Involvement of the Small GTPase Rac in the Defense Responses of Tobacco to Pathogens

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During the hypersensitive response (HR), plants accumulate reactive oxygen species (ROS) that are likely generated at least in part by an NADPH oxidase similar to that found in mammalian neutrophils. An essential regulator of mammalian NADPH oxidase is the small GTP-binding protein Rac. To investigate whether Rac also regulates the pathogen-induced oxidative burst in plants, a dominant negative form of the rice *OsRac1* gene was overexpressed in tobacco carrying the *N* resistance gene. Following infection with *Tobacco mosaic virus* (TMV), DN-*OsRac1* plants developed smaller lesions than wild-type plants, accumulated lower levels of lipid peroxidation products, and failed to activate expression of antioxidant genes. These results, combined with the demonstration that superoxide and hydrogen peroxide levels were reduced in DN-*OsRac1* tobacco developing a synchronous HR triggered by transient expression of the TMV p50 helicase domain or the Pto and AvrPto proteins, suggest that ROS production is impaired. The dominant negative effect of DN-*OsRac1* could be rescued by transiently overexpressing the wild-type *OsRac1* protein. TMV-induced salicylic acid accumulation also was compromised in DN-*OsRac1* tobacco. Interestingly, while systemic acquired resistance to TMV was not impaired, nonhost resistance to *Pseudomonas syringae* pv. *maculicola* ES4326 was suppressed. Thus, the effect DN-*OsRac1* expression exerts on the resistance signaling pathway appears to vary depending on the identity of the inoculated pathogen.

Whether a plant activates an effective defense in response to an attacking pathogen is often determined by the gene-for-gene interaction between the products of a plant resistance (*R*) gene and a pathogen avirulence (*Avr*) gene. If the plant and pathogen carry the cognate *R* and *Avr* genes, the plant often develops a hypersensitive response (HR), in which necrotic lesions form at the sites of pathogen infection (Flor 1971; Hammond-Kosack and Jones 1996). This zone of dead cells is thought to help inhibit proliferation and systemic spreading of the pathogen. The HR also is associated with the accumulation of the defense signal salicylic acid (SA) and increased expression of several families of defense genes, including the pathogenesis-related (*PR*) genes (Dempsey et al. 1999). Subsequent to these events in the inoculated leaf, SA accumulation and *PR* gene expression are usually detected in the uninoculated por-

tions of the plant in conjunction with the development of a broad-based, long-lasting resistance known as systemic acquired resistance (SAR) (Dempsey et al. 1999).

One of the earliest events activated during an incompatible interaction between resistant plants and avirulent pathogens is the accumulation of hydrogen peroxide (H₂O₂) and other reactive oxygen species (ROS), known as the oxidative burst (OB) (Lamb and Dixon 1997). This increase in ROS appears to play several roles in establishing plant resistance. It may directly inhibit pathogen colonization by serving as an antimicrobial agent and by promoting the crosslinking of cell wall glycoproteins (Bradley et al. 1992). In addition, elevated levels of H₂O₂ stimulate multiple defense responses, including *PR* gene expression (Chamnongpol et al. 1998; Neuenschwander et al. 1995), phytoalexin production (Lamb and Dixon 1997), HR-associated cell death (Levine et al. 1994), and enhanced resistance to pathogen infection (Chamnongpol et al. 1998; Takahashi et al. 1997; Wu et al. 1995). H₂O₂ is thought to signal many defense responses via a positive feedback loop that also involves SA and cell death (Draper 1997; Shirasu et al. 1997; Van Camp et al. 1998). Supporting this hypothesis, while H₂O₂ treatment induces SA accumulation in tobacco (Léon et al. 1995; Neuenschwander et al. 1995) and cell death in soybean cells (Levine et al. 1994; Shirasu et al. 1997), SA potentiates both H₂O₂ accumulation and cell death in pathogen-infected soybean suspension cells (Shirasu et al. 1997).

In phagocytic leukocytes, engulfed microorganisms are killed by toxic oxidants generated by NADPH oxidase. This enzyme consists of several 'phox' proteins, including gp91^{phox} and p22^{phox}, which form a b-type cytochrome, as well as p47 and p67 (Babior 2004). Studies during the past several years have suggested that a similar, plasma membrane-localized NADPH oxidase is a major source of ROS production in plants (Lamb and Dixon 1997; Grant and Loake 2000). Plant proteins immunologically related to p47 and p67 have been identified (Dwyer et al. 1996; Xing et al. 1997). In addition, homologs of gp91^{phox}, which encodes the catalytic subunit of the respiratory burst oxidase, have been isolated from several plant species, including rice, *Arabidopsis*, tomato, potato, and tobacco (Amicucci et al. 1999; Groom et al. 1996; Keller et al. 1998; Simon-Plas et al. 2002; Torres et al. 1998; Yoshioka et al. 2001). Intriguingly, while the carboxy termini of the plant gp91^{phox} homologs are similar to human gp91^{phox}, their amino-termini are unique and contain two calcium (Ca²⁺)-binding EF hand motifs. The presence of this motif suggests that Ca²⁺ plays a role in modulating plant NADPH oxidase activity. However, it currently is unclear whether this regulation is mediated via a Ca²⁺-dependent protein kinase (CDPK; Xing et al. 1997; Yoshioka et al. 2001), by direct interaction between Ca²⁺ and the NADPH oxidase complex (Keller et al. 1998; Sagi and Fluhr 2001), or both.

