Plants and animals deploy intracellular immune receptors that perceive specific pathogen effector proteins and microbial products delivered into the host cell. We demonstrate that the ADR1 family of Arabidopsis nucleotide-binding leucine-rich repeat (NB-LRR) receptors regulates accumulation of the defense hormone salicylic acid during three different types of immune response: (i) ADRs are required as "helper NB-LRRs" to transduce signals downstream of specific NB-LRR receptor activation during effector-triggered immunity; (ii) ADRs are required for basal defense against virulent pathogens; and (iii) ADRs regulate microbial-associated molecular pattern-dependent salicylic acid accumulation induced by infection with a disarmed pathogen. Remarkably, these functions do not require an intact P-loop motif for at least one ADR1 family member. Our results suggest that some NB-LRR proteins can serve additional functions beyond canonical, P-loop–dependent activation by specific virulence effectors, extending analogies between intracellular innate immune receptor function from plants and animals.

Plants respond to attempted microbial infection with a two-tiered immune system. In the first tier, extracellular pattern recognition receptors (PRRs) bind conserved microbial-associated molecular pattern (MAMP) ligands, activating a complex host response that results in MAMP-triggered immunity (MTI). Successful pathogens deploy suites of virulence effectors that delay or suppress MTI, allowing infection. In the second tier, plant intracellular immune receptors of the nucleotide-binding leucine-rich repeat (NB-LRR) protein family can be activated either by direct binding of effectors or, alternatively, by effector action on an associated target protein that generates a "modified-self" molecule (1, 2). Effector-mediated NB-LRR activation results in effector-triggered immunity (ETI), a rapid and high-amplitude output triggered by regulation of SA accumulation and subsequent activation of SA-dependent pathways. Furthermore, plants are activated by MAMPs and by modified-self molecules in the form of danger-associated molecular patterns (6) and regulate effector-triggered immunity.

Results

**PHOENIX21 Is a Positive Regulator of lsd1 Runaway Cell Death and Is a Member of the ADR1 Family of CCR-NB-LRR Proteins.** Mutants exist that cannot limit the spread of HR after NB-LRR activation. In the Arabidopsis lsd1 (lesions simulating disease 1) mutant, HR occurs normally, but the oxidative burst generated by pathogen recognition triggers a superoxide-dependent signal leading to "runaway cell death" that spreads beyond infection sites (14). This phenotype requires accumulation of SA and additional components of ETI/MTI signaling (15). In a screen for lsd1 suppressors, we isolated mutations in the PHOENIX (PHX) loci. One recessive complementation group of two alleles (phx21-1 and phx21-2) allowed neither the initiation nor the propagation of SA-induced lsd1 runaway cell death in the Wassilewskija (Ws) ecotype (14) (Fig. S1 A and B). We isolated PHX21 (At5g04720, hereafter, ADR1-L2) by map-based cloning (SI Materials and Methods). ADR1-L2 encodes a CCR-NB-LRR protein belonging to a small clade that includes ADR1 (ACTIVATED DISEASE)
RESISTANCE 1; At1g33560) and ADR1-L1 (At4g33300) (16) (Fig. S2A), previously characterized by the gain-of-function, over-expression phenotype of adr1 (17, 18). Over-expression of ADR1 results in the constitutive activation of the defense responses as well as in drought tolerance; both of these phenotypes are SA-dependent (17, 18). Loss of function for adr1-L1 results in modest suppression of ETI (19). We investigated ADR1 family functions in the Columbia (Col-0) ecotype after observing that an isogenic Col-0 allele (adr1-L2-4) also suppressed Isd1 runaway cell death (Fig. S1 C and D).

ADR1 Proteins Function as Helper NB-LRRs for Some, but Not All, ETI Responses and Are Required for Basal Defense to Virulent Pathogens. We assessed whether the ADR1 proteins can function as helper NB-LRRs for well-defined NB-LRR-mediated ETI responses. We challenged adr1, adr1-L1, and adr1-L2 single knock-out mutants (Fig. S2B), combinatorial double adr1 mutants, and the adr1 adr1-L1 adr1-L2 triple mutant (hereafter “adr1 triple”) with the bacterial pathogen Pseudomonas syringae pv. tomato (Pto) DC3000 expressing either the AvrRpm1 or AvrRpt2 effectors or with two isolates of the obligate biotrophic oomycete Hyaloperonospora arabidopsidis (Hpa isolates Emswal and Cala2). In Col-0, these effectors are recognized by either CC-NB-LRR proteins [RPM1 (20) and RPS2 (21)] or by the Toll/interleukin-1 receptor (TIR)-NB-LRR proteins [RPP4 (22) and RPP2 (23)], respectively. RPS2-mediated HR (Fig. 1A and Fig. S3A) and ETI (Fig. 1B) were significantly compromised in the adr1 triple mutant. RIN4 cleavage by the cysteine protease effector AvrRpt2 (24), which initiates RPS2-mediated ETI, was maintained (Fig. S3B). Hence, the ADR1 genes function downstream of this event in RPS2 signaling. Both RPP4- and RPP2-mediated ETI were also significantly compromised in the adr1 triple mutant (Fig. 1 C and D) and weakly compromised in single and combinatorial mutants. The adr1 triple mutant was nearly as susceptible to infection as the TIR-NB-LRR/basal defense signaling mutant enhanced disease susceptibility 1 (eds1) (25). These results extend evidence that ADR1 and the NRG1 (N requirement gene 1) CCR-NB-LRR proteins are helper NB-LRRs (13). RPM1-mediated ETI was not altered (Fig. S4). Thus, some, but not all, effector-mediated ETI responses tested required ADR1 proteins.

