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Dynamic changes in the subcellular distribution of the tobacco ROS-producing enzyme RBOHD in response to the oomycete elicitor cryptogein

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Abstract

Plant NADPH oxidases, also known as respiratory burst oxidase homologues (RBOHs), have been identified as a major source of reactive oxygen species (ROS) during plant–microbe interactions. The subcellular localization of the tobacco (Nicotiana tabacum) ROS-producing enzyme RBOHD was examined in Bright Yellow-2 cells before and after elicitation with the oomycete protein cryptogein using electron and confocal microscopy. The plasma membrane (PM) localization of RBOHD was confirmed and immuno-electron microscopy on purified PM vesicles revealed its distribution in clusters. The presence of the protein fused to GFP was also seen in intracellular compartments, mainly Golgi cisternae. Cryptogein induced, within 1 h, a 1.5-fold increase in RBOHD abundance at the PM and a concomitant decrease in the internal compartments. Use of cycloheximide revealed that most of the proteins targeted to the PM upon elicitation were not newly synthesized but may originate from the Golgi pool. ROS accumulation preceded RBOHD transcript- and protein-upregulation, indicating that ROS resulted from the activation of a PM-resident pool of enzymes, and that enzymes newly addressed to the PM were inactive. Taken together, the results indicate that control of RBOH abundance and subcellular localization may play a fundamental role in the mechanism of ROS production.

Key words: BY-2 cells, cryptogein, Nicotiana tabacum, protein trafficking, respiratory burst oxidase homolog D (RBOHD), reactive oxygen species, protein trafficking.

Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxide anions (O₂⁻), hydroxyl radicals (OH·), and singlet oxygen (¹O₂) are natural by-products of plant cell metabolism that must be detoxified to protect cellular functions from their strong oxidative properties (Halliwell, 2006). A transient increase in ROS production is observed when a
plant is exposed to a pathogen, and is referred to as the oxidative burst. ROS produced during the oxidative burst are thought to be involved in defence reactions in several ways: directly as antimicrobial agents (Chen and Schofield, 1999) or cross-linking agents during cell wall reinforcement (Bradley et al., 1992), and indirectly as signalling molecules to trigger the hypersensitive response (HR) and systemic acquired resistance (SAR) (Torres, 2010). The oxidative burst is usually biphasic with a rapid and transient phase within 1 h of pathogen recognition followed by a long-lasting second phase a few hours later that is thought to be responsible for the HR (Lamb and Dixon, 1997). Primary ROS production is predominantly apoplastic and, depending on the plant species, is dependent upon plasma membrane (PM) NADPH oxidase, cell wall peroxidase, or both (O’Brien et al., 2012).

Plant NADPH oxidases, also designated as respiratory burst oxidase homologues (RBOHs), belong to a small family of highly conserved proteins with ten members in the model plant Arabidopsis thaliana (Torres et al., 1998; Marino et al., 2011). RBOHs are membrane proteins composed of six transmembrane domains associated with two haem groups, a C-terminal region with NADPH- and FAD-binding domains, and an N-terminal regulatory region with two calcium-binding EF-hands and phosphorylation domains (Suzuki et al., 2011). RBOHs catalyse the formation of the superoxide anion \( \cdot O_2^- \) by transferring an electron from intracellular NADPH to an apoplastic molecule of oxygen (O\(_2\)) (Sagi and Fluhr, 2006). Superoxide anions, which are short-lived radicals, are then rapidly converted to hydrogen peroxide either spontaneously or by superoxide dismutase (Mori and Schroeder, 2004).

The role of RBOHs in plant–pathogen interactions has been investigated in different plant species using knockout mutants and antisense approaches. In Arabidopsis, the RBOHD and RBOHF isoforms are responsible for the ROS burst in response to MAMPs (microbe–associated molecular patterns) (Zhang et al., 2007), DAMPs (damage-associated molecular patterns) (Galletti et al., 2008), and microbial pathogens (Torres et al., 2002; Pogany et al., 2009), but with a prevalent role for RBOHD. In Nicotiana benthamiana the orthologues of AtRBOHD and AtRBOHF, named RBOHB and RBOHA, respectively, are the principal ROS producers in response to infection by Phytophthora infestans (Yoshioka et al., 2003) or Botrytis cinerea (Asai and Yoshioka, 2009), and to elicitors of plant defence reactions (Asai et al., 2008; Zhang et al., 2009). In tobacco (Nicotiana tabacum), RBOHD is the sole isoform responsible for the ROS burst in response to cryptogein (Simon-Plas et al., 2002; Lherminier et al., 2009), a proteinaceous elicitor secreted by the oomycete Phytophthora cryptogea known to induce the HR and SAR (Ricci et al., 1989; Keller et al., 1996).

