RESEARCH PAPER

The Arabidopsis NADPH oxidases RbohD and RbohF display differential expression patterns and contributions during plant immunity

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Received 26 October 2015; Accepted 10 December 2015

Editor: Nick Smirnoff, University of Exeter

Abstract

Plant NADPH oxidases, also known as respiratory burst oxidase homologues (RBOHs), produce reactive oxygen species (ROS) that perform a wide range of functions. RbohD and RbohF, two of the 10 Rboh genes present in Arabidopsis, are pleiotropic and mediate diverse physiological processes including the response to pathogens. We hypothesized that the spatio-temporal control of RbohD and RbohF gene expression might be critical in determining their multiplicity of functions. Transgenic Arabidopsis plants with RbohD and RbohF promoter fusions to β-glucuronidase and Luciferase reporter genes were generated. Analysis of these plants revealed a differential expression pattern for RbohD and RbohF throughout plant development and during immune responses. RbohD and RbohF gene expression was differentially modulated by pathogen-associated molecular patterns. Histochemical stains and in vivo expression analysis showed a correlation between the level of RbohD and RbohF promoter activity, H2O2 accumulation and the amount of cell death in response to the pathogenic bacterium Pseudomonas syringae pv. tomato DC3000 and the necrotrophic fungus Plectosphaerella cucumerina. A promoter-swap strategy revealed that the promoter region of RbohD was required to drive production of ROS by this gene in response to pathogens. Moreover, RbohD promoter was activated during Arabidopsis interaction with a non-virulent P. cucumerina isolate, and susceptibility tests with the double mutant rbohD rbohF uncovered a new function for these oxidases in basal resistance. Altogether, our results suggest that differential spatio-temporal expression of the Rboh genes contributes to fine-tune RBOH/NADPH oxidase-dependent ROS production and signaling in Arabidopsis immunity.

Key words: Arabidopsis, NADPH oxidase, pathogen, RbohD, RbohF, reactive oxygen species.

Introduction

Plants have evolved an inducible and multilayered immune system to face pathogen threats (Jones and Dangl, 2006). The first active layer of the plant immune system is the recognition of pathogen-associated molecular patterns (PAMPs). PAMPs consist of molecules conserved among different pathogen types that are recognized by pattern recognition receptors (PRRs) located...
in plant cell plasma membranes. This initial recognition leads to PAMP-triggered immunity (PTI; Zipfel, 2014). However, some pathogens are able to cause disease because they have evolved effectors that manipulate these initial layers of host defense. In turn, some of these effectors can be recognized, in a direct or indirect manner, by intracellular receptors called resistance proteins. These proteins activate effector-triggered immunity (ETI), a rapid response that amplifies PTI signaling events and limits pathogen spread (Dangl et al., 2013).

Both layers of the immune response share many signaling elements (Dodds and Rathjen, 2010). Among these, apoplastic production of reactive oxygen species (ROS) is one of the fastest physiological responses ubiquitously observed in plants after pathogens are recognized by PRRs (Macho and Zipfel, 2014), or by resistance proteins during ETI. In this latter case, ROS production is often associated with the onset of a hypersensitive response (HR; Torres 2010). In addition to HR, ROS contribute to the regulation of different cellular and physiological processes (Torres, 2010; O’Brien et al., 2012).

NADPH oxidases are conserved enzymes in animals, fungi and plants that are involved in active ROS production (Torres and Dangl, 2005). Their role in plant interactions with a wide range of pathogens and symbionts has been extensively documented (Marino et al., 2012). Plant NADPH oxidases, also known as respiratory burst oxidase homologs (RBOHs), are a family of plasma membrane-localized enzymes with homology to the NADPH oxidase from mammalian phagocytes (Torres and Dangl, 2005; Sumimoto, 2008). These enzymes generate apoplastic superoxide ions (O$_2^-$) that rapidly dismutate to hydrogen peroxide (H$_2$O$_2$) in aqueous solution, either spontaneously or via superoxide dismutase activity. The model plant Arabidopsis thaliana (hereafter Arabidopsis) presents a multigene family of 10 Rboh genes (Torres and Dangl, 2005). Genetic studies revealed that ROS produced by the different RBOHs control a large number of processes in response to both developmental and environmental stimuli (Suzuki et al., 2011).

Some RBOH proteins, such as RBOHC that mediates root hair formation (Takeda et al., 2008), perform specific functions. Other, such as RBOHD and RBOHF, are pleiotropic and mediate diverse functions, ranging from pathogen response or abiotic stress signaling, to lignification and stomatal closure (Suzuki et al., 2011). Specifically, RBOHD is responsible for most of the ROS produced during ETI (Torres et al., 2002), and for the apoplastic ROS burst in response to PAMPs (Nühse et al., 2007; Zhang et al., 2007). It plays a critical role in PAMP-induced stomatal closure as part of pre-invasive immunity (Mersmann et al., 2010) and in cell death regulation in response to necrotrophs (Pogany et al., 2009). In some patho-systems, such as in the response to necrotrophic bacterium Dickeya dadianii, orthologs of RbohD are required for full resistance (Fagard et al., 2007). On the other hand, Arabidopsis RbohF is a crucial modulator of defense-related metabolism upon Pseudomonas syringae infection (Chaouch et al., 2012), and its nearest relative in barley plays a major role in defense response against Blumeria graminis (Proels et al., 2010). Additionally, both oxidases cooperate during cell death signaling (Torres et al., 2002; Zhang et al., 2009), abscisic acid-mediated stomatal closure (Kwak et al., 2003) or the plant response to Sclerotinia sclerotiorum and Glovinomyces chichoracearum (Perchepied et al., 2010; Berrocal-Lobo et al., 2010). Thus, these two pleiotropic oxidases seem to display unequal redundancy, having specific signaling roles that synergize during different physiological responses activated by the plant immune system.