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In mammalian phagocytic cells, activation of NADPH oxidase occurs once p47 and p67 are translocated from the cytoplasm to the plasma membrane, where they form a multimeric complex with gp91^{phox} and gp22^{phox} (Babior 2004; Bokoch and Diebold 2002). In addition, NADPH oxidase activation requires the small GTP-binding protein (G protein) Rac (Bokoch and Diebold 2002). A similar mechanism may regulate activation of NADPH oxidases in plants. In elicitor-treated tomato cells, increased NADPH oxidase activity correlated with translocation to the plasma membrane of proteins that cross-react with antibodies to mammalian p67, p47, and Rac2 (Xing et al. 1997). Furthermore, Hassanain and associates (2000) showed that four related *Rac* genes from maize could function for their mammalian counterparts to activate the NADPH oxidase complex in mouse NIH 3T3 cells, indicating that members of the Rac family are highly conserved and can function in heterologous systems. Expression of a constitutively active form of human *Rac1* in soybean cells or the rice *OsRac1* gene in rice plants or cells enhanced ROS production, whereas expression of dominant negative forms of these proteins suppressed H₂O₂ production (Kawasaki et al. 1999; Park et al. 2000; Suharsono et al. 2002). Strikingly, rice plants expressing constitutively active *OsRac1* also displayed spontaneous HR-like cell death, expression of defense-related genes, and enhanced disease resistance, whereas expression of the dominant negative form of *OsRac1* suppressed pathogen-induced HR formation (Ono et al. 2001). HR formation was similarly suppressed in transgenic tobacco expressing the alfalfa *Ms-rac1* gene in an antisense orientation (Schiene et al. 2000). These results combined with the recent finding that *OsRac1* functions downstream of the heterotrimeric G protein subunit Gα in an *R* gene-mediated resistance pathway (Suharsono et al. 2002) argue that Rac-like proteins regulate ROS production and the activation of disease resistance in plants.

In tobacco, *N* gene-mediated resistance to *Tobacco mosaic virus* (TMV) infection is associated with increased levels of ROS, including superoxide (O₂^{•-}), during HR formation (Doke and Ohashi 1988). Using a novel activity gel assay, Sagi and Fluhr (2001) demonstrated that a tobacco gp91^{phox} homolog displayed NADPH-dependent O₂^{•-}-producing activity that was stimulated by Ca²⁺ in the absence of other cytosolic components, suggesting that it is regulated differently from mammalian gp91^{phox}. To further investigate the regulation of tobacco NADPH oxidase, we generated transgenic tobacco expressing a dominant negative form of the rice *OsRac1* gene. Here, we show that overexpression of this transgene significantly reduced the magnitude of the HR-associated OB. DN-*OsRac1* plants also were compromised for various *N* gene-mediated responses. However, while resistance to the nonhost pathogen *Pseudomonas syringae* pv. *maculicola* ES4326 was impaired, TMV-induced SAR development was not. Taken together, these results suggest that Rac plays an important role in regulating the OB, which presumably modulates many other aspects of the defense signaling pathway. However, whether disease resistance is altered by *DN-OsRac1* expression appears to be a pathogen-specific phenomenon.

RESULTS

Constitutive expression of *DN-OsRac1* reduces the size of TMV-induced lesions.

To assess the role of Rac in *N* gene-mediated resistance to TMV, we constructed transgenic tobacco plants (cv. Xanthi nc [NN]) that expressed a dominant negative form of the small GTP-binding protein *OsRac1* from rice under control of the constitutive *Cauliflower mosaic virus* 35S promoter (Kawasaki et al. 1999). Due to a substitution from threonine to asparagine at

residue 24, the mutated *OsRac1* displays increased affinity for GDP, thereby locking it into an inactive state (Farnsworth and Feig 1991). Seven independent transgenic lines were obtained, and expression of the transgene was monitored by Northern blot analysis (data not shown). Plants from all seven lines displayed normal morphology and fertility, although their growth rate was slightly reduced at the seedling stage. In comparison, the growth of tobacco expressing a *Medicago sativa* *Rac* antisense construct was strongly inhibited (Schiene et al. 2000).

