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RESEARCH PAPER

The role of respiratory burst oxidase homologues in elicitor-induced stomatal closure and hypersensitive response in *Nicotiana benthamiana*

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Abstract

Active oxygen species (AOS) are central components of the defence reactions of plants against pathogens. Plant respiratory burst oxidase homologues (RBOH) of gp91^{phox}, a plasma membrane protein of the neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, play a prominent role in AOS production. The role of two RBOH from *Nicotiana benthamiana*, *NbrbohA* and *NbrbohB* that encode plant NADPH oxidase in the process of elicitor-induced stomatal closure and hypersensitive cell death is described here. *NbrbohA* was constitutively expressed at a low level, whereas *NbrbohB* was induced when protein elicitors exist (such as boehmerin, harpin, or INF1). The virus-induced gene-silencing (VIGS) method was used to produce single-silenced (*NbrbohA* or *NbrbohB*) and double-silenced (*NbrbohA* and *NbrbohB*) *N. benthamiana* plants. The hypersensitive response (HR) of cell death and pathogenesis-related (*PR*) gene expression of these gene-silenced *N. benthamiana* plants, induced by various elicitors, are examined. The HR cell death and transcript accumulation of genes related to the defence response (*PR1*) were slightly affected, suggesting that RBOH are not essential for elicitor-induced HR and activation of these genes. Interestingly, gene-silenced plants impaired elicitor-induced stomatal closure and elicitor-promoted nitric oxide (NO) production, but not elicitor-induced cytosolic calcium ion accumulation and elicitor-triggered AOS production in guard cells. These results indicate that RBOH from *N. benthamiana* function in elicitor-induced stomatal closure, but not in elicitor-induced HR.

Key words: AOS, elicitor, hypersensitive response, *Nicotiana benthamiana*, stomatal closure, virus-induced gene silencing.

Introduction

Plant cell death during the hypersensitive response (HR) has been well studied and has usually been described in incompatible plant–pathogen interactions (Lamb and Dixon, 1997). Defence responses by incompatible pathogens have been modelled using elicitor treatment. Elicitors can induce oxidative burst, which can limit the spread of invading pathogens by generating active oxygen species (AOS) including the moderately reactive radicals hydrogen peroxide (H_2O_2), superoxide (O_2^-), and the highly reactive hydroxyl radical (OH^-). Plants have evolved many AOS-scavenging systems, but cell death may still occur when excessive numbers of AOS are produced (Pitzschke and Hirt, 2006).

Although many enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cell wall peroxidases, amine oxidase, oxalate oxidase, and flavin-containing oxidase are potential H_2O_2 sources (Bolwell and Wojtaszek, 1997; Bolwell *et al.*, 2002), the NADPH oxidase complex is considered as one of the most important sources of oxidative burst (Bolwell *et al.*, 1998; Grant M *et al.*, 2000; Torres and Dangl, 2005). Plant respiratory burst oxidase homologues (RBOH) of gp91^{phox}, a plasma membrane

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protein of the neutrophil NADPH oxidase, are believed to have six transmembrane-spanning domains and two elongation factor (EF) hands in the N-terminal region that may function in Ca^{2+} regulation (Torres and Dangl, 2005).

The RBOH was first isolated from rice (*Oryza sativa*) as a homologue of $gp91^{phox}$ (Groom *et al.*, 1996), and then identified in other plant species including *Arabidopsis*, tomato, tobacco, and potato (Keller *et al.*, 1998; Torres *et al.*, 1998; Amicucci *et al.*, 1999; Yoshioka *et al.*, 2001, 2003; Yoshie *et al.*, 2005). Previous studies have shown that RBOH play a central role in AOS production during biotic and abiotic stress. For example, *rboh*-silenced *Nicotiana tabacum* showed reduced disease resistance to *Phytophthora infestans* (Yoshioka *et al.*, 2003); *rboh* from *Zinnia elegans* was involved in xylem differentiation (Barcelo, 2005); *rboh* knockdowns in tomato resulted in growth anomalies (Sagi *et al.*, 2004); *rbohC* from *Arabidopsis* may have regulated cell expansion during root hair formation (Foreman *et al.*, 2003). Stomatal closure was also severely inhibited in *Arabidopsis rbohD/F* double-mutants after abscisic acid (ABA) treatment (Bright *et al.*, 2006). All of these data suggest that multiple isoforms of RBOH may act in different AOS-dependent functions in different plants.

AOS signalling may also be associated with nitric oxide (NO), a highly reactive nitrogen species produced after pathogen and elicitor recognition (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Lamotte *et al.*, 2004; Zhang *et al.*, 2004; Ji *et al.*, 2005; Asai *et al.*, 2008). NO may work in conjunction with AOS, Ca^{2+}, and protein kinases in plant signalling (Delledonne *et al.*, 2001; Wendehenne *et al.*, 2004; Courtois *et al.*, 2008). Under non-stressed conditions, plants balance the states between AOS and NO. Cytological studies have indicated that AOS and NO determine the fate of the cell, and one signal modulates the accumulation of the other (Tada *et al.*, 2004; Zeier *et al.*, 2004). In addition, both AOS and NO collaborate to mediate ABA-induced stomatal closure in *Arabidopsis* (Garcia-Mata and Lamattina, 2002; Desikan *et al.*, 2002, 2004; Neill *et al.*, 2002*a*; Bright *et al.*, 2006).

Elicitors include a variety of compounds, such as proteins, glycoproteins, glycans, lipids, and synthetic molecules. They may be cell components, pathogen secretions, or substances released by hydrolytic enzymes of pathogens and plants (Garcia-Brugger *et al.*, 2006). The recognition of an elicitor by the plant cell is followed by calcium ion influx, AOS, and NO production. After successive signal transduction, it can induce cell death and stomatal closure (Allan and Fluhr, 1997; Lee *et al.*, 1999; Nürnberger *et al.*, 2004).

During incompatible interactions, *rboh Arabidopsis* mutants with reduced H_2O_2 production had an opposite response to the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (*avrRpm1*) and the oomycete parasite *Peronospora parasitica* (Torres *et al.*, 2002). In addition, *rboh* also played a role in ABA signalling for stomatal closure regulation (Bright *et al.*, 2006). However, research on RBOH in elicitor signalling is still lacking. Two NADPH oxidase catalytic subunit genes, *NbrbohA* and *NbrbohB*, from *Nicotiana benthamiana* were chosen to investigate their function in elicitor-induced plant response and stomatal closure. Transient knock-down via virus-induced gene silencing (VIGS) was performed to assess the role of the two genes.

Materials and methods

Plant materials, elicitors, and treatment protocol

The *N. benthamiana* plants were grown in a controlled growth chamber under a 16/8 h light/dark cycle at 25 °C. Elicitation with the elicitor (50 nM) was conducted on plants by infiltrating an equivalent elicitor solution of 25 µl with a needleless syringe into tiny cuts on the underside of the leaf, thereby flooding the apoplastic space. To prepare *Phytophthora infestans* INF1 and *Phytophthora boehmeriae* boehmerin, overnight cultures of *E. coli* cells, BL21 carrying pET32b with the *inf1* (GenBank accession no. AY830094) or *boehmerin* (GenBank accession no. AY196607) gene, were diluted (1:100) in Luria–Bertani medium containing ampicillin (50 mg ml^{-1}) and incubated at 37 °C. To prepare the *E. coli*-expressed harpin, overnight cultures of *E. coli* cells, BL21 carrying pET30a with the *hrf1* (GenBank accession no. AY875714) gene, were diluted (1:100) in Luria–Bertani medium containing kanamycin (50 mg ml^{-1}) and incubated at 37 °C. When the OD_{600} of cultures reached 0.6, boehmerin, INF1, and harpin were induced in the cultured medium by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside for 6 h. The deposit was harvested by centrifugation, washed repeatedly, stored in 10 mM PBS (pH 6.5), and then broken up by ultrasonification. Supernatants collected by centrifugation (12 000 *g*, 15 min, 4 °C) were dialysed successively against 0.8%, 0.6%, 0.4%, 0.2%, and 0.1% SDS at 15 °C. Finally, supernatants were dialysed against 10 mM PBS (pH 6.5) and stored at –20 °C prior to use. Protein concentrations were determined using Bradford reagent (Qutob *et al.*, 2006), and concentrated stock solutions (500 nM) were prepared.

