Identification of Target Genes of the bZIP Transcription Factor OsTGAP1, Whose Overexpression Causes Elicitor-Induced Hyperaccumulation of Diterpenoid Phytoalexins in Rice Cells

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Abstract
Phytoalexins are specialised antimicrobial metabolites that are produced by plants in response to pathogen attack. Momilactones and phytocassanes are the major diterpenoid phytoalexins in rice and are synthesised from geranylgeranyl diphosphate, which is derived from the methylenerythritol phosphate (MEP) pathway. The hyperaccumulation of momilactones and phytocassanes due to the hyperinductive expression of the relevant biosynthetic genes and the MEP pathway gene OsDXS3 in OsTGAP1-overexpressing (OsTGAP1ox) rice cells has previously been shown to be stimulated by the chitin oligosaccharide elicitor. In this study, to clarify the mechanisms of the elicitor-stimulated coordinated hyperinduction of these phytoalexin biosynthetic genes in OsTGAP1ox cells, transcriptome analysis and chromatin immunoprecipitation with next-generation sequencing were performed, resulting in the identification of 122 OsTGAP1 target genes. Transcriptome analysis revealed that nearly all of the momilactone and phytocassane biosynthetic genes, which are clustered on chromosomes 4 and 2, respectively, and the MEP pathway genes were hyperinductively expressed in the elicitor-stimulated OsTGAP1ox cells. Unexpectedly, none of the clustered genes was included among the OsTGAP1 target genes, suggesting that OsTGAP1 did not directly regulate the expression of these biosynthetic genes through binding to each promoter region. Interestingly, however, several OsTGAP1-binding regions were found in the intergenic regions among and near the cluster regions. Concerning the MEP pathway genes, only OsDXS3, which encodes a key enzyme of the MEP pathway, possessed an OsTGAP1-binding region in its upstream region. A subsequent transactivation assay further confirmed the direct regulation of OsDXS3 expression by OsTGAP1, but other MEP pathway genes were not included among the OsTGAP1 target genes. Collectively, these results suggest that OsTGAP1 participates in the enhanced accumulation of diterpenoid phytoalexins, primarily through mechanisms other than the direct transcriptional regulation of the genes involved in the biosynthetic pathway of these phytoalexins.


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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data for microarray analyses are available from the Gene Expression Omnibus of the NCBI (ID: GSE53414 and GSE53417). All data for Chip-seq analyses are available from the DDBJ Sequence Read Archive (ID: DRA001274).

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Introduction
Phytoalexins are specialised antimicrobial metabolites that are produced by plants in response to pathogen attack [1]. In rice, momilactones and phytocassanes are recognised as the major diterpenoid phytoalexins [2–5].

In plants, isopentenyl diphosphate and dimethylallyl diphosphate, which are the basic C5 precursors for terpenoid biosynthesis, are produced by two distinct pathways: the mevalonate pathway and methylenerythritol phosphate (MEP) pathway [6]. In suspension-cultured rice cells, the MEP pathway genes exhibit elicitor-induced expression, and the MEP pathway is required for the production of sufficient amounts of diterpenoid phytoalexins [7]. However, the transcriptional regulatory mechanisms of the MEP pathway genes remain unknown.

In the biosynthesis of diterpenoid phytoalexins, the common precursor geranylgeranyl diphosphate is sequentially cyclised by OsCPS2, OsCPS4, OsKSL7, and OsKSL4 into two distinct
diterpene hydrocarbons: ent-cassa-12,15-diene and 9βH-pimarane-7,15-diene [8–10]. For momilactone biosynthesis, two P450 monooxygenases (CYP99A2 and CYP99A3) and a dehydrogenase (OsMAS) are involved in the downstream oxidation of 9βH-pimarane-7,15-diene [11,12]. For phytocassane biosynthesis, four P450 monooxygenases (CYP71Z7, CYP76M7, CYP76M8, and CYP701A8/OsKOL4) are involved in the oxidation of ent-cassa-12,15-diene [13–16]. The momilactone and phytocassane biosynthetic genes are localized in narrow regions of chromosomes 4 and 2, respectively, creating functional gene clusters [11,13]. These biosynthetic genes exhibit the temporally coordinated expression of mRNAs after treatment with a biotic elicitor in suspension-cultured rice cells [7,11].

The basic leucine zipper (bZIP) transcription factor OsTGAP1 has been shown to be involved in the regulation of the production of momilactones and phytocassanes. OsTGAP1-overexpressing (OsTGAP1ox) rice cells exhibit the hyperaccumulation of momilactones and phytocassanes as well as the enhanced expression of all momilactone biosynthetic genes, the phytocassane biosynthetic gene OsKSL7, and the MEP pathway gene OsDXS3, upon treatment with an elicitor [17]. However, the details of the regulation of these genes by OsTGAP1 remain unknown.

In Arabidopsis thaliana, 73 members of the bZIP transcription factor family have been identified and classified into ten groups (group A–I and S) based on sequence similarity among their basic regions and the presence of additional conserved motifs [10]. TGA factors (AtTGA1–7), which belong to the group D bZIP transcription factors, regulate pathogenesis-related genes such as PR-1 through binding to the TGACG-motif ([T/G]) on the promoter region and mediate salicylic acid–induced defence responses [19]. The transcriptional regulatory mechanism of PR-1 has been well studied [20]. However, knowledge regarding the genome-wide binding regions of these TGA factors is limited; only AtTGA2 binding regions have been mined. Three TGA factors in rice, viz. rTGA2.1, rTGA2.2, and rTGA2.3, have been identified, among which 14 rTGA factors have been determined. Three TGA factors, including OsTGAP1, have been determined. Three TGA factors in rice, viz. rTGA2.1, rTGA2.2, and rTGA2.3, are involved in the regulation of defence responses against Magnaporthe oryzae and the rice bacterial blight Xanthomonas oryzae pv. oryzae [23–26]. However, the target genes of these three TGA factors remain unknown.

