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Functional analysis of *OsTUB8*, an anther-specific β-tubulin in rice

Guangxiao Yang [a,1], Asad Jan [a,1], Setsuko Komatsu [a,b,*]

[a] *National Institute of Agrobiological Sciences, Tsukuba 305-8602, Japan*
[b] *National Institute of Crop Science, Kannondai 2-1-18, Tsukuba 305-8518, Japan*

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Abstract

Microtubules play important roles in many cellular processes, such as cell division and cell elongation in plants. β-tubulins (TUB), which are one of the basic components of microtubules, are encoded by multigene family in eukaryotes and their nucleotide sequences are highly conserved in protein coding regions. *OsTUB8* that was expressed in rice anthers was characterized with a multi-level approach. At the protein level, OsTUB8 was expressed mainly in anthers compared to callus, root, leaf sheath and leaf blade. *In situ* hybridization and GUS fusion analysis revealed that *OsTUB8* was expressed in vascular bundles of anther filaments and in pollen. *OsTUB8* expression was lower in the anthers of GA-deficient mutants, 'Tanginbozu' and 'Akibrarewaisei', compared to those of their respective wild types. Transgenic rice expressing *OsTUB8* in an antisense orientation were suppressed in the amount of seed set upon maturity. Antisense-transgenic rice plants were 20–60% shorter compared to the vector-only control. These results suggest that *OsTUB8* might be differentially expressed in rice anthers due to the action of GA, and involved in the processes of vegetative growth and seed set in rice.

Keywords: Anther; Gibberellin; Rice; β-Tubulin 8

1. Introduction

Microtubules participate in a wide variety of cellular functions including the development and maintenance of cell shape, movement of chromosomes during mitosis and meiosis and intracellular transport and cell motility [10,24]. Disruption of plant microtubule organization by drugs or through mutation causes defects ranging from changes in cell shape to marked loss of organ form [20,30]. Microtubules are composed of a backbone of tubulin dimers and microtubule-associated proteins (MAPs) [8]. The basic units of microtubules, α- and β-tubulin (TUA and TUB) heterodimers, are polymerized in a head-to-tail manner to form polarized filaments whose assembly is regulated during cell cycle progression and during cell differentiation. TUA and TUB are encoded by multigene families in most organisms [21]. Some tubulin genes are semi-constitutively expressed; others are differentially regulated by developmental cues and external stimuli. The presence of multiple tubulin genes in plants and animals has raised the question of their functional significance [27,15].

Several isotypes of TUA and TUB have been characterized in many plants [5]. Among them, six TUA (AtTUA) and nine TUB (AtTUB) isotypes in Arabidopsis [18,29] and six TUA (ZmTUA) and eight TUB (ZmTUB) isotypes in maize [32,33] have been comprehensively characterized using unique probes for each gene. In rice, there are eight TUB isotypes and the predicted amino acid sequences of the 27 TUBs from rice, Arabidopsis, maize and soybean shared 82–96% identity with most sequence differences occurring in the carboxyl terminals [35]. Despite the striking similarity at the protein sequence level, these genes are differentially expressed, both spatially and temporally, during growth and development [27]. The different patterns of tubulin gene expression suggest unique roles for different tubulin isotypes in plant growth and development.

Dominant-negative amino acid substitutions in the TUA6 and TUA4 of lefty 1 and lefty 2 mutants of Arabidopsis, respectively, cause a right-handed helical orientation of cortical microtubules in elongating cells [30]. A reduction in the expression of *AtTUA* genes in Arabidopsis has been shown to

Abbreviations: TUB8, β-tubulin 8; GA, gibberellin

* Corresponding author at: National Institute of Crop Science, Kannondai 2-1-18, Tsukuba 305-8518, Japan. Tel.: +81 29 838 7142; fax: +81 29 838 8392.
E-mail address: skomatsu@affrc.go.jp (S. Komatsu).
[1] These authors contributed equally to this work.

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affect root growth and morphogenesis [2]. Arabidopsis expressing TUA modified with fused GFP or hemagglutinin at the N-terminus caused right handed helical growth, increased trichome branching, and a shallow left-handed helical array organization [1]. Similarly, disruption of cortical microtubules by over expression of GFP-tagged TUA in Arabidopsis caused a marked reduction in cell wall thickness and consequently alterations in cell morphogenesis and a severe effect on plant growth and development [3].

The effects of different phytohormones on the morphology and spatial localization of microtubules have been examined in stomatal opening, gravitropic bending and some other physiological processes [17]. Phytohormones are also known to affect the stability of various microtubules subpopulations; GA treatment stabilized microtubules in maize suspension cells [13]. Similarly, GA-enhanced transcripts of total *TUB* genes correlated with GA-induced elongation of oat internode segments [22]. Rice anther is an active biosynthesis site of GA_4 and GA_7 [9], emphasizing the role of GA in regulating anther and/or pollen development.

