Expression of OsWRKY71, a rice WRKY gene, was induced by biotic elicitors and pathogen infection. It was also found that OsWRKY71 has features characteristic of a transcriptional repressor. Microarray analysis revealed that several elicitor-induced defense-related genes were upregulated in rice cells overexpressing OsWRKY71. These results indicate that the activation of defense-related genes by OsWRKY71 was probably indirect.

Key words: defense response; elicitor; Oryza sativa L. cv. Nipponbare; WRKY

When plants are attacked by pathogens, they respond with multiple defense reactions, including synthesis of pathogenesis-related (PR) proteins and accumulation of phytoalexins.1) Plants recognize pathogen attack through perception of pathogen-derived elicitors, such as chitin oligosaccharide and fungal cerebroside elicitors, via specific receptors, activating defense genes through intracellular signaling cascades.1–3) In higher plants, transcriptional regulation of defense-related genes is considered to be central to induced disease resistance.

WRKY proteins form a large family of plant-specific transcriptional factors that specifically bind to the W-box elements (T)TGAC(C/T), and they appear to play a regulatory role in a variety of biotic and abiotic stress responses.4–6) They have been investigated extensively for their possible involvement in defense responses against attack by pathogens, mainly in dicotyledonous plants.7,8) The rice genome is predicted to contain over 100 WRKY (OsWRKY) genes.9) It has been found that several WRKY genes are expressed in response to the rice blast fungal elicitor10) and the defense signal molecules salicylic acid and jasmonic acid,11) and that OsWRKY13 and OsWRKY45 are involved in salicylic acid-mediated defense signaling in rice,12,13) but the biological functions of most of the WRKY factors in defense signaling in rice remain unknown.

We have identified OsWRKY53 and OsWRKY71 as chitin oligosaccharide elicitor-induced genes from suspension-cultured rice cells by microarray analysis.14) Recently, we reported that OsWRKY53 functions as a transcriptional activator in the defense responses in rice.15) With regard to OsWRKY71, when we started this study, its involvement in the regulation of α-amylase gene expression in aleurone cells had been reported.16) but the role of OsWRKY71 in defense responses was almost unknown. In this study, we found that OsWRKY71 has features characteristic of a transcriptional repressor, and that overexpression of OsWRKY71 in elicitor-induced defense-related genes. The roles of OsWRKY71 in elicitor-induced defense signaling pathways in rice are discussed below.

We isolated the full-length cDNA of OsWRKY71 from elicited suspension-cultured rice cells by RT-PCR using primers designed based on information from a rice genome database. Calli of Oryza sativa L. cv. Nipponbare were cultured as described previously.15) Four days
after transferring the calli to fresh culture medium, the rice cells were treated with 1 mM of the chitin oligosaccharide elicitor \(N\)-acetylchitooctaose, and were harvested after 1 h. Total RNA was extracted from the elicited rice cells using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s protocol. Reverse transcription was performed with SuperScript III RT (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, using the total RNA (1 \(\mu\)g). The nucleotide sequence of the \(OsWRKY71\) cDNA reported in this paper will appear in the DNA Data Bank of Japan (DDBJ) nucleotide sequence database under accession no. AB190817. All known WRKY proteins contain one or two WRKY domains and are classified based on both the number of WRKY domains and the features of their zinc finger-like motifs. \(^4\) \(OsWRKY71\) belongs to group II, which is characterized by one WRKY domain, which contains the Cys\(_2\)His\(_2\) zinc finger-like motif.

To investigate the involvement of \(OsWRKY71\) in the...
defense response to pathogen infection, we examined its expression using Northern hybridization. The elicitor-induced changes in the steady-state levels of *OsWRKY71* mRNA in suspension-cultured rice cells are shown in Fig. 1A. The mRNA level peaked 0.5 h after the addition of each elicitor (chitin oligosaccharide and fungal cerebroside elicitors), and decreased gradually thereafter. We also analyzed the expression pattern of *OsWRKY71* in rice plants (cv. Nipponbare) inoculated with the fungal pathogen *M. grisea* race 007, which is compatible with cv. Nipponbare. Northern hybridization revealed a significant increase in the level of *OsWRKY71* mRNA at 6 h after inoculation, after which it decreased gradually (Fig. 1B). These results strongly suggest that *OsWRKY71* is involved in the basal defense responses of rice against pathogen infection.17) We also confirmed that sGFP:OsWRKY71 fusion protein localized in the nuclei of onion epidermal cells, and that the recombinant OsWRKY71 protein specifically binds to the double stranded synthetic W-box probe BS65,15) by GMSA analysis (data not shown), suggesting a possible function of OsWRKY71 as a transcriptional regulator.