NB-LRR protein function has been implicated in basal defense (26), which is activated by virulent pathogens on susceptible hosts and can limit pathogen growth (4). We thus tested the ability of the adr1 family mutants to restrict the growth of virulent pathogens. The adr1 triple mutant was more susceptible to Hpa Ems15 and to Pto DC3000 compared with wild-type or

![Image](https://www.pnas.org/ cgi/doi/10.1073/pnas.1111726108)

**Fig. 1.** ADR1 family members function in ETI and basal defense. (A) Pto DC3000 (avrRpt2) or Pto DC3000(EV) (empty vector) was hand-infiltrated into leaves of 4-week-old plants. Leaves were collected and stained with trypan blue to visualize cell death. (B) Twenty-day-old seedlings were dip-inoculated with Pto DC3000 (avrRpt2), and bacterial growth was assessed at 0 and 3 d post inoculation (dpi). (C) Ten-day-old seedlings were inoculated with Hpa Ems15. Sporangiophores per cotyledon were counted at 3 dpi (average of 100 cotyledons per genotype). Cotyledons were classified as supporting no sporulation (0–1), medium sporulation (1–5), or heavy sporulation (>15). Means of sporangiophores/cotyledon for each genotype are noted below. (D) Ten-day-old seedlings were inoculated with Hpa Cala2. Sporangiophores/cotyledon were counted at 6 dpi as described above. (E) Ten-day-old seedlings were inoculated with Hpa Emco5. Sporangiophores/cotyledon were counted at 4 dpi as described above. (F) Twenty-day-old seedlings were dip-inoculated with Pto DC3000 (EV). Bacterial growth was assessed at 0 and 3 dpi. Values in B and F are mean cfu/mg ± 2 × SE (n = 4). Letters indicate a significant difference following post-ANOVA Tukey’s test (α = 0.05).
single adr1 mutants (Fig. 1 E and F). Hence, ADR1 proteins act as redundant regulators of basal defense.

**ADR1 Proteins Regulate SA Accumulation Following an Oxidative Burst.** SA is a key downstream mediator of plant defense against biotrophic pathogens like those used here (27). Systemic acquired resistance (SAR) (28), basal defense (4), MTI (3), and some, but not all, NB-LRR-mediated ETI responses (29) require SA accumulation, which in turn controls transcriptional reprogramming through the TBL/POZ/ankyrin coactivator NPR1 (Nonexpressor of PR genes) (28). For example, RPS2 function is partially compromised in mutants that do not accumulate SA during ETI, but RPM1 is not.

An extracellular burst of superoxide derived from the NADPH oxidase AtbboD and subsequent hydrogen peroxide (H₂O₂) production are also hallmarks of early MTI and ETI responses (30). In wild-type plants, this oxidative burst signals cells surrounding an infection site to up-regulate defense and anti-oxidant gene transcription and to down-regulate cell death (14, 31). Reactive oxygen and SA gradients surrounding an infection site are also part of a signal amplification system (32) that sets a cell death threshold controlled by LSD1 (30). We thus speculated that the ADR1 proteins might regulate SA homeostasis following an oxidative burst. Infection with Pto DC3000 (avrRpt2) triggered an RPS2-dependent oxidative burst in the triple adr1 triple mutant (Fig. 2 A and B). However, both free and total SA levels were poorly induced in this experiment, to levels as low as in either rps2 or eds1, but slightly more than in the SA biosynthetic mutant sid2 (Fig. 2 C).

Provision of an SA analog (benzothiadiazole, or BTH) (33) rescued the defective ETI and basal defense responses of the adr1 triple mutant. Pretreatment with BTH restored RPS2-dependent HR in the adr1 triple mutant and the SA biosynthetic mutant sid2 (Fig. 2 D). BTH also rescued the enhanced susceptibility to Pto DC3000 (EV) detected in both the adr1 triple mutant and the eds1 controls pretreated with water (Fig. 2 E). This phenotype required NPR1. We conclude that these deficient ETI and basal defense responses are the consequences of the adr1 triple mutant’s inability to accumulate SA. Our results suggest that ADR1 proteins are required for SA accumulation following an intact oxidative burst upon effector and MAMP recognition.