In the present study, transcriptome analysis and ChIP analysis with next-generation sequencing (ChIP-seq) using untransformed and elicitor-treated OsTGAP1ox cells were performed, resulting in the identification of OsTGAP1 target genes. Interestingly, the clustered diterpenoid phytoalexin biosynthetic genes were not included among the OsTGAP1 target genes. However, it should be noted that several OsTGAP1-binding regions were found in the intergenic regions among and near the cluster regions. Concerning the MEP pathway genes, it was found that only OsDXS3 possessed an OsTGAP1-binding region in its upstream region and that OsTGAP1 directly regulated OsDXS3 expression, while other MEP pathway genes were not included among the OsTGAP1 target genes. Possible mechanisms by which OsTGAP1 may regulate the production of diterpenoid phytoalexins are also discussed.

### Materials and Methods

**Plants, chemical treatment, and rice transformation**

*Oryza sativa* L. ‘Nipponbare’ was used as the wild-type strain. Suspension-cultured rice cells were maintained as described in a previous paper [8]. N-acetylmuramic acid was prepared and used to treat the rice cells as a chitin oligosaccharide elicitor, as described previously [17,27]. Rice transformation was performed as described previously [17].

**Plasmid construction**

The *Zea mays* polyubiquitin promoter was amplified by PCR using the primers UBQp attB4 F and UBQp attB1 R from pANDA [28] and cloned into pDONR-P4-1R (Invitrogen, CA, USA), resulting in pDONR-UBQp. The polyubiquitin promoter and OsTGAP1 open reading frame (ORF) were then cloned into R4pGWB501 [29] from pDONR-UBQp and pENTR-TGA [17] using LR Clonase II Plus Enzyme mix (Invitrogen). The resulting plasmid was designated as R4pGWB-UBQp-TGA and used for rice transformation.

The 2-kbp upstream region of *OsDXS3* was amplified by PCR using the primers DXS3p 2k F and DXS3p R and cloned into the *Xanthomonas oryzae* KpnI and HindIII sites of the pGL3-Basic vector (Promega, WI, USA), resulting in pGL3-DXS3p-2k. Next, three mutated constructs were generated: one of the two TGACGT sequences was mutated in m1 and m2, while both TGACGT sequences were mutated in m3. The TGACGT sequences in the *OsDXS3* promoter were mutated by PCR using the following primer pairs: DXS3p-m1 F and DXS3p-m1 R, DXS3p-m2 F and DXS3p-m2 R, and DXS3p-m3 F and DXS3p-m3 R. Each mutated *OsDXS3* promoter was then amplified by PCR using the primers DXS3p 2k F and DXS3p R. The amplified DNA fragments were cloned into the *KpnI* and *HindIII* sites of the pGL3-Basic vector and then sequenced, resulting in pGL3-DXS3p-2k-m1, pGL3-DXS3p-2k-m2, and pGL3-DXS3p-2k-m3.

Fragments of the *OsDXS3* promoter (250 bp and 240 bp) were amplified by PCR using the primers DXS3p 250 F and DXS3p R from pGL3-DXS3p-2k. A 250-bp fragment of the *OsDXS3* promoter with mutated TGACGT sequences was also amplified by PCR using the primers DXS3p 250 m1 F, DXS3p 250 m2 F, DXS3p 250 m3 F, and DXS3p R. These amplified DNA fragments were cloned into the *BgIII* and *HindIII* sites of the pGL3-Basic vector and sequenced. The resulting plasmids were designated as pGL3-DXS3p-250, pGL3-DXS3p-250-m1, pGL3-DXS3p-250-m2, and pGL3-DXS3p-250-m3.

The OsTGAP1 ORF was cloned into pUbi_Rfa_Tnos [30] from pENTR-TGA using LR Clonase II Enzyme mix (Invitrogen). The resultant plasmid was designated pUbi_TGA_Tnos and used as the effector plasmid.

A summary of the plasmids used in this study is provided in Table S1, and the sequences of PCR primers used for plasmid construction are provided in Table S2.

**Antibody generation and purification**

An OsTGAP1-specific antibody was generated by immunizing rabbits with the keyhole limpet hemocyanin-coupled synthetic peptide MELYPYGLEDHFNIHK corresponding to the N-terminal peptide sequence (residues 1–16) of OsTGAP1. Cys residues were added to the N-terminus of the peptides to ensure efficient coupling to the keyhole limpet hemocyanin carrier protein. The OsTGAP1-specific antibody was further purified using an antigen affinity column.
Nuclear extraction

