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RESEARCH PAPER

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

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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, H_2O_2 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

Abbreviations: DAB, 3.3′-diaminobenzidine; ETI, effector-triggered immunity; HR, hypersensitive response; PAMP, pathogen associated molecular patterns; Pc, *Plectosphaerella cucumerina*; PRR, pattern recognition receptors; PTI, PAMP-triggered immunity; Pto, *Pseudomonas syringae* pv. tomato; RBOH, respiratory burst oxidase homolog; ROS, reactive oxygen species; TB, trypan blue.

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 ($O_2^{\cdot -}$) that rapidly dismutate to hydrogen peroxide (H_2O_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 dadantii*, 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 sclerotinium* 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 upregulates *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 bacterium *P. syringae* pv. tomato (*Pto*) DC3000 and the necrothrophic 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*

backgrounds) were grown on a soil-vermiculite 3:1 mixture under a 10 h day/14 h night cycle, 24°C day/22°C night temperature, 65% relative humidity and light intensity of 120 mE m^{-2} sec^{-1}. *In vitro* Arabidopsis seedlings growth conditions were: 16 h day/8 h night, on agar plates with Murashige Skoog (MS) salts medium (Duchefa) containing 1% sucrose.

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/R2* reading frame B from the GTW cassette digested with *EcoRV* was cloned into the *SnaBI* site, generating the *pRboh::HA::attR1/R2* entry clones. Subsequently, these cassettes were mobilized using *MfeI* and *XbaI* into a derivative of the pGREEN binary vector with *Eco*RI and *Xba*I sites preceding the NOS term, and the constructs *pRboh::HA::attR1/R2::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.