Opposite results were obtained regarding the effects of the RBOH-mediated ROS burst on the HR and resistance to pathogens. In Arabidopsis, the \( \text{rbohD} \) and \( \text{rbohF} \) double mutant displayed a reduced HR and unaffected pathogen growth after inoculation with an avirulent Pseudomonas syringae strain, but an enhanced HR and resistance to the biotrophic oomycete Peronospora parasitica (Torres et al., 2002). In N. benthamiana, the \( \text{rbohB} \) and \( \text{rbohA} \) double mutant exhibited reduced HR and enhanced susceptibility after infection with the avirulent oomycete Phytophthora infestans (Yoshioka et al., 2003). These diverse effects on the HR and disease resistance suggest that RBOH-derived ROS are not simple toxic compounds triggering cell death, but components of signalling pathways that may have opposite effects on plant defence reactions (Torres et al., 2005; Torres, 2010; Marino et al., 2011). A long-distance signalling function has been demonstrated for RBOHD-derived ROS in Arabidopsis in response to various abiotic stresses (Miller et al., 2009). Signalling activities of RBOH-derived ROS are probably also modulated by other ROS sources (Bindschedler et al., 2006; Yoda et al., 2006; Daudi et al., 2012) and other signalling molecules. One of them is nitric oxide (NO) that is synthesized following pathogen recognition within the same time frame (Romero-Puertas et al., 2004). Both NO and ROS are known to react with each other to produce pro-death molecules such as singlet oxygen or hydroxyl radicals, and a balanced production within intracellular ROS and NO has been shown to be a key determinant for the HR (Delledonne et al., 2001). The recent finding that NO-mediated S-nitrosylation of AtRBOHD governs a negative feedback loop limiting the production of ROS and the HR (Yun et al., 2011) has shed some light on the mostly unknown molecular mechanisms that underpin the interplay between NO and ROS.

To get a better understanding of the cellular and physiological functions of RBOH-derived ROS as ephemeral signal molecules in plant–microbe interactions, it is essential to determine the subcellular localization and dynamics of their producers. Cell fractionation and immunolocalization studies were the first to reveal that RBOHs are intrinsic PM proteins of plant cells (Keller et al., 1998; Sagi and Fluhr, 2001; Simon-Plas et al., 2002), a result later confirmed with the use of GFP-fusions (Kobayashi et al., 2006; Takeda et al., 2008). Proteomic studies also showed that RBOHs are present in detergent-insoluble fractions (DIMs) of the PM thus suggesting that, like their animal counterparts, they could be associated in vivo with sterol- and sphingolipid-enriched domains also known as membrane rafts (Mongrand et al., 2004; Morel et al., 2006; Fujiwara et al., 2009). The non-uniform distribution of several RBOHs within the PM of differentiating cells is in line with this finding (Takeda et al., 2008; Liu et al., 2009; Lee et al., 2013). However, the cell fate of RBOHs after pathogen perception is not documented.

The focus of the present study was to characterize the subcellular localization of RBOHD in tobacco Bright Yellow-2 (BY-2) cells before and after elicitation with cryptogein using confocal and electron microscopy. Two RBOHD isoforms were identified in BY-2 cells. The RBOHDs were found to reside in the PM in the form of small clusters in the Golgi cisternae and in a second, as yet unidentified, intracellular compartment. The RBOHDs partitioning between the PM and endomembranes were further shown to be altered following elicitation. Finally, examination of the relationships between transcriptional control, subcellular dynamics, and activity of RBOHDs revealed that control of RBOH abundance and localization may play a fundamental role in the mechanism of ROS production in the context of plant defence responses.
Materials and methods

Materials

Tobacco BY-2 cells (N. tabacam cv. Bright Yellow-2) were grown at 25°C under continuous light (200 μE m⁻² s⁻¹) on a rotary shaker (140 rpm). The suspensions were sub-cultured every 7 days at 2.80 ml dilution, in MS medium (Murashige and Skoog, 1962) supplemented with 90 mM sucrose, 1.5 mM KH₂PO₄, 0.55 mM inositol, 1 μM 2,4-D, 3 μM thiamine, and 10 mM MES pH 5.6.

ROS determination

Seven-day-old cells were harvested, filtered, and re-suspended (1 g for 10 ml) in buffer (175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, 2 mM MES pH 5.8) for a 3 h equilibration period on a rotary shaker (140 rpm), then elicited by the addition of 50 nM cryptogein. Cryptogein was purified from P. cryptogeia according to Ricci et al. (1989). The production of H₂O₂ was measured by chemiluminescence using luminol and a luminometer (BCL book, Berthold). Every 2 min, a 250 μl aliquot of the cell suspension was added to 50 μl of 0.3 mM luminol and 300 μl of the assay buffer (175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, 50 mM MES pH 6.5).

Plasmid constructions

The RBOHD1 and RBOHD2 cDNAs were obtained by reverse transcription of BY-2 total RNA using the ImProm-II™ reverse transcription system (Promega) and PCR amplification using primer pairs attB1-NotD5/attB2-NotD1-2 and attB1-NotD5/attB2-NotD2-1, respectively (primers are given in Supplementary Table S1 available at JXB online). The PCR products were cloned into Gateway entry vector pDONR221 (Invitrogen) for sequencing. The RBOHD1 gene was PCR-amplified from BY-2 genomic DNA using primer pair attB1-NotbohD4/attB2-NotbohD1 and cloned into Gateway entry vector pDONR-Zeo (Invitrogen). It was then subcloned into plant transformation vector pMD83 (Curtsis and Grossniklaus, 2003) in which the 35S promoter was deleted by digestion with restriction enzymes HindIII/SpeI, blunting of S'-overhangs with Klenow fragment and re-circularization. The Golgi marker Man99-monomeric red fluorescent protein (mRFP) was a gift from Claude Saint-Jore-Dupas (Boulaflous et al., 2009). It corresponds to the first 99 amino acids of Glycine max alpha-mannosidase I fused to mRFP.