DNA constructs and seedling infection for virus-induced gene silencing

Silencing of *NbrbohA* and *NbrbohB* genes in *N. benthamiana* by *Potato virus X* (PVX) VIGS was performed as described by Sharma *et al.* (2003). The *NbrbohA* (GenBank accession no. AB079498) and *NbrbohB* (GenBank accession no. AB079499) inserts were 235 bp and 217 bp and showed 12% and 10% nucleotide identity to the corresponding regions of *NbrbohB* and *NbrbohA*, respectively. The inserts of *NbrbohA* and *NbrbohB* were both derived from the 3′ terminus of the respective open reading frame (ORF), and inserted into the PVX vector separately or simultaneously in the antisense direction to generate PVX.NbrbohA, PVX.NbrbohB, and PVX.NbrbohA/B. The constructs containing the inserts were transformed into *Agrobacterium tumefaciens* strain GV3101. Bacterial suspensions were applied to the undersides of *N. benthamiana* leaves using

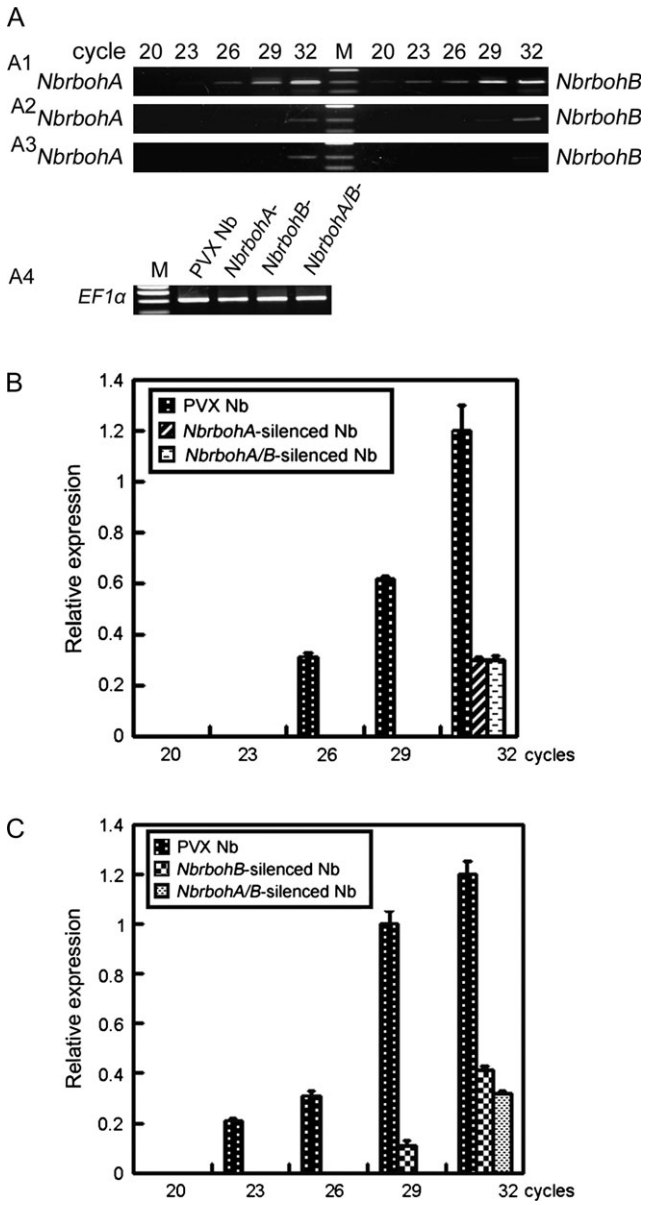

Fig. 1. Evaluation of *NbrbohA* and *NbrbohB* silencing in leaves of *N. benthamiana* infected with PVX, PVX.NbrbohA, PVX.NbrbohB, or PVX.NbrbohA/B. RT-PCR was performed with first-strand cDNA obtained from total RNA derived from various plants silenced for *NbrbohA*, *NbrbohB*, *NbrbohA/B*, and for controls. After a 3-week inoculation, leaf samples were harvested from the third and fourth leaves above the inoculation site, and total RNA was isolated and used for RT-PCR. A 7 μl aliquot was removed from each reaction after three-cycle increments starting after 20 cycles. The aliquots were separated on an agarose gel and stained with ethidium bromide. Equal input of cDNA template for PCR was demonstrated by amplification of the constitutively expressed *EF1α* gene (29 cycles); Lane M shows the DL2000 DNA ladder (TaKaRa, Dalian, China). (A1) The control PVX *N. benthamiana*, (A2) *NbrbohA*-silenced *N. benthamiana* (left) and *NbrbohB*-silenced *N. benthamiana* (right), (A3) both *NbrbohA*- and *NbrbohB*-silenced *N. benthamiana*, and (A4) *EF1α* control. The RT-PCR analysis was repeated for three sets of independently silenced plants in each experiment and in three independent experiments. (B) Relative amount of the transcript accumulation of *NbrbohA* (B) and *NbrbohB* (C) to *EF1α* using software Quantity One, as shown in (A). Values are the mean ±SD from three independent experiments.

a 1 ml needleless syringe. Plants exhibited mild mosaic symptoms 3 weeks after inoculation. The third or fourth leaf above the inoculated one, where silencing was most consistently established, was used for further analysis.

DAB staining

Following the methods of Samuel *et al.* (2005), leaves collected 6 h after elicitor treatment were incubated in diaminobenzidine (DAB) solution for 8 h at 25 °C in light. The leaves were then boiled in 96% ethanol for 10 min to remove the dye. After 4 h of further incubation in ethanol, brown precipitates were observed, indicating H_2O_2 burst. Quantitative scoring of H_2O_2 staining in leaves was analysed using the software Quantity One (Bio-Rad, Milan, Italy).

RNA isolation and RT-PCR analysis

Total RNA was extracted following the Trizol extraction protocol (Invitrogen, Carlsbad, CA) and treated with RNAse-free DNAse I (TaKaRa, Dalian, China). First-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's directions. PCR was performed in 50 μl reactions using 1 μl cDNA template, 1 μM of each gene-specific primer, 2 units of Taq polymerase, and the buffer provided by the manufacturer (containing 1.5 mM $MgCl_2$). To ensure that similar amounts of cDNA were used for silenced and non-silenced plants, parallel reactions were run with elongation factor 1α (*EF1α*) primers as controls (29 cycles). Each PCR cycle included denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 72 °C for 30 s, as described in Zhang *et al.* (2004). The PCR products were analysed on a 1.2% agarose gel stained with ethidium bromide. RT-PCR -specific primers for *NbrbohA*, *NbrbohB*, and *EF1α* are: *NbrbohA* forward primer: 5′-CgTgCTTgATAA-AgAAACACTgA-3′, *NbrbohA* reverse primer: 5′-CCC-ACCCAACCAAAATACgC-3′; *NbrbohB* forward primer: 5′-CgggTgATgCTCgTTCTgCTC-3′, *NbrbohB* reverse primer: 5′-CCAggCgTgTTgTCTTAgTTCTT-3′; and *EF1α* forward primer: 5′-AGACCACCAAGTACTACTGCAC-3′, *EF1α* reverse primer: 5′-CCACCAATCTTGTACACATCC-3′. The RT-PCR primers of defence-related genes are described below. Primer sequences are as follows: *PR-1a*, *PR-1b*, and *PR-1c* forward primer, 5′-ATGCCCATAACAGCTCG-3′; *PR-1a* reverse primer, 5′-GAGGATCATAGTTGCAAGAG-3′; *PR-1b* reverse primer, 5′-GTATGGACTTTGGCCATGAC-3′; and *PR-1c* reverse primer, 5′-GGATCATAGTTGCAA-GAGAC-3′.