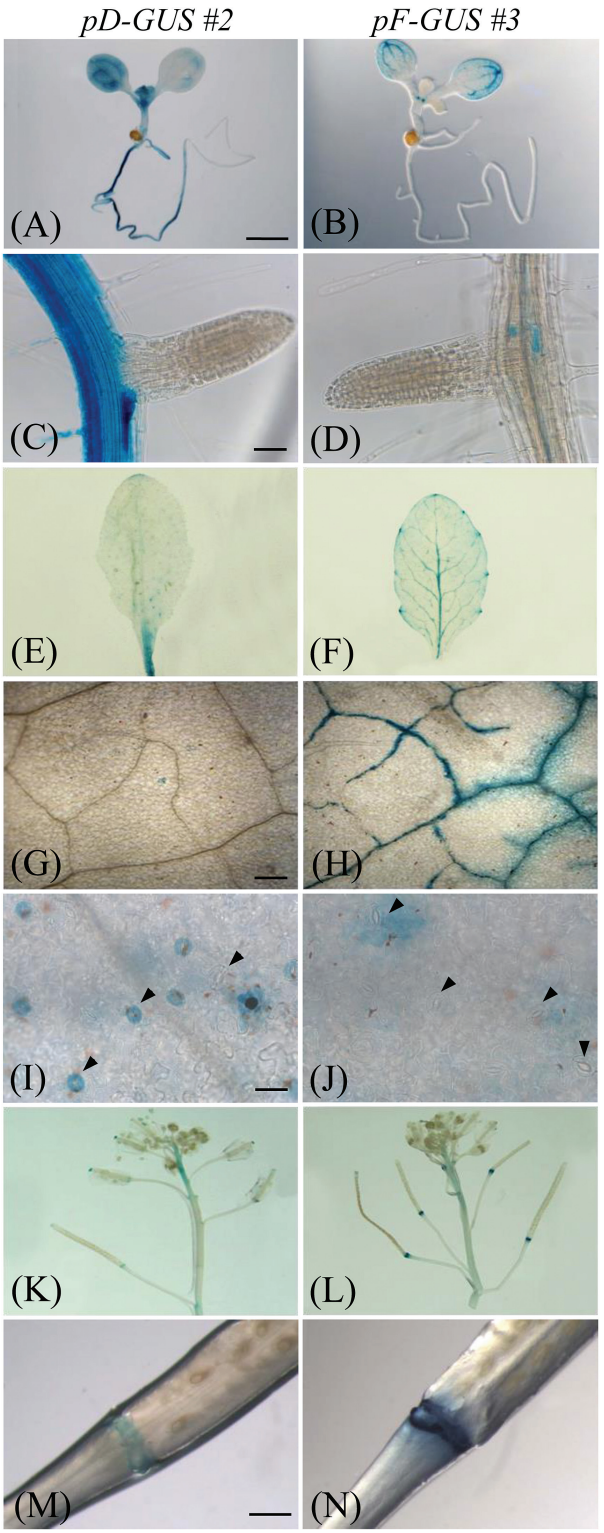

Fig. 1. *RbohD* and *RbohF* present a differential spatial expression pattern during Arabidopsis development. GUS histochemical staining of representative transgenic lines (*pD-GUS#2* and *pF-GUS#3*) in different tissues: (A, B) 6-day-old seedlings; (C, D) secondary root emerging zone; (E, F) 4-week-old 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 nM flg22 (Peptron), 100 nM elf18 (Peptron) or 1 mg ml^{-1} chitin (Yaizu Suisankagaku Industry). For bioluminescence imaging *pD-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 2.5×10^7 cfu ml^{-1} using a 1 ml syringe. For *Pc* inoculation assays, spore suspensions (4×10^6 spores ml^{-1}) of *PcBMM* or *Pc2127* isolates were sprayed on 4-week-old Arabidopsis plants grown on soil. Plants were subsequently kept under high 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 H_2O_2 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-trypan 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 Photek CCD camera (East Sussex, UK). Promoter activity values correspond to photon counts emitted by *Pto* DC3000 (*avrRpm1*) relative to $MgCl_2$ 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 mm^2 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® 269 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: DqRTfw (5′-CGAATGGCATCCTTTCTCAATC-3′) and DqRTrev (5′-GTCACCGAGAGTGCGGATATG-3′) to quantify *RbohD* expression (Penfield *et al.*, 2006); FqRTfw (5′-CTTGGCATTGGTGCAACTCC-3′) and FqRTrev (5′-TCTTTCGTCTTGGCGTGTCA-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 upon flg22 treatment 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.

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 (*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