HrpL-deficient Pto DC3000 ΔhrpL is a potent MTI trigger because it cannot deliver MTI-suppressing effectors to the host (34). Pto DC3000 ΔhrpL induced a weak, but detectable, oxidative burst (Fig. 2 A and B) sufficient to trigger SA accumulation in Col-0 (3), but not in the adr1 triple mutant (Fig. 2 F). Hence, recognition of one or more MAMPs expressed by Pto DC3000 ΔhrpL...
activates MTI responses that result in SA accumulation regulated by the ADR1 proteins.

The two best-characterized MTI responses follow specific recognition of peptides derived from either bacterial flagellin (flg22) or elongation factor (elf18) by the PRRs FLS2 and EFR, respectively (35, 36). The accumulation of a functional epitope-tagged ADR1-L2 protein expressed from its native promoter was up-regulated by both flg22 and elf18 peptide treatments, as well as upon BTH application (Fig. S5), yet the adr1 triple mutant exhibited normal oxidative burst, MAPK activation, and callose deposition following treatment with either peptide (Fig. S6). Collectively, these findings indicate that the ADR1 proteins act in MTI downstream or independently of early events subsequent to EFR or FLS2 activation but upstream of SA accumulation.

An Intact P-Loop Domain Is Dispensable for Any of the ADR1-L2 Functions. STAND proteins bind ATP and most act as ATPases. These include plant NB-LRR proteins (8), a variety of animal NLRs and cell death control proteins (5), and functionally diverse bacterial proteins (9). To date, there is no crystal structure of a full-length NB-LRR or NLR immune receptor. However, structures for both Aapf1 [a functional ATPase (37)] and CED4, which binds ATP (38) but does not hydrolyze it (39), are available (39, 40). Homology modeling of the CC_R-NB domain (residues 40–671) of ADR1-L2 with Aapf1 (residues 1–586) confirmed the location of conserved functionally relevant glycine and lysine residues (GK212/213) analogous to Aapf1 (GK159/160) in the ATP-binding pocket of the P-loop (Fig. S7 A and B). The P-loop directly interacts with the β-phosphate of ADP (41). An invariant GK residue pair is crucial for this interaction, and mutation of these residues abrogates nucleotide binding and/or ATPase activity and functions across kingdoms (41, 42) (Fig. S7 C–E).

We constructed transgenic adr1 triple-mutant transgenic plants expressing wild-type ADR1-L2, ADR1-L2G212A, ADR1-L2K213R, or ADR1-L2AAA (GKT212/213/214AAA) with C-terminal HA-epitope tags under the control of the native promoter (Fig. S7 C–E). Surprisingly, homozygous transgenic adr1 triple-mutant lines expressing these alleles (Fig. 3 A) complemented RPS2-mediated HR (Fig. 3 B), RPP4-dependent ETI (Fig. 3 C), basal defense (Fig. 3 D), and Pto DC3000ΔhrpL-induced SA accumulation (Fig. 3 E) to levels comparable to those observed in the adr1 adr1-L1 double mutant. Thus, an intact P-loop motif is dispensable for

![Fig. 3.](image-url)

**Fig. 3.** An intact P-loop catalytic domain is dispensable for ADR1-L2 to function in ETI, basal defense, and MTI. (A) Protein extracts were sampled from stable homozygous transgenic adr1 triple plants expressing HA-tagged ADR1-L2, ADR1-L2G212A, ADR1-L2K213R, or ADR1-L2AAA (ADR1-L2, G212A, K213R, AAA, respectively). Numbers indicate the identity of the transgenic lines used. An anti-HA antibody was used to detect ADR1-L2 protein accumulation. Equal loading was verified by Coomassie staining (Lower). (B) Pto DC3000(avrRpt2) was hand-infiltrated into leaves from 4-wk-old plants and stained with trypan blue. Numbers indicate how many leaves showed HR out of the total number of leaves analyzed. (C) Ten-day-old seedlings were inoculated with Hpa Emwa1. Sporangiophores/cotyledon were counted at 5 dpi, and cotyledons were classified as in Fig. 1. (D) Ten-day-old seedlings were inoculated with Hpa Emco5. Sporangiophores were counted at 4 dpi as above. (E) Leaves from 4-wk-old plants were hand-infiltrated with Pto DC3000ΔhrpL or MgCl2. Total SA was measured at 9 hpi (mean ± 2 × SE, n = 4) and compared with SA levels from mock-treated plants. Letters indicate a significant difference among genotypes infiltrated with Pto DC3000ΔhrpL following post-ANOVA Student’s t test (α = 0.05). The assays in B–D were repeated three times with similar results.
ADR1-L2 functions in ETI, basal defense, and MTI. Additionally, an allele of ADR1-L2 that lacks a functional P-loop can function in the absence of the other two ADR1 family members.

**Discussion**

We demonstrate that the CC$_R$-NB-LRR ADR1 proteins are functionally distinct innate plant immune receptors. They regulate SA-dependent defense in three contexts: MTI responses against a disarmed pathogen, basal defense against virulent pathogens, and some, but not all, ETI responses. Moreover, at least ADR1-L2 function in these contexts is P-loop–independent. Hence, ADR1-L2 is not activated via the canonical mechanism used by NB-LRR and NLR receptors as microbial sensors, at least for the phenotypes that we describe.