Previously, Yoshikawa et al. [35] reported on the expression of eight *OsTUB* isotypes in rice. By Northern blot analysis, *OsTUB8* was expressed almost exclusively in anthers; however, its precise function in anther is unknown. In the present study, the expression pattern of OsTUB8 protein was analyzed by using a OsTUB8-specific antibody, and expression of *OsTUB8* was examined using *in situ* hybridization and GUS fusion analysis. OsTUB8 protein was further characterized to determine its function.

2. Materials and methods

2.1. Plant materials and treatments

Wild-type rice (*Oryza sativa* L. cv Nipponbare, cv Ginbozu and cv Akibare) and two rice mutants, Tanginbozu and Akibarewaisei, were grown in the granulated nutrient soil (Kureha Chemical, Tokyo, Japan) under white fluorescent light (600 μmol m^{-2} s^{-1}, 12-h light period/day) at 25 °C and 75% relative humidity in a growth chamber. For anther collection, plants were cultivated in controlled green house. Callus was cultured in N_6 liquid medium and sub cultured every other week.

2.2. Preparation of anti-OsTUB8 antibodies and Western blot analysis

For anti-OsTUB8 specific antibodies preparation, a synthetic peptide of 21 amino acid residues from the C-terminal of OsTUB8 was injected into chicken. The anti-serum obtained was used directly in the Western blot experiment. The protein extracts were separated on 15% SDS-PAGE, and were transferred onto polyvinylidene difluoride (PVDF) membrane (Pall, Port Washington, NY, USA). Anti-chicken IgY peroxidase-linked antibody (Sigma–Aldrich, St. Louis, MO, USA) was used as secondary antibody. Binding of antibody was detected using immunostaining HRP-100 detection kit (Konica-Minolta, Tokyo, Japan).

2.3. In situ hybridization

Mature anthers were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde under vacuum. Fixed samples were then dehydrated through a graded ethanol series followed by a *t*-butanol series, and finally embedded in paraplast. Microtome sections (10 μm thick) were mounted on silicon-coated glass slides (Matsunami, Hamamatsu, Japan). Paraplast was removed through a graded ethanol series. Probes for *in situ* hybridization were labeled with digoxigenin11-UTP (Roche Diagnostics, Manheim, Germany). The 3′-UTR region of *OsTUB8* was PCR amplified using primers 5′-TGATCGATCGATCGATCGATCCCT-3′ and 5′-AGTCCCATGAAAATGAAGTAGATA-3′ and cloned into *Bam*HI and *Xba*I site of pBluescript SK plasmid. The pBluescript SK plasmid was either digested with *Bam*HI and transcribed with T7 RNA polymerase (Stratagene, La Jolla, CA; antisense probe) or digested with *Xba*I and transcribed with T3 RNA polymerase (Stratagene; sense probe). Immunological detection was done with an anti-digoxygenin-AP conjugate and 4-nitrobluetetrazolium (Roche Diagnostics [19]).

2.4. Promoter analysis and GUS localization

To amplify *OsTUB8* promoter fragment, rice genomic DNA was extracted from 1-week-old seedlings, grown on MS medium [23] using DNeasy Plant Mini kit (Qiagen, Hilden, Germany). The expected *OsTUB8* promoter fragment was amplified using primer pairs of 5′-CACCCCCATTATTTGGTGACCCGCCACT-3′ and 5′-CTTCGATCGCTTGCACCTCGATCGATCG-3′ by KOD plus (Toyobo, Osaka, Japan) using PCR conditions; 94 °C for 2 min (1 cycle), 94 °C for 15 s, 63 °C for 30 s, 68 °C for 2 min (30 cycles), 68 °C for 7 min (1 cycle). The amplified fragment was purified from gel using Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA). The OsTUB8 promoter fragment was cloned into binary vector pHGWFS7 (Gateway system, Invitrogen, Carsbad, CA, USA). The resulting OsTUB8::GUS fusion plasmid was then transferred into *Agrobacterium tumefacien* strain EHA 105 [12] and transformed into rice as described [31].

GUS assays were conducted according to Jefferson [14]. For histochemical analysis, transgenic rice plants were incubated in 50 mM sodium phosphate buffer (pH 7.2) containing 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc, Wako Pure Chemical) and 5% methanol at 37 °C for 2 h. The reaction was stopped with ethanol.

2.5. Construction of antisense OsTUB8 transgenic rice

In order to construct *OsTUB8* antisense transgenic rice, the full length of *OsTUB8* cDNA in the pBluescript SK+ plasmid was amplified by PCR using primer pairs of 5′-GCTCTAGACTGGAACATCGTGGGGGTATT-3′ (5′-side, underlining *Xba*I site as linker) and 5′-CGCGAGCTCGCTTATCTAAAATCACACCTGA (5′-side, underlining *Sac*I site as a linker). The resulting PCR product was excised, purified and ligated between the CaMV 35S promoter and nopaline synthase