Plant cell transformation

The RBOHD1-GFP and Man99-mRFP constructs were introduced into Agrobacterium tumefaciens strain C58C1 by freeze thawing. RBOHD1-GFP-containing agrobacteria were used to transform BY-2 cells according to Brandizzi et al. (2003). Cells were plated onto agar-MS medium containing 35 mg l⁻¹ hygromycin. Transformed microcalli were transferred into MS liquid medium supplemented with 35 mg l⁻¹ hygromycin and submitted to constant agitation (140 rpm) at 25°C under continuous light to generate cell suspensions. Eight weeks of subculturing were needed before cell suspensions cultures became stable. RBOHD1-GFP cells were retransformed with Man99-mRFP construct and selected onto 100 mg l⁻¹ kanamycin to obtain the doubly transformed cell line.

Real-time qRT-PCR analysis

Total RNA from 7-day-old BY-2 cells was isolated with the RNeasy Plant Mini Kit (Qiagen) and treated with Ambion DNA-free™ DNase (Life Technologies) to eliminate genomic DNA. First-strand cDNA was produced from 1 μg of total RNA using the ImProm-II™ reverse transcription system (Promega). The product was diluted 1:3 with water, and 2 μl was used as a template for RT-qPCR amplification with a GoTaq® qPCR Master Mix (Promega) on an ABI PRISM 7900HT system (Applied Biosystems). The housekeeping genes EF-1α, L25, and PP2A, whose expression has been shown to be stable in tobacco (Schmidt and Delaney, 2010), were used to normalize candidate gene transcripts. Primers were designed using Primer3 software (Untergasser et al., 2012) and are listed in Supplementary Table S1 available at JXB online. Amplifications were carried out for three independent RNA preparations and two technical replicates. Amplification specificity was checked by melting-curve analysis. PCR efficiency was determined using standard curves obtained with serial dilutions of PCR products as templates and shown to be close to 100% for all primer pairs. Data were analysed using the SDS 2.3 software (Applied Biosystems) to obtain cycle threshold values (Ct). Ct values were normalized to the geometric means of three reference genes (ΔCt) and relative expression values were calculated (2⁻ΔCt).

Cell fractionation

Seven-day-old cells were collected by filtration, frozen in liquid nitrogen and homogenized in grinding medium (500 mM sucrose, 20 mM EDTA, 10 mM DTT, 1 mM PMSF, 50 mM Tris-MES pH 8.0). The homogenate was centrifuged at 16 000 g for 20 min. After centrifugation, supernatants were collected, filtered through successive screens (63 and 38 μM) and centrifuged at 96 000 g for 35 min. The microsomal fraction was purified by partitioning in an aqueous two-phase system containing polyethylene glycol 3350/dextran T-500, 6.6% each (Larsson et al., 1994), to obtain the PM fraction, which was re-suspended in storage buffer (250 mM sucrose, 1 mM EDTA, 10 mM DTT, 20% glycerol, 10 μg ml⁻¹ leupeptin, 10 μg ml⁻¹ pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 mM Tris-MES pH 7.3). The amount of protein present in the PM fraction was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

Western blot analysis

Samples of 20 μg protein from PM fractions were solubilized in a buffer containing 40 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 1.5% SDS, 1 mM EDTA, 2 M/l M urea/thiourea, 1% n-octyl glucoside, 10% glycerol, and bromophenol blue for 2 h at room temperature, then loaded on a 4-8% SDS-polyacrylamide gel. After electrophoresis separation (1 h, 40 mA) protein fractions were electroblotted onto nitrocellulose membrane in a buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS (2.5 h, 200 mA). The membrane was blocked with TBS-Tween buffer (150 mM NaCl, 0.05% Tween 20, 20 mM Tris pH 7.6) containing 5% milk. Probing and detection of western blots were performed as described in the ECL Western Blotting detection kit (Amersham). The RBOHD antibody used for western blots and immunogold labelling (see below) was a rabbit polyclonal antibody raised against amino acids 138–152 (CLNKRPIPTGRFDRNK) of RBOHD1 (Simon-Plas et al., 2002). Dot-blot analysis revealed that the antibody only recognized oligopeptide 138–152. The fact that this oligopeptide is also present in the N-terminal region (IAKNKGNKSGSASGGC) of RBOHD1 (Curtsis and Grossniklaus, 2003) is consistent with the antibody being directed against the N-terminal region. The RBOHD antibody used for western blots and immunogold labelling was produced in rabbit and was a polyclonal antibody raised against amino acids 138–152 (CLNKRPIPTGRFDRNK) of RBOHD1 (Simon-Plas et al., 2002). Dot-blot analysis revealed that the antibody only recognized oligopeptide 138–152. The fact that this oligopeptide is also present in the N-terminal region (IAKNKGNKSGSASGGC) of RBOHD1 (Curtsis and Grossniklaus, 2003) is consistent with the antibody being directed against the N-terminal region.

