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Science Signaling **2** (84), ra45. [DOI: 10.1126/scisignal.2000448]

PLANT BIOLOGY

The Plant NADPH Oxidase RBOHD Mediates Rapid Systemic Signaling in Response to Diverse Stimuli

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(Published 18 August 2009; Volume 2 Issue 84 ra45)

Cell-to-cell communication and long-distance signaling play a key role in the response of plants to pests, mechanical wounding, and extreme environmental conditions. Here, we report on a rapid systemic signal in *Arabidopsis thaliana* that traveled at a rate of 8.4 centimeters per minute and was dependent on the *respiratory burst oxidase homolog D* (*RbohD*) gene. Signal propagation was accompanied by the accumulation of reactive oxygen species (ROS) in the extracellular spaces between cells and was inhibited by the suppression of ROS accumulation at locations distant from the initiation site. The rapid systemic signal was triggered by wounding, heat, cold, high-intensity light, and salinity stresses. Our results reveal the profound role that ROS play in mediating rapid, long-distance, cell-to-cell propagating signals in plants.

INTRODUCTION

Due to their sessile lifestyle, higher plants evolved sophisticated mechanisms to cope with biotic or abiotic challenges in their environment. These can be activated locally in tissues that initially interact with the threat, as well as systemically in tissues that were not directly challenged (*1*). The activation of defense or acclimation mechanisms in systemic tissues is often termed systemic acquired resistance (SAR) or systemic acquired acclimation (SAA), respectively, and serves an important role in preventing further infection or damage to the entire plant during stress (*1*–*5*). Signals that mediate systemic plant responses can be divided into (i) slow-moving signals that require several hours to travel and are typically mediated by different plant hormones, such as jasmonic acid (JA), salicylic acid (SA), small peptides, or azelaic acid (*1*, *3*, *6*), and (ii) fast-moving signals that travel within minutes and are thought to be mediated by electric signals (*7*–*9*) or airborne hormones, such as methyl JA (MeJA) or methyl SA (MeSA) [(*2*, *3*); see, however, conflicting results in (*10*)]. Although considerable work has been conducted on SAR, and a number of key players and mutants in this pathway were identified (*4*), little is known about the molecular mechanisms underlying rapid systemic responses, and the signal mediating SAA to a wide range of abiotic stimuli is currently unknown (*11*).

Here, we uncover a rapid systemic signal in the plant *Arabidopsis thaliana* that travels at a rate of up to 8.4 cm min^{-1} and is dependent on the *respiratory burst oxidase homolog D* (*RbohD*) gene. Signal propagation is accompanied by the accumulation of reactive oxygen species (ROS) in the extracellular spaces between cells and by rapid expression of ROS-responsive transcripts. Once initiated, the signal can be blocked by the suppression of ROS accumulation at locations that are distant from the initiation site. Rapid systemic signaling is independent of ethylene, JA, or SA signaling, but can be triggered by wounding, heat, cold, high-intensity light (high light), or salinity stresses. Our results reveal a profound and general role played by ROS in mediating rapid, cell-to-cell–propagating systemic signals in plants and raise the possibility that a similar class of ROS-related signals function in the response of other multicellular organisms to external stimuli.

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RESULTS

Dependence of the rapid systemic signal on *RbohD*

The zinc finger protein Zat12 functions in the response of plants to different environmental conditions, such as intense light, salinity, or osmotic stress (*12*), and the gene encoding Zat12 is expressed within minutes in response to wounding or to the accumulation of ROS (*13*, *14*). To ascertain whether ROS can mediate rapid systemic responses, we used a luciferase (*Luc*) reporter gene fused to the *Zat12* promoter (*Zat12::Luc*). We monitored luciferase expression either in *Zat12::Luc* transgenic plants or, after crossing of the *Zat12::Luc* plants with a mutant deficient in the *RbohD* gene (*15*, *16*), in double homozygous plants (*Zat12::Luc/rbohD*).

Transgenic *Zat12::Luc* plants wounded on three fully expanded rosette leaves exhibited rapid local and systemic responses to mechanical wounding, as measured on wounded or nonwounded leaves (Fig. 1, A and B, and movie S1). In contrast, the local and systemic responses to mechanical wounding of the *Zat12::Luc/rbohD* plants were substantially delayed (Fig. 1, A and B, and movie S1). Imaging of inflorescences from *Zat12::Luc* plants subjected to mechanical wounding ~2 to 3 cm above their base revealed that the systemic signal traveled at a rate of up to 8.4 cm min^{-1} in an up-and-down direction along the wounded inflorescence (Fig. 1C and movie S2). In contrast, the systemic signal detected in *Zat12::Luc/rbohD* plants traveled at a rate of 0.5 cm min^{-1} mainly in the upward direction (Fig. 1C and movie S2).

Accumulation of ROS in local and systemic tissues in response to wounding

Measurements of ROS in wounded and unwounded seedlings revealed that ROS accumulated at the wound site of wild-type seedlings (Fig. 2, A and B, and movie S3). In contrast, ROS accumulation was delayed in *rbohD* seedlings (Fig. 2, A and B, and movie S3). Measurements of ROS in the space between the cell wall and the plasma membrane (the apoplast) with a dye that cannot enter cells (OxyBURST) revealed that ROS accumulation occurred in cotyledons of wild-type seedlings that were wounded at the