Regulation of RBOHD and RBOHF by distinct interacting proteins may account for this RBOH-dependent ROS-signaling specificity (Suzuki et al., 2011). In particular, phosphorylation of their divergent N-termini by different protein kinases appears to be a key activation mechanism of these enzymes in response to different cellular cues (Sirichandra et al., 2009; Drerup et al., 2013; Kadota et al., 2014, 2015; Li et al., 2014). Additionally, differential RbohD and RbohF regulation and spatio-temporal gene expression could account for their specificity of action in different contexts (Adachi et al., 2015).

Analyses of gene spatio-temporal expression patterns can significantly contribute to our understanding of molecular mechanisms of gene function in gene families such as that of Rboh (Niehrs and Pollet, 1999; Song et al., 2013; Valério et al., 2004). Contrary to the other Rboh genes, RbohD expression is strongly upregulated in response to different stresses (Suzuki et al., 2011), further suggesting the relevance that transcription may have on the regulation of RbohD function. It has been shown that RbohD expression is negatively regulated by mitogen-activated protein kinase 8 (Takahashi et al., 2011), whereas activation of the ethylene pathway up-regulates RbohD (Meng et al., 2013) and, to a lesser extent, RbohF (Jakubowizh et al., 2010). Thus, the specific timing and level of RbohD and RbohF transcriptional activation and its correlation with physiological processes that take place in response to pathogens with different lifestyles may contribute to determine the outcome of the plant immune response.

In the present study, the promoters of RbohD (pD) and RbohF (pF) were fused to β-glucuronidase (GUS) and Luciferase (LUC) reporters. These lines were used to analyze the spatio-temporal expression patterns of RbohD and RbohF, both in vivo and ex vivo, in response to PAMPs and to pathogens, including different isolates of the hemibiotrophic bacteria P. syringae pv. tomato (Pto) DC3000 and the necrotrophic fungus Plectosphaerella cucumerina (Pc). These analyses revealed a differential regulation of pD and pF activity that positively correlates spatially and temporally with specific ROS production and cell death during the Arabidopsis immune response to pathogens. Additionally, our spatial and temporal expression analysis helped uncover a novel role for these NADPH oxidases in basal resistance to a non-virulent necrotrophic Pc fungus.

**Materials and methods**

**Plant materials and growth conditions**

Arabidopsis thaliana ecotype Columbia (Col-0), the mutants rbohD, rbohF, rbohD rbohF (Torres et al., 2002), cyp79b2 cyp79b3 (Sánchez-Vallet et al., 2010) and the transgenic lines (Col-0 and rbohD
Molecular cloning

To generate promoter RbohD and RbohF fusions to the β-glucuronidase (GUS) gene, the GUS fragment was amplified by PCR adding EcoRI and BamHI restriction sites to the 5′ and 3′ ends of the amplicons, respectively, and mobilized into derivatives of the SLJ44026 vector (Jones et al., 1992) containing the promoters of RbohD (1877 bp; pD) or RbohF (2002 bp; pF), thus generating the pD::GUS::NOS (pD-GUS) and pF::GUS::NOS (pF-GUS) cassettes. To obtain the pD::Luciferase (pD-LUC) and pF::Luciferase (pF-LUC) constructs, each Rboh promoter was cloned into a Gateway pENTR/D-Topo vector (Invitrogen), and mobilized into the LUCTRAP-3 binary vector (Calderón-Villalobos et al., 2006) using LR CLONASE II enzyme (Invitrogen). To generate RbohD and RbohF promoter-swap constructs, pD and pF DNA fragments were amplified by PCR, adding an MfeI restriction site at the 5′ of the promoters and the HA-tag, flanked by EcoRI at the 5′ and SnaBI and XbaI restriction sites at the 3′ region. Subsequently, the amplicon was cloned into the pENTR/D-Topo vector. In parallel, attR1/attR2 reading frame B from the GTW cassette digested with EcoRV was cloned into the SnaBI site, generating the pRboh::HA::attR1/attR2 entry clones. Subsequently, these cassettes were mobilized using MfeI and XbaI into a derivative of the pGREEN binary vector with EcoRI and XbaI sites preceding the NOS term, and the constructs pRboh::HA::attR1/attR2::NOS were generated. Finally, full-length RbohD and RbohF cDNAs were mobilized from pENTR/D-Topo into the pGREEN derivatives and the four combinations (pD-HA-RbohD, pF-HA-RbohF, pD-HA-RbohF and pF-HA-RbohF) were generated. The genetic constructs generated and primers used are listed in Supplementary Fig. S1 and Table S1, respectively, available at JXB online.

Generation of Arabidopsis transgenic plants

Wild-type Col-0 plants were transformed with the pRboh::GUS and pRboh::LUC constructs (see Supplementary Fig. S1A, B) by the standard floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MS agar plates with 50 μg ml⁻¹ kanamycin and homozygous plants were obtained from lines with single insertion events. For each construct, 5–10 independent transgenic lines were analyzed. All GUS transgenic lines selected and tested (at least four independent lines) gave a consistent pattern of GUS staining during development (Fig. 1 and data not shown), in seedlings after induction with the flg22 bacterial PAMP (Supplementary Fig. S2A) and in 4-week-old plants after treatment with Pc and Pto pathogens (Supplementary Figs S2B, S3). Based on these analyses, lines pD-GUS#2 and pF-GUS#3 were selected for detailed analyses. Similarly, pD-LUC and pF-LUC lines gave a consistent induction and LUC activity in response to flg22 and after inoculation with Pc fungal pathogen (Supplementary Fig. S4). Representative lines pD-LUC#1 and pF-LUC#1 were selected for further analyses. Likewise, Rboh-HA tagged alleles driven by either pD or pF (Supplementary Fig. S1C) were employed to transform rbohD mutant lines. Six or seven transgenic lines for each construct were selected for analyses (Supplementary Table S2). Presence of the transgene was checked by PCR in all these lines, and expression was monitored by qRT-PCR and by western-blot together with complementation assays (Supplementary Fig. S5 and data not shown). Experiments were performed with representative lines. Expression patterns were stable through three generations for all the selected lines.