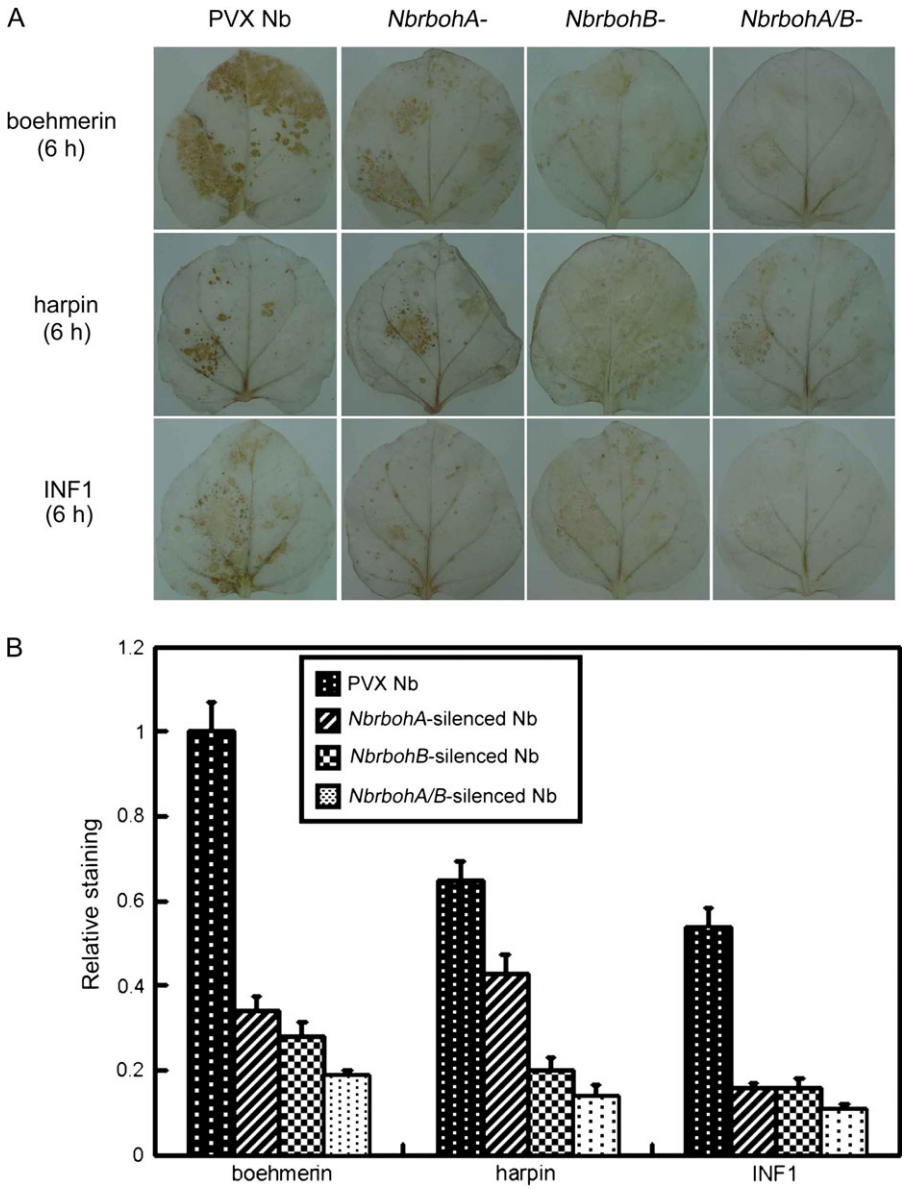

Fig. 2. *In situ* detection of hydrogen peroxide using DAB staining on PVX, PVX.NbrbohA-, PVX.NbrbohB-, and PVX.NbrbohA/B-infected *N. benthamiana* leaves. (A) Photographs of representative leaves ethanol-bleached from control and *Nbrboh*-silenced plants 6 h after boehmerin (50 nM), harpin (50 nM), or INF1 (50 nM) treatment; elicitation with the elicitor was conducted on plants by infiltrating an equivalent elicitor solution of 25 μl; (B) quantitative scoring of staining in leaves of the control and *Nbrboh*-silenced plants with elicitor treatment with the software Quantity One. The analysis was repeated for three sets of independently silenced plants in each experiment; the values shown are the means ±SD of duplicate assays.

Stomatal aperture measurements

Stomatal apertures were measured as described by Chen *et al.* (2004) in 5 mM KCl, 50 mM $CaCl_2$, and 10 mM MES-Tris (pH 6.15).

NO measurement in guard cells

NO accumulation was determined using fluorophore 4, 5′-diaminofluorescein diacetate (DAF-2DA, Sigma-Aldrich) according to Ali *et al.* (2007). Epidermal strips were prepared from control and gene-silenced plants, respectively; the strips were then incubated in 5 mM KCl and 10 mM MES-Tris (pH 6.15) in light for 2 h, followed by incubation in 20 μM DAF-2DA for 1 h in the dark at 25 °C, and finally rinsed three times with 10 mM Tris-HCl (pH 7.4) to wash off excessive fluorophore. Guard cell images were taken 3 h after elicitor treatment, by fluorescence microscopy at 470 nm excitation using a 515 nm emission filter. Fluorescence emission of guard cells was analysed using the software Quantity One.

AOS measurement in guard cells

Dihydrorhodamine 123 (DHR, Merck, Whitehouse Station, NJ) was used to analyse elicitor-induced AOS production in

guard cells. The epidermal strips were incubated in 20 μM DHR for 2 h in the dark at 37 °C and then rinsed three times with PBS (pH 7.4) to wash off excessive fluorophore. Subsequently, 3 h after elicitor treatment, guard cell images were taken by using Adobe Photoshop 5.5 (Mountain View, CA) during a 2 s short UV exposure (one UV exposure per sample) under a fluorescence microscope equipped with a digital camera. Fluorescence emission of the guard cells was analysed using the software Quantity One.

Measurement of Ca^{2+} in guard cells

Following Chen *et al.* (2004), the epidermal strips were peeled gently and incubated in 10 mM 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl) phenoxy]-2-(2-amino-5-methylphenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid and penta-acetoxymethyl ester (fluo-3 AM, Merck, Whitehouse Station, NJ) loading buffer (10 mM MES-Tris, pH 6.15) at 4 °C for 2 h in darkness. Because the activities of esterases at 4 °C were low, fluo-3 AM permeated through the membranes without being hydrolysed by esterases in the cell walls. After washing the strips three times with MES buffer, they were kept at room temperature for 1 h. During this period, fluo-3 AM inside the cell was hydrolysed by intracellular esterases, and the hydrolysed form of fluo-3 AM bound to free Ca^{2+}, indicating dynamic changes in Ca^{2+} in guard cells. Three hours after elicitor treatment, guard cell images were taken with confocal laser scanning microscopy and analysed with the software Quantity One.

Results

NbrbohA *and* NbrbohB *participate in elicitor-induced* H_2O_2 *generation*

It has been reported that *NbrbohA* is expressed constitutively at a low level, whereas the accumulation of *NbrbohB* protein is induced by cell wall elicitors (Yoshioka *et al.*, 2003). To investigate whether agro-infiltrated *N. benthamiana* exhibited lower *rboh* transcription, all inoculated antisense *N. benthamiana* were subjected to semi-quantitative reverse transcriptase (RT)-PCR analysis specific to each gene, using *EF1α* transcript as an expression level control (Fig. 1A). The transcript of *NbrbohA* decreased 75% in both *NbrbohA*-silenced and *NbrbohA*/*B*-silenced plants compared to the control, while the transcript of *NbrbohB* decreased 67% in *NbrbohB*-silenced plants and 75% in *NbrbohA*/*B*-silenced plants compared to the control (Fig. 1B, C). Therefore, it could be concluded that both genes were silenced in all three agro-infiltrated *N. benthamiana lines*.