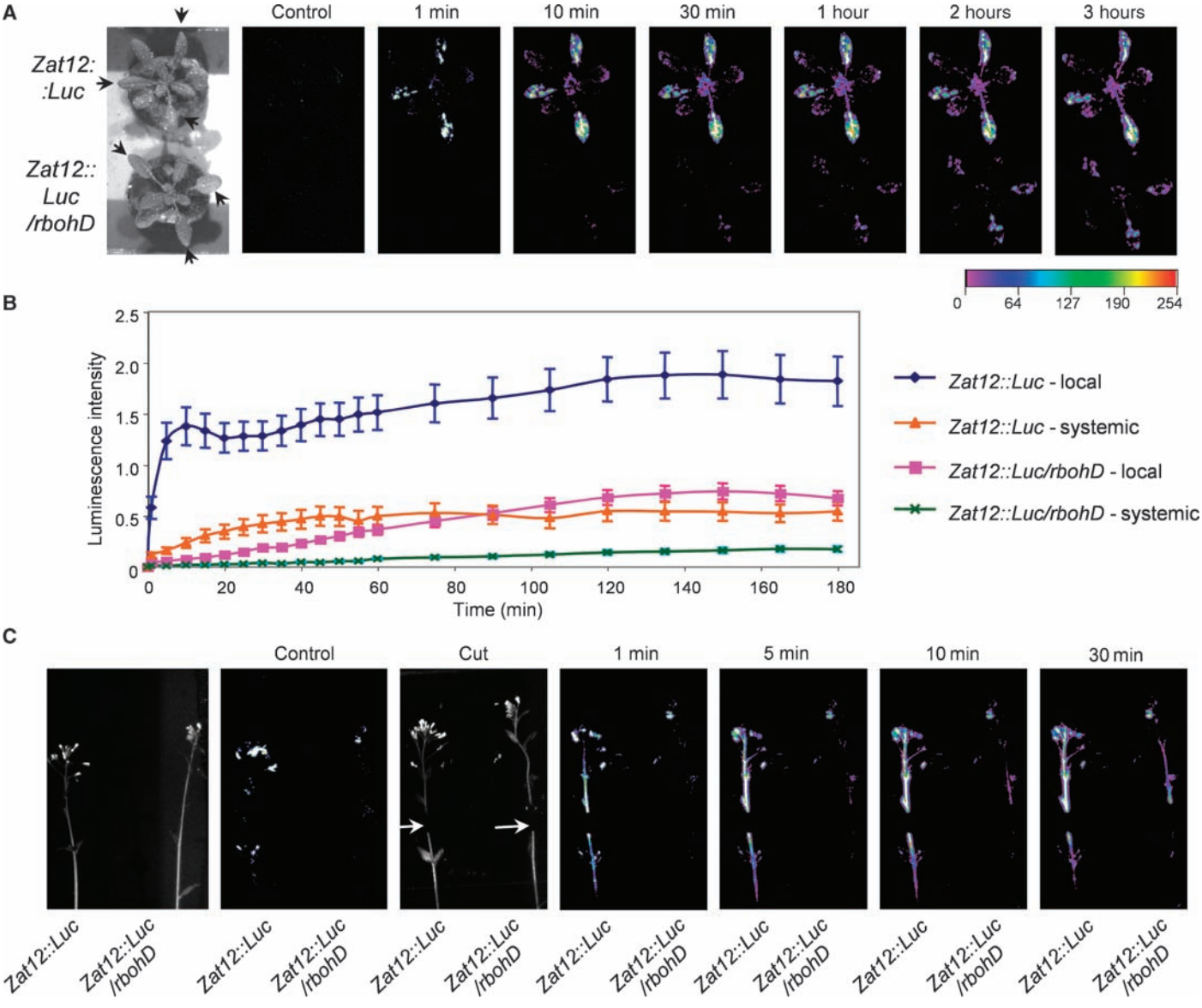

Fig. 1. Suppression of the rapid wound-induced systemic signal in *Zat12::Luc/rbohD*. (**A**) Time course of wound-induced luciferase expression in three wounded rosette leaves (black arrows) and in systemic unwounded leaves at different times after wounding. *Zat12::Luc* plants were wounded immediately after wounding of *Zat12::Luc/rbohD* plants. (**B**) Quantification of the local and systemic wound-induced signals in rosette leaves. Average and SE bars are for three different leaves in four individual biological replicates. (**C**) Time course of wound-induced luciferase expression in the inflorescence of *Zat12::Luc* and *Zat12::Luc/rbohD* plants simultaneously severed ~3 to 4 cm above their base (white arrows). Left and third from left panels are bright-field images.

base of their stems at a rate that was comparable to the advance of the systemic signal detected with the *Zat12::Luc* reporter (Fig. 2, C to F). In contrast, apoplastic ROS accumulation was suppressed in systemic tissues of *rbohD* seedlings (Fig. 2, D to F). Thus, *RbohD* is required for efficient local and systemic wound-induced ROS production.

Inhibition of signal propagation by suppression of ROS accumulation at locations distant from the initiation site

Local application of either of two inhibitors of ROS accumulation, catalase and the NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) flavin–oxidase inhibitor diphenylene iodonium (DPI), at a location ~4 to 5 cm above the wounding site along the inflorescence of *Zat12::Luc* plants (30 min before wounding at the base of the inflorescence stem) suppressed the progression of the systemic signal (Fig. 3, A and B, and movies S4 and S5). In contrast, prior application of inhibitors of calcium signaling, such as 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), EGTA, or $LaCl_3$, at the same location along the inflorescence of *Zat12::Luc* plants had no effect on the rate of advance of the systemic signal (table S1). Application of DPI or $LaCl_3$, but not BAPTA or EGTA, at the wounding site (30 min before wounding) inhibited the initia-

