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Runaway cell death, but not basal disease resistance, in *lsd1* is SA- and *NIM1/NPR1*-dependent

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Summary

***LSD1* was defined as a negative regulator of plant cell death and basal disease resistance based on its null mutant phenotypes. We addressed the relationship between *lsd1*-mediated runaway cell death and signaling components required for systemic acquired resistance (SAR), namely salicylic acid (SA) accumulation and *NIM1*/*NPR1*. We present two important findings. First, SA accumulation and *NIM1/NPR1* are required for *lsd1*-mediated runaway cell death following pathogen infection or application of chemicals that mimic SA action. This implies that *lsd1*-dependent cell death occurs 'downstream' of the accumulation of SA. As SA application triggers runaway cell death in *lsd1* but not wild-type plants, we infer that *LSD1* negatively regulates an SA-dependent signal leading to cell death. Thus SA is both a trigger and a required mediator of *lsd1* runaway cell death. Second, neither SA accumulation nor *NIM1/NPR1* function is required for the basal resistance operating in *lsd1.* Therefore *LSD1* negatively regulates a basal defense pathway that can act upstream or independently of both *NIM1/NPR1* function and SA accumulation following avirulent or virulent pathogen challenge. Our data, together with results from other studies, point to the existence of an SA-dependent 'signal potentiation loop' controlling HR. Continued escalation of signaling in the absence of *LSD1* leads to runaway cell death. We propose that *LSD1* is a key negative regulator of this signal potentiation.**

Keywords: *LSD1*, *NIM1*/ *NPR1*, *NahG*, salicylic acid, hypersensitive response, runaway cell death.

Introduction

Plant disease resistance relies on multilayered processes that ultimately lead to the inhibition of pathogen growth. Some plant defenses are preformed, while others are induced by attempted pathogen ingress. Of the induced responses, resistance (*R*) gene-mediated defenses are the most extensively characterized (reviewed by Dangl and Jones, 2001; Feys and Parker, 2000; McDowell and Dangl, 2000). A plant *R*-gene product recognizes (directly or indirectly) a corresponding pathogen avirulence gene (*avr* gene) product. This recognition is often, but not always, associated with a rapid plant-initiated programmed cell death known as the hypersensitive response (HR) (reviewed by Dangl *et al.*, 1996; Dangl *et al.*, 2000; Heath, 2000; Shirasu and Schulze-Lefert, 2000). At the site of HR, and in surrounding cells, one of the earliest events observed is an oxidative burst whereby reactive oxygen intermediates (ROI) including superoxide (O_2^-) and its dismutation product, hydrogen peroxide (H_2O_2), are produced (Doke, 1983; reviewed by Lamb and Dixon, 1997). Nitric oxide (NO), a redox-active molecule involved in mammalian defense responses (Schmidt and Walter, 1994), is also generated and has been shown to serve as a signaling molecule in plant resistance (Delledonne *et al.*, 1998; Durner *et al.*, 1998). Although the biochemical roles for ROI in plants are unknown, a temporal and stoichiometric balance between NO, ROI and the phenolic signaling molecule salicylic acid (SA) produced early in defense appears to regulate HR (Delledonne *et al.*, 2001; reviewed by Alvarez, 2000; Wendehenne *et al.*, 2001).

Also associated with HR activation is the induction of systemic acquired resistance (SAR) that provides resistance to a broad range of pathogens in distal plant tissues (Ryals *et al.*, 1996). Experiments with transgenic *Arabidopsis thaliana* and tobacco plants expressing a bacterial gene (*NahG*) (encoding salicylate hydroxylase that converts SA to catechol) demonstrated that SA accumulation in distal but not local tissues is essential for SAR (Gaffney *et al.*, 1993; Vernooij *et al.*, 1994). Additionally, local resistance to some, but not all, avirulent pathogens is compromised in *NahG* plants, indicating that certain *R* functions are SA-dependent (Bittner-Eddy and Beynon, 2001; Delaney *et al.*, 1994; McDowell *et al.*, 2000). Application of SA or a functional analogue such as benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) can induce the expression of defense-related genes and confer resistance to some virulent pathogens (Görlach *et al.*, 1996). Further support for SA involvement in SAR comes from mutants that do not accumulate SA in response to infection. These plants display enhanced disease susceptibility (*eds*) phenotypes (Dewdney *et al.*, 2000; Nawrath and Métraux, 1999).

Arabidopsis mutants were isolated that lack the ability to express genes normally activated during SAR (most notably the pathogen-related genes *PR-1* and *PR-2*), or that failed to develop resistance following an SAR-inducing treatment. *nim1/npr1* mutants exhibit significant reduction in *PR* gene expression on treatment with different pathogens or SAR inducing chemicals (Cao *et al.*, 1994; Delaney, 1997). Furthermore, *nim1/npr1* plants exhibit increased susceptibility to several virulent and some avirulent pathogens. The mutant phenotype is not rescued by exogenously applied SA, consistent with a function for *NIM1*/*NPR1* downstream of SA accumulation. NIM1/NPR1 contains ankyrin repeats (Cao *et al.*, 1997; Ryals *et al.*, 1997), and in yeast two-hybrid assays interacts with members of the TGA family of basic leucine zipper transcription factors that bind an SA-responsive element of the *Arabidopsis PR-1* promoter (Zhang *et al.*, 1999; Zhou *et al.*, 2000). Using cDNA micro-arrays, Maleck *et al.* (2000) demonstrated that NIM1/NPR1 is required for upregulation of an SAR gene cluster. Promoters in these genes show an enrichment of potential binding sites for WRKY-transcription factors, a plant-specific family of transcription regulators previously implicated in the control of defense gene expression (Eulgem *et al.*, 2000). Therefore NIM1/NPR1 may regulate expression of SAR-associated genes in conjunction with WRKY and TGA transcription factors. Lastly *SNI1*, whose absence suppresses the *nim1/npr1* phenotype, acts as a negative regulator of *PR* gene expression and potentially SAR (Li *et al.*, 1999). Thus NIM1/NPR1 is a central modulator of defense signals required for timely induction of resistance to some pathogens.