Rice cells (approximately 3 g) were ground and suspended in 30 ml of 70% glycerol buffer (20 mM Hepes/NaOH, pH 7.4, 5 mM MgCl₂, 5 mM KCl, 50 mM saccharose, 70% [v/v] glycerol, 1 mM DTT, and 200 mM phenylmethysulfonyl fluoride) and incubated for 20 min at 4°C under mild shaking. After the filtration of the slurry through a quadrilayer of Miracloth (Merck, UK), the nuclei were collected by centrifugation (4,000 g, 60 min, 4°C) and washed in 10% glycerol buffer (20 mM Hepes/NaOH, pH 7.4, 5 mM MgCl₂, 5 mM KCl, 50 mM saccharose, 10% [v/v] glycerol, 1 mM DTT, and 200 mM phenylmethysulfonyl fluoride) by gentle resuspension followed by centrifugation (4,000 g, 20 min, 4°C). The nuclei were resuspended in 500 µl of TE buffer (pH 8.0) and sonicated for 1 min with a Sonifier 250D (Branson, CT, USA) at output setting 1 with a pulse of 1 s and duty cycle of 50%. The sonicated nuclei were cleared by centrifugation (20,000 xg, 15 min, 4°C) followed by ultracentrifugation (100,000 xg, 60 min, 4°C). The supernatant was then subjected to immunoprecipitation (IP). For protein gel blot analysis, acetone precipitation (95% [v/v]) as the primary antibody and the ECL anti-rabbit IgG was detected using the anti-OsTGAP1 antibody (dilution 1:2,500 [v/v]). The OsTGAP1-binding regions in each sample were identified using the International Rice Genome Sequencing Project genome sequence (build 5) from the Rice Annotation Project Database (RAP-DB: http://rapdb.dna.affrc.go.jp) as the reference genome sequence. The OsTGAP1-binding regions in each sample were subjected by deep sequencing using a Genome Analyzer II (Illumina). These reads were mapped to the rice genome with the Burrows-Wheeler Aligner (BWA) software package [31] using the International Rice Genome Sequencing Project genome sequence (build 5) from the Rice Annotation Project Database (RAP-DB: http://rapdb.dna.affrc.go.jp) as the reference genome sequence. The OsTGAP1-binding regions in each sample were then detected using Partek Genomics Suite (ver. 6.5; http://www.partek.com/, Partek Software, MO, USA) according to the manufacturer’s instructions. In this step, ~350-bp fragments were collected from each sample using E-Gel SizeSelect 2% (Invitrogen). The constructed libraries were subsequently subjected to deep sequencing using a Genome Analyzer II (Illumina). The sequence data were deposited in the NCBI database (accession number SRA064607). The sequence data were analyzed using the following thresholds: window size = 100, peak cut-off false discovery rate (FDR) <0.001, strand separation FDR <0.05, and significant enrichment in ChIP DNA compared to the ‘Input’ control (FDR <0.05). The sequence data were deposited in the NCBI database (accession number SRA064607).
DNA microarray analysis

Wild-type (WT) and OsTGAP1ox rice cells were treated with a chitin oligosaccharide elicitor. Total RNA was then isolated from a small portion of the rice cells using an RNeasy Plant Mini Kit (Qiagen, Germany) at 0, 6, and 24 h after the elicitor treatment and labelled with Cyanine 3 dye (Cy3) or Cyanine 5 dye (Cy5). Aliquots of Cy3-labelled cRNA and Cy5-labelled cRNA (825 ng each) were used for hybridization in a 60-mer rice oligo microarray with 44 k features (Agilent Technologies, CA, USA). Two series of microarray analyses were performed as follow. First, to investigate elicitor responsiveness, a time-course analysis of the elicitor treatment of WT cells was performed. The Cy3-labelled cRNA probe from the 0 h time point of the WT cells was used as a reference, and the Cy5-labelled cRNA probes from the 6 h and 24 h time point samples were compared against the 0 h reference. Second, comparison analysis was performed between the WT and OsTGAP1ox cells. The Cy3-labelled cRNA probes from the WT cells (0, 6, and 24 h after elicitor treatment) were used as references, and the Cy5-labelled cRNA probes from the OsTGAP1ox cells (0, 6, and 24 h after elicitor treatment) were compared against the references at each time point. The glass slides were scanned using a microarray scanner (G2565, Agilent), and the resulting output files were imported into Feature Extraction software (ver. 11; Agilent). These data were deposited into the Gene Expression Omnibus of the NCBI (http://www.ncbi.nlm.nih.gov/geo/; ID: GSE53414 and GSE53417). Statistical analyses were performed using Partek Genomics Suite. The expression levels of the OsTGAP1 target genes at each time point were calculated from fold changes relative to those of the WT cells 0 h after elicitor treatment, and the calculated data were imported into MultiExperiment Viewer (MeV v4.6, http://www.tm4.org/mev/) for cluster analysis. Hierarchical cluster analysis based on the average linkage and cosine correlation was used to cluster the genes on the y-axis using MeV.

ChIP-PCR

Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) on an ABI PRISM 7900 Real-Time PCR System (Applied Biosystems). The DNA amount was determined by generating standard curves using a series of known concentrations of the target sequence. The ratio of DNA present in the ChIP DNA to that in the ‘Input’ control was calculated. The primers DXS3p TGACGT F and DXS3p TGACGT R were used for the OsDXS3 promoter region.

Gel mobility shift assay (GMSA)

The DNA probe was amplified by PCR from pGL3-DXS3p-2k using the primers DXS3p TGACGT F and DXS3p TGACGT R. Mutated probes in the TGACGT sequence (m1, m2, and m3) were also amplified by PCR from pGL3-DXS3p-2k-m1, pGL3-DXS3p-2k-m2, and pGL3-DXS3p-2k-m3, respectively, using the same primers. These amplified probes were end-labelled with 32P by T4 polynucleotide kinase (Takara Bio, Japan). Recombinant N-terminal glutathione S-transferase (GST)-fused OsTGAP1 (GST-OsTGAP1), and GST protein were expressed in *E. coli* Rosetta 2 (DE3) harbouring pDEST15-TGA [17] or pGEX-6p2 (GE Healthcare) and purified using Glutathione Sepharose 4B (GE Healthcare). The reaction mixture comprised 20 mM Hepes (pH 7.6), 1 mM EDTA, 10 mM (NH4)2SO4, 1 mM DTT, 0.2% (v/v) Tween 20, 30 mM KCl, 0.5 pmol of recombinant GST or GST-OsTGAP1, and 0.05 pmol of the probe in a final volume of 20 µl.

