novel function of *EDS1* and *PAD4* is operative when runaway cell death in *lsd1* is initiated through an *R* gene that does not require *EDS1* or *PAD4* for disease resistance. *NDR1*, another component of *R* signaling, also contributes to the control of plant cell death. The roles of *EDS1* and *PAD4* in regulating *lsd1* runaway cell death are related to the interpretation of reactive oxygen intermediate–derived signals at infection sites. We further demonstrate that the fate of superoxide at infection sites is different from that observed at the leading margins of runaway cell death lesions in *lsd1* mutants.

INTRODUCTION

Plants have evolved mechanisms to detect and respond effectively to most pathogens. Analyses of genetic variation in plant responses to pathogens have identified corresponding gene pairs (resistance or *R* genes in the plant and avirulence or *avr* genes in the pathogen) that mediate recognition and cause induction of plant resistance (Staskawicz et al., 1995). These local plant defenses are usually, although not invariably, associated with a form of programmed plant cell death known as the hypersensitive response (HR). The HR can lead to cell death surrounding the infection site (Holub et al., 1994). Localized necrosis also can induce a plant response called systemic acquired resistance, which heightens defenses in uninoculated tissues against a broad spectrum of pathogens (Yang et al., 1997; McDowell and Dangl, 2000).

One of the earliest biochemical changes associated with the HR is an oxidative burst producing reactive oxygen intermediates (ROI), including superoxide anion (O$_2^-$) as a proximal component, which can be dismutated rapidly to hydrogen peroxide (H$_2$O$_2$) (Lamb and Dixon, 1997; Bolwell, 1999; Grant and Loake, 2000). These may serve both as antimicrobial agents and as signaling molecules in local and systemic plant resistance. Nitric oxide (NO), a redox-active molecule with a critical role in the activation of mammalian defense responses (Schmidt and Walter, 1994), also functions as an important signal in plant resistance against pathogens (Delledonne et al., 1998; Durner et al., 1998). Salicylic acid (SA) accumulates in plant tissue responding to pathogen infection and is essential for the induction of systemic acquired resistance as well as being required for some *R* gene–mediated responses, at least in Arabidopsis and tobacco (Gaffney et al., 1993; Delaney et al., 1994; Mur et al., 1997). Recent results suggest that the balance and cooperation between NO, ROI, and SA produced early in the plant resistance response is required for the full expression of the HR (Delledonne et al., 1998, 2001; Klessig et al., 2000). However, little is known about the sequence of events that determines local plant resistance. Also unclear is whether signals are transduced from an infection focus to first initiate, and then dampen, the HR.

Arabidopsis is the key genetic system with which to unravel disease resistance pathways (Glazebrook, 1999; Feys and Parker, 2000). Arabidopsis *R* genes have been cloned that confer specific recognition of viral, bacterial, and oomycete pathogens (Parker et al., 2000). Their products
belong to the most prevalent R protein class identified in a range of plant species that contains a central nucleotide binding (NB) domain and varying numbers of C-terminal leucine-rich repeats (LRRs) (Jones, 2000). NB-LRR proteins were further categorized into those with a coiled-coil (CC) motif at their N termini and those that have N-terminal (TIR) similarity to the cytoplasmic domains of human and Drosophila Toll-like receptors (Jones, 2000).

Mutational analyses in Arabidopsis uncovered genes required as positive regulators of basal defense (Glazebrook, 1999; Feys and Parker, 2000). EDS1 is a necessary component of RPP1- and RPP4-specified resistance to the oomycete pathogen Peronospora parasitica (Pp) (Park et al., 1996; Aarts et al., 1998) and is more generally required for resistance mediated by several tested Arabidopsis R genes encoding TIR-NB-LRR proteins (Aarts et al., 1998). However, EDS1 is not required for resistance conferred by any of the tested CC-NB-LRR R genes (Aarts et al., 1998). Many, but not all, CC-NB-LRR R genes examined are dependent on NDR1, a gene identified through mutational analysis of RPM1-mediated resistance to the bacterial pathogen Pseudomonas syringae expressing avrB (Century et al., 1995). Thus, EDS1 and NDR1 different R gene–mediated events that may, at least in several cases, be conditioned by particular R protein structural types (for the current exceptions, see McDowell et al., 2000). Furthermore, ndr1 mutant plants retain an HR initiated by two R genes, RPM1 and RPS5, even though they fail to prevent bacterial growth, suggesting that resistance and HR are separable (Century et al., 1995). EDS1 encodes a 72-kD lipase-like protein that operates upstream of SA-mediated defenses (Falk et al., 1999), whereas NDR1 encodes a 25-kD protein that has two putative membrane attachment domains (Century et al., 1997).