In an effort to understand plant cell death associated with the HR, mutants were isolated that misregulate plant cell death (summarized by Dangl *et al.*, 1996; Greenberg, 1997). One recessive null mutation defines the *LSD1* gene as a negative regulator of both HR-like cell death and basal defense responses (Dietrich *et al.*, 1994). Following normal induction of the HR by avirulent pathogens on *lsd1* plants, runaway cell death (rcd) spreads to engulf the entire leaf. This rcd phenomenon is dependent on production of O_2^-, but is independent of H_2O_2 production (Jabs *et al.*, 1996). *lsd1* plants challenged in a pre-lesion state are more resistant to some virulent pathogens, suggesting a role for LSD1 in the negative regulation of basal defense. *LSD1* encodes a zinc-finger protein with homology to plant relatives of the GATA-1 transcription factor (Dietrich *et al.*, 1997). Dietrich *et al.* (1997) proposed that LSD1 functions either as a negative regulator of a pro-death signal, or as an activator of plant cell death protection genes.

We recently established that *EDS1* and *PAD4*, two positive regulators of local resistance mediated by a subset of *R* genes, are required for *lsd1*-conditioned rcd (Rustérucci *et al.*, 2001). Suppression of rcd in *eds1 lsd1* or *pad4 lsd1* double mutants occurred in response to all stimuli that induce rcd in *lsd1*. This included inoculation with avirulent pathogens that initiate HR and disease resistance independently of *EDS1* and *PAD4*. Thus the requirements for *EDS1* or *PAD4* in *lsd1* rcd are downstream or independent of the HR. We envisage that *EDS1* and *PAD4* drive a defense-signal potentiation loop by interpreting ROI-derived and other signals emanating from the HR, leading to lesion formation in *lsd1.*

To determine the relationship between *lsd1*-associated rcd and other factors that potentiate defense signaling, we constructed the double-mutant lines *NahG lsd1* and *nim1 lsd1.* Our results surprisingly demonstrate that LSD1 negatively regulates an SA- and NIM1/NPR1-independent basal disease-resistance pathway. In contrast, *lsd1*-induced rcd requires NIM1/NPR1 and SA accumulation. We conclude that both NIM1/NPR1 and SA accumulation contribute to a signal amplification step leading to rcd in *lsd1*.

Results

Complete lsd1 *runaway cell death requires SA accumulation and* NIM1/NPR1

Initially, we assessed the responsiveness of short-day-grown *NahG lsd1* and *nim1 lsd1* double-mutant plants to SA treatment. We used the strong *nim1-1* allele and a strong *NahG* line in combination with the *lsd1* null allele (all in the accession Ws-0). As shown in Figure 1, *lsd1* leaves exhibited visible lesioning 3–4 days after SA application, and complete leaf collapse by day 5. *nim1* and

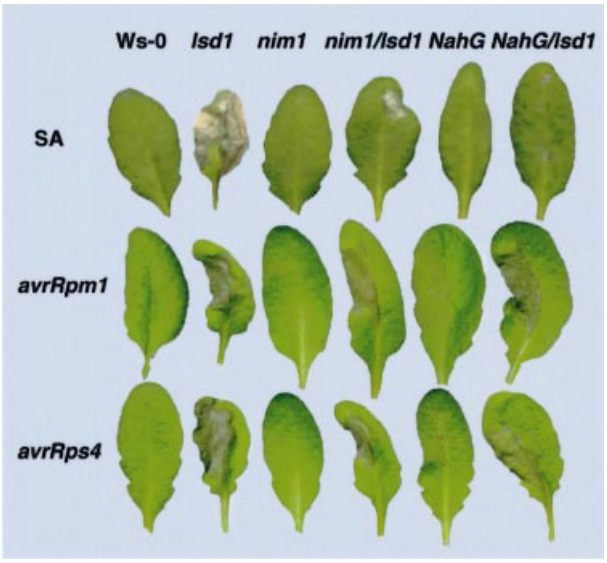

Figure 1. Lesion phenotypes of plant lines after SA treatment or bacterial pathogen inoculation.
Leaves of 4-week-old wild-type, single- or double-mutant plants were sprayed with 2 mM SA or infiltrated on one whole side of the leaf with suspensions (1×10^5 colony-forming units ml^{-1}) *Pseudomonas syringae* pv. DC3000 expressing *avrRps4* or *avrRpm1*. Leaves were photographed at 5 dpi (SA) and at 6 dpi (*Pseudomonas*). Each leaf is a representative of 12 to 15 leaves. All treatments were repeated at least three times with similar results.

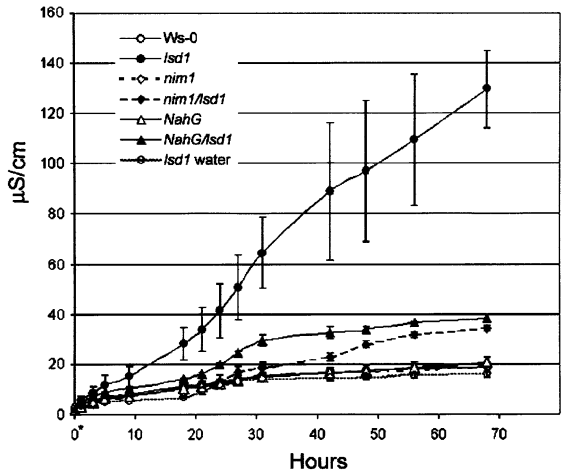

Figure 2. Ion-leakage profiles after SA treatment.
4-week-old plants were sprayed with 2 mM SA to imminent run-off. Ion leakage measurements of leaf discs were started at 3 dpi (0*). Conductivity (µS cm^{-1}) was determined at the time points indicated. Bars represent mean and SD of four independent data points within one experiment. Similar results were obtained in two independent experiments.

NahG did not exhibit the rcd phenotype. SA-treated *nim1 lsd1* plants displayed delayed and significantly reduced lesioning, resulting in an rcd that was incomplete and did not engulf the entire leaf. NahG *lsd1* plants also did not express significant rcd following SA treatment.

