RACK1 Functions in Rice Innate Immunity by Interacting with the Rac1 Immune Complex

Ayako Nakashima,a,1 Letian Chen,a,1 Nguyen Phuong Thao,a Masayuki Fujiwara,a Hann Ling Wong,a Masayoshi Kuwano,b Kenji Umemura,c Ken Shirasu,d,2 Tsutomu Kawasaki,a and Ko Shimamotoa,3

a Laboratory of Plant Molecular Genetics, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma 630-0192, Japan
b Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma 630-0192, Japan
c Agricultural and Veterinary Laboratories, Meiji Seika Kaisha, Kohoku-ku, Yokohama 222-8567, Japan
d Sainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, United Kingdom

A small GTPase, Rac1, plays a key role in rice (Oryza sativa) innate immunity as part of a complex of regulatory proteins. Here, we used affinity column chromatography to identify rice RACK1 (for Receptor for Activated C-Kinase 1) as an interactor with Rac1. RACK1 functions in various mammalian signaling pathways and is involved in hormone signaling and development in plants. Rice contains two RACK1 genes, RACK1A and RACK1B, and the RACK1A protein interacts with the GTP form of Rac1. Rac1 positively regulates RACK1A at both the transcriptional and posttranscriptional levels. RACK1A transcription was also induced by a fungal elicitor and by abscisic acid, Jasmonate, and auxin. Analysis of transgenic rice plants and cell cultures indicates that RACK1A plays a role in the production of reactive oxygen species (ROS) and in resistance against rice blast infection. Overexpression of RACK1A enhances ROS production in rice seedlings. RACK1A was shown to interact with the N terminus of NADPH oxidase, RAR1, and SGT1, key regulators of plant disease resistance. These results suggest that RACK1A functions in rice innate immunity by interacting with multiple proteins in the Rac1 immune complex.

INTRODUCTION

Plants have a number of defense mechanisms to protect them from infection by nematodes, fungi, bacteria, and viruses (Chisholm et al., 2006; Jones and Dangl, 2006). Two systems of plant disease resistance have been extensively studied: pathogen-associated molecular pattern (PAMP)–triggered innate immunity (PTI) and effector-triggered immunity. PTI is induced by recognition of PAMPs, or elicitors, produced by pathogens. The classes of PAMP molecules that stimulate plant defense responses are similar to those that stimulate nonspecific immunity in mammals and include lipopolysaccharides, chitin, and transglutaminase (Zipfel and Felix, 2005; Chisholm et al., 2006). Receptors for plant PAMPs have recently been identified (Zipfel, 2006).

Effector-triggered immunity is specifically induced by the interaction of disease resistance (R) proteins and cognate effectors produced by pathogens. This type of resistance is characterized by hypersensitive cell death in infected plants (Chisholm et al., 2006; Jones and Dangl, 2006). Recognition of specific effectors by nucleotide binding Leu-rich repeat (NB-LRR)–type R proteins, the major class of R proteins, can be either direct or indirect. In an indirect recognition, plant proteins other than R protein are used as sensors for the entry of protein effectors into plant cells (Chisholm et al., 2006). In Arabidopsis thaliana–Pseudomonas syringae interactions, resistance signaling is initiated by changes that occur in host protein RIN4 that are caused by interaction with effectors (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003). The changes in RIN4 are recognized by R proteins, leading to activation of resistance signaling. Direct interactions between effectors and NB-LRR proteins have been observed between the rice (Oryza sativa) NB-LRR protein Pi-ta and its corresponding effector (Jia et al., 2000) and between flax R protein and effectors from flax rust fungus (Dodds et al., 2006).

Neither the mechanisms that allow direct or indirect recognition of effectors nor the mechanisms by which NB-LRR proteins are activated by effectors are known. It is also not known how the immune response is initiated by activated R proteins. R proteins are imported into the nucleus where they are apparently active, suggesting that R proteins may function at multiple sites in the cytoplasm and the nucleus (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007).

Rac/Rop small GTPases constitute the sole group of Rho family small GTPases in plants and have diverse functions in many important cellular activities, such as polar growth, cell differentiation, and stress responses (Yang and Fu, 2007). The roles of the Rac/Rop small GTPases in plant innate immunity have been studied in rice, barley (Hordeum vulgare), and other...
species (Gu et al., 2004; Assmann, 2005; Berken, 2006; Nibau et al., 2006). Rice and Arabidopsis contain seven and 11 Rac genes, respectively (Yang, 2002; Miki et al., 2005), and Os Rac1 is involved in basal and R protein–mediated resistance to rice blast fungus (Magnaporthe grisea) and bacterial blight (Ono et al., 2001; Suharsono et al., 2002). Os Rac1 is involved in the immune response induced by N-acetylchitooligosaccharide and sphingolipids, which act as PAMPs (Ono et al., 2001; Suharsono et al., 2002).

In rice, Rac1 regulates cell death, reactive oxygen species (ROS) production, activation of pathogenesis-related gene expression, and phytoalexin production (Kawasaki et al., 1999). It also regulates lignin production by interacting with and activating cinnamoyl CoA reductase (Kawasaki et al., 2006). Furthermore, PAMP-induced activation of rice MAPK6 requires Rac1, and MAPK6 coimmunoprecipitates with Rac1 protein in rice cell extracts (Lieberherr et al., 2005). A proteomic analysis of proteins induced by the constitutively active (CA) form of Rac1 in cultured rice cells indicates that the majority of proteins induced by sphingolipid elicitors (SE) are also induced by CA-Rac1 (Fujiwara et al., 2006). Rac1 forms a complex with RAR1, HSP90, and HSP70, which are conserved components of plant innate immunity as part of its pathogen defense response (Thao et al., 2007).

The dominant-negative (DN) form of Rac1, the form that suppresses its endogenous activity, inhibits N gene–mediated resistance to tobacco mosaic virus infection in tobacco (Nicotiana tabacum; Moeder et al., 2005). Similarly, Rac/Rop GTPase is involved in ROS production in Arabidopsis (Park et al., 2000). Barley RacB is required for susceptibility to powdery mildew fungus (Schultheiss et al., 2002, 2003). Thus, Rac/Rop GTPases play important roles in innate immunity by regulating a number of downstream components of the immune response. However, how Rac/Rop GTPase acts in the early stages of plant innate immunity signaling is not well understood.