Mutational screens in Arabidopsis identified several other plant defense signaling genes that are components of SA signaling in the plant response against pathogens. For example, PAD4 (Glazebrook et al., 1997; Zhou et al., 1998) and SID1/EDS5 and SID2/EDS16 (Rogers and Ausubel, 1997; Nawrath and Métraux, 1999) function upstream of SA accumulation; whereas NPR1/NIM1 is an important regulator of responses downstream of SA (Cao et al., 1994; Delaney et al., 1995). Significantly, PAD4 encodes a lipase-like protein with catalytic motifs identical to EDS1 (Jirage et al., 1999). EDS1 and PAD4 operate upstream of pathogen-induced SA accumulation, yet their expression can be enhanced by exogenous applications of SA. This finding reinforces evidence of an SA-associated positive feedback loop that may potentiate plant defense (Shirasu et al., 1997; Falk et al., 1999; Jirage et al., 1999). The pad4 mutation affects the same spectrum of R gene functions detailed above for eds1, but the loss of resistance in pad4 is typically not as complete as in eds1 (Glazebrook et al., 1997; Aarts et al., 1998; Feys et al., 2001).

Other Arabidopsis mutations deregulate disease resistance responses and/or HR-like plant cell death responses, suggesting that negative control of plant defense pathways also occurs (Morel and Dangl, 1997). Some of these display a "disease lesion mimic" phenotype that is a feature of several well-characterized crop plant mutants, in which necrotic lesions form spontaneously or can be induced by various biotic or abiotic stresses (Dangl et al., 1996; Büschges et al., 1997; Gray et al., 1997). Importantly, Arabidopsis plants carrying the recessive null lsd1 allele exhibit normal HR after infection by various incompatible pathogens, but runaway cell death (RCD) is initiated subsequently at the margins of these sites (Dietrich et al., 1994). Spreading lesions in lsd1 can be induced by provision of O$_2^-$ (Jabs et al., 1996) in uninfected tissues. This, together with observations that O$_2^-$ accumulation precedes lesion formation (Jabs et al., 1996), suggests that LSD1 responds to a superoxide-dependent signal(s) emanating from an infection site. SA possibly potentiates this pathway, because lsd1 plants are acutely responsive to treatments with SA or chemically active SA analogs (Dietrich et al., 1994; Jabs et al., 1996). Thus, lsd1 lowers the threshold for both initiation and propagation of plant cell death beyond the HR. lsd1 plants also exhibit enhanced resistance to several normally virulent pathogens in a prelesioned state (Dietrich et al., 1994). We infer from these null phenotypes that LSD1 negatively regulates a signaling pathway(s) for basal defense and cell death and thereby may contribute to establishing a boundary to the plant HR (Dietrich et al., 1994). LSD1 encodes a zinc finger protein with homology with GATA-type transcription factors, and it has been suggested that the LSD1 protein functions either to negatively regulate a pro-death pathway component or to activate a repressor of plant cell death (Dietrich et al., 1997).

We constructed double mutant lines between the eds1, pad4, or ndr1 mutations and lsd1 and assessed their effects on RCD and disease resistance phenotypes after pathogen infection, treatment with benzothiadiazole (BTH), a functional SA mimic (Görlach et al., 1996), or a superoxide generator. We demonstrate that lsd1 does not affect the eds1, pad4, and ndr1 pathogen response phenotypes. However, both EDS1 and PAD4 are necessary for lsd1-conditioned RCD initiated by each tested stimulus. In contrast, NDR1 is required for RCD in response to superoxide and partially reduces lsd1 RCD after pathogen inoculation or BTH treatment. The requirement for EDS1 and PAD4 in lsd1 RCD is separable from processes associated with the local HR and disease resistance; therefore, it is likely to operate at the level of defense signal potentiation in cells surrounding an infection site.

**RESULTS**

**EDS1 and PAD4 Are Required for lsd1 RCD Induced by BTH and Pathogens**

We first examined the responses of short-day-grown eds1/lsd1, pad4/lsd1, and ndr1/lsd1c plants to a known inducer of
RCD in lsd1, the SA mimic BTH (all mutants used were null alleles; see Methods). As shown in the top row of Figures 1A and 1B, no phenotype was observed in leaves from either wild-type plants or plants with single mutations in eds1, pad4, or ndr1. Leaves from lsd1 or lsd1c plants, in contrast, formed the expected lesions in response to BTH 3 days after treatment. We did not observe any lesions in leaves of plants with double mutations in eds1/lsd1 and pad4/lsd1, but lesions were observed in leaves of ndr1/lsd1c plants. However, these lesions were not as extensive as those observed in leaves of lsd1 plants (Figure 1B). Thus, mutations in eds1 or pad4 abolish BTH-induced RCD in lsd1 plants. Similar results were observed in these plants after treatment with another inducer of lsd1-mediated RCD, a shift in growing conditions from short-day to long-day conditions (data not shown).