Fig. 1. RbohD and RbohF present a differential spatial expression pattern during Arabidopsis development. GUS histochemical staining of representative transgenic lines (pD-GUS#2 and pF-GUS#3) in different tissues: (A, B) 6-day-old seedlings; (C, D) secondary root emerging zone; (E, F) 4-week-old leaves; (G, H) mesophyll and vasculature from 4-week-old leaves; (I, J) abaxial leaf epidermis (arrowheads highlight several stomata); (K, L) inflorescences and siliques of 6-week-old plants; (M, N) detail of the basal region of the siliques. Bars, 100 μm (C, D, G and H), 2 mm (A, B, M, N), 25 μm (I, J). Six plants per line were analyzed. Representative samples are shown.
Pathogen inoculation assays and PAMP treatments

For PAMP treatment, 6-day-old pD-GUS and pF-GUS plants grown on MS agar plates were transferred to liquid MS medium and kept for 24 h under agitation before treatment with PAMPs at effective concentrations (Schwessinger et al., 2011; Lozano-Durán et al., 2013): 100 mM flg22 (Pepron), 100 mM elf18 (Pepron) or 1 mg ml⁻¹ chitin (Yaizu Suisankagai Industry). For bioluminescence imaging of D-LUC and pF-LUC seedlings were treated with 500 nM flg22 before measurement. All experiments were repeated at least three times with identical results.

Bacterial strains used in this study were Pto DC3000, Pto DC3000 (avrRpm1), and Pto DC3000 hrcC. Four-week-old plants were inoculated with 2.5 × 10⁷ cfu ml⁻¹ using a 1 ml syringe. For Pc inoculation assays, spor suspensions (4 × 10⁶ sporas ml⁻¹) of PcBMM or Pc2127 isolates were sprayed on 4-week-old Arabidopsis plants grown on soil. Plants were subsequently kept on week under relative humidity as described (Sánchez-Rodríguez et al., 2009). Evaluation of basal resistance to Pc2127 was carried out by fungal biomass quantification by qPCR on 2.5-week-old plants (Sánchez-Vallet et al., 2010).

Stains and luciferase activity measurements

Detection of H₂O₂ by 3,3’-Diaminobenzidine (DAB, SIGMA) staining was performed as described in Torres et al. (2002). Dead cells were visualized with a solution of lactophenol-trypsin blue (TB) as described in Slusarenko et al. (1991). Histochemical GUS staining was performed according to Jefferson et al. (1987).

For in vivo quantification of Rboh-promoter driven luciferase activity, pD-LUC and pF-LUC plants were sprayed with a 0.5 mM solution of D-Luciferin (MELFORD). Luminescence produced in response to DC3000 was measured every 15 min for a 10 h period with a Photent CCD camera (East Sussex, UK). Promoter activity values correspond to photon counts emitted by Pto DC3000 (avrRpm1) relative to MgCl₂ infiltrated leaves.

Luciferase activity in response to Pc was quantified using a NightOWL LB 983 in-vivo Imaging System (Berthold). Six fully expanded leaf areas of 135 mm² were measured per plant at 1, 2 and 3 dpi. To monitor luciferase activity in response to flg22, 1-week-old seedlings were transferred to a 96-well plate and kept on liquid MS overnight. Next day, water was exchanged for a solution containing 50 μM D-Luciferin and luminescence (n=8) was measured as relative light units with a TECAN plate reader.

Gene expression analysis by qRT-PCR

RNA extraction was performed as described in Llorente et al. (2005) from in vitro 1-week-old Col-0 seedlings untreated or treated for 90 min with 500 nM flg22. For qRT-PCR, 10 ng of total RNA were used per sample with Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix (Agilent Technologies, USA) in a final volume of 16 μl. Assays were performed in triplicate with a LightCycler 480 II real-time PCR system (Roche). Primers used were: DqRTrw (5’-CGAATGGCATCCTTTTCTCAATC-3’) and DqRTrrev (5’-GTCACCGAGTGGCGGATG-3’) to quantify RbohD expression (Penfield et al., 2006); FqRTrw (5’-CCTGGGATTTGCGAATCC-3’) and FqRTrrev (5’-CTTTCGTCCTGTGCGTGA-3’) for RbohF. Thermal parameters for RT-PCR amplification were: (65°C, 3 min + 50°C, 10 min) for reverse transcription; (95°C, 5 min) for denaturing nucleic acids; 45 cycles (95°C, 20 s; 60°C, 30 s). Amplification with Ubiquitin 10 (At4g05320) primers (He et al., 2006) was used to normalize Rboh transcript level and calculate ΔCt values.

Protein extraction, immunoprecipitation, SDS-PAGE and immunoblotting

Protein extraction and immunoprecipitation from Arabidopsis were performed as described in Schwessinger et al. (2011). For immunodetection, membranes were incubated with antibodies diluted in blocking solution at the following dilutions: anti-HA High Affinity (3F10 monoclonal antibody; Roche) 1:2000, followed by incubation with anti-rat IgG-horseradish peroxidase 1:5000 to detect HA tagged RBOH proteins.

Results

RbohD and RbohF promoters drive reporter gene expression to different tissues during Arabidopsis development

We sought to determine whether RbohD and RbohF present a differential tissue-specific expression pattern that could modulate ROS signaling at a certain time point, in a specific region, and in response to development- or immunity-related cues. Thus, transgenic Arabidopsis (Col-0 background) plants harboring transcriptional fusions between the RbohD (pD) and RbohF (pF) promoters and the β-glucuronidase (GUS) or the firefly Luciferase (LUC) reporter genes were generated (see Supplementary Fig. S1A, B). For pD, the 1877 bp region covering the intergenic region between the preceding gene (At5g47900) and the ATG translation initiation codon of RbohD (At5g47910) was used. For pF, the 2002 bp region upstream of the ATG was selected.