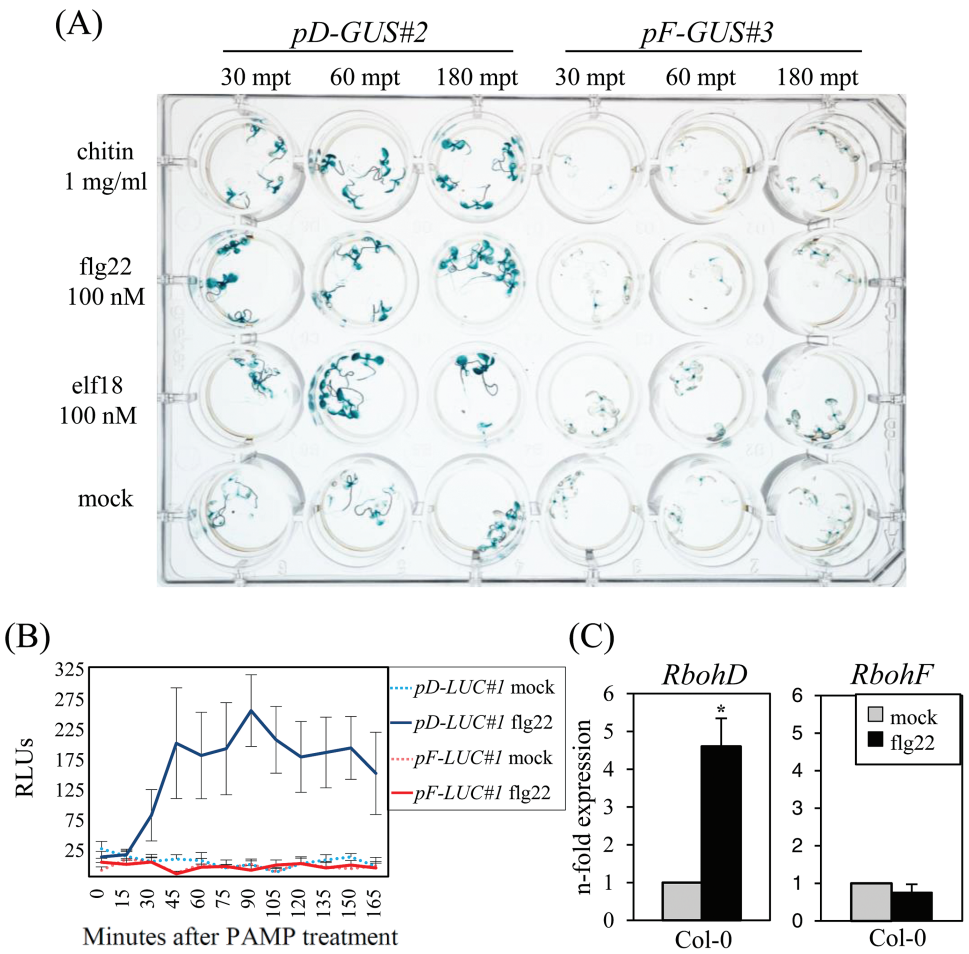

Fig. 2. *RbohD* but not *RbohF* promoter activity is induced by fungal and bacterial PAMPs. (A) GUS histochemical staining of *pD-GUS* and *pF-GUS* in 1-week-old seedlings 30, 60 and 180 min post-treatment (mpt) with the PAMPs chitin, flg22 and elf18 at the indicated concentration. (B) Time course assay 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 *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; $P<0.05$).