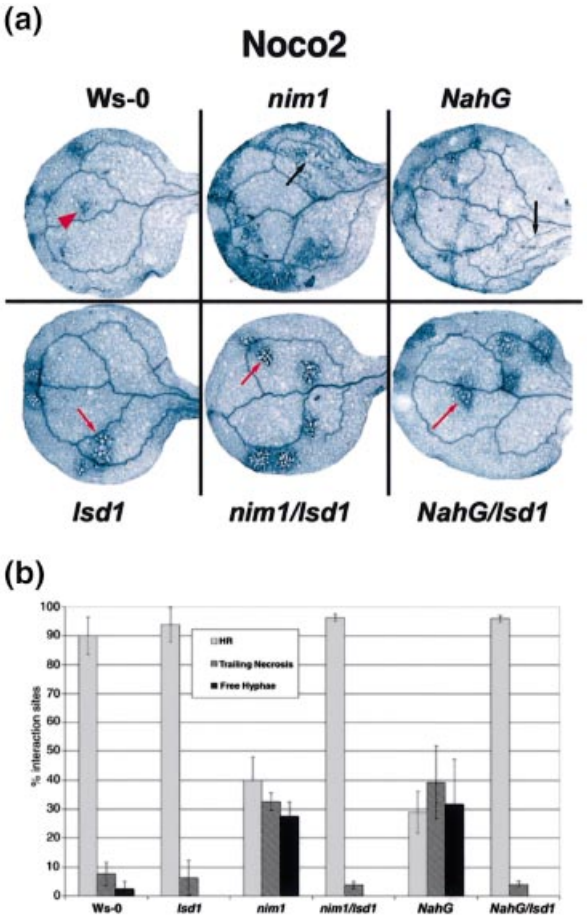

Figure 3. Infection phenotypes and quantification of cotyledons inoculated with *Peronospora parasitica* isolate Noco2.
(a) Cotyledons of 10-day-old seedlings were inoculated with Pp Noco2 (5×10^4 spores ml^{-1}) and stained at 6 dpi with lactophenol trypan blue (TB) to reveal *Pp* mycelium and dead plant cells. Red arrowhead indicates discreet HR; red arrows indicate expanding HR; black arrows indicate free hyphae.
(b) TB-stained cotyledons were harvested at 6 dpi and individual plant–pathogen interaction sites were categorized as HR, trailing necrosis, or free mycelium. Percentages derive from 40–80 cotyledons per experiment. Graphs represent mean and SE from three independent experiments.

To quantify these observations, we utilized the close association between cellular ion leakage and cell death (Baker and Orlandi, 1995; Baker *et al.*, 1991). SA-treated leaves were harvested 3 days after treatment. Subsequently, cell death was monitored for 72 h by ion leakage measurements (see Experimental procedures). As displayed in Figure 2, we observed considerable ion leakage increases in *lsd1.* This correlated with the onset and progression of both lactophenol–trypan blue (TB) staining of cell death and macroscopically visible rcd in these plants (data not shown). In contrast, Ws-0, *nim1, NahG* and water-treated *lsd1* plants did not exhibit cell death as measured by ion leakage in response to SA. Both

nim1 lsd1 and *NahG lsd1* plants, which displayed delayed and less extensive lesioning (Figure 1), also exhibited delayed and appreciably reduced ion leakage.

We next evaluated the phenotypes of Ws-0, single- and double-mutant plants following inoculation with the avirulent bacterial strain *Pseudomonas syringae* pv. *tomato* (DC3000) expressing either *avrRpm1* or *avrRps4*. In Ws-0, resistance to DC3000(*avrRpm1*) or DC3000(*avrRps4*) is mediated by *RPM1* (Grant *et al.*, 1995) and *RPS4* (Gassmann *et al.*, 1999), respectively. *RPM1* requires *NDR1* for its function, whereas *RPS4* requires *EDS1* function (Aarts *et al.*, 1998). As shown in Figure 1, resistant Ws-0 plants exhibited no visible symptoms when inoculated with low levels of inoculum ($\approx 1 \times 10^5$ cfu ml^{-1}) of either DC3000(*avrRps4*) or DC3000(*avrRpm1*). At this low dose of inoculum, no visible HR is observed. These plants do, however, develop visible HR when inoculated with high levels of inoculum ($\approx 5 \times 10^7$ cfu ml^{-1}) of these bacteria (data not shown). *lsd1* plants inoculated with either bacterial strain were resistant as measured by lack of bacterial growth (data not shown), and initiated rcd at 3–4 dpi (Figure 1). Both *nim1* and *NahG* plants, inoculated with either bacterial strain, were fully resistant, and no rcd was observed. As with Ws-0, *nim1* and *NahG* plants developed visible HR when inoculated with high bacterial doses (data not shown). Both *NahG lsd1* and *nim1 lsd1* leaves inoculated with either bacterial strain at low inoculum initiated rcd with the same timing as inoculated *lsd1* plants, even at low inoculum levels. However, as observed with SA treatment, although cell death was evident the spread was slower in *NahG lsd1* and *nim1 lsd1*, and did not engulf the entire leaf. Collectively, these data suggest that SA accumulation and *NIM1/NPR1* are required for timely initiation and/or amplification of a cell death signal(s) generated during the onset of rcd.

lsd1-*mediated basal resistance to the avirulent pathogen Noco2 is independent of* NIM1/NPR1 *and SA accumulation*

We examined resistance responses against the oomycete *Peronospora parasitica* (*Pp*) in the single- and double-mutant lines. Resistance in Ws-0 to the *Pp* isolate Noco2 is conditioned by the *RPP1* locus (Botella *et al.*, 1998). Previously, through genetic studies of *eds1 lsd1*, *pad4 lsd1* and *ndr1 lsd1* double mutants, we demonstrated that *lsd1* retains resistance to Noco2 in cotyledons and leaves, and that rcd is subsequently initiated (Rustérucci *et al.*, 2001). Cotyledons of Ws-0, *lsd1*, *nim1, NahG*, *nim1 lsd1* and *NahG lsd1* were inoculated with Noco2, then stained at 6 dpi with TB to visualize hyphae and dead or dying plant cells (Koch and Slusarenko, 1990). As shown in Figure 3(a), Ws-0 cotyledons developed a typically discrete HR, whereas *lsd1* cotyledons exhibited an expanded HR at pathogen infection foci. There was no visible *Pp* growth in either of these two plant lines. In contrast, inoculated cotyledons of *nim1* and *NahG* supported hyphal growth indicative of susceptibility (Figure 3a). Thus *RPP1* requires SA accumulation and *NIM1/NPR1* for full function, consistent with previous observations (Delaney *et al.*, 1995). Surprisingly, both *nim1 lsd1* and *NahG lsd1*-inoculated cotyledons were as resistant to Noco2 as the *lsd1* mutant (Figure 3a). Although the double-mutant cotyledons developed expanded HR, this cell death never progressed into the rcd seen in *lsd1* cotyledons.