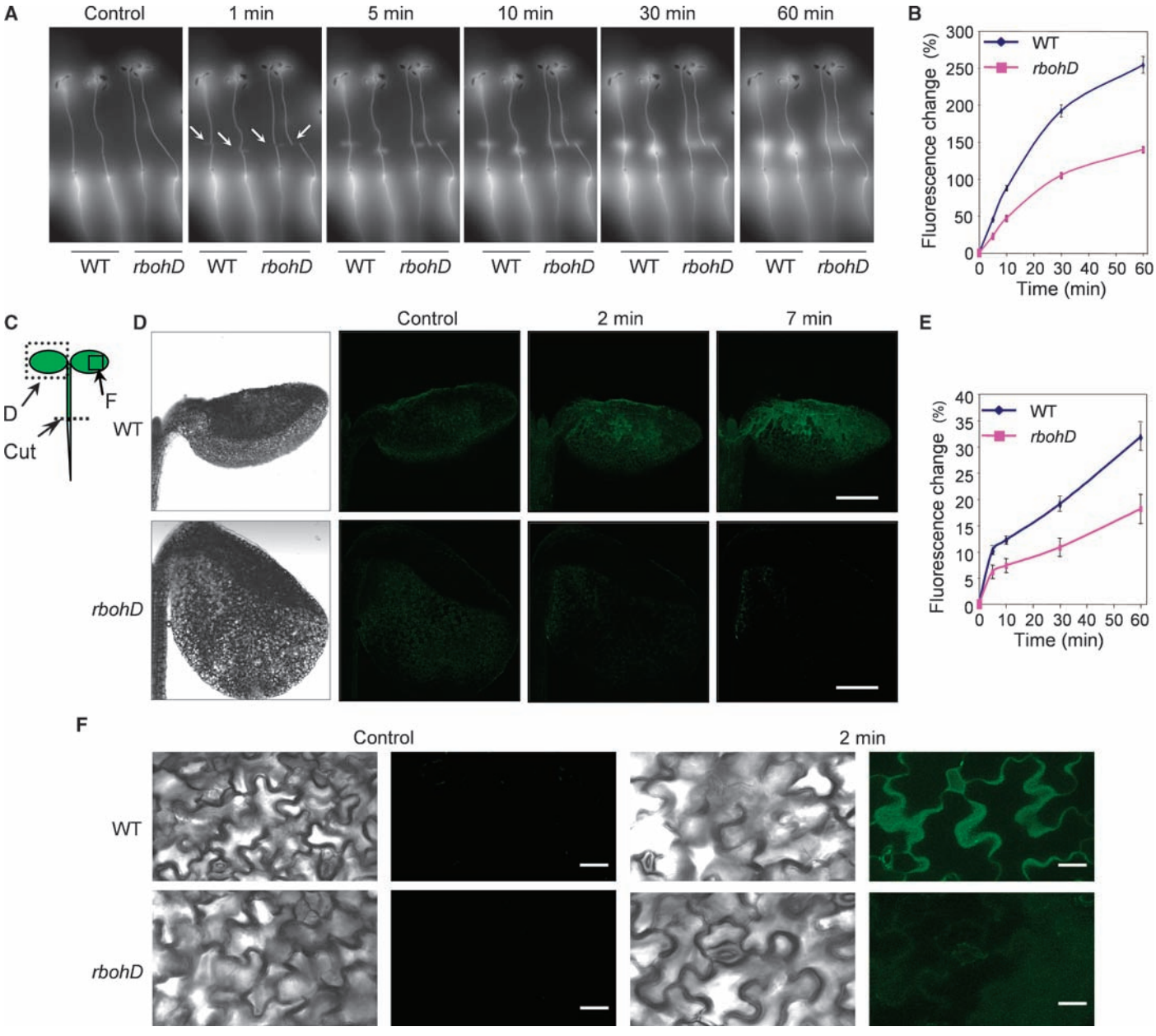

Fig. 2. Wound-induced accumulation of ROS in local and systemic tissues of control and *rbohD* plants. (**A**) Local accumulation of ROS in control and *rbohD* seedlings stained with Amplex Red in response to wounding (white arrows indicate the position at which the seedlings' stems were simultaneously severed). (**B**) Quantification of the injury-induced fluorescence with Amplex Red at the wounding site shown in (A). (**C**) A diagram showing the experimental design used in (D) and (F). OxyBURST-stained seedlings were cut at the base of their stems (arrow pointing to dashed line) and the cotyledons [whole cotyledons in (D) or epidermal cells in (F)] were immediately imaged for fluorescence under a confocal microscope. (**D**) Time course of systemic ROS accumulation in cotyledons of wounded seedlings stained with OxyBURST. Images are representative of at least 10 different seedlings analyzed. Nomarski [differential interference contrast (DIC)] images shown on left (magnification, ×10; bar, 200 μm). (**E**) Quantification of the injury-induced systemic fluorescence with Amplex Red in cotyledons. (**F**) Extracellular ROS accumulation in cells of systemic tissues 2 min after wounding at the base of the seedlings' stems (magnification, ×40; bar, 25 μm). Images are representative of at least 150 different cells imaged in five different seedlings.

tion of the systemic signal (Fig. 3, C and D, and table S1). Hence, the initiation of systemic signaling requires divalent cation (likely calcium) signaling, a relay of the RBOHD-dependent superoxide production, and subsequent H_2O_2 formation. In contrast, propagation of the rapid systemic signal appeared to primarily require RBOHD-dependent accumulation of H_2O_2 (fig. S1). Note, however, that limited diffusion rates might prevent some of the chemicals indicated in table S1 from penetrating cells and inhibiting the rapid systemic signal.

