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NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*

June M.Kwak[1], Izumi C.Mori, Zhen-Ming Pei[2], Nathalie Leonhardt, Miguel Angel Torres[3], Jeffery L.Dangl[3], Rachel E.Bloom, Sara Bodde, Jonathan D.G.Jones[4] and Julian I.Schroeder

Cell and Developmental Biology Section, Division of Biological Sciences and Center for Molecular Genetics, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116, [2]Biology Department, Duke University, Durham, NC 27708, [3]Department of Biology, CB 3280, 108 Coker Hall, University of North Carolina, Chapel Hill, NC 27599-3280, USA and [4]The Sainsbury Laboratory, John Innes Centre, Colney, Norwich NR4 7UH, UK

[1]Corresponding author
e-mail: jkwak@biomail.ucsd.edu

Reactive oxygen species (ROS) have been proposed to function as second messengers in abscisic acid (ABA) signaling in guard cells. However, the question whether ROS production is indeed required for ABA signal transduction *in vivo* has not yet been addressed, and the molecular mechanisms mediating ROS production during ABA signaling remain unknown. Here, we report identification of two partially redundant *Arabidopsis* guard cell-expressed NADPH oxidase catalytic subunit genes, *AtrbohD* and *AtrbohF*, in which gene disruption impairs ABA signaling. *atrbohD/F* double mutations impair ABA-induced stomatal closing, ABA promotion of ROS production, ABA-induced cytosolic Ca^{2+} increases and ABA-activation of plasma membrane Ca^{2+}-permeable channels in guard cells. Exogenous H_2O_2 rescues both Ca^{2+} channel activation and stomatal closing in *atrbohD/F*. ABA inhibition of seed germination and root elongation are impaired in *atrbohD/F*, suggesting more general roles for ROS and NADPH oxidases in ABA signaling. These data provide direct molecular genetic and cell biological evidence that ROS are rate-limiting second messengers in ABA signaling, and that the AtrbohD and AtrbohF NADPH oxidases function in guard cell ABA signal transduction.

Keywords: abscisic acid/calcium channels/guard cell/reactive oxygen species/stomata

Introduction

The plant hormone abscisic acid (ABA) controls important cellular processes including regulation of seed dormancy, seed maturation and vegetative growth during plant development (Koornneef *et al*., 1998; Finkelstein *et al*., 2002). Furthermore, ABA plays a protective role in response to abiotic stresses including drought, salinity and cold (Schroeder *et al*., 2001; Finkelstein *et al*., 2002; Zhu, 2002). ABA regulates the expression levels of a range of genes, and several transcription factors mediating ABA responses have been isolated by analyses of ABA-insensitive mutants (Giraudat *et al*., 1992; Finkelstein *et al*., 1998, 2002). However, surprisingly few signal transduction components have been identified as recessive ABA-insensitive disruption mutants that likely function upstream of transcription during early ABA signal transduction. Cloned signaling genes in which disruption mutations cause recessive ABA insensitivity include the *GPA1* Gα subunit, the *RCN1* protein phosphatase type 2A subunit and the *OST1* protein kinase (Wang *et al*., 2001; Kwak *et al*., 2002; Mustilli *et al*., 2002). In contrast, several negative regulators of ABA signaling have been identified from characterization of ABA hypersensitive mutants (Cutler *et al*., 1996; Pei *et al*., 1998; Lu and Fedoroff, 2000; Hugouvieux *et al*., 2001; Lee *et al*., 2001;Lemichez *et al*., 2001; Xiong *et al*., 2001a,b). The limited number of genetically identified positive ABA transducers is most likely due to redundancy in genes encoding ABA signaling components (Arabidopsis Genome Initiative, 2000).

Under drought conditions, ABA causes closing of stomatal pores that are formed by pairs of guard cells located in the leaf epidermis, which in turn reduces transpirational water loss from plants. The second messenger cytosolic calcium ($[Ca^{2+}]_{cyt}$) mediates ABA signaling in guard cells (McAinsh *et al*., 1990; Schroeder and Hagiwara, 1990). ABA-induced $[Ca^{2+}]_{cyt}$ increases are mediated by Ca^{2+} influx through plasma membrane Ca^{2+}-permeable (I_{Ca}) channels and Ca^{2+} release from internal stores (Wu *et al*., 1997; Leckie *et al*., 1998; Staxen *et al*., 1999; MacRobbie, 2000; Pei *et al*., 2000).

Recently, ABA-regulated hyperpolarization-activated plasma membrane I_{Ca} channels were identified in guard cells of *Vicia* and *Arabidopsis* (Hamilton *et al*., 2000; Pei *et al*., 2000). These I_{Ca} channels were demonstrated to be stimulated by reactive oxygen species (ROS) in *Arabidopsis* and *Vicia* guard cells (Pei *et al*., 2000; Köhler *et al*., 2003). Furthermore, in *Arabidopsis* guard cells, ABA was shown to enhance cellular ROS levels (Pei *et al*., 2000). In addition, the ABA-insensitive mutations *gca2* (Himmelbach *et al*., 1998) and *abi2-1* impaired ROS activation of I_{Ca} channels, providing genetic evidence that I_{Ca} channels are central components of ABA signaling (Pei *et al*., 2000; Murata *et al*., 2001). ABA is capable of increasing H_2O_2 levels in maize embryos and seedlings and in *Vicia* guard cells, further supporting roles of ROS in ABA signaling (Guan *et al*., 2000; Zhang *et al*., 2001; Jiang and Zhang, 2002). Protein phosphatase type 1/2A pharmacological inhibitors activate I_{Ca} channels in *Vicia faba* guard cells (Köhler and Blatt, 2002), and the *ost1* protein kinase mutant (Mustilli *et al*., 2002) and the dominant *abi1-1* mutant (Murata *et al*., 2001) disrupt ABA-induced ROS production, suggesting that protein

phosphorylation functions in ABA-induced ROS production and I_{Ca} channel activation.

I_{Ca} channels have been characterized in tomato suspension cells, *Arabidopsis* root apex cells and in root hair cells, indicating that I_{Ca}-like channels function in various cell types (Gelli and Blumwald, 1997; Kiegle *et al*., 2000; Véry and Davies, 2000). Interestingly, a recent study in *Fucus* rhizoid cells showed that during polar growth, tip-localized ROS production precedes activation of tip-focused Ca^{2+} influx, and that H_2O_2 activates plasma membrane Ca^{2+} channels (Coelho *et al*., 2002). Together with guard cell studies, this indicates that ROS activation of I_{Ca} channels could be of broad significance in plant biology.

Hydrogen peroxide was recently shown to inactivate ABI1 and ABI2 PP2C enzyme activity *in vitro* (Meinhard and Grill, 2001; Meinhard *et al*., 2002). The question whether ROS production is rate limiting for ABA signal transduction has not yet been addressed *in vivo*. Therefore, genetic disruption of ROS-producing enzymes would provide a direct approach to unequivocally test the proposed functions and relative importance of ROS for ABA signal transduction and ABA activation of I_{Ca} channels. However, at least nine different classes of well-characterized cellular proteins can mediate ROS production in plant cells (Lamb and Dixon, 1997; Mittler, 2002; see Discussion), and it remains unknown which enzymatic mechanisms are responsible for ABA-triggered ROS generation in guard cells at the molecular level.