We next assessed the interactions between these double mutant plants and normally avirulent strains of P. syringae pv tomato (DC3000) expressing either avrRpm1 or avrRps4. The different signaling requirements for RPM1 and RPS4 mentioned in the Introduction allowed us to measure the effects of the eds1, pad4, and ndr1 mutations on lsd1-induced phenotypes in the context of both an intact (resistant) and a defective (susceptible) local plant response by using isogenic P. syringae strains differing only in the avr gene they express. Plants were infiltrated with low doses of DC3000/avrRps4 to examine the genetic interactions between eds1 or pad4 in combination with lsd1. As expected, Wassilewskija (Ws-0) and lsd1 plants were resistant (Figure 2A). However, Ws-0 plants exhibited no visible phenotype, whereas lsd1 plants displayed lesions 3 to 4 days postinoculation (DPI) (Figure 1A). In contrast, eds1 and eds1/lsd1 double mutant plants were susceptible (Figure 2A). Additionally, eds1 and eds1/ lsd1 double mutant plants developed characteristic chlorotic disease symptoms, but no spreading lesions were observed in the eds1/lsd1 double mutant plants (Figure 1A). Plants with mutations in pad4 or both pad4 and lsd1 were intermediate; bacterial growth was ~10-fold less than in plants with mutations in eds1 (Figure 2A). However, pad4/lsd1 plants did not exhibit chlorosis associated with disease or pathogen-induced lesioning associated with lsd1 RCD (Figure 1A). Thus, lsd1 does not influence the susceptibility of eds1 or pad4 plants to DC3000/avrRps4.

We then challenged plants with low doses of DC3000/avrRpm1. Wild-type, lsd1, eds1, and pad4 plants responded as expected (see Introduction); all genotypes were resistant, and RCD was visible in lsd1 leaves (Figures 1A and 2C). eds1/lsd1 and pad4/lsd1 double mutants also were resistant, but, surprisingly, they did not exhibit RCD. To confirm this observation, we infiltrated leaves with levels of DC3000/avrRpm1 (10^7/mL) that induce an HR 6 to 8 hr after inoculation (Grant et al., 1995). Plants from all genotypes (Ws-0, lsd1, eds1, eds1/lsd1, pad4, and pad4/lsd1) exhibited an HR. However, spreading lesions were observed in only lsd1 plants and not in eds1/lsd1 or pad4/lsd1 (Table 1). Therefore, EDS1 and PAD4 are required for lsd1 RCD. Importantly, the requirement for EDS1 and PAD4 in RCD is independent of their signaling functions in RPS4-mediated disease resistance and separate from processes controlling RPM1 resistance.

We also infiltrated ndr1/lsd1c double mutants with either DC3000/avrRps4 or DC3000/avrRpm1. Columbia (Col-0) and ndr1 plants were resistant to DC3000/avrRps4 (Figures
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A and 2B), whereas ndr1 plants were moderately susceptible to infection by DC3000/avrRpm1, consistent with previous analyses (Century et al., 1995) (Figures 1B and 2D). ndr1/lsd1c double mutants were resistant to DC3000/avrRps4 and susceptible to DC3000/avrRpm1, as expected. However, we observed a partial suppression of RCD in these plants after inoculation with either bacterial strain (Figure 1B). Therefore, although ndr1 reduced lsd1 RCD, the level of reduction did not correlate with the loss of RPM1 function observed in ndr1 mutants.

We then examined the responses of the eds1/lsd1, pad4/lsd1, and ndr1/lsd1c mutant lines to normally avirulent isolates of the oomycete pathogen Pp. This extends our analysis to an additional pathogen recognized by R genes that differ in their signaling requirements, as outlined in the Introduction. As shown in Figures 3A and 3B, RPP1-mediated resistance to Noco2 in cotyledons of Ws-0 and lsd1 is manifested as HR at points of attempted pathogen penetration 6 DPI. At this time, developing RCD is visible in lsd1 as an enlargement of the trypan blue–stained zone around an infection site (Figure 3A). In contrast, cotyledons of eds1 and eds1/lsd1 plants were susceptible to Noco2; we observed extensive mycelial growth as well as asexual sporulation (Figures 3A and 3B). Noco2 inoculation failed to elicit an HR in eds1/lsd1 plants. pad4 and pad4/lsd1 plants were partially susceptible to Noco2; we observed trailing necrosis in response to Noco2 (Figure 3A), suggesting that HR was elicited but not sufficient to fully restrict pathogen growth. There was no RCD in either double mutant. Therefore, EDS1 and PAD4 are required for lsd1-mediated RCD, using Pp as an RCD inducer.

A similar analysis was performed by inoculating Emoy2 onto cotyledons of Col-0, lsd1c, ndr1, and ndr1/lsd1c plants. RPP4-mediated resistance to Emoy2 in Col-0 and lsd1c was associated with HR and the initiation of RCD in lsd1 cotyledons at 6 DPI (Figure 3A). ndr1 partially suppressed RPP4-mediated resistance to Emoy2; we observed an increased frequency of trailing necrosis (Figures 3A and 3B). lsd1c plants expressed strong resistance to Emoy2, as shown by an increase in the proportion of HR sites, compared with Col-0 (Figure 3B). Surprisingly, ndr1/lsd1c double mutants exhibited an intermediate phenotype (Figure 3B). Therefore, the loss of LSD1 function enhanced host resistance to Pp early in the plant–pathogen interaction independent of NDR1 and presumably independent of the recognition conferred by RPP4.

Figure 2. Bacterial Growth in Wild-Type, Single Mutant, and Double Mutant Plants.

