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The small GTP-binding protein Rac is a regulator of cell death in plants

TSUTOMU KAWASAKI*†, KENJI HENMI*†, EIICHIRO ONO*, SATORU HATAKEYAMA*, MEGUMI IWANO‡, HIKARU SATOH§, AND KO SHIMAMOTO*¶

*Laboratory of Plant Molecular Genetics, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma 630-0101, Japan; ‡Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma 630-0101, Japan; and §Faculty of Agriculture, Kyushu University, Hakozaki, Fukuoka 812-0053, Japan

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ABSTRACT **Cell death plays important roles in the development and defense of plants as in other multicellular organisms. Rapid production of reactive oxygen species often is associated with plant defense against pathogens, but their molecular mechanisms are not known. We introduced the constitutively active and the dominant negative forms of the small GTP-binding protein *OsRac1*, a rice homolog of human Rac, into the wild type and a lesion mimic mutant of rice and analyzed H_2O_2 production and cell death in transformed cell cultures and plants. The results indicate that Rac is a regulator of reactive oxygen species production as well as cell death in rice.**

Cell death is important in the development and defense of multicellular organisms (1). Cell death occurs in normal plant development and during infection by avirulent pathogens (2–5). It plays a key role in plant defense and shares several features with apoptosis in mammalian cells (3, 5, 6). Rapid production of reactive oxygen species (ROS) often is associated with cell death during resistance reactions to pathogens, and a plasma membrane NADPH oxidase similar to the neutrophil enzyme is suggested to be responsible for ROS production (5–7). However, the molecular mechanisms for ROS production and cell death in plants are largely unknown.

In phagocytic cells, activation of NADPH oxidase leads to production of superoxide, which effectively kills invading microorganisms (8, 9). The neutrophil NADPH oxidase is a multicomplex enzyme consisting of two membrane proteins, $gp91^{phox}$ and $p22^{phox}$, and three cytosolic factors, $p47^{phox}$, $p67^{phox}$, and Rac (8, 9), and the plant enzyme is thought to be similar to the neutrophil enzyme (10–13). Genes whose deduced amino acid sequences are similar to those of the Rho/Rac family of the small GTP-binding proteins have been isolated in plants (14–18), and some were shown to play a role in the control of pollen tube growth (19). However, functions of most of these genes are not known. Genes whose amino acid sequences are similar to that of the animal $gp91^{phox}$ also have been isolated from plants (20, 21), but their functions have not been investigated. In this study we analyzed functions of Rac in rice by expressing constitutively active and dominant negative Rac genes in transgenic cell cultures and plants and found that Rac plays an important role in ROS production as well as cell death in rice.

MATERIALS AND METHODS

Biochemical Analysis of Recombinant OsRac1. *OsRac1* cDNA was cloned in an expression vector, pGEX-4T-1 (Pharmacia), and transformed into *Escherichia coli.* The glutathione *S*-transferase (GST)-fusion protein was purified by glutathione-Sepharose beads (Pharmacia) and used for assays of GTP-binding and GTPase activities according to published protocol (22). For the assay of the GTP-binding activity, the binding of [^{35}S]GTPγS to GST-OsRac1 was measured. For the assay of the GTPase activity, GST-OsRac1 was loaded with [γ-^{32}P]GTP and hydrolysis of GTP was measured at different time points.

Rice Transformation. *OsRac1*-G19V was made by substitution of the glycine corresponding to G12 of human Rac1 by valine by the use of an LA PCR *in vitro* Mutagenesis kit (Takara Shuzo, Kyoto). *OsRac1*-T24N similarly was made by substitution of threonine at the 24th position to asparagine. 35S-*OsRac1*-G19V and 35S-*OsRac1*-T24N were introduced in a Ti-based vector, pMSH1, and *Agrobacterium*-mediated transformation of rice callus was performed according to a published protocol (23). Transformed calli were selected by hygromycin resistance, and plants were regenerated from transformed callus cultures. Suspension cell lines were made from transformed calli and maintained in R2S medium (24) for various analyses. The *sl* mutant used in this study carried the CM265 allele in the Kinmaze background.

Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) and Diaminobenzidine (DAB) Staining. TUNEL staining was performed by the use of a fluorescein-dUTP-based *in situ* death detection kit (Boehringer Mannheim) as described previously (25). For DAB staining, the concentration of 1 mg/ml (Wako) was used.

Electron Microscopy. For electron microscopy, cultured cells were fixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.2, and then treated with OsO_4, dehydrated, and embedded in Spurr's resin. Sections were stained with uranylacetate and lead citrate and examined by Hitachi H7100 electron microscopy.

RESULTS AND DISCUSSION

***OsRac1* Encodes a GTPase.** Because Rac plays an important role in the regulation of the NADPH oxidase in phagocytic cells, we sought rice expressed sequence tags that had homology with human Rac and found three such sequences (Fig. 1*A*; ref. 26). They had deduced amino acid sequences that were *ca.* 60% identical with those of human Rac proteins. Comparison of their amino acid sequences with those of other Ras-related GTPase proteins indicated high similarity to Rac; thus, we

Abbreviations: ROS, reactive oxygen species; DPI, diphenylene iodonium; GST, glutathione *S*-transferase; DAB, diaminobenzidine.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB09508, AB029509, and AB029510 for *OsRac1*, *OsRac2*, and *OsRac3*, respectively).

†T.K. and K.H. contributed equally to this work.

¶To whom reprint requests should be addressed. E-mail: simamoto@bs.aist-nara.ac.jp.

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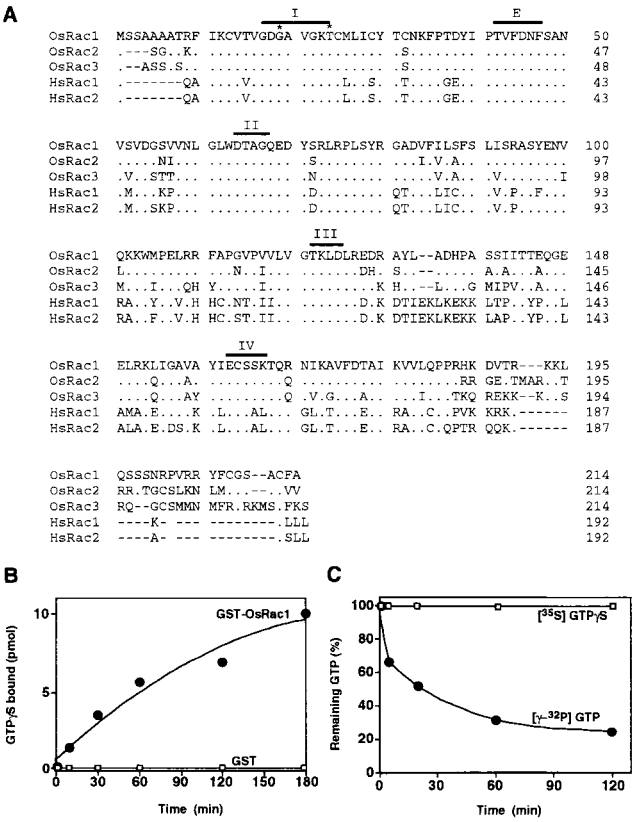

FIG. 1. Deduced amino acid sequences and biochemical analysis of *OsRac*. (*A*) Amino acid alignment of predicted *OsRac* proteins with human Rac proteins (26). Identical residues are indicated by a dot, and gaps are shown by a dash. The conserved regions are indicated by bars above the sequence; regions I and II are the GTPase region, regions III and IV are the GTP/GDP-binding region, and E denotes the effector region. The glycine and threonine residues marked by asterisks in the region I were changed to make a constitutively active and a dominant negative form of *OsRac* genes, respectively. (*B*) GTP-binding activity of the recombinant OsRac1 protein. For the assay, the binding of [^{35}S]GTPγS to GST-OsRac1 was measured at different time points. (*C*) GTPase activity of the recombinant OsRac1 protein. For the assay, GST-OsRac1 was loaded with [γ-^{32}P]GTP and hydrolysis of GTP was measured at different time points.

