

Pathogen-induced, NADPH oxidase–derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis thaliana*

Miguel Angel Torres¹, Jonathan D G Jones² & Jeffery L Dangl^{1,3}

Plant immune responses are usually accompanied by the production of extracellular superoxide at and surrounding infection sites^{1–3}. Extracellular reactive oxygen intermediates (ROIs) in plants were proposed to drive programmed cell death correlated with disease resistance (the hypersensitive response). ROIs derived from this oxidative burst are generated by plasma membrane NADPH oxidases, anchored by gp91^{phox} proteins related to those responsible for the respiratory oxidative burst activated in mammalian neutrophils during infection^{4,5}. Mutation of *Arabidopsis thaliana* respiratory burst oxidase (*Atrboh*) genes eliminated pathogen-induced ROI production but had only a modest effect on the hypersensitive response⁴. We show that *Atrboh* function can be activated by exogenous ROIs. Unexpectedly, the subsequent oxidative burst can suppress cell death in cells surrounding sites of NADPH oxidase activation. This cell death requires salicylic acid, a plant immune system activator⁶. Thus, ROIs generated by *Atrboh* proteins can antagonize salicylic acid–dependent pro-death signals. These results have implications for understanding how salicylic acid activates defense signaling in cells spatially removed from infection sites without causing cell death.

A. thaliana homologs of the mammalian gp91^{phox} respiratory burst NADPH-oxidase subunit are encoded by a ten-member gene family (*AtrbohA–AtrbohJ*)⁷. In leaves, *AtrbohD* is the source of extracellular ROIs after pathogen recognition, yet it contributes only modestly to the hypersensitive response through genetic interactions with *AtrbohF* that are potentially pathogen-dependent⁴. ROI generation is correlated with plant cell death⁸. *AtrbohD* and *AtrbohF* are also required to generate hydrogen peroxide, which triggers elevated Ca²⁺ levels during guard cell signaling⁹, whereas *AtrbohC* is required to generate ROIs and maintain elevated Ca²⁺ levels at the root hair tip during root hair growth¹⁰. To dissect further the role of ROIs in cell death during plant defense, we constructed multiple mutant combinations using *A. thaliana atrbohD*, *atrbohF* and *lsd1*.

The *lsd1* mutant cannot control the extent of the normal hypersensitive response, leading to runaway cell death (RCD) limited to the infected leaf. Thus, the LSD1 zinc-finger protein negatively regulates the spread of cell death to uninfected cells surrounding hypersensitive response sites¹¹. Superoxide generated *in situ* also triggers RCD in *lsd1* plants, as do salicylic acid and salicylic acid analogs¹². Diphenylene iodonium inhibits flavin-containing enzymes, including NADPH oxidases, and diminishes *lsd1* RCD, suggesting that a product of this enzyme is necessary to initiate RCD¹². Disease resistance signaling components, including salicylic acid, contribute to *lsd1*-dependent RCD^{13,14}. Therefore, the *lsd1* mutant can be used to show how ROIs and salicylic acid regulate cell death in cells surrounding infection sites.

We constructed an *lsd1 atrbohD atrbohF* triple mutant, expecting that elimination of extracellular ROIs would suppress *lsd1* RCD¹². Unexpectedly, this triple mutant showed uncontrolled cell death under growth conditions that normally repress *lsd1* RCD (Fig. 1a). Overexpression of *AtrbohD* rescued this phenotype. *lsd1 atrbohD* and *lsd1 atrbohF* double mutants were viable in normal growth conditions, but both showed enhanced cell death after treatment with salicylic acid (data not shown) or the salicylic acid analog benzothiadiazole¹³ (BTH; Fig. 1b). Cell death enhancement was lethal in *lsd1 atrbohD* compared with *lsd1* mutants (Fig. 1b,c). We conclude that *AtrbohD* negatively regulated a cell death process triggered in *lsd1* by BTH and salicylic acid.

We generated extracellular superoxide in approximately one-quarter of each leaf by coapplication of xanthine and xanthine oxidase (X/XO). This led to very low levels of hydrogen peroxide–dependent 3',3'-diaminobenzidine (DAB) staining and cell death (monitored by trypan blue⁴) in the treatment zone in wild-type Col-0 plants (Fig. 2a). DAB staining was reduced in *atrbohD* mutants, but the low level of cell death was not, suggesting that the initial X/XO treatment triggered *AtrbohD*-independent pro-cell death signals. The superoxide generated from X/XO was sufficient to drive increased DAB staining and spreading cell death in the *lsd1* mutant¹². Whereas *lsd1 atrbohD* mutants had essentially no DAB staining, they did have

¹Department of Biology, University of North Carolina, CB# 3280, Coker Hall, Room 108, Chapel Hill, North Carolina 27599-3280, USA. ²Sainsbury Laboratory, John Innes Center, Colney, Norwich NR4 7UH, UK. ³Curriculum in Genetics, Department of Microbiology and Immunology and Carolina Center for Genome Sciences, University of North Carolina, CB# 3280, Coker Hall, Room 108, Chapel Hill, North Carolina 27599-3280, USA. Correspondence should be addressed to J.L.D. (dangl@email.unc.edu).