Confocal microscopy

Seven-day-old cells were mounted in culture medium or in 12 buffer when cryptogein treatment was required. FM4-64 (4.25 μM final) was added to the cell suspension in the dark at 25°C and labelling was imaged at different time points. Brefeldin A (BFA) (40 μM final) was added to the cell suspension 60 min prior to imaging. Cryptogein (50 nM final) and cycloheximide (CHX) (50 μg ml⁻¹ final) were added alone or in combination, and effects were...
observed at different time points. Images were acquired using a Leica TCS SP2-AOBS laser scanning confocal microscope with the 488 nm line of an argon laser for GFP and FM4-64, and with the 594 nm line of a helium-neon laser for mRFP. GFP fluorescence was band-pass filtered between 500 and 550 nm, FM4-64 fluorescence between 660 and 700 nm, and mRFP fluorescence between 620 and 700 nm. Images were processed using Photoshop CS5 (Adobe Systems). The PM fluorescence was quantified using image J software version 1.47h (Schneider et al., 2012) as follows. One-pixel-wide lines (5–15 μm length) were drawn along the PM of at least 30 BY-2 cells per condition and time point, and average fluorescence intensity was measured. Fluorescence intensities were normalized to the time-0 point and graphed as percentages (mean ± SD; n = 5–16). Fluorescent intracellular compartments were scored at the periphery of the PM and scaled to 100 μm of PM using Image J. Compartment numbers were normalized to the time-0 point and graphed as percentages (mean ± SD; n = 6–11).

High-pressure freezing and freeze substitution for transmission electron microscopy

The BY-2 cells suspended in I2 buffer were concentrated by centrifugation (2000g, 10 s), and immediately frozen, without addition of cryoprotectant, in a Leica EM HPM 100 high-pressure freezer. Freeze substitution was subsequently performed using automatic Leica EM AFS1 pre-cooled to −90°C. Samples were substituted in anhydrous acetone containing 0.2% uranyl acetate and 0.1% glutaraldehyde at −90°C for 72 h. The temperature was gradually increased to −50°C (slope 5°C h⁻¹) and kept at this temperature. Samples were rinsed with pure acetone, then pure ethanol for 24 h for each solvent. They were then gradually infiltrated with mixtures of ethanol/Lowicryl HM20 (with increasing concentrations of resin) and finally embedded in pure Lowicryl HM20. Polymerization was carried out at −50°C for 48 h, followed by 24 h at −35°C, and finally 24 h at 0°C. Following polymerization, the blocks of Lowicryl-embedded BY-2 cells were ready for thin sectioning and immunolabelling.

Immunogold labelling of RBOHDs on thin sections of BY-2 cells and on purified PM

Ultrathin sections of tobacco cells were collected onto carbon-collodion-coated nickel grids. Grids were treated with 10 mM PBS pH 7.2, supplemented with 10 mM glucose for 15 min, then with PBS containing 0.5% milk, 10% normal goat serum and Auranin blocking solution for 30 min (Auranin), and then with polyconal anti-RBOHD antibody at a 1:50 dilution in PBS containing 0.1% BSA-c (Aurion) for 1 h at room temperature. Incubation with goat secondary antibody conjugated to 6 nm gold particles (GPs; Sigma-Aldrich) and diluted 1:25 in PBS containing 0.1% BSA-c was then performed for 1 h at room temperature. Purified BY-2 cell PM vesicles, pretreated or not with 20 mM methyl-β-cyclodextrin (MβCD) in buffered conditions (150 mM NaCl, 1 mM PMSF, 20 mM Tris pH 7.6) under constant agitation, were directly deposited onto collodion and carbon-coated microscope nickel grids. 10 μl of PM vesicles (0.2 μg μl⁻¹) were deposited on each grid. Grids were floated, during 30 min at room temperature, on 20 μl droplets of TBS containing 0.1% BSA, 0.1% glycerine, and 5% normal goat serum (NGS), to reduce unspecific background. After three washing steps of 5 min in droplets of TBS, the grids were incubated for 1 h at RT with anti-RBOHD antibody or anti-GFP antibody diluted 1:50 in TBS. Antibody was detected with 5 nm gold-labelled goat anti-rabbit IgG [EM GAR 5 nm, British Biocell International (BBI)] or 5 nm gold-labelled Protein A (EM protein A 5 nm, BBI) at the dilution 1:20 for 45 min at room temperature. After a 10 min fixation in 0.1 M phosphate buffer containing 2.5% glutaraldehyde, preparations were negatively stained during 30 s in 1% ammonium molybdate at room temperature and air-dried. Sections and grids were observed with a Hitachi H7500 transmission electron microscope operating at 80 kV equipped with an AMT camera driven by AMT software (Hitachi).