Growth of P. syringae pv DC3000 expressing avrRps4 or avrRpm1 extracted from leaves at 0 (open bars) and 3 (closed bars) days after inoculation (initial titer, 10^5 colony-forming units/mL). Data from Ws-0 accession lines are presented in (A) and (C), and data from Col-0 accession lines are presented in (B) and (D). Bars represent the mean and ± SD of four independent data points. Similar results were obtained in two independent experiments. cfu, colony-forming units; f.w., fresh weight.
ever, by 3 to 4 DPI, lesions in the rate of initial lesion formation at the boundary of the HR was similar in the area of incompatible oculation. Leaves of Ws-0 or Col-0 in the area of incompatible oculation. Leaves of

leaves infected with P. syringae pv DC3000 expressingavrRps4 or avrRpm1 or treated with a 2-µL droplet of 20 mM RB. Development of the plant HR and accumulation of ROI were scored 4 days, after bacterial inoculation. The scores ( ), +, +, +, and + + + reflect the intensity of staining with lactophenol–trypan blue for the HR and DAB for ROI. They are representative of at least six leaves per treatment. Asterisks denote an expanded, diffuse HR and ROIs.

For the HR and ROI, it is important that the ROIs visualize H2O2 and then inoculated with a 10-µL droplet of avirulent Pp conidia, or they were dipped into suspensions of P. syringae. DAB polymerizes as a brown precipitate on contact with H2O2 in the presence of peroxidase (Shirasu et al., 1997; Thordal-Christensen et al., 1997), thus providing a useful marker for total peroxide accumulation.

The results from this analysis are shown in Figures 4C and 4D and are summarized in Table 1. A plant oxidative burst producing detectable local concentrations of H2O2 was observed only in plant genotypes undergoing an HR. Thus, eds1 (and eds1/lsd1) plants challenged with Pp Noco2 or DC3000/avrRps4, in which resistance is suppressed, failed to elicit an oxidative burst or an HR, pad4 (and pad4/lsd1) plants generated high levels of H2O2 and also developed either trailing necrosis or an HR, depending on the pathogen challenge. We conclude that EDS1 activity is required for the oxidative burst in EDS1-dependent R gene–mediated responses, whereas PAD4 functions either downstream or independently of ROI accumulation in the same responses. Both eds1 and pad4 plants produced a wild-type RPM1-mediated oxidative burst and HR after challenge with DC3000/avrRpm1 (Table 1). Thus, neither EDS1 nor PAD4 is required for the HR-associated oxidative burst in this EDS1-independent pathway. Yet, both are required for RCD in any of the tested contexts. Significantly, therefore, the requirements for EDS1 and PAD4 during lsd1-dependent RCD are unrelated to their effects on local R gene–mediated HR. We conclude from these results that EDS1 and PAD4 provide necessary signaling functions leading to lsd1 RCD that are either downstream or independent of the local HR and associated ROI accumulation.

Interestingly, ndr1 exhibited a reduction in the intensity of HR-associated DAB staining compared with that of Col-0 in response to Pp Emyo2, even though more host cells died, as measured by trypan blue (Figure 4D). This is in contrast to the enhanced H2O2 accumulation in lsd1 and lsd1c (Figures 4C and 4D). The response of nrd1/lsd1c plants was intermediate between that of nrd1 and lsd1c alone, and the RCD boundary was less well defined than it was in wild-type plants (Figure 4D). These data suggest that the reduced ROI production in nrd1 may be responsible for the attenuated RCD observed in the nrd1/lsd1c double mutant.

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<th>Arabidopsis Lines</th>
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Leaves were dipped in suspensions (10⁷ colony-forming units/mL) of P. syringae pv DC3000 expressing avrRps4 or avrRpm1 or treated with a 2-µL droplet of 20 mM RB. Development of the plant HR and accumulation of ROI were scored 4 days, after bacterial inoculation. The scores (+), +, +, +, and + + + reflect the intensity of staining with lactophenol–trypan blue for the HR and DAB for ROI. The leaves of lsd1 produced lesions that spread from the site of the localized resistance response (Figures 4A and 4B). In contrast, assexual sporulation of Pp was observed on infected eds1 and eds1/lsd1 leaves at 6 DPI, and no necrosis is observed. Leaves of pad4 and pad4/lsd1 plants supported some pathogen growth that was accompanied by trailing necrosis (Figure 4A). These results mimic those observed on cotyledons and further support the requirement for EDS1 and PAD4 for lsd1-mediated RCD. Leaves of nrd1 and Col-0 responded in a similar manner to inoculation of Pp Emyo2, although the area of plant tissue undergoing an HR was marginally larger in nrd1 than in Col-0 (Figure 4B; see also Figure 4D). The RCD was severely reduced in nrd1/lsd1c compared with that in lsd1c (Figure 4B). Interestingly, the rate of initial lesion formation at the boundary of the HR was similar in lsd1c and nrd1/lsd1c (data not shown). However, by 3 to 4 DPI, lesions in nrd1/lsd1c ceased to expand, whereas in lsd1c they progressed and consumed the entire leaf by ~6 DPI (Figures 4B and 4D).

