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Original Paper

Function of the rice gp91^{phox} homologs *OsrbohA* and *OsrbohE* genes in ROS-dependent plant immune responses

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Abstract Reactive oxygen species (ROS) are proposed to function as diffusible signaling molecules in plant immune response. Rice respiratory burst oxidase homologs (*Osrboh* genes) are proposed to play a role in ROS generation. We examined a role in rice immune responses of four *Osrboh* homologs, *OsrbohA*, *OsrbohB*, *OsrbohD*, and *OsrbohE*. *OsrbohA* and *OsrbohD* transcripts were induced after inoculation with an incompatible N1141 strain of *Acidovorax avenae*, whereas *OsrbohaB* and *OsrbohE* mRNA levels did not obviously change even after inoculation with the incompatible strain. We examined the function of the *Osrboh* genes in ROS generation and in the plant immune response using RNAi-based knockdown in rice cells. *OsrbohA* and *OsrbohE* knockdown lines showed that rapid H_2O_2 generation is caused by *OsrbohA*, whereas *OsrbohE* is involved in late H_2O_2 production during the immune response. Hypersensitive cell death was decreased only in the *OsrbohA* knockdown line. We further demonstrated that among immune related genes, the induction of *EL2* and *LOX* genes is controlled by ROS generated by *OsrbohE*, whereas expression of *Cht-1* gene is regulated by both *OsrbohA* and *OsrbohE*. These results indicate that the ROS molecules generated by *OsrbohA* and *OsrbohE* regulate different signaling pathways in the plant immune response.

Key words: *Acidovorax avenae*, NADPH-oxidase, Plant immune response, rice, ROS.

Plants are exposed to a variety of potential pathogens during their lifetime, but actual infections only occur in limited cases. Besides preformed physical and chemical barriers to prevent infection, a wide variety of immune responses is induced only after pathogen attack (Heath 2000). One of the earliest responses activated after host recognition is the oxidative burst, in which levels of reactive oxygen species (ROS) rapidly increase (Bolwell et al. 1995; Lamb and Dixon 1997). During the immune response, ROS can inhibit pathogens by strengthening cell walls via oxidative cross-linking or cell wall glycoproteins of by directly killing the pathogen (Bradley et al. 1992; Iwano et al. 2002). ROS could also act as a diffusible signal to induce subsequent immune responses, such as the initiation of hypersensitive cell death and the induction of immune-related genes.

The kinetics and defensive functions of ROS during activation of mammalian neutrophils have served as a models for similar processes in plants. The mammalian NADPH oxidase consists of two plasma membrane proteins, gp91^{phox} and p22^{phox} (phox, phagocyte oxidase), which together form heterodimeric flavocytochrome b558 (Nauseef 1993; Chanock et al. 1994). The four cytosolic regulatory proteins p47^{phox}, p67^{phox}, rac1 and rac2 translocate to the plasma membrane after stimulation to form the active complex (Bokoch et al. 1994). Several lines of evidence demonstrated that the ROS generation during the plant immune response is caused by the activation of an NADPH oxidase (Lamb and Dixon 1997). Chemical inhibitors of NADPH oxidase, such as diphenylene iodonium (DPI), inhibit H_2O_2 production during the immune response induced by pathogen infection in plants (Levine et al. 1994; Auh and Murphy 1995). Enhanced ROS generation is observable in microsomal preparations from pathogen-challenged leaf material (Doke and Ohashi 1988) and the constitutive expression of an active mutant of OsRac, a rice homolog of human Rac, induced H_2O_2 production in cultured rice cells (Kawasaki et al. 1999). Respiratory burst oxidase homologs (rboh) of gp91^{phox}, which is the catalytic subunit of phagocyte NADPH oxidase, were isolated from several plants (Groom et al. 1996; Yoshioka et al. 2003; Torres et al. 2002). Plant rboh is a protein of 105 to 112 kDa, with an approximately 300-residue cytoplasmic amino-terminal extension containing two putative EF-hand motifs. There are 10 *rboh* genes in

Abbreviations: DAB, 3,3′-diaminobenzidine-HCl; DPI, diphenylene iodonium; FAD, flavin adenine dinucleotide; NADPH, nicotine adenine dinucleotide phosphate; NBT, nitroblue tetrazolium chloride; Osrboh, rice respiratory burst oxidase homologs; PCR, polymerase chain reaction; Phox, phagocyte oxidase; ROS, reactive oxygen species.

the *Arabidopsis* genome (Foreman et al. 2003). Of these *rboh* in *Arabidopsis*, *AtrbohD* and *AtrbohF* were shown to be necessary for ROS generation in the plant immune responses (Torres et al. 2002). Furthermore, *NtbohD* from *Nicotiana tabacum* and *NbrbohA* and *NbrbohB* from *N. benthamiana* were required for ROS accumulation and resistance (Simon-Plas et al. 2002; Yoshioka et al. 2003), suggesting that individual isoforms have different functions and participate in multiple distinct signaling pathways.

