Nicotiana benthamiana gp91^{phox} Homologs *NbrbohA* and *NbrbohB* Participate in H$_2$O$_2$ Accumulation and Resistance to *Phytophthora infestans*

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Active oxygen species (AOS) are responsible for triggering defense responses in plants. Respiratory burst oxidase homologs (*rboh* genes) have been implicated in AOS generation. We have isolated two *rboh* cDNAs, *NbrbohA* and *NbrbohB*, from *Nicotiana benthamiana* leaves. *NbrbohA* was expressed constitutively at a low level and the transcripts were increased after mechanical stress of control leaf infiltration, whereas *NbrbohB* was induced specifically by the protein elicitor INF1 from the potato pathogen *Phytophthora infestans*. We examined the function of the *Nbrboh* genes in AOS generation and in the hypersensitive response (HR) using virus-induced gene silencing (VIGS). VIGS indicated that both genes are required for H$_2$O$_2$ accumulation and for resistance to Phytophthora. VIGS of *Nbrboh* genes also led to a reduction and delay of HR cell death caused by INF1. We further demonstrate that the induction of HR-like cell death by overexpression of a constitutively active mutant of a mitogen-activated protein kinase kinase, MEK^{DD}, is compromised by VIGS of *NbrbohB*. We found that MEK^{DD} induced *NbrbohB* but not *NbrbohA*. This work provides genetic evidence for the involvement of a mitogen-activated protein kinase cascade in the regulation of *rboh* genes.

INTRODUCTION

The oxidative burst is a characteristic early feature of the hypersensitive response (HR) during incompatible interactions between resistant plants and avirulent pathogens (Doke, 1983; Dietrich et al., 1996; Jabs et al., 1996; Lamb and Dixon, 1997). The rapid production of active oxygen species (AOS) could inhibit pathogen growth by direct microbicidal action (Peng and Kuc, 1992), by restricting pathogen penetration via cross-linking of cell wall glycoproteins (Bradley et al., 1992), by induction of phytoalexin accumulation or defense-related genes (Apostol et al., 1989; Desikan et al., 2001), and by activating salicylic acid biosynthesis (Léon et al., 1995), which leads to the induction of genes such as *PR-1a* (Chen et al., 1993).

The enzymatic complex of NADPH oxidase found in mammalian neutrophils consists of two plasma membrane proteins, gp91^{phox} and p22^{phox} (phox for phagocyte oxidase). The several cytosolic regulatory proteins, p47^{phox}, p67^{phox}, and the small G protein Rac, translocate to the plasma membrane to form the active complex after stimulation (Bokoch, 1994). Several lines of evidence demonstrated that the oxidative burst in plants is caused by activation of an NADPH oxidase closely resembling that operating in activated neutrophils (Lamb and Dixon, 1997). Incubation of potato tuber slices with an incompatible race of *Phytophthora infestans*, the potato late blight pathogen, or with

a hyphal wall elicitor stimulated NADPH-dependent O$_2^-$-generating activity, which was located predominantly in the plasma membrane fraction (Doke, 1985; Doke and Miura, 1995). Diphenylene iodonium, an inhibitor of the phagocyte NADPH oxidase, blocks the oxidative burst in plant cells (Levine et al., 1994; Auh and Murphy, 1995; Piedras et al., 1998). Homologs of gp91^{phox} (respiratory burst oxidase homolog [*rboh*]), which is the catalytic subunit of phagocyte NADPH oxidase, were isolated from several plants (Groom et al., 1996; Keller et al., 1998; Torres et al., 1998; Amicucci et al., 1999). The Arabidopsis genome contains eight *rboh* genes, suggesting that individual isoforms have different functions and participate in multiple distinct signaling pathways (Torres et al., 2002).

We have isolated *rboh* cDNAs from potato (Yoshioka et al., 2001). The cloning of these cDNAs revealed two isogenes, designated *StrbohA* and *StrbohB*. RNA gel blot analyses showed that *StrbohA* is expressed constitutively, whereas *St-rbohB* is induced by a cell wall elicitor from Phytophthora, salicylic acid, or arachidonic acid in potato tubers. The proteins have an N-terminal extension, which contains two Ca^{2+} binding elongation factor (EF) hands, that is absent in the phagocyte NADPH oxidase (Yoshioka et al., 2001). Earlier work provided evidence that signaling events preceding the burst include the involvement of a plant homolog of human Rac (Kawasaki et al., 1999), which is required for the assembly of an active phagocyte NADPH oxidase (Bokoch, 1994). However, no homologs of the p22^{phox}, p47^{phox}, or p67^{phox} regulators of the phagocyte NADPH oxidase were found in the Arabidopsis genome (Dangl and Jones, 2001). Calcium influx into the cytoplasm (Chandra and Low, 1997; Piedras et al., 1998) and changes in protein

phosphorylation (Kauss and Jeblick, 1995; Miura et al., 1995) are implicated in the activation process of the oxidative burst. To address the function of *rboh* genes in plant defense, we used virus-induced gene silencing (VIGS) in *Nicotiana benthamiana* as an efficient reverse genetics approach (Baulcombe, 1999). This allowed us to circumvent the problem of gene redundancy in plants by potentially silencing redundant members of the *rboh* subfamily.

Phytophthora species produce low molecular mass extracellular proteins known as elicitins, which induce HR and other biochemical changes associated with defense responses in *Nicotiana* species (Ricci et al., 1989; Huet et al., 1994; Kamoun et al., 1999; Sasabe et al., 2000) and some radish and turnip cultivars (Kamoun et al., 1993), presumably after binding to a plasma membrane receptor (Bourque et al., 1999). The perception of elicitin by the plant cell induces an influx of calcium ions (Tavernier et al., 1995), AOS production (Rustérucci et al., 1996), and activation of mitogen-activated protein kinase (MAPK) (Zhang et al., 1998). In contrast to strains of Phytophthora that produce elicitin INF1, strains that are engineered to be deficient in INF1 induce disease lesions on *N. benthamiana*, suggesting that INF1 functions as an avirulence factor that conditions resistance in this species (Kamoun et al., 1998). This finding demonstrates that the *N. benthamiana*–Phytophthora pathosystem is a good model for dissecting the elicitor response and resistance to Phytophthora by VIGS.

Here, we isolated the cDNAs corresponding to *StrbohA* and *StrbohB* of potato from a *N. benthamiana* cDNA library. The *NbrbohA*- and *NbrbohB*-silenced *N. benthamiana* plants were infected with Phytophthora and found to be unable to accumulate AOS during disease resistance reactions to Phytophthora. We demonstrate that the silenced plants were compromised in the INF1-mediated activation of HR. Moreover, we observed that overexpression of a constitutively active mutant of a MAPK kinase (MAPKK) (Yang et al., 2001), which activates endogenous salicylic acid–induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), provoked cell death in *N. benthamiana* plants and also induced the *NbrbohB* gene. These results suggest that the MAPK pathway can regulate *rboh* gene expression.

RESULTS

Two cDNAs for the gp91*phox* Homolog in *N. benthamiana*

We identified two homologs of human gp91*phox*, designated *NbrbohA* and *NbrbohB* (Figure 1). The *NbrbohA* cDNA was 3732 bp in length and had an open reading frame of 962 amino acids with a predicted molecular mass of 109 kD. The longest *NbrbohB* cDNA sequence was 3290 bp in length and had an open reading frame of 939 amino acids with a predicted molecular mass of 106 kD. We cannot exclude the possibility that more *Nbrboh* genes exist. However, the analysis of cDNAs obtained under low-stringency conditions from 6×10^5 plaques of the cDNA library prepared from leaf tissues treated with hyphal wall elicitor suggests that, if there are more gp91*phox* homologs in *N. benthamiana*, they are unlikely to be expressed in elicitor-stimulated leaf tissues.

Although the topology and functions of plant rboh proteins have not been tested experimentally, several features of the amino acid sequence and comparisons with human gp91*phox* allow predictions (Yu et al., 1998). NbrbohA and NbrbohB proteins are predicted to have six transmembrane-spanning domains (TMD-1 to TMD-6) that correspond to those identified in gp91*phox*. TMD-3 and TMD-5 contain pairs of His residues that are likely to be important for heme binding (Finegold et al., 1996). The C-terminal regions of each homolog contain conserved flavin adenine dinucleotide, NADPH-ribose, and NADPH-adenine binding sites, which are likely to be located in the cytoplasm. Moreover, human gp91*phox* amino acid residues Pro-415 and Asp-500, which are indispensable for the catalytic activity (Segal et al., 1992), also are conserved in NbrbohA and NbrbohB. The N-terminal regions of the proteins are hydrophilic and contain two Ca^{2+} binding EF hands, as seen in other plant *rboh* genes. Calcium binding activity of these domains was found experimentally by Keller et al. (1998) for Arabidopsis *AtrbohA*.

Comparison of the Nbrboh Proteins and Genes

Figure 2A shows a phylogenetic tree for polypeptides of NbrbohA, NbrbohB, and the 13 reported plant rboh proteins. The plant rboh proteins, except for AtrbohE, were divided into two groups. The deduced amino acid sequence of NbrbohA had maximum sequence similarity to tobacco NtrbohF (96% identity). On the other hand, NbrbohB was most similar (99% identity) to tobacco NtrbohD.