**Table 1.** Response Phenotypes of Wild-Type, Single Mutant, and Double-Mutant Lines to Inoculation with Avirulent Bacteria or Treatment with RB

An oxidative burst giving rise to local ROI accumulation is an early event associated with the plant HR (Bestwick et al., 1997; Shirasu et al., 1997; Thordal-Christensen et al., 1997). Also, O₂⁻ is necessary and sufficient for lsd1 RCD (Jabs et al., 1996). We examined the production of ROI in wild-type and mutant plants at the point of pathogen penetration to determine whether the effects of eds1, pad4, or nrd1 on lsd1-induced lesion propagation could be related to deficiencies in early ROI accumulation during the HR. Excised leaves were dipped in a solution of 3,3-diaminobenzidine (DAB) to visualize H2O2 and then inoculated with a 10-µL droplet of avirulent Pp conidia, or they were dipped into suspensions of P. syringae. DAB polymerizes as a brown precipitate on contact with H2O2 in the presence of peroxidase (Shirasu et al., 1997; Thordal-Christensen et al., 1997), thus providing a useful marker for total peroxide accumulation.

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Fate of Superoxide at HR Margins and RCD Sites Is Different

Because provision of $O_2^-$ is both necessary and sufficient to induce RCD in $lsd1$ plants (Jabs et al., 1996), we addressed directly whether $EDS1$, $PAD4$, and $NDR1$ act as signaling intermediates between superoxide and the $LSD1$-controlled cell death pathway. We elicited superoxide production by applying a discrete spot of rose bengal (RB) to leaves. In the presence of light, RB generates singlet oxygen that reduces to superoxide, which is rapidly dismutated to the more stable $H_2O_2$ (Knox and Dodge, 1984; Baker and Orlandi, 1995). RB-induced plant cell death was confined to the application site in wild-type Ws-0 leaves, but it induced RCD in $lsd1$ leaves, as shown in trypan blue–stained leaves at 3 DPI (Figure 5A). We assessed $H_2O_2$ accumulation over a time course (3 to 48 hr) of RB treatment. Within 3 hr, we observed intense DAB and trypan blue staining in the area of RB application (Figure 5A). From 27 hr onward, cell death foci were fixed in Ws-0 but expanded in $lsd1$ (Figure 5A). In several independent experiments, RB treatments of wild-type, $eds1/lsd1$, $pad4/lsd1$, and $ndr1/lsd1$ plants failed to elicit RCD (Table 1). The same responses were observed in leaves of all genotypes infiltrated with a xanthine/xanthine oxidase superoxide-generating system that was previously shown to induce lesions in $lsd1$ (Jabs et al., 1996; data not shown).

In the earlier analysis by Jabs et al. (1996), superoxide accumulation was observed in live plant cells bordering the RCD lesions of $lsd1$ leaves. We expected to see DAB precipitation at the leading margins of $lsd1$ lesions that would be generated upon dismutation of $O_2^-$ to $H_2O_2$. However, there was no detectable $H_2O_2$ accumulation associated with $lsd1$ RCD lesions after either RB-induced cell death (Figure 5A) or $Pp$ inoculation (Figure 5B). Superoxide production, measured by nitroblue tetrazolium (NBT) staining, was not detected at any point associated with the $Pp$–induced HR or RB-induced cell death (data not shown). Superoxide accu-
mulation, however, was observed at the boundaries of developing lesions in lsd1, confirming previous results (Jabs et al., 1996; data not shown). These results suggest that the fate of superoxide generated as a component of the R gene–dependent HR is different from that produced in association with RCD in lsd1.

**DISCUSSION**

We demonstrate that EDS1 and PAD4, two positive regulators of plant disease resistance, are essential components of a cell death control pathway regulated by LSD1 in response to pathogen infection, BTH application, or provision of superoxide. Most importantly, the requirement for EDS1 and PAD4 during lsd1 RCD is independent of their roles as mediators of various R gene functions. Additionally, NDR1, a third disease resistance signaling component, contributes to lsd1 RCD during these responses.

**EDS1 and PAD4 Potentiate Plant Defense Signaling**

Our most important conclusion is that the requirements for EDS1 and PAD4 in lsd1 lesion formation are separable from their roles in localized R gene–mediated plant cell death, as shown in the model in Figure 6. For example, neither EDS1
nor PAD4 function in RPM1 resistance, yet both are required for RCD after RPM1 stimulation in lsd1. EDS1, but not PAD4, is necessary for ROI production and HR after local RPS4- or RPP1-mediated pathogen recognition, yet both EDS1 and PAD4 are required for lsd1 RCD in these responses. We suggest that the activities of EDS1 and PAD4 leading to lesion formation in lsd1 are in defense signal potentiation, downstream or independent of the HR (Figure 6). The finding that eds1 and pad4 suppress lsd1 RCD in response to applications of BTH, a functional mimic of the plant resistance signaling molecule SA, is consistent with this idea. Other studies have shown the involvement of SA in signal potentiation during local and systemic plant defenses (Shirasu et al., 1997; Delledonne et al., 1998; Klessig et al., 2000; Martinez et al., 2000). EDS1 (Falk et al., 1999; Feys et al., 2001) and PAD4 (Zhou et al., 1998; Jirage et al., 1999) operate upstream of SA accumulation during resistance responses in which they are required. In these contexts, their expression levels are enhanced by the application of SA, suggesting that EDS1 and PAD4 are regulated by SA-dependent positive feedback (Falk et al., 1999; Jirage et al., 1999; Feys et al., 2001). We suggest that the flux through this feedback is regulated by LSD1 (Figure 6). Experiments are in progress to determine directly the role of SA and the SA response regulator NPR1/NIM1 (Cao et al., 1997; Ryals et al., 1997) in lsd1-dependent RCD. Notably,
nrd1 did not significantly suppress lesioning in lsd1 after BTH treatment. This indicates that, in contrast to EDS1 and PAD4, NDR1 is not essential for BTH signaling (and presumably SA signaling) in relation to LSD1-regulated plant cell death.