called them *OsRac*. Particularly, regions I–IV, which are highly conserved among the small GTP-binding protein family (27), and the effector binding site are extremely conserved between humans and rice (Fig. 1*A*). *OsRac1* and *OsRac3* are expressed in leaves and roots, but *OsRac2* is expressed only in roots (data not shown). The recombinant OsRac1 protein in *E. coli* was found to have both GTP-binding and GTPase activities, confirming that *OsRac1* encodes a GTPase (Fig. 1 *B* and *C*), and its binding was specific to GTP and GDP (data not shown). These results indicate that *OsRac1* codes for a GTPase similar to those in mammals.

Constitutively Active *OsRac* Induces ROS Production in Cultured Rice Cells. To examine whether *OsRac* regulates ROS production in rice, we made a constitutively active form of *OsRac1* by substituting glycine at position 19 corresponding to G12 of the human Rac (Fig. 1*A*) with valine and fused with the CaMV35S promoter. The construct was introduced into seed-derived calli of the wild type (cv. Kinmaze) and a lesion-mimic mutant of rice, *sl* (Sekiguchi lesion), by *Agrobacterium*-mediated transformation (23), and suspension cell cultures were produced from transformed calli. The *sl* mutant is a propagation type of the lesion-mimic mutants, and large, orange lesions are induced in the leaf by a number of biotic and abiotic stimuli (28, 29). In the transformed cell lines of the wild type and the *sl* mutant, *OsRac1*-G19V was overexpressed (Fig. 2*A*). Staining with DAB (30) revealed H_2O_2 production in both transformed wild-type and *sl* cells. It was, however, slightly higher in the *sl* mutant than in the wild type (Fig. 2*B*), suggesting that the *sl* mutant has biochemical alterations upstream of ROS production. The observed H_2O_2 production was inhibited by diphenylene iodonium (DPI), an inhibitor of the neutrophil NADPH oxidase, both in the transformed wild-type and *sl* cells. No H_2O_2 production was detected in the untransformed control cells. No differences in activities of catalase and peroxidase were detected between the untransformed control cells and the transformed cells, suggesting that the H_2O_2 production did not result from decreased scavenging activities (data not shown). These findings indicated that *OsRac1*-G19V induces ROS production in rice cells and suggest that a NADPH oxidase similar to the neutrophil enzyme is involved in Rac-induced H_2O_2 production.

Constitutively Active *OsRac* Induces Cell Death in Cultured Rice Cells. We next analyzed the biochemical and morphological characteristics of the transformed *sl* cells in culture. Terminal deoxynucleotidyltransferase-mediated UTP end labeling signals indicative of nuclear DNA cleavage were observed in the transformed *sl* cells but not in untransformed *sl* cells (Fig. 2*C*). In the wild-type cells transformed with *OsRac1*-G19V, cell death was not observed, suggesting that the *sl* mutation may be required for cell death to occur in the cultured rice cells. Electron microscopy of the transformed *sl* cells suggested the occurrence of cell shrinkage (Fig. 3*B*), condensation of the nucleus (Fig. 3*C*), condensed and fragmented chromatin (Fig. 3*D*), and blebbing of the plasma membrane (Fig. 3*E*). No lysis of the mitochondrial membrane was detected (Fig. 3*F*). None of the morphological changes detected in the transformed *sl* cells were found in the untransformed *sl* cells (Fig. 3*A*). These results suggest that cell death occurs in the *OsRac1*-G19V-transformed *sl* cells and that the observed cell death exhibits a set of morphological changes found in apoptosis in mammalian cells (31). To better understand morphological characteristics of Rac-induced cell death in rice cells, a temporal change of their morphology needs to be studied, and such a study is in progress by the use of an inducible promoter system. Our results also suggest a link between ROS production and cell death during plant–pathogen interactions as has been suggested by previous studies (32–35). They also suggest that *OsRac1*-induced cell death of rice cells occurs through a mechanism similar to that in mammalian cells. However, we were not able to detect laddering or degradation of isolated nuclear DNA in the cultured rice cells undergoing cell death despite repeated experiments (data not shown). The failure to detect any cleavage of isolated nuclear DNA may be due to nonsynchronous cell death occurring in the transformed cells in culture. Alternatively, the type of DNA degradation observed in apoptosis of mammalian cells may not occur in cultured rice cells.