To quantify these resistance responses we classified the observed plant–pathogen interaction sites into three categories: HR, trailing necrosis, or free hyphae (Morel and Dangl, 1998; Rustérucci *et al.*, 2001). As shown in Figure 3(b), 85–100% of the plant–pathogen interactions resulted in HR in both Ws-0 and *lsd1*-inoculated cotyledons. In *nim1* and *NahG*, HR sites comprised only 25–45% of the total, while the majority of interaction sites were classified as trailing necrosis and free hyphae. Both *nim1 lsd1* and *NahG lsd1* plants exhibited *lsd1*-like resistance to Noco2, displaying mostly (>90%) HR sites. Thus, in a genetic context where *RPP1* function is significantly disabled by the lack of either SA accumulation or *NIM1/NPR1*, resistance is conferred by *lsd1*. We will refer to this as *lsd1*-mediated basal resistance. We note that it may or may not be mechanistically the same as the basal resistance observed in *lsd1* challenged with virulent pathogens (Dietrich *et al.*, 1994).

Figure 4 displays a time-course of TB staining of adult wild-type, single- and double-mutant plants following inoculation with Noco2. Ws-0 leaves were fully resistant, exhibiting HR sites and no pathogen growth throughout the time-course. *lsd1* leaves also were resistant, and rcd was initiated at 2 dpi and progressed until the leaf was fully necrotic by 4 dpi. *nim1* leaves were partially susceptible to Noco2 as indicated by the trailing necrosis surrounding parasite hyphae at later time points after infection. As expected, no rcd occurred. *nim1 lsd1* leaves were resistant to Noco2 and, although rcd was initiated in these plants, its expansion was delayed and less extensive than in similarly treated *lsd1* plants. *NahG* leaves, although also partially susceptible to Noco2, appeared more resistant than *nim1* leaves. *NahG lsd1* leaves were also resistant to Noco2, but rcd in these leaves was completely suppressed. The HR sites evident in treated *NahG lsd1* leaves were larger than comparably treated Ws-0 leaves (Figure 4). Thus SA depletion suppressed *lsd1*-mediated rcd more strongly than *nim1*. From the combined data in Figures 3 and 4, we conclude that *lsd1*-mediated resistance to Noco2 in either cotyledons or adult leaves does not require SA accumulation or *NIM1/NPR1*. In contrast, however, both SA and *NIM1/NPR1* are required for full expression of *lsd1* rcd.

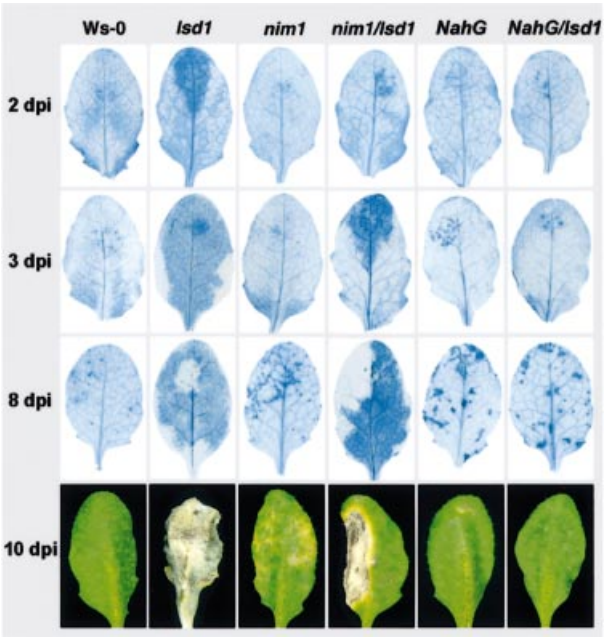

Figure 4. Time-course of infection phenotypes of adult leaves inoculated with *Peronospora parasitica* isolate Noco2.
Leaves of 4-week-old plants were inoculated with a 10 µl droplet of *Pp* Noco2 (5×10^4 spores ml^{-1}) on the top half of each leaf. Macroscopic phenotypes and corresponding TB-staining of plant–pathogen interaction sites are shown for adult leaves. Pictures are representative of four independent experiments using at least five leaves per genotype per experiment.