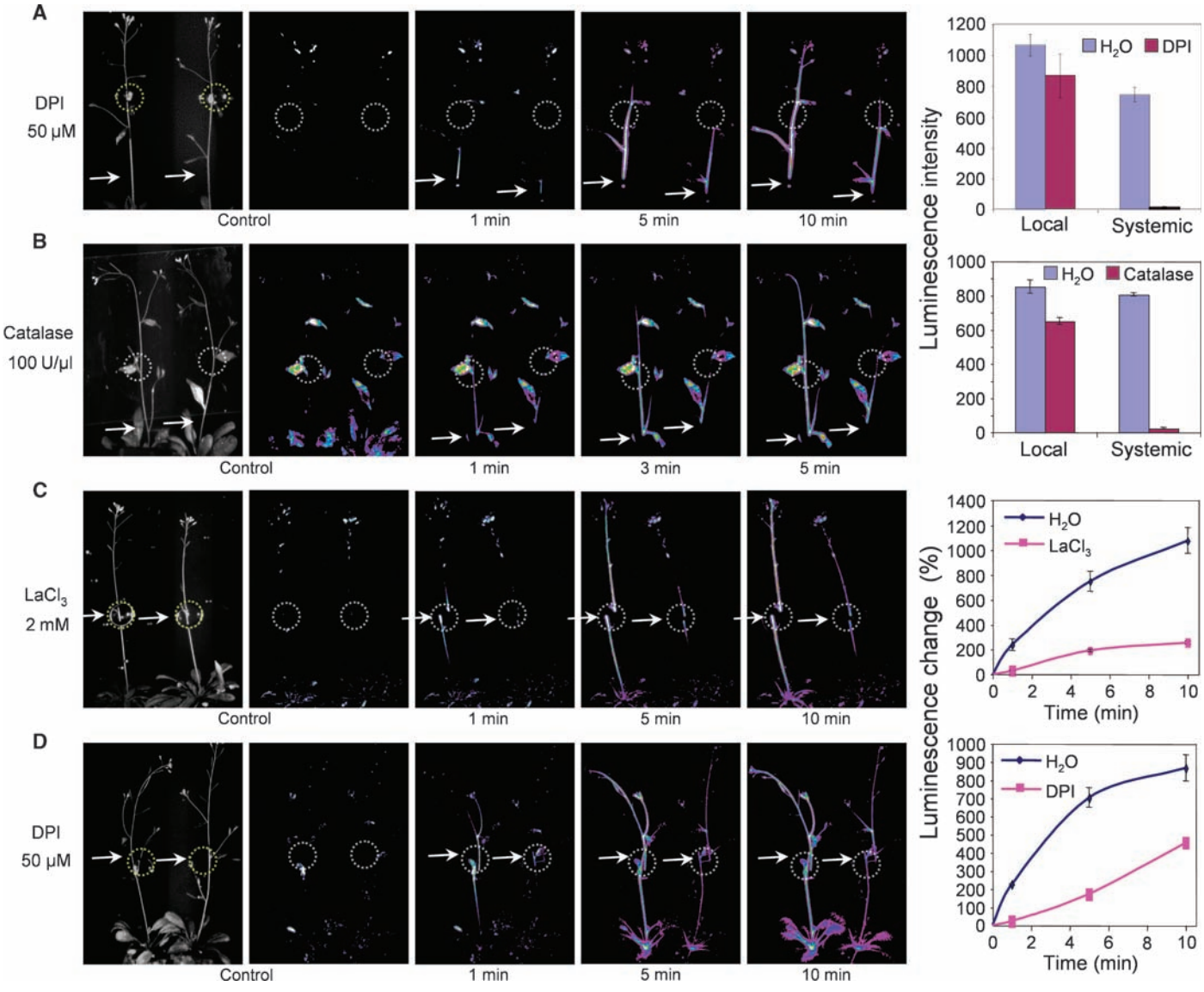

Fig. 3. Suppression of ROS accumulation in local or distal locations delays the progression of the rapid systemic signal. (**A**) *Zat12::Luc* plants were pretreated at a defined location (dashed circle) with a drop of water (left plant) or DPI (right plant) 30 min before wounding. Plants were simultaneously severed at the base of their inflorescence stems (white arrows) and the progression of the signal was monitored at different times after wounding (left panel shows a bright-field image and panels 2 through 5 from left show luciferase activity). Right panel shows quantification of luminescence intensities at the wounding site (Local) or in systemic tissue (~2 to 3 cm above the drop of water or DPI) 10 min after wounding. (**B**) *Zat12::Luc* plants were pretreated at a defined location (dashed circle) with a drop of water (left plant) or catalase (right plant) 30 min before wounding and then treated and monitored as in (A). Quantitation is shown to the right. (**C**) *Zat12::Luc* plants were pretreated at the wounding site (dashed circle) with a drop of water (left plant) or $LaCl_3$ (right plant) 30 min before wounding; the treated the stems were severed simultaneously and signal progression was monitored as in (A) and (B). The panel on the right shows a time course of luminescence intensity at the wounding site. (**D**) The plants were pretreated at the wounding site with a drop of water (left plant) or with a drop of DPI (right plant) and then treated and monitored as in (C).

Transcriptional microarray analysis of the rapid systemic signal

Microarray analysis revealed that the expression of 81 different transcripts was rapidly elevated (Fig. 4), and that of 50 different transcripts was rapidly decreased (table S2), in systemic tissues 10 min after wounding. In agreement with the wound-induced rapid accumulation of ROS in systemic tissue (Fig. 2, C to F), 84% of the transcripts elevated in systemic tissues in response to wounding were previously reported to be H_2O_2-responsive (*12*, *13*) (Fig. 4). These included at least three proteins implicated in calcium signaling, a number of ROS-responsive transcription factors, and several proteins with putative kinase activity, including two receptor-like kinases. In contrast, there was little overlap with genes

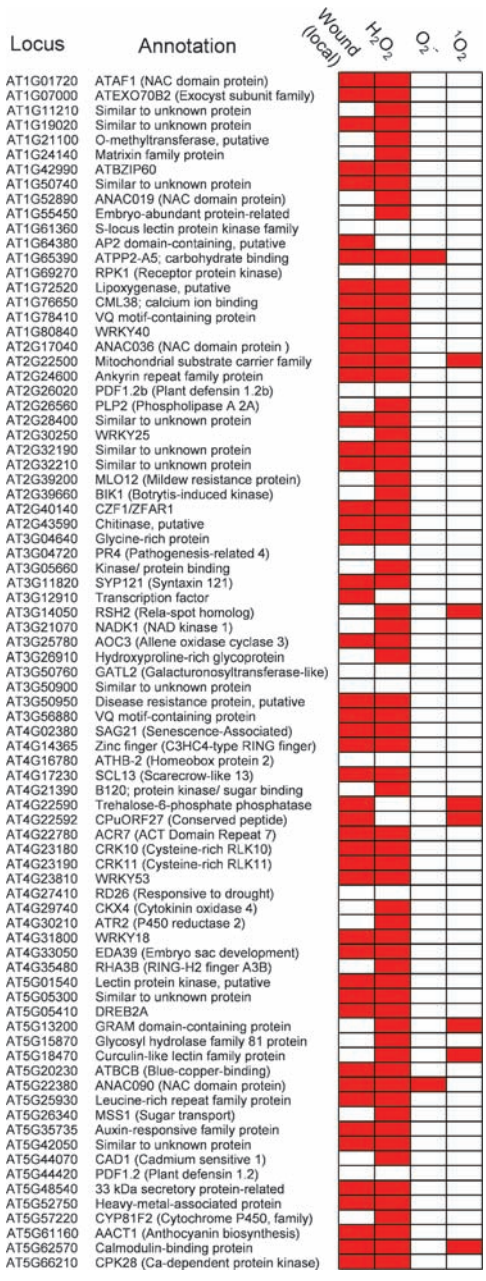

Fig. 4. GeneChip analysis reveals that transcripts increased in systemic tissue by wounding largely overlap with those increased by H_2O_2 treatment. Transcripts listed are all of those that were elevated in systemic tissue of wild-type plants 10 min after wounding. The accession number of the gene loci, a brief transcript annotation, and previously published early (15 to 60 min) expression in response to different treatments are indicated by the red boxes. Wound, local tissue wounding (*35*); H_2O_2, treatment of seedlings with H_2O_2 (*12*) or internal accumulation of H_2O_2 in leaves of mature plants (*13*); O_2^-, treatment of plants with the superoxide-generating agent methyl viologen (*35*); $^1O_2$, response of mutants impaired in chlorophyll biosynthesis (*flu*) to singlet oxygen accumulation (*36*).

reportedly activated by other types of ROS, including superoxide (O_2^-) and singlet oxygen ($^1O_2$).

Analysis of the rapid systemic signal in various mutant backgrounds

At least two different *RBOH* transcripts are expressed in mature leaves of *Arabidopsis* (*RBOHD* and *RBOHF*) (*13*, *15*, *16*). In addition, *RbohC* (*17*) has a key role in regulating ROS signaling in roots (*18*, *19*). Nonetheless, neither *RbohF* nor *RbohC* was required for the rapid systemic response of Zat12 (Fig. 5A), or several other systemic transcripts (fig. S2), showing