NB-LRR pairs acting in ETI have been reported (12). The lack of physical interaction between the pairs analyzed to date supports a scenario in which the helper NB-LRRs might constitute convergence points in defense responses downstream of recognition and oxidative burst mediated by either PRRs or by NB-LRR sensors activated via effector-driven, P-loop–dependent conformational changes. Our analysis of the CC$_R$-NB-LRR ADR1 proteins is consistent with their evolutionary history and divergence from other CC-NB-LRR family members (13).

We speculate that the CC$_R$-NB-LRR ADR1 proteins might function as signaling scaffolds and regulators of signal transduction processes leading to SA accumulation and consequent defense outputs. This is reminiscent of the function of NLRC5 and NLRP12. NLRs that do not function as microbial sensors per se but rather regulate intracellular signaling pathways (43). These proinflammatory NLRs might function in conjunction with additional NLRs, as suggested by the physical interaction of NLRP12 with NOD2 (44). By analogy, the plant CC$_R$-NB-LRR ADR1 proteins might mediate signal transduction in response to common upstream stimuli. Consequently, the microbial sensor function of immune receptors might be the result of the coordination between effector-mediated sensor activation and a more general signaling function provided by CC$_R$-NB-LRR ADR1 proteins.

The expanded CC$_R$-NB-LRR functions that we describe for at least ADR1-L2 require neither the canonical P-loop motif nor the other two full-length CC$_R$-NB-LRR ADR1 family members. We note ADR1-L2 is encoded by ADR1-L2 in the Col-0 genome (At5g47280), although it lacks approximately the first 190 amino acids at its N terminus and has no reported phenotype. Nevertheless, this protein could play a unique and equally noncanonical role in the phenotypes that we describe. We suggest that the unique signaling functions for at least ADR1-L2 are a consequence of its association with one or more yet-to-be-defined defense machines. The functions that we define for CC$_R$-NB-LRR ADR1 proteins do not preclude an additional, undiscovered, P-loop–dependent function as an effector sensor that, in this context, may associate with and guard the hypothetical signaling machine. Interestingly, a rice protein was recently described that lacks an intact ATP-binding domain and functions in disease resistance against rice blast (45), suggesting a mode of action similar to the CC$_R$-NB-LRR ADR1 proteins. We speculate that additional immune receptors of the NB-LRR and NLR classes might have the following mechanistically separable functions: (i) canonical P-loop–dependent activation via microbial perturbation of the inter- and intramolecular architectures, unusual phylogenetic patterns, and evolution by horizontal gene transfer. 1. Jones JD, Dangl JL (2006) The plant immune system. Nature 444:323–329.
5. Leipe DD, Koonin EV, Aravind L (2004) STAND, a class of P-loop NTPases including animal and plant regulators of programmed cell death: Multiple, complex domain interactions that repress nucleotide exchange and/or hydrolysis and (ii) recognition-independent, P-loop–independent scaffold functioning as part of stress response machinery.

**Materials and Methods**

ADR1 CC$_R$-NB-LRR Protein Family Nomenclature. ADR1 (At1g33560) was identified via its ectopic over-expression phenotype, which resulted in constitutive SA-dependent defense gene activation and consequent induction of disease resistance, a common NB-LRR protein gain-of-function phenotype. ADR1-L1 (At4g33300) and ADR1-L2 (At5g04720) were identified by homology (16).

**Plant Lines and Pathogens Strains.** T-DNA insertion lines in the Arabidopsis thaliana Col-0 accession were from public collections and were identified by searching the SIGnAL database (http://signal.salk.edu). Details of the mutants, the pathogen strains, and their growth quantification used in this study are provided in SI Materials and Methods.

**DNA Manipulations.** Standard techniques of DNA manipulation were used.

**Cell Death Assays.** Leaves were harvested and cell death was assessed by Trypan blue staining to visualize dead cells or by conductivity measurements as described in SI Materials and Methods.

**Immunoblot Analysis.** Leaves from 4-wk-old plants were harvested, and total proteins were extracted by grinding frozen tissue in a buffer containing 20 mM Tris HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.1% SDS, 10 mM DTT, and plant protease inhibitor mixture (Sigma-Alrich). Samples were centrifuged at 14,000 × g for 15 min at 4 °C to pellet the debris. Proteins (75 μg) were separated on 7.5% or 12% SDS/PAGE for detection of ADR1-L2 or RIN4, respectively. Proteins were transferred to polyvinylidene difluoride membranes, and Western blots were performed using standard methods. Monoclonal anti-HA antibody (Santa Cruz Biotechnology) antibody was used at a 1:3,000 dilution, whereas anti-RIN4 serum was used at 1:2,000. Signals were detected by enhanced chemiluminescence using ECL Plus (Amersham Biosciences).