To further understand the role of Rac/Rop GTPase and the molecular components of rice innate immunity, we applied a proteomic approach to identify Rac1-interacting proteins in rice. Among the selected Rac1-interacting proteins, we selected RACK1, which has been extensively studied and has been shown to be involved in many cellular activities in animals. RACK1 interacts with many signaling proteins in animals (Schechtman and Mochly-Rosen, 2001; Yaka et al., 2002; Patterson et al., 2004; Lopez-Bergami et al., 2005) and based on its structure (McCahill et al., 2002) is considered to be a scaffolding protein in a number of signaling pathways. In addition to its signaling roles, it is associated with 80S ribosomes and is involved in translational regulation in yeasts and animals (Link et al., 1999; Ceci et al., 2003; Sengupta et al., 2004). Recently it was also shown that RACK1 is associated with cytosolic ribosomes in Arabidopsis (Chang et al., 2005; Giavalisco et al., 2005). Thus, it is evident that RACK1 plays multiple roles in the cellular activities of eukaryotes. We have previously shown that rice RACK1 plays a key role in the production of ROS and disease resistance and binds RAR1 and SGT1, two important regulators of plant innate immunity (Shirasu and Schulze-Lefert, 2003). Here, we show that RACK1 is positively regulated by Rac1 at both the transcriptional and posttranscriptional levels, that it interacts with other proteins in the Rac1 immune complex, and that it is involved in resistance to rice blast infection.

RESULTS
Isolation of RACK1 by Rac1 Affinity Column Chromatography

To understand the molecular mechanisms of disease resistance induced by Rac GTPase, an affinity method successfully used for the isolation of Rho GTPase-interacting proteins in mammals (Amano et al., 1996) was adapted to isolate Rac1-interacting proteins using GST-Rac1 affinity chromatography. A simplified methodology for Rac1 affinity column chromatography is shown in Figure 1A. Affinity columns were loaded with GST, GDPβS-GST-Rac1, or GTPγS-GST-Rac1 (GTPγS is a nonhydrolyzable GTP analog), and protein extracts obtained from rice cell cultures treated with a sphingolipid elicitor were passed through the columns. To obtain proteins with various functions, both GTP and GDP forms of Rac1 were used. Bound proteins were eluted with 500 mM NaCl (Figure 1B). A total of 21 proteins were identified by mass spectrometry (Table 1; see Supplemental Tables 1 to 3 online), of which seven may play a role in rice innate immunity (Table 1). Five were identified as NBLR-type proteins, which are the largest class of plant R proteins (Dangl and Jones, 2001). In addition, proteins homologous to RACK1 and stress-inducible protein 1 (Sti1), which are involved in cellular signaling in animals and other organisms were found. Sti1 (also known as Hop in mammalian cells) is a cochaperone for the HSP90 and HSP70 chaperones (Pratt and Toft, 2003) and has three tetra-tricopeptide repeat domains (Smith, 2004). None of these identified proteins were previously shown to interact with Rac/Rop GTPase. We chose RACK1 for further study since its role in plant innate immunity has not been studied in any system.

Rac1 Interacts with WD Repeats 1 and 2 of RACK1A

RACK1 was originally isolated as a receptor for activated C-kinase 1, and it has homology with the G protein β-subunit (Ron et al., 1994). RACK1 contains seven WD40 repeats and serves as an adaptor protein by binding with protein kinases, phosphatases, transcription factors, and membrane receptors in various mammalian signaling pathways (Schechtman and Mochly-Rosen, 2001; Yaka et al., 2002; Patterson et al., 2004; Lopez-Bergami et al., 2005). In rice, a RACK1 homolog was isolated as a homolog of Gβ and designated RWD (Iwasaki et al., 1995), but its function has not been studied. Rice has two copies, and Arabidopsis has three copies of the RACK1 homologs (Chen et al., 2006). To follow the nomenclature used in Arabidopsis (Chen et al., 2006), we have used RACK1A and RACK1B for the two rice RACK1 genes in this article. RACK1A and RACK1B share 82% amino acid identity (Figure 2A). Rac1 affinity chromatography identified RACK1A but not RACK1B (Figure 2A).

Since rice RACK1A was isolated from the affinity columns bound with both GDPβS-GST-Rac1 and GTPγS-Rac1, we examined interactions of RACK1A and RACK1B with Rac1 by yeast two-hybrid assays (Figure 2B). The CA form of Rac1 could bind
both RACK1A and RACK1B, but RACK1A interacted with CA-Rac1 with higher affinity (Figure 2B), consistent with the finding that only RACK1A was isolated using affinity chromatography (see boxed peptides in Figure 2A). The DN form of Rac1 did not interact with RACK1A or RACK1B, indicating that only RACK1A is an effector of Rac1 and thus may be involved in Rac GTPase signaling in rice. Analysis using various fragments of RACK1A showed that WD40 repeats 1 and 2 interacted with Rac1 (Figure 2C). RACK1A also binds CA-Rac1 with higher affinity than RACK1B, as measured using WD40 repeats 1 and 2 (Figure 2D). The other six Rac genes present in the rice genome (Miki et al., 2005) were also tested for interactions with RACK1A using the split ubiquitin yeast two-hybrid system (Kim et al., 2002) that detects interaction at the periphery of the plasma membrane. In addition to Rac1, CA forms of Rac3 and Rac6 clearly interacted with RACK1A, and Rac5 and Rac7 weakly interacted with RACK1A (Figure 2E). Rac4 did not interact with RACK1A.

Intracellular Localization of RACK1A

To test whether Rac1 associates with RACK1A in rice cell extracts, we performed coimmunoprecipitation experiments using extracts from transgenic rice cell cultures expressing myc-CA-Rac1, myc-DN-Rac1, or myc-C212S-Rac1 (a construct in which the C-terminal Cys was exchanged with Ser to block the plasma membrane localization of myc-CA-Rac1), all under the control of the constitutive maize (Zea mays) Ubiquitin promoter (Lieberherr et al., 2005). The RACK1 antibody used in this study preferentially recognized RACK1A (see Supplemental Figure 1 online). RACK1A coimmunoprecipitated with myc-CA-Rac1 but not with DN or CS mutant Rac1 protein, indicating that RACK1A associates with the GTP form of Rac1 in rice cell extracts (Figure 3A), consistent with the yeast two-hybrid assay results. Endogenous Rac1 could not be immunoprecipitated with Rac1-specific antibody (Lieberherr et al., 2005) (data not shown).