The above mixed samples were incubated for 20 min at room temperature, and then 5 µl of loading buffer (0.25 × TBE buffer, 40% [w/v] glycerol, 0.2% [w/v] bromophenol blue) was added. The samples were separated on a 4% polyacrylamide gel in 0.5 × TBE at room temperature, and the bands were visualized by autoradiography.

Transactivation assay

pUb_GUS_Tnos [30] and pUb_OsTGAP1_Tnos, in which β-glucuronidase (GUS) and OsTGAP1, respectively, are under the control of the polyubiquitin promoter, were used as the effector plasmids. pGL3-DXS3p-2k, pGL3-DXS3p-250, pGL3-DXS3p-240, pGL3-DXS3p-250-m1, pGL3-DXS3p-250-m2, and pGL3-DXS3p-250-m3, in which the OsDXS3 promoter was fused to the firefly luciferase (FLUC) gene, were used as the reporter plasmids. The plasmid pPTRL [32], which contains the *Renilla* luciferase (*RLUC*) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter, was used as an internal control. Particle bombardment of rice cells was conducted as previously described [17]. In the cotransfection assays, 0.1 µg or 1 µg of the effector plasmid, 1.0 µg of the reporter plasmid, and 0.5 µg of pPTRL were used for each bombardment. Luciferase (LUC) assays were performed as previously described [17]. The ratio of LUC activity (FLUC/RLUC) was calculated to normalize the values after each assay.

Results

Genome-wide identification of in vivo OsTGAP1-binding regions

To locate in vivo OsTGAP1-binding regions, ChIP-seq analysis was performed. An antipeptide antibody directed against the N-terminal peptide sequence (residues 1–16) of OsTGAP1 was generated, as this sequence has high immunogenicity and low similarity to other proteins in rice. To assess the specificity of the generated anti-OsTGAP1 antibody, protein gel blot analysis of the nuclear extracts from the WT and OsTGAP1ox cells was performed. A single band (~45 kD) was detected from the nuclear extract of the WT cells, and this band was strengthened in the nuclear extract of the OsTGAP1ox cells (Figure S1A). IP, using the anti-OsTGAP1 antibody, was also performed from the nuclear extracts of the WT cells. When rabbit normal IgG was used for IP, no bands were detected by protein gel blot analysis using the anti-OsTGAP1 antibody. In contrast, a single band thought to represent OsTGAP1 was detected after IP by the anti-OsTGAP1 antibody (Figure S1B). These results indicated that the anti-OsTGAP1 antibody generated in this study had sufficient specificity.

The OsTGAP1ox cells were treated with or without the chitin elicitor for 6 h and then collected for ChIP-seq analysis using the anti-OsTGAP1 antibody. Two biological replicates were performed for each sample. DNA libraries of ChIP DNA from the untreated and elicitor-treated samples were then generated. DNA libraries of the ‘Input’ controls were also generated from each sample. These libraries were then subjected to next-generation sequencing using a Genome Analyzer II (Illumina). A total of 4.5 to 10 million reads (100 bp per read) were obtained from each library. These reads were mapped to the rice genome by BWA [31]. OsTGAP1-binding regions were then detected in each sample. Two biologically independent ChIP-Seq experiments were compared, and the regions that showed enrichment in both datasets were identified as OsTGAP1-binding regions. The results identified 2,763 and 2,777 binding regions from the untreated and elicitor-treated samples, respectively (Table S3, Table S4).
comparison of the binding regions in the two conditions revealed that a common set of 2,803 regions (approximately 70%) were bound by OsTGAP1 both with and without elicitation.

Subsequent analysis revealed that among all of these OsTGAP1-binding regions, 32% (i.e. 880 and 901 regions) were assigned to the upstream regions of particular rice genes (within 2 kbp of the transcription start site) in the untreated and elicitor-treated conditions, respectively; moreover, 10% and 9% (i.e. 272 and 248 regions) were assigned to the gene regions of particular rice genes (from the transcription start site to the transcription termination site), and 18% and 16% (i.e. 491 and 452 regions) were assigned to the downstream regions of particular rice genes (within 2 kbp of the transcription termination site) in the untreated and elicitor-treated conditions, respectively. Intriguingly, the remaining 41% and 42% of the regions (i.e. 1,120 and 1,176) were assigned to the intergenic regions in the untreated and elicitor-treated conditions, respectively. This proportion is larger than those of the other plant transcription factors (HY5, PIL5, AGL15, and BZR1) whose genome-wide binding regions have previously been reported [33–36]. These results are summarized in Figure 1A. The locations of the OsTGAP1-binding regions assigned to the upstream or downstream regions of particular genes were significantly concentrated within 400-bp of the transcription start sites (Figure 1B). This distribution pattern implies that OsTGAP1-binding regions located within 400-bp of the transcription start sites has the significant role on the transcriptional regulation of downstream genes. Therefore, this study focused on the genes that possessed an OsTGAP1-binding region within the 400-bp upstream region in either the untreated or elicitor-treated condition. A total of 693 genes were identified as potential OsTGAP1 target genes (Table S5).

Analysis of OsTGAP1-binding motifs

Overrepresented motifs in the OsTGAP1-binding regions were analysed using Partek Genomics Suite based on Gibbs motif sampler [37]. The TGACGT sequence was found in both the untreated and elicitor-treated condition (Figure 1C). These results are consistent with those of a previous report, which observed that OsTGAP1 binds to the TGACGT sequence in vitro [17]. We also analyzed whether TGACGT sequences are overrepresented in the OsTGAP1-binding regions compared to the rest of genome. As a result, TGACGT sequences are enriched approximately 8-fold in the OsTGAP1-binding regions compared to the rest of genome, although known binding motifs of other transcription factors were not enriched. (Figure S2). Furthermore, analysis of the distribution pattern of the TGACGT sequence around the OsTGAP1-binding regions (~1,000 to1,000 bp of each predicted binding region) revealed that the occurrence of the TGACGT sequence peaks at the predicted OsTGAP1-binding regions in both the untreated and elicitor-treated condition (Figure 1D).