Analysis of plants from the T₃ generation revealed that lines 1 and 7 expressed high levels of the *DN-OsRac1* transgene (Fig. 1). Thus, all experiments were performed with these lines. To analyze the effect of *DN-OsRac1* expression on defense responses, plants from lines 1 and 7 were infected with TMV. Lesion formation was detected in both wild-type (wt) plants and the DN-*OsRac1* transgenic lines approximately 48 h postinoculation (hpi) with TMV. However, lesion expansion on the DN-*OsRac1* transgenic plants occurred more slowly throughout the experiment (up to 2 weeks). Thus, by 6 days postinoculation (dpi), lesion diameters on the transgenic plants were 40 to 70% smaller than those exhibited by wt plants (Fig. 2A and B).

Since *OsRac1* is involved in regulating NADPH oxidase activity, it is possible that reduced ROS production led to the smaller lesions developed by DN-*OsRac1* transgenics. To assess this possibility, the extent of lipid peroxidation, which is frequently used as a measure of oxidative stress, was monitored (Larkindale and Knight 2002; Ranieri et al. 1996). Following TMV infection, the level of lipid peroxidation (based on the production of thiobarbituric acid-reactive substances, expressed as malondialdehyde [MDA] equivalents) in wt plants more than doubled by 120 hpi (Fig. 2C). In contrast, MDA levels in DN-*OsRac1* plants increased only slightly, correlating with the smaller lesion size. Based on this result, DN-*OsRac1* transgenic plants appear to undergo less oxidative stress than wt plants following TMV infection.

Expression of *DN-OsRac1* delays lesion formation and suppresses ROS accumulation.

To determine whether *DN-OsRac1* expression affected the timing of the TMV-induced oxidative burst, wt and DN-*OsRac1* plants were inoculated with *Agrobacterium tumefaciens* carrying a binary plasmid with the TMV helicase p50 domain-encoding gene (Erickson et al. 1999). Unlike TMV inoculation, which stimulates an asynchronous HR in a gradually expanding number of cells, this method induces a synchronous HR in the infiltrated area of wt plants by approximately 48 h postinfiltration (Fig. 3A) (Erickson et al. 1999). By contrast, the HR in comparably infiltrated DN-*OsRac1* plants was not detected until between 60 and 72 h postinfiltration (Fig. 3A). A similar delay in HR formation was observed in DN-*OsRac1* plants developing a synchronous HR due to *A. tumefaciens*-mediated transient coexpression of *AvrPto* and *Pto* (Fig. 3B) (Sessa et al. 2000).

Fig. 1. Expression of *DN-OsRac1* in transgenic tobacco lines. The expression of the transgene was confirmed by Northern analysis in the T₃ generation. RNA (8 µg) was loaded per sample and probed with the *DN-OsRac1-T24N* cDNA. Ethidium bromide-stained rRNA served as a control for gel loading. The wild-type control (wt.) was an untransformed tobacco plant.

Fig. 2. Lesion formation and accumulation of lipid peroxidation products after *Tobacco mosaic virus* (TMV) infection of wild-type (wt) and DN-OsRac1 plants. **A**, Lesion formation in DN-OsRac1 and wt plants. The photograph was taken 6 days postinoculation (dpi). **B**, The average lesion diameter was determined at 6 dpi for TMV-inoculated wt and DN-OsRac1 plants. The data represent the mean $\pm$ standard deviation of at least 40 lesions chosen randomly from several leaves. **C**, Accumulation of thiobarbituric acid-reactive substances (as malondialdehyde [MDA] equivalents) in wt (open bars) and DN-OsRac1 plants (solid bars) at various times after TMV infection. The experiment was done three times with similar results for both lines 1 and 7.

Whether the delayed HR in DN-OsRac1 plants correlated with delayed ROS accumulation was then tested. In wt plants, large patches of cells containing $O_2^{\bullet-}$ (stained with nitro blue tetrazolium [NBT]) and H_2O_2 (stained with 3,3'-diaminobenzidine [DAB]) were detected at 43 h postinfiltration, which slightly preceded lesion formation (Fig. 3C). However, in DN-OsRac1 plants, only weak NBT and DAB staining was observed at this time. To quantify ROS levels, leaves from wt and DN-OsRac1 transgenics were infiltrated with the oxidation-sensitive dye phenol red (Schraudner et al. 1998). A 3.5-fold increase in ROS levels was detected in wt plants by 43 h postinfiltration, whereas only a twofold increase was observed in DN-OsRac1 plants (Fig. 3D).

The dominant negative effect of DN-OsRac1 can be rescued by overexpression of the *OsRac1* wt gene.