Labelling analysis and spatial statistics

In order to characterize PM vesicle labelling, the density of labelling was evaluated by counting the number of GPs per square micrometre of PM. When groups of GPs were identified on a vesicle, distances between all GPs in a group were measured with AMT software and the proportion of GPs in groups (2, 3, and 4 particles) was compared to total labelling. Counting and measurement were performed on three repetitions for each time of treatment and 30 PM vesicles per sample were observed. For spatial statistics, coordinates of the GPs and vesicle contours were determined using ImageJ software version 1.47h (Schneider et al., 2012). The GP patterns within PM vesicles were considered as realizations of a stationary point process observed in windows of varying sizes and shapes. The spatial distribution of a stationary point process can be quantified based on Ripley’s K-function (Ripley, 1976). For a given radius r, K(r) = N(r)/λ² where N(r) is the expected number of neighbours lying within distance r from a typical point and λ is the mean number of points per unit area. For a completely random (Poisson) point process, K(r) = πr². When K is above πr², a point process is considered as spatial clustering. When K is below πr², it is considered as spatially repulsive. Estimation of the K-function was performed using the translation correction (Ohsler, 1983). Instead of computing individual estimates on each realization (PM vesicle), a global estimation was performed by pooling together all observed patterns using the approach proposed by Baddeley et al. (1993). Computation of the global estimates was implemented as a modified version of the Kest function provided by the R spatstat package (Baddeley et al., 1993; R Development Core Team, 2009). Simulation envelopes of the K-function under the Poisson hypothesis were computed from a modified version of the envelope function of spatstat.

Results and discussion

Two RBOHD genes are expressed in BY-2 cells

In order to express an RBOHD-GFP fusion protein at native level in tobacco BY-2 cells, a BLAST search of the SOL genomic network (SGN) database was performed with RBOHD cDNA (accession number AJ309006.1) to identify the tobacco RBOHD gene. The search identified two copies of the RBOHD gene, which were named RBOHD1 and RBOHD2 (Fig. 1). N. tabacum is a young allotetraploid species resulting from the hybridization of the diploid species N. tomentosiformis and N. sylvestris less than 200 000 years ago (Leitch et al., 2008). A second BLAST search of the SGN database identified a single RBOHD gene in the N. tomentosiformis genome (NtRBOHD) and N. sylvestris genome (NsRBOHD). The intron insertion pattern was identical in all four genes (Fig. 1). Intron length was different for RBOHD1 and RBOHD2, but almost identical for RBOHD1 and NsRBOHD, and for RBOHD2 and NtRBOHD. The cDNAs for RBOHD1 and RBOHD2 were successfully isolated from BY-2 cells. Both sequences shared 98.4% identity at the amino-acid level and 96.7% identity at the nucleotide level in their coding regions (Supplementary Figure S1 available at JXB online). Sequence comparison with N. tomentosiformis and N. sylvestris RBOHD coding regions showed that RBOHD2 shared 100% identity at the nucleotide level with...
**NtoRBOHD** and **RBOHD1** displayed a single mismatch with **NsRBOHD** (data not shown). Altogether, the results indicate that **RBOHD1** and **RBOHD2** are two homeologous genes originating from *N. sylvestris* and *N. tomentosiformis*, respectively, that are transcribed in BY-2 cells.

RBOHDs localize to the PM and endomembranes

The **RBOHD1** gene including a ~2-kb fragment upstream of the start codon was fused to GFP coding sequence and used to transform BY-2 cells. GFP fluorescence was detected at the PM and also intracellularly in the form of dots and rings (Fig. 2A–C). Similar fluorescence patterns were observed with 35S-driven fusion constructs **RBOHD1-GFP**, **GFP-RBOHD1**, and **RBOHD2-GFP** suggesting that cell localization of the fusion protein is not significantly influenced by its expression level, the position of the fluorescent tag, or the identity of the isoform (data not shown).

Unlike their animal counterparts (Ushio-Fukai, 2009), plant NADPH oxidases have not been reported to be associated with intracellular compartments. This prompted us to identify the ones that are labelled by RBOHD1-GFP.

Fluorescent rings were previously observed in plant cells expressing Golgi fusion proteins (Robinson et al., 2008). RBOHD1-GFP rings were identified as Golgi from the following results. Firstly, RBOHD1-GFP co-localized with the Golgi marker Man99-mRFP (Boulafflous et al., 2009) and formed a rim around the Man99-GFP signal, suggesting a location at the periphery of the Golgi cisternae (Fig. 2D–F). Secondly, the RBOHD1-GFP signal was found in close apposition with that of FM4-64, an endocytic tracer that labels the *trans*-Golgi network (TGN) and the *trans* side of the Golgi stack 30 min after internalization (Bolte et al., 2004; Lam et al., 2007) (Fig. 2G–I). Thirdly, RBOHD1-GFP labelling was sensitive to treatment with BFA, a fungal toxin that causes aggregation of Golgi stacks and TGN into BFA bodies (Ritzenthaler et al., 2002). Upon BFA treatment, RBOHD1-GFP relocated to large aggregates that were also labelled by FM4-64 30 to 45 min after uptake (Fig. 2J–L).

RBOHD1-GFP-labelled dots were not identified in the course of our study. Co-localization studies performed with FM4-64 over a 1 h time course always showed the continuous presence of a population of GFP-only labelled dots (Fig. 2M–O). At time 60 min, FM4-64 labelled the tonoplast (Fig. 2O). This result suggests that the unidentified compartment does not lie on the endocytic pathway and could correspond to an exocytic compartment that delivers RBOHDs from the Golgi to the PM via a TGN-independent route. A similar exit route from the Golgi complex has been suggested for the cellulose synthase complex in *Arabidopsis* (Crowell et al., 2009).