**EDS1, PAD4, and NDR1 Mediate ROI-Dependent Signaling**

It was shown previously that RCD in lsd1 plants can be triggered by superoxide furnished by local applications of xanthine and xanthine oxidase (Jabs et al., 1996). Also, superoxide accumulation proceeded lesion formation in lsd1 tissue and was detectable in cells bordering the developing lesion by specific NBT staining (Jabs et al., 1996). Thus, accumulation preceded lesion formation in thine and xanthine oxidase (Jabs et al., 1996). Also, superoxide generated by superoxide furnished by local applications of xanthine oxidase (Jabs et al., 1996).

We suggest that EDS1 and PAD4, and interestingly also NDR1, mediate the ROI-derived signal leading to lsd1 RCD. The most compelling evidence for this is the failure of the eds1/lsd1, pad4/lsd1, and nrd1/lsd1c plants to initiate spreading lesions after local provision of superoxide, supplied either by RB (Table 1) or xanthine/xanthine oxidase applications. These data imply that all three disease resistance regulators express this particular function in unchallenged cells.

The activities of EDS1 and PAD4 in ROI signaling leading to RCD, therefore, are genetically distinct from their roles during the oxidative burst associated with a pathogen-induced HR (Figure 6). This finding strengthens the notion that EDS1 and PAD4 have a second function operating downstream or independently of the HR. We postulate that this second function helps establish the signal normally required to initiate lsd1 RCD. However, three observations suggest a different role for NDR1 in ROI signaling. (1) nrd1 attenuated the oxidative burst during the HR through RPS4 or RPM1, whereas it enhanced the oxidative burst during the HR through RPM1 pathogen recognition. (2) In all of these plant–pathogen combinations, nrd1 diminished lsd1 RCD. (3) NDR1 is not required for lsd1 lesions in response to BTH but is required for lesion development in response to ROI provision. These three points lead us to conclude that NDR1 is important in regulating the local ROI status (Figure 6). Imbalances in this system are likely to affect the efficiency of the HR and consequent local signaling and probably drive RCD in lsd1. Recent studies reveal that the balance of ROI, most particularly O$_2^{-}$, H$_2$O$_2$, and NO, is crucial for the establishment of the HR (Delledonne et al., 1998, 2001; Klessig et al., 2000).

We propose that EDS1 and PAD4 are regulators of ROI- and SA-dependent signaling in a plant defense potentiation circuit. We suggest that NDR1 is required more proximally for the control of ROI generation and the transduction of a ROI-derived signal at the initial interaction site. In this respect, it is interesting that EDS1 and PAD4, but not NDR1, are components of a basal resistance pathway that limits the growth of virulent pathogens in the absence of plant cell death.

**ROI Requirements Differ between the HR and LSD1-Controlled Plant Cell Death**

Our analysis of ROI accumulation suggests that the nature of ROIs produced by cells undergoing the HR is different from that of ROIs associated with signaling from those cells, and monitored by LSD1. We detected superoxide, but not H$_2$O$_2$, in living cells bordering spreading lsd1 lesions, as shown previously (Jabs et al., 1996). Our failure to observe H$_2$O$_2$ at these margins was surprising, because superoxide would be expected to dismutate to H$_2$O$_2$. LSD1 is required for the SA-dependent induction of antioxidant copper-zinc...
superoxide dismutase (Cu-Zn SOD) (Kliebenstein et al., 1999) and potentially other antioxidant genes. Thus, a simple explanation is that there is no, or there is delayed, accumulation of Cu-Zn SOD in lsd1 and hence no dismutation. This simple model is weakened by the unlikelihood that Cu-Zn SOD operates in the apoplast, where $\text{O}_2^{-}$ is first produced during the oxidative burst (Bolwell, 1999).