Table 1. Proteins Identified by GST-Rac1 Affinity Column Chromatography

<table>
<thead>
<tr>
<th>MW</th>
<th>Homologous Protein</th>
<th>GTP/GDP</th>
<th>Accession Number</th>
<th>Mascot Score</th>
<th>No. of Peptides</th>
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<tr>
<td>p230</td>
<td>NB-LRR protein</td>
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<td>gi 115443833</td>
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<td>2</td>
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<tr>
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<td>gi 14589374</td>
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<tr>
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<td>gi 115447567</td>
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<td>2</td>
</tr>
<tr>
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<td>WD repeat–containing receptor of activated C kinase 1</td>
<td>GTP</td>
<td>gi 1346109</td>
<td>162</td>
<td>3</td>
</tr>
</tbody>
</table>

a GTP and GDP indicate affinity columns bound to GTP-Rac1 and GDP-Rac1, respectively, identified in the corresponding protein.

b Numbers of matching peptides identified in the amino acid sequence of assigned protein.
To determine the intracellular localization of RACK1A in rice cells, we isolated proteins from the cytosolic and microsomal fractions of rice cell cultures expressing CA, DN, and the C212S mutant form of Rac1 and immunoblotted with anti-RACK1A antibody (Figure 3B). RACK1A protein was found in both cytosolic and microsomal fractions of the wild type and in the three mutant transgenic cell culture lines. RACK1A–green fluorescent protein (GFP) fluorescence was mainly detected in the cytoplasm (Figure 3C). RACK1-GFP localization in rice cells is similar to mammalian cells (Parent et al., 2008).

**Figure 2.** Interaction of RACK1 and Rac1 in Yeast Two-Hybrid Assays.

(A) Alignment of the amino acid sequences of rice RACK1A and RACK1B. The peptide fragments identified by mass spectrometry are boxed. Asterisks represent amino acids conserved in RACK1A and RACK1B.

(B) Interaction of RACK1A and RACK1B with CA- and DN-Rac1 in yeast two-hybrid assays using a LexA-VP16 system. This two-hybrid system detects the protein–protein interaction in nuclei. Since the C-terminal Cys of Rac1, which is required for plasma membrane localization, was considered to inhibit transfer of Rac1 into nuclei, the Cys residue was exchanged with Ser. Growth on selective plates without His (−H) or without His plus 3-aminotriazole (3AT) indicates a positive interaction.

(C) Determination of specific WD40 repeats that interact with Rac1 using yeast two-hybrid assays.

(D) Difference of binding activities of RACK1A and RACK1B to Rac1.

(E) Interaction of RACK1A and the CA form of Rac family proteins in split-ubiquitin two-hybrid assays. Growth on selective plates with 5-fluoroorotic acid (5-FOA) indicates a positive interaction.

**RACK1A Expression Is Induced by an Elicitor and Plant Hormones in Rice Cell Cultures**

To study the function of RACK1A, its expression was studied in wild-type and transgenic rice cell cultures expressing CA-Rac1 (Figure 4). RACK1A was induced in CA-Rac1 cell cultures by an SE (Figure 4A) that has been previously shown to induce a variety of defense responses in rice (Koga et al., 1998; Suharsono et al., 2002). However, there was no induction of RACK1A expression by SE in wild-type cell cultures (Figure 4A). These results show that RACK1A expression is induced by an elicitor in CA-Rac1 cell
cultures but not in wild-type cell cultures, suggesting that RACK1A expression may be positively regulated by the elicitor and active Rac1.

Since T-DNA insertion mutants of RACK1A, one of three RACK1 homologs in Arabidopsis, were shown to have altered sensitivity to multiple hormones (Chen et al., 2006), we tested the effects of plant hormones on RACK1A expression in cell culture. Methyl jasmonate, indole-3-acetic acid (IAA), and abscisic acid (ABA) all induced RACK1A expression in wild-type cell cultures (Figures 4B to 4D), suggesting that RACK1A expression is not only induced by an elicitor but phytohormones in rice cell culture. The mechanisms of RACK1 induction appear to be different, since RACK1 induction by the three phytohormones did not

Figure 3. Intracellular Localization of RACK1A.

(A) RACK1A communoprecipitates with Rac1 in a GTP-dependent manner. Total protein extracts from transgenic cell cultures expressing Rac1 mutant proteins (CA, DN, or C212S) were incubated with anti-myc antibody and protein A Sepharose beads. Precipitates were washed, collected by centrifugation, and separated by SDS-PAGE. Total extracted and immunoprecipitated samples from nontransformed (NT) cell cultures were used. Immunoblot analyses were performed with anti-RACK1A and antitubulin antibodies. RACK1A was detected in CA immune complexes but not in DN or C212S-Rac1.

(B) Intracellular localization of RACK1A. Soluble and microsomal membrane fractions were prepared from transgenic Rac1 cell cultures and subjected to immunoblot analysis. PIP is a marker for the plasma membrane. The bottom panel shows protein staining by CBB (Coomassie blue).

(C) Localization of RACK1A-GFP in rice protoplasts. Bars = 5 μm.

Figure 4. RACK1A mRNA Expression Is Induced by SE and Plant Hormones.

(A) Transcript levels for RACK1A were measured by real time RT-PCR in nontransgenic rice cell cultures (NT) and transgenic cell cultures expressing CA-Rac1 after treatment with SE.

(B) to (D) Transcript levels for RACK1A were measured by real time RT-PCR in nontransgenic rice cell cultures with plant hormones jasmonic acid (B), IAA (C), and ABA (D). Real time RT-PCR data were normalized to Ubiquitin (Ubq) transcript and shown as relative to that of RACK1A transcript in nontransgenic rice cell cultures without any treatments. Bars represent the means ± SD calculated using three biological replicates, where each biological replicate consists of two technical replicates.
require the presence of active Rac1, whereas the elicitor response did.

RACK1A Regulates ROS Production and Defense Gene Expression in Rice Cell Culture

To examine the possible functions of RACK1A in rice, RACK1A-RNA interference (RNAi) cell cultures were produced in which RACK1A was specifically suppressed by the target gene 3' untranslated region (Figure 5A). Though RACK1B RNA was not suppressed, RACK1 protein levels were strongly reduced, suggesting that anti-RACK1A antibody did not appreciably detect RACK1B protein (Figure 5B; see Supplemental Figure 1 online). No plants could be generated from >50 independently transformed RACK1A-RNAi calli. Shoots were occasionally obtained from transformed calli, but they became necrotic and did not grow further, suggesting that RACK1A has critical functions for the regeneration of plants from rice callus. Therefore, analysis of RACK1A function was limited to RACK1A-RNAi cell cultures.

Expression of RACK1A is a positive regulator of ROS production in rice. In three RACK1A-RNAi cell cultures were produced in which RACK1A mRNA, which in turn induce various defense responses, was not transcribed (Figure 6G), consistent with the results obtained from RACK1A-RNAi cell cultures tested (Figure 5D). These results indicate that RACK1A contributes to ROS production and defense gene expression in rice cell cultures.