Knowing that the location of a fusion protein may be different from that of its native form, ultrathin sections of BY-2 cells were immunogold-labelled with an antipeptide antibody that recognized the two RBOHD isoforms. Gold particles were observed at the PM, at the periphery of the Golgi cisternae, and associated with vesicle-like compartments (Fig. 2P–R), supporting the results of the live cell fluorescence microscopy studies. It should be mentioned that overall labelling density was low, either arguing for low abundance of the native RBOHDs or for poor epitope accessibility.

Altogether, the confocal and electron microscopy studies revealed that RBOHD isoforms were partitioned between the PM and endomembranes in BY-2 cells.

RBOHDs are organized in clusters within the PM

Despite the low density of the labelling observed on BY-2 cell sections, GPVs linked to the anti-RBOHD antibody mostly occurred as groups of 2–4 on cell PM and endomembranes (Fig. 2P–R). Purified PM vesicles of BY-2 cells were probed with the anti-RBOHD antibody to analyse the distribution pattern of RBOHDs on the PM surface (Fig. 3A). The mean density of the labelling over three independent biological
Fig. 2. RBOHDs mostly localize to the PM and Golgi in BY-2 cells, as shown by confocal microscopy of RBOHD1-GFP-expressing BY-2 cells (A–O) and immunogold labelling of RBOHDs on ultrathin sections of wild-type cells (P–R). (A–C) Cells expressing RBOHD1-GFP and stained with the endocytic tracer FM4-64 for 5 min. Shown are GFP fluorescence alone (A, C) and an overlay of GFP and FM4-64 fluorescence (B). GFP signals were observed at the PM [white arrowhead in (A)] and in intracellular dots and rings [yellow arrowhead and yellow arrow, respectively, in (C)]. (D–F) Cells co-expressing RBOHD1-GFP and the Golgi marker Man99-mRFP. Shown are GFP fluorescence (D), mRFP fluorescence (E), and overlay (F). The insets show that RBOHD1-GFP labelled the margin of the Golgi. (G–I) Cells expressing RBOHD1-GFP and stained with the endocytic tracer FM4-64 for 30 min. Shown are GFP fluorescence (G), FM4-64 fluorescence (H), and overlay (I). The inset in (I) shows tricoloured labelling due to partial overlap. (J–L) Cells expressing RBOHD1-GFP, treated with BFA for 60 min and with FM4-64 for 30 min. Shown are GFP fluorescence (J), FM4-64 fluorescence (K), and overlay (L). The yellow arrowhead indicates a BFA body. (M–O) RBOHD1-GFP-expressing cells were stained with FM4-64 for 10 min (M), 20 min (N), and 60 min (O). Shown are overlays of GFP and FM4-64 fluorescence. Yellow arrow and arrowheads indicate FM4-64-labelled tonoplast and green-only dots, respectively. (P–R) Immunogold-labelled sections of BY-2 cells performed with anti-RBOHD antibody. Black arrowheads indicate GPs associated with the PM (P), the margin of the Golgi (Q), and a vesicle-like compartment (R). Scale bar represents 10 µm (A–O), 2 µm (insets in panels D–I), 500 nm (P–R), and 100 nm (insets in panels P–R).
the spatial distribution of animal membrane proteins (Prior et al., 2003; Lillemeier et al., 2006). K-function analysis confirmed that the gold pattern was aggregated, since K(r) values laid clearly above the simulation envelope for the K-function of completely random patterns (Fig. 3C, left panel). To rule out the possibility that groups might correspond to the binding of several secondary antibodies to a single primary antibody, a fourth experiment was performed with protein A-gold conjugates (PAG). The number of particles within groups was only slightly reduced (by 10%) in PAG-labelled vesicles compared to secondary antibody-labelled vesicles (Fig. 3B), clearly indicating that RBOHs are organized in clusters at the PM. Labelling of PM vesicles prepared from RBOHD1-GFP cells with an anti-GFP antibody revealed that the fusion protein forms similar clusters in the PM (Supplementary Figure S3 available at JXB online), but with a lower density that could be due to the lower sensitivity of the GFP antibody and/or the lower abundance of the antigen.

Similarly, NADPH oxidase from human neutrophil was reported to be distributed in clusters within the PM (Wientjes et al., 1997). In line with this finding are the non-uniform distribution of several plant RBOHs in the PM of differentiating cells (Takeda et al., 2008; Liu et al., 2009; Lee et al., 2013) and the demonstration that H$_2$O$_2$ resulting from the activity of RBOHs upon cryptogein treatment was observed as discrete patches along the PM of BY-2 cells (Lherminier et al., 2009).

Such a clustering is also in agreement with previous experiments showing that RBOHs are found in the DIM fractions of tobacco (Mongrand et al., 2004; Morel et al., 2006) or rice (Fujiwara et al., 2009). Indeed, preferential association of a protein (or lipid) with sterol- and sphingolipid-enriched DIM fractions has been proven to be indicative of its presence in subdomains of the membrane in numerous studies (reviewed by Simons and Gerl, 2010). Like RBOHs, the lipid PIP$_2$, and the proteins remorin and flotilin, have been found predominantly associated with the DIM fractions of plant PM and detected by immuno-electron microscopy in PM nanodomains of 25, 70, and 100 nm, respectively (Raffaele et al., 2009; Furt et al., 2010; Li et al., 2012).