Repression of RBOH polypeptide expression may imply a reduction in the constitutive level of AOS. RBOH produce superoxide radicals (Sagi and Fluhr, 2001), and staining for H_2O_2 produced by endogenous superoxide dismutation of superoxide radicals has been used to quantify RBOH activity (Yoshioka *et al.*, 2003). Elicitor-induced H_2O_2 production measurements were performed using DAB staining, which indicated H_2O_2 accumulation by the formation of a brown precipitate. Figure 2A shows the development of the DAB-H_2O_2 reaction product in leaves of the control and *Nbrboh*-silenced plants 6 h after elicitor treatment. Brown precipitate in control leaves triggered by boehmerin was highest among the three elicitors. However, the brown precipitate decreased, with lighter colouring and lower distribution, in *Nbrboh*-silenced leaves after various elicitor treatments (Fig. 2A). Results of further quantitative analysis, using the software Quantity One, revealed that *Nbrboh*-silencing attenuated elicitor-induced H_2O_2 production (Fig. 2B). The results suggest that NADPH oxidases mediate elicitor-induced AOS generation in *N. benthamiana*, and that *NbrbohA* and *NbrbohB* may be the major catalytic subunits in this response.

RBOH are not involved in elicitor-triggered HR

Gene-silenced plants were selected for further evaluation of elicitor-triggered HR. Photographs of representative (control and *Nbrboh*-silenced plants) leaves infiltrated with the elicitor are shown in figure 3. After inoculation, all three elicitors rapidly induced a water-soaked appearance of the leaves (12 h), followed by brown-pigmented necrosis characteristic of HR (12–24 h). Necrosis was restricted to the inoculated area of the leaf, and the lesion became fully desiccated 2–3 d after inoculation. Inoculation of serially diluted elicitor solutions indicated that minimal threshold concentrations of 1–10× were necessary for HR induction by boehmerin, harpin, and INF1. No obvious difference was observed among the elicitors in the specificity and severity of HR induction on the controls or the *Nbrboh*-silenced *N. benthamiana*. These results indicate that

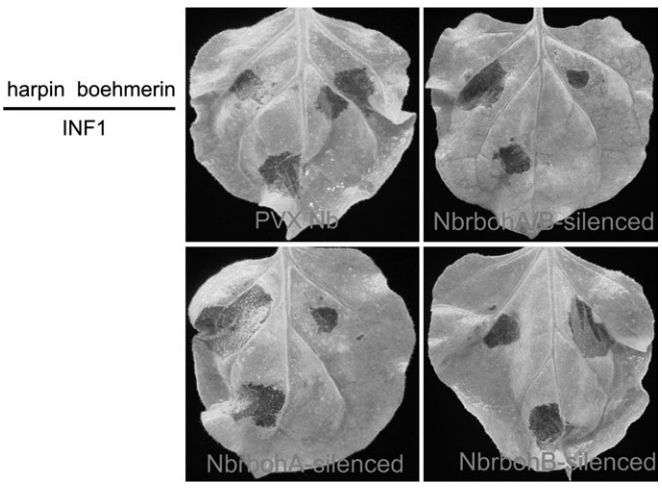

Fig. 3. Local induction of hypersensitivity responses with boehmerin (50 nM), harpin (50 nM), and INF1 (50 nM). A leaf (representative of three replicate treatments) was infiltrated with the three elicitors simultaneously. (A) Control PVX *N. benthamiana*, (B) *NbrbohA/B* dual-silenced *N. benthamiana*, (C) *NbrbohA*-silenced *N. benthamiana*, (D) *NbrbohB*-silenced *N. benthamiana*. Leaves were removed from plants after 3 d of treatment and bleached in ethanol.