Detailed GUS histochemical staining with selected pD-GUS#2 and pF-GUS#3 homozygous lines was performed throughout different stages of Arabidopsis development (Fig. 1). pD-GUS plants displayed a high level of expression in roots and aerial parts of seedlings, whereas pF-GUS showed a lower expression level in this stage, being often restricted to the plant vessels. Interestingly, in roots, GUS activity driven by pD appeared widespread throughout most tissues including root hairs, while in pF-GUS lines GUS expression was mostly restricted to areas where secondary roots develop (Fig. 1C, D). In leaves from 4-week-old plants, pF drove GUS expression to the vascular tissue and hidatodes, while pD directed GUS expression to disperse areas of the leaf lamina (Fig. 1E-H) and to stomatal guard cells (Fig. 1I-J). In the inflorescence, pF activity was localized at the basal region of siliques, whereas pD drove a lower GUS expression in these areas (Fig. 1K-N). These analyses revealed that the promoters of RbohD and RbohF specifically drive expression of the GUS reporter gene to different tissues in response to developmental cues.

PAMPs differentially modulate Rboh promoter activity

Since RbohD and RbohF function has been related to pathogen response, we addressed whether bacterial or fungal PAMPs transcriptionally up-regulate pD and pF activity. Seedlings of pD-GUS#2 and pF-GUS#3 lines were treated with effective concentrations of 3 different PAMPs: flg22 (active epitope of bacterial flagellin), elf18 (elicitor peptide from bacterial elongation factor EF-tu) and chitin (a component of the fungal cell wall). Analysis of GUS activity in these seedlings revealed that RbohD was notably up-regulated by these three PAMPs, whereas RbohF promoter activity remained unaltered compared with mock treated plants (Fig. 2A). This differential, PAMP-mediated...
regulation of \(pD\) and \(pF\) was further confirmed with additional transgenic lines (Supplementary Fig. S2A).

Time course quantification of LUC activity using \(pD\)-LUC\#1 and \(pF\)-LUC\#1 lines revealed that \(pD\) up-regulation initiated 15 min after treatment with flg22, reaching a maximum activity at 1–1.5 h, whereas \(pF\) activity was not detected throughout the experiment (Fig. 2B; Supplementary Fig. S4A). These results were confirmed by qRT-PCR analysis of \(RbohD\) and \(RbohF\) expression in wild-type plants, which revealed a differential expression of \(RbohD/RbohF\) genes (Fig. 2C). These data are in line with the known role of \(RbohD\) in early immune signaling responses (Zhang et al., 2007), further indicating that transcriptional regulation of \(RbohD\) is relevant for an effective immune response to a pathogen threat.

\textbf{RbohD and RbohF promoters are differentially activated in response to Pto DC3000}

We next studied whether \(pD\) and \(pF\) exerted a differential transcriptional regulation of RBOH-NADPH oxidases during plant interaction with phytopathogenic bacteria, and if this expression correlated with modulation of ROS production and cell death at different stages of the immune response. To that aim, the spatial and temporal pattern of GUS expression was examined in several \(pD\)-GUS and \(pF\)-GUS lines after infiltration with different strains of the hemibiotrophic bacterial pathogen \(Pto\) DC3000 (Fig. 3A; Supplementary Fig. S3). In parallel, \(H_2O_2\) accumulation and cell death were monitored in the infiltrated leaves by diaminobenzidine (DAB) and Trypan Blue (TB) stains, respectively (Fig. 3A and data not shown). After infiltration with avirulent \(Pto\) DC3000 (\textit{avrRpm1}), that activates ETI, a strong \(pD\) activity was detected 4 h post-inoculation (hpi) in those areas where a massive accumulation of peroxides occurs and cell death takes place, whereas \(pF\) was slightly activated at the leaf vasculature (Fig. 3A; Supplementary Fig. S3). On the other hand, after infiltration with virulent strain \(Pto\) DC3000, which leads to a compatible interaction with no ROS production or cell death at the initial stage of infection, a mild activation of both \(Rboh\) promoters was observed (Fig. 3; Supplementary Fig. S3). A similarly

![Fig. 2.](http://jxb.oxfordjournals.org/)

\(RbohD\) but not \(RbohF\) promoter activity is induced by fungal and bacterial PAMPs. (A) GUS histochemical staining of \(pD\)-GUS and \(pF\)-GUS in 1-week-old seedlings 30, 60 and 180 min post-treatment (mpt) with the PAMPs chitin, flg22 and elf18 at the indicated concentration. (B) Time course assay showing LUC activity driven by \(pD\) and \(pF\) in 1-week-old seedlings treated with 500 nM flg22. Each time point represents the mean luminescence (Relative Light Units, RLUs) of 8 seedlings. (C) \(RbohD\) and \(RbohF\) expression in 1-week-old Col-0 seedlings 90 min after flg22 (500 nM) treatment. Expression was normalized using \textit{Ubiquitin10} gene as endogenous control. Bars represent n-fold relative expression of the indicated genes relative to the control sample (Col-0 mock=1). Data represent the mean ±SE of three biological replicates. Asterisks indicate statistically significant differences (Welch Student two sample t-test; \(P<0.05\)).
weak activation was observed after challenging $pD$-GUS and $pF$-GUS plants with mutant strain $Pto$ DC3000 $hrcC$ that lacks a functional type III secretion system and activates PTI with only a mild accumulation of peroxides and no cell death (Fig. 3 and data not shown). Thus, strong activation of $pD$ parallels high levels of ROS production and cell death during ETI.