To determine whether OsWRKY71 acts as either an activator or a repressor of gene transcription, we constructed an effector plasmid that contained the CaMV 35S promoter driving a gene that encodes a fusion protein of the DNA-binding domain of the yeast transcriptional activator GAL4 (GAL4 DB) and full-length OsWRKY71 (Fig. 1C). This plasmid, or a control plasmid encoding GAL4 DB alone, were cotransfected into rice cells along with the reporter plasmid 35S-GAL4-TATA-LUC-NOS by particle bombardment, as previously described.15) Compared with rice cells expressing GAL4 DB alone, the level of expression of the reporter gene was reduced by 90% when the effector that encodes full-length OsWRKY71 was co-expressed with the reporter plasmid (Fig. 1C). Similarly, when the effector plasmid GAL4 DB-SRDX, which encodes a chimeric repressor,19) was co-expressed with the reporter plasmid, the level of expression of the reporter gene was reduced by 75% (Fig. 1C). Thus we demonstrated that OsWRKY71 has transrepression potential. This is supported by the result reported by Liu et al. that OsWRKY71 protein did not show transactivation activity in a yeast activation activity assay.20)

To gain clues to understanding the roles of OsWRKY71 in defense responses in rice, we attempted comprehensively to identify defense-related genes whose expression is altered in OsWRKY71-overexpressing transgenic rice cells. For this purpose, we decided to use the Agilent Rice Oligo DNA Microarray (Agilent Technologies, Palo Alto, CA). To identify the genes upregulated in the OsWRKY71-overexpressing transgenic rice cells, three independent OsWRKY71-overexpressing transgenic lines (lines A, C, and E in Fig. 2A) were used in each experiment. Total RNA was used to prepare Cy5- and Cy3-labeled cRNA probes. Sample amplification, labeling, and hybridization were performed following the protocol recommended by Agilent Technologies. Genes showing a signal value below 500 in the Cy3 channel of the control cells were excluded from analysis. Feature extraction and image analysis software (version A.6.1.1, Agilent Technologies) were used to locate and delineate each spot in the array and to integrate each spot’s intensity, filtering, and normalization.

As the results of microarray analysis, a total of 64 genes were down-regulated, and 21 genes of the 64 genes were also down-regulated by chitin oligosaccharide elicitor treatment.21) One-third of the 21 down-regulated genes are of unknown function, and the other two-thirds included genes likely to be related to metabolism, regulation of gene expression, and signal transduction, but the biological functions of these down-regulated genes in defense responses remain unknown. On the other hand, a total of 200 genes were up-regulated in the OsWRKY71-overexpressing transgenic rice cells, and 146 of the 200 up-regulated genes were also up-regulated by chitin oligosaccharide elicitor treatment.21) One-third of the 146 up-regulated genes are of unknown function, and the other two-thirds include genes likely to be related to metabolism, regulation of gene expression, defense, transport, and signal transduction. Because WRKY proteins have been investigated extensively for their possible involvement in defense responses, we focused next on defense-related genes among the 146 up-regulated genes. Table 1 shows the six most up-regulated defense-related genes in the OsWRKY71-overexpressing transgenic rice cells. All of these six genes, (AK063939, AK102505, AK073267, AK101155, AK071453, and AK064281) encode chitinases. The up-regulation of the six chitinase genes in five independent OsWRKY71-overexpressing transgenic rice lines (line A, B, C, D, and E in Fig. 2A) was confirmed using real-time RT-PCR (Fig. 2B).

During the course of this study, it was reported that overexpression of the OsWRKY71 gene in rice resulted in enhanced resistance to the virulent bacterial pathogen *Xanthomonas oryzae pv. oryzae* (Xoo) 13751.20) In the OsWRKY71-overexpressing transgenic rice cells, in addition to the chitinase genes, the other defense-related genes, such as PR-5 (AK102970) and peroxidases

### Table 1. Up-Regulated Defense-Related Genes in OsWRKY71-Overexpressing Transgenic Rice Cells

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Annotation</th>
<th>Fold change*</th>
</tr>
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<tbody>
<tr>
<td>AK063939</td>
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<td>7.19</td>
</tr>
<tr>
<td>AK102505</td>
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<td>6.05</td>
</tr>
<tr>
<td>AK073267</td>
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</tr>
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</tr>
<tr>
<td>AK071453</td>
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<td>3.83</td>
</tr>
<tr>
<td>AK064281</td>
<td>Chitinase</td>
<td>3.81</td>
</tr>
</tbody>
</table>

*Each value represents the geometric mean of ratios obtained in three microarray analyses.*
(AK058883 and AK064360), were also up-regulated. Since chitinases generally show antifungal activity, these defense-related genes other than the chitinase genes might contribute to enhanced disease resistance to virulent bacterial pathogen.

The mechanism by which overexpression of OsWRKY71 activates defense-related genes remains to be clarified, but considering that OsWRKY71 probably...
functions as a repressor, we speculate that the activation of defense-related genes by overexpressed OsWRKY71 is indirect. In a previous microarray analysis using suspension-cultured rice cells treated with chitin oligosaccharide elicitor, it was found that the mRNA levels of genes encoding the above chitinases, PR-5, and peroxidases showed changes similar to that of OsWRKY71, or delayed changes, further confirming the involvement of OsWRKY71 in the activation of defense-related genes.

Recently, on the other hand, HvWRKY1/2, homologs of OsWRKY71 in barley, were reported to be negative regulators of the basal defense responses in barley. To clarify further the roles of OsWRKY71 in defense responses to pathogen infection in rice, the identification of downstream target genes of OsWRKY71 is required.

Acknowledgments

We thank Dr. T. Nakagawa (Shimane University) for the gift of pGWB2, Dr. M. Takagi and Dr. M. Shikata (National Institute of Advanced Industrial Science and Technology) for the gift of 35S-GAL4DB, 35S-GAL4DB-SRDX, 35S-GAL4-TATA-LUC-NOS, and pPTRL plasmids, and Ms. R. Motoyama for technical assistance in microarray analysis. This work was supported in part by Grants-in-Aid for Scientific Research (nos. 12460051 and 15380080) to H.Y. from the Japanese Society for the Promotion of Science, and by the Program for Promotion of Basic Research Activities (PROBRAIN).

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