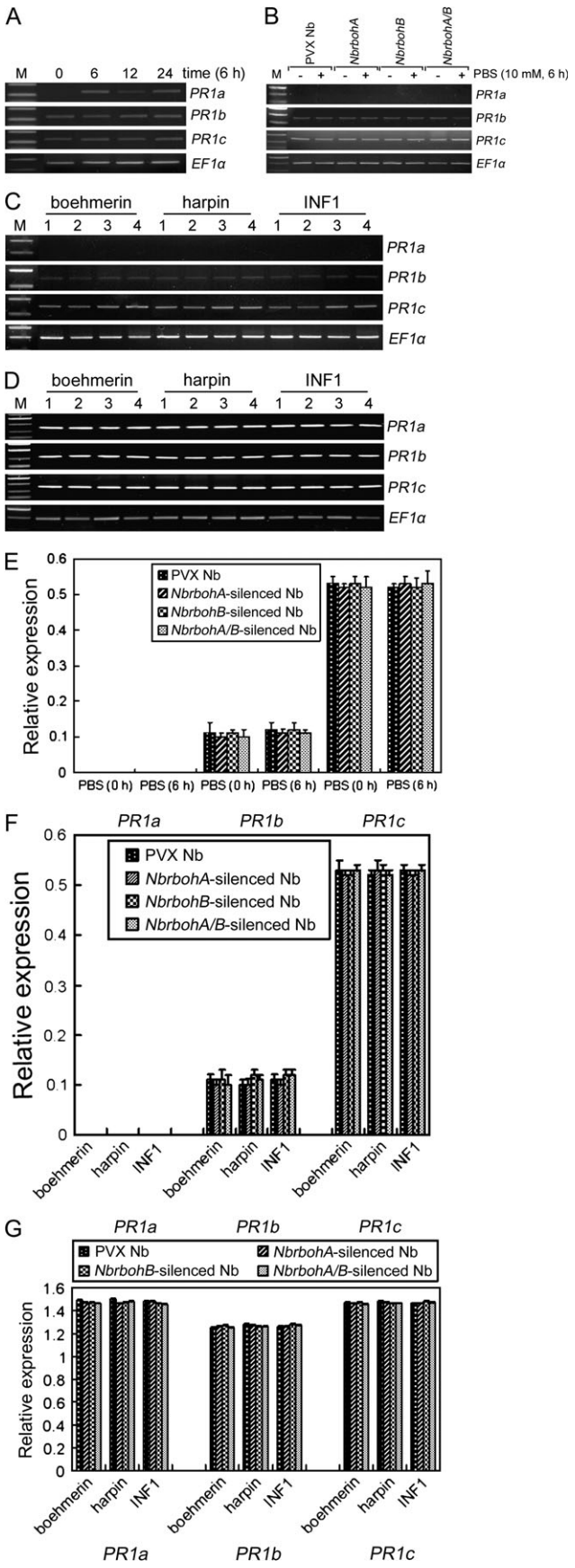

Fig. 4. Response of *PR1a*, *PR1b*, and *PR1c* genes in elicitor-treated *N. benthamiana* leaves using semi-quantitative RT-PCR. RT-PCR was performed with RNA isolated from *N. benthamiana* plants with primers specific for *PR1a*, *PR1b*, *PR1c*, and *EF1α* of *N. benthamiana* after PBS (10 mM), boehmerin (50 nM), harpin (50 nM), and INF1 (50 nM) treatment, respectively. PCR conditions, ranging from 20 to 40 amplification cycles were tested in both cases. Presented here are 29 cycles corresponding to the log-linear phase of amplified PCR product in *N. benthamiana*. (1) Control PVX *N. benthamiana*, (2) *NbrbohA*-silenced *N. benthamiana*, (3) *NbrbohB*-silenced *N. benthamiana*, (4) *NbrbohA/B*-dual-silenced *N. benthamiana*. The RT-PCR analysis was repeated for three independent control plants in each experiment and in three independent experiments. (A) Time-course accumulation of transcripts of *PR1a*, *PR1b*, and *PR1c* genes in boehmerin-treated leaves of PVX-infected *N. benthamiana*. The elicitor-treated leaves were removed at the indicated time point. (B) RT-PCR analysis to examine transcript levels of defence-related genes in *N. benthamiana* leaves after PBS treatment. (C) RT-PCR analysis to examine transcript levels of defence-related genes in *N. benthamiana* leaves 0 h after elicitor treatment. (D) RT-PCR analysis to examine transcript levels of defence-related genes in *N. benthamiana* leaves 6 h after elicitor treatment. (E) Relative transcript accumulation of *PR1a*, *PR1b*, and *PR1c* to *EF1α* according to the software Quantity One as shown in (B). Values are the mean ±SD from three independent experiments. (F) Relative transcript accumulation of *PR1a*, *PR1b*, and *PR1c* to *EF1α* with the software Quantity One as shown in (C). Values are the mean ±SD from three independent experiments. (G) Relative transcript accumulation of *PR1a*, *PR1b*, and *PR1c* to *EF1α*, calculated by the software Quantity One as shown in (D). Values are the mean ±SD from three independent experiments.

NbrbohA and *NbrbohB* may not be the key contributors to HR caused by these elicitors.

Elicitor-induced PR gene expression is regulated in an RBOH-independent manner

Incompatible interactions can induce not only HR cell death but also systemic acquired resistance (SAR), which requires both local and systemic salicylic acid (SA) accumulation, and the induction of a subset of pathogenesis-related (*PR*) genes (Grant and Lamb, 2006). To investigate whether *Nbrboh* deficiency affects transcript accumulation of the *PR* gene, semi-quantitative RT-PCR was performed to monitor transcript accumulation of defence genes including *PR1a*, *PR1b*, and *PR1c* after treatment with boehmerin, harpin, and INF1. In control plants, boehmerin induced rapid transcript accumulation of *PR1a*, *PR1b*, and *PR1c* 6 h after inoculation (Fig. 4A). This result is consistent with other reports that transcripts of *PR-1* genes began to accumulate after 6 h during *N* gene-mediated HR (Seo *et al.*, 2000; Hatsugai *et al.*, 2004). There is no obvious increased transcript accumulation of *PR1a*, *PR1b*, and *PR1c* in both gene-silenced and control plants after PBS treatment (Fig. 4B, E). RT-PCR was performed to analyse transcript accumulation of *PR1a*, *PR1b*, and *PR1c* in *N. benthamiana* plants infected with PVX, PVX.NbrbohA, PVX.NbrbohB, or PVX.NbrbohA/B at certain time points (0 h, 6 h) after

various elicitor treatments (Fig. 4C, D). The results show increased transcript accumulation of *PR1a*, *PR1b*, *and PR1c* 6 h after elicitor treatment, but no obvious difference among the *Nbrboh* single-silenced, dual-silenced, or control plants (Fig. 4F, G). The experimental data suggest that *Nbrboh* may have a slight effect on the transcription of *PR* genes upon elicitor induction, which is consistent with HR cell death.

Elicitor-induced stomatal closure is impaired in Nbrboh-*silenced* N. benthamiana

Stomata are specialized epidermal structures formed by two guard cells surrounding a pore, through which carbon dioxide (CO_2) for photosynthesis is absorbed and water evaporates. The stomatal pores open in light and close in response to water stress through the action of ABA (Pei *et al.*, 2000). Because elicitor PB90 from *P. boehmeriae* induces stomatal closure (Zhang *et al.*, 2007) and *rbohD/F* from *Arabidopsis* has an effect on inhibiting ABA-induced stomatal closure, guard cells display a classic innate immune response to both pathogen-associated molecular pattern (PAMP) compounds and pathogens (Lee *et al.*, 1999; Wright *et al.*, 2000). Elicitor-induced stomatal closure analysis was performed with *Nbrboh* single- and dual-silenced *N. benthamiana*. As shown in figure 5, harpin induced stomatal closure of control leaves, which was inhibited in the *NbrbohA/B* dual-silenced *N. benthamiana*. *Nbrboh* dual-silenced *N. benthamiana* significantly inhibited boehmerin-induced stomatal closure compared to the *Nbrboh* single-silenced *N. benthamiana* and controls (Table 1A; P=0.01). The results of harpin treatment were the same as those with boehmerin (Table 1B; P=0.01). However, INF1 treatment led to significantly different results between the gene-silenced plants and controls, but not between the dual-silenced and single-silenced *N. benthamiana* (Table 1C, P=0.01). These results suggest that *NbrbohA* and *NbrbohB* function in elicitor-induced stomatal closure. The impact of *Nbrboh* dual-silencing and single-silencing on stomatal closure induced by boehmerin, harpin, and INF1 is somewhat different.

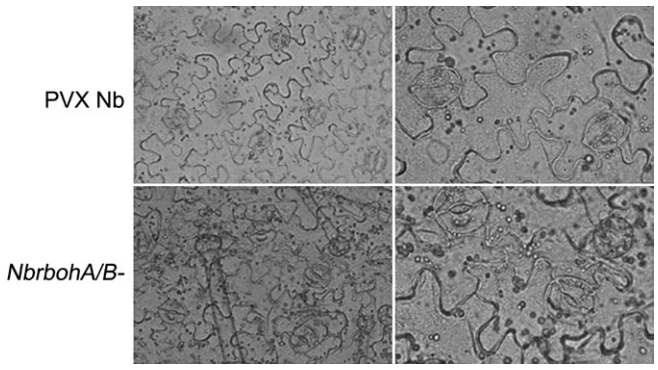

Fig. 5. Elicitor-induced stomatal closure is impaired in the *NbrbohA/B* dual-silenced plants after harpin treatment. Leaf epidermal peels prepared from control (top panels) and *NbrbohA/B* dual-silenced plants (bottom panels) were incubated in harpin (50 nM).

NO is associated with elicitor-induced stomatal closure

NO co-ordinates HR and plant innate immunity, serving as a cellular signalling molecule in a wide range of organisms including plants, especially in stomatal guard cells (Dangl, 1998; Ali *et al.*, 2007). NO is involved in ABA-induced stomatal closure (Neill *et al.* 2002*a*). To determine whether NO plays a role in the inhibition of elicitor-induced stomatal closure, NO generation was compared in guard cells isolated from controls and *Nbrboh*-silenced *N. benthamiana* 3 h after treatment with boehmerin, harpin, and INF1. As shown in figure 6A, PBS-treated guard cells showed almost no fluorescence in guard cells of the control and gene-silenced plants. Elicitor treatment evoked NO generation in guard cells of the control plants, and this response was inhibited in both single- and dual-silenced *N. benthamiana*. Results of further quantitative analysis of *Nbrboh*-silencing effects on elicitor-induced NO production, with the software Quantity One, are shown in figure 6B. NO production in the dual-silenced plants decreased severely after elicitor treatment compared to the controls, suggesting that NO is associated with elicitor-induced stomatal closure.

The increase of cytosolic calcium induced by elicitors is independent or acts upstream of Nbrboh

Cytosolic Ca^{2+} quickly increases upon pathogen infection (Garcia-Brugger *et al.*, 2006), and Ca^{2+} influx is necessary for AOS production after elicitation (Blume *et al.*, 2000; Grant M *et al.*, 2000). The interplay between Ca^{2+} influx through channels and Ca^{2+} efflux from pumps and carriers will determine the form of a Ca^{2+} spike that is potentially

Table 1. Stomatal aperture measurements show that elicitor-induced stomatal closure is partially reduced in *Nbrboh* single-silenced and dual-silenced *N. benthamiana*

Stomatal aperture was measured 3 h after incubation in boehmerin (50 nM) (A), harpin (50 nM) (B), and INF1 (50 nM) (C). Data were compared by using the DPS at the 95% significance level.