Received 18 April; accepted 18 July; published online 18 September 2005; doi:10.1038/ng1639

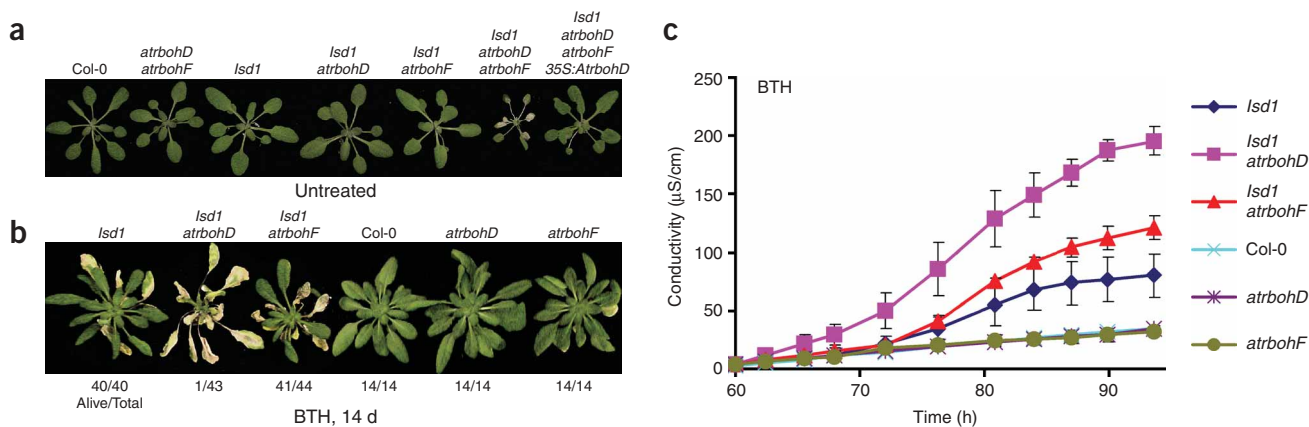


Figure 1 Plant NADPH oxidase gp91^{phox} can negatively regulate cell death. (a) The *Lsd1 atrbohD atrbohF* triple mutant was not viable; seedlings invariably died of spreading cell death in conditions otherwise permissive for *Lsd1* growth. Untreated 24-d-old control plants of the relevant genotypes (listed above) are shown. (b) *atrbohD Lsd1* and *atrbohF Lsd1* mutants had enhanced cell death after BTH treatment. Genotypes are listed above; numbers of plants alive 14 d after BTH treatment and the total numbers of plants treated are listed below. (c) Quantification of cell death from the experiment in b by electrolyte leakage. Mean \pm 2 s.e. Experiment repeated three times with similar results.

enhanced cell death compared with *Lsd1* mutants, consistent with the observed phenotypes (Fig. 1). Quantification of cell death after *in situ* generation of superoxide (Fig. 2b) confirmed that both AtrbohD and AtrbohF negatively regulate *Lsd1* RCD.

Overexpression of *AtrbohD* enhanced ROI generation in the treatment zone after X/XO application, with no alteration in the low number of dead cells. No DAB stain was observed in the untreated leaf area of these plants (Fig. 2a). AtrbohD is therefore part of an NADPH oxidase that is not constitutively active but can be activated by exogenous ROIs generated by X/XO application. This suggests that AtrbohD is limiting for enhanced ROI production after a primary ROI-generating stimulus, consistent with the bimodal oxidative burst often observed in plants cells after pathogen recognition or ozone treatment^{3,15}. *AtrbohD* overexpression reduced *Lsd1* RCD after X/XO treatment (Fig. 2a,c). We measured RCD beyond the X/XO application zone for 7 d (Fig. 2d,e). We observed minimal cell death in the application zone and no spread in wild-type Col-0, *atrbohD* and *atrbohF* plants (data not shown). By contrast, cell death spread beyond the application zone in 42% of *Lsd1* leaves, and this was enhanced to 76% in *Lsd1 atrbohD* leaves (Fig. 2e). Overexpression of *AtrbohD* in *Lsd1* suppressed this phenotype; we observed spread of cell death in only ~10% of these leaves. Therefore, loss of function and overexpression analyses established that ROIs derived from AtrbohD, and to a lesser extent AtrbohF, negatively regulated a superoxide-triggered cell death program uncovered in the absence of LSD1. Because cell death is enhanced in *Lsd1 atrbohD* mutants, and because both alleles are null, we infer that AtrbohD and LSD1 act independently to negatively regulate the same cell death signaling pathway.