In this report, we identify two NADPH oxidase catalytic subunit genes, *AtrbohD* and *AtrbohF*, which function in ABA-induced ROS production in guard cells, and demonstrate that ROS production is rate-limiting for ABA signal transduction *in vivo*.

Results

AtrbohD and AtrbohF are guard cell-expressed NADPH oxidase genes

NADPH oxidases are plasma membrane proteins (Keller *et al*., 1998) that may produce ROS in the vicinity of plasma membrane ion channels. However, 10 NADPH oxidase catalytic subunit genes exist in the *Arabidopsis* genome. Assuming redundancy in these 10 genes, 45 unique double mutant combinations could be analyzed. To test the hypothesis that NADPH oxidases may function in I_{Ca} channel regulation and ABA-induced stomatal closing, we first pursued isolation of ROS-producing NADPH oxidase genes that are expressed in guard cells. To identify guard cell-expressed NADPH oxidase catalytic subunit genes, *gp91^{phox}* homologous sequences (Torres *et al*., 1998; Bánfi *et al*., 2000) were aligned. Degenerate oligomers were used to amplify guard cell-expressed *Atrboh* genes using enriched (>95% purified) *Arabidopsis* guard cell cDNA libraries (Kwak *et al*., 2002). A guard cell-expressed *gp91^{phox}* homologous gene, *AtrbohD*, was identified from guard cell cDNA libraries. In addition, Genechip experiments were performed with RNA that was independently prepared from highly purified guard cells (>98% pure) and mesophyll cells (>96% pure), and also led to identification of *AtrbohD* and another guard cell-expressed *gp91^{phox}* homolog, *AtrbohF*. Furthermore, microarray results suggested that *AtrbohA*, *AtrbohC*, *AtrbohE*, *AtrbohG* and *AtrbohI* genes are not expressed in guard cells, irrespective of ABA treatment. *AtrbohB* was potentially expressed in guard cells at low levels but ABA treatment abrogated gene expression in guard cells. *AtrbohH* and *AtrbohJ* are not present on the Genechip we used.

Genechip microarray expression analyses showed that expression of both *AtrbohD* and *AtrbohF* mRNAs is upregulated by ABA in guard cells (Figure 1A). Without ABA treatments, these two genes were expressed in guard cells based on chip hybridization signals (Figure 1A; see Materials and methods). *AtrbohD* was highly expressed in mesophyll cells with and without ABA treatment (Figure 1A). In contrast, *AtrbohF* was not detectable in mesophyll cells before ABA treatment, and ABA treatment led to a low expression level of *AtrbohF* in mesophyll cells (Figure 1A). Transgenic plants expressing the β-glucuronidase (GUS) reporter under the control of a 1536 bp *AtrbohD* promoter fragment also showed that *AtrbohD* is expressed in guard cells (Figure 1B) and mesophyll cells (data not shown). To further examine the expression of *AtrbohD* and *AtrbohF*, we carried out RNA blot analyses with total RNA extracted from leaves treated with ABA before RNA isolation. Interestingly, *AtrbohD* expression reached the highest induction by ABA at 30 min and then decreased back to basal levels after 120 min (Figure 1C). Under the same conditions, *AtrbohF* was not detectable on RNA blots containing total leaf RNA, which correlates with the low level of *AtrbohF* expression in mesophyll cells (Figure 1A; Keller *et al*., 1998).

ABA-induced stomatal closing is partially impaired in the atrbohF single mutant and more strongly impaired in atrbohD/F double mutants

To analyze whether NADPH oxidases function in ABA signaling and more specifically whether the *AtrbohD* and *AtrbohF* genes contribute to ABA responses, we isolated mutants carrying *dSpm* insertions in each gene using PCR-based screening of a *dSpm* insertion mutant population (Tissier *et al*., 1999). The *atrbohD* and *atrbohF* alleles used here show lack of expression in these genes (Torres *et al*., 2002). Moreover, an *atrbohE* mutant carrying a *dSpm* insertion was also isolated for control experiments. Because NADPH oxidases are only one of several possible enzymatic sources of ROS production in plant cells, we subsequently performed ABA-induced stomatal closing analyses with *Atrboh* single mutants. As shown in Figure 2A, stomatal closure in response to ABA in the *atrbohD* single mutant did not show any difference from the response in wild type (WT; $P > 0.29$ at 10 μM ABA). However, ABA-induced stomatal closure in the *atrbohF* single mutant was partially reduced compared with WT (Figure 2A; $P < 0.0005$ at 10 μM ABA). *atrbohD/F* double mutant lines were obtained from crosses between homozygous *atrbohD* and *atrbohF* mutants. Interestingly, in the *atrbohD/F* double mutant, ABA-induced stomatal closing was clearly impaired compared with both WT and *atrbohF* ($P < 0.0001$ at 10 μM ABA; for *atrbohD/F* versus *atrbohF*). In controls, neither *atrbohE* nor *atrbohD/E* mutants showed any altered ABA responses in stomatal closing assays compared with WT plants (data not shown). These results show that the *atrbohD* mutation contributes to increased ABA insensitivity of the *atrbohD/F* double mutant, and