The sterol-chelating agent MjβCD was used on PM vesicles to assess the possible sterol-dependency of the clustered distribution. A 30-min treatment with MjβCD, which removed ~60% of PM sterols (data not shown), increased by 16% the number of isolated particles but did not modify either the labelling density or the size of the GP groups (Fig. 3B). Accordingly, K-function analysis of MjβCD-treated vesicles indicated that the distribution of GPs was still clustered, although the amplitude of K curves was lower than for untreated vesicles, suggesting a decrease in the degree of aggregation after treatment with MjβCD (Fig. 3C, right panel). This indicates that neither the association of RBOH proteins to the PM nor their pattern within the membrane is fully dependent upon PM sterol content. Similar results have been obtained for several raft markers such as ganglioside M1 in mouse fibroblast PM (Fujita et al., 2007), a tyrosine kinase lek-derived oligopeptide in mouse T-cell PM (Lillemeier et al., 2006), and PIP$_2$ in tobacco cell PM (Furt et al., 2010).
The small and homogeneous size of the RBOHDs clusters, together with their relative resistance to cyclodextrin, suggests that RBOHDs might be present in plant membranes as oligomers. Interestingly, Nox5, an animal NADPH oxidase which is structurally more closely related to plant RBOHs than any of the other animal isoforms (Suzuki et al., 2011), forms an active oligomer in the PM (Kawahara et al., 2011). Moreover, structure and physiological studies of rice RBOHB revealed that it could form a functional dimer (Oda et al., 2010). This does not question the fact that RBOHD clusters might reside in particular sterol-rich domains of the membrane. Indeed, in animal cells, several classes of proteins have been demonstrated to be associated with membrane domains as oligomers. For instance, the matrix protein VP40 from the Ebola virus is found essentially in the DIM fractions as oligomers whereas the low amount of the protein present in the soluble fraction consists mostly of monomers (Panchal et al., 2003). For members of the flotillin family, found associated to membrane rafts in various models and proven to form oligomers, oligomerization is necessary to mediate association with DIMs (Neumann-Giesen et al., 2004). Furthermore, oligomerization of the amyloid β-peptide is ganglioside-dependent and MβCD-insensitive within lipid rafts of CHO cells (Kim et al., 2006). Finally, a very interesting study with glycosylphosphatidylinositol-anchored proteins in MDCK epithelial cells identified oligomerization as one of the determinants of their association to sterol-rich domains in the Golgi and their polarized transport to the apical cell membrane (Paladino et al., 2004).

All these data are thus consistent with the hypothesis that RBOHDs might reside as oligomers within sterol-rich domains of the tobacco PM. Further investigations such as blue native gel and radiation inactivation analyses will be required to ascertain the oligomeric nature of RBOHDs.

**Intracellular and PM-associated pools of RBOHDs are differentially affected following cryptogein treatment**

Elicitation of tobacco cells by cryptogein induces a rapid and transient production of ROS (Viard et al., 1994) that depends upon the activity of RBOHDs (Simon-Plas et al., 2002). In BY-2 and RBOHD1-GFP-expressing cells, ROS production peaked 10–15 min after cryptogein addition then decreased to a level slightly above basal levels at 30 min (Supplementary Figure S2 available at JXB online). ROS production was consistently higher in RBOHD1-GFP-expressing cells suggesting that the fusion protein retained some enzymatic activity as previously reported for an N-terminal fusion (Takeda et al., 2008). As this is not direct evidence for activity, one has to keep in mind that the fusion enzyme could exhibit a reduced enzymatic activity due to the presence of the fluorescent tag. To better understand the transient production of ROS, the subcellular distribution of RBOHDs was investigated within the first hour of elicitation.

The first obvious change observed in elicited RBOHD1-GFP cells was an increase in PM fluorescence (Fig. 4A). PM fluorescence intensity of RBOHD1-GFP cells was significantly different from that of untreated cells at 30 min and was ~40% higher at 60 min (Fig. 4B). PM accumulation of the fusion protein was also shown by western blot analysis of PM-enriched fractions using GFP antibody (Fig. 4C). Furthermore, immunodetection with an antibody recognizing both RBOHD1 and RBOHD2 revealed that native isoforms accumulate at the PM 60 min after elicitation (Fig. 4C). Immunolabelling of PM vesicles also showed a 50% increase in the number of GPs (Fig. 4D). All these approaches thus yielded a similar result, namely an increase of about 50% of the amount of RBOHDs present on the PM after 1 h of cryptogein treatment, and further confirmed that the fusion protein is a faithful reporter for monitoring RBOHD subcellular dynamics.

Simon-Plas and collaborators previously showed that cryptogein upregulates RBOHD transcription in tobacco cells (Simon-Plas et al., 2002). This was confirmed in the present study using real-time PCR. RBOHD1 and RBOHD2 transcript levels were upregulated ~1.5-fold 30 min after elicitation and ~2.3-fold at time 60 min (Fig. 5). Transcriptional upregulation could explain the 50% increase in abundance of RBOHDs at the PM. However, treatment of RBOHD1-GFP-expressing cells with the protein synthesis inhibitor CHX had very little effect on the cryptogein-triggered increase in PM fluorescence (Fig. 6A). This indicates that RBOHD1-GFP accumulation at the PM after exposure to cryptogein is not mainly due to the delivery of newly synthesized proteins.