RACK1A Plays a Role in Resistance to Rice Blast Infection

Transgenic rice plants overexpressing RACK1A (RACK1A ox; Figures 6A and 6B) had increased resistance to a compatible race of rice blast fungus compared with the wild type (Figures 6C and 6D). Since RACK1A-RNAi plants could not be regenerated, leaky-but-viable RACK1A mutants may be needed to test the role of RACK1A in incompatible interactions. Levels of Rac1 and PBZ1 mRNA were also much higher than in the wild type in the absence of pathogen infection (Figures 6E and 6F). These results suggest that RACK1A overexpression increases levels of Rac1 mRNA, which in turn induce various defense responses, including increased PBZ1 expression, leading to increased resistance to rice blast infection.

To test whether RACK1A overexpression influences ROS production in seedlings, T1 seedlings of RACK1A ox plants were stained with nitroblue tetrazolium (NBT), a pale yellow dye that becomes dark blue when reduced by free radicals and is used to detect superoxide production. There was more intense NBT staining in the scutellum and roots than in nontransformed controls (Figure 6G), consistent with the results obtained from RACK1A-RNAi cell cultures (Figure 5). It is thus likely that RACK1A plays a role in rice innate immunity, including as a regulator of ROS production in rice.

Rac1-RNAi Decreases Levels of RACK1A Protein and PBZ1 Expression in Rice Cell Cultures

Our analysis of RACK1A indicates that it functions in ROS production and innate immunity in rice, and it is possible that RACK1A function in rice innate immunity is dependent on Rac1. These observations prompted an analysis of the influence of Rac1 on RACK1A transcription and translation using Rac1-RNAi cell cultures (Miki et al., 2005). As reported previously, Rac1 transcription was decreased in the Rac1-RNAi cell cultures (Figure 7A), and, interestingly, RACK1A transcription was also significantly lower (Figure 7B). Furthermore, RACK1A protein levels were reduced to a barely detectable level, suggesting that Rac1 also regulates RACK1 posttranscriptionally. PBZ1 expression was also reduced in Rac1-RNAi cell cultures (Figure 7D). Together, these results indicate that RACK1A expression is positively regulated by Rac1 at both the transcriptional and translational levels, which would make Rac1 a key regulator of its effector, RACK1A.

RACK1A Interacts with RAR1 and SGT1 and the N Terminus of Rboh

In Arabidopsis and other species, RAR1 and SGT1 play critical roles in R protein–mediated disease resistance (Shirasu and Schulze-Lefert, 2003). Recent studies indicate that they may function as molecular chaperones to stabilize NB-LRR type R proteins (Bieri et al., 2004; Holt et al., 2005; Leister et al., 2005; Azevedo et al., 2006), though their biochemical functions are not well understood. It was recently found that RAR1 and HSP90 complex with Rac1 and are involved in rice innate immunity (Thao et al., 2007), suggesting that RAR1, SGT1, and HSP90 could also form a complex with RACK1A. Therefore, we examined whether RACK1A directly interacts with RAR1, SGT1, and HSP90. Results of two-hybrid assays indicate that RACK1A interacts with RAR1 and SGT1 but not with HSP90 (Figure 8A). These results were confirmed by split ubiquitin two-hybrid assays in yeast (see Supplemental Figure 2 online). We further analyzed the interaction of RACK1A with RAR1 and SGT1 by dissecting RACK1A. We found that both RAR1 and SGT1 interacted with WD40 repeats 1 and 2 (Figure 8B), Rice RAR1 and SGT1 interact with each other (Wang et al., 2008), as has been shown in other plants (Azevedo et al., 2002; Liu et al., 2002).

To determine whether Rac1 could interact with SGT1 and Rac1 in rice cell extracts, we performed communoprecipitation experiments using wild-type and Rac1-RNAi cell cultures (Thao et al., 2007). RACK1A communoprecipitated with Rac1, but only a very faint band was detected in the control experiments using Rac1-RNAi cell cultures, confirming that Rac1 interacts with RACK1A (Figure 8C). Similar results were obtained for SGT1 (Figure 8D), though we were not able to use SGT1-RNAi cell cultures (Thao et al., 2007), presumably because rice has a single SGT1 gene and SGT1-RNAi cell cultures were nonviable. These results showed that both SGT1 and Rac1 interact with RACK1A in rice cells.

Because we have recently shown that Rac1 directly interacts with the N terminus of the NADPH oxidase (Rboh) and stimulates ROS generation (Wong et al., 2007), we tested whether RACK1A interacts with the N terminus of rice RbohB in split ubiquitin two-hybrid assays. The results indicate that they interact with each other (Figure 8E). We tested whether interaction of RACK1A with RbohB is influenced by Rac1 and found that neither the CA nor the DN form of Rac1 affects interaction of RACK1A and RbohB (Figure 8E). Full-length RbohB did not interact with RACK1A,
suggesting that the C terminus of RbohB has an inhibitory effect
on the interaction (see Supplemental Figure 3 online). Thus,
RACK1A functions in ROS production by directly interacting with
the N terminus of Rboh, and this interaction is not dependent on
Rac1.

DISCUSSION

Rac1 Affinity Column Chromatography

A method originally used for the isolation of interacting proteins
of the small GTPase Rho in mammals (Amano et al., 1996) was
modified to isolate Rac1-interacting proteins. A number of pro-
teins that could have roles in innate immunity were identified in
the screen, including NB-LRR proteins and Sti1 (Table 1). Affinity
column chromatography may thus be a promising method for the
isolation of protein interactors in plants, particularly for small
GTPases. We have previously used a yeast two-hybrid screen
with CA-Rac1 to isolate Rac1-interacting proteins and found that
cinnamoyl-CoA reductase (CCR) acts as an effector (Kawasaki
et al., 2006). However, no other important interactors were
identified in the previous yeast two-hybrid screen (K. Hasegawa
and K. Shimamoto, unpublished results). Therefore, with respect
to the efficiency of the identification of Rac1 effectors, affinity
column chromatography may be an alternative and superior
screen for interactors, though a direct and robust comparison of
the methods is difficult.