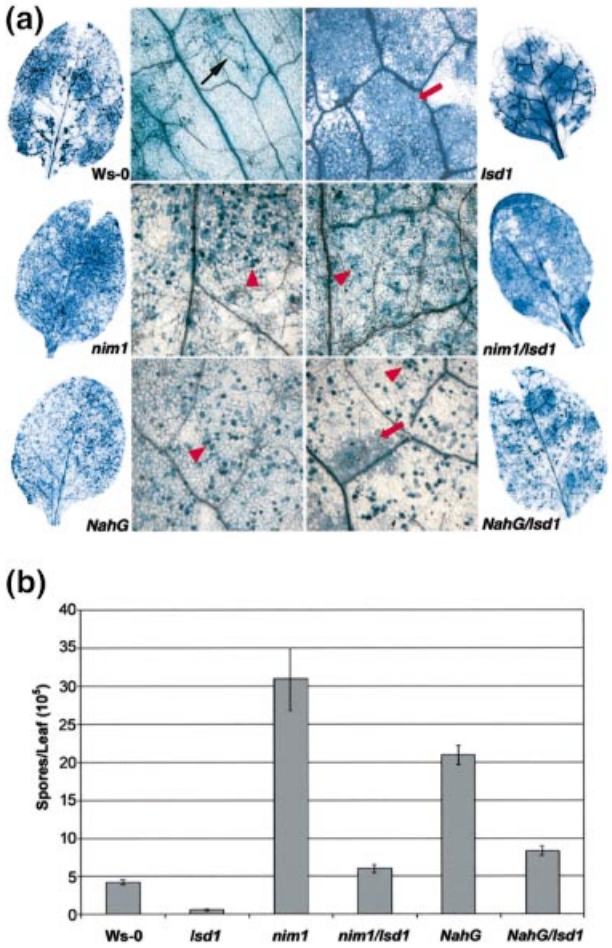

Figure 5. Trypan blue and spore count of Emco5-infected plants.
Leaves of 4-week-old plants were inoculated with *Pp* Emco5 (5×10^4 spores ml^{-1}) and 5 dpi leaves were harvested and either stained with TB or used to determine spore concentration.
(a) Trypan blue-stained full and 40× magnified views of infected leaves. Black arrow indicates growing hyphae; red arrowheads indicate oospores; red arrows indicate rcd.
(b) Spore concentrations of infected leaves (see Experimental procedures).

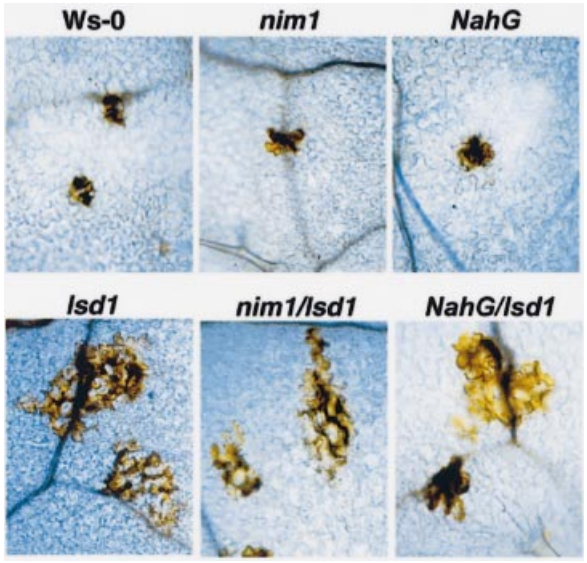

Figure 6. Localized H_2O_2 accumulation at *Peronospora parasitica*–plant interaction sites.
Leaves of 4-week-old plants were inoculated with a 10 µl droplet of *Pp* Noco2 (5×10^4 spores ml^{-1}). At 2 dpi, H_2O_2 accumulation was detected using DAB staining. Pictures shown are representative of three independent experiments using eight leaves per genotype per time point.

lsd1-*mediated basal resistance to the virulent* Pp *pathogen Emco5 is largely independent of* NIM1/NPR1 *and SA accumulation*

Previously, *lsd1* was shown to display a heightened resistance (compared to wild-type) to the virulent *Pp* isolate Emwa1 and to the virulent bacterial strain *Pseudomonas syringae* PsmM4 (Dietrich *et al.*, 1994). To determine the effects of the *nim1* mutation and *NahG* transgene on *lsd1*-mediated resistance to a virulent *Pp* strain, we treated adult leaves with Emco5 and performed trypan staining and spore counts (see Experimental procedures). Spore counts allow accurate quantification of pathogen reproduction, especially under conditions of high sporangiophore coverage of the leaf, as expected

following infection with virulent isolates of *Pp* such as Emco5. As expected, Ws-0 plants permitted heavy sporulation and resulted in $\approx 4.2 \times 10^5$ spores per leaf (Figure 5a,b). Also, as expected, *lsd1* plants exhibited a typical rcd, and spore production was significantly reduced to $\approx 6.1 \times 10^4$ spores per leaf. Both *NahG* and *nim1* plants supported significantly enhanced Emco5 growth compared to Ws-0 (Figure 5a,b).

NahG lsd1 plants exhibited a strong reduction in spore production compared to *NahG* plants (Figure 5b). Thus *lsd1*-mediated basal resistance against a virulent *Pp* isolate is functional largely in the absence of SA accumulation, as observed following infection with an avirulent *Pp* isolate (Figure 3). *NahG lsd1* plants also did not display obvious rcd, although TB staining of infected tissue revealed some microscopic zones of cell death that never progressed into rcd (Figure 5a). This is consistent with data presented above (Figures 1, 3 and 4), furthering the argument that SA accumulation is required for complete rcd. *nim1 lsd1* plants also exhibited reduced spore production compared to *nim1* plants (Figure 5b). In both double-mutant contexts *Pp* growth was not reduced to the levels observed in *lsd1*. Cumulatively, these data imply that *lsd1*-mediated basal resistance to the tested virulent *Pp* isolate is largely maintained in the double mutants. In contrast, *lsd1* rcd is not triggered in tissues of *nim1 lsd1* plants, and is strongly attenuated in *NahG* plants.

H_2O_2 production during the oxidative burst induced by the avirulent pathogen Noco2 is independent of SA accumulation or NIM1/NPR1

One of the earliest events to occur at an HR site is a local oxidative burst that gives rise to ROI production. We therefore examined production of local ROI in wild-type and mutant plants following spot inoculation of adult leaves with the incompatible *Pp* isolate Noco2. Figure 6 shows that the extent of diaminobenzidine (DAB) staining after *Pp* inoculation is similar in Ws-0, *nim1* and *NahG.* Thus, although *NahG* and *nim1* plants are partially susceptible to Noco2 infection (Figures 3 and 4), a localized oxidative burst was still initiated in these plants. This result is consistent with retention of *RPP1* recognition function, but suppression of downstream signaling in *nim1* and *NahG*. DAB staining of *lsd1, NahG lsd1* and *nim1 lsd1* revealed more extensive areas of ROI accumulation than observed in Ws-0, *NahG* or *nim1.* We conclude that increased H_2O_2 production seen in *lsd1* during Noco2-induced HR is independent of SA accumulation and *NIM1/NPR1*. The enhanced oxidative burst in *lsd1*, *nim1 lsd1* and *NahG lsd1* correlated with enhanced cell death and increased resistance to Noco2 in the same lines (Figures 3 and 4).