The altered defense response exhibited by DN-OsRac1 plants likely is due to the overexpressed DN-OsRac1 protein, which competes with the endogenous Rac proteins for an essential cofactor such as a guanidine nucleotide exchange factor by forming a nonproductive complex (Farnsworth and Feig 1991; Jung et al. 1994). However, the altered phenotype might also be due to reduced activity of another protein, such as another GTPase, which shares the essential cofactor with the Rac1 protein. To address this possibility, the wt *OsRac1* gene was co-expressed with the TMV helicase domain p50 in DN-OsRac1 plants to determine if it could repress the delayed HR phenotype. If the alternative explanation that reduction in activity of another protein besides Rac1 is responsible for the altered phenotype is correct, then overexpression of the wt *OsRac1* gene should not rescue or overcome the delayed HR observed in DN-OsRac1 plants and might even enhance this phenotype, due to even more competition from the wt as well as the from the mutant OsRac1 protein for the limited cofactor. In contrast, we found that wt *OsRac1* overexpression partially rescued the delayed HR phenotype (Fig. 3E), indicating that the altered defense response is primarily due to the lack of active Rac1 protein. The HR was clearly developed in wt plants by 60 hpi, whereas the DN-OsRac1 plants did not show any lesion development (data not shown) (note that the timing of HR development was delayed compared with previous experiments shown in 3A, due to different growth chamber conditions). At 72 hpi, DN-OsRac1 plants still did not show the p50-induced HR (Fig. 3E, section 1). However, the HR formation was partially rescued by co-expressing the wt *OsRac1* gene with p50. The HR formation was recovered in a dose-dependent manner. At a 1:1 ratio of p50 to OsRac1, a partial HR was observed (Fig. 3E, section 4), whereas at a 1:2 ratio an almost complete HR was seen (Fig. 3E, section 5). At 96 hpi a full HR was visible in all infiltrated areas (data not shown).

Expression of DN-OsRac1 alters TMV-induced gene induction.

In TMV-inoculated tobacco, the HR is associated with activation of a wide variety of genes. Some of these genes, such as the *PR* genes, are thought to provide pathogen resistance, whereas others, like ascorbate peroxidase (*APX*) and epoxide hydrolase (*EH-1*) may detoxify deleterious compounds generated during the OB (Guo et al. 1998, 2000). Since DN-OsRac1 plants display a delayed HR, reduced oxidative stress, and decreased ROS levels as compared with those in wt plants, we tested whether expression of *APX* and catalase (*CAT*), which encode the two major H_2O_2 scavenging enzymes of the plant cell, were altered. Both DN-OsRac1 and wt plants contained similar levels of *CAT* and *APX* transcripts at 0 hpi (Fig. 4A). Following TMV infection, *CAT* mRNA levels decreased in

both sets of plants. However, while *CAT* transcripts in wt plants returned to their original levels by 48 hpi, their level in DN-*OsRac1* plants remained low. TMV-induced accumulation of *APX* mRNAs also was suppressed in DN-*OsRac1* plants. Induction of *EH-1*, which converts epoxides to diols, also was suppressed in TMV-inoculated DN-*OsRac1* lines as compared with comparable wt plants (Fig. 4B). Unexpectedly, *PR-1* expression was unaffected by expression of DN-*OsRac1*; similar levels of transcripts for this gene were detected in wt and transgenic plants throughout the time course (Fig. 4B). The apparent earlier induction of *PR-1* expression (24 hpi) in DN-*OsRac1* plants is due to overloading of the gel. Analyses of other DN-*OsRac1* lines indicate that *PR-1* expression is not induced at 24 hpi.

TMV-induced SA accumulation is suppressed in DN-*OsRac1* transgenic plants.

SA is a critical signal for activating both local and systemic defense responses following pathogen infection (Dempsey et al. 1999). In tobacco, *N* gene-mediated resistance to TMV is associated with large increases in both free SA and its conjugate, SA glycoside (SAG) (Malamy et al. 1992). To assess whether this phenomenon is affected in DN-*OsRac1* plants, we measured the levels of free SA and SAG following TMV infection. As expected, SA and SAG levels in wt plants increased substantially by 72 hpi. Increased levels of SA and SAG also were detected in TMV-inoculated plants from DN-*OsRac1* lines 7-5 and 1-3; however, they were significantly lower than those observed in wt plants (Fig. 5A and data not shown).

Fig. 3. Effects of transient expression of the *Tobacco mosaic virus* (TMV) helicase domain (p50) and *AvrPto* plus *Pto* in wild-type (wt) and DN-*OsRac1* plants. *Agrobacterium tumefaciens* carrying p50 was infiltrated into leaves of DN-*OsRac1* and wt plants at a concentration at an optical density at 600 nm of 0.5. **A**, Hypersensitive response (HR) formation in wt and DN-*OsRac1* plants at 48, 60, and 72 h postinoculation (hpi) of p50. + and - indicate the strength of its HR: - = none, ± = very weak, + = modest, ++ = strong. **B**, HR formation in wt and DN-*OsRac1* plants at 24, 48, and 72 hours after coinfiltration of *AvrPto* and *Pto*. **C**, The accumulation of $O_2^{\bullet-}$ and H_2O_2 was visualized by staining leaf segments from wt or DN-*OsRac1* plants with nitro blue tetrazolium or diaminobenzidine, respectively, at 43 hpi (p50). **D**, The production of reactive oxygen species was quantified at 0 and 43 hpi with p50 by infiltrating leaves of DN-*OsRac1* and wt plants with the oxidation-sensitive dye phenol red. The experiment was done three times with several plants (line 7-5) per experiment. **E**, Coexpression of the wt *OsRac1* protein with p50 in DN-*OsRac1* plants partially rescued the delayed HR phenotype. *A. tumefaciens* carrying the p50 and *A. tumefaciens* carrying either an empty vector (pMSH-1) or the wt *OsRac1* gene were coinfiltrated into leaves of DN-*OsRac1* plants (line 7-5) in different ratios: 1, p50 only; 2, p50 empty vector ratio 1:1; 3, p50 empty vector 1:2; 4, p50 wt *OsRac1* 1:1; and 5, p50 wt *OsRac1* 1:2. The photo was taken at 72 hpi.