**Detection of H$_2$O$_2$.** H$_2$O$_2$ was visualized in situ by 3,3’-diaminobenzidine (DAB) staining as described (30). Leaves from 4-wk-old plants were hand-infiltrated with Pto DC3000(avrRpt2) or Pto DC3000ΔhrpL at 10$^7$ cfu/mL and collected 10 h after infiltration. Leaves were vacuum-infiltrated with a solution containing 1 mg/mL DAB and placed in a dark plastic box under high humidity for an additional 8 h. Leaves were then destained in a solution of 3:1:1 ethanol/lactic acid/glycerol.

**Free and Total SA Measurement.** Leaves from 4-wk-old plants were hand-infiltrated with Pto DC3000(avrRpt2) at 10$^7$ cfu/mL or with Pto DC3000ΔhrpL at 5 × 10$^7$ cfu/mL free SA and glucose-conjugated SA (SAG) (SA + SAG) measurements were performed as described in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Plant Lines and Propagation. All Arabidopsis mutant lines are in the Columbia (Col-0) ecotype except lsd1-1 (1), which is in the Wassilewskija (Ws) accession. adr1-L2-4 (At5g074720, SALK_126422), adr1-L2-6 (SALK_150245), adr1-L1 (At1g33560, SAIL_S42_B50), and adr1-L1-1 (At4g33300, SAIL_302_C06) were obtained from the Arabidopsis Biological Resource Center. atrobohD (2), lsd1-2 (3), npri1-1 (4), rps2-101C (5), rpm1-3 (6), sid2-1 (7), fts2 (8), and efr (8) are described elsewhere. The Col-0 eds1-2 introgressed line was provided by Jane Parker (Max-Planck-Institut for Plant Breeding Research, Cologne, Germany).

Planck-Institut for Plant Breeding Research, Cologne, Germany.

Mapping, phx21-2 was mapped by map-based cloning. A mapping population was generated from the F2 plants derived from a cross between the Col-0 introgressed lsd1 (9) and phx21-2 lsd1-1. A total of 100 F2 plants were obtained by precrossing with benzothiadiazole (BTH) spraying. Genetic analysis showed that phx21-2 was recessive (130 wild type, 54 mutant, \( \chi^2 = 1.86, P > 0.2 \)). The F2 suppressor was allowed to self and was confirmed in the F3 generation. DNA from 10 F2 individuals was used in PCR amplification of known PCR-based molecular markers (http://www.arabidopsis.org) to obtain approximate mapping positions. This interval was refined using molecular markers that we developed (available upon request). We used DNA from F2 individuals to find a clear linkage of phx21-2 to the top arm of chromosome 5 between the molecular markers F15F17-5 and ciw18. Proof of isolation was obtained via sequence analysis of independent mutant alleles, as noted in the main text.

DNA Manipulations and Generation of Transgenic Plants. For expression of ADR1-L2, ADR1-L2(2214A), ADR1-L2(2136R), or ADR1-L2(2444A) under the control of its native promoter, ADR1-L2-HA, ADR1-L2(2214A)-HA, ADR1-L2(2136R)-HA, or ADR1-L2(2444A)-HA cDNAs were fused by PCR to ADR1-L2 promoter (500 bp) and cloned in the pGWB1 Gateway vector (10) for expression in the triple adr1 mutant. Arabidopsis transgenics were generated using Agrobacterium (GV3101)-mediated transformation by floral dip (11).

Pathogen Strains, Inoculation, and Growth Quantification. Halo- peronospora arabidopsidis (Hpa) isolates Emw1 and Emco5 were propagated on the susceptible Arabidopsis ecotype Ws, whereas Cala2 was propagated on Ler. Conidiospores of Hpa Emw1, Cala2, and Emco5 were resuspended in water at a concentration of 10^4 spores/mL and spray-inoculated onto 14-d-old seedlings (12). Inoculated plants were covered with a lid to increase humidity and grown at 19 °C under a 9-h light period.

Pto DC3000 (EV) was resuspended in 10 mM MgCl₂ to a final concentration of 2.5 × 10⁷ cfu/mL, whereas Pto DC3000 (avrRpt2) and Pto DC3000 (avrRpm1) were resuspended to 2.5 × 10⁷ cfu/mL. Twenty-day-old seedlings were dipped in the bacterial solution and growth was assessed as described (13). To test BTH-mediated acquired resistance, 4-wk-old plants were sprayed with a solution of 300 μM BTH or H₂O containing 0.005% Silwet. Leaves were hand-infiltrated with Pto DC3000 (EV) to 5 × 10⁴ 2 d post application (dpa), and bacterial growth was measured as described (14). To test the hypersensitive cell death response (HR), bacteria were resuspended in 10 mM MgCl₂ to 5 × 10⁴ cfu/mL and hand-infiltrated in leaves from 4-wk-old plants. The RPM1- and RPS2-mediated HR was assessed at 5 and 10 h post inoculation (hpi), respectively.