RACK1A Is a Key Effector of Rac GTPase

We show here that RACK1A specifically binds a GTP-bound form
of Rac1. RACK1A contributes to ROS production, defense gene
expression, and disease resistance; all of which are regulated by
Rac1 (Kawasaki et al., 1999). Thus, RACK1A is apparently an
important effector of Rac1 function in rice innate immunity.
Though numerous proteins have been identified as interactors
with RACK1 in mammalian cells (McCahill et al., 2002; Yaka et al.,
2002; Patterson et al., 2004; Lopez-Bergami et al., 2005; Liu
et al., 2007; Parent et al., 2008), Rho-type GTPase apparently is
not an interactor. By contrast, we have shown that Rac1 is a Rho
GTPase that does interact with RACK1 in rice. We have previ-
ously shown that CCR is an effector of Rac1 and that CCR has
increased enzymatic activity when bound to Rac1, thus stimu-

Figure 5. RACK1A-RNAi Rice Cell Cultures.

(A) Transcript levels for RACK1A and RACK1B were measured by RT-
PCR in RACK1A-RNAi rice cell cultures and nontransgenic rice cell
cultures (NT). Numbers 1 to 3 indicate independent RNAi transgenic
lines. Ubq is used as an internal control.

(B) Protein levels of RACK1A in RACK1A-RNAi cell cultures. RACK1A
protein levels were analyzed by immunoblotting with anti-RACK1A
antibody, and an antitubulin antibody was used as a loading control.

(C) Reduction of H$_2$O$_2$ induction by SE in RACK1A-RNAi rice cell
cultures. Numbers 1 to 3 indicate independent transgenic lines. Bars
represent the means ± SD of four independent experiments.

(D) Reduction of PBZ1 mRNA induction by SE in RACK1A-RNAi rice cell
cultures. Numbers 1 to 3 indicate independent transgenic lines. Tran-
script levels were normalized to Ubq transcript. Bars represent the
means ± SD calculated using three biological replicates, where each
biological replicate consists of two technical replicates.
Figure 6. RACK1A-Overexpressing Rice Plants.

(A) Expression of RACK1A in T1 RACK1A-ox and in nontransgenic (NT) rice plants. RACK1A mRNA levels were measured by real time PCR. Numbers 1 to 4 indicate independent transgenic lines. Transcript levels were normalized to Ubq transcript. Bars represent the means ± SD calculated using three biological replicates, where each biological replicate consists of two technical replicates.

(B) Expression of RACK1A protein in T1 RACK1A-ox rice plants. Levels of RACK1A protein were measured by immunoblotting with anti-RACK1A antibody. Numbers 1 to 4 indicate independent transgenic lines.

(C) Increased resistance of T1 RACK1A-ox plants to infection by a compatible race of rice blast fungus 2 weeks after infection. Numbers 1 to 4 indicate independent transgenic lines.

(D) Quantitative analysis of increased resistance of T1 RACK1A-ox plants to infection by a compatible race of rice blast fungus. Numbers 1 to 4 indicate the independent transgenic lines shown in (C). Bars represent the means ± SD calculated using >15 independent inoculated regions for each line.

(E) Expression of Rac1 mRNA in T1 RACK1A-ox plants. Levels of Rac1 mRNA were measured by real-time RT-PCR. Numbers 1 to 4 indicate independent transgenic lines. Transcript levels were normalized to Ubq transcript. Bars represent the means ± SD calculated using three biological replicates, where each biological replicate consists of two technical replicates.

(F) Expression of PBZ1 mRNA in T1 RACK1A-ox transgenic rice in the absence of elicitor. Levels of PBZ1 mRNA were measured by real-time RT-PCR. Numbers 1 to 4 indicate independent transgenic lines. Transcript levels were normalized to Ubq transcript. Bars represent the means ± SD calculated using three biological replicates, where each biological replicate consists of two technical replicates.

(G) ROS production in T1 RACK1A-ox rice seedlings. T1 RACK1A-ox seeds were germinated for 5 d and stained with NBT for ROS production. Arrows indicate sites of increased ROS production. NT, nontransgenic control.
cell integrity, and vesicle trafficking. It can interact with multiple proteins, thus potentially functioning as a scaffold within protein complexes. Our study shows that RACK1A interacts with Rac1, RAR1, and SGT1; thus, it could act as a scaffolding protein in plants. These three proteins seem to interact with WD repeats.

Figure 7. RACK1A Expression in Rac1-RNAi Cell Cultures.
(A) Transcript levels for Rac1 were measured by real time RT-PCR in Rac1-RNAi rice cell cultures and nontransgenic rice cell cultures (NT). Numbers 1 and 2 indicate independent RNAi transgenic lines. Transcript levels were normalized to Ubq transcript. Bars represent the means ± SD calculated using three biological replicates, where each biological replicate consists of two technical replicates.
(B) Transcript levels for RACK1A were measured by real time RT-PCR in Rac1-RNAi rice cell cultures. Transcript levels were normalized to Ubq transcript. Bars represent the means ± SD calculated using three biological replicates, where each biological replicate consists of two technical replicates.
(C) Levels of RACK1A protein were measured by immunoblotting with specific antibodies. Numbers 1 and 2 indicate independent transgenic lines. NT, nontransgenic rice cell cultures. 1-2 indicates independent RNAi transgenic lines.
(D) Expression of PBZ1 in Rac1-RNAi rice cell cultures in the absence of elicitor treatment. Levels of PBZ1 mRNA were measured by real-time RT-PCR. Numbers 1 and 2 indicate independent transgenic lines. Transcript levels were normalized to Ubq transcript. Bars represent the means ± SD calculated using three biological replicates, where each biological replicate consists of two technical replicates.

Figure 8. Interaction of RACK1A with RbohB, RAR1, and SGT1 in Yeast Two-Hybrid Assays.
(A) Interaction of RACK1A with Rac1, SGT1, and RAR1 but not with cytosolic HSP90 in yeast two-hybrid assays. SGT1 and RAR1 are both single-copy genes in rice.
(B) Determination of the WD40 repeats of RACK1A that are involved in interaction with SGT1 and RAR1.
(C) RACK1A coimmunoprecipitated with RAR1 in extracts from wild-type rice cell cultures. A very faint band was detected in RAR1-RNAi cell cultures. SGT1-RNAi cell culture was nonviable since SGT1 is a single-copy gene in rice. Control SGT1-RNAi cultures were thus not available for the analysis.
(D) RACK1A coimmunoprecipitated with SGT1 in extracts from wild-type rice cell cultures. SGT1-RNAi cell culture was nonviable since SGT1 is a single-copy gene in rice. Control SGT1-RNAi cultures were thus not available for the analysis.
(E) Split-ubiquitin two/three-hybrid assays of RACK1A, RbohB-N, and Rac1. RbohB-N contains the N-terminal region (amino acids 1 to 355) of RbohB, which interacts with Rac1 (Wong et al., 2007). The bait and prey vectors were selected on plates with His (−H) and Trp (−T), respectively. The pDEST32 vectors carrying Rac1 were selected by removal of Leu (−L) from the plates. Growth on selective plates with 5-fluroorotic acid (5-FOA) indicates a positive interaction.
1 and 2 of RACK1A. RACK1A that is already bound with the active form of Rac1 may thus not be able to interact with RAR1 or SGT1. However, since RACK1 forms a homodimer (see Supplementary Figure 4 online) and potentially interacts with two different proteins by its recognition of the same motif (Thornton et al., 2004; Liu et al., 2007), RACK1A could possibly interact with two or three interactors simultaneously. Furthermore, WD repeats 1 and 2 of RACK1A could interact with distinct proteins. Whether RACK1A indeed interacts with multiple proteins simultaneously remains to be studied.