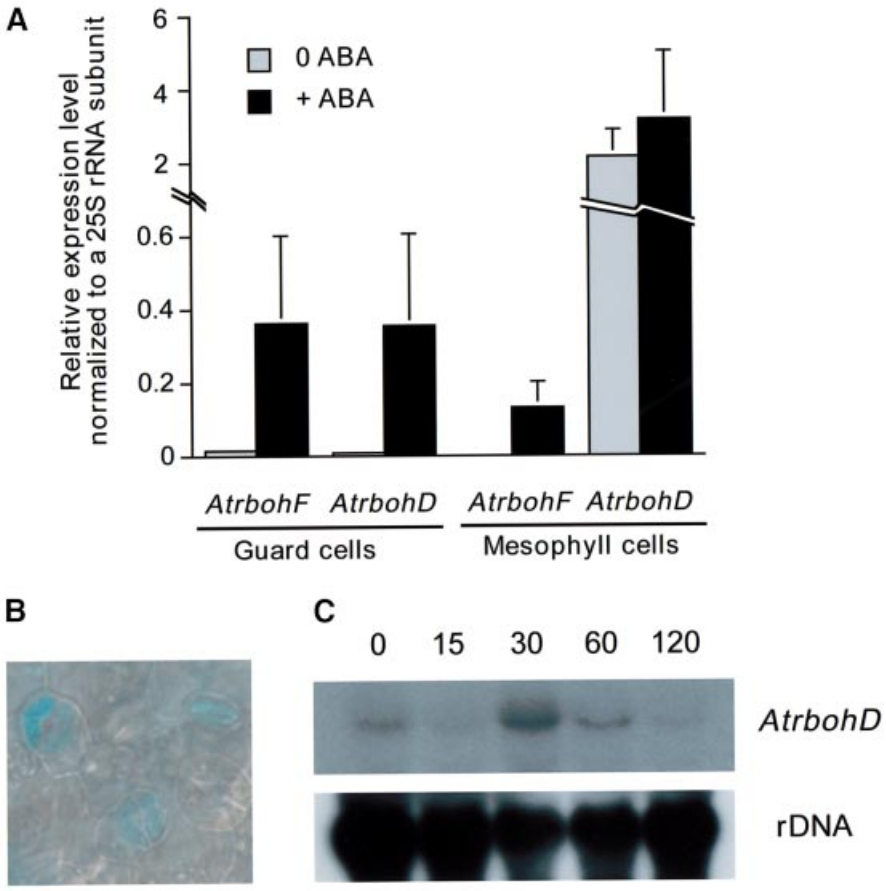

Fig. 1. *AtrbohD* and *AtrbohF* genes are upregulated by ABA in guard cells. (**A**) Genechip experiments show expression levels of *AtrbohD* and *AtrbohF* mRNA in guard cells and mesophyll cells. Gene chip experiments were performed with guard cell and mesophyll cell RNA that was extracted from WT plants sprayed with 100 μM ABA or water 4 h prior to RNA isolation. (**B**) *AtrbohD* promoter drives GUS expression in guard cells of WT plants expressing the AtrbohD::GUS fusion construct. (**C**) WT plants were sprayed with 100 μM ABA for 15, 30, 60 or 120 min prior to RNA isolation. Total RNA (25 μg) extracted from rosette leaves was separated in a denaturing gel and then transferred onto a nylon membrane. The blot was hybridized with ^{32}P-labeled *AtrbohD* or *AtrbohF* cDNA. The same blot was probed with ^{32}P-labeled 18S rDNA to show relative amounts of RNA samples.

suggest that AtrbohD and AtrbohF show partial overlap in their functions in the guard cell ABA response.

ABA-induced ROS production is impaired in atrbohD/F double mutants

We carried out ABA-induced ROS production measurements using the fluorescent dye 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) in guard cells of *atrbohD/F* double mutants in order to determine whether NADPH oxidases are the major enzymatic source of ABA-induced ROS generation in guard cells. As previously reported, ABA treatment produced increases in fluorescence in WT guard cells (Figure 2B; $n = 41$ guard cells, $P < 0.005$). In contrast, 50 μM ABA treatment failed to induce increases in ROS levels in *atrbohD/F* guard cells (Figure 2B), suggesting that NADPH oxidases mediate ABA-induced ROS generation in guard cells and that AtrbohD and AtrbohF are the major catalytic subunits in this response.

Exogenous ROS rescue stomatal closing in atrbohD/F double mutants

To further test whether lack of ABA-induced ROS (Figure 2B) can be linked to ABA insensitivity of the *atrbohD/F* double mutant, we examined whether exogenously applied ROS can induce stomatal closure in *atrboh* mutants. Stomatal apertures were measured at two different concentrations of exogenously applied H_2O_2. Figure 3 shows that ROS-induced stomatal closing in the *atrbohD/F* double mutant exhibited no significant difference to WT ($P > 0.28$ at 100 μM H_2O_2). Furthermore, in *atrbohD* and *atrbohF* single mutants, ROS-induced stomatal closure showed no difference to WT (data not shown). These results indicate that H_2O_2 can rescue WT stomatal responses in the *atrbohD/F* double mutant.

ABA-induced cytosolic calcium increases are significantly reduced in atrbohD/F

To analyze the relative contributions of NADPH oxidases and ABA-induced ROS production to $[Ca^{2+}]_{cyt}$ elevations, we carried out calcium imaging analysis on guard cells in intact epidermal strips using WT and *atrbohD/F* plants expressing the cytosolic Ca^{2+} reporter yellow cameleon 2.1 (Miyawaki *et al*., 1997; Allen *et al*., 1999). Two independent homozygous WT and *atrbohD/F* plant lines expressing yellow cameleon 2.1 were used for ratiometric calcium imaging experiments.

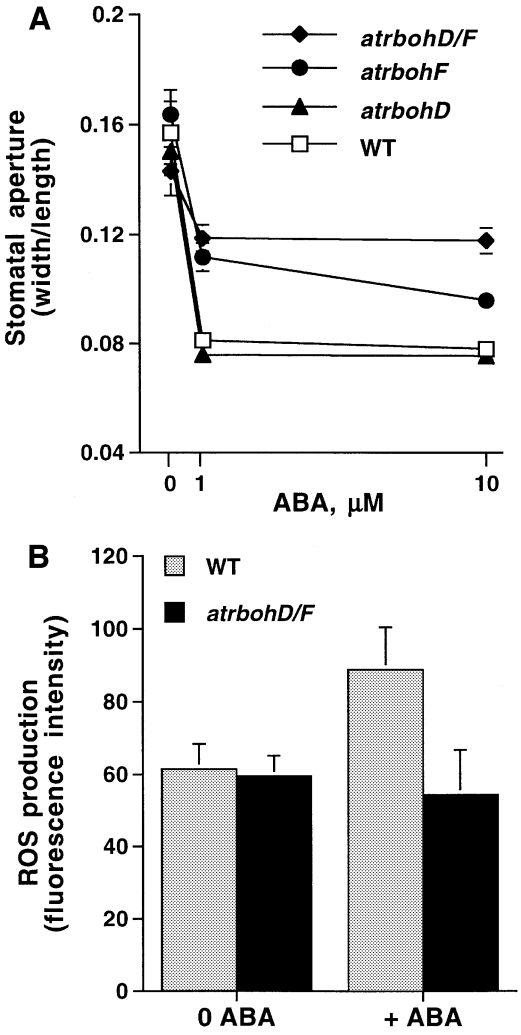

Fig. 2. ABA-induced stomatal closing and ABA-induced ROS generation are disrupted in the *atrbohD/F* double mutant. (**A**) Stomatal aperture measurements show that ABA-induced stomatal closing is partially reduced in *atrbohF* and *atrbohD/F* double mutants. (**B**) ABA (50 μM) induces ROS increases in guard cells of WT (three experiments; $n = 44$ guard cells before ABA treatment, $n = 41$ guard cells after ABA treatment). ABA failed to induce an increase in ROS levels in guard cells of *atrbohD/F* double mutant (three experiments; $n = 59$ guard cells before ABA treatment, $n = 39$ guard cells after ABA treatment). In (A), $n = 6$ experiments (120 stomatal apertures per data point) for WT; $n =$ four experiments (80 stomatal apertures) for *atrbohD/F*; $n = 3$ experiments each (60 stomatal apertures each) for *atrbohD* and for *atrbohF*. Starting stomatal apertures were: 3.46 ± 0.63 μm (WT), 3.88 ± 0.37 μm (*atrbohD*), 3.36 ± 0.48 μm (*atrbohF*), 3.16 ± 0.44 μm (*atrbohD/F*). Error bars represent ± SEM relative to number of experiments. Error bars are smaller than symbols when not visible.

