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# Characterization of β-tubulin 4 regulated by gibberellins in rice leaf sheath

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## Abstract

Tubulins are basic components of microtubules and are encoded by a multigene family in eukaryotes. The expression of *OsTUB4,* one of eight β-tubulin isotypes identified in the rice genome, was characterized. *OsTUB4* was expressed in root primodia and the shoot apical meristem in basal parts of the leaf sheath in rice seedlings. *OsTUB4* transcript abundance in leaf sheath increased by treatment with gibberellic acid ($GA_3$) in a dose- and time-dependent manner. *OsTUB4* transcript levels in gibberellin (GA)-deficient mutants were less than those of wild type rice. An *OsTUB4* promoter*::GUS* assay also confirmed the responsiveness of *OsTUB4* to exogenous $GA_3$, suggesting that *OsTUB4* expression was regulated by GA and may be involved in GA-regulated leaf sheath growth. In addition, OsTUB4 could interact with different specific proteins *in vitro* as assayed by a yeast two-hybrid system, indicating that OsTUB4 may have diverse functions through interaction with different proteins.

*Additional key words:* gene expression, GUS assay, *Oryza sativa*.

## Introduction

Microtubules are the basic components of the eukaryotic cell cytoskeleton and are involved in many cellular processes including cell division, cell elongation, intracellular transport, cell motility and the control of cell shape (Goddard *et al.* 1994, Nick 1998). Tubulin is an important protein in microtubules, which are composed of repeating heterodimers of α-tubulin (TUA) and β-tubulin (TUB). Both forms exist as many isotypes encoded by different genes. TUA and TUB are polymerized to form a filamentous structure and their assembly is regulated during the cell cycle and differentiation. It has been predicted that microtubule assembly as well as stability is regulated through transcription of different isotypes of tubulin, the folding of tubulin monomers, formation of functional dimers and also through post-translational modifications (Nogales 2000). Several lines of evidence have shown that re-alignment of the microtubule cortical array can be induced by a wide range of factors, such as light, plant hormones, cold and wounding (Nick 1998, Wasteneys 2004).

Many genes for TUA and TUB isotypes have been identified in plants (Luduena 1998). Among them, six *AtTUA* and nine *AtTUB* in *Arabidopsis* (Kopczak *et al.* 1992, Snustad *et al.* 1992), six *ZmTUA* and eight *ZmTUB* in maize (Villemur *et al.* 1992, 1994), five *TUA* isotypes in barley (Schroder *et al.* 2001) and eight *TUB* isotypes in rice (Yoshikawa *et al.* 2003) have been comprehensively characterized using unique probes for each gene. Some isotypes of *TUA* and *TUB* displayed tissue-specific expression while others were expressed almost constitutively. *AtTUA* was the most divergent from other members and was expressed preferentially in pollen (Carpenter *et al.* 1992). *AtTUB1* was shown to accumulate primarily in roots and *AtTUB9* was expressed in floral tissue with high levels of expression observed in pollen, elongating pollen tubes and ovules (Cheng *et al.* 2001). Furthermore, in Norway maple, the activation of the cell cycle and the β-tublin accumulation were associated with embryo dormancy breaking (Pawlowski *et al.* 2004).

The effects of different plant hormones on the stability and orientation of microtubules and expression of *TUA* and *TUB* genes have also been examined. Gibberellins

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*Abbreviations*: ABA - absisic acid, BA - 6-benzyladenine; BL - brassinolide; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA - gibberellins; $GA_3$ - gibberellic acid; TUB4 - β-tubulin 4; WT - wild type.