Another possibility is that superoxide produced by cells at HR margins, where LSD1 is proposed to function, is converted to something other than $\text{H}_2\text{O}_2$. This could reflect an interplay between $\text{O}_2^{-}$ with other ROI molecules, SA, or antioxidant systems. In animal cells, superoxide can react with NO to produce peroxynitrite (ONOO$^-$), a highly reactive redox species that serves as a signal or as a cytotoxic agent, depending on its level and the availability of other redox molecules (Bonfoco et al., 1995; Lin et al., 1995). Delledonne et al. (2001) propose that $\text{O}_2^{-}$ production and its dismutation to $\text{H}_2\text{O}_2$ regulate a balance of $\text{H}_2\text{O}_2$/NO that, when disturbed, leads to HR. They argue against a direct role in cell killing for ONOO$^-$. An alternate explanation is that the $\text{H}_2\text{O}_2$ produced is locally unavailable for polymerization with DAB. This could be caused by changes in the cellular pH specific to these mutant backgrounds (DAB staining is effective only at pH values between 5.5 and 6.0; Thordal-Christensen et al., 1997) or by a surge of ROI scavenging enzymes (Vanacker et al., 2000). Cells are permeable to DAB and $\text{H}_2\text{O}_2$ (Thordal-Christensen et al., 1997), ruling out the possibility that $\text{H}_2\text{O}_2$ generated within the cell would be inaccessible for detection. Our data clearly suggest that the fate of superoxide produced in cells undergoing an HR is different from that generated locally during lsd1 lesion development. This implies that signaling extending from infected cells is controlled differently than it is in the infected cells themselves.

Conversely, we observed $\text{H}_2\text{O}_2$, but not superoxide, at infection foci. Superoxide is an unstable redox molecule that rapidly dismutates enzymatically or nonenzymatically to $\text{H}_2\text{O}_2$ (Lamb and Dixon, 1997). Overwhelming evidence suggests that production of superoxide at the cell surface is the proximal event in the plant oxidative burst (Bolwell, 1999). However, other extracellular and intracellular mechanisms may contribute to ROI generation during the oxidative burst (Allan and Fluhr, 1997; Martinez et al., 1998; Bolwell, 1999). The transience of the oxidative burst and the inherent instability of superoxide may account for our failure to observe NBT-reactive material at infection sites or in cells supplied with superoxide by exogenous RB application. RB was applied onto the leaf surface and therefore would release superoxide into the plant apoplast that would be accessible to NBT (Baker and Orlandi, 1995).

**Putative Signaling Functions of EDS1, PAD4, and LSD1**

Our results draw an important genetic link between the disease resistance–promoting functions of EDS1, PAD4, and LSD1 and the negative regulation of plant cell death exerted by LSD1 (Figure 6), raising questions about the biochemical roles of these proteins in healthy and pathogen-challenged plants. LSD1 encodes a zinc finger protein with similarity to the GATA-type family of transcription factors. EDS1 and PAD4 share homology with the catalytic domains of eukaryotic lipases (Falk et al., 1999; Jirage et al., 1999), although hydrolytic activities have not been demonstrated. It is possible, therefore, that they process ROI-activated signal intermediates spreading from infected to surrounding noninfected cells to perpetuate plant defense responses. In animals (Serhan et al., 1996; Stafforini et al., 1997) and plants (Farmer et al., 1998; Sanz et al., 1998; Rusterucci et al., 1999), activated fatty acids are important signaling molecules produced in response to certain pathogens and after wounding. Thus, EDS1 and PAD4 may potentiate resistance by processing ROI- and SA-activated molecules. The production of such molecules, whether lipid based or otherwise, would normally lead to cell death only if their levels passed a cell death control threshold. Obviously, in an lsd1 null mutant, these levels need not be high to initiate RCD. The biochemical role of NDR1 also remains to be resolved, although its potential membrane association (Century et al., 1997) may be important in regulating cellular communication between external and internal redox systems. Elucidating the activities, cellular localization, and molecular associations of all of these signaling components should provide important insights into their precise functions in plant disease resistance.

**METHODS**

**Plant Material and Cultivation**

The origins of eds1-1 (Parker et al., 1996) and lsd1 (Dietrich et al., 1997) in accession Wassilewskijia (Ws-0) have been described previously. The pad4-5 T-DNA insertion mutant also was isolated in Ws-0 (Feyes et al., 2001). The T-DNA is inserted 35 bp 5′ to the end of the single intron in the PAD4 gene. The ndr1-1 mutant line in accession Columbia (Col-0) (Century et al., 1997) was kindly provided by Dr. Brian Staskawicz (University of California, Berkeley). Seed were sown on low nutrient compost and grown in a chamber under a light period of 8 hr (∼160 μE·m$^{-2}·$sec$^{-1}$) at 22°C and 65% relative humidity (RH).

F2 plants derived from selfed F1 plants were genotyped for the lsd1 mutation by polymerase chain reaction (PCR) using a triple primer set (5′-ACCTAAAGAAAAAGAGTGTTGGAGG-3′, 5′-ATATAAAACCCTACTAGCTAAACAGG-3′, and 5′-CTGCTACTTTTACACAAAACGAGTC-3′). The wild-type LSD1 allele produces a 940-bp product, whereas lsd1 gives a 600-bp product. The Col-0 allele of lsd1 (lsd1c) was constructed by introgressing the Ws-0 allele into a Col-0 line over seven generations and selecting for the mutant allele using the lsd1 PCR described above. The ndr1/lsd1c double mutant was constructed by crossing ndr1-1 F1 plants with lsd1c, selfing the F1 plants, and genotyping the segregating F2 plants for the lsd1 mutation (described above) and the ndr1-1 mutation by using the primer set 5′-GGGACGGTCTATTCTGATAGA-3′ and 5′-CGAGATTG-
TCATTGCCATTGG-3’. The eds1-1 mutation was detected in eds1-1 × lsd1 F2 plants using the primer set 5’-GGATAGAGATGAAATACAAGGC-3’ and 5’-ACCTAAGTCTCGTTACTGCTG-3’. PCR products were digested for at least 4 hr with Mse1, and products were resolved on a 2% agarose gel. Cleavage of wild-type EDS1 produces three visible bands of 280, 180, and 150 bp, whereas eds1-1 gives visible products of 240, 180, and 150 bp. PCR-based selection of the pad4-5 mutant allele in pad4-5 × lsd1 F2 plants was as described (Feyts et al., 2001). In the initial characterization of mutant phenotypes, we examined several independent mutant lines. All behaved similarly; hence, more detailed analyses were performed with one representative single and double mutant per genotype.