Cell Death Assays. Leaves were harvested and stained with lactophenol trypan blue to visualize dead cells as described (12, 15). For the conductivity measurements (3, 16), leaves of 4-wk-old plants were infiltrated with the bacterial pathogens (see below) and at 1 hpi four leaf discs were cored (7-mm diameter), floated in water for 30 min, and subsequently transferred to tubes containing 6 mL distilled water. Conductivity of the solution was determined with an Orion conductivity meter at the indicated time points.

Free and Total Salicylic Acid Measurements. Free salicylic acid (SA) and glucose-conjugated SA (SAG) measurements were performed as described (17). Leaves (100 mg) were collected at 24 hpi [Pto DC3000 (avrRpt2)] or 9 hpi [Pto DC3000 ΔavrRpmL] and frozen in liquid nitrogen. Samples were ground and tissue was homogenized in 200 μL 0.1 M acetic buffer (pH 5.6). Samples were then centrifuged for 15 min at 16,000 × g at 4 °C. Supernatant (100 μL) was transferred to a new tube for free SA measurements, and 10 μL was incubated with 1 μL 0.5 U/mL β-glucosidase for 90 min at 37 °C for total SA measurement. After incubation, plant extracts were diluted fivefold with 44 μL acetic buffer for free SA measurement. Sixty microliters of LB media, 5 μL of plant extract (treated or not with β-glucosidase), and 50 μL of Acinetobacter sp. APDWH-lux (OD = 0.4) was added to each well of a black 96-well plate. The plate was incubated at 37 °C for 60 min, and luminescence was read with the Spectra Max M5 (Molecular Devices) microplate reader.

For the standard curve, 1 μL of the known amount of SA stock (0–1,000 μg/mL) was diluted 10-fold in sid2-1 plant extract, and 5 μL of each standard (undiluted for free SA measurement, or fivefold diluted for total SA) was added to the wells of the plate containing 60 μL of LB and 50 μL of Acinetobacter sp. APDWH-lux (OD = 0.4). SA standards were read in parallel with the experimental samples.

Bioassays for Microbial-Associated Molecular Pattern-Induced Responses. The oxidative burst upon microbial-associated molecular pattern (MAMP) application was performed essentially as described previously (8). Leaf discs (3.8-mm diameter) excised from leaves of 4-wk-old plants were floated in water overnight. Water was replaced by a solution containing 100 nM flg22 or elf18, 17 μg/mL luminol (Sigma) and 10 μg/mL HRP (Sigma). Luminescence was recorded over time using the Spectra Max M5 (Molecular Devices) microplate reader. The MAPK activation assay was performed as described (18) with some modifications. Seven-day-old seedlings were grown in Murashige and Skoog (MS) agar plates and transferred for one additional week to MS liquid culture. The media were replaced with fresh liquid MS containing 1 μM of either flg22 or elf18. Seedlings were collected at the indicated time points, and proteins were extracted in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 15 mM EDTA, 100 mM NaCl, 2 mM DTT, 1 mM NaF, 1 mM Na₂MoO₄, 0.5 mM Na₃VO₄, 30 mM β-d-glucose-phosphate, and 0.1% Nonidet P-40. Samples were centrifuged at 16,000 × g for 30 min at 4 °C, and the supernatant was filtered through a layer of miracloth. Protein (40 μg) was loaded on a 12% SDS/PAGE. Membranes were probed with anti-phospho-p44/p42 MAPK antibody (Cell Signaling).

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MAMP-triggered callose deposition was also performed as described (19). fg22 and elf18 were applied at 2 or 1 μM, respectively, for 20 h on 2-wk-old seedlings, grown under the same conditions as for the MAPK activation assay. Seedlings were vacuum-infiltrated with 3:1 ethanol/glacial acetic acid and left shaking with several changes of solution for 8 h until cotyledons were translucent. Seedlings were then rehydrated in 70% ethanol for 1 h and in 50% ethanol for an additional 1 h, washed in water twice, and left shaking in water overnight. The following day seedlings were washed twice in H2O and incubated in 150 mM K2HPO4 (pH 9.5) containing 0.01% aniline blue for 4 h. Stained cotyledons were mounted on slides with 50% glycerol and viewed on a Nikon Eclipse E800 microscope under UV illumination with a broadband DAPI filter set. Flg22 and elf18 peptides were synthesized at the University of North Carolina (Chapel Hill, NC) Microprotein Sequencing and Peptide Synthesis core facility using the following sequences: ORLSTGSRINSAKDDAAGLQIA (flg22) and acetyl-SKEFKERTKPHNVNGITIG (elf18).