We tested whether the spread of cell death triggered by plant pathogens was also regulated by AtrbohD and AtrbohF. We infected plants with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000(*avrRpm1*). There was little or no change in the hypersensitive response in any of the mutant combinations infected ~6 h after inoculation⁴, despite the diminution and enhancement of DAB staining in *atrbohD* mutants and *AtrbohD* overexpressing plants, respectively (Fig. 3a). By 48 h after inoculation, we observed RCD in one-third of inoculated *Lsd1* leaves and 79% of *Lsd1 atrbohD* leaves, despite the absence of DAB staining in the latter (Fig. 3b). Overexpression of *AtrbohD* in *Lsd1* suppressed the spread of cell death but

did not alter the hypersensitive response compared with wild-type plants (Fig. 3a,b). These results confirm that activated AtrbohD generates negative regulatory signals in cells surrounding a normal hypersensitive response site. Further, the increased levels of ROIs are sufficient to protect those cells from death in the absence of LSD1. This suggests that the negative regulatory function of ROIs generated by AtrbohD is downstream or independent of LSD1.

We infected plants with a weakly pathogenic strain of the necrotrophic pathogen *Botrytis cinerea*, which is thought to generate its own ROIs as a trigger to initiate host cell death¹⁶. We observed low level DAB staining in wild-type leaves. This was eliminated in *atrbohD* mutants and enhanced by overexpression of *AtrbohD* in wild-type plants, without changes in cell death (Fig. 3c). Infection of *Lsd1* resulted in more ROI production than in wild-type, and in RCD, as expected. This phenotype was enhanced markedly in *Lsd1 atrbohD* double mutants but was not accompanied by fungal growth (data not shown). We observed enhanced DAB staining in *Lsd1* lines that overexpressed *AtrbohD* after infection, but RCD was eliminated. Thus, our pathology data support the contention that AtrbohD, and ROIs derived from it, negatively regulate cell death in cells surrounding the hypersensitive response or in disease lesions around infection sites.

Salicylic acid levels increase markedly in infected cells and in cells immediately surrounding infection sites¹⁷. This generates a salicylic acid-dependent signaling gradient leading to defense gene activation around infection sites¹⁸. Additionally, exogenous hydrogen peroxide can trigger salicylic acid accumulation, and these signals can synergize to drive cell death^{19,20}. These experiments relied on exogenous application or generation of either hydrogen peroxide or salicylic acid. Hence, there has been no genetic analysis of the interplay in cell death control between the AtrbohD-dependent ROIs generated after pathogen recognition and salicylic acid accumulation.

We used *eds16*, which has a mutation in the isochorismate synthase gene of the salicylic acid biosynthetic pathway, to address the interaction of salicylic acid and ROIs in cell death control. *eds16* retains basal salicylic acid levels, but additional salicylic acid does not accumulate upon infection²¹. *Lsd1* does not overaccumulate salicylic acid, but RCD probably requires salicylic acid accumulation¹⁴. *Lsd1 eds16* mutants did not undergo RCD after ROI generation (Fig. 4a). The enhanced cell death observed after ROI-generating treatment in *Lsd1*

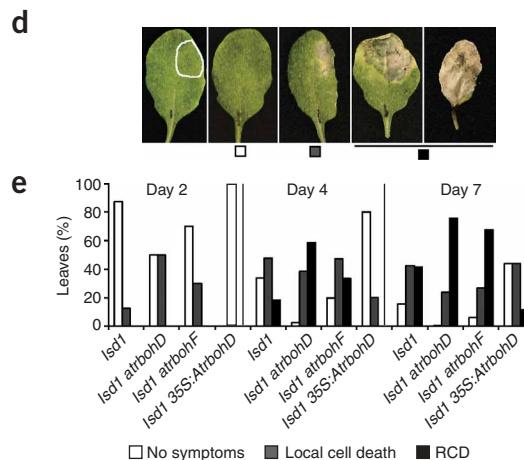
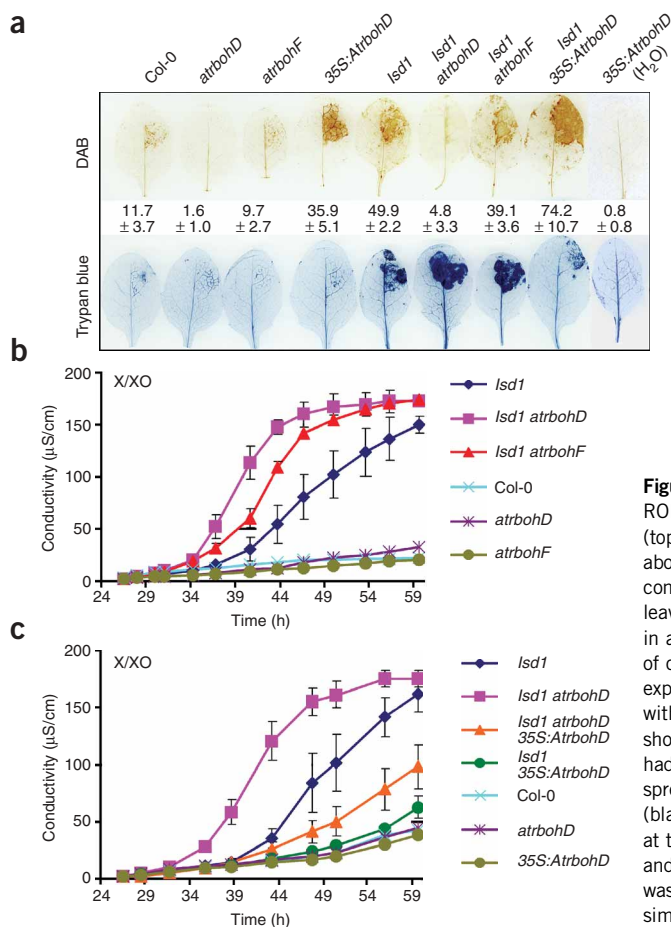