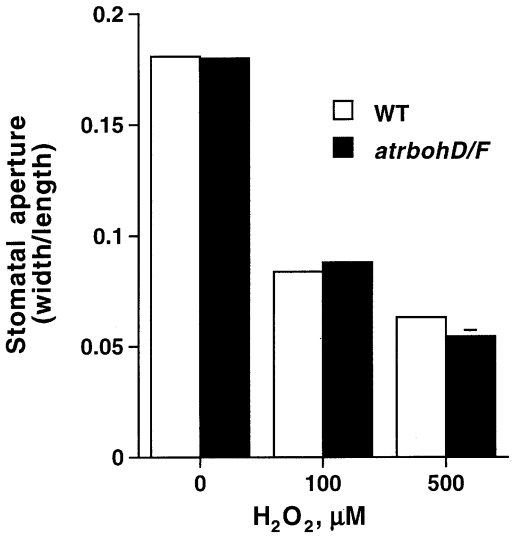

Fig. 3. Exogenous H_2O_2 rescues stomatal closing in *atrbohD/F* double mutant. H_2O_2 induces stomatal closing both in *atrbohD/F* and WT. Stomatal apertures were measured 3 h after addition of 100 or 500 μM H_2O_2. $n = 3$ experiments (60 stomatal apertures) for WT; $n = 3$ experiments (60 stomatal apertures) for *atrbohD/F*. Starting stomatal apertures: 3.81 ± 0.20 μm (WT), 3.40 ± 0.04 μm (*atrbohD/F*). Error bars represent ± SEM relative to three independent experiments. Error bars are smaller than symbols when not visible.

ABA was applied to guard cells that showed stable Ca^{2+} baselines to analyze ABA-dependent $[Ca^{2+}]_{cyt}$ changes. When treated with 5 μM ABA, 85.7% of WT guard cells showed ABA-induced $[Ca^{2+}]_{cyt}$ elevations during recordings (Figure 4A, top trace and C). Among them, 54.3% of WT guard cells showed three or more $[Ca^{2+}]_{cyt}$ transients ($n = 19$ of 35 cells; Figure 4C) and 31.4% showed one or two $[Ca^{2+}]_{cyt}$ transients ($n = 11$ of 35 cells; Figure 4C) within the recording period. In contrast, in *atrbohD/F* only 45.5% of guard cells exhibited ABA responsiveness in $[Ca^{2+}]_{cyt}$ elevations (Figure 4B, top trace and C). Among them, 27.3% of *atrbohD/F* guard cells showed three or more $[Ca^{2+}]_{cyt}$ transients ($n = 9$ of 33 cells; Figure 4C) and 18.2% showed a single or two $[Ca^{2+}]_{cyt}$ transients ($n = 6$ of 33 cells; Figure 4C). Moreover, 54.5% of *atrbohD/F* guard cells showed no measurable ABA-induced $[Ca^{2+}]_{cyt}$ elevations ($n = 18$ of 33 cells; Figure 4B, bottom trace and C), whereas only 14.3% of WT guard cells showed no response to 5 μM ABA ($n = 5$ of 35 cells; Figure 4A, bottom trace and C). Statistical analyses indicate that the number of cells showing ABA-induced $[Ca^{2+}]_{cyt}$ increases was significantly reduced in *atrbohD/F* guard cells ($\chi^2 = 12.16$, $P < 0.0005$), suggesting that the *atrbohD/F* mutation partially decreases the responsiveness of guard cells with respect to ABA-induced cytosolic calcium elevations.

atrbohD/F mutation abolishes ABA but not H_2O_2 activation of plasma membrane I_{Ca} channels

To determine whether the AtrbohD and AtrbohF NADPH oxidase subunits function in ABA activation of plasma membrane I_{Ca} channels, we tested ABA activation of I_{Ca} channels in *atrbohD/F* by patch–clamping guard cells. ABA clearly activated I_{Ca} channels in 10 of 18 WT guard cells (Figure 5A and B). The average response of all 18 cells, including non-responsive cells, showed a significant ABA activation of I_{Ca} channels in WT (Figure 5B; $P < 0.05$ at –196 mV). In contrast, as shown in Figure 5C and D, ABA activation of I_{Ca} channels was completely abolished in *atrbohD/F* guard cells ($n = 10$ of 10 cells, $P > 0.99$ at –196 mV).

We further tested whether H_2O_2, a rapid turnover product of superoxide produced by NADPH oxidases, can bypass the *atrbohD/F* double mutation and activate I_{Ca} channels in *atrbohD/F* guard cells. ROS activation of I_{Ca} channels occurred both in WT (Figure 6A and B; $n = 10$) and *atrbohD/F* guard cells (Figure 6C and D; $n = 7$). Impairment in ABA- but not in ROS-activation of I_{Ca}

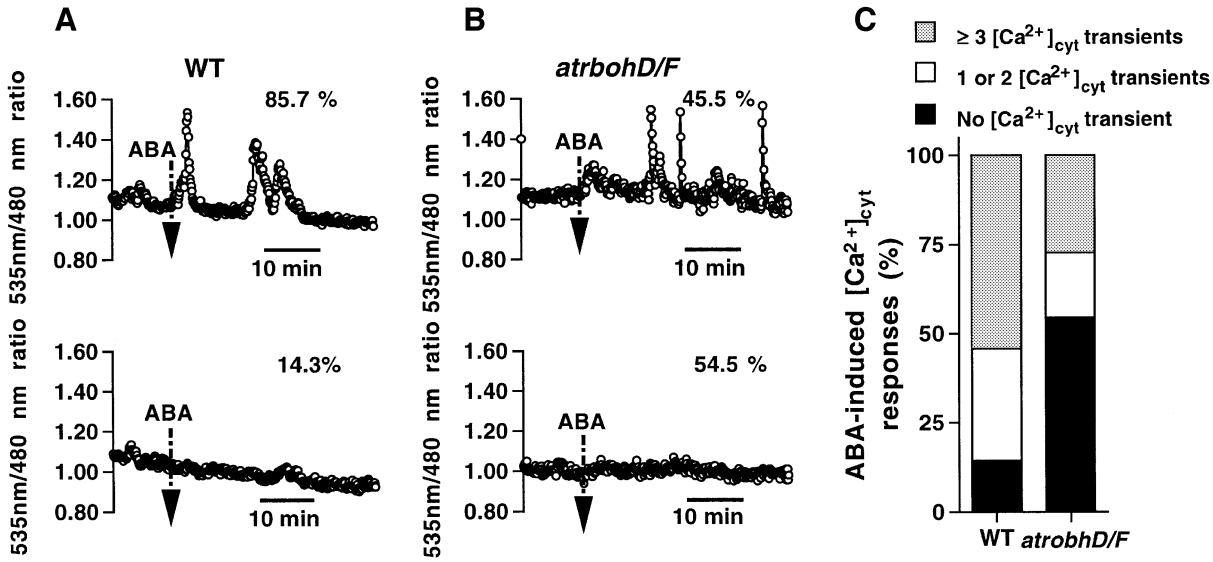

Fig. 4. *atrbohD/F* mutation impairs ABA-induced $[Ca^{2+}]_{cyt}$ elevations in guard cells. (**A**) Fluorescence emission ratio (535/480 nm) shows responsiveness of ABA-induced $[Ca^{2+}]_{cyt}$ elevations at 5 μM ABA in WT guard cells. Thirty of 35 cells (85.7%) showed ABA-mediated $[Ca^{2+}]_{cyt}$ elevations whereas five of 35 cells (14.3%) showed no response. (**B**) Fluorescence emission ratio (535/480 nm) shows examples of ABA-induced $[Ca^{2+}]_{cyt}$ elevations at 5 μM ABA in *atrbohD/F* guard cells (15 of 33 cells = 45.5%). Traces showing ABA-induced $[Ca^{2+}]_{cyt}$ transients are shown in the top panels of (A) and (B), and those with no clear ABA-induced $[Ca^{2+}]_{cyt}$ elevations are shown in the bottom panels of (A) and (B). Vertical arrow lines indicate when guard cells were challenged with 5 μM ABA. $[Ca^{2+}]_{cyt}$ transients were counted when changes in $[Ca^{2+}]_{cyt}$ ratios were ⩾0.1 U. (**C**) Stack column representation of number of ABA-induced $[Ca^{2+}]_{cyt}$ transients recorded in WT (n = 35) and *atrbohD/F* (n = 33) guard cells at 5 μM ABA.

channels (Figures 5 and 6) is consistent with the stomatal phenotype of *atrbohD/F* that showed ABA-insensitive but H_2O_2-responsive stomatal movements (Figures 2 and 3). These data suggest that the AtrbohD and AtrbohF catalytic subunits of NADPH oxidases function in the signaling pathway that mediates ABA activation of I_{Ca} channels, and provide direct genetic evidence that ROS function as rate-limiting second messengers in ABA signaling.

