OsTGAP1, a bZIP Transcription Factor, Coordinately Regulates the Inductive Production of Diterpenoid Phytoalexins in Rice

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Production of major diterpenoid phytoalexins, momilactones and phytocassanes, is induced in rice upon recognition of pathogenic invasion as plant defense-related compounds. We recently showed that biosynthetic genes for momilactones are clustered on rice chromosome 4 and co-expressed after elicitation, mimicking pathogen attack. Because genes for most metabolic pathways in plants are not organized in gene clusters, examination of the mechanism(s) regulating the expression of such clustered genes is needed. Here, we report a chitin oligosaccharide elicitor-inducible basic leucine zipper transcription factor, OsTGAP1, which is essential for momilactone biosynthesis and regulates the expression of the five genes in the cluster. The knock-out mutant for OsTGAP1 had almost no expression of the five clustered genes (OsCPS4, OsKSL4, CYP99A2, CYP99A3, and OsMAS) or production of momilactones upon elicitor treatment. Inductive expression of OsKSL7 for phytocassane biosynthesis was also largely affected in the osgap1 mutant, although phytocassane accumulation still occurred. Conversely, OsTGAP1-overexpressing lines exhibited enhanced expression of the clustered genes and hyperaccumulation of momilactones in response to the elicitor. Furthermore, enhanced expression of OsKSL7 and hyperaccumulation of phytocassanes was also observed. We also found that OsTGAP1 overexpression can influence transcriptional up-regulation of OsDXS3 in the methylerythritol phosphate pathway, eventually leading to inductive production of diterpenoid phytoalexins. These results indicate that OsTGAP1 functions as a key regulator of the coordinated transcription of genes involved in inductive diterpenoid phytoalexin production in rice and mainly exerts an essential role on expression of the clustered genes for momilactone biosynthesis.
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Plant Material and Chemical Treatment—Purified chitooctaose (Yaizu Suisankagaku Industry Co., Ltd., Tokyo, Japan) was re-N-acetylated to give N-acetylchitooctaose, as described (22), and used as a chitin oligosaccharide elicitor throughout this study. *Oryza sativa* L. cv. Nipponbare was used as the wild type. Calli of *O. sativa* L. cv. Nipponbare were cultured as described previously (13). Six days after transfer to fresh culture medium, the cultured rice cells were used for assays with a chitin oligosaccharide elicitor treatment (N-acetylchitooctaose, 1 ppm) throughout this study. *Tos17*-inserted mutants of H0155 (for AK073715) and NC0005 (for AK102690) were obtained from the National Institute of Agrobiological Sciences of Japan (*Tos17* mutant panel project; available on the World Wide Web). The insertion site of *Tos17* is indicated in supplemental Fig. S2A. The *Tos17* homozygous mutants were selected by PCR genotyping, and generated cultured cells of the mutant were used for the experiments.

Cloning and Transformation—The *OsKSL4* promoter fragments were amplified by PCR, using genomic DNA prepared from suspension-cultured rice cells (cv. Nipponbare) as template. The mutated constructs were generated via a two-step PCR process (primer overlapping mutagenesis), and the core TGAC sequence was converted to CCTA. The *OsKSL4* promoter deletions and mutated constructs were cloned into the KpnI and XhoI sites of the pGL3 basic vector (Promega, Madison, WI) containing the firefly luciferase gene (*LUC*).