Figure 2 The *AtrbohD*-dependent oxidative burst is activated by exogenous ROIs and negatively regulates superoxide-induced RCD in *lsd1*. **(a)** DAB (top) and trypan blue (bottom) staining of leaves from the genotypes listed above 3 d after injection with X/XO. The leaf on the far right is a water control. Numbers represent quantification of DAB staining from 12–20 leaves per phenotype measured using the luminosity function in Photoshop in arbitrary units with mean (top) ± 2 s.e. (bottom). **(b,c)** Quantification of cell death by electrolyte leakage (mean ± 2 s.e.) from independent experiments treated with X/XO as in **a**. **(d)** Example of representative leaves with different levels of cell death 7 d after X/XO treatment. The left leaf shows the limited area typically inoculated (bordered in white). Other leaves had no symptoms (unfilled), local necrosis (gray filled) or cell death spreading beyond the application site and extending across the leaf mid-rib (black filled). **(e)** Quantification of cell death spread in the genotypes shown at the bottom, using the scale defined and color-coded in **d**. Between 40 and 47 leaves of each genotype were inoculated with X/XO, and cell death was monitored for 7 d. The experiment was repeated three times, using a similar number of leaves per genotype, with similar results.

atrbohD was suppressed in *lsd1 atrbohD eds16*. After the hypersensitive response, salicylic acid accumulates in plant vascular tissues and is required for transmission of long-distance signals in the establishment of systemic acquired disease resistance⁵. *lsd1* RCD is normally limited to the leaf where it is initiated¹¹. After spot application of X/XO onto *lsd1 atrbohD* leaves, we noted unexpectedly that ~40% of the meristems were dead 12 d later, whereas no meristems were dead in *lsd1* or *atrbohD* (**Fig. 4b,c**). This systemic cell death was greatly reduced in *lsd1 atrbohD eds16*. Therefore, salicylic acid accumulation is required for both local and systemic cell death spread in *lsd1*. We infer that the negative regulatory function of *AtrbohD* in signaling beyond the original site of ROI generation acts on a salicylic acid-dependent cell death pathway that is also negatively regulated by *LSD1*.

Plasma membrane-bound NADPH oxidases in plants and animals were thought originally to generate antimicrobial ROIs. We now know that the function of the mammalian phagocyte NADPH oxidase in antimicrobial defense is as a signal for pH changes in the phagocyte vacuole leading to protease activation²². In plants, the sum of pharmacological data to date suggests that superoxide and nitric oxide collaborate to control the hypersensitive response^{23,24}. It was presumed that the superoxide was derived from Rboh action because of its inhibition by diphenylene iodonium in plasma membrane preparations²⁵. Our genetic analysis showed, however, that although RbohD is the source of DAB-stained peroxides, it is not a key contributor to the hypersensitive response⁴. Because diphenylene iodonium can reduce both the hypersensitive response and spreading cell death in *lsd1*¹², there is probably at least one other flavin-

containing oxidase that contributes to cell death in plants. This proposed enzyme is unlikely to be the *A. thaliana* nitric oxide synthase (AtNOS1) implicated in defense responses, as it does not contain a flavin-adenine dinucleotide²⁶. Our results identify an unexpected function for the *Atrboh*-containing plant NADPH oxidase after infection: preventing the relay of salicylic acid-dependent cell death signals to cells surrounding an infection site. This salicylic acid-dependent pro-death pathway is also negatively regulated by *LSD1* in a seemingly independent fashion.