	Nb	Stomatal aperture (μm) P=0.01
(A)	PVX Nb	0.41±0.08 c
	NbrbohA-silenced Nb	1.37±0.40 bc
	NbrbohB-silenced Nb	3.01±0.86 bc
	NbrbohA/B-silenced Nb	3.56±1.03 a
(B)	PVX Nb	0.40±0.24 b
	NbrbohA-silenced Nb	1.20±0.94 b
	NbrbohB-silenced Nb	1.31±0.77 b
	NbrbohA/B-silenced Nb	3.65±0.40 b
(C)	PVX Nb	0.35±0.42 b
	NbrbohA-silenced Nb	0.88±0.64 ab
	NbrbohB-silenced Nb	2.21±1.10 ab
	NbrbohA/B-silenced Nb	2.70±1.11 a

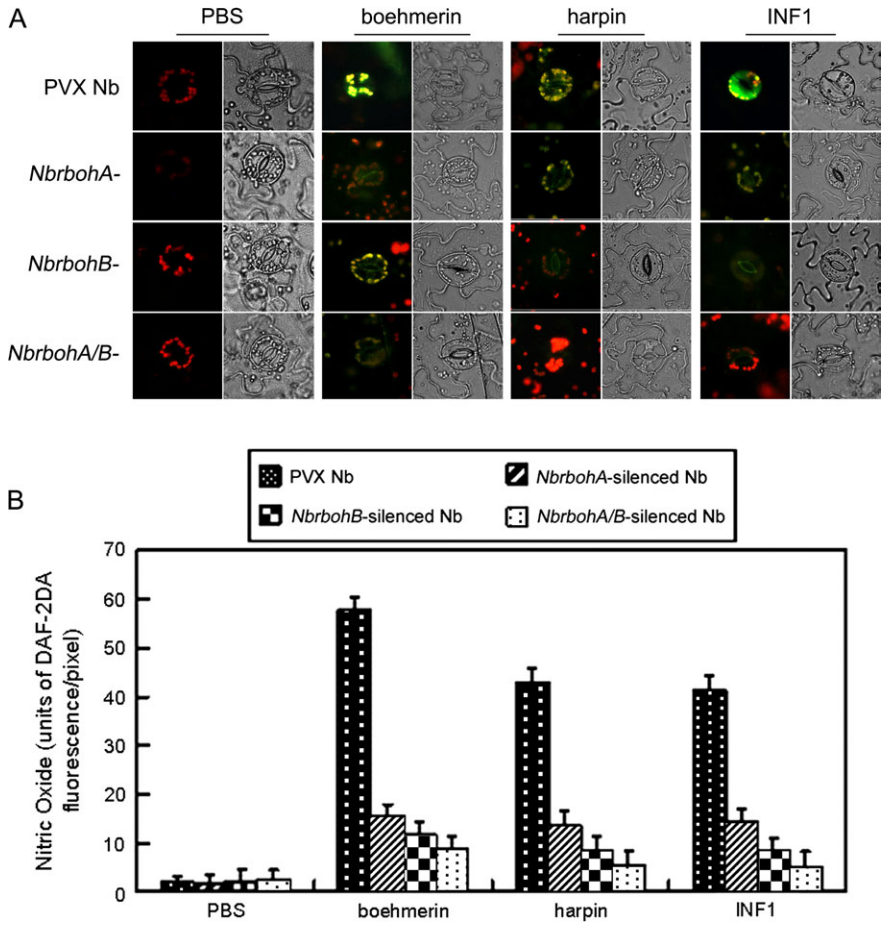

Fig. 6. Elicitor activation of NO is reduced in guard cells of *Nbrboh*-silenced plants. (A) In all cases, NO-sensitive dye DAF-2DA was loaded into cells of the epidermal peels, and fluorescence was measured after addition of PBS (10 mM), boehmerin (50 nM), harpin (50 nM), and INF1 (50 nM). For each treatment, fluorescence and bright-field images are shown. Results from several experiments are compiled in this figure. Experiments were repeated at least three times, and representative images are shown. (B) Quantitative analysis of *in vivo* NO generation monitored using DAF-2DA fluorescence as shown in (A). Results are presented as the mean ($n \geqslant 3$) fluorescence intensity per pixel.

specific to relevant sensors (Sanders *et al.*, 2002). To evaluate the relative contributions of NADPH oxidases and whether elicitor-induced AOS production has an effect on $[Ca^{2+}]_{cyt}$ elevation, calcium fluorescence imaging analysis of guard cells from intact epidermal strips was conducted. In control PBS-treated guard cells of gene-silenced and control plants, there was almost no fluorescence. Elicitors were applied to guard cells that showed obvious Ca^{2+} fluorescence. Cytosolic Ca^{2+} fluorescence in guard cells from both the controls and *Nbrboh*-silenced plants was altered slightly after elicitor treatment for 3 h (Fig. 7A). Further quantification using the software Quantity One revealed that *Nbrboh*-silencing caused little alterations of Ca^{2+} fluorescence intensity (Fig. 7B). This result indicates that the elicitor-induced calcium spike is independent of oxidative burst or acts upstream of the oxidative burst induced by Nbrboh. This finding is consistent with reports that a cytosolic Ca^{2+} spike precedes NADPH oxidase (NOX) activation as part of the elicitor-induced defence response (Nürnberger and Scheel, 2001; Zhao *et al.*, 2005).

AOS, apart from H_2O_2 and NO, are not involved in elicitor-induced stomatal closure but are related to elicitor-induced HR

Reduced DAB-H_2O_2 production was observed in leaves and decreased NO fluorescence in guard cells of *Nbrboh*-silenced *N. benthamiana*. However, the accumulation of AOS is characteristic of the HR in plant tissues and functions as a second signal mediating plant HR (Lamb and Dixon, 1997; Gechev and Hille, 2005; Li *et al.*, 2006; Gan *et al.*, 2009). To evaluate whether other AOS were involved in elicitor-induced stomatal closure, AOS such as peroxide and peroxynitrite were analysed by incubation with DHR, which is oxidized to the fluorochrome rhodamine 123 in the presence of AOS (Schulz *et al.*, 1996). All epidermal peels (controls, single-silenced, and dual-silenced *N. benthamiana*) showed similar, bright fluorescence after elicitor treatments. By contrast, the control guard cells showed almost no fluorescence (Fig. 8A, B), indicating that *Nbrboh*-silencing has little effect on AOS production (except H_2O_2 and NO) in guard cells. Another enzyme might account for H_2O_2