Acidovorax avenae is a Gram-negative bacterium that causes a seedling disease characterized by the formation of brown stripes on the sheaths of infected plants (Kadota et al. 1991). *A. avenae* can infect a wide range of monocotyledonous plants, including rice, oats, Italian millet, and maize. However, individual strains of the pathogen can infect only one or a few host species (Nishiyama et al. 1979; Kadota et al. 1991; Kadota et al. 1996). We recently reported that several immune responses, such as H_2O_2 generation, hypersensitive cell death accompanied by clear 180-bp nucleosomal DNA laddering, and immune-related genes were induced when cultured rice cells were inoculated with a rice-incompatible strain of *A. avenae*. These responses were completely abolished when a rice-compatible strain of *A. avenae* was used as the inoculating pathogen (Che et al. 1999; Tanaka et al. 2001; Iwano et al. 2002; Tanaka et al. 2003; Fujiwara et al. 2004). To identify the specific elicitor related to the induction of these immune responses in cultured rice cells, a strain-specific antibody was raised against the incompatible strain (N1141) and then absorbed by the compatible strain (H8301). The specific antibody detected flagellin protein, a component of the flagellum of bacteria, which exhibits structural differences between the compatible and incompatible strains of *A. avenae* (Che et al. 2000). Flagellin purified from the incompatible strain induced rice immune responses in cultured rice cells, such as rapid H_2O_2 generation and hypersensitive cell death, whereas flagellin purified from the compatible strain induced no such responses. Furthermore, a flagellin-deficient N1141 strain lost the ability to induce several type of immune responses. These data indicate that flagellin from the incompatible strain of *A. avenae* can be characterized as a specific elicitor in immunity in cultured rice cells. This finding demonstrates that the cultured rice cells and *A. avenae* (or its flagellin) constitute is a good model for dissecting the mechanism of ROS generation during immune responses and the function of ROS in the plant immune response.

In this study, we examined the mechanism of ROS generation during immune responses, and the role of each rice *rboh* homologs in the induction of plant immune responses. When cultured rice cells were inoculated with the incompatible strain of *A. avenae*, NADPH-dependent oxidase activity in the plasma membranes of rice cells increased, whereas this phenomenon was not observed when rice was inoculated with the compatible strain, suggesting that ROS generation in cultured rice cells is also caused by activation of *rbohs* in rice. Among *Osrboh* homologs, four *rboh* homologs of rice, which designated *OsrbohA*, *OsrbohB*, *OsrbohD* and *OsrbohE*, were selected to examine a role in rice immune responses. Using RNAi-based knockdown system, we isolated *OsrbohA* and *OsrbohE* knockdown transformants and demonstrated that the *OsrbohA*-RNAi transformants largely eliminated rapid H_2O_2 generation during immune responses in cultured rice cells, while late H_2O_2 generation was decreased in the *OsrbohE*-RNAi transformants. We also observed reduced hypersensitive cell death, as visualized by Evans blue stain, in *OsrbohA*-RNAi transformants after inoculation with the *A. avenae* incompatible strain. Interestingly, real-time RT-PCR analysis showed that the expression patterns of several immune-related genes were different between the *OsrbohA* and *OsrbohE* transformants following inoculation with the incompatible strain, suggesting that *OsrbohA* and *OsrbohE* regulate the different signal pathways in immune system.

Materials and methods

Plants and bacteria

Suspension cultures of rice cells, line Oc (Baba et al. 1986), were grown at 30°C under light irradiation. The cells were diluted in fresh medium every seven days, and all experiments were performed four days past-transfer.

Acidovorax avenae K1, isolated from rice, and strain N1141 (MAFF 301141), isolated from finger millet, were used as previously described (Kadota et al. 1996; Che et al. 2000). Each *A. avenae* strain was maintained at 30°C on Pseudomonas F agar plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) as described (Che et al. 1999).

H_2O_2 detection and quantification

The generation of reactive oxygen species in the medium of cultured cells was monitored by the DAB staining method (Iwano et al. 2002) and the luminol method (Schwacke and Hager 1992).

The DAB staining protocol was as follows: Cultured rice cells were incubated with bacteria (10^8 cfu ml^{-1}) at 30°C for variable periods after inoculation. Following two washes in PBS, cultured cells were incubated for 20 min in DAB solution (1 mg ml^{-1} 3,3′-diaminobenzidine-HCl, 50 mM Tris-HCl, pH 6.0) (Sigma Chemical Co., St. Louis, MO, U.S.A.), and then washed extensively with distilled water. Samples were further prepared by incubation in 0.5 ml 30% lactic acid and 30% phenol for 10 min. Cells were observed by microscopy

(AxiophotoII, Carl Zeiss, Germany) with an attached CCD camera (Progress 3012, Carl Zeiss, Germany). For quantitative analysis of the DAB assay, color images were acquired using the CCD camera and converted to grayscale images using 256 shades of gray with IPLab Spectrum 2.4 software (Signal Analysis Co.). We then estimated the intensity of DAB staining in these images.

The protocol for the luminol method was as follows: One hundred mg of cultured cells were transferred to 1 ml of fresh media and pre-incubated for 3–8 h at 30°C. The cultured cells were incubated with various flagellin proteins ($<100\,\mu$l) at 30°C for variable periods after treatment. Next, 10 μl of the reaction medium was mixed with 160 μl of 50 mM potassium phosphate buffer (pH 7.9), 10 μl of 1.1 mM luminol, and 20 μl of 14 mM potassium ferricyanide, and immediately analyzed for chemiluminescence with a Genelight55 lumi-counter (Microtec Co., Ltd., Chiba, Japan). An equal volume of sterile, distilled H_2O was measured as a control. The amount of reactive oxygen was determined using a standard curve of known concentrations of H_2O_2

Detection of hypersensitive cell death

Detection of hypersensitive cell death in cultured rice cells was performed using Evans blue staining as previously described (Che et al. 1999).