Our data suggest that antagonism between ROIs and *LSD1* on one hand and salicylic acid accumulation on the other controls cell death in cells surrounding infection sites after successful recognition of pathogens (**Fig. 4d**). We speculate that signals contributing to the hypersensitive response, including elevated salicylic acid levels, hydrogen peroxide generated by sources in addition to the Rboh-containing NADPH oxidase²⁷, nitric oxide²³ and calcium influx²⁸ collectively surpass a threshold for cell death in these cells that is negatively regulated by *LSD1*. Supporting this model, conditional overexpression of *LSD1* dampens the hypersensitive response (H. Kaminaka, P. Epplé & J.L.D., unpublished data). We propose that *LSD1*, *AtrbohD* and *AtrbohF* respond to signals emanating from cells undergoing the hypersensitive response (in addition to their possible functions in those cells). This model is compatible with our previous finding that *AtrbohD* and *AtrbohF* might interact to fine-tune the spatial control of ROI production and the hypersensitive response in cells in and around infection sites⁴. This model is also consistent with the finding that superoxide can titrate the pro-cell death functions of nitric oxide

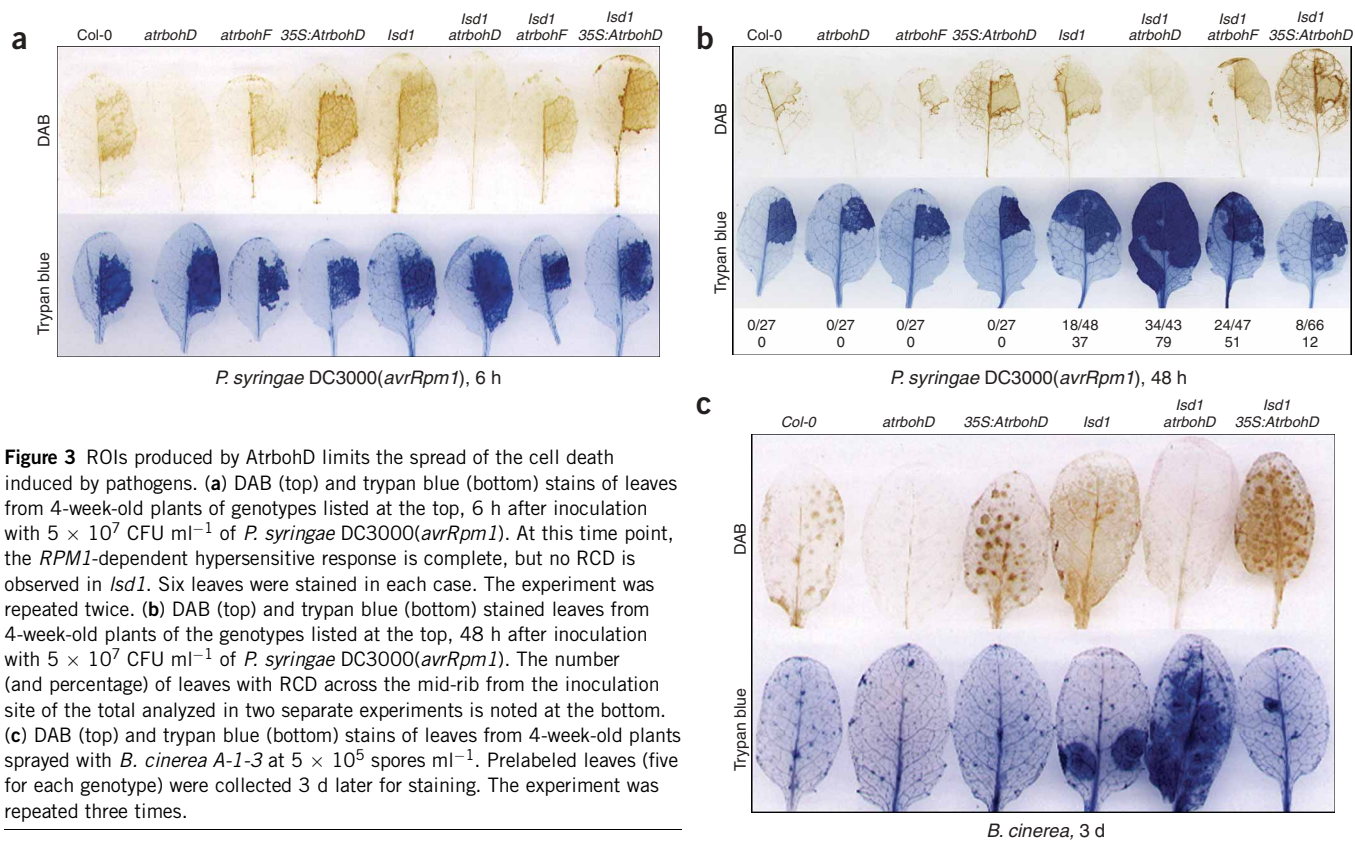


Figure 3 ROIs produced by *AtrbohD* limits the spread of the cell death induced by pathogens. **(a)** DAB (top) and trypan blue (bottom) stains of leaves from 4-week-old plants of genotypes listed at the top, 6 h after inoculation with 5×10^7 CFU ml^{-1} of *P. syringae* DC3000(*avrRpm1*). At this time point, the *RPM1*-dependent hypersensitive response is complete, but no RCD is observed in *Lsd1*. Six leaves were stained in each case. The experiment was repeated twice. **(b)** DAB (top) and trypan blue (bottom) stained leaves from 4-week-old plants of the genotypes listed at the top, 48 h after inoculation with 5×10^7 CFU ml^{-1} of *P. syringae* DC3000(*avrRpm1*). The number (and percentage) of leaves with RCD across the mid-rib from the inoculation site of the total analyzed in two separate experiments is noted at the bottom. **(c)** DAB (top) and trypan blue (bottom) stains of leaves from 4-week-old plants sprayed with *B. cinerea* A-1-3 at 5×10^5 spores ml^{-1} . Pre-labeled leaves (five for each genotype) were collected 3 d later for staining. The experiment was repeated three times.