Discussion

We recently demonstrated that two positive regulators of *R*-gene function, *EDS1* and *PAD4*, are also required for both *lsd1*-mediated rcd and *lsd1*-mediated basal resistance (Rustérucci *et al.*, 2001). EDS1 and PAD4 are essential for SA accumulation in certain *R* gene responses (Feys *et al.*, 2001). However, their activities in *lsd1* rcd are not associated with induction of the plant HR, rather the processing of SA- and ROI-generated signals downstream or independently of the HR. In this study we directly assessed the roles of SA accumulation and the central SAR regulator *NIM1/NPR1* on rcd and disease resistance in *lsd1*. We present two important findings. First, *NIM1/NPR1* and SA accumulation are required for *lsd1*-mediated rcd. Second, *NIM1/NPR1* function and SA accumulation are dispensable for the basal resistance operating in *lsd1* against tested avirulent or virulent pathogens. Our data define two distinct roles for *LSD1* in controlling cell death and basal resistance responses, and add to the growing body of evidence supporting an SA-dependent signal amplification system operating to control HR and defense responses.

LSD1 *regulates a cell-death signal*

We previously suggested that *LSD1* functions to monitor the levels of a superoxide-dependent cell-death signal initiated during the HR (Jabs *et al.*, 1996). We proposed that cell-death signal concentration is highest at the site of HR. Signal levels there were proposed to rise above a threshold, and cell death occurs. In cells surrounding the HR site, the cell-death signal is below the threshold, and *LSD1* in those cells negatively regulates the local propagation of a cell-death signal. This limits the spread of the HR. In the *lsd1* null mutant, even low levels of signal spreading from the cells undergoing HR can initiate cell death; these dying cells presumably generate more unchecked signal, and rcd ensues. Under the conditions tested here, the lack of both SA accumulation and *NIM1/NPR1* activity significantly reduced initiation and extent of *lsd1*-mediated rcd. Yet it is clear that applied SA can induce rcd (Dietrich *et al.*, 1994). Thus we infer that *LSD1* both generates and responds to SA-mediated signals and acts in ROI-dependent signal potentiation (*sensu* Shirasu *et al.*, 1997).

The *nim1-1* allele has a single nucleotide inserted at position 3579, resulting in a seven amino acid frame-shift, premature truncation, and a deletion of 349 of 549 amino acids (Ryals *et al.*, 1997). Thus it is formally possible that residual activity from the truncated protein may account for the cell-death initiation seen in the double mutants (Figures 1–4), although this allele confers a strong loss of BTH-induced SAR function. (Lawton *et al.*, 1996). Likewise, the sites of cell death evident in the *NahG lsd1*-treated

plants may result from residual levels of SA remaining in the *NahG* plants. It is therefore likely that wild-type expression of *NIM1/NPR1*, and the ability of plants to accumulate high levels of SA, are two requisites for maximal *lsd1*-mediated rcd. This assigns roles for *NIM1/NPR1* and SA accumulation in the generation and/or amplification of the rcd signal downstream from ROI accumulation. In the absence of signal amplification, there is no requirement for negative regulation and, likewise, no rcd expected, even in the absence of *LSD1*. Both positive (*LSD6*) and negative regulators (*SSI1*) of SA-dependent cell death have been identified, supporting our conclusion of the requirement of SA accumulation for full expression of *lsd1*-mediated rcd (Greenberg, 2000).

nim1/npr1 plants cannot be rescued by SA application, suggesting that *NIM1/NPR1* functions downstream of SA. *NahG* plants are also not rescued by SA (reviewed by Ryals *et al.*, 1996). Our data suggest that *lsd1*-mediated rcd is downstream of both *NIM1*/*NPR1* function and SA accumulation. This implies that *LSD1* regulates rcd downstream of these signal intermediates. However, we previously demonstrated that SA causes rcd in *lsd1* plants (Dietrich *et al.*, 1994). Because SA application does not cause rcd in wild-type plants, we inferred in those studies that *LSD1* negatively regulates an SA-dependent signal leading to cell death. Thus SA is both a trigger and a required mediator of *lsd1* rcd. Hence our data support a model where SA is both necessary and sufficient for rcd in *lsd1.*

These genetic inferences are further supported by pharmacological studies, some in conjunction with *NahG* plants. Shirasu *et al.* (1997) demonstrated that ROI and SA signals work synergistically to induce HR in soybean cell cultures. They further demonstrated that SA functions to 'potentiate' ROI production, HR and SA levels. These authors demonstrated that SA production occurs in a biphasic manner, in parallel to the well known biphasic induction of the oxidative burst (reviewed by Baker and Orlandi, 1995; Lamb and Dixon, 1997). More importantly, the initial rise in SA levels influenced both the timing and ultimate levels of ROI during the oxidative burst (Shirasu *et al.*, 1997). These data, together with our results, suggest an SA-dependent signal 'amplification loop' that leads to rcd if *LSD1* is absent. *lsd1* does not express elevated levels of SA in its non-lesioned state (data not shown). Thus *LSD1* may monitor SA levels directly or indirectly.

The notion of overlapping, and independent, controls of spatial and temporal signal amplification at infection sites is supported by experiments in locally stimulated leaf tissue (Costet *et al.*, 1999; Dorey *et al.*, 1997; Dorey *et al.*, 1999; Draper, 1997; Mur *et al.*, 1996; Mur *et al.*, 1997). In particular, Mur *et al.* (1997) used transgenic tobacco lines expressing a novel salicylate hydroxylase isozyme (SH-L) under the control of promoters that preferentially express SH-L constitutively, early or late during tobacco mosaic virus infection. They showed that the critical period for SA action in HR is during the early, pre-necrotic phase, and occurs at the actual site of pathogen infection. This implies that inhibition of early SA accumulation prevents signal amplification and consequently delays both HR and resistance. Their data are also consistent with a model in which *LSD1* could perceive and control the spread of an SA-dependent signal.