To monitor in vivo the promoter activity of both $Rboh$ genes and to determine their temporal regulation and expression level during ETI, representative transgenic lines $pD$-LUC#1 and $pF$-LUC#1 were challenged with $Pto$ DC3000 ($avrRpm1$) (Fig. 3B; Supplementary Videos S1 and S2). Interestingly, we detected a wave of $RbohD$ promoter activity between 3 and 7 hpi, with a maximum at ~5 hpi. A similar trend was observed in the $pF$-LUC transgenic line, although $RbohF$ promoter activity was ~8-fold lower than that of $RbohD$. The comparison between the promoter activities at 5 hpi in the leaf area infiltrated with $Pto$ DC3000 ($avrRpm1$) and control leaves infiltrated with MgCl$_2$ revealed a 4.3 ± 0.9-fold increase in $pD$ activity and a 2.6 ± 0.3-fold increase in $pF$ activity. These results point towards a putative role for transcriptional regulation of these NADPH oxidases at the site of interaction, especially for $RbohD$, during ETI signaling.

**RbohD and RbohF promoters determine a differential expression pattern during Arabidopsis immune response to the necrotrophic fungus* P. cucumerina**

We studied the spatio-temporal expression pattern and activity of $Rboh$ promoters in response to the necrotrophic fungus *P. cucumerina* (*Pc*). A spore suspension of the *Pc* virulent isolate BMM (*PcBMM*) was sprayed on different $pD$-GUS and $pF$-GUS lines. Histochemical GUS staining revealed that $pD$ activity is strongly up-regulated in leaves during the infection process in a pattern that spatially parallels the areas of the leaf lamina where $H_2O_2$ accumulated and cell death took place (Fig. 4A, left panels; Supplementary Fig. S2B). Likewise, an increase in $pF$ activity was observed, mostly associated to leaf veins.
To further explore if the up-regulation in Rboh expression was just a consequence of the necrosis process, GUS activity was monitored in plants sprayed with isolate Pc2127, that infects Viola spp. in nature and does not cause necrosis nor colonize Arabidopsis (Col-0) wild-type plants, but infects a cyp79b2 cyp79b3 double mutant, impaired in both tryptophan metabolite biosynthesis and non-host resistance (Sánchez-Vallet et al. 2010; Ramos et al., 2013). Interestingly,
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**Fig. 5.** The double mutant rbohD rbohF shows weakened basal resistance to the non-virulent Pc2127 isolate. (A) Symptoms of 3-week-old wild-type Col-0 plants compared with those of the listed mutant genotypes at 7 dpi with Pc2127. (B) Quantification of Pc2127 biomass. Fungal DNA (P. cucumerina β-tubulin) was quantified by qPCR at 7 dpi using specific primers for Pc β-tubulin and normalised to Arabidopsis Ubiquitin10. Bars represent averages (±SE) of fungal DNA levels relative to Col-0 plants, from one of two independent repetitions of the assay. Statistical analysis was performed by ANOVA 5%, corrected with LSD post hoc test.

PD-GUS, but not PF-GUS plants displayed an enhanced GUS activity that parallels a low level of ROS accumulation, although no macroscopic cell death was observed (Fig. 4A, right panels). These results suggest that induction of RbohD expression could be triggered by molecular determinants of the pathogen recognized by the plant. In contrast, activation of PF might be associated with the necrosis process.

The pattern and level of LUC expression driven by PD and PF was monitored in vivo during interaction of several transgenic lines with the PcBMM isolate (Fig. 4B, C; Supplementary Fig. S4B). After fungal inoculation, LUC activity driven by PD promoter was already up-regulated at 24 hpi, and increased to higher levels with progression of the infection (Fig. 4B, C). Interestingly, PD promoter activity extended from the areas of fungal growth through the petioles reaching the whole plant rosette. In contrast, LUC activity driven by PF was just limited to locally infected areas in leaves (Fig 4B, C). We also tested the promoter activities in response to the non-adapted isolate Pc2127. PD-LUC plants displayed a LUC activity that was lower than that observed after PcBMM inoculation, whereas just a weak, localized LUC activity was detected in PF-LUC plants. Hence, the level of Rboh promoter activity, mainly that of RbohD, correlates with the levels of ROS and cell death produced in these two plant-fungal interactions.

Loss of RbohD and RbohF function alters basal resistance to the non-adapted Pc2127 fungus

To determine whether the RbohD up-regulation detected after inoculation with Pc2127 implicated a physiological role for Rbohs in basal resistance, rbohD and rbohF single and rbohD rbohF double mutants were inoculated with this fungal isolate. Fungal growth in these mutants was compared with that in wild-type plants and in the cyp79b2 cyp79b3 double mutant. At 7 days post-inoculation (dpi), rbohD rbohF plants displayed more macroscopic disease symptoms and supported more fungal growth, quantified by qPCR, than wild-type plants, whereas fungal growth and disease symptoms in rbohD and rbohF single mutants were similar to those of resistant wild-type plants (Fig. 5). Susceptibility of rbohD rbohF was, however, lower than that of cyp79b2 cyp79b3 plants, impaired in non-host resistance (Sánchez-Vallet et al., 2010). These results demonstrate that RbohD and RbohF are necessary for full basal resistance to the non-virulent isolate Pc2127.

Differences between RbohD and RbohF promoters control ROS production during Arabidopsis response to P. cucumerina

To assess the role that RbohD and RbohF promoters play in determining functional specificity of these oxidases, we evaluated the capability of Rboh promoter-swap constructs to complement rbohD mutant immunity-related phenotypes. The following own-promoter and promoter-swap HA-tagged constructs were generated: PD::HA-RbohD (PD-HA-D), PD::HA-RbohF (PD-HA-F), PF::HA-RbohD (PF-HA-D) and PF::HA-F (PF-HA-F; Supplementary Fig. S1C). These constructs were transformed into rbohD Arabidopsis mutant
lines, and homozygous lines were obtained (Supplementary Table S2).