To further examine whether ABA-activated hyperpolarization-induced currents in WT were carried by the same channels as ROS-activated currents, we analyzed the cation selectivity of both currents. As reported previously, both ABA- and ROS-activated currents were Ba^{2+} permeable (Figures 5 and 6). In further experiments, we analyzed the Na^+ conductance through both ABA- and ROS-regulated currents with 200 mM extracellular Na^+. Both ABA (Figure 7A; n = 6 cells) and H_2O_2 (n = 7 cells) activated hyperpolarization-induced currents (Figure 7B). In the absence of ABA and ROS, a constitutive background inward Na^+ current was also observed in guard cells, indicating the presence of an additional type of plasma membrane Na^+ current in *Arabidopsis* guard cells (Figure 7A, no ABA). These data show that I_{Ca} currents in guard cells are permeable to the monovalent cation Na^+, as well as the divalent cations Ca^{2+}, Mg^{2+}, Cd^{2+} and Ba^{2+} (Pei *et al*., 2000; Perfus-Barbeoch *et al*., 2002).

ABA inhibition of root growth and seed germination is impaired in atrbohD/F

To test whether disruption mutations in *atrboh* genes affect other ABA responses, we examined ABA inhibition of root growth and seed germination. Root elongation was measured over a 5 day period (7- to 12-day-old seedlings) in WT, *atrbohD*, *atrbohF* and *atrbohD/F* grown on medium containing 0 and 10 μM ABA. Root lengths of *atrbohF* and *atrbohD/F* mutant seedlings grown on Murashige and Skoog (MS) medium containing no ABA were smaller than those of WT and *atrbohD* seedlings (Figure 8; $P < 0.02$). Roots of *atrbohF* and *atrbohD/F* were insensitive to ABA compared with WT (Figure 8; $P < 0.006$, *atrbohF*; $P < 0.001$, *atrbohD/F*), whereas the roots of *atrbohD* showed a similar ABA sensitivity compared with WT ($P > 0.93$), suggesting that NADPH oxidases may contribute to ABA regulation of root elongation. Despite differences in root lengths of seedlings, root elongation rates during 5 days (from 7- to 12-day-old seedlings) on ABA-free medium did not show significant differences between WT, *atrbohF* and *atrbohD/F* mutants (190% growth for WT, 201% growth for *atrbohF*, 172% growth for *atrbohD/F*), further indicating that mutants may be less ABA sensitive.

To determine whether NADPH oxidase mutation affects ABA regulation of seed germination in *Arabidopsis*, we examined seed germination of *atrbohD/F* double mutants. ABA inhibition of seed germination shows a partial ABA insensitivity in the *atrbohD/F* mutant compared with WT (Figure 8B; $P < 0.02$ at 0.5 μM ABA, $P < 0.0005$ at 1 μM ABA). These results show that NADPH oxidases affect ABA inhibition of seed germination in *Arabidopsis* as well as ABA inhibition of root elongation.

Discussion

Here, we directly demonstrate through molecular genetic, cell biological and biophysical analyses that ROS production is required for ABA signal transduction in guard cells, and that AtrbohD and AtrbohF are major NADPH oxidase catalytic subunits that mediate ABA-induced ROS production, ABA activation of I_{Ca} channels and ABA-induced stomatal closure. Furthermore, H_2O_2 activates I_{Ca}

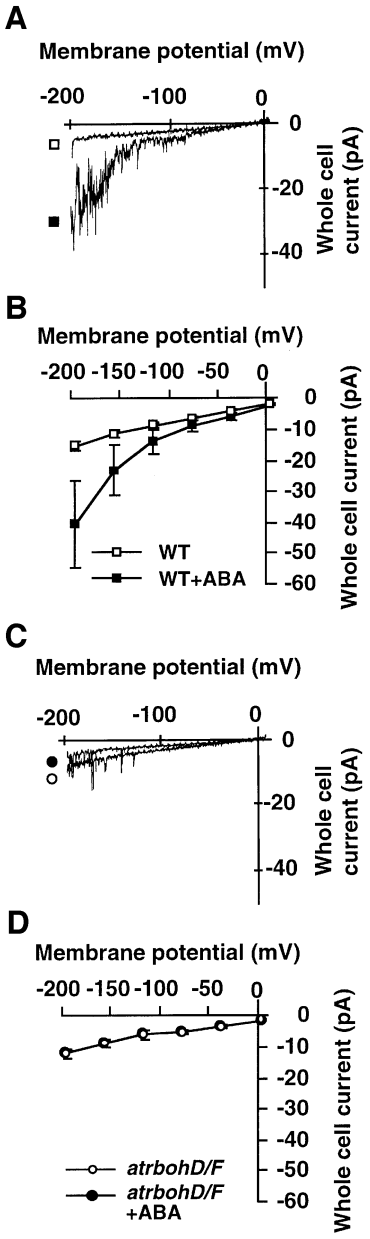

Fig. 5. ABA-activation of I_{Ca} channels is abolished in *atrbohD/F* guard cells. (A and B) ABA (50 μM) activated I_{Ca} channel currents in WT guard cells. Current traces before and after ABA activation of I_{Ca} channels are shown in a representative cell in (**A**) and average current–voltage curves of 18 cells are shown in (**B**). (C and D) ABA failed to activate I_{Ca} channel currents in *atrbohD/F* guard cells. A response in a representative cell is shown in (**C**) and average current–voltage curves (n = 10 cells) are shown in (**D**). Symbols of the *atrbohD/F* guard cells perfused with ABA overlap with *atrbohD/F* control guard cell symbols when not visible.

