The role of respiratory burst oxidase homologues in elicitor-induced stomatal closure and hypersensitive response in \textit{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 gp91phox, 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 \textit{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) \textit{N. benthamiana} plants. The hypersensitive response (HR) of cell death and pathogenesis-related (PR) gene expression of these gene-silenced \textit{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 \textit{N. benthamiana} function in elicitor-induced stomatal closure, but not in elicitor-induced HR.

Key words: AOS, elicitor, hypersensitive response, \textit{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 ($\text{H}_2\text{O}_2$), superoxide ($\text{O}_2^-$), and the highly reactive hydroxyl radical ($\text{OH}^-$). Plants have evolved many AOS-scapenging 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 $\text{H}_2\text{O}_2$ sources (Bolwell and Wojtaszek, 1997; Bolwell \textit{et al.}, 2002), the NADPH oxidase complex is considered as one of the most important sources of oxidative burst (Bolwell \textit{et al.}, 1998; Grant \textit{et al.}, 2000; Torres and Dangl, 2005). Plant respiratory burst oxidase homologues (RBOH) of gp91phox, a plasma membrane
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 gp91phox (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, rbhol-silenced Nicotiana tabacum showed reduced disease resistance to Phytophthora infestans (Yoshioka et al., 2003); rbhol from Zinnia elegans was involved in xylem differentiation (Barcelo, 2005); rbhol knockdowns in tomato resulted in growth anomalies (Sagi et al., 2004); rbholC from Arabidopsis may have regulated cell expansion during root hair formation (Foreman et al., 2003). Stomatal closure was also severely inhibited in Arabidopsis rbholD/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., 2002a; 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, rbhol Arabidopsis mutants with reduced H$_2$O$_2$ 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, rbhol 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 boehmeria, overnight cultures of E. coli cells, BL21 carrying pET32b with the inf1 (GenBank accession no. AY830094) or boehmeria (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 hrfl (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, boehmeria, 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
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 H₂O₂ burst. Quantitative scoring of H₂O₂ 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 MgCl₂). 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′-CTGCTTGAATAGAACACCTGGA-3′;
- *NbrbohA* reverse primer: 5′-CCCGGGTATCTCGTTTCTGTC-3′;
- *NbrbohB* forward primer: 5′-CCAGCGGTTTTGCTCAAGTTTCA-3′; and
- *EF1α* reverse primer: 5′-CCACAAATCTTGTACACATCC-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′-ATGCCCTAACAGCTGC-3′; *PR-1a* reverse primer: 5′-GAGATCATAGTTTGCAAGAG-3′; *PR-1b* reverse primer: 5′-GTATGGCCTTGTGCATGAC-3′; and *PR-1c* reverse primer: 5′-GGATCATAGTTTGCAAGAG-3′.

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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.
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

![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.](https://academic.oup.com/jxb/article-abstract/60/11/3109/531503)
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) phenoxyl]-2-(2-amino-5-methylphenoxy) ethane-N,N,N',N'-tetra-acetic acid and penta-acetoxyethyl 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 \(\text{H}_2\text{O}_2\) generation

It has been reported that \(\text{NbrbohA}\) is expressed constitutively at a low level, whereas the accumulation of \(\text{NbrbohB}\) protein is induced by cell wall elicitors (Yoshioka et al., 2003). To investigate whether agro-infiltrated \(N.\) benthamiana exhibited lower \(\text{rboh}\) transcription, all inoculated antisense \(N.\) benthamiana were subjected to semi-quantitative reverse transcriptase (RT)-PCR analysis specific to each gene, using \(\text{EF1}\) \(\alpha\) transcript as an expression level control (Fig. 1A). The transcript of \(\text{NbrbohA}\) decreased 75% in both \(\text{NbrbohA}\)-silenced and \(\text{NbrbohA/B}\)-silenced plants compared to the control, while the transcript of \(\text{NbrbohB}\) decreased 67% in \(\text{NbrbohB}\)-silenced plants and 75% in \(\text{NbrbohA/B}\)-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 \(\text{H}_2\text{O}_2\) produced by endogenous superoxide dismutation of superoxide radicals has been used to quantify RBOH activity (Yoshioka et al., 2003). Elicitor-induced \(\text{H}_2\text{O}_2\) production measurements were performed using DAB staining, which indicated \(\text{H}_2\text{O}_2\) accumulation by the formation of a brown precipitate. Figure 2A shows the development of the DAB-\(\text{H}_2\text{O}_2\) reaction product in leaves of the control and \(\text{Nbrboh}\)-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 \(\text{Nbrboh}\)-silenced leaves after various elicitor treatments (Fig. 2A). Results of further quantitative analysis, using the software Quantity One, revealed that \(\text{Nbrboh}\)-silencing attenuated elicitor-induced \(\text{H}_2\text{O}_2\) production (Fig. 2B). The results suggest that NADPH oxidases mediate elicitor-induced AOS generation in \(N.\) benthamiana, and that \(\text{NbrbohA}\) and \(\text{NbrbohB}\) 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 \(\text{Nbrboh}\)-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 nM 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 \(\text{Nbrboh}\)-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) \(\text{NbrbohA/B}\) dual-silenced \(N.\) benthamiana, (C) \(\text{NbrbohA}\)-silenced \(N.\) benthamiana, (D) \(\text{NbrbohB}\)-silenced \(N.\) benthamiana. Leaves were removed from plants after 3 d of treatment and bleached in ethanol.](https://example.com/fig3.png)
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 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.
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.