Expression of *DN-OsRac1* does not affect SAR but suppresses resistance to a nonhost pathogen.

Since many defense responses are either delayed, suppressed, or both in DN-*OsRac1* transgenics, we tested whether SAR also was affected. Analysis of DN-*OsRac1* plants at 7 dpi failed to detect TMV in the uninoculated, systemic leaves (data not shown), indicating that the virus was unable to spread out of the inoculated leaves. Six days after the primary infection, previously uninfected leaves were challenged with TMV. The lesion size and number were reduced after the secondary challenge with TMV as compared with those produced after a primary infection (Fig. 6A). As noted previously for the primary lesions, the secondary lesions developed by DN-*OsRac1* plants were significantly smaller than those formed by challenge-inoculated wt plants. Thus, the robust resistance to TMV mediated by the *N* gene was not compromised by expression of *DN-OsRac1*. We then tested whether resistance to the nonhost

bacterial pathogen *P. syringae* pv. *maculicola* ES4326 was affected in DN-*OsRac1* plants. Inoculation of wt plants with *P. syringae* pv. *maculicola* ES4326 induced a visible HR by 24 hpi (Fig. 6B). By contrast, this HR was either completely absent or strongly reduced in DN-*OsRac1* plants. Consistent with the lack of HR development, bacterial growth was about 10-fold higher in DN-*OsRac1* plants than in wt plants at 3 dpi (Fig. 6C). Taken together, these results indicate that expression of *DN-OsRac1* suppresses nonhost resistance to *P. syringae* pv. *maculicola* ES4326.

DISCUSSION

Mounting evidence suggests that Rac-like proteins play an important role in regulating ROS production and defense signaling in plants. Here, we demonstrate that overexpression of a dominant negative form of the rice *OsRac1* gene in tobacco leads to a significant reduction in pathogen-induced ROS production. Furthermore, these transgenic plants exhibit smaller TMV-induced lesions, altered defense gene expression, suppressed SA accumulation, and decreased resistance to *P. syringae* pv. *maculicola* ES4326.

Similar to these results, rice expressing a dominant negative form of *OsRac1* and tobacco expressing an antisense construct of alfalfa *Ms-rac1* displayed suppressed elicitor- or pathogen-induced HR formation (Ono et al. 2001; Schiene et al. 2000). Additionally, soybean and rice cells expressing dominant negative forms of human *Rac1* or *OsRac1*, respectively, displayed reduced ability to generate ROS in response to elicitor treatment (Ono et al. 2001; Park et al. 2000; Suharsono et al. 2002). Unlike these analyses, our efforts to detect reduced H₂O₂ production in TMV-inoculated DN-*OsRac1* plants were unsuccessful; reduced ROS generation was therefore demonstrated indirectly by monitoring lipid peroxidation levels. A likely explanation for this result is that only a gradually increasing number of cells respond hypersensitively in TMV-inoculated leaves, whereas all of the suspension cells respond synchronously to elicitor treatment. Consistent with this conclusion, DN-*OsRac1* plants in which a synchronous HR was activated, due to transient expression of the TMV p50 helicase domain or co-expression of *Pto* and *AvrPto*, accumulated substantially lower levels of H₂O₂ than comparable wt plants.

Fig. 4. Expression of antioxidant genes is suppressed in DN-*OsRac1* plants. **A**, Induction of the *CAT* and *APX* genes in DN-*OsRac1* (line 7-5) and wild-type (wt) plants after *Tobacco mosaic virus* (TMV) infection. Total RNA was isolated and used for semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). The RT-PCR products resulting from 23 and 21 amplification cycles for *CAT* and *APX*, respectively, were loaded. Equal loading of cDNA was monitored by amplification of β -*tubulin* (β -*tub*, 28 cycles). **B**, Induction of *EH-1* and *PR-1* genes in DN-*OsRac1* (line 1-3) and wt plants after TMV infection. Gene expression was monitored by Northern blot analysis using 8 μ g of RNA per sample. Ethidium bromide-stained rRNA served as a control for gel loading.