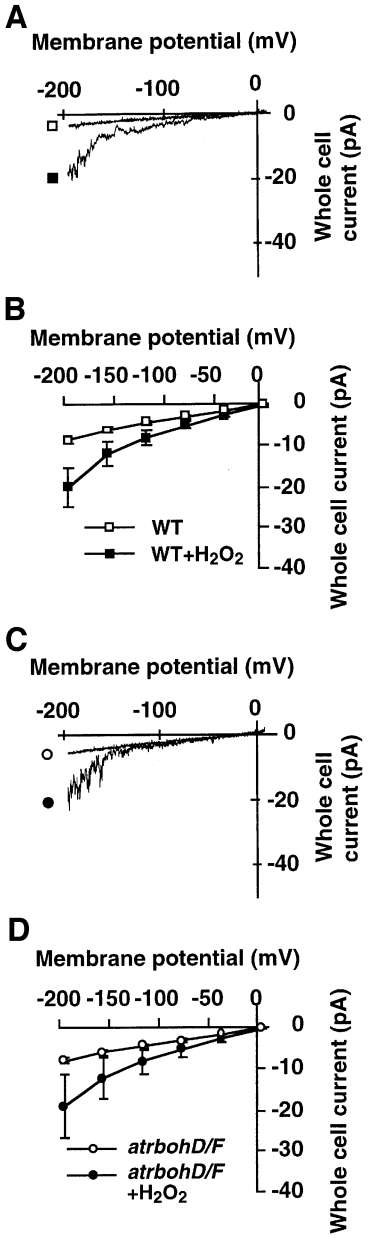

Fig. 6. ROS activate plasma membrane I_{Ca} channels both in *atrbohD/F* and WT guard cells. (**A** and **C**) H_2O_2 activation of I_{Ca} channels in a representative cell of WT and *atrbohD/F*, respectively. (**B** and **D**) Average current–voltage curves (WT, n = 10; *atrbohD/F*, n = 7). Error bars represent SEM. Error bars are smaller than symbols when not visible.

channels and rescues stomatal closing in *atrbohD/F* double mutants, indicating that AtrbohD and AtrbohF NADPH oxidase catalytic subunits act upstream of plasma membrane I_{Ca} channels in this recently identified ABA signaling branch. These results unequivocally demonstrate that ROS function as rate-limiting second messengers and that NADPH oxidases function as a central positive transducer in ABA signal transduction in guard cells.

Redundancy in ABA signaling and cell-type specific function

Given that many gene families in the *Arabidopsis* genome have large numbers of homologs relative to other sequenced genomes (Arabidopsis Genome Initiative, 2000), the relatively low number of recessive ABA-insensitive mutants (see Introduction) is most likely due to redundancy in genes encoding ABA transducers. This explains why conventional genetic screens have not identified many recessive ABA-insensitive mutants. Ten *Atrboh* genes are present in the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000). Therefore, deter-

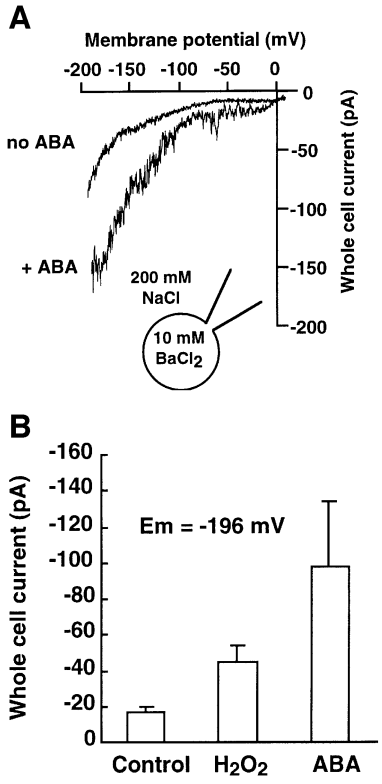

Fig. 7. H_2O_2- and ABA-activated I_{Ca} currents are Na^+ permeable in guard cells. (**A**) Whole-cell current recordings without (no ABA) and with 50 μM ABA (+ ABA) in the same guard cell bathed in 200 mM NaCl. (**B**) Average Na^+ currents at –196 mV show that both ABA and H_2O_2 activated inward Na^+ currents in *Arabidopsis* guard cells (H_2O_2, $n = 7$ cells; ABA, $n = 6$ cells).

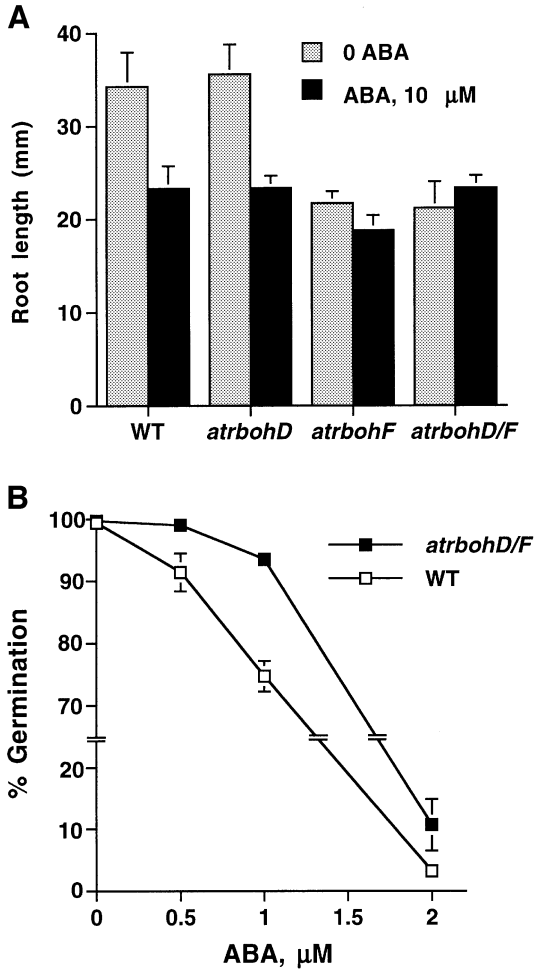

Fig. 8. Root growth and seed germination in *atrbohD/F* show reduced ABA sensitivity. (**A**) Seven-day-old WT, *atrbohD*, *atrbohF* and *atrbohD/F* seedlings were placed on MS medium supplemented with 0 and 10 μM ABA. Root elongation was measured after 5 days. Each data point represents the mean value of 12–15 seedlings. Error bars represent ±SEM. (**B**) The *atrbohD/F* mutant shows partial reduction in ABA sensitivity of seed germination inhibition. Error bars are smaller than symbols when not visible. Error bars represent ±SE of $n = 3$ independent experiments; >360 seeds at each data point.

mination of which two *Atrboh* genes are highly expressed in a single cell type (guard cells) was pivotal for functional analyses (Figures 1 and 2). By pursuing single-cell functional genomics, two of the NADPH oxidase catalytic subunit genes were identified as positive ABA signal transducers.

ROS-mediated Ca^{2+} channel activation may be of general importance in plant signal transduction

ABA inhibition of root elongation is reduced in *atrbohD/F* and *atrbohF* (Figure 8), suggesting that ROS production by the NADPH oxidase AtrbohF may be of broader significance for ABA signal transduction. In addition, ABA inhibition of seed germination is reduced in *atrbohD/F* double mutants (Figure 8B). A recent study in *Fucus* rhizoid cells showed that tip-localized ROS production and plasma membrane I_{Ca} channel activation precede polar growth (Coelho *et al.*, 2002). Additionally, ROS were shown to activate plasma membrane Ca^{2+} channels in plant root cells (Demidchik *et al.*, 2003). Therefore, it appears that the ROS-mediated plasma membrane Ca^{2+} channel activation pathway may be of more general importance in plant signal transduction and development. It is conceivable that different NADPH oxidase genes or combinations may contribute to this signaling branch in distinct responses and cell types.