For transactivation assays, the *OsTGAP1* coding sequence was amplified using *OsTGAP1* cDNA obtained from the Rice Genome Resource Center. The *OsTGAP1* cDNA was fused to the DNA-binding domain (1–147 amino acids) of the yeast transcription factor GAL4 (GAL4-DBD) to prepare the effector construct. *LUC*, which is under the control of a promoter containing six repeats of the GAL4 binding site (6×UAS), was used as a reporter (23). For the production of recombinant GST-*OsTGAP1* protein and the generation of T7-

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3 The abbreviations used are: bZIP, basic leucine zipper; MEP, methylerythritol phosphate; RT, reverse transcription; qRT, quantitative reverse transcription; GST, glutathione S-transferase; GC, gas chromatography; MS, mass spectrometry; LC, liquid chromatography; LUC, luciferase; TGA, TGACG-sequence-specific binding protein.
OsTGAP1-overexpressing plants, the OsTGAP1 coding sequence was cloned into pENTR/D/Topo (Invitrogen). For protein purification, the entry clone was confirmed by sequencing, recombined (with Invitrogen LR GATEWAY recombination) into pDEST15 (Invitrogen), and introduced into Escherichia coli strain Rosetta (DE3). The entry clone was also recombined into pGBW27 (24) and used for generating transgenic rice cells by Agrobacterium-mediated transformation, as described by Kaku et al. (25). Overexpression of T7-OsTGAP1 was confirmed by qRT-PCR and immunoblot analysis with an anti-T7 antibody (Chemicon, Temecula, CA).

Expression Analysis—RT-PCR and qRT-PCR were used to confirm the levels of expression. For the RT reaction, a SuperScript reverse transcriptase kit (Invitrogen) and Quantitect kit (Qiagen K.K., Tokyo, Japan) were used. qRT-PCR was performed using SYBR Green technology on an ABI PRISM 7300 real time PCR system (Applied Biosystems, Foster City, CA). Raw data from qRT-PCR were analyzed using the ACT (difference in threshold cycles) method, and the results were expressed as relative mRNA values normalized to the expression level of UBQ, as described previously (21).

Luciferase Activity Assay—Particle bombardment was carried out with the Biolistic PDS-1000/He particle delivery system (Bio-Rad) according to the manufacturer’s protocol. Suspension-cultured rice cells, 6 days after transfer to fresh culture medium, were used for bombardment. The OsKSL4 promoter-LUC reporter construct was mixed in a 2:1 molar ratio with the internal control and precipitated onto gold particles (1.6-μm diameter). Approximately 1 μg of DNA was delivered into the rice cells per bombardment. Following bombardment, the samples were incubated in N6 medium with or without N-acetylchitoolactosamine (1 mg/liter) for 15 h at 25 °C in darkness, collected, and then homogenized in cell lysis buffer (Promega). The firefly LUC and Renilla LUC luminescence was monitored using a Centro LB960 plate reader (Berthold Japan, Tokyo, Japan) according to the manufacturer’s instructions. To normalize the values after each assay, the ratio of LUC activity (firefly LUC/Renilla LUC) was calculated.

Electrophoretic Mobility Shift Assays—Double-stranded oligonucleotides spanning the TGACG motif upstream of OsKSL4 were synthesized, annealed, and digoxigenin-labeled. The OsTGAP1 protein was overexpressed in E. coli as a glutathione S-transferase (GST) fusion protein (GST-OsTGAP1). The E. coli Rosetta-gami (DE3), harboring a plasmid for the expression of GST-OsTGAP1 (pDEST17-OsTGAP1), was cultured in Overnight Express Instant TB medium (30 °C, 24 h) (Merck). The fusion protein was affinity-purified on glutathione-Sepharose 4B (Amersham Biosciences) and used for electrophoretic mobility shift assays. Probe fragments were amplified by PCR using the −1928 construct (for normal probes) or the m3 construct (for mutated probes) as template. Electrophoretic mobility shift assays were carried out using a digoxigenin gel shift kit, second generation (Roche Applied Science) according to the manufacturer’s instructions.

Metabolite Analysis—Diterpene hydrocarbons were extracted from elicitor-induced suspension-cultured rice cells using ethyl acetate and detected by gas chromatography-mass spectrometry (GC-MS) as described previously (13). Phytoalexins were extracted from suspension-cultured rice cells after elicitation and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (26).

Oligonucleotide Primers—The oligonucleotides used for genotyping, RT-PCR, qRT-PCR, and cloning are listed in supplemental Table S1.