Gel blot hybridizations were performed with *N. benthamiana* genomic DNA samples under high-stringency conditions using regions of the cDNAs that encode the N-terminal portions of NbrbohA and NbrbohB as gene-specific probes (Figures 1 and 2B). The hybridization patterns of the blots were different, indicating that these probes detect each *Nbrboh* sequence. Moreover, each isogene-specific probe was shown to hybridize to another paralog in the genome, suggesting that there are two copies of *NbrbohA* and *NbrbohB*. Smith (1979) reported that the *N. benthamiana* genome contains 19 chromosomes (1*n* value), in contrast to diploid members of the Solanaceae, which have 12 chromosomes. DNA gel blot analysis is consistent with the proposed duplication of *Nbrboh* isogenes.

Induction of *NbrbohB* by INF1 and Salicylic Acid

We estimated changes over time in the transcript levels of *NbrbohA* and *NbrbohB* in *N. benthamiana* leaves after elicitation with INF1 *S3* (HR−) or INF1 (HR+), salicylic acid (SA), or methyl jasmonic acid (MeJA) or after mechanical stress caused by infiltrating water into leaves. INF1 *S3* (HR−) contains a mutated INF1 (HR+) sequence in which the Cys at position 3 of the processed protein is mutated into a Ser and serves as a negative control (Kamoun et al., 1997). The probes from the entire sequences of *NbrbohA* and *NbrbohB* were not sensitive enough to detect a signal with RNA gel blot analysis (data not shown). Therefore, reverse transcriptase–mediated (RT) PCR with gene-specific primers was performed. We also used primers for *hsr203J* (Tronchet et al., 2001) and *EF-1α* as controls for an induced and a constitutively expressed gene, respectively (Figure 3).
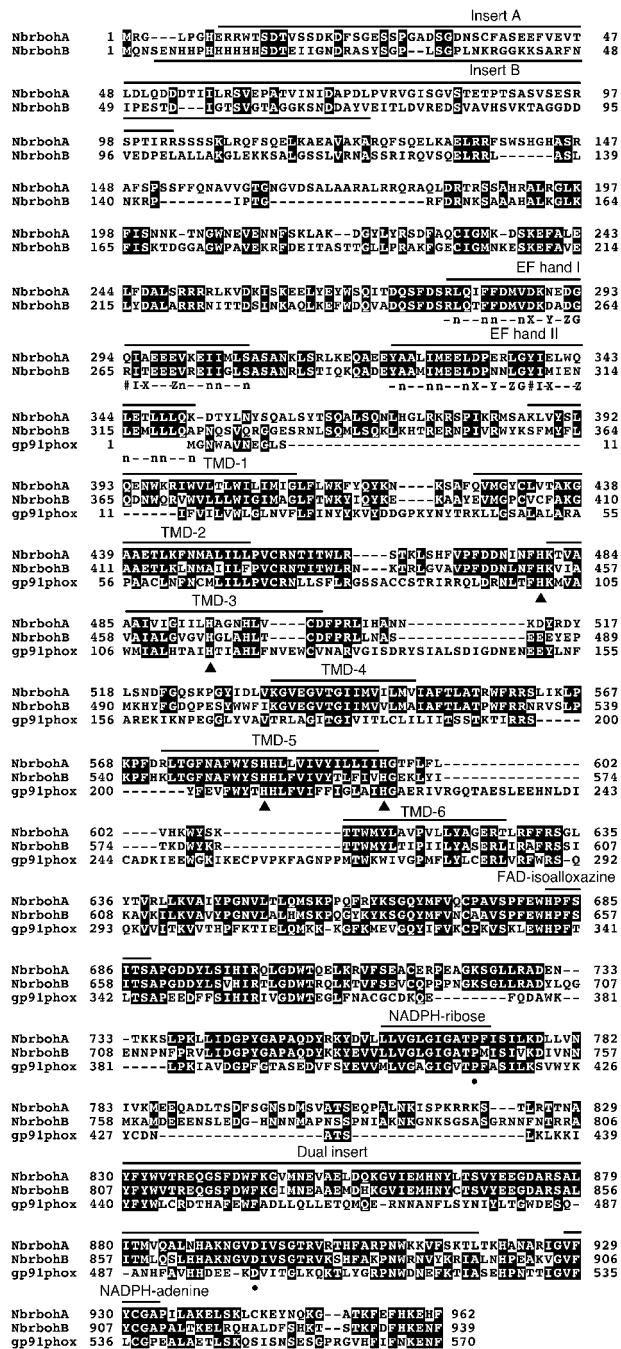
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                                        Insert A
NbrbohA    1  MRG---LPGHERRWTSDTVSSDKDFSGESSPGADSGDNSCFASEEFVEVT  47
NbrbohB    1  MQNSENHHPHHHHHSDTEIIGNDRASYSGE--LSGPLNKRGGKKSARFN   48
                                        Insert B
NbrbohA   48  LDLQDDDTILLRSVEPATVINIDAPDLPVRVGISGVSTETPTSASVSESR  97
NbrbohB   49  IPESTD---IGTSVGTAGGKSNDDAYVEITLDVREDSVAVHSVKTAGGDD  95

NbrbohA   98  SPTIRRSSSSKLRQFSQELKAEAVAKARQFSQELKAELRRFSWSHGHASR  147
NbrbohB   96  VEDPELALLAKGLEKKSALGSSLVRNASSRIRQVSQELRRL------ASL  139

NbrbohA  148  AFSPSSFFQNAVVGTGNGVDSALAARALRRQRAQLDRTRSSAHRALRGLK  197
NbrbohB  140  NKRP--------IPTG----------------RFDRNKSAAAHALKGLK   164

NbrbohA  198  FISNNK-TNGMNEVENNFSKLAK--DGYLYRSDFAQCIGMK-DSKEFALE  243
NbrbohB  165  FISKTDGGAGMPAVEKRFDEITASTTGLLPRAKFGECIGMKNKSKEFAVE  214
                                                           EF hand I
NbrbohA  244  LFDALSRRRRLKVDKISKEELYEYWSQITDQSFDSRLQIFFDMVDKNEDG  293
NbrbohB  215  LYDALARRRNITTDSINKAQLKEFWDQVADQSFDSRLQTFFDMVDKDADG  264
                 -n--nn--nX-Y-ZG
                                                          EF hand II
NbrbohA  294  QHAEEEVKEIIMLSASANKLSRLKEQAEYAALIMEELDPERLGYIELWQ   343
NbrbohB  265  RITEEEVREIIGLSASANRLSTIQKQADEYAAMIMEELDPNNLGYLMIEN  314
              #I-X--Zn--nn--n              -n--nn--nX-Y-ZG#I-X--Z
NbrbohA  344  LETLLLQK-DTYLNYSQALSYTSQALSQNLHGLKRKSPIKRMSAKLVVSL  392
NbrbohB  315  LEMLLLLQAPNQSVQRFGGESRNLSQMLSQKLKHTFERMPIVRWYKSFMVFL 364
gp91phox   1  MGNWAVNEGLS-----------------------  11
              n--nn--n  TMD-1
                                      TMD-1
NbrbohA  393  QENWKRIWVLTLWILIMIGLFLWKFYQYKN-----KSAFQVMGYCLVTAKG 438
NbrbohB  365  QDNWQRVWVLLLWIGIMAGLFTWKYIQYKE----KAAYEVMGPCVCPAKG  410
gp91phox  11  ------IFVILVMLGLNVFLFINYLKVLDDGPKYNYTRKLLGSALALARA  55
                                      TMD-2
NbrbohA  439  AAETLKFNMALILLPVCRNTITWLR----STKLSHFVPFDDNLNFHKTVA  484
NbrbohB  411  AAETLKLNMAILILPVCRNTITWLRN---KHRLGVAVPFDDNLNFHKVIA  457
gp91phox  56  PAACLNFNCMLILLPVCRNLLSFLRGSSACCSTRIRRQLDRNLTFHKMVA  105
                                      TMD-3
NbrbohA  485  AAIVIGIILHAGNHLV----CDFPRLIHANN------------KDYRDY  517
NbrbohB  458  VAIAIGVGVHGLAHLT----CDFPRLLNAS-------------EEYEP   489
gp91phox 106  WMIALHTAIHTIAHLFNVEWCVNARVGISDRYSIALSDIGDNENEEYLNF  155
                                      TMD-4
NbrbohA  518  LSNDFGQSKPGYIDLKVGVEGVTGIIMVILNVIAFTLATRMFRRSLIKLP  567
NbrbohB  490  MKHYFGDQPESVWWFIKVGVEGVTGIIMVVLNAIAFTLATPMFRRNRVSLP 539
gp91phox 156  AREKIKNPEGGLYVAVTRLAGITGIVITLCLILITSSTKTIRRS-----  200
                                      TMD-5
NbrbohA  568  KPFDRLTGFNAFWYSHHLLVIVYILLIHGTPLFL----------------  602
NbrbohB  540  KPFHKLTGFNAFWYSHHLFVIVYTDFIVHGEKLYI---------------  574
gp91phox 200  -------YFEVFWYTHHLFVIFFIGLAIHGAERIVRGQTAESLEEHNLDI  243
                                      TMD-6
NbrbohA  602  ----VHKTYSK-------------TTWMYLAVPVLLYAGERTLRFFRSGL  635
NbrbohB  574  ----TKDTYKR-------------TTWMYLPIPILILYASSRLIRAFRSSI 607
gp91phox 244  CADKIEEWGKIKECPVPKFAGNPPMTWKWIVGPMFLYLCERLVRFWRS-Q  292
                                      FAD-isoalloxazine
NbrbohA  636  YTVRLLKVAIYPGNVLTLQMSKPPQFRYKSGQYMFVQCPAVSPFEWHPFS  685
NbrbohB  608  KAVKFLKVAVYPGNVLALHMSKPQGYKYKSGQYMFVNCAAVSPFEWHPFS  657
gp91phox 293  QKVVITKVVTHPFKTIELQMKK-KGFKMEVGQYIFVKCPKVSKLEWHPFT  341