Activity staining of NADPH-dependent oxidase

For activity staining of NADPH-dependent oxidase, 9 g of cultured rice cells were homogenized with a French press in 50 mM Tris-HCl buffer (pH 7.5), 250 mM sucrose, 5 mM EDTA, 5 mM EGTA, 1 mM $CaCl_2$, 1 mM DTT, 3.6 mM L-Cys and 0.1 mM PMSF. The homogenates were passed through four layers of gauze and subjected to differential centrifugation at $9{,}000\times g$ for 30 min and $140{,}000\times g$ for 90 min. The supernatant was considered the cytoplasmic fraction and the pellet was considered the total membrane fraction. The plasma membrane fraction was further purified from the total membrane fraction by a two-phase preparation method using Dextran-polyethylene glycol. Native-PAGE was performed with 10% acrylamide gels containing 10% Chaps. The running buffer was 25 mM Tris base, 192 mM glycine, and 10% Chaps. The gels were run at 25 mA for 2 h. After electrophoresis, the gels were stained for detection of dehydrogenase activity, which contained 0.5 mg NBT/ml and 0.2 mM NADPH in 50 mM Tris-HCl buffer (pH 8.5).

RNA isolation and real time RT-PCR

Total RNA was isolated from cultured rice cells using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Real-time PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) using a QuantiTect SYBR Green RT-PCR Kit (QIAGEN) with the following gene-specific primers. *OsrbohA*: forward, 5′-ATGGACAGGAGATAGCAAGG-3′ and reverse, 5′-TCCATCATGGCTTTTGCAGC-3′; *OsrbohB*: forward, 5′-GTTGGAGTTTTCTACTGCG-3′ and reverse, 5′-GCTTCCATTTCCACAAGGAG-3′; *OsrbohD*: forward, 5′-GGTACAAAATATATAGGACA-3′ and reverse, 5′-TGATTGGGTCCAGATTGGTG-3′; *OsrbohE*: forward, 5′-GTCTTCACTAGAATTGCTT-CCA-3′ and reverse, 5′-CATTTCCACAAGGAGTAC-TTCT-3′; *Cht-1*: forward, 5′-AACATCATCAACGGC-GGCGT-3′ and reverse, 5′-GCTAGAACGAGCTATTA-GGAGTT-3′; *EL2*: forward, 5′-GCTGCAGGAAGCTC-CTCTTCGA-3′ and reverse, 5′-GAAGTGCAGTGAG-GTCAGGTAT-3′; *LOX*: forward, 5′-CAGACGAGGC-CTGGAACAGCGA-3′ and reverse, 5′-TTCATCAG-CTGGTACGGCAGGAT-3′; *CCD1*: forward, 5′-TGT-TCATAGCATCAGGATTCATCA-3′ and reverse, 5′-TGAGAGTACAAATGCGATTGGAA-3′; *EREBP1*: forward, 5′-GCAGCAGCTCCCTTCAAGAT-3′ and reverse, 5′-GCGTCGAGATCATGAGCACTT-3′. The fluorescence data produced sigmoidal amplification plots in which the number of cycles was plotted against fluorescence. Quantification of each mRNA was calculated from threshold points located in the log-linear range of the RT-PCR. Standard samples of known template amounts were used to quantify the PCR products.

Generation of transgenic rice cells using RNAi constructs of OsrbohA *and* OsrbohE

A pANDA vector (Miki and Shimamoto 2004) was used to express inverted repeat sequences of a 194 bp fragment of the *OsrbohA* gene and a 186 bp fragment of the *OsrbohE* gene. The target sequence was amplified using PCR primers. *OsrbohA*: forward, 5′-CACCGAAGACCTTGAGCTTCTTCTG-3′ and reverse, 5′-AGTGAAAAGGGAAAGGTATAC-3′; *OsrbohE*: forward, 5′-CACCAGGCACAAGGTCAGGACACA-3′ and reverse, 5′-ACTCGCTTCCATTTCCACAA-3′. Each PCR product was cloned into the pENTR/D-TOPO cloning vector (Invitrogen) and the target sequence transferred into a pANDA vector by LR clonase reaction. Each construct then introduced into *Agrobacterium tumefaciens* EHA105 by electroporation. *Agrobacterium*-mediated transformation of rice was carried out as described by Toki (1997).

Isolation and purification of flagellin

Flagellin was purified from the incompatible N1141 strain of *A. avenae* as previously described method (Tanaka et al. 2003).

Results

Activity staining of NADPH-dependent oxidase

In cultured rice cells inoculated with the incompatible strain of *A. avenae*, precipitates of DAB, indicating the presence of H_2O_2, were located predominantly on the plasma membrane and occasionally within the cell walls (Figure 1A). In contrast, cultured rice cells inoculated with the compatible strain did not accumulate H_2O_2 to the same degree (data not shown).

To examine the ROS-generating system located on the plasma membrane, we tested several protein extracts from cultured rice cells using in-gel activity staining. As shown in Figure 1B, one significant activated one band was observed in plasma membrane extracts from the cultured rice cells inoculated with the incompatible N1141 strain. When NADPH was removed during the staining, the activation of the band disappeared (data not shown), indicating that activation of the ROS-generating system during the rice immune response is dependent upon NADPH.