Fig. 5. Salicylic acid (SA) accumulation in *Tobacco mosaic virus* (TMV)-infected wild-type (wt) and DN-*OsRac1* plants. The content of free SA (solid bars) and SA glycoside (open bars) was determined in TMV-infected DN-*OsRac1* (line 7-5) and wt plants at 0 and 72 h postinoculation. The data represents the mean $\pm$ standard deviation of three independent samples. The experiment was done twice with similar results. FW = fresh weight. Similar results were obtained with line 1-3.

The correlation between reduced ROS accumulation and decreased lesion size in DN-OsRac1 tobacco suggests that wt levels of ROS are required to develop full-sized lesions. Supporting this hypothesis, ROS have been implicated in activating HR cell death in soybean suspension cells (Levine et al. 1994; Shirasu et al. 1997). Furthermore, TMV-induced lesion formation was abolished in tobacco grown under low oxygen conditions, which presumably suppresses the production of ROS (Mittler et al. 1996). When intracellular oxygen levels were increased slightly by supplementing the low oxygen conditions with carbon dioxide, which allowed photosynthesis, these plants developed very small lesions after TMV infection. Lesion size also was reduced in TMV-inoculated tobacco plants that contain reduced levels of ROS due to overexpression of mitochondrial alternative oxidase (Ordog et al. 2002).

A likely explanation for the observed effects of overexpression of a dominant negative form of Rac is that it competes with endogenous Rac proteins for an essential cofactor such as guanine nucleotide exchange factor (Farnsworth and Feig 1991; Jung et al. 1994). This possible depletion of a cofactor may also affect other unrelated Rac proteins or GTPases. However, the effect of the dominant negative mutant could be rescued by overexpressing the wt *OsRac1* gene in DN-OsRac1 plants. This shows that the rice Rac protein is functional in tobacco and argues that the altered defense response in the DN-OsRac1 plants is primarily due to lack of active Rac protein. Other groups (Hassanain et al. 2000; Park et al. 2000) also reported that Rac proteins from one species can function in another and that dominant negative versions reduce ROS production, whereas constitutive active forms lead to an increase in ROS production, indicating that the regulation of NADPH oxidase by Rac is quite conserved. Recently, Morel and associates (2004) isolated five *Rac* cDNAs from tobacco. Interestingly, *NtRac5* was repressed after treatment with the elicitor cryptogein, and overexpression of this gene led to a reduction in elicitor-induced ROS formation, showing that Rac proteins may also function as a negative regulator of NADPH oxidase. However, *NtRac5* belongs to the Rac/Rop group IV, whereas *OsRac1* belongs to group II. The closest tobacco family member in group II is *NtRac1* (Morel et al. 2004). While these studies, including that reported here, implicate NADPH oxidase, the evidence is indirect, and we can not rule out the possibility that the reduction in Rac activity affects other proteins besides NADPH oxidase.

The reduced levels of ROS in TMV-inoculated DN-OsRac1 plants are likely responsible for the decreased accumulation of SA and SAG. Previous studies have demonstrated that SA accumulation is induced by H₂O₂ treatment (Léon et al. 1995; Neuenschwander et al. 1995) or by the elevated ROS levels found in catalase antisense plants (Chamnongpol et al. 1998; Takahashi et al. 1998). Surprisingly however, while SA-deficient NahG tobacco developed significantly larger lesions than wt plants after TMV infection (Gaffney et al. 1993; Mur et al. 1997), those developed by DN-OsRac1 plants were smaller. Perhaps in the case of the DN-OsRac1 plants, production of ROS rather than SA is the limiting factor for expansion of cell death. Since *PR-1* expression and SAR were not affected in TMV-inoculated DN-OsRac1 plants, the modest increase in SA levels was sufficient for their activation, although it presumably was insufficient for activation of the SA-inducible *APX* and *EH-1* genes (Guo et al. 1998; 2000). Similar to our results, *PR-1* expression and SAR were not affected in tobacco maintained under low oxygen pressure, despite the lack of HR formation and reduced SA accumulation (Mittler et al. 1996).

To assess whether pathogen resistance was affected in DN-OsRac1 plants, their response to inoculation with various

Fig. 6. Disease resistance response in DN-OsRac1 plants. **A**, Development of systemic acquired resistance in DN-OsRac1 plants. The size of primary lesions developed after *Tobacco mosaic virus* (TMV)-inoculation of wild-type (wt) and DN-OsRac1 plants (line 7-5) was measured at 6 days postinoculation (dpi). Six days after the primary TMV infection, the upper previously uninoculated leaves received a secondary inoculation with TMV. The diameter of the lesions was then measured 6 days after the secondary infection. Each value represents the average size (in millimeters $\pm$ standard deviation) of 50 lesions. (1° = primary, 2° = secondary infection) **B** and **C**, Hypersensitive response (HR) formation and bacterial growth in DN-OsRac1 and wt plants after infection with *Pseudomonas syringae* pv. *maculicola* ES4326. Plants were infiltrated with a concentration of 1×10^7 CFU ml⁻¹ of bacteria. **B**, Nonhost HR formation in wt and DN-OsRac1 line 7-5 plants at 24 hpi. **C**, Bacterial titer in the leaves of DN-OsRac1 (line 7-5, ■) or wt plants (○). Leaf disks were harvested at 0, 1, and 3 dpi, and the number of CFU per disk was determined. The values are the mean $\pm$ standard deviation of samples (each from three leaf disks) from three independent plants. The experiment was done three times with similar results.