NbrbohA  686  ITSAPGDDYLSIHIRQLGDWTQELKRVFSEACERPEAGKSGLLRADEN--  733
NbrbohB  658  ITSAPGDDYLSVHIRTLGDWTQLRTVFSEVCQPPPNGKSLLRADYLQG    707
gp91phox 342  LTSAPEEDFFSIHIRIVGDWTEGLFNACGCDKQE--------FQDAWK--  381
                                      NADPH-ribose
NbrbohA  733  -TKKSLPKLLIDGPYGAPAQDYRKYDVLLLVGLGIGATPFISILKDLLVN  782
NbrbohB  708  ENNPNFPRVLIDGPYGAPAQDYKKYEVVLLVGLGIGATPMISIVKDIVNN  757
gp91phox 381  -----LPKIAVDGPFGTASEDVFSYEVVMLVGCAGIGVTPFASILKSVWYK 426

NbrbohA  783  IVKMEEQADLTSDFSGNSDMSVATSEQPALNKISPKRRKS---TLRTTNA  829
NbrbohB  758  MKAMDEEENSLEDG-HNNNMAPNSGPNIAKNKGNKSGSASGRNNNFNRRA  806
gp91phox 427  YCDN-----------------ATS------------------LKLKKI   439
                                      Dual insert
NbrbohA  830  YFYWVTREQGSFDWFKGVMNEVAELDQKGVIEMHNYLTSVYEEGDARSAL  879
NbrbohB  807  YFYWVTREQGSFDWFKGIMNEAAEMDHKGVIEMHNYCTSVYEEGDARSAL  856
gp91phox 440  YFYWLCRDTHAFEWFADLLQLLETQMQE-RNNANFLSYNIYLTGWDESQ-  487

NbrbohA  880  ITMVQALNHAKNGVDIVSGTRVRTHFARPNWKKVFSKTLTKHANARIGVF  929
NbrbohB  857  ITMLQSLHAKNGVDIVSGTRVKSHFAKPNWRNVYKRIALNHPEAKVGVF   906
gp91phox 487  -ANHFAVHHDEE-KDVITGLKQKTLYGRPNWDNEFKTIASEHPNTTIGVF  535
             NADPH-adenine
NbrbohA  930  YCGAPILAKELSKLCKEYNQKG--ATKFEFHKEHF  962
NbrbohB  907  YCGAPALTKELRQHALDFSHKT--STKFDFHKENF  939
gp91phox 536  LCGPEALAETLSKQSISNSESCGPRGVHFIFNKENF 570
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Figure 1. Alignment of the Predicted Amino Acid Sequences of NbrbohA, NbrbohB, and Human gp91*phox*.

Amino acids that are conserved are boxed in black. Six potential transmembrane-spanning domains (TMD-1 to TMD-6) are indicated with overlines. Conserved His residues, demonstrated to bind heme in yeast FRE1 (Finegold et al., 1996), are indicated by solid triangles. Dots under the sequences indicate amino acid residues involved in *N*-glycosylation. EF hand motifs in the N-terminal domain of Nbrboh are overlined. Beneath these motifs is the sequence of a canonical EF hand (Kretsinger, 1996). n is usually a hydrophobic residue. Dashes indicate variable amino acid residues. X, Y, Z, and −X, contain oxygen within their side chains. Carbonyl oxygen of # serves as a ligand. −Z is usually Glu. Re-

NbrbohA mRNA was expressed constitutively at a low level before injection. The increase in the transcript level was detected 9 to 24 h after all injections, including water control and INF1 *S3* (HR−), except injection of MeJA. This induction could be caused by the mechanical stress of the infiltration procedure. MeJA suppressed the infiltration stimulus–induced *NbrbohA* transcript accumulation. On the other hand, RT-PCR of *NbrbohB* and *hsr203J* showed that the infiltration of INF1 (HR+) led to specific elicitation in the transcript level. Moreover, exogenous SA stimulated the accumulation of *NbrbohB* and *hsr203J* transcripts at a concentration of 0.5 mM, which is sufficient to induce the oxidative burst in plant cell suspensions (Kauss and Jeblick, 1995; Shirasu et al., 1997) and plant tissues (Yoshioka et al., 2001). By contrast, the transcript level of *EF-1α*, an abundant protein of the cell, is unaltered under these conditions.

Isogene-Specific VIGS of the *Nbrboh* Subfamily

Because the N-terminal regions of NbrbohA and NbrbohB are distinct and the C-terminal regions are homologous, we conducted isogene-specific and isogene-nonspecific gene silencing using *NbrbohA*- and *NbrbohB*-specific regions or the conserved region as a dual insert for *Potato virus X* (PVX) vector (Figure 1). The *NbrbohA* and *NbrbohB* inserts were 278 and 197 bp and showed 41 and 40% nucleotide identity to the corresponding regions of *NbrbohB* and *NbrbohA*, respectively. For the dual insert, the silencing construct was generated by inserting a 234-bp *NbrbohA* cDNA fragment, which constituted most of the conserved region of *rboh*, into the viral vector. This fragment showed 80% nucleotide identity to *NbrbohB*.

Two to 3 weeks after inoculation of leaves with PVX-NbrbohA (PVX:A), PVX-NbrbohB (PVX:B), PVX-NbrbohA/B dual-silencing insert (PVX:A/B), or PVX (no insert), plants were analyzed for *NbrbohA* and *NbrbohB* transcripts 24 h after treatment with INF1 (HR+) or Phytophthora (Figure 4). The transcript levels were determined by RT-PCR using gene-specific primers that amplify regions that do not contain insert sequences for PVX vector. The transcript accumulation of *NbrbohA* was reduced dramatically in PVX:A- and PVX:A/B-inoculated plants but was unaffected in *NbrbohB*-silenced plants. On the other hand, *NbrbohB* was silenced in PVX:B- and PVX:A/B-inoculated plants but not in *NbrbohA*-silenced plants. These data indicate that the *NbrbohA* or *NbrbohB* isogene was silenced specifically when insert A or insert B was used, respectively, and that both genes were silenced when the dual insert was used. We estimated the relative amounts of *NbrbohA* and *NbrbohB* transcripts by densitometric scanning of the negative films from the DNA gels of PCR products. In all cases, >80% of the transcripts were reduced by VIGS. The coat protein (CP) transcript

gions used for VIGS and DNA gel blot analysis are indicated by insert A and insert B. Dashes indicate gaps in the sequence to allow for maximal alignment. Multiple alignment of the protein sequence was made using the CLUSTAL W program (Thompson et al., 1994). FAD, flavin adenine dinucleotide.
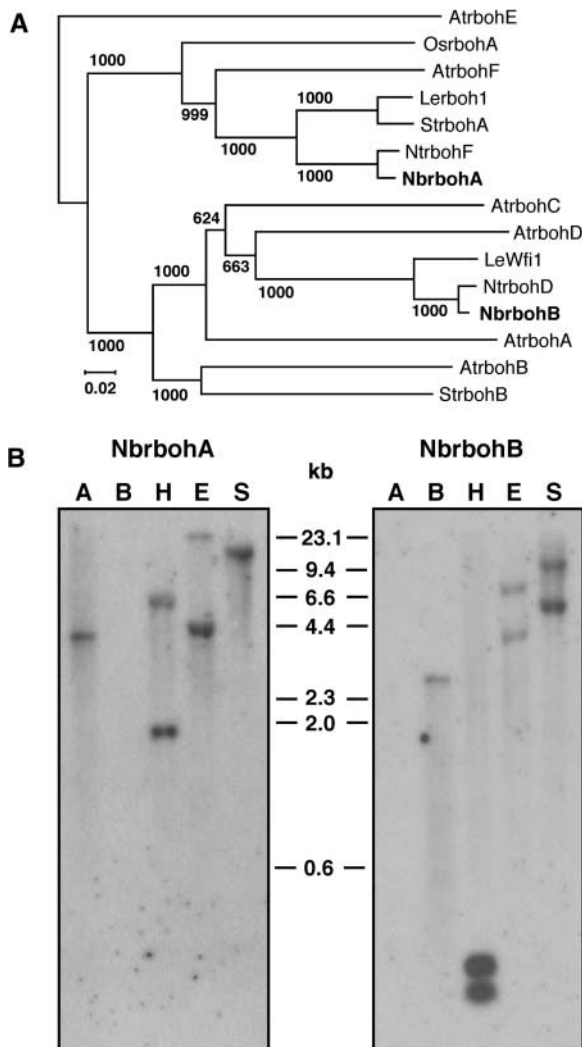
Figure 2. *Nbrboh* Gene Organization.