Many mammalian RACK1-interacting proteins interact with RACK1 on repeats 4, 5, 6, and 7 (McCahill et al., 2002), but the three intracellular loops of the thromboxane A2 receptor (TPβ) interact with RACK1 at WD40 repeats 1 to 3, and the C terminus of TPβ interacts with WD40 repeats 3 and 7 (Parent et al., 2008). As more plant proteins that interact with RACK1 are found, specific WD40 repeats that preferentially interact with other proteins will be clarified.

RACK1A Function in Rice Innate Immunity

Based on the results obtained in our study, two functions of RACK1 in rice innate immunity can be envisaged (Figure 9). One is that it constitutes a component of the Rac1 complex consisting of Rac1, RAR1, SGT1, HSP90, and HSP70 and functions as a scaffolding protein for the immune complex. We have previously postulated that all of these proteins could form a protein complex (Thao et al., 2007). The abundance of each of the cochaperones (RAR1, HSP90, and HSP70) present in the immune complex may need to be finely regulated to ensure a rapid and stable response to pathogen attack. However, whether these components are always present in a complex or whether some components associate with the complex only transiently remain to be studied. Another hypothesis is that RACK1A constitutes a component of the NADPH oxidase complex together with Rac1 and regulates ROS production at an early stage of immune responses since RACK1A interacts with the N-terminal region of NADPH oxidase (Rboh) (Figure 8E). How these functions of RACK1A are regulated or how its interactions with other proteins are temporally and spatially regulated after pathogen infection remain to be studied in the future (Figure 9).

One recent development in the study of plant innate immunity is that the NB-LRR type R proteins are translocated to the nucleus and function there in immunity (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007). Thus, it is possible that some components of the initial immune complex thought to be present at the periphery of the plasma membrane may move to the nucleus. In this regard RACK1 may be an interesting adaptor protein since it functions in various intracellular compartments in mammalian cells, including the plasma membrane, the nucleus, and the endoplasmic reticulum, and it is able to interact with cell surface receptors, protein kinases, transcription factors, and transcription-associated proteins (Schechtman and Mochly-Rosen, 2001; Yaka et al., 2002; Patterson et al., 2004; Lopez-Bergami et al., 2005; Liu et al., 2007; Parent et al., 2008). A possible function of RACK1 in plant immune responses may be that it facilitates translocation of components present in the immune complex, which is initially present at the periphery of the plasma membrane, to various subcellular compartments where it would stimulate or complete immune responses. Alternatively, as shown in the study of the effect of RACK1 on the TPβ receptor, it could function to transport membrane receptors from the endoplasmic reticulum to the cell surface (Parent et al., 2008). Studies on the function of RACK1A in various steps in innate immune responses will be the subjects of future studies.

Regulation of RACK1A Expression by Rac1

Our results indicate that Rac1 regulates RACK1A expression at the transcriptional as well as posttranscriptional levels (Figure 7B) and possibly vice versa (Figure 6E). We have previously found positive transcriptional regulation of RAR1 and SGT1 by Rac1 in rice (Thao et al., 2007). Rac1 exerts posttranscriptional regulation of RAR1 as it does on RACK1A. These results suggest that Rac1 regulates the expression levels of RACK1A, RAR1, and SGT1 in the rice immune complex and that this could be a means to balance abundance of components in the immune complex during defense responses.

RACK1 in Phytohormone and Immune Signaling

RACK1 homologs have been isolated from several plant species (Ishida et al., 1993; Iwasaki et al., 1995; McKhann et al., 1997; Perennes et al., 1999). However, in all previous studies, RACK1 expression patterns in different tissues and the effects of a variety of exogenous factors on expression have been the
principle focus. More recently, T-DNA insertion mutants of RACK1A, one of three RACK1 homologs in Arabidopsis, were shown to exhibit altered sensitivity to multiple hormones (Chen et al., 2006). Arabidopsis rack1a mutants showed reduced sensitivity to GA during seed germination and hypersensitivity to ABA during seed germination and early seedling growth. Furthermore, rack1a mutants are hypersensitive to auxin during root formation. Our analysis showed that RACK1A expression is induced by methyl jasmonate, ABA, and IAA (Figure 4).

Arabidopsis G protein complex mutant proteins are hypersensitive to ABA responses (Pandey et al., 2006). A recent proteomic study of the rice dwarf1 mutant lacking the Gx subunit of heterotrimeric G protein indicates that, in the embryo at least, RWD (RACK1A) protein levels are reduced compared with the wild type (Komatsu et al., 2005). Heterotrimeric G proteins regulate innate immunity through Rac1 (Suharsono et al., 2002) and hormone signaling (Fujisawa et al., 2001) in rice. Therefore, it is possible that RACK1A function in rice innate immunity may be partly mediated through heterotrimeric G proteins (Figure 9). A recent study on the role of yeast RACK1 in the regulation of heterotrimeric G proteins and Rho-type small GTPase may help to shed light on the signaling pathway involving these three groups of proteins (Zeller et al., 2007). The yeast RACK1 ortholog Asc1 was found to associate with one of the two Gx proteins in a GDP-dependent manner, indicating that it acts as a negative regulator of G protein signaling in response to glucose. Interestingly, yeast RACK1 has another role in glucose signaling. It inhibits the Cdc42-mediated MAPK pathway by suppressing Ste20 kinase, which is positively regulated by Cdc42 (Zeller et al., 2007). Therefore, in the G protein signaling of the glucose response in budding yeast, RACK1 suppresses Gx and Cdc42-regulated Ste20 kinase, two different proteins (Zeller et al., 2007). RACK1 may thus also be involved in both Rac/Rop-mediated innate immunity and hormone signaling.