Increased ROI production in lsd1 *is not impaired by* nim1 *or* NahG

Our ROI studies identified identical DAB staining patterns in wild type, *nim1* and *NahG* plants infected with the incompatible *Pp* isolate Noco2. Therefore ROI production in an oxidative burst is not sufficient for resistance to Noco2 in the absence of *NIM1/NPR1* and SA accumulation. This is consistent with several studies suggesting that SA accumulation and *NIM1/NPR1* function are downstream of the oxidative burst (reviewed by Dong, 1998). ROI production around *Pp* infection sites in *lsd1, nim1 lsd1* and *NahG lsd1* plants is more extensive. As discussed above, these plants exhibited heightened resistance compared to *nim1* and *NahG*, respectively. This exaggerated ROI production could be the cause, or simply a consequence, of the basal resistance mechanism operating in *lsd1* independently of SA and *NIM1/NPR1.*

SA accumulation and ROI production have long been established as important signaling molecules. NO has recently been implicated as a key component in the regulation of both HR cell death and disease-resistance responses. HR is dependent on the proper timing and extent of production of these three factors, without which defense genes are not activated and cell death does not occur (Delledonne *et al.*, 2001). We are currently conducting experiments to investigate the effects of NO generation and NO depletion in conjunction with SA accumulation and ROI production on the *lsd1* phenotype.

lsd1-*mediated basal disease resistance functions independently of requirements for SA and NIM1/NPR1*

Our most striking finding is that when *nim1* and *NahG* disable *RPP1*-mediated resistance, basal resistance in *lsd1* is still functional in the corresponding double mutants. We confirmed that *RPP1*-mediated resistance to Noco2 requires both *NIM1/NPR1* function and SA accumulation (Figures 3 and 4; Delaney *et al.*, 1995). In effect, this pathogen is rendered virulent on these two single-mutant plant lines. The *nim1 lsd1* and *NahG lsd1* double mutants, however, express the resistance phenotype of *lsd1* (Figure 3). This is all the more striking as there is no demonstrable rcd in these lines. Thus the resistance cannot be a simple

consequence of ectopic cell death in *lsd1*. We infer that the *lsd1* mutation results in the activation of defense, upstream or independent of both *NIM1/NPR1* function and SA accumulation following avirulent pathogen challenge.

We further observed that SA accumulation and *NIM1/NPR1* are not required for *lsd1*-mediated basal resistance against a virulent *Pp* isolate (Figure 5). Leaves of both *nim1 lsd1* and *NahG lsd1* plants were, unlike the single *nim1* and *NahG* parents, able to generate obvious resistance against Emco5 (Figure 5). Furthermore, full rcd did not occur in the double mutants, consistent with data from Figures 1–3 showing that SA and NIM1/NPR1 are generally required for full rcd. This confirms results published earlier using *lsd1* plants (Dietrich *et al.*, 1994).

Enhanced ROI production was observed in all lines containing *lsd1* following infection with either the avirulent *Pp* isolate (Figure 6) or virulent *Pp* isolate (data not shown). We note that ROI production in *lsd1* genotypes following infection with a virulent *Pp* isolate is rapid and complete before the onset of rcd (D.H.A. and J.L.D., unpublished results). In contrast, there is no early recognition of the virulent *Pp* isolate and no ROI produced in the wild type (M. Torres and J.L.D., unpublished results). Thus the excess ROI associated with *lsd1* may suffice to initiate basal resistance in the *lsd1* genotypes. If so, the mechanism of basal resistance might reflect prolonged signaling, akin to the potentiation of HR by SA and ROI discussed above. In this scenario, basal resistance would be mediated by excess ROI that accumulates because of the *lsd1* mutation. Alternatively, the basal resistance we observed in the *lsd1* lines may be independent of ROI accumulation.

The collective data on requirements for SA and *NIM1/NPR1* in basal resistance signaling do not illuminate our understanding of how basal resistance in *lsd1* might be controlled. Basal resistance mechanisms were originally defined by mutants that were more susceptible to virulent pathogens than the wild type (enhanced disease susceptibility, *eds* mutants). Some can be rescued by SA (such as *EDS1* and *PAD4*; Parker *et al.*, 1996; Zhou *et al.*, 1998), implying that these loci control basal resistance upstream or independently of SA accumulation. Yet SA- and *NIM1/NPR1*-independent defense pathways are not uncommon (e.g. Bittner-Eddy and Beynon, 2001; Delaney *et al.*, 1994; McDowell *et al.*, 2000). The *HRT R* gene mediating resistance to turnip crinkle virus requires SA accumulation but does not require *NIM1/NPR1* (Cooley *et al.*, 2000; Kachroo *et al.*, 2000). Similarly, disease resistance in some of the *cpr* (constitutive expresser of *PR* genes) mutants was found to be SA-dependent but *NIM1/NPR1*-independent (Clarke *et al.*, 2000). In contrast, induced systemic resistance (ISR), established by colonization of roots by certain rhizosphere bacteria, has been shown to require *NIM1/NPR1* but not SA accumulation. Thus the differential dependence of the *HRT, cpr* or ISR pathways on either SA or *NIM1/NPR1* discriminates these from the defense pathway operating in *lsd1.* Resistance to *Botrytis cinerea* and *Alternaria* is dependent on ethylene- and jasmonic acid (JA)-mediated pathways (Thomma *et al.*, 2001). It will therefore be informative to address whether the basal resistance to Noco2 that we observed is mediated by JA or ethylene.

Divergent phenotypes represent the dual functions of LSD1

The discussion above suggests that the two proposed functions for *LSD1*, negative regulation of rcd and of basal disease resistance, are separable. Other mutants also distinguish between cell death and resistance. *dnd1* exhibits inhibition of HR cell death while preserving disease resistance, although maintenance of resistance may be due to constitutive expression of some defense genes (Yu *et al.*, 1998). Conversely, *acd5* mutant plants exhibit spontaneous, SA-dependent cell death, but increased susceptibility to the bacterial pathogen *P. syringae. nim1/npr1* inhibits the *acd5* spontaneous cell death, supporting the contention that *NIM1/NPR1* functions to control cell death during pathogen infection (Greenberg, 2000).

