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OsDOG, a gibberellin-induced A20/AN1 zinc-finger protein, negatively regulates gibberellin-mediated cell elongation in rice

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ABSTRACT

The A20/AN1 zinc-finger proteins (ZFPs) play pivotal roles in animal immune responses and plant stress responses. From previous gibberellin (GA) microarray data and A20/AN1 ZFP family member association, we chose *Oryza sativa dwarf rice with overexpression of gibberellin-induced gene* (*OsDOG*) to examine its function in the GA pathway. *OsDOG* was induced by gibberellic acid (GA_3) and repressed by the GA-synthesis inhibitor paclobutrazol. Different transgenic lines with constitutive expression of *OsDOG* showed dwarf phenotypes due to deficiency of cell elongation. Additional GA_1 and real-time PCR quantitative assay analyses confirmed that the decrease of GA_1 in the overexpression lines resulted from reduced expression of *GA3ox2* and enhanced expression of *GA2ox1* and *GA2ox3*. Adding exogenous GA rescued the constitutive expression phenotypes of the transgenic lines. *OsDOG* has a novel function in regulating GA homeostasis and in negative maintenance of plant cell elongation in rice.

Introduction

The A20 zinc-finger domain was first identified in the protein encoded by a tumor-necrosis-factor-inducible gene, A20, in human endothelial cells (Opipari et al., 1990). The AN1 domain was first found at the C terminus of AN1, a ubiquitin-like protein from *Xenopus laevis* (Rebagliati et al., 1985). Proteins containing A20 and/or AN1 zinc-finger domains, referred to as the A20/AN1 zinc-finger proteins (ZFPs), have been found in 22 organisms, including protists, fungi, plants and animals (Vij and Tyagi, 2008). In animals, the role of some A20/AN1 ZFPs in regulating immune responses has been well characterized, including ZNF216 and AWP1 in humans (Duan et al., 2000; Huang et al., 2004; Scott et al., 1998), and ZNF216 in mice (Hishiya et al., 2006).

In plants, genome-wide surveys have revealed several plant species with A20/AN1 ZFP genes inducible by abiotic stresses; examples are 18 rice genes, 14 Arabidopsis genes and 13 tomato genes encoding A20/AN1 ZFPs and 19 poplar genes and 11 maize genes encoding proteins containing at least one AN1 zinc finger (Jin et al., 2007; Solanke et al., 2009; Vij and Tyagi, 2006). Some of the A20/AN1 ZFP genes play an important role in stress tolerance. For example, transgenic plants overexpressing *OsiSAP1* or *OsiSAP8* conferred tolerance to cold, drought and salt stresses (Kanneganti and Gupta, 2008; Mukhopadhyay et al., 2004). Moreover, overexpression of the rice A20/AN1 ZFP gene *ZFP177* in tobacco enhanced tolerance to both low and high temperature stresses while increasing sensitivity to salt and drought stresses (Huang et al., 2008). Although abundant research has shown that A20/AN1 ZFPs may play crucial roles in plant stress responses, little is known about their functions in regulating plant development.

Gibberellins (GAs) regulate several plant growth and development processes such as seed germination, stem elongation, leaf expansion and reproductive development (Sun and Gubler, 2004). Dwarfism is a typical phenotype for mutants with defects in GA-synthesis enzymes such as GA20ox and GA3ox (Sakamoto et al., 2004). Overexpression of *GA2ox1* or *GA2ox3* can inhibit stem elongation in transgenic rice (Sakai et al., 2003; Sakamoto et al., 2001). Moreover, factors that modify GA expression may also affect plant height. For example, *elongated uppermost internode* (*EUI*) encodes a cytochrome P450 monooxygenase that epoxidizes GAs; and *eui1* plants show greatly elongated internodes, especially the uppermost ones (Luo et al., 2006; Zhu et al., 2006). Mutations affecting GA signaling pathways may also alter plant height—mutants with impaired GA signaling are dwarf, whereas those with constitutively

Abbreviations: EST, expressed sequence tag; GA, gibberellin; GUS, β-glucuronidase; OsDOG, Oryza sativa dwarf rice with overexpression of gibberellin-induced gene; PAC, paclobutrazol; PI, propidium iodide; RNAi, RNA interference; ZFP, zinc-finger protein.

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active GA responses display a slender phenotype (Sun and Gubler, 2004).

In this paper, we describe an A20/AN1 ZFP gene from rice, *Oryza sativa dwarf rice with overexpression of gibberellin-induced gene* (*OsDOG*), which negatively regulates GA-mediated cell elongation in rice.

Materials and methods

Plant materials and treatments

Rice variety Zhonghua 10 (*Oryza sativa* L. ssp. *japonica* cv. Zhonghua 10, ZH10) was used in this study. Rice plants were grown on half-strength Murashige and Skoog (MS) medium with 0.7% agar (pH 5.8) under continuous light at 30 °C in a plant growth-chamber or in the field under natural conditions. T_2 and T_3 generations of transgenic rice lines were used in the phenotype analysis and physiological experiments.