Rice rboh homologs

Several lines of evidence have shown that plant rboh, a homolog of the mammalian gp91^{phox} (the catalytic subunit of phagocyte NADPH oxidase), functions as a generator of ROS during the plant immune response (Torres et al. 2002). The *Arabidopsis* genome contains at least ten *rboh* genes, suggesting that individual isoforms have different functions and participate in multiple distinct signaling pathways. Because one *rboh* gene (*OsrbohA*) in rice has been reported (Groom et al. 1996; Accession AK103747), we first tried to select other *Osrboh* genes from rice EST database and cDNA cloning. Three genes were identified as *OsrbohA* homologs, (Accession No. AK120739, AK120905, XM_482730), and each was predicted to have six transmembrane-spanning domains corresponding to those identified in gp91^{phox}. Transmembrane domains 3 and 5 contain pairs of His residues that are likely important for heme binding (Finegold et al. 1996). The C-terminal regions of each homolog contains conserved flavin adenine dinucleotide, NADPH-ribose, and NADPH-adenine binding sites, which are likely located in the cytoplasm. Moreover, the N-terminal regions of these proteins are hydrophilic and contain two Ca^{2+} binding EF hands. All these features of the homologs are characteristics of plant rboh, and we therefore designated them *OsrbohB* (Accession AK120739), *OsrbohD* (Accession AK120905), and *OsrbohE* (Accession XM_482730). The deduced amino acid sequence of *OsrbohA* had maximum sequence similarity of 60% identity to *OsrbohB*, 91% identity to *OsrbohD*, and 55% identity to *OsrbohE*.

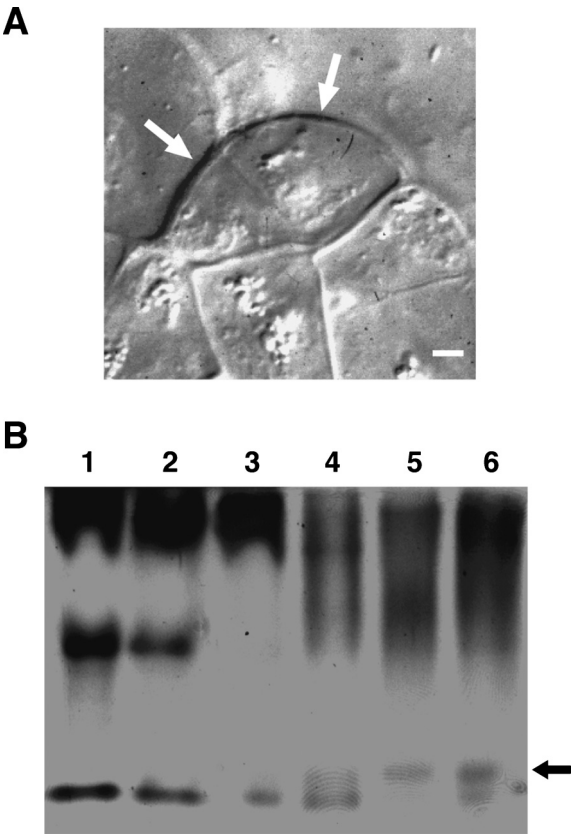

Figure 1. Active staining NADPH-dependent oxidase. (A) Localization of H_2O_2 detected by DAB staining in cultured rice cells 6 h after inoculation with the incompatible N1141 strain. The arrowhead indicates the H_2O_2 accumulation position, and the bar represents 5 mm. (B) NBT in-gel staining of native PAGE. 20 μg protein from each fraction was separated by native PAGE and then stained with NBT solution containing NADPH. Lane 1, cytosolic fraction isolated from control cells; lane 2, cytosolic fraction isolated from cultured cells 6 h after inoculation with the incompatible N1411 strain; lane 3, total membrane fraction isolated from control cells; lane 4, total membrane fraction isolated from cultured cells 6 h after inoculation with the incompatible N1411 strain; lane 5, plasma membrane fraction isolated from control cells; and lane 6, plasma membrane fraction isolated from cultured cells 6 h after inoculation with the incompatible N1411 strain. The arrow indicates the activated band after inoculation with the N1141 strain.

Induction of Osrboh genes in cultured rice cells inoculated with an A. avenae *incompatible strain*

We examined the time course of accumulation of the *OsrbohA*, *OsrbohB*, *OsrbohD* and *OsrbohE* mRNAs in cultured rice cells after inoculation with the incompatible N1141 and the compatible K1 strains by real-time RT-PCR analysis using specific primer sets. Cultured rice cells were inoculated with either the N1141 and K1 strains, and total RNA was extracted 0, 1, 3 and 6 h after inoculation. The *OsrbohA* mRNA was found to be induced 6 h after incubation with the N1141 strain, whereas no significant increase in *OsrbohA* mRNA was observed 6 h after inoculation with the compatible K1 strain (Figure 2). The same induction patterns were observed when the *OsrbohD* primer set was used,