Identification of OsTGAP1 target genes

To identify the genes regulated by OsTGAP1, a genome-wide DNA microarray analysis was performed using the WT and OsTGAP1ox cells, and gene expression was compared between these cells at 0, 6, and 24 h after the chitin elicitor treatment. Four biological replicates were used for each time point. Statistical analysis was performed using ANOVA-FDR (q value=0.05), and genes with changes in expression were identified as those that experienced a two-fold increase or decrease of expression level in the OsTGAP1ox cells at least one time point compared to the expression levels in the WT cells. Based on this criterion, 1,352 genes, 1,539 genes, and 1,267 genes were upregulated in the OsTGAP1ox cells at 0, 6, and 24 h after the elicitor treatment, respectively. By combining these three groups, 2,268 genes were identified that were upregulated in the OsTGAP1ox cells at least one time point (Figure S3A, Table S6). Conversely, 1,270 genes, 1,307 genes, and 1,624 genes were downregulated in the OsTGAP1ox cells at 0, 6, and 24 h after the elicitor treatment, respectively. By combining these three groups, 2,276 genes were identified that were downregulated in the OsTGAP1ox cells at least one time point (Figure S3B, Table S7).

To determine the OsTGAP1 target genes, the transcriptome data and ChIP-seq data were compared. Among the 2,763 upregulated genes, 86 (3.1%) possessed OsTGAP1-binding regions in each 400-bp upstream region (Figure 2A, Table S8). Moreover, among the 2,777 downregulated genes, 36 (1.3%) possessed OsTGAP1-binding regions in each 400-bp upstream region (Figure 2B, Table S9). Therefore, these 122 genes were tentatively identified as OsTGAP1 target genes.

Next, the 86 upregulated genes and 36 downregulated genes were subjected to hierarchical clustering. Based on their expression patterns in the WT and OsTGAP1ox cells, the 86 upregulated genes were classified into two groups: group I, whose expression decreased in WT cells after elicitor treatment; and group II, whose expression increased in WT cells after elicitor treatment (Figure 2C). Most of the genes (approximately 88%) were included in group II. The 36 downregulated genes were classified into three groups: group I, whose expression increased in WT cells after elicitor treatment; group II, whose expression was not largely changed in WT cells after elicitor treatment; and group III, whose expression decreased in WT cells after elicitor treatment (Figure 2D). Group I included 14 genes (39%), group II included 17 genes (47%), and group III included 5 genes (14%).

Furthermore, functional classification of these OsTGAP1 target genes was performed. The genes were classified into the following categories according to function: biosynthetic enzyme, defence response, transcription factor, protein kinase, transporter, other function, and unknown function. The results of the functional classification are summarized in Figure 2E. The 86 genes that were upregulated in the OsTGAP1ox cells were classified as follows: biosynthetic enzyme, 24 genes (27.9%); defence response, 6 genes (7.0%); transcription factor, 7 genes (8.1%); protein kinase, 8 genes (9.3%); transporter, 6 genes (7.0%); other function, 13 genes (15.1%); unknown function, 22 genes (25.6%). The 36 genes that were downregulated in the OsTGAP1ox cells were functionally classified as follows: biosynthetic enzyme, 12 genes (33.3%); defence response, 3 genes (8.3%); transcription factor, 9 genes (25%); protein kinase, 3 genes (8.3%); transporter, 1 gene (2.8%); other function, 4 genes (11.1%); unknown function, 4 genes (11.1%).

OsTGAP1-binding regions in phytoalexin biosynthetic gene clusters

Although nearly all of the momilactone and phytocassane biosynthetic genes were confirmed to be hyperinducively expressed in the elicitor-stimulated OsTGAP1ox cells through transcriptome analysis (Table S8), none of the momilactone and phytocassane biosynthetic genes in the clusters were identified as OsTGAP1 target genes (Figure S4 and S5). These results suggest that OsTGAP1 does not directly regulate the expression of these genes by binding to their promoter regions. Intriguingly, several OsTGAP1-binding regions were found in intergenic regions among and near the cluster regions (Figure 3). This result raises the possibility that OsTGAP1 positively effects the expression of clustered genes by binding to these regions, although it may also indirectly regulate the expression of these genes via other transcription factors.
Figure 1. Overview of the results of ChIP-seq. (A) Distribution of OsTGAP1-binding regions in the rice genome. The upstream region includes the binding regions within 2 kbp of the transcription start site. The gene region includes the binding regions that are located between the transcription start site and the transcription termination site. The downstream region includes the binding regions within 2 kbp of the transcription termination site. The remaining binding regions were assigned to intergenic regions. (B) Distribution of OsTGAP1-binding regions in the upstream and downstream regions. Red lines indicate the average of number of OsTGAP1-binding regions. Statistical analysis was performed for each 100-bp region, and significantly enriched regions were indicated by asterisks (P < 0.01 the binomial test and the Bonferroni correction). (C) Overrepresented motifs in the OsTGAP1-binding regions analysed in the untreated and elicitor-treated conditions using Partek Genomics Suite based on Gibbs motif sampler [37]. (D) Distribution of TGACGT sequences around the OsTGAP1-binding regions.