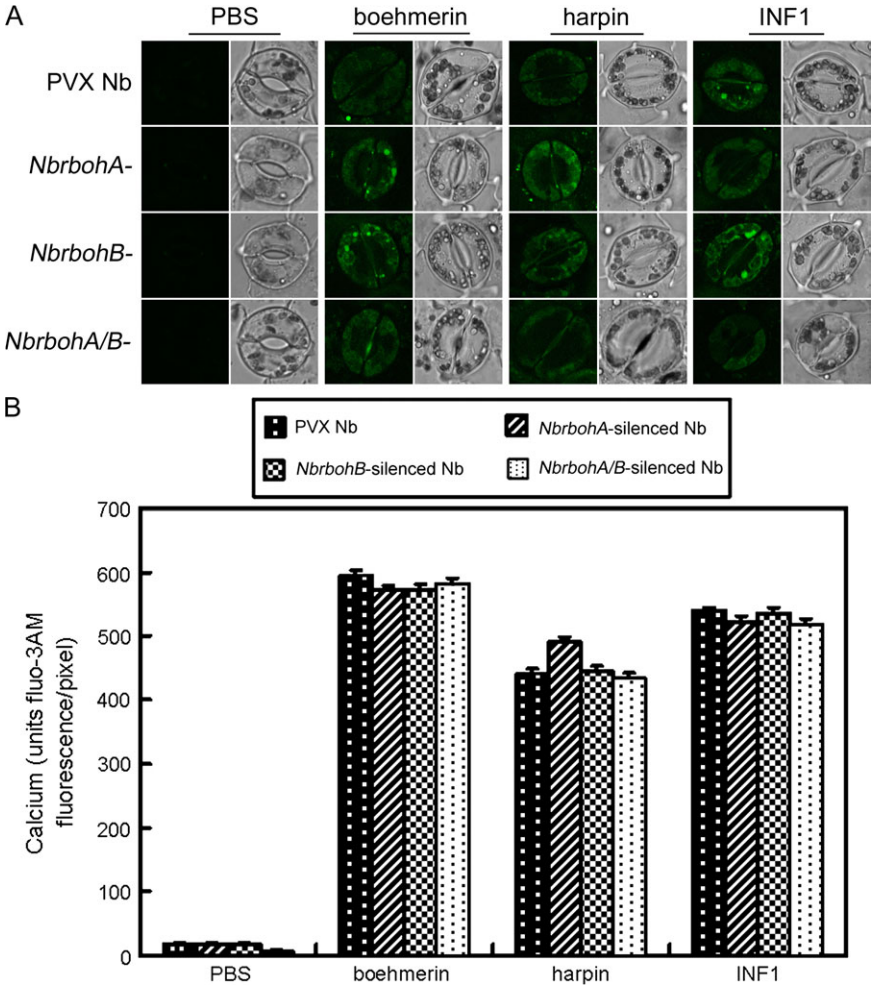

Fig. 7. Elicitor activation of Ca^{2+} generation in guard cells of control and *Nbrboh*-silenced plants. (A) Leaf epidermal peels prepared from the control (top panels) or *Nbrboh*-silenced plants were loaded with the Ca^{2+} dye fluo-3 AM prior to incubation in PBS (10 mM), boehmerin (50 nM), harpin (50 nM), and INF1 (50 nM). In each case, corresponding fluorescence and bright-field images are shown. The areas of the peel subjected to analysis are greater than those shown in the figure. This experiment was repeated three times. Representative cells from one of three experiments are shown. In each experiment, a minimum of three epidermal peels were used as treatment replicates. (B) Quantitative analysis of *in vivo* Ca^{2+} generation monitored using fluo-3 AM fluorescence as shown in (A). Results are presented as the mean ($n \geqslant 3$) fluorescence intensity per pixel.

production apart from superoxide and hydroxyl radical production. The observed AOS production is consistent with elicitor-induced HR, and *Nbrboh* was not sufficient for elicitor-induced HR. A growing body of evidence indicates that a balance between H_2O_2 and NO is key , and that the redox state determines the fate of cell death (Zeier *et al.*, 2004; Frank and Dat, 2006). Moreover, AOS may serve as a secondary message, contributing to the establishment of defence (Torres *et al.*, 2006).

Discussion

Our results indicate that AOS production is required for elicitor signal transduction in guard cells. *NbrbohA* and *NbrbohB* are the main genes that mediate elicitor-induced H_2O_2 production in leaves and affect the aperture of guard cells of *N. benthamiana* upon elicitor treatment. The inhibition of elicitor-induced stomatal closure was accompanied by less NO generation, and cytosolic calcium induced by the elicitor increased to the same level in both controls and the *Nbrboh*-silenced plants. These results suggest that H_2O_2 and NO are signalling molecules for elicitor-activated signal transduction in guard cells.

RBOH are the main contributors to elicitor-induced AOS production

NbrbohA-, *NbrbohB*-, and *NbrbohA*/*B*-silenced *N. benthamiana* showed impaired elicitor-induced H_2O_2 production in leaves. Upon elicitor treatment, the production of H_2O_2 was obviously decreased, but some brown precipitate remained in the silenced leaves. This result indicates that RBOH are the major H_2O_2 source. Extracellular AOS production by *NbrbohA* and *NbrbohB* is required for elicitor-induced AOS production. Our data do not exclude the possibility that

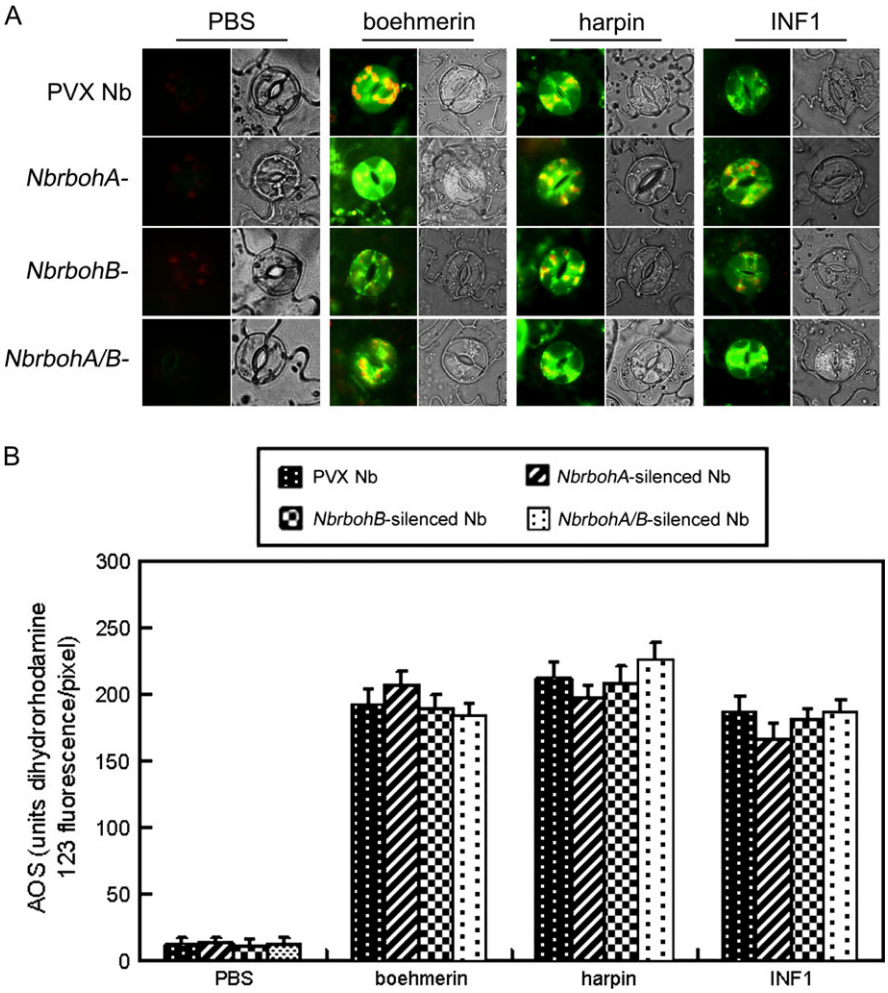

Fig. 8. Elicitor-induced AOS increase in guard cells of the control and *Nbrboh*-silenced plants. (A) In all cases, AOS dye DHR was loaded into cells of epidermal peels, and fluorescence was detected after incubation in PBS (10 mM), boehmerin (50 nM), harpin (50 nM), and INF1 (50 nM). For each treatment, fluorescence and bright-field images are shown. Results from several experiments are compiled in this figure. Experiments were repeated at least three times, and representative images are shown. (B) Quantitative analysis of *in vivo* AOS generation monitored using DHR fluorescence as shown in (A). Results are presented as mean ($n \geqslant 3$) fluorescence intensity per pixel.

other *Nbrboh* or AOS-producing/scavenging genes contribute to the elicitor response. Other proteins (peroxidases, amine oxidase, oxalate oxidase, and flavin-containing oxidase) may account for reactive oxygen burst (Bolwell *et al.*, 2002), and other cellular mechanisms can generate AOS in guard cells. AOS are associated with photosynthesis, chloroplasts, and cell wall peroxisomes (Grant JJ *et al.*, 2000; Bolwell *et al.*, 2002; Karpinski *et al.*, 2003; Apel and Hirt, 2004). Distinct sources of elicitor-induced oxidative bursts may differentiate according to catalase sensitivity (Allan and Fluhr, 1997). On the other hand, various AOS-scavenging systems, including ascorbate peroxidases, glutathione, superoxide dismutases, and catalases, maintain AOS homeostasis in different compartments of the plant cell (Mittler *et al.*, 2004) and may also be regulated by elicitors.