Complementation of the rbohD phenotype was tested in 4-week-old transgenic plants with an rbohD mutant background. Plants were sprayed with a suspension of PcBMM spores, and leaves were stained with DAB 24 hpi to detect RbohD-dependent H$_2$O$_2$ accumulation (Fig. 6A). Only constructs harboring the RbohD gene under the control of its cognate promoter (pD-HA-D) were able to restore H$_2$O$_2$ production in response to the necrotrophic fungus, whereas transgenic lines harboring RbohD driven by the RbohF promoter (pF-HA-D) or RbohF driven by the RbohD promoter (pD-HA-F) failed to restore ROS production in the rbohD mutant background (Fig. 6A; Supplementary Table S2). Expression of HA-tagged RBOH proteins was monitored in representative lines by immunodetection using an anti-HA antibody. We found that HA-RBOHD accumulation was enhanced in complementation lines driven by pD, as compared with that of HA-RBOHF or HA-RBOHD in complementation lines driven by pF (Fig. 6B and data not shown). These results suggest that both the promoter and the transcribed RbohD sequences are necessary to achieve the high levels of RbohD-dependent ROS production observed after successful pathogen recognition.

**Discussion**

RBOHD and RBOHF play many different signaling roles in plant immunity (Suzuki et al., 2011; Marino et al., 2012). However, the precise way in which these two oxidases are regulated to achieve their functional specificity is not fully understood. Since we hypothesized that transcriptional regulation of these oxidases might contribute to specify the signaling role of these enzymes, we used RbohD and RbohF promoter fusions to GUS and LUC reporter genes to investigate in detail the expression pattern of these two genes during different events of the Arabidopsis immune response. Furthermore, an RbohD/RbohF promoter-structural gene swap strategy was carried out in order to explore the relevance of promoter regulatory regions in determining the specificity of ROS production in response to pathogens.

![Fig. 6. Functional complementation of rbohD by Rboh promoter-swap constructs after PcBMM inoculation.](http://jxb.oxfordjournals.org/)

(A) Complementation of H$_2$O$_2$ accumulation 24 hpi with PcBMM. H$_2$O$_2$ accumulation was detected by DAB staining in leaves from 4-week-old wild-type Col-0 and rbohD plants and three independent transgenic lines of each HA-tagged promoter-swap construct in rbohD background (Supplementary Table S2): pD-HA-D, pD-HA-F, pF-HA-D and pF-HA-F. (B) Microscopic details of H$_2$O$_2$ accumulation at the leaf lesions are shown; bar, 500 μM. (C) Immunodetection by western blot of HA-RBOH proteins using anti-HA antibody. Total protein extraction was performed from 4-week-old own-promoter and promoter-swap transgenic plants 24 hpi with PcBMM. Molecular mass is indicated. CBB, Comassie Brilliant Blue.
RbohD and RbohF promoters drive distinct spatio-temporal expression patterns in response to PAMPs and bacterial pathogens

Histochemical staining of transgenic lines with Rboh-promoter fusions to the GUS reporter gene revealed that RbohD and RbohF promoters direct a differential spatial expression pattern in different stages of Arabidopsis development (Fig. 1). This is congruent with the importance that the spatio-temporal control of RBOH-dependent ROS production might have to specify the multiplicity of functions of these oxidases (Suzuki et al., 2011). However, abnormal development phenotypes related to these expression patterns were not observed in the single mutants rbohD or rbohF, and only the double mutant rbohD rbohF exhibits reduced rosette growth and some necrotic lesions (Torres et al., 2002). Functional redundancy could explain the absence of development phenotypes in the individual mutants, given that Arabidopsis encodes 10 Rboh genes (Torres and Dangl, 2005), some of which mediate plant development processes (Suzuki et al., 2011). The RbohD promoter activity observed in root tissues and guard cells and the RbohF expression in leaf hydathodes (Fig. 1), all of them areas of pathogen penetration into plants (Dodman, 1979; Hallman, 2001), is congruent with the known role of these genes as components of the plant immune response (Torres, 2010; Marino et al., 2012).

RbohD promoter activity was markedly higher than that of RbohF in seedling tissues and across the responses analyzed in this study, in agreement with previous data of RbohD and RbohF mRNA steady-state levels and microarray analyses (Torres et al., 1998; Suzuki et al., 2011). Only RbohD promoter is transcriptionally up-regulated in response to the PAMPs tested (Fig. 2; Supplementary Figs S2A, S4A). In response to Pto DC3000 (avrRpm1) both RbohD and RbohF promoters were also differentially up-regulated, with RbohD activation being 8-fold stronger than that of RbohF (Fig. 3; Supplementary Fig. S3). This up-regulation takes place in the areas where ROS accumulate and HR occurs (Fig. 2A). This is in agreement with most of the apoplastic ROS generated during ETI being RBOHD-dependent and with the additive role of both oxidases in the control of the HR (Torres et al., 2002). These results also suggest that, in addition to the known post-translational regulation of these NAPDH oxidases (Kadota et al., 2014, 2015; Li et al., 2014), RbohD and RbohF transcriptional activation might be necessary to achieve the high amount of H2O2 that occurs after successful recognition of avirulent pathogens during ETI.

Infiltration with the virulent Pto DC3000 wild type or with the hrcC mutant strain did not trigger a clear induction of pD- or pF-driven GUS or LUC activity (Fig. 3A; Supplementary Fig. S4; data not shown). However, seedlings treated with flg22 or elf18 bacterial PAMPs showed an up-regulation of RbohD (Fig. 2A, C). Differences in the developmental stage of the plants and in the amount of available PAMPs to be sensed by PRRs might explain these contradictory results. Alternatively, additional signals may be present in the microbe that dampen promoter up-regulation after PAMP recognition. The fact that PAMPs triggered pD but not pF activity is in agreement with a role of RbohD in later events of the plant immune response (Zhang et al., 2007; Kadota et al., 2014), while RbohF activity could be more related to HR execution (Torres et al., 2002).