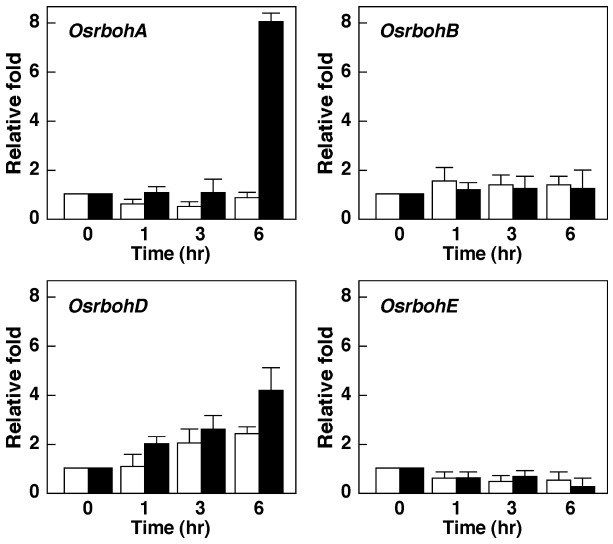

Figure 2. Expression analysis of *OsrbohA*, *OsrbohB*, *OsrbohD*, and *OsrbohE* in cultured rice cells inoculated with the incompatible N1141 or the compatible K1 strains using real-time RT-PCR. The amount of each mRNA was calculated from the threshold point located in the log-linear range of RT-PCR. Standard samples with known template amounts were used for quantification of each mRNA. Open bars, K1 inoculation; solid bars, N1141 inoculation. The y-axis represents fold-change relative to the amount of mRNA in water-treated cultured rice cells. The error bars indicate standard deviation of the mean of three experiments.

indicating that the *OsrbohA* and *OsrbohD* transcripts are induced during the incompatible interaction. Real-time RT-PCR reactions using the specific primer sets of *OsrbohB* and *OsrbohE* showed that these mRNA did not change in cultured rice cells infected with either the compatible K1 or the incompatible N1141 strains, suggesting that the *OsrbohB* and *OsrbohE* transcripts in rice are not induced after *A. avenae* pathogen infection (Figure 2).

Immune responses in OsrbohA *and* OsrbohE *RNAi knockdown transformants*

To examine the contributions of *Osrboh* homologs in ROS generation during the incompatible interaction and their functions in the immune response in rice, two *Osrboh* knockdown lines were made using RNAi technology: *OsrbohA*, which is induced during the incompatible interaction, and *OsrbohE*, which is non-inducible during the compatible and incompatible interactions. The RNAi constructs expressing an inverted repeat sequence of *OsrbohA*, *OsrbohE*, or the control construct were transformed into rice by *Agrobacterium*-mediated transformation. As a result, two RNAi knockdown transformants, *OsrbohA*-RNAi3 and *OsrbohE*-RNAi2, and one control transformant were obtained. Real-time RT-PCR using specific primer sets for *OsrbohA*, *OsrbohB*, *OsrbohD*, and *OsrbohE* showed that mRNA levels of *OsrbohA* in *OsrbohA*-RNAi3 were decreased compared to the control cells, whereas no

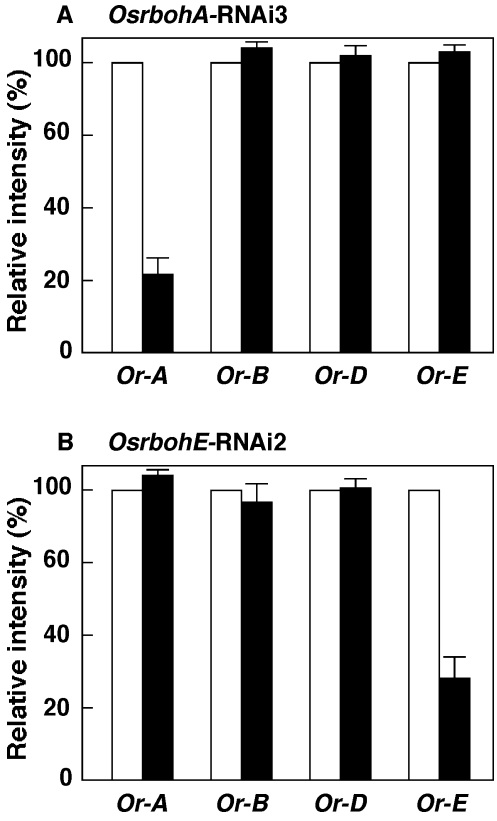

Figure 3. Expression levels of *OsrbohA* (*Os-A*), *OsrbohB* (*Os-B*), *OsrbohD* (*Os-D*), and *OsrbohE* (*Os-E*) in *OsrbohA*-RNAi3 (A) and *OsrbohE*-RNAi2 (B). The amount of each mRNA was calculated from the threshold point located in the log-linear range of RT-PCR. Open bars, control cultured cells; solid bars, each RNAi-transformant cultured cells. Standard samples with known template amounts were used for quantification of each mRNA. The y-axis represents % change relative to the amount of mRNA in control cultured rice cells. The error bars indicate standard deviation of the mean of three experiments.

change in *OsrbohB*, *OsrbohD*, and *OsrbohE* expression levels were observed in *OsrbohA*-RNAi3 (Figure 3A). *OsrbohE* expression levels were also decreased in *OsrbohE*-RNAi2; however, mRNA transcripts of the other *Osrboh* homologs were the same as in control cells (Figure 3B).