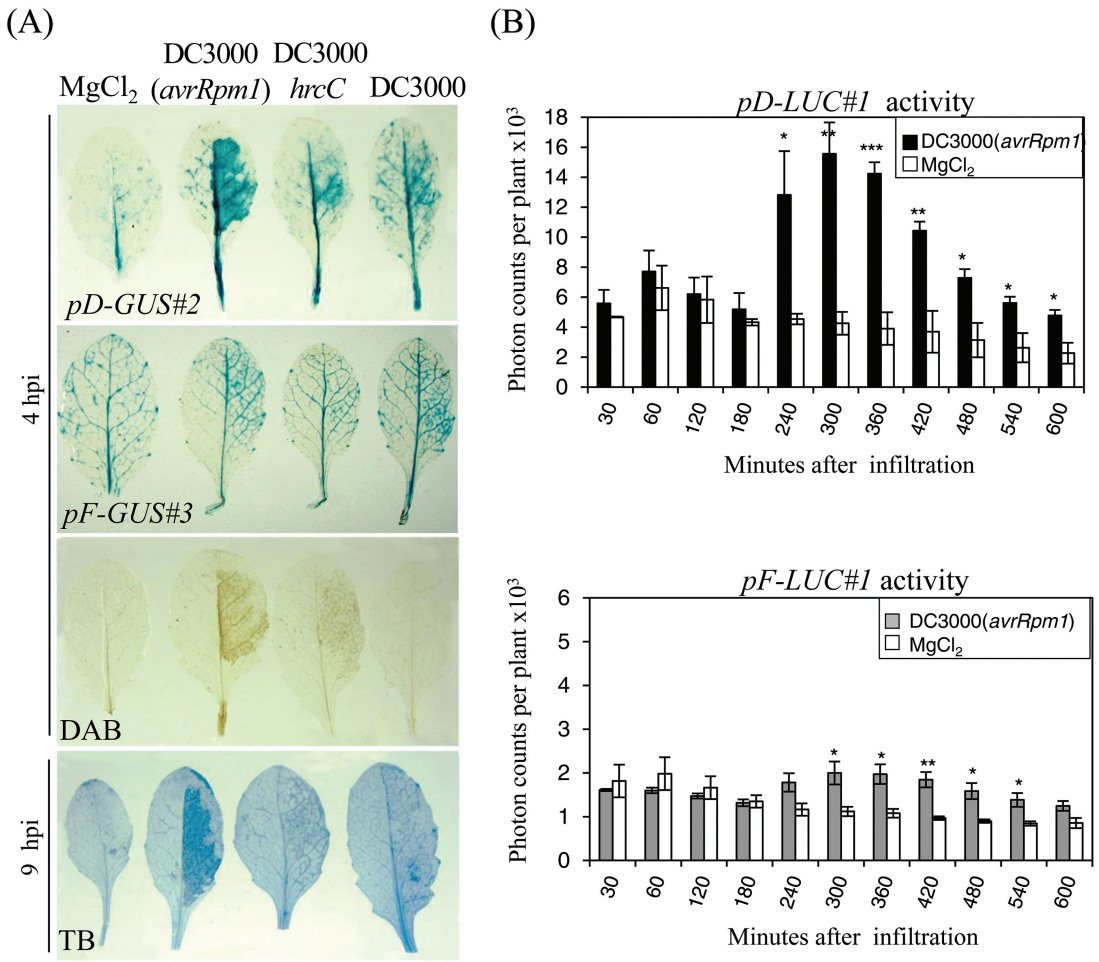

Fig. 3. *RbohD* and *RbohF* expression pattern in Arabidopsis immune response to different *Pto* DC3000 strains. (A) Histochemical stain of leaves from 4-week-old plants after injection with 10^7 cfu ml^{-1} of different *Pto* strains, (DC3000(*avrRpm1*), DC3000 *hrcC* or DC3000), or $MgCl_2$ (control): GUS stain on *pD-GUS* and *pF-GUS* plants 4 hpi, DAB stain on Col-0 plants 4 hpi and TB stain on Col-0 plants 9 hpi. (B) *In vivo* quantification of luciferase activity detected in 4-week-old *pD-LUC* and *pF-LUC* transgenic lines after infiltration with DC3000(*avrRpm1)* or $MgCl_2$. Bars represent the mean (±SE) of total photon counts emitted by the rosettes of three plants with two infiltrated leaves per treatment. Asterisks indicate significant differences at 5 hpi (Welch Student two sample *t*-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.005$). Note that the Y-axis in the *RbohF* promoter activity graphic is three times smaller than that in *RbohD*.

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 necrothophic 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.