METHODS

Affinity Chromatography and Mass Spectrometry

Rice (Oryza sativa) cells were homogenized in TEDM buffer (20 mM Tris/ HCl, pH 7.5, 1 mM DTT, 5 mM EDTA, and 10 mM MgCl2) containing 1 μg/ mL leupeptin and 10% sucrose and filtered through four layers of gauze. The homogenate was centrifuged at 6000 g for 30 min at 4°C to remove cellular debris, and the supernatant was clarified by centrifugation at 100,000 g for 60 min at 4°C. The soluble proteins were used for affinity chromatography. The GST-fused rice Rac1 protein was purified as described previously (Kawasaki et al., 2006). Guanine nucleotide-bound forms of GST-Rac1 were made by incubating purified protein for 1 h at 30°C with GDPβS or GTPγS (Sigma-Aldrich) in a reaction buffer (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 10 mM MgCl2, and 5 mM MgCl2). The GTPγS- or GDPβS-bound GST-Rac1 proteins were immobilized on glutathione Sepharose 4B beads (GE Healthcare). The soluble proteins were incubated with glutathione Sepharose 4B beads to remove the native rice GST proteins and then loaded onto a GST-Rac1 affinity column, washed with a TEDM buffer, and eluted three times with an elution buffer (20 mM Tris/HCl, pH 7.5, 10 mM EDTA, 1 mM DTT, 5 mM MgCl2, and 500 mM NaCl).

Eluted proteins were separated by 7.5 and 12% SDS-PAGE and stained with silver nitrate. The silver staining was performed without glutaraldehyde fixation as described by Shevchenko et al. (1996). Protein bands that interacted specifically with GTP- or GDP-bound Rac1 were excised from the gels, washed twice with HPLC-grade water containing 30% acetonitrile (Wako), washed with 100% acetonitrile, and dried in a vacuum concentrator. The dried gel pieces were treated with 2 μL of a 0.5 μg/μL trypsin (Promega) in 50 mM ammonium bicarbonate (Shevchenko, 2001) and incubated at 37°C for 16 h. The digested peptides in the gel pieces were extracted twice with 20 μL of 5% formic acid in 50% acetonitrile, combined, and dried in a Speedvac evaporator. Trypsin-digested peptides were separated by HPLC using a PEPMAP12 column (5 μm, 75-μm internal diameter, 15 cm; Dionex) in the CapLC system (Waters). Buffers were 0.1% HCOOH in water (A) and 0.1% HCOOH in acetonitrile (B). A linear gradient from 5 to 45% B for 25 min was applied, and peptides eluted from the column were introduced directly into a Q-TOF Ultima mass spectrometer (Waters) with a flow rate of 100 nL/min. Ionization was performed with a potential of 2200 V applied to a Picotip nanospray source (New Objective). For the survey scan, mass spectrometry (MS) spectra were acquired for mass-to-charge ratios of 400 to 1500, and MS/MS spectra were acquired for the two most intense ions from the precursor ion scan. For collision-induced dissociation, the collision energy was set automatically according to the mass and charge state of the precursor peptides. MS/MS spectra were uploaded to the MASCOT server (Matrix Science, version 2.0) and compared against a protein database (NCBI, 200304050) from the National Center for Biotechnology Information (NCBI). The NCBI’s taxonomy used was O. sativa. To remove weak matching peptides, the threshold was set at 0.05 in the ion score cut off during the MASCOT search. The following parameters were used for database searches: peptide tolerance at ±0.4 D and MS/MS tolerance at ±0.2 D; peptide charge of 2+ or 3+; trypsin as enzyme allowing up to one missed cleavage; carbamidomethylation on Cys residues as a fixed modification and oxidation on Met as a variable modification.

Yeast Two-Hybrid Assay Using a LexA-VP16 System

Bait vectors carrying CA and DN mutants of rice Rac1 have been described previously (Kawasaki et al., 2006). This two-hybrid system detects the protein–protein interaction in nuclei. Since the C-terminal Cys of Rac1, which is required for plasma membrane localization, was considered to inhibit transfer of Rac1 into nuclei, the Cys residue was exchanged with Ser. The bait and prey vectors containing different fragments of RACK1A were made using pBYM116 and pVP16, respectively (Kawasaki et al., 2006). Prey vectors containing rice SGT1, rice RAR1, and barley (Hordeum vulgare) HSP90 (Takahashi et al., 2003) were cloned into pVP16. Combinations of bait and prey vectors were introduced into cells of Saccharomyces cerevisiae L40. The interaction was analyzed based on the requirement of His for growth as described previously (Kawasaki et al., 2006).

Split-Ubiquitin Two/Three-Hybrid Assay

CA mutants of six members (Rac2 to Rac7) of the rice Rac family were produced by base substitution so that their amino acids corresponded to the CA mutant of Rac1. The resultant mutants were designated as Rac2-G16V, Rac3-G17V, Rac4-G17V, Rac5-G15V, Rac6-G15V, and Rac7-G15V. The coding regions of rice RACK1, RACK1B, and SGT1, CA-Rac1-7, and DN-Rac1 were cloned into the pMet-GWY-Cub-RUra3A bait vector or pCup-Nul-GWY-Cyc1 prey vector through the gateway system (Kim et al., 2002). Split-ubiquitin two-hybrid system assays were performed according to the published protocol (Kim et al., 2002). A split-ubiquitin three-hybrid system was modified from the split-ubiquitin two-hybrid system by introducing a third pDEST32 (Invitrogen) construct carrying Rac1 into yeast host strain DJ32. Yeast cells expressing two (bait and prey) or three (bait, prey, and pDEST32) constructs were grown to 5 × 10^6 cells/mL and spotted in serial dilutions on selective
agaroze plates with or without 5-fluoroorotic acid. At least three independent colonies were repeated for each two-hybrid pair.

**Plasmid Constructs and Rice Transformation**

To produce transgenic plants that overexpress RACK1A, the protein coding region was amplified by PCR using the primer set (forward, 5'-CACCCTATGCCCAGCAGAGTCT-3'; reverse, 5'-CTAGCGGCGTGACTGAAAACC-3'). The amplified fragment was cloned into the pENTR/D-TOPO cloning vector (Invitrogen) and transferred into the p2K1 vector by an LR clonase reaction. Expression of RACK1A is under the control of the maize (Zea mays) Ubp1 promoter (Miki and Shimamoto, 2004). For the RNAi construct of RACK1A, a 328-bp RACK1A cDNA region covering the C-terminal region (73-bp upstream of the stop codon) and a 255-bp fragment of the 3' untranslated region was amplified using the primer set (forward, 5'-CACCCCTATGCGGTGTTACAC-3'; reverse, 5'-AAAAAGAGAGAAGCACCATG-3') and cloned in inverse orientation into the pANDA vector (Miki and Shimamoto, 2004).