**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. 2002a). 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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stomatal aperture (( \mu )m)</th>
<th>( P=0.01 )</th>
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<tr>
<td>(A)</td>
<td></td>
<td></td>
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<tr>
<td>PVX Nb</td>
<td>0.41±0.08 c</td>
<td></td>
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<tr>
<td>( NbrbohA )-silenced Nb</td>
<td>1.37±0.40 bc</td>
<td></td>
</tr>
<tr>
<td>( NbrbohB )-silenced Nb</td>
<td>3.01±0.86 bc</td>
<td></td>
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<tr>
<td>( NbrbohA/B )-silenced Nb</td>
<td>3.56±1.03 a</td>
<td></td>
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<tr>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVX Nb</td>
<td>0.40±0.24 b</td>
<td></td>
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<tr>
<td>( NbrbohA )-silenced Nb</td>
<td>1.20±0.94 b</td>
<td></td>
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<tr>
<td>( NbrbohB )-silenced Nb</td>
<td>1.31±0.77 b</td>
<td></td>
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<tr>
<td>( NbrbohA/B )-silenced Nb</td>
<td>3.65±0.40 b</td>
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<td>(C)</td>
<td></td>
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<tr>
<td>PVX Nb</td>
<td>0.35±0.42 b</td>
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<tr>
<td>( NbrbohA )-silenced Nb</td>
<td>0.88±0.64 ab</td>
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<tr>
<td>( NbrbohB )-silenced Nb</td>
<td>2.21±1.10 ab</td>
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<td>( NbrbohA/B )-silenced Nb</td>
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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 \([\text{Ca}^{2+}]_{\text{cyt}}\) 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 \(\text{H}_2\text{O}_2\) and NO, are not involved in elicitor-induced stomatal closure but are related to elicitor-induced HR

Reduced DAB-H\(_2\)O\(_2\) 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; Geczy 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 \(\text{H}_2\text{O}_2\) and NO) in guard cells. Another enzyme might account for \(\text{H}_2\text{O}_2\)
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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ production in leaves. Upon elicitor treatment, the production of H$_2$O$_2$ was obviously decreased, but some brown precipitate remained in the silenced leaves. This result indicates that RBOH are the major H$_2$O$_2$ source. Extracellular AOS production by NbrbohA and NbrbohB is required for elicitor-induced AOS production. Our data do not exclude the possibility that
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**

H₂O₂ and NO function as stress signals in plants, mediating a range of responses to environmental stress (Neill et al., 2002b). Nbrboh-silenced plants showed less H₂O₂ 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 H₂O₂ was neither necessary nor sufficient for HR cell death, PAL activation, or SA accumulation in cultured tobacco cells. Although both H₂O₂ 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 H₂O₂ in response to pathogen attack and mediates defence responses similar to observations following H₂O₂ 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

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**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 ≥3) fluorescence intensity per pixel.
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\textsuperscript{2+} acts upstream of \textit{Nbrboh}

All elicitors in this study induced bright fluorescence, which
is consistent with the early elicitor-induced Ca\textsuperscript{2+} spike
reported by Garcia-Brugger et al. (2006). There was little
visible difference in Ca\textsuperscript{2+} fluorescence between the controls
and the \textit{rboh}-silenced plants 3 h after elicitor treatment.
Apoplastic Ca\textsuperscript{2+} influx is important to the oxidative burst,
and Ca\textsuperscript{2+} can activate RBOH proteins \textit{in vitro} in tobacco
and tomato (Miura et al., 1995, 1999; Sagi and Fluhr,
2001). Overexpression of TPC1 from \textit{O. sativa}, a putative
voltage-gated Ca\textsuperscript{2+}-permeable channel, enhances elicitor-
induced oxidative burst (Kurusu et al., 2005). Ecotopic
expression of \textit{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 \textit{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\textsuperscript{2+} signalling.

\textit{RBOH}-silencing with decreased H\textsubscript{2}O\textsubscript{2} and NO
production affects elicitor-induced stomatal closure, but
not HR

It was found that \textit{Nbrboh}-silenced plants decreased H\textsubscript{2}O\textsubscript{2}
production and that inhibition of elicitor-induced stomatal
closure was associated with less NO fluorescence in guard
cells of \textit{Nbrboh}-silenced \textit{N. benthamiana}, which is consistent
with other reports. Elicitor-induced H\textsubscript{2}O\textsubscript{2} production leads
to stomatal closure (McAinsh et al., 1996; Lee et al., 1999),
while inhibition of H\textsubscript{2}O\textsubscript{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 \textit{Arabidopsis}
NADPH oxidase (\textit{rbohD}) double mutants, suggesting
that endogenous H\textsubscript{2}O\textsubscript{2} production elicited by ABA is
required for NO synthesis (Bright et al., 2006). ABA, which
induces stomatal closure in a Ca\textsuperscript{2+} and cGMP-dependent
manner, stimulates NO synthesis in guard cells, indicating
that NO is an even earlier secondary messenger in this
response pathway.

\textit{Nbrboh}-silencing did not affect elicitor-induced HR.
Similar and bright fluorescence was observed in \textit{Nbrboh}-
silenced and control plants stained with DHR. The results
suggest that \textit{Nbrboh} is not the key contributor to elicitor-
induced HR. AOS (other than H\textsubscript{2}O\textsubscript{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).

Based on these results, a simple model of elicitor signal-
ing is presented (Fig. 9). This model considers how RBOH,
AOS, and NO production (including the balance between
AOS and NO), and Ca\textsuperscript{2+} are associated with elicitor-
induced stomatal closure and HR cell death. Elicitors may
trigger a Ca\textsuperscript{2+} spike activating upstream of RBOH and
induce NO-associated stomatal closure. On the other hand,
AOS of another origin and the balance between both AOS and NO determine the fate of
the cell.

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