pathogens was assessed. As previously indicated, *N* gene-mediated induction of SAR was unaffected. Basal resistance to virulent *P. syringae* pv. *tabaci* also was not disrupted (data not shown). By contrast, nonhost resistance to *P. syringae* pv. *maculicola* ES4326 was impaired; HR formation was strongly suppressed and 10-fold greater bacterial growth was detected as compared with wt plants. Similar to these results, rice expressing *DN-OsRac1* were compromised not only for ROS production and HR development but also for resistance to an avirulent race of *Magnaporthe grisea* (Ono et al. 2001). *Nicotiana benthamiana* in which *Nbrboh A* or *B*, two orthologs of *gp91^{phox}*, were silenced also displayed reduced ROS production and enhanced disease symptoms following inoculation with *Phytophthora infestans* (Yoshioka et al. 2003). Moreover, *Arabidopsis atrbohD* and *atrbohF* single and double mutants, which contain null alleles for these *gp91^{phox}* orthologs, displayed a varied response to infection with different pathogens (Torres et al. 2002). These mutants failed to accumulate ROS and displayed reduced HR cell death following inoculation with avirulent or virulent strains of *P. syringae* pv. *tomato*, although bacterial growth was comparable to that in wt plants. However, in response to avirulent *Peronospora parasitica* isolate Emco5, these mutants displayed heightened HR cell death and increased resistance despite suppressed ROS production. By contrast, they displayed no change in either the amount of cell death or resistance following inoculation with other avirulent *Peronospora parasitica* isolates. Taken together, these results suggest that altered ROS production may exert different effects depending on the plant-pathogen system.

In summary, our analysis of DN-*OsRac1* tobacco suggests that Rac is an important regulator of ROS production. Whether it exerts its effects by modulating NADPH oxidase activity, analogous to the role Rac plays in mammalian neutrophils, is currently not known. A tomato protein that cross-reacts with antibodies to mammalian Rac2 was shown to move to the plasma membrane following elicitor treatment and this corresponded with increased NADPH oxidase activity (Xing et al. 1997). In addition, *OsRac1* fusion proteins were recently localized to the plasma membrane of rice protoplasts, which presumably is where NADPH oxidase is located (Ono et al. 2001). By contrast, NADPH oxidase activity in isolated tobacco and tomato membranes was shown to be directly stimulated by Ca^{2+} , raising the possibility that this plant enzyme is regulated via a different mechanism than *gp91^{phox}* (Sagi and Fluhr 2001). Further studies are clearly required to determine whether both Ca^{2+} and a membrane-localized Rac work together to activate NADPH oxidase activity or whether Rac regulates ROS levels via a separate mechanism.

MATERIALS AND METHODS

Plant growth and pathogen treatments.

Tobacco plants *Nicotiana tabacum* cv. Xanthi nc [NN] were grown as described by Malamy and associates (1992). All infection experiments were done with 5- to 6-week-old plants. TMV inoculations were performed with 1 μg of TMV (U1 strain) per ml in 10 mM MES buffer, pH 7.0, in the presence of Carborundum. At each timepoint, half a leaf was harvested, was ground in liquid nitrogen, and was used for all further analyses. For the analysis of SAR, previously uninfected leaves of TMV-treated plants were inoculated with TMV six days after the primary infection. *P. syringae* pv. *maculicola* ES4326 infection was performed by injecting the bacteria at a concentration of 1×10^7 CFU ml^{-1} in 10 mM MgCl_2 into the abaxial surface of several leaves. At 0, 1, and 3 dpi, leaf disks were collected and

homogenized in 10 mM MgCl_2 (3 disks per sample, 7-mm diameter). The homogenate was then plated on King's B medium agar plates in appropriate dilutions. After incubation at 28°C for two days, the number of colonies was determined. The experiment was repeated three times.

Tobacco transformation.

The *OsRac1-T24N* transgene was obtained from K. Shimamoto (Nara Institute of Science and Technology, Takayama, Ikoma, Japan). Details of the constructs were described in Kawasaki and associates (1999). *A. tumefaciens*-mediated transformation of *Nicotiana tabacum* cv. Xanthi nc. [NN] plants was done using the leaf disk method, as described by Horsch and associates (1985). Three weeks after infection with *A. tumefaciens* LB4404, regenerated tobacco shoots were picked from Murashige and Skoog (MS) shoot regeneration medium plates containing 100 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxim and were transferred to MS medium without phytohormones but containing the antibiotics for further selection. About two weeks after the second selection, kanamycin-resistant plants were obtained. Seeds of DN-*OsRac1* transgenic and untransformed plants were germinated on MS medium with or without 100 $\mu\text{g ml}^{-1}$ kanamycin, respectively. Kanamycin-resistant DN-*OsRac1* seedlings were transferred to soil. The expression of the transgene in each plant was confirmed by reverse transcription-polymerase chain reaction (RT-PCR).