RESULTS

Identification of an Elicitor-inducible cis-Acting Element in the OsKSL4 Gene Promoter—To identify regulating factors for momilactone biosynthesis, we first examined a chitin oligosaccharide elicitor-inducible promoter activity within a 2-kb region upstream of the OsKSL4 gene, responsible for the first committed step specific to momilactone biosynthesis, using a LUC reporter assay. Fig. 2A shows that deletion from −1224 to −991 bp and further deletion to −414 bp rendered the pro-
were selected, based on their expression profiles after elicitor treatment (21). From more than 100 transcription factors having features of bZIP transcription factors, 10 candidate genes were selected, based on their expression profiles after elicitor treatment (Fig. 3A). Among them, the AK073715, AK102690, and AK106988 proteins were TGA-type bZIP transcription factors, having the typical conserved motifs of TGA factors (supplemental Fig. S1). Arabidopsis TGA factors can bind to the TGACG motif and are involved in defense responses (29). Thus, we further analyzed these three TGA-type bZIP transcription factors as promising candidates to regulate OsKSL4 gene expression. Expression analyses of these genes by qRT-PCR revealed that the AK073715 gene was maximally induced 4 h after elicitor treatment (slightly earlier than OsKSL4), expression returned to basal levels by 8 h, and the AK102690 gene was induced for 4–8 h after treatment, similar to OsKSL4 (Fig. 3B). We could not clearly detect inducible expression of the AK106988 gene and thus discarded it as a candidate. Phylogenetic analysis revealed that the AK073715 product was relatively close to the node containing soybean sTGA1, tobacco TGA1a, and Arabidopsis TGA1 and TGA4 and that the AK102690 product was classified in the node containing AtbZIP21 (supplemental Fig. S1). Tobacco TGA1a is shown to bind the as-1 element involved in salicylic acid-induced activation of transcription (30), and TGA4 is involved in defense responses (31), but the function of AtbZIP21 is still unknown. Nuclear localization of the AK073715 and AK102690 products was confirmed using green fluorescent protein as a reporter (Fig. 3C). Thus, we focused on the AK073715 and AK102690 genes to determine whether they were responsible for the inducible expression of OsKSL4.

OsTGAPI Is Essential for Elicitor-inducible OsKSL4 Gene Expression and Production of Momilactones—We used the rice Tos17 insertion mutants H0155 (for AK073715) and NC0005 (for AK102690) to examine their physiological functions. Two mutants obtained from the Rice Genome Research Program contained the Tos17 insertion in the first intron of AK073715 and the fifth intron of AK102690 (supplemental Fig. S2A) (32–34). They were both shown to be null mutants, lacking expression in response to elicitation (supplemental Fig. S2B).

LC-MS/MS of phytoalexins accumulated in the culture medium 0, 48, and 72 h after elicitor treatment revealed that the level of momilactones in H0155 mutant cells severely decreased to less than 20% of that in wild-type cells (Fig. 4A), whereas the phytocassanes levels were almost the same between the mutant and wild-type cells at 48 h; thereafter, the phytocassanes levels were somewhat higher than that in wild-type cells at 72 h in the H0155 mutant (Fig. 4B). The inductive expression of OsKSL4 after elicitation was severely suppressed in the H0155 mutant compared with wild-type cells (Fig. 4C). The inductive expression of OsKSL7 after the elicitation seen in wild-type cells was also weakened in the H0155 mutant, but the expression levels at 0 h (basal level) and 24 h after the elicitation were rather higher than that of wild-type cells (Fig. 4D). In addition to expression analysis of specific genes for phytoalexin biosynthesis, the upstream gene expression responsible for plastidial geranylglycerol diphosphate substrate production was also analyzed in the H0155 cells. The elicitor-induced expression of OsDXS3 in rice, which encodes deoxyxylulose phosphate synthase involved in the first committed step in the MEP pathway in plastids, was significantly suppressed in the H0155 mutant (Fig. 4E).

