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

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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.

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
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 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.

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.

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 LaCl₃, 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 LaCl₃, but not BAPTA or EGTA, at the wounding site (30 min before wounding) inhibited the initia-
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$_2$O$_2$ formation. In contrast, propagation of the rapid systemic signal appeared to primarily require RBOHD-dependent accumulation of H$_2$O$_2$ (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.
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$_2$O$_2$-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. 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 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).
reportedly activated by other types of ROS, including superoxide (O$_2^-$) and singlet oxygen ($^1$O$_2$).

**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...
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 micro-
array analysis shows that transcripts responsive to H$_2$O$_2$ 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$_2$O$_2$, and the propagation of the systemic
signal was suppressed by catalase that decomposes H$_2$O$_2$ (Fig. 3B). It is
therefore likely that superoxide produced by RBOHD is rapidly dismutated
to H$_2$O$_2$ either spontaneously or through an apoplastic-localized super-
oxide dismutase (22) such that the ROS that primarily mediates the rapid
systemic signal is H$_2$O$_2$. Our inability to initiate the rapid systemic signal
by applying H$_2$O$_2$ (table S1), combined with our finding that RbohD is spe-
cifically 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 prop-
agates at a rate comparable to that of the signal reported here (7, 9, 23).
Because propagation of the systemic signal described in this work re-
quires continued RBOHD-dependent ROS production along its systemic
path (Figs. 2 and 3 and fig. S1), and subsequent dismutation of super-
oxide produced by RBOHD to H$_2$O$_2$ could cause depolarization of
membrane potential (8), it is possible that activation of RBOHD in differ-
cent 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 pro-
duction at the root hair tip (18, 19, 24). In contrast to previous impli-
cations of RBOH proteins in local responses to stress, pathogens, or develop-
ment 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 RBOHD-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 re-
sponse 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 en-
hanced 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$_2$O$_2$ (11, 20). In addition, only 8 of the tran-
scripts identified in our analysis of the wound-induced rapid systemic re-
sponse (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 system-
ic 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 ap-
lications, as well as detailed studies of the heat stress signal transduc-
 tion pathway.

ROS were previously shown to function as downstream secondary mes-
 sengers 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 deriv-
atives, or both, as mediators of cell-to-cell communication over long dis-
 tances 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 sa-
linity 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 per-
formed 30 min after dipping of local leaves revealed that relative NaCl
concentrations in the systemic leaves remained ambient. The ratio of so-
dium to magnesium in local salt-treated leaves was 1.776 ± 0.232, where-
as 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.

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 at 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). ΔΔC<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.

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<thead>
<tr>
<th>Product of target gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Zat12</td>
<td>TGGGAAAGAGATGGCTGTGT</td>
<td>TAAACGTTTCTTCAAGCTCCA</td>
</tr>
<tr>
<td>Ankyrny</td>
<td>TTGGTGCCACTCACAGGAT</td>
<td>CCATCGAGTTCGGGAAAT</td>
</tr>
<tr>
<td>WRKY18</td>
<td>ATGCCTGTTTGGACCTTCT</td>
<td>AGCATCCCTTCAAGAGATT</td>
</tr>
<tr>
<td>WRKY40</td>
<td>ACGAGCCAACAGTCAAGAAT</td>
<td>TCCGGTAACAGTCTGCTA</td>
</tr>
<tr>
<td>WRKY53</td>
<td>GCCATTTCCAAGGCAACAAA</td>
<td>GGCGTATCGGGAACGAGAA</td>
</tr>
<tr>
<td>EF1-α</td>
<td>GAGCCCAAGTTTTTGAAAGA</td>
<td>CTAACGCGAAGACCTG</td>
</tr>
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(tolerance threshold) values for Zat12 (At5g59820), ankyrny repeat family protein (AT2G24600), WRKY18 (AT4G318000), WRKY40 (AT1G80840), and WRKY53 (AT4G23810) were calculated with the CT 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, ankyrny repeat family protein, WRKY18, WRKY40, and WRKY53 in wild type and mutants deficient in RBOH-D (rbohD), RBOHF (rbohF), or RBOHC (rbohC).

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 inoressences 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.

REFERENCES AND NOTES


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