(A) Phylogenetic tree of various plant respiratory burst oxidase homologs. An unrooted tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). The length of the horizontal lines connecting the sequences is proportional to the estimated genetic distance between these sequences. The number above or below each horizontal line is the frequency with which a given branch appeared in 1000 bootstrap replications. Accession numbers are listed at the end of Methods. **(B)** DNA gel blot hybridization of genomic DNA. Genomic DNA (10 μg) was digested with HindIII (H), EcoRI (E), or SpeI (S) and hybridized with the ^{32}P-labeled N-terminal region of *NbrbohA* or *NbrbohB* cDNA as indicated in Figure 1. Lane A, full-length *NbrbohA* cDNA (30 pg); lane B, full-length *NbrbohB* cDNA (30 pg). DNA molecular mass markers are indicated.

of PVX was amplified as a marker for viral infection. *EF-1α* mRNA was unaltered in all plants.

Reduction of the Oxidative Burst and Disease Resistance of *Nbrboh*-Silenced *N. benthamiana* to Phytophthora

We evaluated whether silencing of *Nbrboh* genes had effects on the plant's resistance response against Phytophthora (Fig-

ure 5). The zoospores of Phytophthora race 0 could not infect the PVX control–inoculated leaves of *N. benthamiana*, and the leaf displayed macroscopic HR at 12 days after inoculation (Figure 5A, left). In some cases, no visible response was observed in the spots inoculated with Phytophthora. To determine the cytological basis of the resistance of *N. benthamiana* to Phytophthora, we observed the interaction microscopically using lactophenol-trypan blue–stained discs of inoculated leaves. After 3 days of inoculation, granular cytoplasm was observed in the responding cells, suggesting that a typical HR occurred in some but not all cases (Figure 5B, left). In most cases, the primary infection vesicle inside the host cell was not formed, suggesting that the pathogen is restricted to the cell during attempted penetration (Figure 5C, left). Moreover, we conducted 3,3′-diaminobenzidine (DAB) staining, which shows H_2O_2 accumulation visualized as a brown precipitate (Thordal-Christensen et al., 1997). Strongly polymerized DAB was detected 24 h after inoculation in the inoculated areas of whole leaf (Figure 5D, left) and in attacked cells (Figure 5E, left).

We performed similar analyses with PVX:A-, PVX:B-, and PVX:A/B-inoculated plants at both the macroscopic and microscopic levels, as illustrated in Figure 5. The typical water-soaked Phytophthora lesions appeared within 7 days after inoculation, and the expanding disease lesions around the inoculated spot were observed in all silenced plants at 12 days after inoculation (Figure 5A). As early as 3 days after inoculation, primary infection vesicles and secondary hyphae were observed in the infected cells at a lower rate (Figures 5B and 5C), indicating a partial loss of resistance. Importantly, brownish polymerized DAB was not detected at 24 h after inoculation in the inoculated cells, indicating that both *NbrbohA* and *NbrbohB* are required for the oxidative burst in response to Phytophthora (Figures 5D and 5E). *Nbrboh* silencing resulted in an increase in appressoria formation compared with that in the PVX control. These results suggest that the oxidative burst is involved in the early process of plant resistance to microbial pathogenesis. On the underside of the leaves (the leaf surface opposite to the inoculated side), sporangiophores emerging from stomata and numerous sporangia were readily observed in PVX:A-, PVX:B-, and PVX:A/B-inoculated *N. benthamiana* (Figure 5F).

VIGS of *Nbrboh* Compromises INF1-Mediated HR

To determine whether the HR cell death observed in *N. benthamiana* after recognition of the protein elicitor INF1 could involve an *rboh*-mediated oxidative burst, *inf1* was expressed under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV) using an agroinfiltration method in *Nbrboh*-silenced plants (Figure 6, top row). Because Phytophthora transformants deficient in INF1 induce disease lesions when inoculated on *N. benthamiana*, it has been suggested that INF1 functions as an avirulence factor in the interaction (Kamoun et al., 1998). At 3 days after infiltration, the necrotic reaction induced by INF1 was observed in PVX control–inoculated leaves of *N. benthamiana*, indicating that a typical HR response had occurred. By contrast, PVX:A-, PVX:B-, and PVX:A/B-inoculated plants displayed significantly reduced and delayed necrotic symptoms by INF1. This finding indicates that HR caused by INF1 is dependent on both *NbrbohA* and *NbrbohB*.
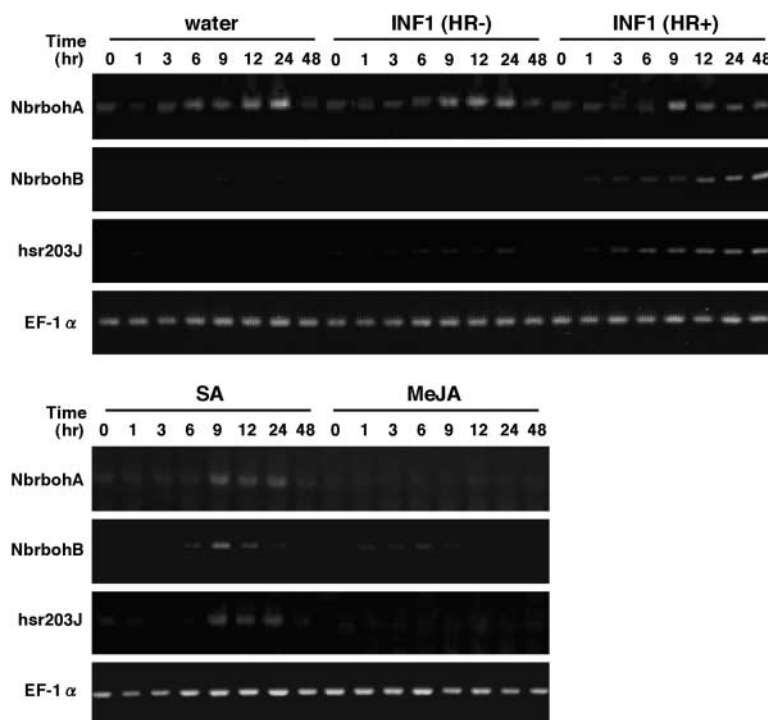
Figure 3. Expression Patterns of *NbrbohA* and *NbrbohB* after Elicitation by Wound or Pathogen Signals.

N. benthamiana leaves were treated with water, 10 μg/mL FLAG-INF1 *S3* (HR−), 10 μg/mL FLAG-INF1 (HR+), 0.5 mM SA, and 0.1 mM MeJA. At the times indicated, leaf samples were harvested. Total RNA was isolated and used for RT-PCR as described in Methods, applying 35, 29, and 35 amplification cycles with specific primers for *NbrbohA*, *NbrbohB*, and *hsr203J*, respectively. Equal loading of cDNA was monitored by amplification of a constitutively expressed *EF-1α* gene (29 cycles).

VIGS of *NbrbohB* Compromises MEK^DD-Mediated HR

Recent studies have indicated that expression of a constitutively active mutant of MAPKK, MEK^DD, activates endogenous SIPK and WIPK and leads to an oxidative burst (Ren et al., 2002). To address how the MAPK cascade regulates rboh-mediated HR cell death, *MEK^DD* was expressed by agroinfiltration in *Nbrboh*-silenced *N. benthamiana*. As shown in Figure 6 (bottom row), HR-like cell death initiated by *MEK^DD* was significantly reduced and delayed, and it developed only around the infiltration sites in PVX:B- and PVX:A/B-inoculated plants but not in PVX:A-inoculated plants. In most cases, the HR-like cell death initiated by *MEK^DD* in PVX:B- and PVX:A/B-inoculated plants occurred 2 to 3 days later than that observed in PVX control–inoculated leaves. These results indicate that the cell death that resulted from the activation of the MAPK pathway is dependent on active NbrbohB.

Induction of *NbrbohB* by MAPKK Activity

To address the question of how the MAPK cascade initiates HR-like cell death, we examined the transcript levels of *NbrbohA* and *NbrbohB* in leaves of *N. benthamiana* after infiltration of *Agrobacterium tumefaciens* harboring *MEK^DD* or *β-glucuronidase* (*GUS*) as a control under the control of the 35S

promoter of CaMV (Figure 7). *NbrbohA* was expressed constitutively at a low level before injection. The increase in the transcript levels of *NbrbohA* and *hsr203J* was detected 24 h after injection, except for in the control. This increase likely was caused by the mechanical stress of the infiltration procedure (Figure 3) or by infection stimulus from *A. tumefaciens*. Conversely, expression of *MEK^DD* resulted in specific elicitation of *NbrbohB* transcript within 24 h after infiltration. The transcript level of *EF-1α*, an abundant protein of the cell, was unaltered under these conditions. When the leaves were infiltrated with *A. tumefaciens* harboring *MEK^DD*, a maximum accumulation of the transcript was detected within 24 h, after which it declined. A massive H_2O_2 accumulation was detected by DAB staining, and HR-like necrosis appeared within 24 h (data not shown), as reported by Ren et al. (2002). Thus, we found that *NbrbohB* mRNA levels are influenced by a MAPK cascade.