In previous work, we showed that flagellin purified from the incompatible N1141 strain of *A. avenae* induced the rapid H_2O_2 generation in cultured rice cells. (Che et al. 2000; Tanaka et al. 2003). We first examined whether the knockdown of *OsrbohA* and *OsrbohE* genes had any effects on the H_2O_2 generation induced by the incompatible flagellin using a luminol-chemiluminescence assay (Schwacke and Hager 1992). As shown in Figure 4A, rapid H_2O_2 generation was decreased in *OsrbohA*-RNAi3 compared to that in the control cells. In *OsrbohE*-RNAi2, no significant change was observed compared to that of control cells. We also examined late H_2O_2 generation induced by the incompatible N1141 strain of *A. avenae* using DAB staining (Iwano et al. 2002). Interestingly, DAB

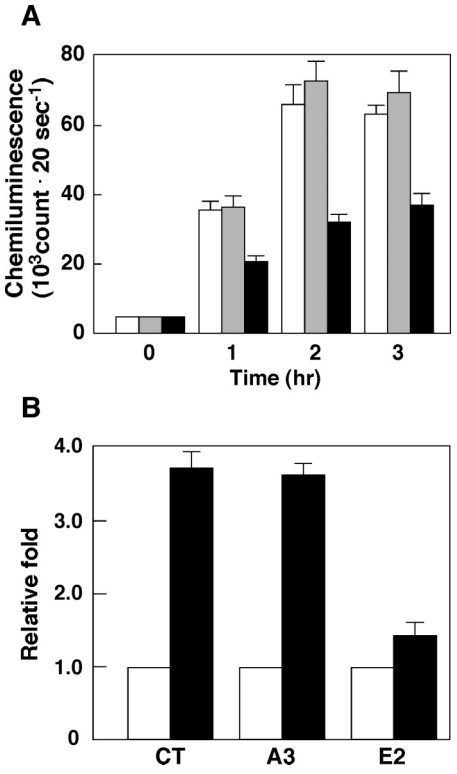

Figure 4. ROS generation in *OsrbohA*-RNAi3 and *OsrbohE*-RNAi2 after treatment with flagellin (A) and the incompatible N1141 strain (B). (A) ROS generation was assayed by luminol-chemiluminescence. Open bars, control cultured cells; gray bars, *OsrbohE*-RNAi2; solid bars, *OsrbohA*-RNAi3. The y-axis represents fold-change relative to the amount of ROS in cultured cells 0 h after inoculation. (B) ROS generation in control cells (CT), *OsrbohA*-RNAi3 (A3) and *OsrbohE* RNAi2 (E2) after inoculation with the N1141 strain, as detected by DAB staining. Open bars, 0 h after inoculation; solid bars, 6 h after inoculation. The y-axis represents fold-change relative to the amount of ROS in cultured cells 0 h after inoculation. The error bars indicate standard deviation of the mean of three experiments.

precipitation, indicating H_2O_2 generation, was decreased in *OsrbohE*-RNAi2 but not in *OsrbohA*-RNAi3 (Figure 4B). These findings suggest that rapid H_2O_2 generation by the incompatible strain is caused by *OsrbohA* activation, and *OsrbohE* contributed to the late H_2O_2 generation.

Evans blue stain is excluded from viable cells but not from dead cells that have lost a function of their plasma membranes. Therefore, this stain can be used to monitor cell death. We looked for effects on hypersensitive cell death induced by the incompatible strain in *Osrboh*-RNAi. After 6 h co-incubation of *OsrbohA*-RNAi2 cultured rice cells with the incompatible N1141 strain, hypersensitive cell death decreased (Figure 5). In contrast, the same induction pattern of cell death was observed between *OsrbohE*-RNAi3 and the control cells, indicating that HR cell death in cultured rice cells induced by the incompatible strain is caused by *OsrbohA*-mediated ROS (Figure 5).

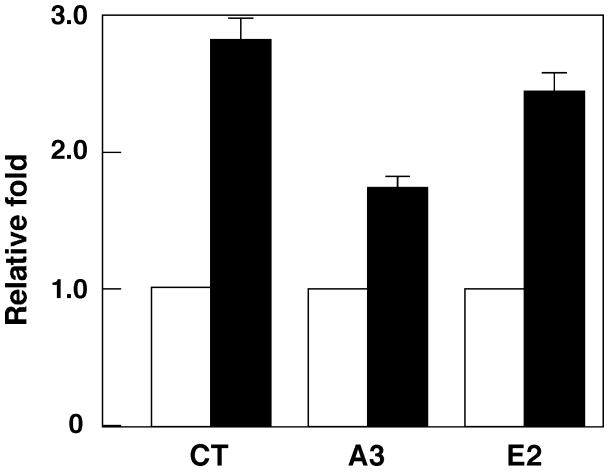

Figure 5. Induction of hypersensitive cell death in control cells (CT), *OsrbohA*-RNAi3 (A3) and *OsrbohE* RNAi2 (E2) after inoculation with the incompatible N1141 strain of *A. avenae*. Open bars, 0 h after inoculation; solid bars, 6 h after inoculation. The y-axis represents fold-change relative to the amount of hypersensitive cell death in cultured cells 0 h after inoculation. The error bars indicate standard deviation of the mean of three experiments.

Immune related-genes regulated under OsrbohA *and* OsrbohE

Recently, we reported that 136 rice genes are upregulated during the incompatible interaction, as indicated by a rice cDNA microarray, and these 136 genes are likely relevant to the induction of immunity as determined by clustering analysis (Fujiwara et al. 2004). Of the 136 genes, five genes— *EL2*, *Cht-1*, *EREBP1*, *CCD1*, and *LOX*— were selected and we examined the induction patterns of these genes in *OsrbohA*-RNAi3 and *OsrbohE*-RNAi2 cultured rice cells by real-time RT-PCR.