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Identification of OsTGAP1 Target Genes

A. Genes that possess OsTGAP1-binding region within 500 bp upstream region
   693

   607 \[ \bigcap \] 86 \[ \bigcup \] 2,182
   Up-regulated genes in OsTGAP1ox
   2,268

B. Genes that possess OsTGAP1-binding region within 500 bp upstream region
   693

   657 \[ \bigcap \] 36 \[ \bigcup \] 2,240
   Down-regulated genes in OsTGAP1ox
   2,276

C. Heat map and timecourse analysis of gene expression

D. Heat map and timecourse analysis of gene expression

E. Functional annotation of up-regulated and down-regulated genes

   Up-regulated
   86 genes
   22
   Group I
   (10 genes)
   24
   Group II
   (76 genes)

   Down-regulated
   36 genes
   12
   Group I
   (14 genes)
   9
   Group II
   (17 genes)
   3
   Group III
   (5 genes)

   - Biosynthetic enzyme
   - Defense response
   - Transcription factor
   - Protein kinase
   - Transporter
   - Other function
   - Unknown function
OsTGAP1 binds to the OsDXS3 promoter via the TGACGT sequence

OsDXS3, which encodes 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (EC 2.2.1.7), was included among the OsTGAP1 target genes. The MEP pathway is involved in diterpenoid phytoalexin production in rice [7], and DXS catalyses a key step of this pathway [38]. Therefore, we dissected the transcriptional regulation of OsDXS3 to understand the regulation of diterpenoid phytoalexin production by OsTGAP1.

OsTGAP1 bound to an approximately 100-bp upstream region of the transcription start site of OsDXS3, both with and without elicitation, in the ChiP-seq analysis (Figure 4A). This binding was confirmed by quantitative PCR using ChiP DNA from another biological replicate. The OsDXS3 upstream region was enriched in the ChiP DNA immunoprecipitated by the anti-OsTGAP1 antibody compared to that immunoprecipitated by the normal rabbit IgG (Figure 4B), indicating that OsTGAP1 binds to the OsDXS3 promoter. This OsTGAP1-binding region contained two TGACGT sequences (Figure 5A). GMSAs were performed using a DNA probe containing the OsDXS3 promoter to investigate whether OsTGAP1 recognized these two TGACGT sequences. As shown in Figure 5B, the GST-fused OsTGAP1 (GST-OsTGAP1) recombinant protein could bind the DNA probe containing the OsDXS3 promoter. When either TGACGT sequence in this region was mutated (m1 and m2), the binding of GST-OsTGAP1 to the DNA probe was weakened. When both TGACGT sequences were mutated (m3), GST-OsTGAP1 no longer bound to the DNA probe (Figure 5B). Taken together, these results indicate that OsTGAP1 binds to the OsDXS3 promoter via two TGACGT sequences.

OsTGAP1 directly regulates OsDXS3 expression

To further investigate whether the two TGACGT sequences on the OsDXS3 promoter are involved in the transcriptional regulation of OsDXS3, a transactivation assay was performed. Fragment of 250 bp upstream from the ATG translation start site of OsDXS3 was cloned and fused to the FLUC gene to produce a reporter plasmid (Figure 6A). Effector plasmids were constructed that contained a GUS or OsTGAP1 gene under the control of the maize ubiquitin promoter. The reporter plasmid and either the GUS or OsTGAP1 effector plasmid were delivered into cultured rice cells, along with an internal control plasmid that contained the RLUC gene under the control of the CaMV 35S promoter, by particle bombardment. The LUC activities were quantified and calculated as described in the ‘Materials and Methods’.

Rice cells with the reporter and OsTGAP1 effector plasmids showed 7-fold higher LUC activity than those with the reporter and GUS effector plasmids (Figure 6B). Because OsDXS3 mRNA level is increased by elicitor treatment, LUC activities after elicitor treatment were also measured. Although the enhancement of LUC activity by OsTGAP1 was detected, no change to LUC activity was observed following elicitor treatment (Figure 6B). These results suggest that a 250-bp fragment of the OsDXS3 promoter contains elements contributing to the OsTGAP1-dependent induction of OsDXS3 expression but not to the gene’s responsiveness to the elicitor. A transactivation assay was performed using a fragment of 2 kbp upstream from the ATG translation start site of OsDXS3. Rice cells with the reporter plasmid containing a 2-kbp fragment of the OsDXS3 promoter and the OsTGAP1 effector plasmid showed higher LUC activity than those with GUS effector plasmid. However, LUC activity showed no change under elicitor treatment, as was similar to the results observed for the 250-bp fragment of the OsDXS3 promoter (Figure 6C).

Next, a series of three mutants of the 250-bp fragment of the OsDXS3 promoter fused to FLUC (m1, m2, and m3) were constructed. In m1 and m2, one of the two TGACGT sequences was mutated, while in m3, both TGACGT sequences were mutated. A FLUC reporter plasmid containing a 240-bp fragment of the OsDXS3 promoter was also constructed, and this reporter plasmid did not contain any TGACGT sequences. This series of reporter plasmids was introduced to the cultured rice cells along with either the GUS or OsTGAP1 effector plasmids, and the LUC activities were measured after incubation. The rice cells with m1 or m2 and the OsTGAP1 effector plasmid exhibited almost the same level of LUC activity as those with the 250-bp promoter construct. However, rice cells with m3 or the 240-bp promoter construct showed significantly lower LUC activity compared to those with the 250-bp promoter construct (Figure 6D). These results indicate that the two TGACGT sequences on the OsDXS3 promoter are involved in the OsTGAP1-dependent induction of OsDXS3 expression. Taken together with the fact that OsTGAP1 binds to the OsDXS3 promoter via these two TGACGT sequences, the results strongly suggest that OsTGAP1 directly regulates OsDXS3 expression.