DISCUSSION

Function of *Nbrboh* Genes in the Oxidative Burst and Defense Responses

We used VIGS in *N. benthamiana* to define biological functions for two *Nbrboh* genes in the oxidative burst during the defense
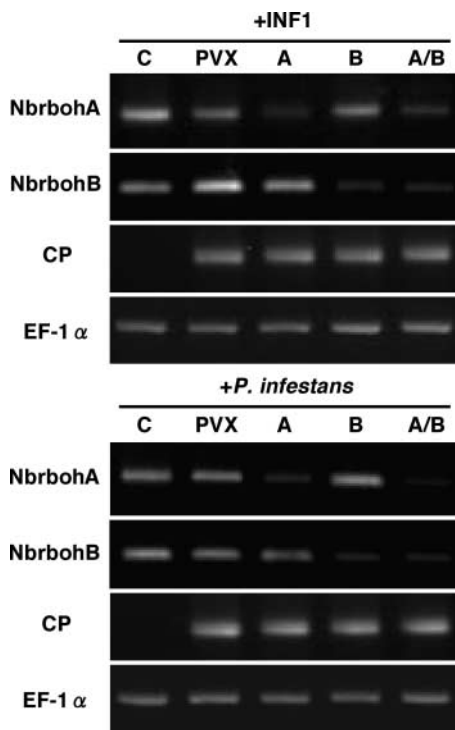
Figure 4. Effect of VIGS on the Transcription of *Nbrboh* Genes.

N. benthamiana leaves were inoculated with PVX, PVX:A (A), PVX:B (B), or PVX:A/B (A/B) or treated with water (C). Total RNA was isolated from leaves at 24 h after inoculation with zoospores of Phytophthora or injection with 10 μg/mL FLAG-INF1 (HR+) and used for RT-PCR as described in Methods, applying 35, 29, and 30 amplification cycles with specific primers for *NbrbohA*, *NbrbohB*, and *CP*, respectively. Equal loading of cDNA was monitored by amplification of a constitutively expressed *EF-1α* gene (29 cycles).

response. We observed that *NbrbohA-*, *NbrbohB-*, and *NbrbohA/B*-silenced plants show no DAB staining, a standard marker of H_2O_2 accumulation, after challenge with the nonpathogen Phytophthora (Figures 5D and 5E), suggesting that the enzyme that generates AOS in *N. benthamiana* is similar to phagocyte NADPH oxidase in mammalian cells. Furthermore, we found that *Nbrboh*-silenced *N. benthamiana* plants became susceptible to Phytophthora and exhibited enhanced sporangia and disease lesion formation by the pathogen (Figure 5). In PVX:A-, PVX:B-, and PVX:A/B-inoculated plants, necrotic symptoms caused by INF1 were significantly reduced and delayed (Figure 6, top row), consistent with the result that Phytophthora could infect those leaves.

The results presented here indicate that *NbrbohA* and *NbrbohB* share functions in H_2O_2 generation and defense responses, although the expression profiles of these genes are different in response to various stimuli. *NbrbohA* was expressed constitutively at a low level and was induced by the control leaf infiltration, whereas *NbrbohB* showed pathogen

signal–specific elicitation in response to INF1 and exogenous SA (Figure 3). It is possible that in plants, elicitor induction of *rboh* mRNA is one of the mechanisms that control the oxidative burst machinery, perhaps leading to a long-term generation of AOS that could be responsible for cell death. Such elicitation of Arabidopsis *AtrbohD* and potato *StrbohB* has been reported (Desikan et al., 1998; Yoshioka et al., 2001). Recently, Simon-Plas et al. (2002) showed that tobacco *NtrbohD*, which is homologous with *NbrbohB*, is induced by the elicitin cryptogein and that tobacco cells transformed with antisense constructs of the gene abolished elicitor-stimulated AOS production. Their results coincide with our finding that *NbrbohB*-silenced leaves could not produce AOS during defense.

NbrbohA and NbrbohB are homologous with AtrbohF and AtrbohD, respectively, compared with Arabidopsis rboh proteins (Figure 2A). Using a *dSpm* insertion mutagenesis system, Torres et al. (2002) showed that *atrbohD/F* double mutations largely eliminate AOS accumulation during disease resistance reactions of Arabidopsis to avirulent *Pseudomonas syringae* pv tomato DC3000 and *Peronospora parasitica*. They also demonstrated that each mutation plays a different role in the defense of the plant. For instance, the *atrbohD* mutation eliminated the majority of AOS production, whereas *atrbohF* exhibited a strong effect on HR cell death in response to avirulent bacteria. Our analysis of the VIGS of *Nbrboh* genes suggested that the silencing of either *NbrbohB* or *NbrbohA* is enough to eliminate the oxidative burst in response to Phytophthora (Figures 5D and 5E). How can silencing of only one *rboh* gene lead to the suppression of detectable AOS production and HR in response to pathogen attack? One possible explanation is provided by Delledonne et al. (2001). Nitric oxide (NO) has been implicated in the activation of disease resistance mechanisms in plants (Noritake et al., 1996; Delledonne et al., 1998; Durner et al., 1998). Bacteria-induced HR in soybean suspension cells requires a fine balance between NO and H_2O_2 (Delledonne et al., 2001). Depletion of AOS production or administration of NO at high concentrations blocks HR cell death triggered by avirulent bacterial pathogens. NO and O_2^-, which is a precursor of H_2O_2, are produced independently (Durner et al., 1998) and could form peroxynitrite ($ONOO^-$) after pathogen attack. However, HR does not appear to be mediated by $ONOO^-$. Reduction of total AOS levels in *Nbrboh*-silenced plants would cause a reduction of positive signal (H_2O_2) for HR by a scavenging effect of NO on O_2^- and result in an imbalance of those molecules.

In plants, several kinds of enzyme are implicated in O_2^- generation in response to pathogen signals, such as NADPH oxidase in the plasma membrane, peroxidase in the cell wall, and oxalate oxidase (Kawano et al., 1998; Zhou et al., 1998). Other recent experiments using pharmacological agents suggested that at least two distinct sources, NADPH oxidase and peroxidase-type enzymes, contribute to the generation of redox cues (Allan and Fluhr, 1997; Grant et al., 2000). These reports indicate that the dominant source of AOS generation depends on the experimental system. Recently, Sagi and Fluhr (2001) confirmed that a putative plasma membrane NADPH oxidase from tobacco can produce O_2^- using a novel activity gel assay. Here, we provide genetic evidence for a dominant role of
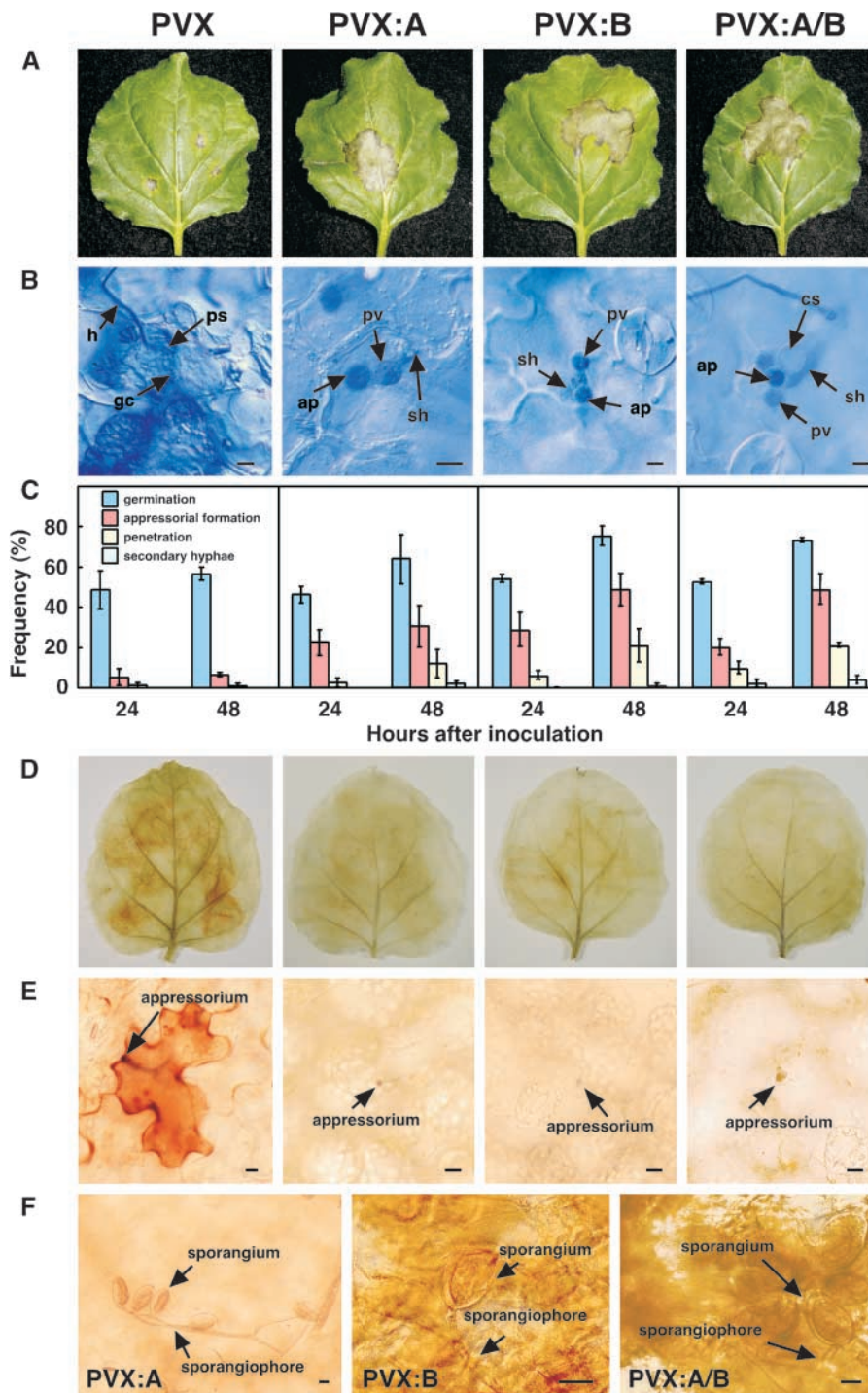
Figure 5. A Representative Set of *Nbrboh*-Silenced Leaves after Inoculation with Phytophthora.