Real-time RT-PCR using an *EL2* specific primer set showed that *EL2* mRNA was induced in the control cultured cells 6 h after inoculation with the incompatible N1141 strain. When *OsrbohE*-RNAi2 cultured rice cells was inoculated with the N1141 strain, no significant increase in *EL2* mRNA was observed 6 h after inoculation (Figure 6). When the *Cht-1* primer set was used, induction of *Cht-1* was observed in the control cultured cells, whereas expression levels of *Cht-1* did not change in *OsrbohA*-RNAi3 and *OsrbohE*-RNAi2 6 h after inoculation with the incompatible N1141 strain (Figure 6).

Expression levels of the *EREBP1* and *CCD1* transcripts were increased in the control cells up to 6 h after inoculation. These induction patterns of both genes after inoculation with the N1141 strain were also observed in *OsrbohA*-RNAi3 and *OsrbohE*-RNAi2 cultured rice cells (Figure 6), suggesting that induction of *EREBP1* and *CCD1* are not regulated by ROS generated by *OsrbohA* and *OsrbohE*.

Real-time-RT PCR using a LOX-specific primer set

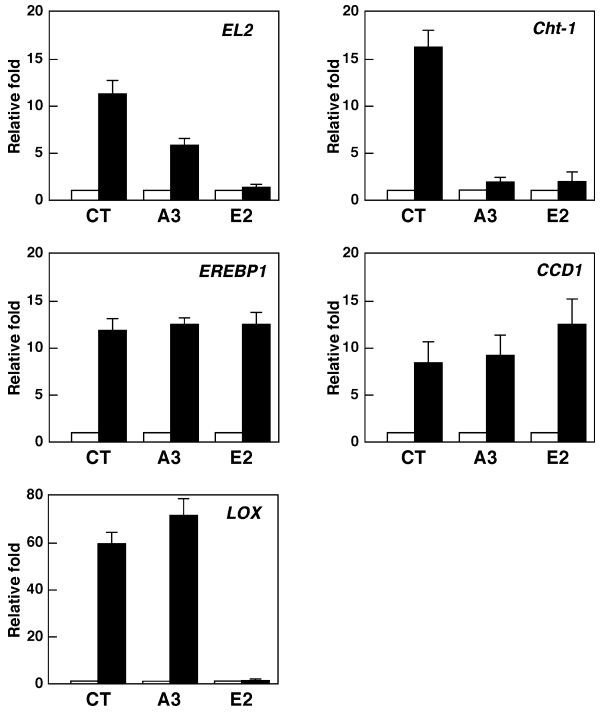

Figure 6. mRNA accumulations of *EL2*, *Cht-1*, *EREBP1*, *CCD1* and *LOX* in control cells (CT), *OsrbohA*-RNAi3 (A3) and *OsrbohE*-RNAi2 (E2). The amount of each mRNA was calculated from the threshold point located in the log-linear range of RT-PCR. Standard samples with known template amounts were used for quantification of each mRNA. Open bars, 0 h after inoculation; solid bars, 6 h after inoculation. The y-axis represents fold-change relative to the amount in cultured cells 0 h after inoculation. The error bars indicate standard deviation of the mean of three experiments.

revealed that expression of *LOX* in the control cells increased 6 h after inoculation with the incompatible N1141 strain. The same induction pattern was observed in *OsrbohA*-RNAi3, whereas no significant induction was observed in *OsrbohE*-RNAi2 cultured rice cells (Figure 6). These results suggest that induction of *LOX* expression is caused by *OsrbohA*-mediated ROS, but not *OsrbohE*-mediated ROS.

Discussion

We provide molecular evidence that *OsrbohA*, *OsrbohB*, *OsrbohD*, and *OsrbohE*, which encode probable components of a plant NADPH oxidase, are responsible for the ROS produced in the plant immune system. It has been previously reported that the genome of *Arabidopsis thaliana* contains at least ten *Atrboh* genes (Foreman et al. 2003). All known Atrbohs have six putative transmembrane domains, four histidine residues important for heme binding, an NADPH-ribose binding site, an NADPH-adenine binding site, FAD binding site, and two EF hand motifs (Keller et al. 1998). These characteristic structural domains were also found in all Osrbohs in this study. Among these conserved structures, the EF hand motif is predicted with a Ca^{2+} binding site, and it can therefore be presumed that intracellular Ca^{2+} is involved in regulation of Osrboh activity. Our previous study showed that infection of cultured rice cells with the incompatible N1141 strain of *A. avenae* induces ROS generation preceding hypersensitive cell death (Iwano et al. 2002). Furthermore, chelation of extracellular Ca^{2+} completely blocked the hypersensitive cell death in cultured rice cells induced by the incompatible strain inoculation (data not shown). Kwak et al. (2003) also reported that the *AtrbohD* and *AtrbohF* function in guard cell ABA signal transduction, and that Ca^{2+} is important in *AtrbohD*-and *AtrbohF*-mediated signal transduction of ABA. Their results together with our findings, indicate that activation and regulation of *Osrboh* genes involves Ca^{2+}.