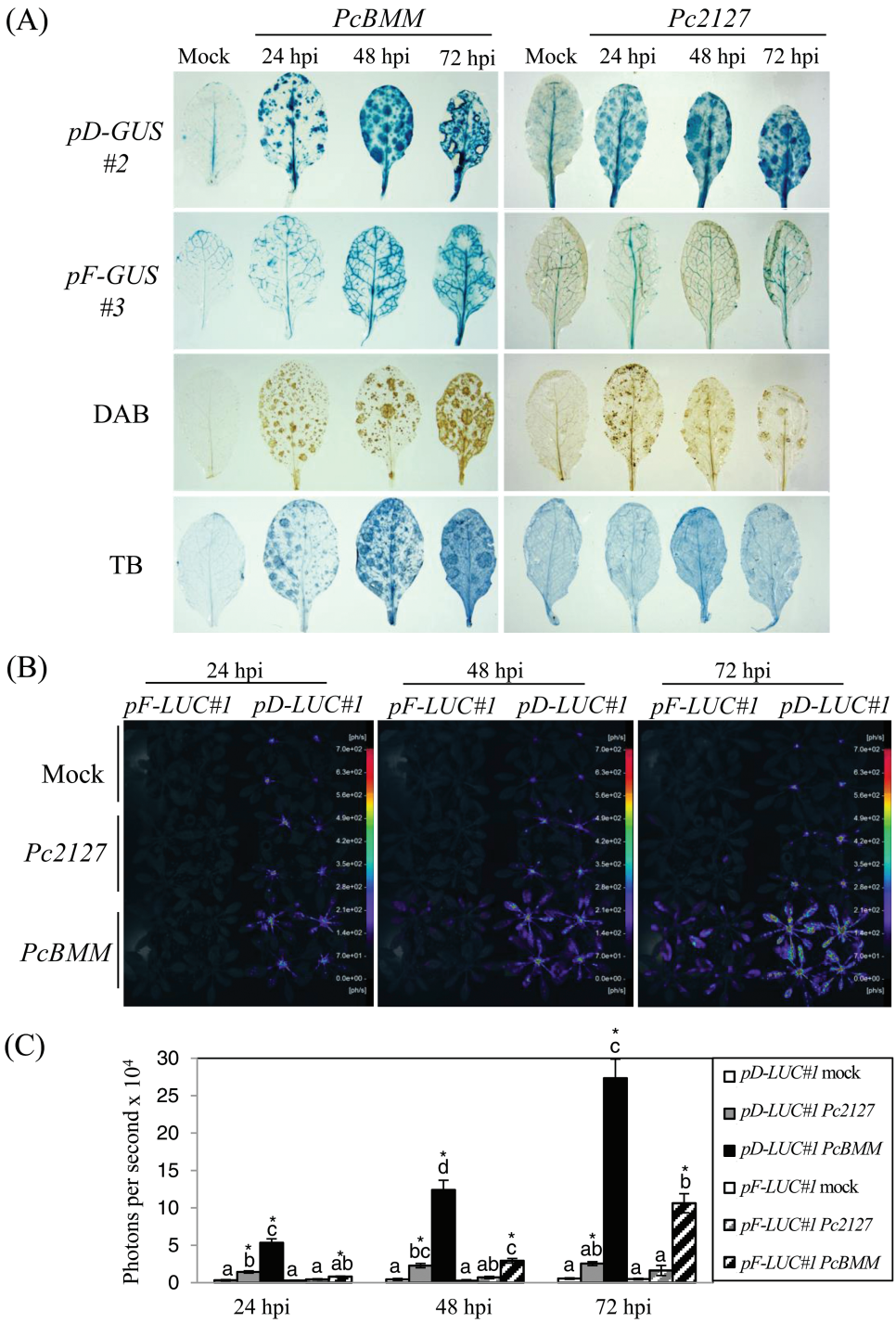

Fig. 4. Spatio-temporal expression pattern driven by *pRbohD* and *pRbohF* during Arabidopsis interaction with *P. cucumerina* isolates *PcBMM* and *Pc2127*. (A) Histochemical staining (24, 48 and 72 hpi) of 4-week-old plants sprayed with 4×10^6 spores ml^{-1} of the virulent isolate *PcBMM* (left panels) and the non-adapted isolate *Pc2127* (right panels), From top to bottom: GUS stain on *pD-GUS* and *pF-GUS* plants, DAB stain on Col-0 plants and TB stain on Col-0 plants. (B) *In vivo* bioluminescence generated by 4-week-old *pD-LUC* and *pF-LUC* transgenic plants sprayed with *PcBMM*, *Pc2127* (4 x 10^6 spores ml^{-1}) or mock-treated. (C) Quantification of *Rboh*-promoter driven luciferase activity. Bars represent means (±SE) of total photon counts over 20′ emitted by 135 mm^2 areas from plant leaves shown in B (n=12). Statistical analysis for each time point was performed by analysis of variance (ANOVA) 5%, corrected with LSD *post hoc* test. Letters indicate groups with homogenous means. Asterisks indicate statistically significant differences compared with mock.

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,

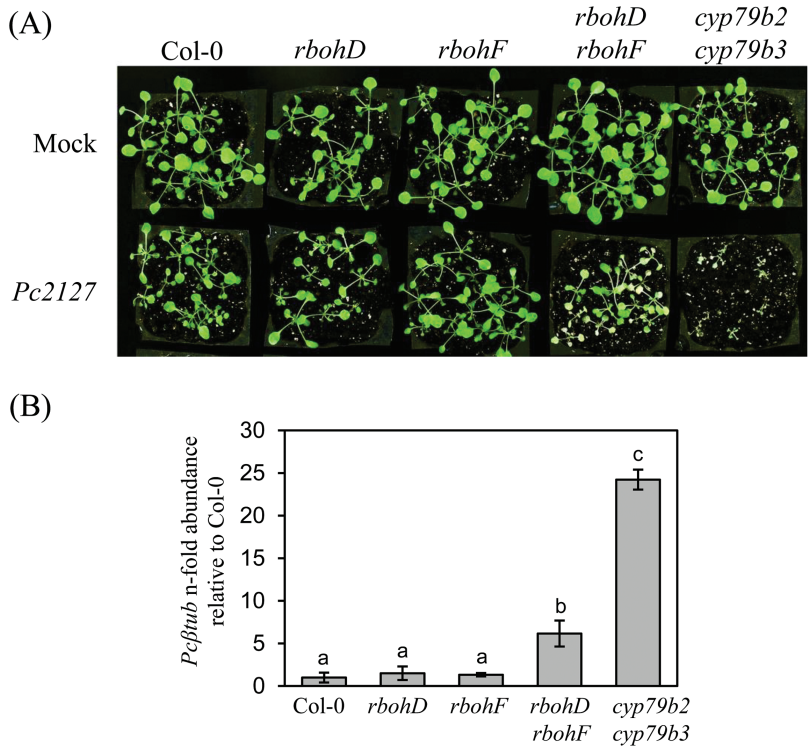

Fig. 5. The double mutant *rbohD rbohF* shows weakened basal resistance to the non-virulent *Pc2127* isolate. (A) Symptoms of 3-week-old wild-type Col-0 plants compared with those of the listed mutant genotypes at 7 dpi with *Pc2127*. (B) Quantification of *Pc2127* biomass. Fungal DNA (*P. cucumerina* 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_2O_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_2O_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.