(A) After 3 weeks of inoculation with PVX (control), PVX:A, PVX:B, or PVX:A/B, *N. benthamiana* leaves were inoculated with four drops of Phytophthora zoospores. The *Nbrboh*-silenced leaves showed typical water-soaked lesions from the pathogen. Photographs were taken at 12 days after the inoculation.

(B) *N. benthamiana* leaf discs were stained with lactophenol-trypan blue at 3 days after inoculation with Phytophthora. ap, appressorium; cs, cystospore; gc, granular cytoplasm; h, hyphae; ps, penetration site; pv, primary vesicle; sh, secondary hyphae. Bars = 5 μm.

(C) Infection behavior of Phytophthora on *Nbrboh*-silenced *N. benthamiana*. Frequencies for cystospore germination, appressoria formation, penetration, and secondary hyphae formation of Phytophthora were assessed on leaf surfaces of *N. benthamiana*.

(D) H_2O_2 accumulation visualized by DAB in *N. benthamiana* leaves at 24 h after inoculation with Phytophthora. Detached leaves were stained with DAB solution as described in Methods.

(E) Microscopic observations of the DAB-stained leaves of *N. benthamiana* shown in **(D)**. Bars = 10 μm.

(F) Sporulation was observed on *Nbrboh*-silenced *N. benthamiana* leaves at 12 days after inoculation with Phytophthora. Bars = 10 μm.
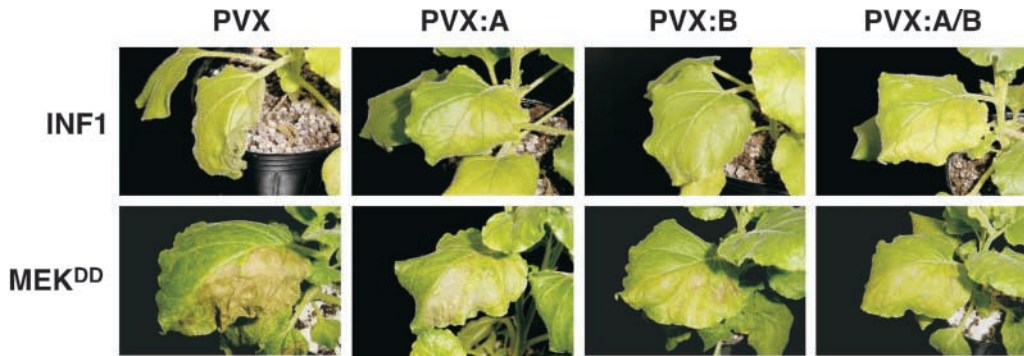
Figure 6. HR Induction in *N. benthamiana* Leaves Silenced with *Nbrboh*.

N. benthamiana leaves were inoculated with PVX (control), PVX:A, PVX:B, or PVX:A/B. At 3 weeks after inoculation, a silenced leaf above the primary infected leaf was infiltrated with *A. tumefaciens* expressing INF1 or MEK[DD] as indicated carrying PR-1a:INF1 or the constitutive active mutant of MAPKK on one leaf half. Photographs of INF1 and MEK[DD] leaves were taken at 3 and 5 days after infiltration, respectively.

NADPH oxidase in the generation of AOS during the defense response in *N. benthamiana* leaves.

MAPK Pathway–Mediated HR-Like Cell Death Is Dependent on NbrbohB

MAPK cascades are known as major pathways by which extracellular stimuli are transduced into intracellular responses in plants (Asai et al., 2002). Recent studies have indicated that expression of a constitutively active mutant of MAPKK, MEK[DD], activates endogenous SIPK and WIPK and leads to an HR that includes defense gene activation, such as Phe ammonia-lyase and 3-hydroxy-3-methylglutaryl CoA reductase (Yang et al., 2001). Ren et al. (2002) reported that the activation of endogenous Arabidopsis MAPKs by *MEK[DD]* transgenes under induced conditions leads to HR-like cell death, which is preceded by the generation of H_2O_2. They demonstrated that the oxidative burst might be the result of prolonged activation of a MAPK pathway. Both the activation of MAPKs and the burst precede and correlate with the HR, indicating that MAPK-induced HR-like cell death might be mediated by AOS. We demonstrated previously that two hyphal wall elicitors of Phytophthora, arachidonic acid and SA, activate a 51-kD MAPK (which may be a SIPK ortholog of tobacco) in potato tubers (Katou et al., 1999) and that these pathogen signals also stimulated both the oxidative burst and *StrbohB* (Yoshioka et al., 2001). Here, we showed that cell death resulting from the activation of the MAPK pathway is compromised in *NbrbohB*-silenced plants (Figure 7), suggesting that MAPK-induced HR-like cell death might be mediated by AOS produced by NbrbohB.

We have demonstrated that MEK[DD] induced the *NbrbohB* gene at the transcriptional level and HR-like cell death (Figures 6 and 7). However, the oxidative burst has been shown to be controlled through phosphorylation activation by its upstream kinase (Levine et al., 1994; Miura et al., 1995; Piedras et al., 1998) and dephosphorylation inactivation by its negative regulator, phosphatase. Treatment of cultured rice cells with the phosphatase inhibitor calyculin A without elicitor rapidly stimulates a diphenylene iodonium–sensitive oxidative burst (Kawasaki

et al., 1999), suggesting that a small portion of its upstream kinase is active. A sudden increase in a large amount of NbrbohB by the long-lasting activation of MAPKs disrupts the balance in the cells. After the newly synthesized NbrbohB becomes activated by the basal level of upstream kinase, there is not enough
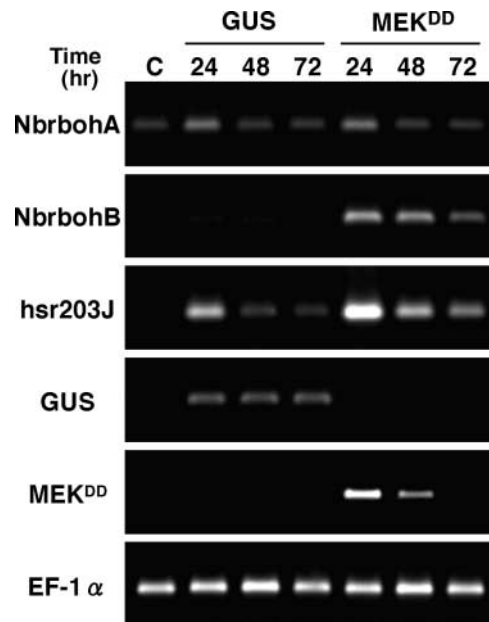

Figure 7. Effect of the Overexpression of *MEK[DD]* on the Transcription of *Nbrboh* Genes.

N. benthamiana leaves were infiltrated with *A. tumefaciens* harboring *MEK[DD]* or *GUS* (control) under the control of the 35S promoter of CaMV. Total RNA was isolated from leaves at 1 to 3 days after agroinfiltration or from nontreated leaves (C) and used for RT-PCR as described in Methods, applying 35, 29, and 29 amplification cycles with specific primers for *NbrbohA*, *NbrbohB*, and *hsr203J*, respectively. Transgene efficiency was monitored by amplification of *GUS* and *MEK[DD]* mRNAs (25 cycles). Equal loading of cDNA was monitored by amplification of a constitutively expressed *EF-1α* gene (29 cycles).

phosphatase activity to inactivate NbrbohB, which leads to lethal levels of oxidative burst. Alternatively, in mammalian cells, MAPK also is implicated in the activation of phospholipase A_2, protein kinase C, and Ras upstream of the MAPK to stimulate the signaling network (Bhalla et al., 2002), suggesting that a potential role of the MAPK cascade is not only the transcriptional activation of a number of defense genes but the post-translational activation of enzymes. In fact, a highly specific inhibitor of MAPKK, U0126, suppresses the rapid AOS generation induced by the Avr9 elicitor in *Cf-9*–expressing tobacco cell cultures (O. Rowland, and J.D.G. Jones, unpublished results). It is possible that MEK^{DD}-mediated sustained SIPK and WIPK activities provoke the post-translational activation of NbrbohB in addition to an increase in the transcript level.