Constitutively Active *OsRac* Induces ROS Production and Cell Death in the Leaf of Transgenic Rice. To test whether H_2O_2 production and cell death also can be induced at the whole plant level, we introduced 35S-*OsRac1*-G19V into wild-type and *sl* plants by *Agrobacterium*-mediated transformation of rice callus and generated 12 and 14 independent transgenic plants of the wild type and the *sl* mutant, respectively. DAB staining of leaf sheath cells of transgenic wild-type plants showed H_2O_2 production, which appeared to be localized at the intercellular space (Fig. 4*Ab*). In addition, appearance of cytoplasmic granules (Fig. 4*Ac*), which is the first sign of hypersensitive cell death that occurs after infection of resistant rice cultivars with the avirulent blast fungus (36), also was observed in transgenic rice plants. Neither H_2O_2 production nor the appearance of cytoplasmic granules was observed in untransformed control plants (Fig.

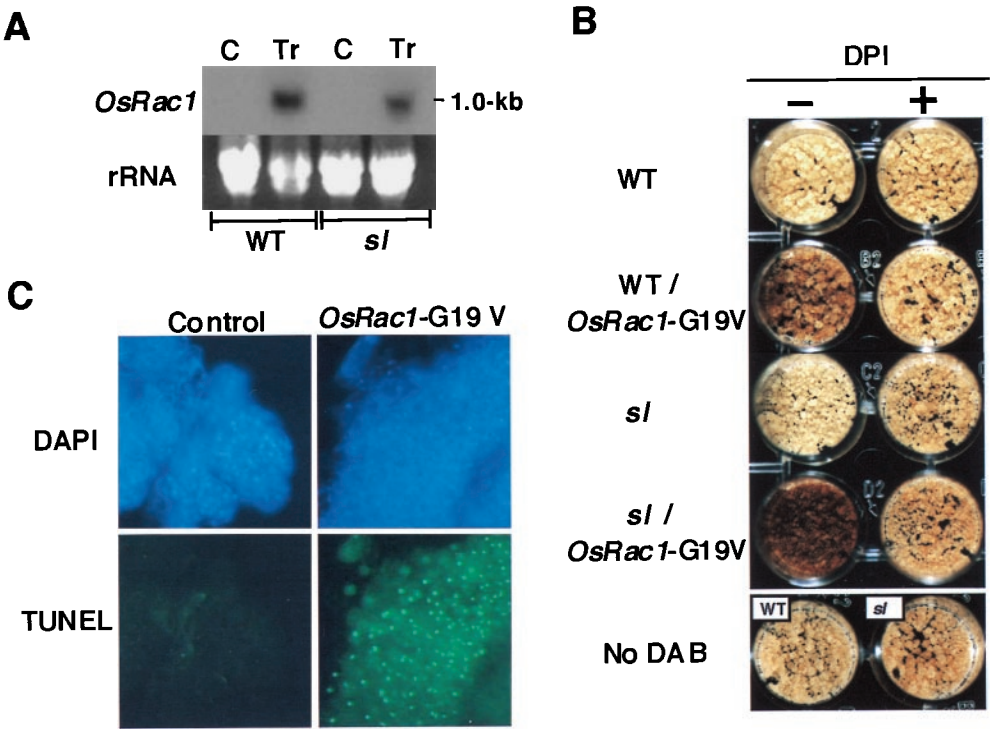

FIG. 2. H_2O_2 production in suspension-cultured rice cells transformed with 35S-*OsRac1*-G19V. (*A*) Overexpression of the *OsRac1* in transformed wild-type and *sl* cells. The probe used was specific to *OsRac1*. (*B*) H_2O_2 production by suspension-cultured rice cells transformed with 35S-*OsRac1*-G19V. Brown color developed after staining with DAB indicates production of H_2O_2, and light-brown color shows the background. DPI was added to the cell suspension 30 min before H_2O_2 detection at a concentration of 50 μM. (*C*) DNA cleavage in *sl* cells transformed with 35S-*OsRac1*-G19V.