Correlation of Rboh spatio-temporal expression pattern with ROS production, cell death and basal resistance to P. cucumerina

Analysis of RbohD and RbohF spatio-temporal expression pattern in response to plant infection with the necrotrophic fungal pathogen PcBMM revealed a strong up-regulation of RbohD promoter at the leaf lamina and petioles, whereas RbohF is mainly up-regulated at the mid-rib and leaf veins (Fig. 4A, B; Supplementary Fig. S2B). This Rboh up-regulation, which increases with progression of the infection, is in agreement with their implication in the immune resistance response against this necrotrophic fungus as well as with the additive role of these oxidases for a full immune response to different pathogens (Marino et al., 2012; Torres and Dangl, 2005). The concomitance between levels of RbohD promoter activation, H2O2 accumulation and pathogen colonization (Fig. 4A) is coincident with the pre-eminent role that this NADPH oxidase has in early events of the plant immune response (Kadota et al., 2014; Zhang et al., 2007). In accordance with this, the fungal PAMP chitin also triggered up-regulation of the RbohD promoter but not that of RbohF (Fig. 2A), further suggesting that transcriptional up-regulation of RbohD could be a determining factor for the large accumulation of ROS detected after pathogen recognition.

The association between the staining pattern defined by these promoters and the progress of the necrosis also suggests a role for these oxidases in cell death. The large increase in RbohD promoter activity observed at the leaf areas surrounding necrotic lesions induced by PcBMM (Fig. 4A, B) is congruent with the existence of cell death related cues that might modulate the strong level of Rboh-promoter activation and subsequent high H2O2 accumulation that possibly impacts cell death levels along necrosis progression. RbohD was proposed to control cell death during Alternaria brassicicola infection by triggering damaged cell death, while simultaneously inhibiting it in neighboring cells through a negative feedback regulation of salicylic acid and ethylene (Pogany et al., 2009). In addition, RbohF promoter activity, which is very low at early stages of the infection, increases substantially when cell death is in progress. This is consistent with the additive role that these two oxidases display in cell death modulation (Torres et al., 2002, 2005).

ROS production by these oxidases is required for full resistance to PcBMM (Torres et al., 2013). However, some studies report that pathogens with a necrotrophic phase could benefit from ROS produced by the host to promote cell death, thereby contributing to disease progression (Torres, 2010). While the double mutant rbohD rbohF is also more susceptible to Sclerotinia sclerotiorum compared with wild type (Perchepied et al., 2010), rbohD was shown to be equally resistant to Botrytis cinerea (Galletti et al., 2008) and even more resistant to Alternaria brassicicola (Pogany et al., 2009).
Thus, although RBOHD and RBOHF contribute to the immune signaling in response to necrotrophs, their roles in disease progression may vary depending on the pathosystem analyzed and even on plant growth conditions or on inoculation methods. In view of the role of RBOH-dependent ROS in the response to necrotrophic pathogens, it might be useful to consider the ROS produced in response to external threats as signal transducers that balance the cell fate between life and death rather than noxious chemicals that just kill the pathogen (Torres, 2010; Coll et al., 2011; O’Brien et al., 2012).

Non-host resistance is a durable and broad spectrum defense response, that shares some molecular mechanisms with plant basal resistance (Niks and Marcel, 2009; Schulze-Lefert and Panstruga, 2011). Understanding basal resistance mechanisms against non-adapted pathogens can contribute to reveal the molecular basis behind non-host resistance. Interestingly, we found that RbohD promoter was activated upon infection with the non-virulent fungal isolate Pc2127, which is unable to successfully colonize Arabidopsis wild-type plants, but that infected cyp79b2 cyp79b3 plants impaired in non-host resistance (Fig. 4). These results point to a putative role for RbohD in resistance to this fungal isolate, possibly during recognition of the fungus, since RbohD promoter was transcriptionally regulated by chitin. As a matter of fact, after inoculation with Pc2127, double mutants rbohD rbohF, but not the single ones, supported enhanced fungal growth compared to wild-type plants (Fig. 5). This shows the relevance of these two plant NADPH oxidases for full basal resistance to non-virulent fungi, and indicates some partial functional redundancy between both oxidases, as previously reported in some biotic interactions (Marino et al., 2012). This result is in agreement with the enhanced susceptibility of Nicotiana benthamiana to non-virulent Phytophthora infestans upon silencing of NbRbohA and NbRbohB (Yoshioka et al. 2003). In contrast, just RbohF function has already been associated with Arabidopsis non-host resistance to Magnaporthe oryzae (Nozaki et al., 2013). Thus, it is likely that, depending on the interaction, RbohD, RbohF or both oxidases might be relevant for some non-host resistance responses, as occurs in the orchestration of the full immune response in different pathosystems (Marino et al., 2012; Torres et al., 2013).

**Intrinsic differences between RbohD and RbohF promoters differentially modulate the spatial pattern of ROS production during Arabidopsis immune response**

RbohD is the NADPH oxidase responsible for the generation of ROS in the response of Arabidopsis to all pathogens tested (Marino et al., 2012; Suzuki et al., 2011). To help deciphering the relevance of transcriptional regulation of RbohD and RbohF to specify ROS production during Arabidopsis immune response, we set up a promoter-swap strategy. This strategy revealed that the promoter of RbohD is necessary to drive RbohD-dependent ROS production, since only constructs harboring the RbohD structural part of the gene under the control of RbohD promoter (pD-HA-D) restored DAB staining after Pc-BMM inoculation in rbohD mutant background, whereas constructs with the RbohF promoter driving RbohD (pF-HA-D) failed to complement the mutant phenotype (Fig. 6; Supplementary Table S2). This data suggests that the differential pattern and level of expression of these Rboh genes, which is determined by their promoters, is required to achieve their specific function in immunity.