Crosstalk between the Oxidative Burst and the MAPK Cascade

It has been reported that overexpression of constitutively active deletion mutants of a MAPKK kinase activated AtMPK3 (a WIPK ortholog) and AtMPK6 (a SIPK ortholog) of the six co-overexpressed MAPKs tested in Arabidopsis protoplasts exposed to acute H_2O_2 stress (Kovtun et al., 2000). Moreover, exposure of Arabidopsis leaves to H_2O_2 by glucose/glucose

oxidase infiltration activates 46- and 48-kD MAPKs orthologous with WIPK and SIPK of tobacco, respectively (Grant et al., 2000). Those authors also indicated that treatment with the MAPKK inhibitor PD98059 completely suppresses the activation of both MAPKs induced by H_2O_2. Here, we showed that prolonged activation of the MAPK pathway in leaf cells activated the *NbrbohB* gene (Figure 7), suggesting that the MAPK cascade–dependent increase of *rboh* mRNA levels could be an amplification circuit in the induction of defense responses (Figure 8). We confirmed that VIGS of SIPK/WIPK compromised INF1-mediated HR cell death in *N. benthamiana* (N. Ikeda, O. Rowland, J.D.G. Jones, and H. Yoshioka, unpublished results). However, the frequencies for the germination, appressoria formation, primary infection vesicle, and secondary hyphae in the *Nbrboh*-silenced leaves were low, suggesting that the silenced plants remain partially resistant to the pathogen. We speculate that the MAPK pathway could be activated after the perception of the pathogen in the absence of H_2O_2, which leads to partial resistance, or that Nbrboh needs to be fully silenced (no transcript) to complete the loss of resistance. Alternatively, other mechanisms may be activated for plant defense responses (Sasabe et al., 2000; Peart et al., 2002). Further investigation of the upstream and downstream regulatory components of the oxidative burst should clarify the interlinked signal network of defense pathways in plants.
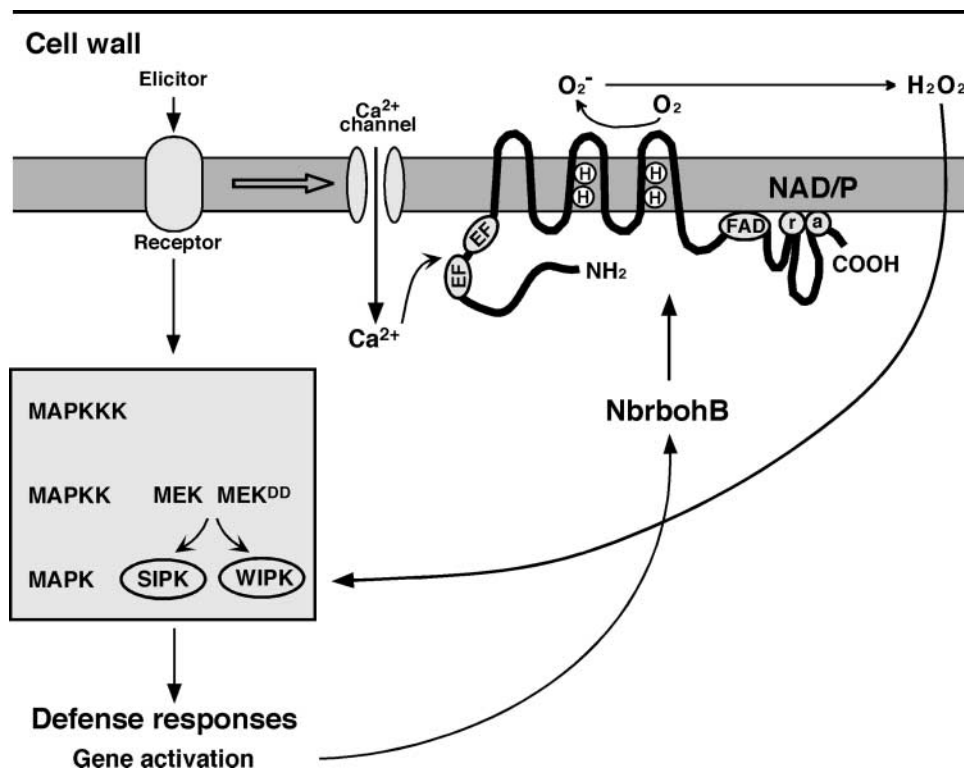

Figure 8. Scheme of the NbrbohB Protein and Proposed Model for the Regulatory Mechanism of *NbrbohB* Gene Expression.

The elicitor induces various defense-related genes, including *NbrbohB*, through the MAPK cascade. H_2O_2 produced by NbrbohB activates SIPK and WIPK. Nbrboh contains the membrane with six transmembrane domains and the putative positions of the two heme binding sites (H, histidine), as indicated in Figure 1, the flavin adenine dinucleotide (FAD), and NAD/P (a, adenine; r, ribose) binding sites, and the EF hands.

METHODS

Plant Materials, INF1 Elicitor, Chemicals, and Treatment Protocols

Nicotiana benthamiana plants were grown in environmentally controlled growth cabinets under a 16-h photoperiod and an 8-h dark period at 25°C. Elicitation with INF1 was conducted on 4-week-old plants by infiltrating INF1 solution with a needleless syringe into tiny cuts at the underside of the leaf, thereby flooding the apoplastic space. The response to osmotic stress (without the elicitation component) was investigated by infiltration of water. To prepare *Phytophthora infestans* elicitin FLAG-INF1 (HR+) and FLAG-INF1 *S3* (HR−), in which the Cys at position 3 of the processed protein is mutated to a Ser (Kamoun et al., 1997), overnight cultures of *Escherichia coli* DH5α carrying either pFB52 (−) or pFB53 (+) were diluted (1:100) in Luria-Bertani medium containing ampicillin (50 μg/mL) and incubated at 37°C. When the OD_{600} of cultures reached 0.6, isopropylthio-β-galactoside was added to a final concentration of 0.4 mM, and the cultures were grown for 3 to 4 h to induce the elicitin into cultured medium. The *E. coli* cells were pelleted by centrifugation, and the supernatant was filtrated through a 0.45-μm pore filter (Kurabo, Osaka, Japan) to eliminate *E. coli*; this preparation then was used as elicitin. Plants were treated with salicylic acid or methyl jasmonic acid (Sigma, St. Louis, MO) infiltration with a needleless syringe as described above for INF1 infiltration.

Zoospore Inoculation and Plant Assays

Race 0 of Phytophthora was maintained on susceptible potato (*Solanum tuberosum*) tubers. A suspension of Phytophthora zoospores was prepared as described previously (Yoshioka et al., 1999). Ten-microliter droplets containing ∼500 zoospores were applied to the upper side of the attached leaves under high humidity at 20°C. In the case of RNA isolation, *N. benthamiana* leaves were inoculated with 1×10^4 Phytophthora zoospores/mL using a lens paper to disperse the zoospores. For the microscopic observation, leaf discs containing the inoculum were excised at various times after inoculation with Phytophthora and then fixed on the filter paper by immersion in a 3:1 solution of ethanol:acetic acid. The fixed samples were stained carefully with 0.1 μg/mL trypan blue solution to avoid washing spores away and then examined by microscopy for plant responses and growth of Phytophthora.

Isolation of gp91*phox* Homologs from *N. benthamiana*

Two cDNAs for potato *rboh*, *StrbohA* and *StrbohB*, were used as probes to screen an oligo(dT)-primed Uni-ZAP XR library (Stratagene, La Jolla, CA), which was constructed from poly(A)+ RNAs of *N. benthamiana* leaves treated with hyphal walls of Phytophthora for 6 h. From 6×10^5 plaques of the library, six positive clones were isolated after three rounds of plaque hybridization under low-stringency conditions by each probe (12 positive clones total). Positive phage plaques were excised directly in vivo into pBluescript SK− phagemid according to the manufacturer's instructions (Stratagene). The nucleotide sequences were determined for both strands using the Prism Dye Termination Cycle Sequencing Ready Reaction Kit and a DNA sequencing system (model 310; Applied Biosystems, Foster City, CA). The positive clones obtained by the *StrbohA* probe contained one *NbrbohA* and five *NbrbohB* cDNAs, whereas the *StrbohB* probe detected six *NbrbohB* cDNAs. The partial sequences of 11 *NbrbohB* cDNAs were identical.