Although features of the LSD1 primary structure suggest a role in transcription regulation, little is known about its direct mode of action. The data presented here demonstrate that SA and *NIM1/NPR1* are components of a pro-cell-death pathway repressed by *LSD1.* Previous reports associate *NIM1/NPR1* with gene regulation in many disease-resistance responses. An intriguing possibility is that *LSD1* antagonizes the *NIM1/NPR1*-dependent pro-death pathway directly at the level of gene regulation. Assignment of a role for *LSD1* as a transcriptional repressor can be further bolstered by recent evidence of nuclear localization and direct interaction with known transcriptional regulators (M. Ellerström, B.F.H. and J.L.D., unpublished results).

Experimental procedures

Plant material and cultivation

nim1-1, NahG (gifts from Syngenta Biotechnology, Inc., Research Triangle Park, NC) and *lsd1* have been described previously (Dietrich *et al.*, 1994). Seeds and plants were grown on a mixture of four parts Promix (Premier Horticulture Inc., Red Hill, PA), two parts sand and one part vermiculite. Plants were cultivated under a 9 h light; 15 h dark schedule with a daytime temperature of 24°C and a night-time temperature of 20°C, and maintained under a relative humidity of 60% with a light intensity of 120 μE m^{-2} sec^{-1}.

Double mutants were PCR screened for homozygosity of the respective mutation with the primer set 5′-ACC TAA CAA AAA

GAA AAG TGT GTG AGG-3′, 5′-ATA ATA AAC CCT ACT AGC TCT AAC AAG-3′ and 5′-CTG CTA CTT TCA TCC AAA C-3′ (*lsd1*); and primer set 5′-CAC GAA TTC AGC ATG AAA AAC-3′ and 5′-GTC GAA TCC GCG ATC GGT G-3′ (*NahG*). *nim1-1* lines were selected using the primer set 5′-ATT TGG CTT TCA TCT TCA-3′ and 5′-GAT CAT GAG TGC GGT TCT-3′. Subsequent sequencing of the PCR product confirmed the presence of the mutation.

Pathogen isolates and growth determinations

The *Pp* isolates Noco2 and Emco5 were maintained on the genetically susceptible *Arabidopsis* accessions Col-0::*RPP8* (McDowell *et al.*, 1998) and Ws-0, respectively (Dangl *et al.*, 1992). To determine disease symptom development, *Pp* conidiospores were suspended in water (5×10^4 spores ml^{-1}) and sprayed onto 10-day-old (cotyledon assays) or 3–4-week-old (leaf assays) plants. Inoculated plants were kept under a sealed lid to achieve high relative humidity in a growth chamber at 19°C under an 8 h light period (100–160 μE m^{-2} sec^{-1}).

Spore-count assay

Ten plants of each line were inoculated with Emco5. Leaves (5 dpi) were harvested and three replicate samples per plant line of 10 leaves each were placed in 15 ml tubes containing 5 ml distilled water. Tubes were vortexed, and spore concentrations were determined using a haemocytometer (Reichert, Buffalo, NY). All plants were of equivalent age and size, and were grown under identical conditions. Data are presented as spores per leaf. Mean and SE were calculated based on these three replicate samples for each genetic background.

Induction of lsd1 *runaway cell death*

For chemical induction of rcd leaves, 4-week-old plants were sprayed with 2 mM SA. Plants were maintained under normal growth conditions and inspected for lesion development over 5 days. Bacterial induction of rcd was measured by infiltration of *Pseudomonas syringae* DC3000(*avrRps4*) or DC3000(*avrRpm1*) suspensions (1×10^5 cfu ml^{-1}) into one side of the leaf using a needle-less syringe. Plants were inspected for disease symptoms and/or rcd formation over 6 days under the same conditions as described above. Alternatively, a 10 μl droplet of *Pp* conidiospores (5×10^4 spores ml^{-1}) was placed on the leaf surface, and plants were incubated for up to 10 days under the same conditions as used for *Pp* growth assays.

Ion leakage measurement

Four-week-old plants were sprayed with 2 mM SA. Four leaf discs (3 dpi) were punched out using a 3 mM diameter cork borer. Cores were taken from one side of the leaf between the central vein and leaf margin. Leaf discs were floated in 20 ml distilled H_2O for 30 min to eliminate signal derived from wounded cells. Four discs from each line were then placed in tubes containing 6 ml fresh distilled H_2O, and measurements of solution conductivity were taken at the indicated time points using a conductivity meter (Orion, Beverly, MA).

Trypan blue staining

Cell death induced by pathogen inoculation or chemical treatment, as well as the development of *P. parasitica* mycelium on cotyledon or leaf tissues, was monitored by staining with lactophenol–trypan blue (TB) and destaining in saturated chloral hydrate as described (Koch and Slusarenko, 1990).

Histochemical detection of H_2O_2 at interaction sites

Hydrogen peroxide (H_2O_2) production was detected by endogenous peroxidase-dependent *in situ* histochemical staining using 3,3-diaminobenzidine (DAB) in a protocol modified from Thordal-Christensen *et al.* (1997). Leaves of 4-week-old plants were drop-inoculated with a 10 μl droplet of *Pp* conidiospores (5×10^4 spores ml^{-1}). Leaves were then removed from the plant and supplied through the cut petiole with a solution of 1 mg ml^{-1} DAB for 8 h in light (100–160 μE m^{-2} sec^{-1}) or in darkness, under the same conditions as used for determining *Pp* growth. Subsequently, the DAB solution was replaced with water and leaves were maintained under the same conditions as before. At different times after pathogen inoculation, leaves were cleared for 5 min in boiling acetic acid/glycerol/ethanol (1/1/3, v/v/v) solution. Material was mounted on slides in 60% glycerol and examined using a light microscope (Zeiss, Axiophot). H_2O_2 is detectable as reddish-brown coloration.

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