For the RNAi construct of RACK1A, a 451-bp cDNA fragment covering the C-terminal region (214-bp upstream of the stop codon) and a 234-bp fragment of the 3' untranslated region was amplified using specific primers (forward, 5'-CTGGAATGAGCCTAGGGTTTG-3'; and reverse 5'-GACAAGTCTCCACACACA-3'). The amplified fragment was cloned into pENTR/D-TOPO (Invitrogen) and subcloned into pANDA in the inverse orientation by one LR reaction.

**Elicitor Treatment, Real-Time RT-PCR, and Quantification of ROS**

Cultured rice cells expressing CA-Rac1, RACK1A, and RACK1A RNAi were collected after treatment with 5 μg/mL of an SE prepared from Magnaporthe grisea (Koga et al., 1998; Umemura et al., 2002). Total RNA was extracted using the RNAeasy plant mini kit (Qiagen) and treated with DNase I (Invitrogen). cDNA was synthesized from total RNA using Superscript II reverse transcriptase (Invitrogen) and used for quantitative analysis of gene expression performed with SYBR Green PCR master mix (Applied Biosystems) with the gene-specific primers listed in Supplemental Table 2 online. Data were collected using the ABI PRISM 7000 sequence detection system according to the instruction manual. Quantification of SE-induced hydrogen peroxide was performed as described previously (Wong et al., 2004). After a 24-h incubation at 30°C, the protoplasts were examined under a confocal microscope (LSM510; Carl Zeiss).

**Pathogen Inoculation**

Rice blast fungus (M. grisea) strain 2403-1 (race 007) is virulent and TH67-22 (race 031) is avirulent on cv Kinmaze. The growth conditions of blast fungus and punch infection of leaf blades were performed as described previously (Wong et al., 2004). Superoxides generated during seedling stages were detected by staining with 0.1% (w/v) nitroblue tetrazolium. Preinoculation of SE-induced hydrogen peroxide was performed as described previously (Wong et al., 2004). After a 24-h incubation at 30°C, the protoplasts were examined under a confocal microscope (LSM510; Carl Zeiss).

**Antibody, Immunoblot Analysis, and Coimmunoprecipitation Assays**

To produce His-tagged RACK1A protein, RACK1A cDNA was cloned into pET15b (Novagen). The recombinant protein was produced in Escherichia coli and purified with a HiTrap chelating HP column according to the manufacturer's instructions (GE Healthcare) to be used as an antigen to raise antibodies in rabbits. To purify the antibody, GST-fused RACK1A protein was prepared by cloning RACK1A cDNA into pGEX-4T-1 (GE Healthcare). A 10-mL aliquot of antiserum was incubated at 25°C for >4 h with a membrane containing bound recombinant GST-RACK1A protein. The membrane was then washed five times with Tris-buffered saline buffer (137 mM NaCl, 2.68 mM KCl, and 25 mM Tris, pH 7.4). RACK1A-specific antibody was eluted with 2 mL of 0.1 M glycine/0.15 M HCl, pH 2.5–3.0, and neutralized with 450 μL of 0.5 M HEPES/NaOH, pH 8.5. Coimmunoprecipitation and immunoblotting experiments were performed using transgenic rice cells expressing myc-CA-Rac1, myc-DN-Rac1, myc-C212S-Rac1, or RACK1A, and transgenic rice cells suppressing expression of Rac1 or RACK1A by RNAi. Rice cells were homogenized as above. Total proteins were prepared by removing insoluble debris by centrifugation at 2000g for 10 min at 4°C. Soluble and microsomal membrane proteins were separated by centrifugation at 100,000g for 1 h. The proteins were separated by SDS-PAGE and electrotransferred onto an Immobilon-P membrane (Millipore) for immunoblot detection. The membrane was blocked for 1 h in PBS (137 mM NaCl, 8.1 mM NaHPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄) containing 5% skim milk, washed three times with PBS, and incubated for 1 h with purified anti-RACK1A, anti-α-tubulin, anti-RAR1 (Thao et al., 2007), or anti-SGT1 antibodies (Azevedo et al., 2002). After washing with PBS containing 0.1% Tween, the membranes were incubated for 1 h with anti–rabbit IgG conjugated to horseradish peroxidase (GE Healthcare). Detection was performed using ECL protein gel blot detection reagents (GE Healthcare), Coimmunoprecipitation assays with anti-myc, anti-SGT1, or anti-RAR1 antibodies were performed as described previously (Lieberherr et al., 2005).

**Transient Expression in Rice Protoplasts**

The GFP sequence derived from sGFP-S65T (Chiu et al., 1996) was fused to the C terminus of RACK1A. Expression of the GFP control and RACK1A-GFP was driven by the maize ubiquitin promoter. Protoplast isolation and electroporation were performed as described previously (Wong et al., 2004). After a 24-h incubation at 30°C, the protoplasts were examined under a confocal microscope (LSM510; Carl Zeiss).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: Os Rac1 (AB029508), Os Rac2 (AB029509), Os Rac3 (AB029510), Os Rac4 (AK061102), Os Rac5 (AK067504), Os Rac6 (AK0100842), Os Rac7 (AK058414), Os RACK1A (D38231), Os RACK1B (AK121567), Os SGT1 (AAF18438), Os RAR1 (AK111981), PBZ1 (D38170), and barley HSP90 (AY325266).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** The anti-RACK1A Antibody Showed Much Lower Affinity for the RACK1B Protein.

**Supplemental Figure 2.** RAR1 and SGT1 Interact with RACK1A in a Split-Ubiquitin Two-Hybrid Assay.

**Supplemental Figure 3.** Interaction between RACK1A and RbohB in a Split-Ubiquitin Two-Hybrid Assay.

**Supplemental Figure 4.** Dimerization of RACK1A and RACK1B.

**Supplemental Table 1.** List of Proteins Identified by GST-Rac1 Affinity Column Chromatography.

**Supplemental Table 2.** MS Data of Identified Proteins.

**Supplemental Table 3.** List of Other Candidate Proteins.

**Supplemental Table 4.** Primers Used in This Study.
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Ayako Nakashima, Letian Chen, Nguyen Phuong Thao, Masayuki Fujiwara, Hann Ling Wong,
Masayoshi Kuwano, Kenji Umemura, Ken Shirasu, Tsutomu Kawasaki and Ko Shimamoto

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