Reverse Transcriptase–Mediated PCR

Total RNA from leaf tissues was prepared as described previously (Yoshioka et al., 1996). Reverse transcriptase–mediated (RT) PCR was

conducted using a commercial kit (r*Tth* DNA polymerase RT-PCR high-*Plus*-; Toyobo Co., Osaka, Japan). cDNA was synthesized from total RNA (1 μg). After the cDNA synthesis reaction, PCR was performed with a denaturing temperature of 94°C for 1 min and annealing plus extension temperatures of 53°C for 1.5 min, for 29 to 35 cycles. Gene-specific primers of each sequence were as follows: *NbrbohA* (forward, 5′-CTG-CTCAGGGCTGACGAAAACACCAAGAAA-3′; reverse, 5′-AGTACTCTT-TCTCCTTTTCGGAGAAATCTT-3′), *NbrbohB* (forward, 5′-TTTCTCTGA-GGTTTGCCAGCCACCACCTAA-3′; reverse, 5′-GCCTTCATGTTGTTG-ACAATGTCTTTAACA-3′), *hsr203J* (forward, 5′-TGTGTCAGCCATGCT-GATTG-3′; reverse, 5′-CCGATAGGACCGCACGAAAC-3′), and *CP* (forward, 5′-TGGGACTTAGTCAGACACTG-3′; reverse, 5′-CCCCTCTTT-GGGCATGATGG-3′). As a control for equal cDNA amounts in each reaction, PCR was performed with primers for *EF-1α* (forward, 5′-CTTCCTACC-TCAAGAAGGTAGGATACAAC-3′; reverse, 5′-TGCCTCCTGAAGAGC-TTCGTGGTGCAT-3′).

In the case of *β-glucuronidase* (*GUS*) or *MEK^DD* transcript amplification, cDNA was synthesized with oligo(dT) primer to distinguish plant RNA from bacterial RNA. RT-PCR was conducted using a commercial kit (High-Fidelity RNA PCR; Takara, Kusatsu, Japan). PCR was performed with denaturing, annealing, and extension temperatures of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, respectively, for 25 cycles. Gene-specific primers of each sequence were as follows: *GUS* (forward, 5′-GAT-GTGCTGTGCCTGAACCGTTATTACGGA-3′; reverse for 3′ untranslated region sequence derived from binary vector, 5′-GCTTGACTCTAGAGG-ATCTTATGCGTAGTC-3′) and *MEK^DD* (forward, 5′-CCTTCTCTGTGG-GGAGACAAGGAGACTGGG-3′; reverse for 3′ untranslated region sequence derived from binary vector, 5′-GCTTGACTCTAGAGGATCTTA-TGCGTAGTC-3′). The gene-specific primers of *NbrbohA*, *NbrbohB*, *hsr203J*, *GUS*, *MEK^DD*, *CP*, and *EF-1α* generate expected product sizes of 291, 228, 354, 492, 347, 297, and 347 bp, respectively. PCR products were separated on a 1.8% agarose gel and visualized after ethidium bromide staining.

DNA Constructs and Seedling Infection for Virus-Induced Gene Silencing

A 278-bp cDNA fragment of the isogene-specific N-terminal region of *NbrbohA* was amplified with the following primers: *NbrbohA*-SalI (5′-GTCGACGCCGGTGGACATCCGATACGGTAT-3′) and *NbrbohA*-ClaI (5′-ATCGATTCGTCGGCGATCGAGATTCCGATA-3′) (restriction sites are underlined). A 197-bp cDNA fragment of an isogene-specific region of *NbrbohB* was amplified with primers *NbrbohB*-SalI (5′-GTC-GACAATCATCATCCGCACCACCATCAC-3′) and *NbrbohB*-ClaI (5′-ATCGATACGCATCATCATTGGACTTGCCGC-3′). A 234-bp cDNA fragment for a conserved region of *Nbrboh* was amplified with primers *NbrbohD*-SalI (5′-GTCGACATATTTTTATTGGGTGACCCGGGA-3′) and *NbrbohD*-ClaI (5′-ATCGATCTAGCAAAATGTGTCCTCACCCTG-3′) using *NbrbohA* cDNA as a template. These cDNA fragments were subcloned into the TA cloning site of pGEM-T Easy (Promega, Madison, WI) and ligated as a ClaI-SalI fragment into the *Potato virus X* (PVX) vector pPC2S (Baulcombe et al., 1995). The constructs contained these inserts in the antisense orientation and were designated PVX-NbrbohA (PVX:A), PVX-NbrbohB (PVX:B), and PVX-NbrbohA/B dual-silencing insert (PVX:A/B). PVX that does not contain any inserts was used as a control. Synthesis of virus RNA from the construct and inoculation onto a leaf of *N. benthamiana* were as described by Chapman et al. (1992). Second leaves of 2-week-old *N. benthamiana* seedlings were inoculated with viral RNA using Carborundum. After an additional 2 to 3 weeks, the fourth and fifth leaves above the inoculated leaves of each plant were analyzed for transcript levels of various genes and used for *Agrobacterium tumefaciens*–mediated transient expression.

Transient Expression of INF1 and StMEK^{DD} in *N. benthamiana*

For agroinfiltration experiments, we used the binary vector p35S-INF1 containing a fusion between the signal peptide of tobacco (*Nicotiana tabacum*) *PR-1a* and the Phytophthora *inf1* gene driven by the 35S promoter of *Cauliflower mosaic virus* (Kamoun et al., 1999; S. Kamoun, personal communication).

To clone the ortholog of tobacco *NtMEK2* (Yang et al., 2001) from potato, we performed PCR with primers StMEK-F (5′-ATGCGACCTCTT-CAACCACCCCCACCAG-3′) and StMEK-R (5′-TTCTCCAAAAATAAA-ATCCAACCACCC-3′) using a potato cDNA library as a template. The amplified 1258-bp (full-length) fragment was subcloned into the TA cloning site of pGEM-T Easy (Promega) and sequenced. The constitutively active mutant of *StMEK1*, *StMEK^{DD}*, in which the conserved Thr-228 and Ser-234 residues are replaced by Asp (Yang et al., 2001), was generated by Mutan-Super Express Km site-directed mutagenesis (Takara) and confirmed by sequencing. *StMEK^{DD}* was inserted into ClaI-BamHI sites of the vector pSLJ4K1 (Jones et al., 1992). The HindIII-EcoRI fragment then was cloned into the pGreen binary vector (Hellens et al., 2000). The *StMEK^{DD}* was preceded by the 35S promoter of *Cauliflower mosaic virus*, the 5′ untranslated region was replaced with the ω sequence from *Tobacco mosaic virus*, and the nopaline synthase terminator region was on the 3′ end of the gene.

These binary plasmids were transformed into *A. tumefaciens* strain GV3101, which harbors the transformation helper plasmid pSoup (Hellens et al., 2000). Overnight cultures were harvested by centrifugation, and cells were resuspended in 10 mM MgCl$_2$, 10 mM Mes, pH 5.6, and 150 μM acetosyringone to an OD$_{600}$ of 0.5, incubated for 2 to 5 h at room temperature, and then infiltrated into leaves of *N. benthamiana* using a needleless syringe (Romeis et al., 2001). To analyze for defense-related phenotypes, the silenced fourth and fifth leaves were infiltrated with *A. tumefaciens* expressing *PR-1a:inf1* or StMEK^{DD} on one leaf half.

DNA Gel Blot Hybridization

Total genomic DNA of *N. benthamiana* was extracted from leaves according to the method described by Murray and Thompson (1980). Ten micrograms of DNA was digested with HindIII, EcoRI, or SpeI and then separated by electrophoresis on a 0.8% agarose gel. The separated DNA was transferred from gels to nylon membranes (Hybond N$^+$; Amersham, Arlington Heights, IL) and hybridized according to the manufacturer's recommendations. The cDNA probes for *NbrbohA* (278 bp) and *NbrbohB* (197 bp) that were used for virus-induced gene silencing were labeled with ^{32}P-dCTP using a random-primed DNA labeling kit (Takara).

H$_2$O$_2$ Detection by the 3,3′-Diaminobenzidine Uptake Method

To visualize H$_2$O$_2$ in the infection site of Phytophthora, 3,3′-diaminobenzidine staining was performed as described by Thordal-Christensen et al. (1997). *N. benthamiana* leaves were inoculated with 1×10^4 Phytophthora zoospores/mL using a lens paper to disperse the zoospores at 20°C. Detached leaf samples were collected at 8 h after incubation with 1 mg/mL 3,3′-diaminobenzidine solution. Leaves then were fixed with a 3:1:1 solution of ethanol:acetic acid:glycerol.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

The accession numbers for the *NbrbohA* and *NbrbohB* cDNA sequences are AB079498 and AB079499, respectively. The accession numbers for the other sequences mentioned in this article are as follows: potato MAPKK (*StMEK1*), AB091780; Arabidopsis respiratory burst oxidase homologs (*AtrbohA* to *AtrbohF*), AF055353, AF055354, AF055355, AF055357, AF055356, and AB008111, respectively; rice *rboh* (*OsrbohA*), AP003560; tomato *rboh* (*Lerboh1*), AF088276; *Wfi1* (tomato), AF147783; potato *rboh* (*StrbohA*), AB050660; *StrbohB*, AB050661; tobacco *rboh* (*NtrbohD*), AJ309006; and *NtrbohF*, AJ320505. Arabidopsis *AtrbohA* (AF015301), referred to by Keller et al. (1998), corresponds to *AtrbohF* (AB008111), referred to by Torres et al. (1998).

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