Differences between the cis-regulatory DNA elements (CREs) present in the 5’UTR sequences of RbohD and RbohF might be responsible for their differential transcriptional regulation. In agreement with this hypothesis, we predicted the presence of common and distinct putative CREs in RbohD and RbohF promoters that might be potentially involved in their transcriptional reprogramming upon pathogen challenge (Supplementary Fig. S6). In *silico* phylogenetic shadowing data point towards a potential role of WRKY transcription factors (TFs) in RbohD transcriptional regulation during plant immunity. Notably, it has been recently reported that WRKY TFs regulate the activity of NbRbohB, the AtRbohD ortholog in Nicotiana benthamiana, through binding to a W-box present in its promoter (Adaichi et al., 2015). Moreover, potential binding sites for GT-1 transcriptional promoters are predicted in RbohD and RbohF promoters (Supplementary Table S3), suggesting that these TFs may mediate the expression of these Rboh in response to pathogen or salt stresses. Further experimental work will be required to validate these *in silico* data.

Our data indicate that RbohD promoter is not sufficient to drive ROS production and to complement the rbohD deficient immune response, since pD-HA-F construct failed to restore the wild-type response in this mutant (Fig. 6; Supplementary Table S2). Therefore, the RBOH proteins themselves must harbor important determinants that contribute to the functional specificity of RBOHD and RBOHF. It is known that the N-terminal cytosolic extensions of these proteins contain regulatory regions, among them calcium-binding EF-hands and phosphorylation motifs, important for the activation and regulation of these plant oxidases (Suzuki et al., 2011; Kadota et al., 2014; Kaya et al., 2014; Li et al., 2014). Thus, differences between both RBOHs, mainly at the N-termini, might also contribute to fine-tune ROS production by these isoforms in the context of the defense response.

We observed that in promoter-swap transgenic lines the level of immunodetected HA-RBOHF tagged protein is invariably lower than that of HA-RBOHD, regardless of the promoter driving RbohD expression and the pathogen used for challenging: Pc-BMM (Fig. 6) or Pto DC3000 (avrRpm1) (Supplementary Fig. S5B). However, transcription of HA-RbohD and HA-RbohF transgenes in PAMM-treated pD and pF lines paralleled transcription of RbohD and RbohF genes in wild-type plants (Fig. 2C; Supplementary Fig. S5A). This result points to a lower stability of HA-RBOHF in comparison to HA-RBOHD, although a differential stability of transcripts cannot be fully excluded. In the present study, the constructs employed for the complementation assays were generated using Rboh cDNA, and it has been shown that introns are key elements for correct expression of proteins in Arabidopsis (Karve et al., 2011). Since RbohF harbors 14 introns and RbohD only 7 (Torres et al., 2002), it is plausible that transcripts or proteins derived from our RbohF cDNA...
constructs present lower stability than the endogenous ones, therefore lowering their ROS production ability. Congruent with this, it has been proposed that alternative splicing in another NADPH oxidase gene, AtRbohBr, could play an important role in the regulation of seed dormancy and after ripening (Müller et al., 2009).

In conclusion, through the analysis of transgenic lines with RbohD or RbohF promoters driving the GUS and LUC reporter genes during the plant immune response to both hemibiotrophic bacteria and necrotrophic fungal pathogens, we have shown that these oxidases present differential spatio-temporal expression patterns and gene expression levels during plant immune responses. Interestingly, these expression studies uncovered a new function of these pleiotropic oxidases in basal resistance to non-virulent fungi. The correlation between RbohD and RbohF promoter activation with the level of ROS production and cell death, suggests that transcriptional regulation of these oxidases contributes to modulate the strength of the immune response to different microbial threats. In fact, our promoter-swap experiments indicate that location and level of expression of RbohD driven by its own promoter is required to fine-tune H2O2 accumulation in response to pathogens. This study exemplifies the complex regulation of multigenic families that achieve many specialized physiological functions using a common enzymatic mechanism.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Schematic representation of the genetic constructs with Rboh promoter fused to reporter genes generated in this study.

Figure S2. Characterization of different pD-GUS and pF-GUS transgenic lines generated in this study in response to a bacterial PAMP and a necrotrophic pathogen.

Figure S3. Characterization of different pD-GUS and pF-GUS transgenic lines generated in this study in response to Pto DC3000.

Figure S4. Characterization of different pD-LUC and pF-LUC transgenic lines generated in this study in response to a bacterial PAMP and a necrotrophic pathogen.

Figure S5. Detection of transgenic transcripts and HA-tagged RBOH proteins in transgenic plants with RbohD and RbohF own-promoter and promoter-swap constructs generated in this study.

Figure S6. Prediction of common and distinct potential cis-regulatory DNA elements (CREs) involved in RbohD and RbohF transcriptional control upon pathogen challenge.

Table S1. Primers used in cloning and expression procedures.

Table S2. Homozygous Arabidopsis transgenic lines with Rboh-own promoter and promoter-swap genetic constructs in rbohD genetic background generated in this work.

Table S3. Sequence and location of the conserved putative CREs found in RbohD and RbohF promoters.

Acknowledgements

This work was supported by funding from Grant ‘(2007)D/5629’ from the International Reintegration Program from the European Union (to MAT), Grant ‘BI02012-32910’ from the Spanish Ministry of Economy and Competitiveness (to AM), the Gatsby Charitable Foundation (to CZ) and the European Research Council (to CZ). JM was supported by a doctoral fellowship from Universidad Politécnica de Madrid and an EMBO short term fellowship (ASTF317-2013). YK received a Special Postdoctoral Research Fellowship, an Excellent Young Researcher Overseas Visit Program and a Uehara Memorial Foundation. We thank Dr Juan Imperial (Centro de Biotecnología y Genómica de Plantas-UPM-INIA) for critical reading of the manuscript.

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Video S1. RbohD promoter drives a dramatic increment of luciferase activity during ETI.

Video S2. RbohF promoter drives a small increment of luciferase activity during ETI.


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