4*Aa*). Furthermore, necrotic lesions were found in the leaves of all 12 transgenic wild-type plants at a young stage. During maturation, discrete lesions developed in the leaves (Fig. 4*B*), and they frequently were observed at the junction of the blade and the sheath of the leaves (Fig. 4*Ba*) and in the midrib region (Fig. 4*Bc*). This was observed in 8 of 12 transgenic plants. In transgenic *sl* mutants, large orange lesions, a characteristic of the *sl* mutants (28, 29), appeared in leaves of very young plants (Fig. 4*Ca*), and the lesions progressively spread over the entire plants when they matured (Fig. 4*Cb*) and then died before the emergence of the spikelet. This was observed in all 14 transgenic *sl* plants analyzed. In untransformed *sl* plants, no lesions were detected in plants at the young stage, and spikelets developed normally when they matured. These findings indicated that *OsRac1*-G19V induces H_2O_2 production as well as cell death in the leaf of rice plants.

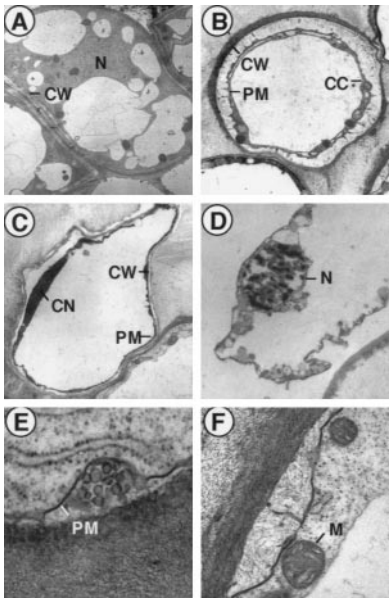

FIG. 3. Apoptosis-like cell death in suspension-cultured rice cells transformed with 35S-*OsRac1*-G19V. (*A*) Untransformed *sl* cells. N, nucleus; CW, cell wall. (*B*) Shrinkage of the plasma membrane (PM) observed in the transformed *sl* cells. CC, condensed cytoplasm. (*C*) Condensed nucleus in the transformed *sl* cells. CN, condensed nucleus. (*D*) Condensed and fragmented chromatin found in the nucleus of a dead *sl* cell transformed with 35S-*OsRac1*-G19V. (*E*) Blebbing of the plasma membrane found in the transformed *sl* cells. (*F*) Intact mitochondrial membrane (M) in a dead, transformed *sl* cell.

Dominant Negative *OsRac1* Blocks H_2O_2 Production and Cell Death in Transgenic Lesion-Mimic Mutants. To examine further the role of *OsRac1* in ROS production and cell death in rice, we produced a dominant negative form of *OsRac1* by changing threonine at the 24th position to asparagine (Fig. 1*A*), fused with the 35S promoter, and introduced it into the wild type and the *sl* mutant. In untransformed *sl* cell cultures H_2O_2 production was induced by calyculin A (CA), a protein phosphatase inhibitor, and the induction was inhibited by DPI (Fig. 5*A*), as shown previously with other lesion-mimic mutants of rice (25). In contrast, the CA-induced H_2O_2 production was inhibited in the *sl* cells transformed with *OsRac1*-T24N, indicating that *OsRac* was required for activation of H_2O_2 production in the *sl* cells. To examine whether the dominant negative *OsRac1* suppresses lesion formation in *sl* plants, leaves of untransformed and transformed *sl* plants were inoculated with the rice blast fungus, *M. grisea*. It was shown previously that infection of the *sl* leaf with a blast fungus rapidly induced characteristic lesions distinct from the blast lesion (28, 29). Lesion formation was inhibited strongly in transformed plants (Fig. 4*B*), and the mean area of lesions developed in leaves of four independent transgenic plants was 10–20% of