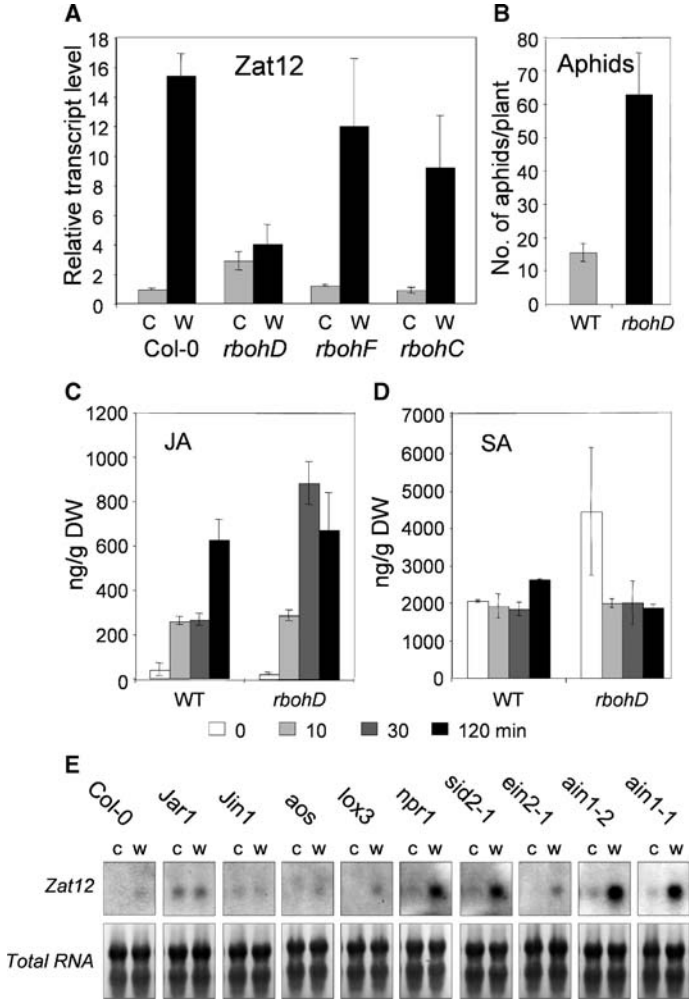

Fig. 5. Signal specificity to *RbohD*, increased sensitivity to aphid infection, and altered JA signaling in *rbohD* mutants. (**A**) qRT-PCR analysis of Zat12 rapid systemic expression in wild type and mutants deficient in RBOHD (*rbohD*), RBOHF (*rbohF*), or RBOHC (*rbohC*) 10 min after wounding. (**B**) Increased susceptibility of *rbohD* plants to aphid infection. (**C** and **D**) Accumulation of JA or SA in local wounded leaves of wild-type and *rbohD* plants. (**E**) RNA blot analysis of *Zat12* rapid systemic expression in wild-type plants and plants with mutations affecting JA, SA, or ethylene perception (JA, *Jar1*, *Jin1*; SA, *npr1*; ethylene, *ein2-1*, *ain1-2*) or synthesis (JA, *aos*, *lox3*; SA, *sid2-1*; ethylene, *ain1-1*). C, control; W, systemic leaves of wounded plants 10 min after injury.

a specific function for *RbohD* in this response. Interestingly, the expression of several wound-induced transcripts appeared to be elevated in the *RbohD* background in the absence of wounding (fig. S2).

The inability of *rbohD* plants to mediate rapid systemic responses correlated with higher sensitivity of these plants to aphid infection (Fig. 5B), suggesting a biological role for *RbohD* in the defensive response to insects. JA and SA are well-known mediators of the plant response to herbivory and wounding. Therefore, we measured JA and SA accumulation in local tissues of wild-type and *rbohD* plants. JA accumulation in wild-type plants peaked at 120 min after wounding. Surprisingly, wound-induced JA accumulation was accelerated and peaked at 30 min after wounding in *rbohD* plants (Fig. 5C). Compared to wild-type plants, SA concentrations were slightly higher in *rbohD* and the concentration of SA decreased after wounding (Fig. 5D). Despite the enhanced concentrations of wound-induced JA, *rbohD* plants were more susceptible to aphids than were wild-type plants (Fig. 5B). Thus, the JA-mediated wound signaling in the response of plants to insect feeding (*6*) appears to require *RbohD*. Despite the altered accumulation of SA and JA in the local tissues of *rbohD* plants (Fig. 5, C and D), the rapid systemic induction of Zat12 in response to wounding was not suppressed in different mutants impaired in ethylene, SA, or JA signaling (Fig. 5E). In mutants impaired in SA or ethylene signaling, *Zat12* expression was actually facilitated in systemic tissues in response to wounding, whereas in two mutants impaired in JA signaling (*aos* and *lox3*), *Zat12* expression was not suppressed in systemic tissues in response to wounding (Fig. 5E). In two other mutants impaired in JA signaling (*Jar1* and *Jin1*), *Zat12* was constitutively expressed (Fig. 5E). These results suggest that signaling through the SA, JA, and ethylene pathways might not be directly required for the wound-induced rapid systemic response.

Activation of the rapid systemic signal by heat and other abiotic stresses

We defined a surprisingly broad requirement for *RbohD* in rapid systemic signaling on the basis of the expression of the *Zat12::Luc* construct in responses to cold, heat, wounding, high light, and salinity (Figs. 1 and 6, A to D, and fig. S3). Wounding and high-light systemic responses transcriptionally regulate different systemic target genes (*2*–*5*, *20*); systemic responses to heat stress are less well known. Two heat stress response proteins [MBF1c and HSP17.3 (*21*)] were detected in systemic tissues of wild-type, but not *rbohD*, plants in response to local application of heat stress (Fig. 6E). Interestingly, the abundance of MBF1c and HSP17.3 was enhanced in the local tissues of *rbohD* plants compared to wild type (Fig. 6E).

DISCUSSION

Systemic signals play a key role in the defense or acclimation of plants to a large array of biotic and abiotic challenges (*1*–*5*). Here, we report that plants can mediate rapid cell-to-cell communication over long distances, and that this communication requires enhanced production of ROS by different cells along the path of the signal, which is dependent on RBOHD (Figs. 1 to 3). The rapid rate of the ROS signal (up to 8.4 cm min^{-1}) and its ability to travel at the same rate in the up or down direction along the plant's stem (Fig. 1) suggest that the signal is independent of normal diffusion rates and is actively propagating. The inhibition of the signal by suppressing ROS accumulation at locations distant from the initiation site further indicates that the signal requires continuous production of ROS by individual cells along its path (that is, the signal is autopropagating) (Fig. 3 and fig. S1).