Discussion

Genome-wide analysis of OsTGAP1 target genes

In this study, ChiP-seq and transcriptome analyses were performed to identify the OsTGAP1 target genes. From the ChiP-seq analysis, the TGACGT sequence was found to be an overrepresented motif in the OsTGAP1-binding regions (Figure 1C, Figure S2). However, there also be the OsTGAP1-binding regions which did not contain any TGACGT sequences (Table S3, Table S4), suggesting that OsTGAP1 may bind to these regions through other motifs. OsTGAP1 may also interact with a DNA-binding protein that recognizes other motifs. However, known binding motifs of other transcription factors were not found in this study (Figure S2). To investigate how OsTGAP1 binds to these regions, a more detailed functional analysis of OsTGAP1 using a biochemical approach is required.

The locations of the OsTGAP1-binding regions assigned to the upstream region were found to be concentrated within 400 bp of the transcription start sites (Figure 1B), suggesting that OsTGAP1 binds near the transcription start sites and regulates the expression of downstream genes. Therefore, this study focused on those genes that possessed an OsTGAP1-binding region within 400 bp of the upstream region and identified the OsTGAP1 target genes.
through comparisons to transcriptome data. A total of 86 upregulated genes, along with 36 downregulated genes, were identified in OsTGAP1ox cells (Table S8, Table S9). These target genes were classified based on their expression patterns. Most of the 86 upregulated genes (group II) exhibited an elicitor-induced expression pattern in WT cells. Moreover, this elicitor-induced expression was enhanced in OsTGAP1ox cells (Figure 2C). In light of a previous report that OsTGAP1 shows transactivational activity [17], OsTGAP1 may function to enhance the expression of these genes, including OsDXS3. However, the regulatory
mechanisms of the 36 genes that were downregulated in OsTGAP1ox cells remain unclear. As these 36 genes could be classified into three groups based on their expression patterns (Figure 2D), each group may be regulated by OsTGAP1 in a different manner. Recent studies have reported that several transcription factors in plants act as both activators and repressors [39,40]. These transcription factors are thought to interact with coactivators and corepressors, thereby altering these functions. OsTGAP1 may interact with as yet unknown corepressors to suppress the expression of these genes.

A substantial number of the OsTGAP1 target genes encode enzymes of biosynthetic pathways other than terpenoid biosynthesis, such as flavonoid biosynthesis and lipid metabolism (Figure 2E, Table S8, Table S9). This fact suggests that OsTGAP1 is involved not only in the regulation of diterpenoid phytoalexin production but also in the regulation of other biosynthetic processes. In addition, the 86 genes that were upregulated in OsTGAP1ox cells include several defence-related genes, such as glucanase (Os07g0168600) and cystatin (Os01g0803200 and Os01g0915200; Figure 2E, Table S8). Furthermore, OsPLDbeta1 (Os10g0524400) and OsWRKY76 (Os09g0417600) were included among the OsTGAP1 target genes that were downregulated in OsTGAP1ox cells (Table S9). As OsPLDbeta1 and OsWRKY76 negatively regulate the rice defence response against M. oryzae [41,42], OsTGAP1 may contribute to rice defence responses against pathogens by regulating the expression of these genes. In addition, the OsTGAP1 target genes upregulated in OsTGAP1ox cells included several genes related to plant hormone signalling: DWARF AND LOW-TILLERING/OsGRAS32 (Os06g0127800), which relates to brassinosteroid signalling [43], and OsBIF2 (Os12g0614600), which relates to auxin signalling [44] (Table S8). These results raise the possibility that OsTGAP1 impacts the signalling of these plant hormones.

Figure 4. OsTGAP1-binding regions in the OsDXS3 upstream region. (A) The mapped Chip-seq reads in untreated (-, grey) and elicitor-treated (+, black) conditions were visualized using Partek Genomics Suite. Two biological replicates were performed. Black bars indicate the positions of TGACGT sequences. The gene structure of OsDXS3 is shown in the bottom row. Open and closed squares indicate untranslated and coding regions, respectively. Lines indicate introns. (B) Chip-PCR was performed using Chip DNA immunoprecipitated by the anti-OsTGAP1 antibody and normal rabbit IgG. Values indicate the ratio of the amount of DNA in the Chip DNA to the amount in the ‘Input’ control (n = 3); bars indicate the standard deviation of the mean.

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Regulation of MEP pathway genes by OsTGAP1
OsTGAP1 binds to the OsDXS3 promoter via two TGACGT sequences (Figure 5B), and these two TGACGT sequences are involved in the OsTGAP1-dependent induction of OsDXS3 expression (Figure 6C). These results strongly suggest that OsTGAP1 directly regulates OsDXS3 expression. However, a 250-bp fragment and a 2-kbp fragment of the OsDXS3 promoter including the OsTGAP1-binding region exhibited no responsiveness to the elicitor (Figure 6B, Figure S6), despite the increase in OsDXS3 mRNA level due to the elicitor treatment [7]. The regulatory mechanism of the elicitor-induced expression of OsDXS3 requires further explanation.

Figure 5. Gel mobility shift assay using the OsDXS3 upstream region. (A) DNA probes used in GMSA. Closed triangles indicate TGACGT sequences. (B) GMSA was performed using purified recombinant GST-fused OsTGAP1 (GST-OsTGAP1) protein and 32P-labelled DNA probes containing the TGACGT sequences in the OsDXS3 promoter. WT: wild-type probe, m1–m3: mutated probes.

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Identification of OsTGAP1 Target Genes
The rice genome encodes three DXS genes: *OsDXS1*, *OsDXS2*, and *OsDXS3* [45]. Among these three *DXS* genes, *OsDXS3* is the only one upregulated by elicitor treatment [17]. This observation implies that *OsDXS3* is responsible for diterpenoid phytoalexin production and that *OsDXS1* and *OsDXS2* are involved in the biosynthesis of terpenoids for primary metabolism. The *OsTGAP1* target genes identified in this study included *OsDXS3* but not *OsDXS1* and *OsDXS2*. The expression of *OsDXS1* and *OsDXS2* was also unchanged in *OsTGAP1ox* cells (Table S10). These results suggest that the transcriptional regulation of the MEP pathway by *OsTGAP1* mainly contributes to diterpenoid phytoalexin production and that its contribution to the biosynthesis of terpenoids for primary metabolism is limited.

