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Overexpression of the bZIP transcription factor OsbZIP79 suppresses the production of diterpenoid phytoalexin in rice cells

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ABSTRACT

Phytoalexins are antimicrobial specialised metabolites that are produced by plants in response to pathogen attack. Momilactones and phytocassanes are major diterpenoid phytoalexins in rice that are synthesised from geranylgeranyl diphosphate that is derived from the methylerythritol phosphate (MEP) pathway. We have previously reported that rice cells overexpressing the basic leucine zipper (bZIP) transcription factor OsTGAP1 exhibit a hyperaccumulation of momilactones and phytocassanes, with hyperinductive expression of momilactone and phytocassane biosynthetic genes and MEP pathway genes, upon response to a chitin oligosaccharide elicitor. For a better understanding of OsTGAP1-mediated regulation of diterpenoid phytoalexin production, we identified OsTGAP1-interacting proteins using yeast two-hybrid screening. Among the OsTGAP1-interacting protein candidates, a TGA factor OsbZIP79 was investigated to verify its physical interaction with OsTGAP1 and involvement in the regulation of phytoalexin production. An *in vitro* pull-down assay demonstrated that OsTGAP1 and OsbZIP79 exhibited a heterodimeric as well as a homodimeric interaction. A bimolecular fluorescence complementation analysis also showed the interaction between OsTGAP1 and OsbZIP79 *in vivo*. Intriguingly, whereas OsbZIP79 transactivation activity was observed in a transient reporter assay, the overexpression of *OsbZIP79* resulted in suppression of the elicitor-inducible expression of diterpenoid phytoalexin biosynthetic genes, and thus caused a decrease in the accumulation of phytoalexin in rice cells. These results suggest that OsbZIP79 functions as a negative regulator of phytoalexin production triggered by a chitin oligosaccharide elicitor in rice cells, although it remains open under which conditions OsbZIP79 can work with OsTGAP1.

Introduction

When plants are attacked by pathogenic microorganisms they respond with a variety of defensive reactions, including the production of antimicrobial secondary metabolites known as phytoalexins (Ahuja et al., 2012). In rice, momilactones and phytocassanes are major diterpenoid phytoalexins (Kato et al., 1973; Cartwright et al., 1981; Okada, 2011; Yamane, 2013).

In plants, isopentenyl diphosphate and dimethylallyl diphosphate (the basic C5 precursors for terpenoid biosynthesis) are produced via two distinct pathways: the mevalonate pathway, and the methylerythritol phosphate (MEP) pathway (Lichtenthaler et al., 1997). MEP pathway genes exhibit elicitor-induced expression, and the MEP pathway is required for the production of sufficient amounts of diterpenoid phytoalexins in rice (Okada et al., 2007).

Abbreviations: BiFC, bimolecular fluorescence complementation; bZIP, basic leucine zipper; CaMV, Cauliflower Mosaic Virus; CBB, Coomassie Brilliant Blue; FLUC, firefly luciferase; GAL4DBD, GAL4 DNA-binding domain; GST, glutathione *S*-transferase; LUC, luciferase; MBP, maltose binding protein; MEP, methylerythritol phosphate; ORF, open reading frame; qRT-PCR, quantitative RT-PCR; RLUC, *Renilla* luciferase; UBQ, ubiquitin; WT, wild-type; YFP, yellow fluorescent protein.

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In the biosynthesis of diterpenoid phytoalexins, OsCPS2, OsCPS4, OsKSL7, and OsKSL4 catalyse sequential cyclisation of the common precursor, geranylgeranyl diphosphate, to two distinct diterpene hydrocarbons: *ent*-cassa-12,15-diene and 9βH-pimara-7,15-diene (Cho et al., 2004; Otomo et al., 2004a,b). For momilactone biosynthesis, two P450 monooxygenases (CYP99A2 and CYP99A3) and a dehydrogenase (OsMAS) are involved in the downstream oxidation steps of 9βH-pimara-7,15-diene (Shimura et al., 2007; Wang et al., 2011). For phytocassane biosynthesis, four P450 monooxygenases (CYP71Z7, CYP76M7, CYP76M8, and CYP701A8/OsKOL4) are involved in the oxidation of *ent*-cassa-12,15-diene (Swaminathan et al., 2009; Wu et al., 2011; Wang et al., 2012a,b). Interestingly, momilactone and phytocassane biosynthetic genes, except for *OsKOL4*, are localised in narrow regions of chromosomes 4 and 2, respectively, creating functional gene clusters (Shimura et al., 2007; Swaminathan et al., 2009).