The dependence of the rapid systemic signal on RBOHD could suggest that superoxide radicals that are produced by this enzyme are the

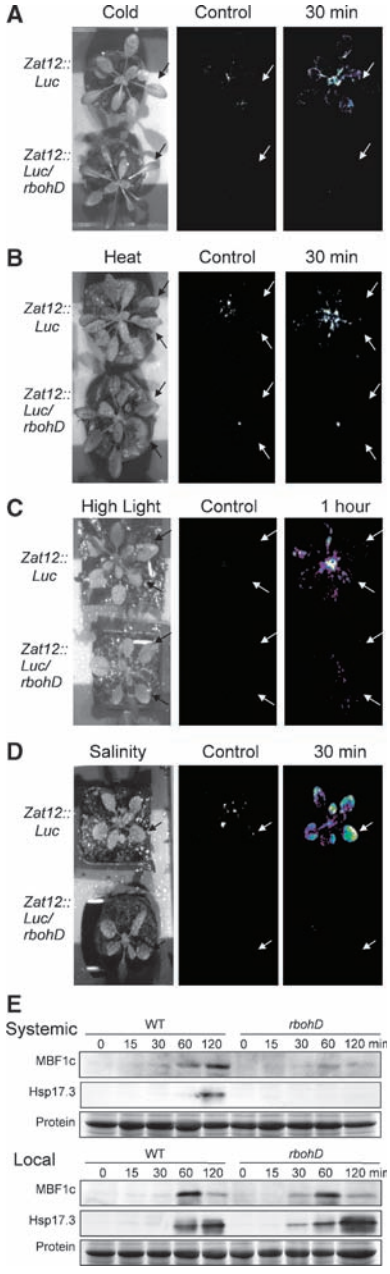

Fig. 6. RBOHD is involved in the systemic response to diverse abiotic stresses. (**A**) Triggering of the *RbohD*-dependent rapid systemic response by cold stress. The arrow indicates the leaf treated with ice water. (**B**) Triggering of the *RbohD*-dependent rapid systemic response by heat stress. The arrow indicates the leaf treated with hot water. (**C**) Triggering of the *RbohD*-dependent rapid systemic response by high-intensity light (high light). Arrows indicates the leaves exposed to high light intensity. (**D**) Triggering of the *RbohD*-dependent rapid systemic response by salinity stress. The arrow indicates the leaf treated with high salt condition. (**E**) Time course protein blot analysis showing accumulation of heat response proteins in systemic or local leaves of wild-type and *rbohD* plants subjected to heat stress. All experiments were repeated at least three times with similar results. Control treatment with 21°C water and detailed time courses for heat and cold stresses are shown in fig. S3.

primary ROS involved in mediating the signal. However, transcript microarray analysis shows that transcripts responsive to H_2O_2 account for most transcripts up-regulated in systemic tissues (Fig. 4). Moreover, Amplex Red and OxyBURST that were used in our imaging analysis (Fig. 2) are both primarily responsive to H_2O_2, and the propagation of the systemic signal was suppressed by catalase that decomposes H_2O_2 (Fig. 3B). It is therefore likely that superoxide produced by RBOHD is rapidly dismutated to H_2O_2 either spontaneously or through an apoplastic-localized superoxide dismutase (*22*) such that the ROS that primarily mediates the rapid systemic signal is H_2O_2. Our inability to initiate the rapid systemic signal by applying H_2O_2 (table S1), combined with our finding that *RbohD* is specifically required for the initiation, as well as the propagation of the rapid systemic signal (Figs. 1 to 4), suggest that RBOHD activation along the path of the systemic signal is essential for signal propagation.

Electric signals, such as system potentials, are a type of signal that propagate at a rate comparable to that of the signal reported here (*7*, *9*, *23*). Because propagation of the systemic signal described in this work requires continued RBOHD-dependent ROS production along its systemic path (Figs. 2 and 3 and fig. S1), and subsequent dismutation of superoxide produced by RBOHD to H_2O_2 could cause depolarization of membrane potential (*8*), it is possible that activation of RBOHD in different cells along the path of the systemic signal can amplify or facilitate wound-induced electric signals. Further studies are required to address this potentially important link.

Our findings are in agreement with previous studies that showed the regulation of RBOH proteins by calcium and their function in mediating the growth of root hairs by generating local waves of enhanced ROS production at the root hair tip (*18*, *19*, *24*). In contrast to previous implications of RBOH proteins in local responses to stress, pathogens, or development and growth (*18*, *19*, *24*, *25*), our findings demonstrate that RBOHD can mediate cell-to-cell communication over long distances in plants. A front of RBOH-dependent ROS production can therefore propagate across the entire plant and mediate responses to diverse stimuli (Figs. 1 to 4 and figs. S1 and S3).

High light intensity–induced SAA is perhaps the most comparable response to the rapid systemic signal described in this work. It was initially discovered in 1999 by Karpinski *et al.* (*5*), who showed an increase in transcript expression and acclimatory changes in photochemistry of distal shaded leaves of an *Arabidopsis* plant that was partially exposed to high-light stress. Recently, it was reported that 86% of transcripts up-regulated in leaves exposed to high-light stress are also expressed in distal shaded leaves and, as a consequence, both exposed and shaded leaves have enhanced tolerance to oxidative stress (*11*, *20*, *25*). Similar to the signal described in this study, high light intensity–induced SAA is independent of JA or SA signaling and occurs within 15 min of high-light application to local leaves. Nevertheless, the signal mediating high-light SAA was not identified, and in contrast to the signal reported here, it did not involve systemic accumulation of H_2O_2 (*11*, *20*). In addition, only 8 of the transcripts identified in our analysis of the wound-induced rapid systemic response (Fig. 4) were among the 360 transcripts up-regulated in systemic tissues of plants after high light intensity–induced SAA (*20*).

Heat stress plays a major role in yield reduction in agriculture and is a critical stress for plant reproduction (*26*). In contrast to SAA due to high light intensity or different types of SAR responses, the heat stress systemic response is virtually unknown at present. Nevertheless, the same logic that applies to high light intensity–induced SAA that is a requirement for a rapid systemic acclimatory mechanism to stresses triggered by local changes in light intensity in plants (*5*, *11*, *20*) would also apply to the rapid SAA due to heat stress [with the further importance of heat stress tolerance to plant reproductive tissues (*26*)]. Our findings that local heat stress application results in a systemic heat stress response and that this response is dependent on RBOHD function (Fig. 6 and fig. S3) could therefore open the way for the development of new biotechnological applications, as well as detailed studies of the heat stress signal transduction pathway.

ROS were previously shown to function as downstream secondary messengers of the wound response (*27*, *28*), as well as early local wound or pathogen response mediators (*8*). Our findings that ROS accumulation is required along the path of rapid systemic signaling (Figs. 3 and 4) and that *RbohD* is required for this process (Figs. 1 to 3, 5, and 6) reveal a previously unknown role for the superoxide generated by RBOHD or its reactive derivatives, or both, as mediators of cell-to-cell communication over long distances in plants (fig. S1). ROS accumulation along a systemic signal front is therefore essential for long-distance signaling in plants in response to diverse environmental stimuli.