in plant cells²³. We propose that ROIs, derived from activation of *Atrboh* proteins, is required to trigger local elevations in Ca^{2+} concentration in cells surrounding hypersensitive response sites²⁸ and, as shown for *Atrboh* proteins, in both guard cell function and root hair tip growth^{9,10}. *AtrbohD* is required to maintain high levels of ascorbate peroxidase in response to high light stress²⁹, and *LSD1*

is required for accumulation of a particular superoxide dismutase isoform during cell death spread³⁰, suggesting that *AtrbohD* and *LSD1* might ultimately protect cells from death by regulating ROI metabolism. The result is suppression of unwanted cell death in the face of the increasing salicylic acid levels required to initiate appropriate defense responses beyond the initial infection site⁶.

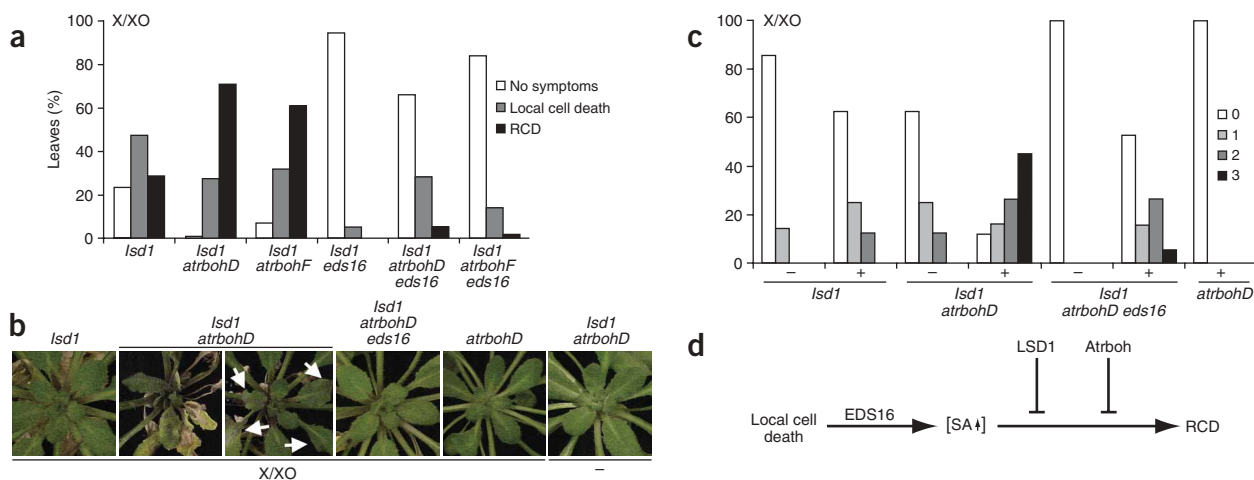


Figure 4 *LSD1* and *AtrbohD* antagonize a salicylic acid–dependent pro-death pathway. **(a)** Quantification of local cell death spread in the genotypes shown at the bottom, 7 d after treatment with X/XO (as in Fig. 2d,e). Between 36 and 40 leaves of each genotype were inoculated. The experiment was repeated twice, with similar results. **(b)** Systemic spread of cell death requires salicylic acid accumulation. Twelve days after inducing local cell death in fully expanded mature leaves of the genotypes listed at the top with X/XO, cell death appears in some untreated young leaves of *Lsd1 atrbohD*. Arrows indicate points of cell death. **(c)** Quantification of systemic cell death 12 d after treatment of fully expanded leaves with X/XO (+ or –, below): 0, no cell death; 1, systemic cell death in one or two leaves; 2, systemic cell death in three or more leaves; 3, cell death throughout the meristem. Analysis of 28–48 plants for each genotype from two independent experiments. **(d)** *LSD1* and *Atrboh* function additively to regulate a salicylic acid (SA)–dependent cell death pathway subsequent to localized cell death.