Two transcription factors are involved in the regulation of phytoalexin production in rice. The basic leucine zipper (bZIP) transcription factor OsTGAP1 is involved in the transcriptional regulation of MEP pathway genes and momilactone and phytocassane biosynthetic genes, in chitin oligosaccharide elicitor-treated rice cells (Okada et al., 2009). OsTGAP1 directly regulates the expression of *OsDXS3*, which encodes a key enzyme of the MEP pathway by binding to an *OsDXS3* promoter, and probably indirectly regulates other MEP pathway genes (Miyamoto et al., 2014). OsTGAP1 binds to intergenic regions in and near the phytoalexin biosynthetic gene clusters, suggesting that OsTGAP1 does not directly regulate the expression of these biosynthetic genes by binding to each promoter region, and that the bindings of OsTGAP1 to the intergenic regions may play a particular role in the transcriptional regulation of these biosynthetic genes (Miyamoto et al., 2014). The transcription factor OsWRKY76 negatively regulates phytoalexin production in *Magnaporthe oryzae*-infected rice leaves (Yokotani et al., 2013). However, the detailed regulatory mechanism involved in the expression of clustered genes by OsTGAP1 and OsWRKY76 is still unknown.

In *Arabidopsis thaliana* and rice, 75 and 89 members, respectively, of the bZIP transcription factor family have been identified (Jakoby et al., 2002; Nijhawan et al., 2008). *Arabidopsis* bZIP transcription factors have been classified into ten groups (groups A–I and S), based on the sequence similarity of the basic region and the presence of additional conserved motifs (Jakoby et al., 2002). In *A. thaliana*, ten TGA factors (AtTGA1–7, 9, and 10, and PERIANTHIA), which belong to group D bZIP transcription factors, have been reported (Gatz, 2013). Among them, AtTGA1–7 regulates pathogenesis-related genes, such as *PR-1*, and mediates salicylic acid-induced defence responses (Alves et al., 2013). In contrast, AtTGA 9 and 10, and PERIANTHIA, are involved in flower organ development (Gatz, 2013). Regarding rice bZIP transcription factors, 14 members, including OsTGAP1, share conserved motifs with *Arabidopsis* group D bZIP transcription factors (Nijhawan et al., 2008). TGA factors have been found to bind to DNA as homodimers or heterodimers (Katagiri et al., 1992; Niggeweg et al., 2000), although there is a report demonstrating that the closely related TGA factor AtTGA2 and AtTGA3 do not form heterodimers (Johnson et al., 2003). In contrast, our knowledge of the proteins, which interact with rice TGA factors, is limited.

Here, yeast two-hybrid screening was conducted to identify OsTGAP1-interacting proteins. Among the OsTGAP1-interacting protein candidates, we focused on a TGA factor OsbZIP79 and investigated its involvement in the regulation of phytoalexin production. Whereas OsbZIP79 transactivation activity was observed in a transient reporter assay, the overexpression of *OsbZIP79* resulted in suppression of the elicitor-inducible expression of diterpenoid phytoalexin biosynthetic genes, and thus caused a decrease in the accumulation of phytoalexin in rice cells.