NADPH oxidase-mediated ROS production, a rate-limiting ABA signal transduction branch

ROS were shown to induce cytosolic calcium elevations in tobacco seedlings and guard cells (Price *et al.*, 1994; McAinsh *et al.*, 1996; Pei *et al.*, 2000). A recent study has led to the suggestion that ROS may not be a critical second messenger for ABA signaling in guard cells (Köhler *et al.*, 2003). However, this study focuses on exogenous H_2O_2 regulation of K^+ channels including the Ca^{2+}-independent outward K^+ channels, which show differences compared with ABA regulation. Note that previous studies have shown that exogenous H_2O_2 application causes partial stomatal closing responses compared with ABA (Pei *et al.*, 2000), and that responses to exogenous H_2O_2 or ozone application are not equivalent to ABA responses (Torsethaugen *et al.*, 1999; Allen *et al.*, 2000; Pei *et al.*, 2000). Differences between (i) biological localized enzymatic ROS production and exogenous global H_2O_2 application, (ii) ion channel micro-environments,

(iii) temporal ROS production and (iv) a model in which parallel ROS-independent ABA signaling mechanisms were proposed (see figure 5F in Pei *et al*., 2000) may contribute to differences in exogenous H_2O_2 application and multiple roles for ROS in biological signaling.

Our results show that ABA-induced cytosolic Ca^{2+} elevations, ABA activation of I_{Ca} channels as well as ABA-induced stomatal closing are impaired in *atrbohD/F* mutants (Figures 2A, 4 and 5). However, 45% of *atrbohD/F* guard cells still show ABA-induced cytosolic Ca^{2+} elevations (Figure 4), suggesting that other ROS-independent parallel pathways as proposed by Pei *et al*. (2000) are functional in *atrbohD/F* and contribute to ABA-induced cytosolic Ca^{2+} increases. This result supports the current model for guard cells in which ABA signaling is mediated by parallel Ca^{2+} influx and Ca^{2+} release pathways (MacRobbie, 2000), and correlates with the partial ABA-induced stomatal closing response observed in *atrbohD/F* double mutants (Figure 2A). Note that our data do not exclude the possibility that one of the parallel ABA signaling branches may be able to partially activate I_{Ca} channels via a parallel ROS-independent branch in intact *Arabidopsis* guard cells. Ion channels often function as integrating targets of multiple signaling branches (Hille, 2001). Despite these possibilities, our findings directly demonstrate that ABA-induced ROS are rate limiting for functional ABA signaling *in vivo*, and illustrate the relative contribution of AtrbohD and AtrbohF to ROS-mediated ABA signaling.

Although the *atrbohD/F* double mutation impairs ABA-induced ROS production, there is a background level of cellular ROS in guard cells before ABA treatment, which suggests the existence of other cellular mechanisms generating ROS in guard cells (Figure 2B). Multiple enzymes and reactions are known to cause ROS production, including the mitochondrial respiration electron transport chain, chloroplast photosynthetic electron transport, oxalate oxidases, glycolate oxidases, xanthine oxidases, fatty acid β-oxidation, amine oxidases and cell wall-bound peroxidases (Mittler, 2002). On the other hand, ROS scavenging enzymes including catalases, superoxide dismutases, glutathione peroxidases, thioredoxin peroxidases and ascorbate peroxidases remove cellular ROS and thus contribute to ROS homeostasis in plant cells (Mittler, 2002) and may also be ABA regulated. It is interesting that in light of these diverse activities, extracellular ROS production by AtrbohD and AtrbohF is a requirement for ABA-induced ROS production and ABA activation of plasma membrane Ca^{2+} channels. Our data do not exclude additional contributions of further *Atrboh* or ROS producing and scavenging genes to ABA responses.

NADPH oxidase regulation

NADPH oxidases in mammals are composed of two plasma membrane proteins, $gp91^{phox}$ and $p22^{phox}$, which form a heterodimeric flavocytochrome$_{-558}$ (Bokoch, 1994). During activation, two cytosolic proteins, p47 and p67, and the small G protein Rac translocate to the plasma membrane, resulting in the formation of the active NADPH oxidase complex in neutrophils (Bokoch, 1994; Diekmann *et al*., 1994). In plants, AtrbohF was shown to be a plasma membrane protein (Keller *et al*., 1998), and a plasma membrane NADPH oxidase was shown to produce superoxide (Sagi and Fluhr, 2001). Therefore, plant NADPH oxidases may produce ROS in the vicinity of plasma membrane I_{Ca} channels, thus regulating I_{Ca} channels. Moreover, plant NADPH oxidases do not require additional cytosolic factors for enzymatic activity (Sagi and Fluhr, 2001), suggesting that plant NADPH oxidases differ from the mammalian NADPH oxidases in their functional composition. This is further supported by the finding that no p47 and p67 homologs of the mammalian NADPH oxidase are found in the *Arabidopsis* genome (Torres *et al*., 2002). It will be of interest to investigate AtrbohD- and AtrbohF-interacting proteins *in vivo*.

Dual functions of NADPH oxidases in ABA signal transduction and in plant defense response

ROS are known to control programmed cell death and pathogen defense in plants (Lamb and Dixon, 1997; Mittler, 2002). Cell wall-bound peroxidases and plasma membrane NADPH oxidases have been proposed to be the main ROS sources in plant defense responses (Lamb and Dixon, 1997). A recent study showed that the AtrbohD and AtrbohF NADPH oxidase catalytic subunits contribute to pathogen-induced ROS production in *Arabidopsis*. ROS production induced by bacterial and fungal infection was reduced in *atrbohD/F* double mutants (Torres *et al*., 2002). However, unexpectedly, despite reduction in ROS generation, enhanced cell death was observed in *atrbohD/F* mutants in response to oomycete pathogen, whereas bacteria-induced cell death was reduced in *atrbohD/F* mutants (Torres *et al*., 2002). These data indicate that other genes may mediate fungal pathogen-induced cell death. Together with this study, our results indicate that these NADPH oxidases have a dual function in mediating ABA signal transduction and in contributing to pathogen-associated ROS production.

Materials and methods

Identification of Atrboh genes in guard cells

To identify guard cell-expressed *Atrboh* genes, we carried out degenerate oligomer-based RT–PCR using enriched *Arabidopsis* guard cell cDNA libraries (Kwak *et al*., 2002). Six sequences of *Atrboh* genes, which were available at the outset of this project, were aligned to design degenerate oligomers, and then two highly conserved regions were selected from the protein sequences: VCRNTITW (for sense primer) and GLGIGATP (for antisense primer). The degenerate oligomers from these sequences were 5′-GTITGYMGIAAYACIATHACITGG-3′ (sense primer) and 5′-GGIGTIGCICCDATICCIARICC-3′ (antisense primer). Total RNA was extracted from guard cell-enriched epidermal strips as described previously (Kwak *et al*., 2002), and then 2 μg of total RNA were converted to cDNA using the First-strand cDNA Synthesis Kit (Amersham-Pharmacia Biotech). PCR reactions were performed as described previously (Kwak *et al*., 2002). PCR products were purified, cloned into the pGEM-T Easy vector (Promega) and subjected to sequencing reactions to obtain the insert sequence as described previously (Kwak *et al*., 1997). *Arabidopsis* Genome Initiative numbers for *Atrboh* genes are *AtrbohA* (At5g07390), *AtrbohB* (At1g09090), *AtrbohC* (At5g51060), *AtrbohD* (At5g47910), *AtrbohE* (At1g19230), *AtrbohF* (At1g64060), *AtrbohG* (At4g25090), *AtrbohH* (At5g60010), *AtrbohI* (At4g11230), and *AtrbohJ* (At3g45810).