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7,15-diene, a precursor for momilactones, was observed only in the wild-type cells, whereas accumulation of ent-cassa-12,15-diene, a precursor for phytocassanes, was detected to the same extent in both the H0155 mutant and wild-type cells (Fig. 4).

These results suggest that AK073715 is essential for the elicitor-inductive momilactone biosynthesis through the up-regulation of OsKSL4 gene expression and has a role in regulation of OsKSL7 and OsDXS3 expression induced by elicitor treatment, which normally leads to transient production of phytocassanes after the elicitor recognition. Hence, the AK073715 gene was designated OsTGAP1 (Oryza sativa TGA factor for phytoalexin production 1).

In the NC0005 mutant, both diterpenoid phytoalexin production and the biosynthetic gene expression (OsKSL4) were comparable with that in wild-type cells (supplemental Fig. S3), suggesting that AK102690 is unlikely to be involved in regulating phytoalexin biosynthesis.

**FIGURE 4. Physiological function of AK073715 in momilactone biosynthesis.** A, accumulation of momilactones in wild-type and H0155 mutant cells after elicitor treatment. B, accumulation of phytocassanes in wild-type and H0155 mutant cells after elicitor treatment. Phytoalexin levels in the culture medium collected 0, 48, and 72 h after elicitor treatment were determined by LC-MS/MS. The results are the average of at least three independent experiments. Bars, mean ± S.D. C, expression analysis of OsKSL4 in elicitor-treated cells, by qRT-PCR. Total RNA was prepared 0, 6, 12, and 24 h after elicitor treatment. The results are the average of at least three independent experiments, and values for OsKSL4 mRNA expression were normalized to the expression of the UBQ gene. Bars, means ± S.D. D, expression analysis of OsKSL7 in elicitor-treated cells after qRT-PCR as in C. E, expression analysis of OsDXS3 in elicitor-treated cells after qRT-PCR as in C. F, GC-MS analysis of accumulated diterpene hydrocarbons in the mutant and wild-type cells after elicitation. Selected ion chromatograms at a mass/charge ratio of 272 showing 9βH-pimara-7,15-diene and ent-cassa-12,15-diene are indicated.
OsTGAP1 Requires the TGACG Motif to Bind to the OsKSL4 Promoter and Has Transactivation Capacity— Gel mobility shift assays revealed that GST-tagged OsTGAP1 could bind the DNA fragment containing the TGACG motif (TGACGT) in the OsKSL4 promoter, in a TGACG motif-dependent manner (Fig. 5A). We also found that OsTGAP1 functioned as a transcriptional activator, using a transient assay system in rice cells with or without elicitor. The data are means ± S.D. of three replicates.

OsTGAP1 Coordinately Regulates the Clustered Genes for Momilactone Biosynthesis—As previously discussed, the momilactone biosynthetic genes are organized in a gene cluster, exhibiting coordinated, inducible expression upon elicitor treatment. OsTGAP1 overexpression led to constitutive production of momilactones even without elicitation, indicating that the five momilactone biosynthetic genes, including OsKSL4, may be coordinately regulated by OsTGAP1. To verify this, we analyzed expression of the genes in the momilactone biosynthetic gene cluster (with neighboring genes) in both the ostgap1 mutant and the T7-OsTGAP1-overexpressing lines. Elicited expression of the five momilactone biosynthetic genes (OsCP54, OsKSL4, CYP99A2, CYP99A3, and OsMAS) was severely suppressed in ostgap1 cells, whereas expression of the Os04g0177600 gene (encoding actin protein) and the Os04g0180900 gene (unknown function), both of which are located outside the cluster, were virtually unaffected (Fig. 7). The clustered genes, but not the neighboring genes, were hyperinductively expressed in the overexpressing cells stimulated by an elicitor (Fig. 7). The results indicate that clustered genes for momilactone biosynthesis are coordinately regulated by OsTGAP1.