Materials and methods

Plants, chemical treatment, and rice transformation

Oryza sativa L. *japonica* 'Nipponbare Kanto BL no. 2' (No. 8935; Plant Variety Protection, the Ministry of Agriculture, Forestry and Fisheries of Japan) was used as the wild-type strain. Suspension-cultured rice cells were maintained as described in a previous paper (Cho et al., 2004). *N*-Acetylchitooctaose was prepared, and treated to rice cells as a chitin oligosaccharide elicitor, as described previously (Ito et al., 1997; Okada et al., 2009). Rice transformation was performed as described previously (Toki et al., 2006). *OsbZIP79*-overexpressing (OsbZIP79ox) rice cells were induced from T1 seeds.

Plasmid construction

The *OsTGAP1* open reading frame (ORF) was amplified by PCR using the primers OsTGAP1 ORF pSos F and OsTGAP1 ORF pSos R. The amplified DNA fragment was directly cloned into the pZErO2 vector (Invitrogen, CA, USA) and sequenced, resulting in pZErO-OsTGAP1. After performing a sequence check, the DNA fragment was excised from pZErO-OsTGAP1 by *Sal*I and *Mlu*I digestion, and was inserted between the corresponding sites of the pSos vector (Agilent Technologies, CA, USA). The resultant plasmid was designated as pSos-OsTGAP1, and used for yeast two-hybrid screening as bait vector.

The *OsbZIP79* ORF was amplified by PCR using the primers OsbZIP79 ORF F and OsbZIP79 ORF R, from pFLC-AK102690 obtained from the Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp/index.html). The amplified DNA fragment was cloned into pENTR/D-TOPO (Invitrogen) according to the manufacturer's protocol and sequenced, resulting in pENTR-OsbZIP79.

The *OsbZIP79* ORF was cloned into pDEST15 (Invitrogen) from pENTR-OsbZIP79 using LR clonase II Enzyme mix (Invitrogen). The resultant plasmid was designated as pDEST15-OsbZIP79, and used for the expression of recombinant N-terminal glutathione *S*-transferase (GST)-fused OsbZIP79 (GST-OsbZIP79).

The *OsTGAP1* and *OsbZIP79* ORFs were amplified by PCR using the following primer pairs: OsTGAP1 ORF pMAL F and OsTGAP1 ORF pMAL R, and OsbZIP79 ORF pMAL F and OsbZIP79 ORF pMAL R. The amplified DNA fragments were cloned into the *Eco*RI and *Xba*I sites of the pMAL-c2x (New England Biolabs, MA, USA), and sequenced. The resultant plasmids were designated as pMAL-OsTGAP1 and pMAL-OsbZIP79, and used for the expression of recombinant N-terminal maltose binding protein (MBP)-fused OsTGAP1 and OsbZIP79 (MBP-OsTGAP1 and MBP-OsbZIP79).

To construct the C-terminal fragment of yellow fluorescent protein (YFP)-fused *OsTGAP1* gene, *OsTGAP1* ORF was cloned into pcYGW (Hino et al., 2011) from pENTR-OsTGAP1 (Okada et al., 2009) using LR clonase II Enzyme mix (Invitrogen). The resultant plasmid was designated as pcYFP-OsTGAP1. To construct the N-terminal fragment of YFP-fused *OsbZIP79* gene, *OsbZIP79* ORF was cloned into pnYGW (Hino et al., 2011) from pENTR-OsbZIP79, using LR clonase II Enzyme mix (Invitrogen). The resultant plasmid was designated as pnYFP-OsbZIP79. These plasmids were used for a bimolecular fluorescence complementation (BiFC) analysis.

To construct the plasmid in which DsRed1 was under the control of the Cauliflower Mosaic Virus (CaMV) *35S* promoter, the green fluorescent protein gene of pTH121 (Zhu et al., 1997) was substituted to the *DsRed1* gene. The resultant plasmid was designated as pTH121R.

To construct the DNA-binding domain of the yeast transcriptional activator GAL4 (GAL4DBD)-fused *OsbZIP79* gene, the *OsbZIP79* ORF was amplified from pENTR-OsbZIP79 by PCR, using the primers OsbZIP79 GAL4 F and OsbZIP79 GAL4 R. The amplified DNA fragment was cloned into the 430T1.2 (Hiratsu et al., 2004) using an