RNA extraction and semi-quantitative or real-time PCR

RNA was extracted from rice tissues by use of TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). RT-PCR involved a two-step method: synthesizing the first-strand cDNA with AMV or M-MLV reverse transcriptase (Promega, USA) following the manufacturer's instructions, and amplifying the cDNAs with specific primers. LA Taq polymerase (Takara Bio Inc., Japan) was used for semi-quantitative RT-PCR, and SYBR® Green Realtime PCR Master Mix (Toyobo Co., LTD., Japan) was used for real-time PCR. The primers for *OsDOG* were 5′-CCTCGCTAACCCATTCCCAAAA-3′ and 5′-CCCTCTTTCCATCCAACTTCC-3′, and for *Tubulin* were 5′-TCAGATGCCCAGTGACAGGA-3′ and 5′-TTGGTGATCTCGGCAACAGA-3′. The real-time PCR primers for *OsDOG* were 5′-TGTGAATTGCCCTGACAGCT-3′ and 5′-TGGACCTACCAATAATGCCAG-3′. The real-time PCR primers for *GA3ox2*, *GA2ox1*, *GA2ox2* and *OsActin1* (the internal reference) were the same as those used by Dai et al. (2007).

Construction of OsDOG-overexpressing and RNA-interference (RNAi) rice lines

To generate the overexpression rice lines, the cDNA fragment of *OsDOG* containing the full-length open reading frame (ORF) was cloned with the primers 5′-GGATCCTCTCTCGGA TTCGATCAT-3′ (*Bam*H I site underlined) and 5′-GGTACCCCCTTGTATTATCCTTTTAG-3′ (*Kpn* I site underlined) and were inserted downstream of a maize Ubiquitin promoter in the pUN1301 vector (Ge et al., 2004).

For the RNAi rice construct, the specific fragment of *OsDOG* was amplified with the primers 5′-GGGTACCACTAGTCGTTAGGTTCTAAAAGGA-3′ (*Kpn* I and *Spe* I restriction site underlined) and 5′-GGATCCGAGCTCTTTCCATCCAACTTC-3′ (*Bam*H I and *Sac* I restriction site underlined) and was then inserted into the RNAi vector pTCK303 (Wang et al., 2004). The rice transformants were created as described (Ge et al., 2004).

Cell-length analysis in rice internodes

The middle parts of the internodes were separated from culms of mature rice and stained with propidium iodide (PI). The inner layer of parenchyma cells of the internodes was examined under a laser scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany).

Construction of pOsDOG::GUS and histochemical localization of GUS

The promoter 2 kb upstream of the start codon of *OsDOG* was amplified with the primers 5′-GAGCTCGAAAGGGAGCAGAAGCAGCAG-3′ (*Sac* I site underlined) and 5′-TCTAGAGAGG CGAGGAGAGGGTGAGTC-3′ (*Xba* I site underlined) and then inserted into the 5′-end of the β-glucuronidase (GUS) gene (gusA) in pCAMBIA1301 to generate a vector containing *pOsDOG::GUS*. The construct was then introduced into rice plants and GUS signals were detected as described (Ge et al., 2004).

Quantitative analysis of endogenous GA_1

Shoots of four-leaf-stage seedlings of *OsDOG*-overexpressing lines and the wild type were harvested and ground with use of a mortar and pestle. The homogenized tissue was incubated in 80% acetone for 1 h at 25 °C. The extract was centrifuged at 10,000 × *g* for 5 min, and the supernatants were evaporated under vacuum to a volume of 50 μL (aqueous phase). After adding 350 μL distilled water, the aqueous phase was extracted with ethyl acetate. Particles were removed by centrifugation, and the ethyl acetate-soluble fraction containing GAs underwent high-performance liquid chromatography (Agilent Technologies 1200 series) for purification with a reverse-phase column (Eclipse XDB-C18). After silylation with 20 μL pyridine and 40 μL N,O-bis-(trimetylsilyl) trifluoroacetamide for 40 min at 65 °C, the purified fraction underwent gas chromatography–mass spectrometry with selected ion monitoring with use of a gas chromatography–mass spectrometer (Trace 2000 series, ThermoQuest) equipped with a capillary column (DB-5; Agilent Technologies) after derivatization. The GA_1 level was analyzed by use of ^{2}H-labeled GA_1 as described (Varbanova et al., 2007).

Effect of exogenous GA on the elongation of the second-leaf sheaths, shoots and internodes

Rice seeds were surface sterilized and planted on half-strength MS medium containing optimal concentrations of GA_3 or paclobutrazol (PAC) and incubated under continuous light at 30 °C for 8 d before the length of the second leaf sheath or the shoot length was measured or under continuous darkness at 30 °C for 14 d before the length of the second lower internodes was measured.

Results

Analysis of OsDOG characteristics

Using cDNA microarray analysis, we previously discovered that GA treatment in rice increased the expression of the expressed sequence tag (EST) p782a09 (Wang et al., 2005). This EST corresponds to LOC_Os08g39450 on chromosome 8 at location 24, 821, 089-24, 822, 306 in the rice genome and was designated *Oryza sativa dwarf rice with overexpression of gibberellin-induced gene* (*OsDOG*) on the basis of the phenotypes of its transgenic rice lines. We further confirmed the effect of GA on the expression of *OsDOG* using real-time PCR. The mRNA level of *OsDOG* increased with GA_3 treatment and decreased with PAC, an inhibitor of gibberellin synthesis (Fig. 1A and B). The putative transcript of *OsDOG* is 1218 bp and consists of a 513-bp coding region, a 73-bp 5′ untranslated region and a 632-bp 3′ untranslated region. The putative OsDOG protein contains 170 amino acids (aa) with a predicted molecular weight of 18.144 kDa (http://www.gramene.org). Because A20/AN1 ZFPs exist universally across diverse organisms (Vij and Tyagi, 2008), we analyzed the phylogenetic relation of OsDOG and its orthologs using Mega 4.0 software (Tamura et al., 2007) and found that A20/AN1 ZFPs from plants and animals