A. tumefaciens-mediated transient expression.

A. tumefaciens (strain GV2260) carrying the TMV helicase domain (p50) in a binary plasmid (Erickson et al. 1999) was grown overnight in LB medium, 10 mM MES, pH 5.7, 20 μM acetosyringone. Following centrifugation, bacteria were resuspended in infiltration medium (10 mM MgCl_2 , 10 mM MES, pH 5.7, 150 μM acetosyringone) at a concentration of 0.5 OD₆₀₀ (optical density at 600 nm) and were infiltrated into leaves using a syringe without the needle. The experiment was repeated three times with several plants per line. The procedure for transient expression of *Pto* and *AvrPto* was essentially the same; however, the final concentration of bacteria was 0.065 OD₆₀₀ for each strain (total OD₆₀₀ was 0.13; Sessa et al. 2000). For the competition experiment the different *Agrobacterium* cultures carrying either the helicase domain or the empty binary vector pMSH-1 or the wt *OsRac1* gene in the pMSH-1 vector were mixed just prior to infiltration.

Northern blot analysis.

Total RNA extractions were carried out using the TRIzol reagent (Gibco BRL, Rockville, MD, U.S.A.), according to the manufacturer's instructions. RNA was separated on a 1.5% denaturing agarose gel, was transferred onto a Hybond-NX membrane (Amersham Pharmacia, Piscataway, NJ, U.S.A.), and was hybridized with radiolabeled random primed probes, as described previously (Shah et al. 1999).

RT-PCR.

The cDNA for RT-PCR was generated using Superscript reverse transcriptase (Invitrogen Life technologies, San Diego, CA, U.S.A.), according to the manufacturer's instructions. PCR was performed using Advantage 2 polymerase (Clontech, Palo Alto, CA, U.S.A.). The gene-specific primers used for RT-PCR analysis were as follows: for *APX*, APX-F (5'-CCCACTGTAAGCGAGGAGTAC-3') and APX-R (5'-GAGGAGAGCCTGTCTGAAGG-3'); for *CAT1*, CAT-F (5'-TTGTTTCATGCCCGAGGTGCTA-3') and CAT-R (5'-ACATGTGGGCTTCCAGTGAA-3'); for β -*tubulin*, Tub-F (5'-CTGCATTGGTACACTGGTGAAGG-3') and Tub-R (5'-CAATACTTCATCATCATAAAAC-3').

Extraction and analysis of SA.

SA and SAG were extracted from 0.5 to 1.0 g of leaf tissue, and their levels determined as described by Bowling and associates (1994).

Thiobarbituric acid-reactive substances assay.

The level of lipid peroxides formed in TMV-inoculated plants was evaluated by measuring the concentration of thiobarbituric acid-reactive substances (TBA-RS), essentially as described by Heath and Packer (1968). Leaf tissue was frozen in liquid nitrogen and was homogenized in 0.1% trichloroacetic acid and centrifuged for 10 min at $10,000 \times g$. Supernatant (250 μ l) was incubated with 1 ml of 0.5% TBA in 20% trichloroacetic acid at 95°C for 30 min and then was cooled in an ice bath. After centrifugation (10 min at $10,000 \times g$), the absorbance of the supernatant was read at 532 nm and was corrected for the nonspecific background at 600 nm. The amount of TBA-RS was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Determination of ROS production.

The production of ROS was determined using the phenol red method as described in Schraudner and associates (1998). After infiltrating leaves with *A. tumefaciens* carrying the TMV helicase fragment (43 h), leaves were infiltrated with 0.14 mM phenol red in 10 mM MES, pH 6.5. After 1.5 h of incubation in petri dishes in the light at 25°C, the leaves were gently rolled and centrifuged for 15 min at $1,000 \times g$. A concentration of 1 M NaOH (20 μ l/ml) was added to the obtained apoplastic washing fluid. The oxidized phenol red was quantified by monitoring the absorbance at 610 nm. The concentration of ROS was calculated using a calibration curve with known concentrations of H_2O_2 (Schraudner et al. 1998).

Histochemical staining for ROS accumulation.

Leaf segments were infiltrated with 0.1% (wt/vol) DAB, 10 mM MES, pH 4, or 0.1% (wt/vol) NBT, 10 mM sodium azide, 50 mM potassium phosphate, pH 6.4 (Schraudner et al. 1998). After a 30-min incubation in the light, leaves were cleared in ethanol/lactic acid/glycerol (4:1:1).

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