Of the MEP pathway genes, only *OsDXS3* was included among the *OsTGAP1* target genes. Nevertheless, two MEP pathway genes (*OsHDR* and *OsHDS*) were found among the upregulated genes in *OsTGAP1ox* cells (Table S3). Other MEP pathway genes (*OsDXR*, *OsCMS*, *OsCMK*, and *OsMCS*) were also upregulated by approximately 1.5-fold 24 h after elicitor treatment in the *OsTGAP1ox* cells compared to their expression after treatment in WT cells, although these genes were eliminated by the threshold of the data analysis (Table S10). These results indicate that the expression of all seven MEP pathway genes is affected by *OsTGAP1*. However, *OsDXS3* was more strongly upregulated than were the other MEP pathway genes in *OsTGAP1ox* cells. Therefore, the enhanced expression of MEP pathway genes by *OsTGAP1*, except that of *OsDXS3*, may be indirect.

Possible mechanism for the regulation of diterpenoid phytoalexin biosynthetic gene clusters by *OsTGAP1*

*OsTGAP1ox* cells exhibit enhanced expression of all monolactone biosynthetic genes and the phytocassane biosynthetic gene *OsKSL7* [17]. In this study, other phytocassane biosynthetic genes in the cluster (*OsCPS2*, *CYP76M5*-8, and *CYP71Z6*) were found to be upregulated in the *OsTGAP1ox* cells (Table S3), suggesting that *OsTGAP1* functions in the transcriptional regulation of the diterpenoid phytoalexin biosynthetic gene clusters. However, a simple model in which *OsTGAP1* directly regulates the expression of these biosynthetic genes through binding to each promoter region followed by transactivation was not supported by the ChIP-seq analysis (Figure 3, Figure S4 and Figure S5).

Several transcription factor genes were found among the candidate *OsTGAP1* target genes, including *OsWRKY76* (Figure 2E, Table S8, Table S9). Recently, *OsWRKY76* has been reported to negatively regulate diterpenoid phytoalexin biosynthetic genes [42]. Therefore, the downregulation of *OsWRKY76* expression may be among the mechanisms explaining the enhanced expression of diterpenoid phytoalexin biosynthetic genes in *OsTGAP1ox* cells.

Another hypothesis is that the binding of *OsTGAP1* to the intergenic regions of the gene clusters plays a particular role in the transcriptional regulation of diterpenoid phytoalexin biosynthetic genes. In this study, at least 40% of the *OsTGAP1-binding* regions were located within intergenic regions (Figure 1A). This proportion is larger than those observed for the other plant transcription factors (HY5, PIL5, AGL15, and BZR1) whose genome-wide binding regions have been previously reported [33–36]. However, in the case of FHY3, which is a component of phytochrome A signalling, approximately 40% of FHY3-binding regions are located in intergenic regions, and a large portion of these intergenic binding regions are localized in the centromeric regions of the genome [46]. FHY3 binds to the promoter regions of its target genes, thereby regulating phytochrome A signalling and the circadian clock. FHY3 also binds to the centromeric repeats, suggesting that FHY3 has a function beyond regulating the expression of target genes via binding to their promoter regions. Similar to that of FHY3, the binding of *OsTGAP1* to intergenic regions may also have unknown but essential functions. The information presented in this study regarding *OsTGAP1-binding* regions near and in the diterpenoid phytoalexin biosynthetic gene cluster regions will contribute to future research investigating the regulation of these gene clusters by *OsTGAP1*.

Supporting Information

Figure S1  Specificity of anti-*OsTGAP1* antibody.

(PDF)
Figure S2  The enrichment of each motif in OsTGAP1-binding regions. (PDF)

Figure S3  Summary of genes whose expression was altered in OsTGAP1-overexpressing rice cells. (PDF)

Figure S4  OsTGAP1-binding regions around monomilactone biosynthetic genes. (PDF)

Figure S5  OsTGAP1-binding regions around phytothiocassane biosynthetic genes. (PDF)

Figure S6  Transactivation assay using the 2-kbp fragment of the OsDXS3 promoter. (PDF)

Table S1  Plasmids used in this study. (DOCX)

Table S2  Primers used in this study. (DOCX)

Table S3  List of OsTGAP1-binding regions in untreated condition. (XLSX)

Table S4  List of OsTGAP1-binding regions in elicitor-treated condition. (XLSX)

Table S5  List of genes that possess OsTGAP1-binding site within 400 bp upstream region. (XLSX)

References


Table S6  List of genes whose expression is significantly upregulated in OsTGAP1-overexpressing rice cells. (XLSX)

Table S7  List of genes whose expression is significantly downregulated in OsTGAP1-overexpressing rice cells. (XLSX)

Table S8  List of OsTGAP1 target genes whose expression is upregulated in OsTGAP1-overexpressing rice cells. (XLSX)

Table S9  List of OsTGAP1 target genes whose expression is downregulated in OsTGAP1-overexpressing rice cells. (XLSX)

Table S10  Expression profiles of MEP pathway genes from microarray analysis. (XLSX)

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Author Contributions

Conceived and designed the experiments: KM HN H. Yamane KO. Performed the experiments: KM TM AO KK TC. Analyzed the data: KM TM. Contributed reagents/materials/analysis tools: H. Yoshikawa. Contributed to the writing of the manuscript: KM TC H. Yamane KO.