Molecular Modeling. A homology model for ADR1-L2 (residues 40–671) was constructed using an alignment to the crystal structure of ABP1-bound Apaf-1 (residues 1–586) (Protein Data Bank ID 1Z6T) generated by the BioInfoBank Meta Server, a prediction-based server for protein structure and function (http://meta.bioinfo.pl) (20) and the MODELLER 9v8 comparative modeling program (http://www.salilab.org/modeller/about_modeller.html) (21).
Fig. S1. ADR1-L2 positively regulates lsd1 runaway cell death in Ws and Col-0. (A and C) Four-week-old plants were sprayed with 300 μM BTH, and pictures of the plants were taken 5 dpa. Untreated control plants of the same genotype are shown on the left. (B and D) Representative leaves of the plants shown in A and C, respectively, were stained with trypan blue to visualize the cell death at 5 dpa. All of the genotypes in A and B are in the Ws ecotype, whereas the genotypes in C and D are in the Col-0 accession.
Fig. S2. ADR1-L2 is part of a small clade of coiled-coil nucleotide-binding leucine-rich repeat (CC-NB-LRR) proteins. (A) Alignment of the Col-0 ADR1 family deduced protein sequences. Sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/), and the alignment was edited with Jalview (http://www.jalview.org/index.html). Amino acids shaded in dark gray are invariant, whereas residues shaded in light gray are conserved in >60% of the sequences. The red boxes represent previously reported conserved motifs on the NB domains (22). (B) Mutations of ADR1-L2, ADR1, and ADR1-L1 genes. The translated parts of exons (boxes), as well as intron sequences (lines), are depicted. For each T-DNA mutant used in this study, the insertion site and the orientation of the T-DNA are indicated. The corresponding lines were identified in the SALK or SAIL T-DNA collections and are in the Col-0 background: \( \text{adr1-L2-4, SALK}_126422 \); \( \text{adr1-L2-6, SALK}_150245 \); \( \text{adr1-1, SAIL}_842\_B05 \); and \( \text{adr1-L1-1, SAIL}_302\_C06 \). The T-DNA insertions are not drawn to scale. \( \text{phx21-2} \) is the original EMS allele isolated in the Ws background (23), which carries a point mutation at the position 2,817 (G > A) that results in a premature stop codon. The numbers on top represent the position of the insertion or the mutation.
Fig. S3. ADR1 proteins function downstream of RIN4 cleavage in RPS2-mediated effector-triggered immunity (ETI). (A) Ion leakage measurements following inoculation with Pto DC3000 carrying EV, avrRpt2, or buffer only in Col-0 (Upper) or the triple adr1 mutant (Lower). Following infiltration, four leaf discs per treatment were washed with H2O for 30 min and then transferred to fresh H2O where conductance was measured (μSiemens/cm) over time. Values are means ± 2×SE (n = 5). The experiment is representative of three replicates. (B) Col-0 or triple adr1 plants were hand-infiltrated with Pto DC3000(avrRpt2) (+) or 10 mM MgCl2 (−). The cysteine protease type II effector AvrRpt2 targets and cleaves RIN4, leading to RPS2-mediated ETI (24, 25). Samples were collected at 24 hpi, and total protein extracts were subjected to anti-RIN4 Western blot to detect RIN4 cleavage.
Fig. S4. ADR1 proteins are not required for RPM1-mediated effector-triggered immunity. (A) Pto DC3000(EV), or expressing avrRpm1, was hand-infiltrated into leaves from 4-wk-old plants. Leaves were collected at the indicated time and HR cell death was stained with TB. Leaves infiltrated with Pto DC3000(EV) do not show any cell death; thus the stain is specific for HR. (B) Ion leakage measurements following inoculation with Pto DC3000 carrying EV, avrRpm1, or buffer only in Col-0 (Upper) or the triple adr1 mutant (Lower) were performed as in Fig. S3. Values are means ± 2SE (n = 5). The experiment is representative of three replicates. (C) Fourteen-day-old seedlings of the genotypes listed were dip-inoculated with Pto DC3000 expressing avrRpm1, and bacterial growth was assessed at 0 and 3 dpi. Values are mean cfu/mg ± 2SE (n = 4). Letters indicate a significant difference following post-ANOVA Tukey’s test (α = 0.05).