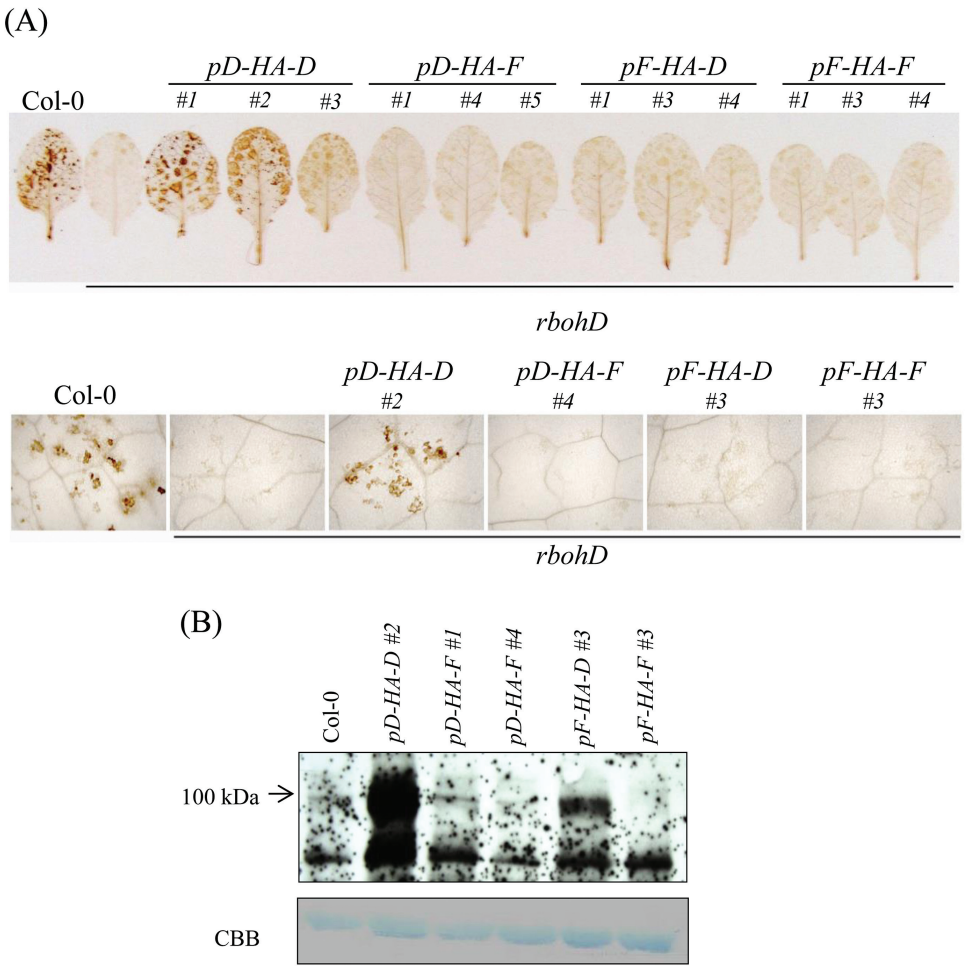

Fig. 6. Functional complementation of *rbohD* by *Rboh* promoter-swap constructs after *PcBMM* inoculation. (A) Complementation of H_2O_2 accumulation 24 hpi with *PcBMM*. H_2O_2 accumulation was detected by DAB staining in leaves from 4-week-old wild-type Col-0 and *rbohD* plants and three independent 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_2O_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 NADPH oxidases (Kadota *et al.*, 2014, 2015; Li *et al.*, 2014), *RbohD* and *RbohF* transcriptional activation might be necessary to achieve the high amount of H_2O_2 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, H_2O_2 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 H_2O_2 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 been already 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 *PcBMM* inoculation in *rbohD* mutant background, whereas constructs with the *RbohF* promoter driving *RbodD (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 (Adachi *et al.*, 2015). Moreover, potential binding sites for GT-1 transcriptions 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 *RbohF* expression and the pathogen used for challenging: *PcBMM* (Fig. 6) or *Pto* DC3000 (*avrRpm1*) (Supplementary Fig. S5B). However, transcription of *HA-RbohD* and *HA-RbohF* transgenes in PAPM-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, *AtRbohB*, 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 H_2O_2 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.

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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