DISCUSSION

In this study, we identified an elicitor-inducible rice bZIP transcription factor, OsTGAP1, which is essential for elicitor-inducible production of momilactones and which coordinately regulates the expression of all five genes in the momilactone biosynthetic gene cluster. OsTGAP1 was also shown to be involved in the transcriptional regulation of OsKSL7 for phytocassane biosynthesis and OsDXS3 in the MEP pathway. Overexpression analysis clearly demonstrated that OsTGAP1 can influence both momilactone and phytocassane production through up-regulation of both the biosynthetic genes and the upstream MEP pathway gene under the elicitor treatment. Since OsTGAP1 expression itself is induced by treatment with a chitin oligosaccharide elicitor, OsTGAP1 would be a crucial master regulator that controls the inducible expression of biosynthetic genes and
upstream pathway genes required for diterpenoid phytoalexin production as part of the plant’s defensive response, acting through the detection of a chitin oligosaccharide elicitor (25).

In Arabidopsis, genes encoding enzymes that biosynthesize the phytoalexin camalexin are coordinately expressed, and their expression is probably the result of an unidentified key transcription factor(s) (35). Although the transcription factor AtWRKY33, which regulates the expression of the two P450 genes (PAD3 and CYP71A13) involved in camalexin biosynthesis, has been reported (36), whether a key transcription factor exists that is involved in the coordinated up-regulation of multiple camalexin biosynthetic genes is still unknown. Additionally, the PAD3 and CYP71A13 genes are not located in a gene cluster. Thus, such a function of OsTGAP1 in regulating chitin oligosaccharide-inducible expression of the momilactone biosynthetic gene cluster seems to be characteristic of rice plants.
Phylogenetic analysis showed that OsTGAP1 is located close to the node, including Arabidopsis OBF4/TGA4 (sharing 58% amino acid identity), which has been shown to positively regulate basal resistance in Arabidopsis (31). The similarity suggested that OsTGAP1 may function in the defensive response in rice. As a result of a homology search using the RICE cDNA database and the Knowledge-based Oryza Molecular Biological Encyclopedia (KOME; available on the World Wide Web), at least 13 TGA-type transcription factor genes are expressed in rice. Of these 13, only three genes, including OsTGAP1, were selected as having elicitor responsiveness by our microarray analysis. Thus, our attempt to narrow down the candidate elicitor-inducible TGA factors regulating the OsKSL4 expression using the microarray data was apparently valid in this case.

We used the rice Tos17 insertion mutant H0155 for physiological analysis of OsTGAP1. Although a genetic complementation test of H0155 has not yet been carried out, the phenotype of the mutant is most likely linked to a mutation of the OsTGAP1 locus, because four ostgap1/ostgap1 homozygous plants that we tested, segregated by self-pollination of a heterozygous OsTGAP1/ostgap1 plant, all exhibited the momilactone phenotype. Loss-of-function analyses showed that OsTGAP1 was responsible for elicitor-inducible production of momilactones and the expression of the biosynthetic gene. Because accumulation of momilactones 48 h after the elicitation, but not phytocassanes, was affected by the OsTGAP1 mutation, OsTGAP1 was shown to exert a strong influence on momilactone biosynthetic gene expression; in fact, OsKSL4 gene expression was severely suppressed up to 24 h after elicitation in the ostgap1 mutant, whereas OsKSL7 expression in the mutant was still observed 6 h after the elicitation, which was about 50% suppression compared with the expression level in wild-type cells. Moreover, the basal level of OsKSL7 expression was detected in the mutant without elicitation. Although why the basal level of OsKSL7 expression in the ostgap1 mutant increases is unknown at present, this constitutive OsKSL7 expression with slight up-regulation is consistent with the accumulation of phytocassanes in the ostgap1 mutant (Fig. 4, B and D). With regard to expression of OsDXS3 in the upstream MEP pathway, significant but incomplete suppression of OsDXS3 expression was detected in the ostgap1 mutant after elicitation. Thus, the inductive expression of momilactone biosynthetic genes appears to be regulated by OsTGAP1 in a manner different from that of OsKLS7 and OsDXS3 gene expression.