METHODS

Plant material and cultivation. We grew *A. thaliana* accession Col-0 and mutants derived from it in conditions previously described¹⁴. We used the following mutant alleles: *atrbohD*, *atrbohF*⁴ and *lsd1-1*^{W^s-0} introgressed into Col-0 over seven generations to create *lsd1-1*^{Col-0} (ref. 13). RCD is less severe in both *lsd1-1*^{Col-0} and an isogenic Col-0 mutant line with a T-DNA insertion in *lsd1* (*lsd1-2*; SALK_042687). Images presented show *lsd1* introgressed into Col-0. We also generated genotype combinations using *lsd1-2*, *atrboh* and *eds16-1*; these had the same phenotypes as this introgression line. The *eds16-1* (ref. 21) mutant was provided by F.M. Ausubel (Harvard University). All mutants were confirmed by PCR to have the mutations or transgenes (primers and conditions are available on request).

To generate plants overexpressing *AtrbohD*, we carried out PCR amplification of the first-strand cDNA from total Col-0 RNA (primer sequences available on request), which produced a fragment containing the *AtrbohD* coding region with a *Clal* site at the ATG and a *BamHI* site after the stop codon. After sequence verification, we ligated the *Clal*-*BamHI* fragment between the CaMV 35S promoter and the NOS terminator into the binary vector pSLJ7291 generating plasmid 231. We transformed Col-0 or *atrbohD* plants using *Agrobacterium tumefaciens* with this construct and identified Km resistant plants. We verified complementation by restoration of ROI production in response to *P. syringae* pv. *tomato* (*Pto*) DC3000(*avrRpm1*). The 35S:*AtrbohD* transgenic line used in these experiments (line 234.1) expresses approximately four times more *AtrbohD* than wild type (data not shown).

Cell death induction. We used plants aged 4–5 weeks for all experiments. We injected 100 μM xanthine and 0.05 U ml⁻¹ xanthine oxidase (X/XO) into fully expanded leaves. To monitor the spreading of cell death, we injected one-quarter of the leaf, for three leaves per plant, and monitored cell death for 7 d. We sprayed a suspension of *Botrytis cinerea* strain A-1-3 spores (5 × 10⁵ spores per ml in 2% glucose) onto 4-week-old plants and monitored the plants for 1 week. We sprayed BTH (0.35 mM in distilled water and 0.005% silwet) or 0.5–1 mM salicylic acid in water onto fully expanded leaves. We injected the avirulent bacterium *Pto* DC3000(*avrRpm1*) at a concentration of 5 × 10⁷ colony-forming units (CFU) per ml in 10 mM MgCl₂. For studies of systemic cell death, we sprayed fully expanded leaves from 4-week-old plants with X/XO, analyzed them for 12 d and then evaluated the appearance of necrosis in uninoculated leaves and the center of the rosette.

Stains and cell-death quantification. We stained peroxides *in situ* with DAB as described⁴. We visualized dead cells with lactophenol-trypan blue⁴. We carried out electrolyte leakage assays as described⁴. We inoculated fully expanded leaves from 4-week-old plants with X/XO. After 24 h, we collected 7.5-mm leaf discs and washed them extensively with distilled water for 1 h. We placed four leaf discs in a tube with 6 ml of distilled water (four replicates per treatment) and measured conductivity over time using an Orion (Boston) conductivity meter (model 130). Alternatively, we sprayed BTH onto leaves and collected the leaf discs 60 h later.

ACKNOWLEDGMENTS

We thank K. Overmyer and J. McDowell for comments on the manuscript. This work was supported by grants from the US National Science Foundation and the US National Institutes of Health to J.L.D. The Sainsbury Laboratory is supported by the Gatsby Charitable Trust.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at <http://www.nature.com/naturegenetics/>