The results presented here indicate that expression patterns of *OsrbohA*, *OsrbohB*, *OsrbohD* and *OsrbohE* are different during the incompatible interaction. Expression of *OsrbohA* and *OsrbohD* was induced by inoculation with the incompatible strain, whereas *OsrbohB* and *OsrbohE* were expressed constitutively and the expression patterns did not change after infection with the incompatible strain. Two such types of induction patterns of plant *rboh* genes have been reported. Among potato *rboh* homologs, *StrbohA* is constitutively expressed at a low level, whereas *StrbohB* is induced by hyphal wall components (HWC elicitor) from the potato pathogen *Phytophthora infestans* in potato tubers (Yoshioka et al. 2001). Two $gp91^{phox}$ genes, *NbrbohA* and *NbrbohB* were isolated from *Nicotiana benthamiana* and *NbrbohB* was induced specifically by the protein elicitor INF1 from *Phytophthora infestans*, while expression of *NbrbohA* did not increase after treatment with the elicitor (Yoshioka et al. 2003). NADPH oxidases in mammals are composed of two plasma membrane proteins, $gp91^{phox}$ and $p22^{phox}$ (Bokock 1994). During activation, two cytosolic proteins, p47 and p67, and the small G protein Rac translocate to the plasma membrane, resulting in the formation of the active NADPH oxidase complex in neutrophils (Diekmann et al. 1994). However, no p47 and p67 homologs of the mammalian NADPH oxidase are found in plant genomes, such as *Arabidopsis* and rice (Torres et al. 2002). Moreover, plant NADPH oxidases do not require additional cytosolic factors for their enzymatic activity (Sagi and Fluhr 2001), suggesting that plant NADPH oxidases differ from the mammalian NADPH oxidases at least in their activation mechanisms. Transcriptional control of plant *rbohs* may be a control systems for the plant oxidative burst.

We used RNAi technology in rice to define biological functions for two *Osrboh* genes in the oxidative burst during the immune response. An *OsrbohA*-knockdown line showed that the rapid H_2O_2 generation induced by a

specific elicitor, flagellin isolated from the incompatible N1141 strain of *A. avenae*, was decreased compared to that of the control cells, whereas the late H_2O_2 generation was decreased in an *OsrbohE*-knockdown rice line (Figure 4). Inoculation of soybean cells with avirulent *Pseudomonas syringae* pv. *glycinea* evoked massive H_2O_2 production and was sustained for several hours (Levine et al. 1994). In addition, treatment of soybean cells with a glucan elicitor caused a rapid oxidative burst following a lag of about 10 min, and this burst was transient and began to decline after 40–50 min. These rapid oxidative bursts and late oxidative bursts function as different diffusible signals for subsequent plant immune responses, such as cell wall protein cross-linking, hypersensitive cell death, and systemic acquired resistance (Alvarez et al. 1998). Our results with *OsrbohA* and *OsrbohE*-knockdown transformants showed that the primary and secondary oxidative bursts are caused by different H_2O_2 generation machinery. Moreover, different expression profiles of *OsrbohA* and *OsrbohE* during the immune response suggested that the rapid and late oxidative bursts are regulated by different mechanisms. It will be of interest to investigate the correlation between the primary and secondary ROSs.

Among the *Arabidopsis rboh* homologs, the *AtrbohD* gene is required for most of the ROS generation observed after inoculation with an incompatible pathogen, whereas *AtrbohF* makes a more limited contribution. Although *AtrbohF* exhibits minor diminution of ROS production, it expresses strongly enhanced cell death phenotypes. Using *OsrbohA*-RNAi3 and *OsrbohE*-RNAi2, we observed that the *OsrbohA* gene is required for the induction of hypersensitive cell death. Interestingly, *OsrbohA* is homolog to *AtrbohF*, as compared to the *Arabidopsis rboh* genes (data not shown). Induction of hypersensitive cell death may be caused by *OsrbohA*-type *rboh* in the plant kingdom.

Several genes have been identified from various plant species whose expression is transcriptionally up- or downregulated during the plant immune response (Rushton and Somssich 1998; Fujiwara et al. 2004). We have reported that 136 rice genes are upregulated in cultured rice cells inoculated with the incompatible N1141 strain, using a rice cDNA microarray (Fujiwara et al. 2004). Of the 136 genes, five genes—*EL2*, *Cht-1*, *EREBP1*, *CCD1*, and *LOX*—were selected, and we examined the induction pattern of these genes in *OsrbohA*-RNAi3 and *OsrbohE*-RNAi2 cultured rice cells. We demonstrated here that induction of the *EL2* (elicitor early responsible gene) and *LOX* (Lipoxygenase) genes is controlled by ROS generated by *OsrbohE*, and *Cht-1* (chitinase) gene expression is regulated by both *OsrbohA* and *OsrbohE*. EREBPs bind GCC boxes in several pathogenesis-related (PR) protein gene promoters, and higher levels of transcription are induced by incompatible pathogens (Durrant et al. 2000). *CCD1* involved in calcium signaling in the plant immune response; therefore, the *EREBP1* and *CCD1* genes are known to be important for the induction of plant immunity. However, these two genes were not regulated by ROS generated by either *OsrbohA* or *OsrbohE*. These results indicate that several signal molecules including ROS generated by *OsrbohA* and *OsrbohE* play an important factor for induction of plant immune responses. It will be of interest to investigate the character of these signal molecules and the regulatory mechanisms of various plant immune signalings.

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