The ostgap1 mutant also showed that all five genes for momilactone biosynthesis in the cluster were regulated by OsTGAP1 upon elicitor treatment, whereas expression of genes located outside of the cluster (Os04g0180900 and Os04g0177600) was virtually unaffected. Coordinated regulation of the clustered genes for momilactone biosynthesis by OsTGAP1 was also shown using T7-OsTGAP1-overexpressing lines. These results imply that the OsTGAP1 has a profound effect on regulating the gene cluster for momilactone biosynthesis.

We found that OsTGAP1 overexpression can also influence transcriptional up-regulation of the phytocassane biosynthetic gene (OsKSL7) and the MEP pathway gene (OsDXS3) under elicitation, eventually leading to diterpene phytoalexin production. Since OsTGAP1 was shown to function as a transcriptional activator (Fig. 5B), OsTGAP1 may be able to influence expression of these genes by binding to their regulatory elements. In fact, several TGACG motifs can be found in the possible promoter regions of OsKSL7 and OsDXS3 (supplemental Fig. S4). Although the physical binding of OsTGAP1 to the regulatory elements remains to be demonstrated, our data support the idea that OsTGAP1 could be the master regulator for the production of diterpenoid phytoalexins and transcriptionally controls both phytoalexin biosynthetic genes and the upstream MEP pathway gene responsible for supplying geranylgeranyl dipiphosphate, the precursor of diterpene phytoalexins, after elicitor recognition.

Determining how OsTGAP1 synchronously controls expression of the clustered genes for momilactone biosynthesis will also be important. A survey of TGACG motifs existing in the broad region encompassing the clustered genes revealed more than 100 possible TGACG motifs that could be occupied by OsTGAP1. However, we also found that a large number of TGACG motifs exist in other regions extending from the momilactone cluster in both directions, indicating that the possible promoter regions (~3 kb) of not only the five momilactone biosynthetic genes but also non-elicitor-inducible genes contain TGACG-motifs (supplemental Fig. S4). Therefore, OsTGAP1 may bind to the putative promoter regions of all clustered genes to regulate their expression, but it is also possible that OsTGAP1 regulates expression of the clustered genes for momilactone biosynthesis by unknown mechanisms other than binding to all TGACG motifs existing in promoter regions of the clustered genes. Confirmation of OsTGAP1-binding sites on the momilactone biosynthetic gene cluster would be the next step leading to understanding of the regulation mechanism.

Overexpression of T7-OsTGAP1 also revealed that OsTGAP1 itself was not sufficient to fully activate the expression of genes for diterpenoid phytoalexin biosynthesis. This may suggest that a posttranslational modification of OsTGAP1 and/or the involvement of an unknown protein factor that synergistically functions with OsTGAP1 upon elicitor treatment can explain the elevated activation of biosynthetic gene expression (Fig. 6, D–F). Redox regulation of Arabidopsis TGA1 has been shown to modify the activity of this protein (37) and might also represent a way to modify OsTGAP1 activity. However, cysteine residues that are involved in TGA1 redox control are not conserved in OsTGAP1 (supplemental Fig. S1), suggesting that this is not the case for OsTGAP1 modification. Identification of unknown factors, which are required to modulate gene expression for diterpenoid phytoalexin biosynthesis with OsTGAP1, would provide potential clues for understanding the mechanisms of OsTGAP1-based regulation. Although the way in which OsTGAP1 coordinately transactivates all genes involved in diterpenoid phytoalexin production, including the momilactone biosynthetic gene cluster, is currently unknown, our results provide evidence for the presence of an effective coordinated regulation system by OsTGAP1 to produce defensive compounds in rice.

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