Fig. S5. ADR1-L2 expression is up-regulated by flg22, elf18, and BTH. (A) Seven-day old seedlings were grown on MS plates and transferred to liquid media for an additional week. A total of 1 μM flg22 or elf18 was added to fresh media, and proteins were sampled at 24 hpa. adr1-L2 ADR1-L2-HA 44.12 represents a homozygous transgenic line of adr1-L2 expressing a native copy of a C-terminally HA-tagged ADR1-L2 allele. Anti-HA antibody was used to detect ADR1-L2 protein accumulation. Equal loading was verified by Coomassiee staining (Lower). (B) Four-week-old plants were sprayed with 300 μM BTH or mock-sprayed, and proteins were sampled at 24 hpa. Anti-HA was used to detect ADR1-L2 protein accumulation. Equal loading was ensured by Coomassiee staining (Lower).
Fig. 5.6. Early responses to flg22 and elf18 do not require the ADR1 proteins. (A) Oxidative burst in leaves of the indicated genotypes after addition of 100 nM flg22 (Left) or elf18 (Right). Results are means ± 2 × SE (n = 8). (B) MAPK activation in seedlings of the indicated genotypes upon application of 1 μM of flg22 or elf18 over time. The identity of each band is shown on the left. Equal loading was verified by Coomassie stain. (C) Callose deposits stained with aniline blue on the cotyledons of seedlings treated with water (Top panels), 1 μM flg22 (Middle panels), or 2 μM elf18 (Bottom panels) for 24 h. Images are representative of 20 individuals.
Fig. S7. ADR1-L2 is structurally similar to Apaf-1. (A) Homology model of the CC-NB domain (residues 40–671) of ADR1-L2 (brown) based on the crystal structure of ADP-bound Apaf-1 (26) (gray). (B) The location of the conserved functionally relevant G212, K213, and T214 of ADR1-L2 is illustrated in blue inside the ADP-binding pocket. (C) Model of the structural reorganization caused by the substitution of G212A in ADR1-L2. G212 maps to the Apaf-1 G159 and forms a polar contact (orange dashed line) to a neighboring residue in the polypeptide backbone. A mutated ADR1-L2G212A (green) forms an additional contact (dark brown dashed line) with another neighboring residue, which is likely to result in a different secondary structure or in the elongation of the α-helix. (D) Model of the structural reorganization caused by the substitution of K213 in ADR1-L2. K213 maps to the Apaf-1 K160, and in both cases the Lys residue is predicted to coordinate ADP via two hydrogen bonds (dashed lines). A mutated ADR1-L2K213R (green) loses the coordination of ADP. (E) Model of the structural reorganization caused by the substitution of T214 in ADR1-L2. T214 maps to the Apaf-1 T205, and in both cases the Thr residue is predicted to form a hydrogen bond with ADP (dashed line). (F) Model of the structural reorganization caused by the substitution of G212A in ADR1-L2. G212 maps to the Apaf-1 G159 and forms a polar contact (orange dashed line) to a neighboring residue in the polypeptide backbone. A mutated ADR1-L2G212A (green) forms an additional contact (dark brown dashed line) with another neighboring residue, which is likely to result in a different secondary structure or in the elongation of the α-helix. (G) Legend continued on following page
organization caused by the substitution of GKT212/213/214AAA in ADR1-L2. (F) Schematic representation of ADR1-L2 showing the P-loop mutations described in this study. The amino acid changes are shown in relation to their linear position. The CC domain is shown in green, the NB domain in red, and the C-terminal LRR domain in blue. (G) Alignment of the P-loop domain from various plant, animal, and bacterial STAND proteins and ATPases for which mutations in this motif result in loss of function. The consensus sequence is displayed on the bottom. Residues highlighted in red represent essential amino acids required for the coordination of ATP/ADP and their mutations (substitutions are shown on the right) resulted in loss-of-function proteins [references are listed in parentheses in the protein column (27–47)]. Green indicates that the mutation does not cause loss of function.
Correction

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The authors note that Fig. 2 appeared incorrectly. The corrected figure and its legend appear below.

Fig. 2. ADR1 proteins are required for effector-independent SA accumulation following a superoxide burst. (A) Leaves from 4-week-old plants were hand-infiltrated with Pto DC3000( avrRpt2), Pto DC3000ΔhrpL, or Pto DC3000(EV). H$_2$O$_2$ accumulation was monitored by 3′,3′-diaminobenzidine (DAB) staining at 5 h post inoculation (hpi). Leaves are representative of 10 individuals. (B) DAB staining shown in A was quantified (mean ± 2 × SE, n = 5). Letters indicate a significant difference following post-ANOVA Student’s t test (α = 0.05). (C) Leaves from 4-week-old plants were hand-infiltrated with Pto DC3000( avrRpt2) or with MgCl$_2$. Free (Left) and total SA (Right) were measured at 24 hpi (mean ± 2 × SE, n = 4). (D) Pto DC3000( avrRpt2) was hand-infiltrated into leaves from 4-week-old plants pretreated with either H$_2$O (Upper) or BTH (Lower) 24 h before bacterial infiltration. Leaves were collected 10 hpi and stained with trypan blue. Leaves are representative of 20 individuals. Numbers indicate how many leaves showed HR out of the total number of leaves analyzed. (E) Four-week-old plants were sprayed with either H$_2$O or BTH. Leaves were hand-infiltrated with Pto DC3000(EV) 2 d post application (dpa). Bacterial growth was monitored at 0 and 3 dpi, mean ± 2 × SE (n = 4). (F) Leaves from 4-week-old plants were hand-infiltrated with Pto DC3000ΔhrpL, Pto DC3000(EV), or MgCl$_2$. Total SA was measured at 9 hpi (mean ± 2 × SE, n = 4) and compared with SA levels from uninfiltrated plants. Letters indicate a significant difference among genotypes infiltrated with Pto DC3000ΔhrpL following post-ANOVA Student’s t test (α = 0.05). The experiments in A–F were repeated three times with similar results.