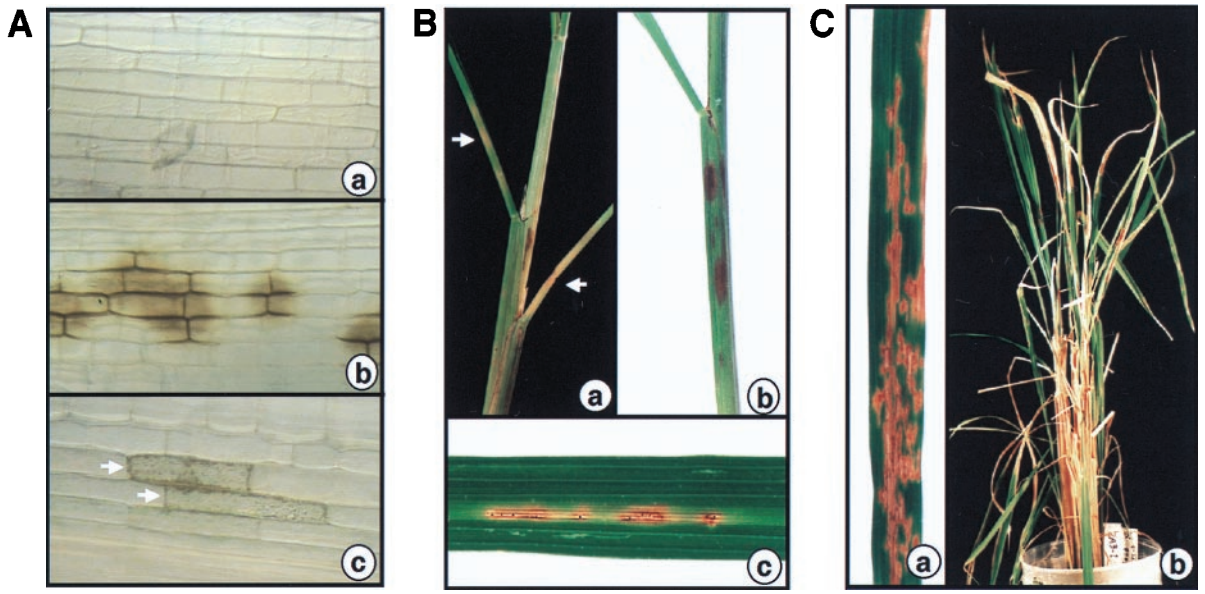

FIG. 4. Cell death and H_2O_2 production in transgenic rice plants transformed with 35S-*OsRac1*-G19V. (*A*) H_2O_2 production (*b*) and cytoplasmic granulation (*c*) in leaf sheath cells of transformed wild-type plants. (*a*) Untransformed plant. (*B*) Cell death in wild-type plants transformed with 35S-*OsRac1*-G19V. Discrete lesions (cell death) were observed at the junction of the blade and the sheath of the leaf (*a*), part of the leaf blade close to the spikelet (*b*), and the midrib region (*c*). (*C*) Cell death in transformed *sl* plants. (*a*) Lesions that showed the size and color characteristics of the Sekiguchi lesion. (*b*) Lesions developed over the entire plant.

that found in the untransformed *sl* mutant (Fig. 5 *B* and *C*). Transgenic wild-type plants expressing *OsRac1*-T24N showed no apparent phenotypic effects (data not shown). These results strongly suggest the role of *OsRac* in the induction of H_2O_2 production and cell death in the *sl* mutant.