Genechip experiments and GUS activity analysis

Total RNA (10 μg) from two independently extracted guard cell and two mesophyll cell protoplast preparations (Kwak *et al*., 2002) was pooled and used for experiments with DNA chip (Affymetrix), which represents ~8000 *Arabidopsis* genes. Probe labeling and hybridization of the chips were performed at the UC San Diego and UC Irvine Gene Chip Cores.

Data normalization and analysis were carried out using Affymetrix GeneChip Suite 4.0 software. After performing standard normalization of scanned chip images relative to whole-chip intensities (Zhu *et al*., 2001), expression levels of *AtrbohD* and *AtrbohF* in guard cells and mesophyll cells were further normalized relative to the 25S rRNA expression levels in each cell type. GUS activity was assayed in epidermal strips of 7-day-old seedlings as described previously (Hugouvieux *et al*., 2001).

Isolation of atrbohD and atrbohF null mutants

Homozygous knockout plants carrying a single *dSpm* transposon insertion in *AtrbohD* and *AtrbohF* genes were isolated in which no full-length transcript was detected, as described previously (Torres *et al*., 2002). *atrbohD/F* double mutant plants were obtained by crossing *atrbohF* and *atrbohD* single mutants and confirmed by PCR.

Stomatal aperture measurements

Stomatal apertures were measured as described previously (Kwak *et al*., 2002) in 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES–Tris pH 6.15. Stomatal apertures were measured 3 h after ABA (1 and 10 μM) or H_2O_2 (100 and 500 μM) was added. Student's *t*-test (two-tailed distribution, two-sample assuming equal variance) was used to determine the statistical significance of the data.

RNA isolation and RNA blot analyses

Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies) from rosette leaves of 5-week-old WT plants sprayed with 100 μM ABA 0, 15, 30, 60 or 120 min prior to RNA extraction. Total RNA samples were separated as described previously (Kwak *et al*., 2002). The blots were hybridized with ^{32}P-labeled *AtrbohD, AtrbohF* cDNA or 18S rDNA and washed as described previously (Kwak *et al*., 2001).

ROS production measurements in guard cells

H_2DCF-DA was used to analyze ABA-induced ROS production in guard cells as described previously (Murata *et al*., 2001), with a slight modification. Epidermal strips were prepared from 4- to 5-week-old WT and *atrbohD/F* plants using a blender. The epidermal strips were incubated in 5 mM KCl, 10 mM MES–Tris pH 6.15 in the light with a fluence rate of 95 μE m^{-2} s^{-1} for 2 h. Fifty micromolar H_2DCF-DA was added to the solution containing the epidermal strips for 30 min on an orbital shaker (70 r.p.m.). Then 50 μM ABA or 0.1% ethanol (control) was added to the incubation medium after dye loading. Guard cell images were taken using Adobe Photoshop 5.5 (Mountain View, CA) during short 2 s UV exposures (one UV exposure per sample) under a fluorescence microscope equipped with a digital camera (Murata *et al*., 2001). Fluorescence emission of guard cells was analyzed using Adobe Photoshop 5.5.

Calcium imaging analyses

To perform calcium imaging analyses, WT and *atrbohD/F* plants were transformed with the pH-insensitive yellow cameleon construct p35SYC2.1 (Allen *et al*., 1999). Homozygous WT (two independent lines) and *atrbohD/F* (two independent lines) expressing yellow cameleon were used to measure $[Ca^{2+}]_{cyt}$ changes as described previously (Allen *et al*., 1999; Hugouvieux *et al*., 2001; Kwak *et al*., 2002). Guard cells that showed spontaneous $[Ca^{2+}]_{cyt}$ transients (Allen *et al*., 1999; Staxen *et al*., 1999) were excluded from analyses. $[Ca^{2+}]_{cyt}$ transients were counted when the change in $[Ca^{2+}]_{cyt}$ ratios was ⩾0.1 U above the baseline. Background fluorescence was measured in guard cell-less epidermal domains and was subtracted from the epidermal field prior to ABA application (Allen *et al*., 1999; Hugouvieux *et al*., 2001; Kwak *et al*., 2002). Statistical analyses showed that the average of raw data fluorescence baseline ratios prior to ABA application was not significantly different in WT (1.13 ± 0.19, $n = 35$) and *atrbohD/F* double mutant (1.05 ± 0.20, $n = 33$; $P > 0.086$). The fluorescence baseline of the top trace in Figure 4B was reduced by 0.05 U subtraction. Cells showing stable $[Ca^{2+}]_{cyt}$ ratios during the first 10 min of recordings (continuous bath perfusion) were perfused with the stomatal opening solution containing 5 μM ABA. The recording continued for 50 min. The *P* value was calculated from the χ^2-test for a 2 × 2 contingency table containing ABA-responsive cells versus non-responsive cells of WT and *atrbohD/F* obtained from calcium imaging analyses.

Patch–clamp analyses

Guard cell protoplasts were enzymatically isolated from *Arabidopsis* leaf epidermal strips of 4- to 6-week-old WT and *atrbohD/F* double mutant plants as described previously (Murata *et al*., 2001). Whole-cell patch–clamp recordings of guard cells were performed as described previously (Murata *et al*., 2001). The pipette solution was composed of 10 mM $BaCl_2$, 0.1 mM DTT, 4 mM EGTA and 10 mM HEPES–Tris pH 7.1. The bath solution contained 100 mM $BaCl_2$ and 10 mM MES–Tris pH 5.6. To measure Na^+ currents, 100 mM $BaCl_2$ in the bath solution was replaced with 200 mM NaCl. When ABA activation of plasma membrane I_{Ca} channels was measured, 1 mM NADPH was supplemented in the pipette solution, whereas 0.1 mM DTT was additionally added in the bath solution when H_2O_2 activation of plasma membrane I_{Ca} channels was measured. Osmolalities were adjusted to 500 mmol/kg (pipette solution) and 485 mmol/kg (bath solution). Seal resistance was >10 GΩ and liquid junction potentials were corrected (Ward and Schroeder, 1994). Whole-cell leak currents were not subtracted. The standard voltage protocol ramped from +14 to –196 mV (ramp speed 210 mV/1.5 s). ABA and H_2O_2 were applied by continuous bath perfusion during whole-cell recordings.

Root growth and seed germination measurements

To measure root growth in the presence of ABA, seeds of WT, *atrbohD*, *atrbohF* and *atrbohD/F* were plated on one-fourth-strength MS medium. Plates containing seeds were stratified at 4°C for 4 days and then transferred to a growth chamber (22°C under a 16 h light/8 h dark regime) in a vertical orientation. Seedlings grown for 7 days were transferred to plates containing one-fourth-strength MS medium with 0 or 10 μM ABA. Seedlings were further incubated in the growth chamber for another 5 days and then root length was measured. To measure seed germination rate, seeds of WT and *atrbohD/F* were plated on one-fourth-strength MS medium containing 0, 0.5, 1 or 2 μM ABA. Plates containing seeds were stratified at 4°C for 4 days and then transferred to a growth chamber (22°C under a 16 h light/8 h dark regime). Seed germination rates were scored after 5 days in the growth chamber.

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Foreman *et al.* (2003) recently reported a requirement for an NADPH oxidase in root hair growth (*Nature*, **422**, 442–446).