Pathogen Isolates and Growth Determinations

Pseudomonas syringae was tested on leaves using 5 × 10⁸ colony-forming units (CFUs)/mL of P. syringae pv tomato DC3000 expressing either avrRps4 or avrRpm1 into one side of the leaf using a 1.5-mL needleless syringe. Plants were inspected for disease symptoms and/or spreading lesion formation over 6 days under the same conditions as for the bacterial growth assays. Hypersensitive response (HR) tests were performed using 5 × 10⁵ colony-forming units/mL. Growth of P. syringae pv tomato DC3000 expressing avrRps4 or avrRpm1 in the various lines was determined by dip inoculation and subsequent growth analysis essentially as described (Innes et al., 1993) with modifications (P. Tomera and J.L. Dangl, unpublished data). Briefly, pots containing 2-week-old plants were immersed for 10 to 15 sec in a suspension containing 2.5 × 10⁵ colony-forming units/mL (OD₆₀₀ = 0.05) and Silwet (200 μL/L). Plants were kept under high humidity for 1 hr, after which time measure-ment zero was taken. At time 0 and 3 days, bacteria were extracted from the plant tissue and grown on selective agar plates to determine concentration.

Benzo(thi)odiazole Induction of lsd1 RCD

For chemical induction of RCD, leaves of 4-week-old plants were sprayed with 0.35 mM benzo(thi)odiazole (BTH), which was provided as a gift from Syngenta (Research Triangle Park, NC). Plants were maintained under normal growth conditions and inspected for lesion development over 6 days.

Histochemical Analysis of Plant Cell Death and Pp Development

Plant cell necrosis induced by pathogen inoculation or chemical treatment, as well as the development of Pp mycelium inside cotyle-don or leaf tissues, was monitored by staining with lactophenol-blue and destaining in saturated chloral hydrate as described (Koch and Slusarenko, 1990). Material was mounted on a slide in 60% glycerol and examined using a light microscope (Axioskop; Zeiss, Jena, Germany). Excised leaves were manipulated in parallel with those used for detection of hydrogen peroxide (H₂O₂) and maintained under the same conditions (see below).

Histochemical Detection of H₂O₂ at Interaction Sites

Detection of H₂O₂ was by endogenous peroxidase-dependent in situ histochemical staining using 3,3-diaminobenzidine (DAB) in a proto-col modified from Thordal-Christensen et al. (1997). Leaves of 4-week-old plants were inoculated with a 10-µL droplet of Pp conidiospores placed on the leaf surface. Leaves were then excised and supplied through the cut petiole with a solution of 1 mg/mL DAB for 8 hr in light (100 to 160 μE·m⁻²·sec⁻¹) or in darkness under the same conditions used to determine Pp growth. Subsequently, the DAB solution was replaced with water, and leaves were maintained under the same conditions as before. For assessment of H₂O₂ accumulation at P. syringae infection sites, excised leaves were allowed to take up DAB solution for 8 hr and then were dipped in bacterial suspensions and incubated as described for the bacterial growth assays except that leaves were kept in the dark. At different times after pathogen inoculation, leaves were cleared for 5 min in boiling acetic acid/glyc- erol/ethanol (1:1:3 [v/v/v]) solution. Material was mounted on a slide in 60% glycerol and examined using a light microscope (Axioskop; Zeiss). H₂O₂ was detectable as reddish brown coloration.

Chemical Provision of ROI in Leaves

Rose bengal (4,5,6,7-tetrachloro-2’,4’,5’,7’-tetraiodofluorescein [RB]; Sigma) is an efficient singlet molecular oxygen (O₂·) producer in aqueous solution (Knox and Dodge, 1984). O₂· gives rise to radical anion superoxide (O₂⁻) and subsequently to H₂O₂. RB was applied as a droplet of 10 µL (20 mM solution) onto the surface of excised leaves of 4-week-old plants. These were placed in a growth chamber in the light (160 to 200 μE·m⁻²·sec⁻¹) for at least 3 hr after RB treatment and maintained for several days under an 8-hr photoperiod at 19°C and 65% RH. Xanthine and xanthine oxidase coinfiltration in leaves of 4- or 5-week-old plants was used to generate superoxide, as described previously (Jabs et al., 1996). Infiltrated plants were maintained under normal plant growth conditions.

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