RBOH may not be a critical factor in elicitor-induced HR

H_2O_2 and NO function as stress signals in plants, mediating a range of responses to environmental stress (Neill *et al.*, 2002*b*). *Nbrboh*-silenced plants showed less H_2O_2 production in leaves and less NO fluorescence in guard cells, but normal HR cell death upon treatment with elicitors. This result indicates that *Nbrboh* is not necessary for elicitor-triggered HR, but *PR* gene expression is accompanied by HR cell death. Our findings are consistent with those of Dorey *et al.* (1999), who reported that H_2O_2 was neither necessary nor sufficient for HR cell death, PAL activation, or SA accumulation in cultured tobacco cells. Although both H_2O_2 and NO play a role in the HR of plants infected by bacteria and viruses (Delledonne *et al.*, 1998; Durner *et al.*, 1998), a critical balance between AOS and NO determines the fate of the cell. NO is generated at the same time as H_2O_2 in response to pathogen attack and mediates defence responses similar to observations following H_2O_2 generation. Planchet *et al.* (2006) argued that the role of NO in HR should be reconsidered. In addition to AOS- and NO-scavenging systems, Rho-family GTPase (rac) isoforms may also regulate a cell response. A combination of rac isoforms with specific RBOH isoforms may mediate

differential regulatory outcomes, which could explain the different functions of NADPH oxidases in regulating cell death. In rice (*O. sativa*) rac1 is a positive regulator of AOS production and cell death (Ono *et al.*, 2001), whereas in tobacco (*N. tabacum*) rac5 acts as a negative regulator of AOS production via *rbohD* (Morel *et al.*, 2004).

Ca^{2+} acts upstream of Nbrboh

All elicitors in this study induced bright fluorescence, which is consistent with the early elicitor-induced Ca^{2+} spike reported by Garcia-Brugger *et al.* (2006). There was little visible difference in Ca^{2+} fluorescence between the controls and the *rboh*-silenced plants 3 h after elicitor treatment. Apoplastic Ca^{2+} influx is important to the oxidative burst, and Ca^{2+} can activate RBOH proteins *in vitro* in tobacco and tomato (Miura *et al.*, 1995, 1999; Sagi and Fluhr, 2001). Overexpression of TPC1 from *O. sativa*, a putative voltage-gated Ca^{2+}-permeable channel, enhances elicitor-induced oxidative burst (Kurusu *et al.*, 2005). Ectopic expression of *Arabidopsis* CDPK (*calcium*-dependent protein kinase) in tomato protoplasts elevates plasma membrane-associated NADPH oxidase activity (Xing *et al.*, 2001). Transient expression of the constitutive active form of CDPK2 in *N. benthamiana* leads to oxidative burst-mediated cell death against hypo-osmotic stress (Ludwig *et al.*, 2005). In potato, CDPK5 activates rbohB by phosphorylation of the N-terminal region and regulates oxidative burst (Kobayashi, 2007). Therefore, plant NADPH oxidases may be regulated by Ca^{2+} signalling.

RBOH-silencing with decreased H_2O_2 and NO production affects elicitor-induced stomatal closure, but not HR

It was found that *Nbrboh*-silenced plants decreased H_2O_2 production and that inhibition of elicitor-induced stomatal closure was associated with less NO fluorescence in guard cells of *Nbrboh*-silenced *N. benthamiana*, which is consistent with other reports. Elicitor-induced H_2O_2 production leads to stomatal closure (McAinsh *et al.*, 1996; Lee *et al.*, 1999), while inhibition of H_2O_2 production compromises ABA-induced stomatal closure (Shintaro *et al.*, 2007). AOS and NO collaborate to mediate ABA-induced stomatal closure (Desikan *et al.*, 2004). NO synthesis and stomatal closure in response to ABA are severely reduced in *Arabidopsis* NADPH oxidase *(rbohD/F)* double mutants, suggesting that endogenous H_2O_2 production elicited by ABA is required for NO synthesis (Bright *et al.*, 2006). ABA, which induces stomatal closure in a cADPR and cGMP-dependent manner, stimulates NO synthesis in guard cells, indicating that NO is an even earlier secondary messenger in this response pathway.

Nbrboh-silencing did not affect elicitor-induced HR. Similar and bright fluorescence was observed in *Nbrboh*-silenced and control plants stained with DHR. The results suggest that *Nbrboh* is not the key contributor to elicitor-induced HR. AOS (other than H_2O_2 and NO) and the balance between AOS and NO participate in elicitor-triggered HR. Other enzymes (such as cell wall peroxidases, amine oxidase, oxalate oxidase, and flavin-containing oxidase) may account for AOS production (Bolwell and Wojtaszek, 1997; Bolwell *et al.*, 2002; Tada *et al.*, 2004; Zeier *et al.*, 2004).

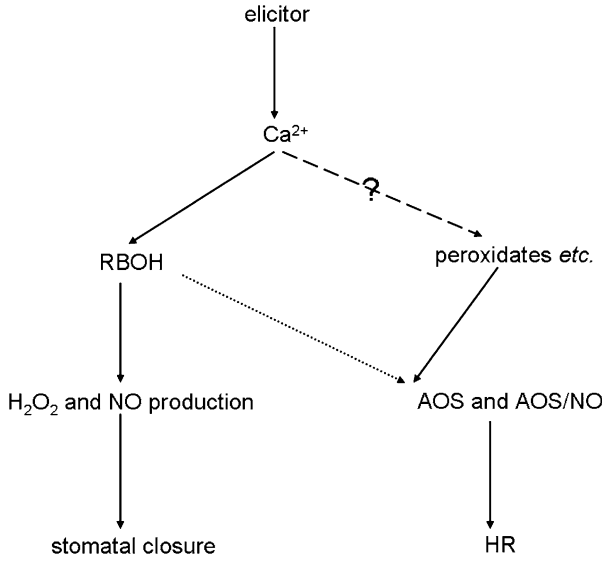

Fig. 9. A simple model of the elicitor signalling of stomatal closure and HR. Elicitor-induced Ca^{2+} generation, the branch point of stomatal closure signalling, and HR signalling. *rboh*-silencing, which disrupts elicitor-induced H_2O_2 and NO production affected elicitor-induced stomatal closure, but not HR ('·····>' shows that RBOH are not the key contributors to elicitor-induced HR because *rboh*-silencing did not effect elicitor-triggered HR). In plants, plant cell wall peroxidases, amine oxidase, oxalate oxidase, and flavin-containing oxidase, which may act downsteam of Ca^{2+} generation, account for AOS production. RBOH-dependent AOS production is not the key contributor to elicitor-induced HR. AOS of other origin and the balance between both AOS and NO determine the fate of the cell.

Based on these results, a simple model of elicitor signalling is presented (Fig. 9). This model considers how RBOH, AOS, and NO production (including the balance between AOS and NO), and Ca^{2+} are associated with elicitor-induced stomatal closure and HR cell death. Elicitors may trigger a Ca^{2+} spike activating upstream of RBOH and induce NO-associated stomatal closure. On the other hand, AOS of another origin and the balance between AOS and NO may be associated with elicitor-triggered HR cell death. These results are in agreement with previous evidence that AOS are activated in response to elicitors, and that H_2O_2 is crucial to the regulation of stomatal movements, thus shedding further light on this complex and important topic.

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