Concluding Remarks. Results of our investigations suggest that the small GTP-binding protein Rac regulates the ROS production in rice most likely through a NADPH oxidase, although existence of other routes for ROS production in plants has been suggested (38). Our results also suggest the conservation of the role of Rac to regulate ROS production between plants and animals. They also show that Rac induces cell death in rice cells and that it has biochemical and morphological features similar to apoptosis in mammalian cells. However, the failure to observe laddering or degradation of isolated nuclear DNA in transgenic rice cells might indicate the existence of multiple types of cell death in plants (3, 5). The present findings support a model in which ROS produced by a DPI-inhibitable NADPH oxidase triggers cell death during the defense response of plants (5, 6). Whether the Rac-induced cell death in rice plants has a role in disease resistance remains to be investigated. Analysis of disease resistance in Rac transformants should clarify whether cell death has a causal role in disease resistance in rice. ROS appear to activate cell death in mammalian cells under some circumstances (39). Thus, plants and animals may have in common certain signals for cell death.

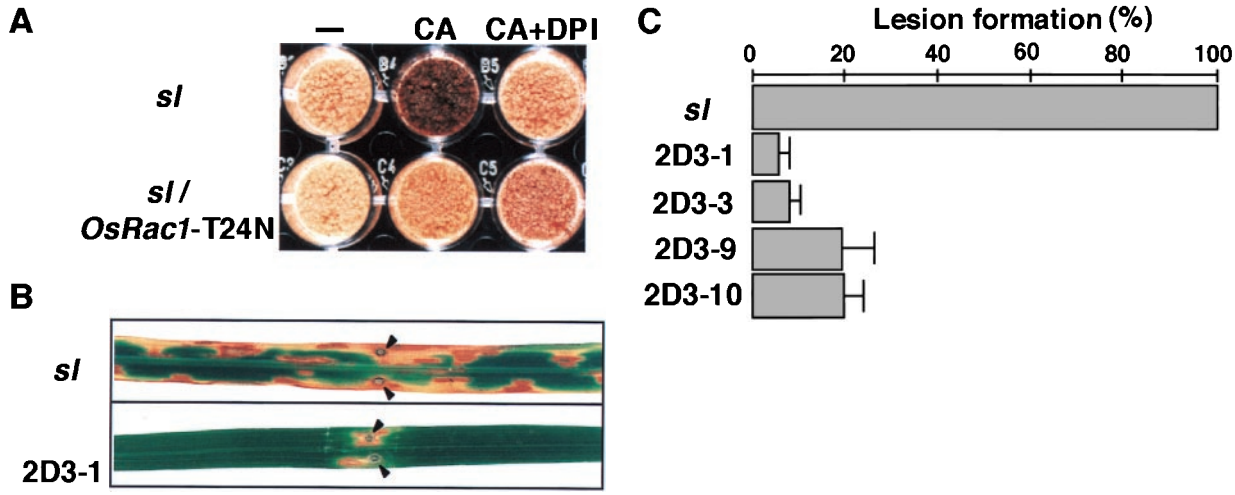

FIG. 5. Suppression of H_2O_2 production and cell death in the *sl* rice transformed with the dominant negative *OsRac1*. (*A*) Inhibition of calyculin A-induced H_2O_2 production in *sl* cells transformed with 35S-*OsRac1*-T24N. Calyculin A (2 μM; Wako) was added to the cell suspensions 30 min before DAB staining. (*B*) Inhibition of lesion formation in the leaf of the *sl* plants transformed with 35S-*OsRac1*-T24N. Inoculation of leaves with the blast fungus was performed on press-injured spots made by a specially designed pressing machine (Fujihara, Osaka, Japan). A piece of agar covered with spores was placed on the injured spots (37). A strain of *Magnaporthe grisea*, TH67–22 (race 031), which is incompatible with cv. Kinmaze, was used in this experiment. Arrows indicate the sites of inoculation. (*C*) Quantitative assessment of lesion formation in the leaf of the *sl* plants transformed with 35S-*OsRac1*-T24N. Areas of lesions developed were measured with three to five leaves for each transgenic *sl* plant at 7 days after inoculation.

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