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

1. Doke, N. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiol. Plant Pathol.* **23**, 345–357 (1983).

2. Bestwick, C.S., Brown, I.R., Bennett, M.H.R. & Mansfield, J.W. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolicola*. *Plant Cell* **9**, 209–221 (1997).
3. Apel, K. & Hirt, H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **55**, 373–399 (2004).
4. Torres, M.A., Dangl, J.L. & Jones, J.D.G. *Arabidopsis* gp91-phox homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc. Natl. Acad. Sci. USA* **99**, 523–528 (2002).
5. Cross, A.R. & Segal, A.W. The NADPH oxidase of professional phagocytes—prototype of the NOX electron transport chain systems. *Biochim. Biophys. Acta* **1657**, 1–22 (2004).
6. Durrant, W.E. & Dong, X. Systemic acquired resistance. *Annu. Rev. Phytopathol.* **42**, 185–209 (2004).
7. Torres, M.A. & Dangl, J.L. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr. Opin. Plant Biol.* **8**, 397–403 (2005).
8. Levine, A., Tenhaken, R., Dixon, R. & Lamb, C.J. H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79**, 583–593 (1994).
9. Kwak, J.M. *et al.* NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. *EMBO J.* **22**, 2623–2633 (2003).
10. Foreman, J. *et al.* Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* **422**, 442–446 (2003).
11. Dietrich, R.A., Richberg, M.H., Schmidt, R., Dean, C. & Dangl, J.L. A novel zinc-finger protein is encoded by the *Arabidopsis* *lsd1* gene and functions as a negative regulator of plant cell death. *Cell* **88**, 685–694 (1997).
12. Jabs, T., Dietrich, R.A. & Dangl, J.L. Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* **273**, 1853–1856 (1996).
13. Rusterucci, C., Aviv, D.H., Holt, B.F. III, Dangl, J.L. & Parker, J.E. The disease resistance signaling components *EDS1* and *PAD4* are essential regulators of the cell death pathway controlled by *LSL1* in *Arabidopsis*. *Plant Cell* **13**, 2211–2224 (2001).
14. Aviv, D.H. *et al.* Runaway cell death, but not basal disease resistance, in *lsd1* is SA- and *NIM1/NPRI*-dependent. *Plant J.* **29**, 381–391 (2002).
15. Joo, J.H., Wang, S., Chen, J.G., Jones, A.M. & Fedoroff, N.V. Different signaling and cell death roles of heterotrimeric G protein (alpha) and (beta) subunits in the *Arabidopsis* oxidative stress response to ozone. *Plant Cell* **17**, 957–970 (2005).
16. Govrin, E. & Levine, A. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* **10**, 751–757 (2000).
17. Enyed, A.J., Yalpani, N., Silverman, P. & Raskin, I. Localization, conjugation and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc. Natl. Acad. Sci. USA* **89**, 2480–2484 (1992).
18. Dorey, S. *et al.* Spatial and temporal induction of cell death, defense genes, and accumulation of salicylic acid in tobacco leaves reacting hypersensitively to a fungal glycoprotein elicitor. *Mol. Plant Microbe Interact.* **10**, 646–655 (1997).
19. Shirasu, K., Nakajima, H., Rajasekar, V.K., Dixon, R.A. & Lamb, C.J. Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. *Plant Cell* **9**, 261–270 (1997).
20. Draper, J. Salicylate, superoxide synthesis and cell suicide in plant defense. *Trends Plant Sci.* **2**, 162–165 (1997).
21. Wildermuth, M.C., Dewdney, J., Wu, G. & Ausubel, F.M. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**, 562–565 (2001).
22. Reeves, E.P. *et al.* Killing activity of neutrophils is mediated through activation of proteases by K⁺ flux. *Nature* **416**, 291–297 (2002).
23. Delledonne, M., Zeier, J., Marocco, A. & Lamb, C. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proc. Natl. Acad. Sci. USA* **98**, 13454–13459 (2001).
24. Wendehenne, D., Durner, J. & Klessig, D.F. Nitric oxide: a new player in plant signalling and defence responses. *Curr. Opin. Plant Biol.* **7**, 449–455 (2004).
25. Auh, C.-K. & Murphy, T.M. Plasma membrane redox enzyme is involved in the synthesis of O₂⁻ and H₂O₂ by *Phytophthora* elicitor-stimulated rose cells. *Plant Physiol.* **107**, 1241–1247 (1995).
26. Guo, F.Q., Okamoto, M. & Crawford, N.M. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* **302**, 100–103 (2003).
27. Allan, A.C. & Fluhr, R. Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell* **9**, 1559–1572 (1997).
28. Levine, A., Pennell, R., Palmer, R. & Lamb, C.J. Calcium-mediated apoptosis in a plant hypersensitive response. *Curr. Biol.* **6**, 427–437 (1996).
29. Davletova, S. *et al.* Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of *Arabidopsis*. *Plant Cell* **17**, 268–281 (2005).
30. Kliebenstein, D.J., Dietrich, R.A., Martin, A.C., Last, R.L. & Dangl, J.L. *LSL1* regulates salicylic acid induction of copper-zinc superoxide dismutase in *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* **12**, 1022–1026 (1999).