At the same time as the increase in PM fluorescence, a decrease in the number of fluorescent intracellular compartments was observed in elicited cells. Indeed, cryptogein induced a 2-fold reduction in the number of these compartments 30 min after treatment (Figs 4A and 6B). A mechanistically relevant explanation is that cryptogein triggers the targeting to the PM of RBOHDs released from intracellular stores. The combination of cryptogein and CHX led to a decrease in the number of fluorescent intracellular compartments 60 min after elicitation which was much more pronounced than with cryptogein alone, suggesting that internal stores are both the early source of RBOHD proteins accumulating at the PM and the late destination of newly synthesized proteins arriving from the endoplasmic reticulum.

Altogether these results are in favour of a distribution of RBOHDs between PM and Golgi pools that is shifted in favour of the PM pool following elicitation, first by the delivery of proteins already present in the Golgi, and then by the synthesis of new proteins. This raises the question of the Golgi as a genuine reservoir for RBOHDs. There is no report in the literature of any Golgi-localized NADPH oxidase in plant or animal cells. However, there are several examples of PM-associated proteins that cycle between the PM and the Golgi (Nichols et al., 2001; Le and Nabi, 2003; Milhas et al., 2010). Interestingly, these proteins have been found associated with lipid rafts. Raft assembly is presumed to be initiated at the ER with proteins being incorporated at the Golgi complex (Brown and London, 1998; Heino et al., 2000). Nichols and collaborators have raised the possibility that recycling of PM raft components could play a role in raft assembly and in the regulation of signalling pathways that are lipid raft-dependent (Nichols et al., 2001). Further experiments
Subcellular dynamics of RBOHD upon elicitation

Involving pharmacological and/or genetic alterations of the endocytic and exocytic pathways, coupled with the use of photoswitchable fluorescent proteins, will be required to demonstrate that a similar cycling operates in plant cells to regulate the abundance of RBOHD at the PM.

Insights on the regulation of RBOHD activity

Since RBOHD-mediated ROS production was observed a few minutes after elicitation with cryptogein (Fig. 5), we asked whether RBOHD activation was associated with their redistribution in the PM. The percentage of GPs within groups and the size of the groups did not change significantly 5 min after elicitation (Fig. 4D), indicating that activation of the protein that occurs within this time frame does not result from the aggregation of enzyme-containing nanodomains to generate large redox platforms as documented for animal cells (Jin et al., 2011). Our results, however, do not rule out the possibility that signalling platforms are formed from the coalescence of nanodomains containing different sets of signalling components including RBOHDs, or targeting of regulatory proteins to RBOHD nanodomains. Consistent with

Fig. 4. PM abundance of RBOHDs increases upon elicitation by cryptogein. (A) RBOHD1-GFP-expressing cell before (Ctrl) and after 60 min incubation with cryptogein (Cry 60 min). Scale bars = 10 µm. (B) Quantification of the PM fluorescence in RBOHD1-GFP cells. Values are means ± SD of 5–16 independent experiments. An asterisk indicates a difference of statistical significance between non-elicited and elicited cells for each time point, Mann-Whitney test (P < 0.05). (C) Western blot analysis of PM fractions from BY-2 and RBOHD1-GFP cells with anti-RBOHD and anti-GFP antibodies. Cells were untreated (Ctrl) or cryptogein-treated for 5 min (Cry5) or 60 min (Cry60). CB: Coomassie blue staining as a loading control. (D) RBOHD distribution on PM vesicles from elicited BY-2 cells. Values for non-elicited cells are the same as the ones reported in Fig. 2B. Abbreviation: n, number of experiments.

Fig. 5. Kinetics of ROS production and RBOHD gene expression following elicitation. The kinetics of ROS production, measured by chemiluminescence (dashed line, left axis), is plotted against the kinetics of RBOHD1 and RBOHD2 mRNA accumulation, measured by qRT-PCR (solid lines, right axis), ROS kinetics is representative of three independent experiments. Expression values are means ± SEM of log2-transformed mRNA levels relative to the geometric mean of reference genes EF-1α, L25, and PP2A (n = 3). Values are expressed in arbitrary units (a.u.).
The catalytic core of the phagocytic NADPH oxidase cycles between the PM and internal reservoirs in macrophages, and it has been proposed that cycling could represent a mechanism by which superoxide production is regulated (Casbon et al., 2009; Ejlerskov et al., 2012).

Conclusion

The results presented here allow a new hypothesis concerning the regulation of plant NADPH oxidases during the set-up of plant defence. Until now, this regulation has mainly been addressed by considering variation of gene expression, post-translational modifications, or regulation by other proteins or secondary messengers such as calcium or phosphatidic acid. It now seems of interest to consider subcellular trafficking as a potential determinant of RBOH activity. Future prospects will be to decipher the mechanisms underlying the subcellular dynamics of RBOHs and to evaluate the roles of the different pools of RBOHs in the signalling process associated with plant defence.

Supplementary material

Supplementary data can be found at JXB online.

Supplementary Table S1. Primers used in this study.

Supplementary Figure S1. Alignment of RBOHD1 and RBOHD2 amino acid sequences.

Supplementary Figure S2. Kinetics of ROS production upon elicitation by cryptogein.

Supplementary Figure S3. RBOHD-GFP protein forms clusters in the PM.

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