MATERIALS AND METHODS

Plant material and growth conditions

A. thaliana (cv. *Colombia*) Col-0, *rbohF*, *rbohC*, *rbohD* (*15*), transgenic *Arabidopsis* expressing luciferase (*Luc*) under the control of the *Zat12* promoter (*12*) (*Zat12::Luc*) and *Zat12::Luc/rbohD* crosses were grown for 3 weeks at 23°C under constant light (50 μmol m^{-2} s^{-1}) either on peat pellets (Jiffy 7) or on soil mixture (MetroMix 200, SUN GRO) in 20-cm^2 inserts as described (*12*).

Aphid infection

Mature green peach aphids *Myzus persicae* (Sulzer) raised on cabbage leaves were transferred onto leaves of bolting wild-type or *rbohD* plants (3.5 to 4 weeks old) in separate containers (*29*), 2 aphids per plant, and the total number of aphids on each plant was determined 10 days later.

Wounding

For wounding (*12*), two different experimental systems were used: (i) Three rosette leaves in each plant were pricked 10 times with the tip of a scalpel. (ii) The inflorescence of bolting plants was severed with a scalpel.

Abiotic stress treatments

Plants were exposed to heat stress. Two (for luciferase imaging) or three (for protein extraction) leaves of each plant were dipped in a water bath (42°C) for either 5 min (for imaging) or 15, 30, 60, and 180 min (for proteins extraction). Protein extraction and protein blot analysis were performed according to (*21*). Plants were exposed to cold stress. One or two leaves were covered with ice water for 5 min. Plants were exposed to high-intensity light. Plants were covered with foil, exposing only two to three leaves to 500 μmol m^{-2} s^{-1} for 60 min. Plants were exposed to salinity stress. A single leaf was dipped in 100 mM NaCl and 1 mM luciferin solution in a 2-ml tube for 30 min. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) analysis (*30*) of systemic leaves performed 30 min after dipping of local leaves revealed that relative NaCl concentrations in the systemic leaves remained ambient. The ratio of sodium to magnesium in local salt-treated leaves was 1.776 ± 0.232, whereas the same ratio in systemic leaves was 0.846 ± 0.153, and in control untreated leaves 0.783 ± 0.095 (data represent the average and SE of three biological repeats).

Luciferase imaging

Plants were sprayed with 1 mM luciferin (sodium salt, GOLD BioTechnology, USA) solution before and after wounding or the different stress treatments

and immediately placed on a Kodak image station 2000MM for imaging (*12*). Sequential imaging mode was used for image capturing consisting of 1-min exposure time per capture. Luminescence intensity measurements of locally injured and systemic rosette leaves were performed with the Kodak MI software as recommended by the manufacturer.

Pharmacological treatments

Bolting *Zat12::Luc* and *Zat12::Luc/rbohD* plants with an inflorescence stem of 15 to 20 cm were sprayed with luciferin and placed horizontally on the Kodak imager station. A drop of 1 ml of water containing 1 mM luciferin supplemented with the different inhibitors or treatments (table S1) was placed on the inflorescence stem for 30 min before wounding. The stem was then severed within the drop or 4 to 5 cm below it. All cuts were made in the presence of a drop of 1 mM luciferin.

JA and SA analysis

SA was quantitated with deuterated d_6-salicylic acid as internal standard (*21*). For detection of JA, the mass spectrometer was operated in single-reaction monitoring (SRM) mode with negative electrospray ionization. Ionization voltage was 3.8 kV with sheath and auxiliary gas flow rates of 30 and 10 units, respectively. Capillary temperature was 270°C and the collision gas pressure (helium) was 1.5 mtorr. Jasmonic acid was monitored by SRM transitions of 209→59.1 at collision energy of 22 eV. For JA quantitation, a calibration curve was prepared by injecting solutions containing known concentrations of JA (3.81 to 50,000 ng/ml), and extraction recovery was determined by spiking a set of duplicated samples with known amounts.

ROS imaging and microscopy

For Amplex Red staining, wild-type and *rbohD* plants were germinated side by side on ½ MS agar (0.8%) containing 1% sucrose. Plates were placed vertically in the dark for 5 days and then transferred to light (50 μmol m^{-2} s^{-1}) for 5 more days. Seedlings were covered for 30 min with 2 μM Amplex-Red (Molecular Probes, Invitrogen, or MBL Intl Corp) in 50 mM sodium phosphate buffer (pH 7.4). The excess Amplex Red was drained and fluorescence was imaged on a Kodak Image Station 2000MM camera, with 535 and 600 nm excitation and emission filters, respectively (*31*). For CM-H_2DCFDA and OxyBURST-H2HFF staining, 5-day-old seedlings grown vertically on ½ MS agar (0.8%) plates or in liquid ½ MS (*12*) were gently transferred to 2-ml tubes containing either 5 μM CM-H_2DCFDA or OxyBURST-H2HFF (10 μg/ml; Molecular Probes, Invitrogen) in ½ MS and incubated for 30 min under constant shaking. Fluorescence of cotyledons was detected before and after injury with an Olympus confocal microscope (FV1000) at 488 and 535 ± 10 nm excitation and emission, respectively.

Systemic wounding microarray experiment

Three-week-old transgenic *Arabidopsis Zat12::Luc* plants, grown in a growth chamber under constant light conditions (50 μmol m^{-2} s^{-1}), were wounded as described above. Systemically wounded leaves (W) were collected 10 min after injury. Control (C) unwounded leaves of similar sizes and developmental stages were sampled in parallel. For each treatment, three independent biological replicates, each composed of leaves pooled from 20 plants, were taken. Isolated total RNA (*12*) was treated with deoxyribonuclease I (DNaseI; Turbo DNA free, Ambion Inc. Austin, TX) and cleaned on RNeasy Mini columns (Qiagen, ML, USA). These RNA samples were used to perform chip hybridization and analysis of transcriptional measures (*Arabidopsis* ATH1 chips; Affymetrix, Santa Clara, CA) at the Nevada Genomics Center at the University of Nevada, Reno, as previously described (*12*, *13*). Some of the results were confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) as described below.

Microarray data processing

In this study, three biological triplicates were included per experimental condition (Wounded and Control). Expression data were first subjected to a series of rigorous quality control steps to ensure data reproducibility and overall quality. Average background and noise metrics were examined for consistency across all six arrays, as indicated by the Affymetrix GeneChip Operating Software user's guide. Average background ranged from 44 to 59 when run on 10% PMT (photomultiplier tube) scanner settings. RawQ noise levels fell between 1.9 and 2.5 with a mean value of 2.13 and SD of 0.2. Present call rates were consistent across all arrays, ranging from 51 to 63% (mean rate 58%). The hybridization controls BioB, BioC, BioD, and Cre were present 100% of the time. Additionally, it was verified that signal intensities of BioC, BioD, and Cre increased, respectively. Lastly, 3′-to-5′ ratios of both actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were verified to be within Affymetrix guidelines: All actin ratios were less than 1.5; GAPDH ratios were consistently below 1.1. Images of all arrays were examined, and no obvious scratches or spatial variation were observed. Digestion curves describing trends in RNA degradation between the 5′ end and the 3′ end in each probe set were examined and all proved similar. Correlations among biological replicates were high: Spearman coefficients ranged from 0.984 to 0.992; Pearson coefficients ranged between 0.987 and 0.994.

Raw intensity values were processed first by RMA (*32*) (robust multi-array average) with the R package Affy (*33*). Upon application of preprocessing and normalization, all arrays exhibited consistent expression distributions. Data from the 15,325 noncontrol probe sets that were found to be present in at least one of the six array measurements were retained for further analyses. To ensure strict reproducibility standards, the triplicated expression measurements of these remaining probe sets were inspected individually. Any set of triplicates in which one of the measures exhibited a SD of more than 1.14 (the maximum possible SD for three measures is 1.1547) and a coefficient of variation of greater than 0.5 for the triplicate set was scrutinized. If one single measure was near 1.1547, this indicated that the remaining two measures were similar, and that the third triplicate was as its maximum outlying capacity; thus, this one triplicate value was removed. This procedure left two replicates within the set of which the mean was used for subsequent analyses. Only 326 measurements (0.4% of all measurements) were excluded for this rule. Additionally, any remaining triplicates that exhibited a coefficient of variation of greater than 0.75 were removed. This included only 10 sets of triplicated measures (0.03% of all triplicated measurements) and reduced the mean coefficient of variation of all triplicates to 0.11. We found that these thresholds allowed us to identify gross outlying individual measurements within a triplicate set.

A simple set of Student's *t* tests was applied to all probe sets that exhibited a twofold or greater differential expression between the two experimental conditions. Upon a multiple testing correction (*34*) [false discovery rate (FDR)], we found that 129 probe sets exhibited a statistically significant differential expression of twofold or higher. These probe sets can be found in Fig. 4 and table S2.

cDNA synthesis and qRT-PCR analysis

Total RNA was obtained from three independent biological repeats (each pooled from leaves of 5 plants) of control and systemically wounded Col-0, *rbohD*, *rbohF*, and *rbohC* plants. First strand complementary DNAs (cDNAs) were produced after DNaseI treatment from 1.3 μg of total RNA with SuperScript III (Invitrogen). qRT-PCR was performed in an optical 96-well plate with the ABI 7500 Fast Real-Time PCR System and the Fast SYBR Green Master Mix (Applied Biosystems, Scoresby, Victoria, Australia). For all PCR reactions the following standard thermal profile was used: 40 cycles of 95°C for 15 s and 60°C for 1 min. The RT-PCR data were analyzed with the 7500 Software v2.0.1 (Applied Biosystems). $\Delta\Delta C_T$

Table 1. Primer pairs for qRT-PCR. RNA blot analysis and Zat12 probing presented in Fig. 5E were performed as previously described (*12*, *13*). Total RNA was stained with methylene blue dye.

Product of target gene	Forward primer	Reverse primer
Zat12	TGGGAAGAGAGTGGCTTGTTT	TAAACTGTTCTTCCAAGCTCCA
Ankyrin	TTGGTGGCCACTCACAGGAT	CCATCGAGTTCCGGGAAAAT
WRKY18	ATGCTCGTTTGCACCGTCTT	AGCATCCCCTTCAGAAGCATT
WRKY40	ACGAGCCCAACGTCAAGAAT	TCCGGTAACAGCTGCTGCTA
WRKY53	GCCATTACCCAAAAGCCAAA	GGCGTATCAGGGAACGAGAA
EF1-α	GAGCCCAAGTTTTTGAAGA	CTAACAGCGAAACGTCCCA

(threshold cycle) values for Zat12 (At5g59820), ankyrin repeat family protein (AT2G24600), WRKY18 (AT4G31800), WRKY40 (AT1G80840), and WRKY53 (AT4G23810) were calculated with the C_T of EF1-α (AT5G60390) as internal control. The primer pairs used for amplification are listed in Table 1.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/2/84/ra45/DC1

Fig. S1. A model of the ROS-mediated autopropagating long-distance rapid signal in plants.

Fig. S2. qRT-PCR analysis of the rapid systemic expression of Zat12, ankyrin repeat family protein, WRKY18, WRKY40, and WRKY53 in wild type and mutants deficient in RBOHD (*rbohD*), RBOHF (*rbohF*), or RBOHC (*rbohC*).

Fig. S3. Detection of the rapid systemic signal in response to local application of high light intensity, cold, heat, or salinity stresses.

Table S1. Pharmacological treatments used to study the rapid systemic signal with the experimental design shown in Fig. 3.

Table S2. Transcripts down-regulated in systemic leaves of Arabidopsis plants 10 min after wounding

Movie S1. Suppression of the rapid wound-induced systemic signal in *Zat12::Luc* and *Zat12::Luc/rbohD* rosette leaves.

Movie S2. Rapid wound-induced systemic signal in inflorescences of control *Zat12::Luc* and *Zat12::Luc/rbohD* plants.

Movie S3. Local wound-induced accumulation of ROS in wild-type and *rbohD* plants.

Movie S4. Delay in the progression of the rapid systemic signal by application of DPI at distal location.

Movie S5. Delay in the progression of the rapid systemic signal by application of catalase at distal location.

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37. Supported by funding from The National Science Foundation (IBN-0420033, NSF-0431327, IOS-0639964 and IOS-0743954), The Nevada Agricultural Experimental Station, NIH grant P20 RR-016464 from the INBRE Program of the National Center for Research Resources, Israel Science Foundation grant 214/08, and European Union grant FP7—MARRIE CURIE 447. We thank G. Jander, Boyce Thompson Institute for Plant Research, for the gift of aphids. Author contributions: G.M. designed, conducted and analyzed experiments. K.S. performed biostatistical analysis of microarray results. R.T. conducted experiments. D.C. and V.S. performed and analyzed metabolic analysis. G.M., M.A.T., J.L.D., and R.M. designed experiments, analyzed data, and participated in writing the manuscript.

Submitted 26 May 2009
Accepted 30 July 2009
Final Publication 18 August 2009
10.1126/scisignal.2000448
Citation: G. Miller, K. Schlauch, R. Tam, D. Cortes, M. A. Torres, V. Shulaev, J. L. Dangl, R. Mittler, The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. *Sci. Signal.* **2**, ra45 (2009).