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(GA) and abscisic acid (ABA) caused reorientation of cortical microtubules in epicotyl cells of the decapitated dwarf pea (Sakiyama-Sogo *et al.* 1993). Similarly, GA treatment stabilized microtubules in maize suspension cells against cold and acetylation of TUA (Huang and Lloyd 1999). It has been reported that GA treatment enhanced the transcript abundance of total *TUB* genes in oat that correlated with GA-induced elongation of oat internode segments (Mendu and Silflow 1993). Brassinolide (BL) also up-regulated the mRNA amount of total TUA and TUB, and *AtTUB1* in *Arabidopsis* (Catterou *et al.* 2001). In rice, ABA repressed all transcript levels of TUA and TUB and *OsTUB16* in particular (Giani and Breviario 1996). Although indole-3-acetic acid (IAA) alone did not affect the total TUB transcript abundance in rice, it restored the ABA-mediated decrease in tubulin mRNA levels (Giani *et al.* 1998). The transcripts of *OsTUB5*, *OsTUB6* and *OsTUB7* genes in rice were enhanced by GA treatment, but all eight *OsTUB* genes were repressed by ABA (Yoshikawa *et al.* 2003). This result indicates that various isotypes of TUA and TUB along with microtubule assembly are regulated by different hormones.

Yoshikawa *et al.* (2003) reported the identification of eight *OsTUB* isotypes in the rice genome and the transcripts of these Os*TUB* isotypes showed different expression patterns. In this study, *OsTUB4,* an isotype that was up-regulated by exogenous $GA_3$ in rice leaf sheath, was characterized for its role in the elongation of leaf sheath in rice.

## Materials and methods

**Plants and treatment:** The rice (*Oryza sativa* L. cv. Nipponbare) was used in this study. GA deficient rice mutants Tanginbozu, Syojyotamanishiki, Akibarewaisei, and their wild types Ginbozu, Tamanishiki and Akibare were also used. They were grown in the granulated nutrient soil (*Kureha Chemical*, Tokyo, Japan) under at irradiance (white fluorescent tubes) of 600 μmol $m^{-2}$ $s^{-1}$, 12-h photoperiod, temperature of 25 °C and relative humidity of 75 % in a growth chamber. For anther collection, plants were cultivated in controlled greenhouse. Rice callus (cv. Nipponbare) was cultured in $N_6$ liquid medium and subcultured every other week (Komatsu *et al.* 1996). $GA_3$, ABA, 6-benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D) (*Wako Pure Chemical*, Osaka, Japan) and BL (*Fuji Chemical*, Toyama, Japan) were applied to the substrate. Plant hormone stock solutions were made with dimethyl sulfoxide and control treatment contained the same amount of dimethyl sulfoxide (0.1 % final concentration).

**Antibody preparation and Western blot analysis:** For OsTUB4 specific antibody preparation, a synthetic peptide of 21 amino acid residues to the C-terminal of OsTUB4 was injected into chicken using the standard protocol. The anti-serum obtained was used directly in the Western blot experiment. The protein extracts were separated on 15 % SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane. Anti-chicken IgY peroxidase-linked antibodies (*Sigma-Aldrich*, St. Louis, MO, USA) were used as secondary antibodies. Binding of antibodies were detected using immunostaining *HRP-100* detection kit (*Konica-Minolta*, Tokyo, Japan).

***In situ* hybridization:** Shoot tissues taken from 10-d-old seedlings 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 Co*., Hamamatsu, Japan). Paraplast was removed through a graded ethanol series. Probes for *in situ* hybridization were labeled with digoxigenin-11-UTP (*Roche*, Mannheim, Germany). 3'-UTR fragment was PCR-amplified using primers of 5'-TAAGGTGGCTTTTGCTTGGTGGTT-3' and 5'-AACGCGGAAAGGTAAAATTCATGG-3' into *Bam*HI and *Xba*I site of pBluescript SK plasmid. The plasmid was either treated with *Bam*HI or transcribed with T7 RNA polymerase (*Stratagene*, Cedar Creek, TX, USA) (antisene probe) or digested with *Xba*I and transcribed with T3 RNA polymerase (*Stratagene*) (sense probe). Immunological detection was done with an anti-digoxigenin-AP conjugate and 4-nitroblue tetrazolium (*Roche*) (Kouchi and Hata 1993).

**RNA extraction and Northern blot analysis:** Tissue samples were quick-frozen in liquid nitrogen. Samples were ground to powder using a mortar and pestle, and total RNAs were isolated according to the procedure described by Chomczynski and Sacchi (1987). For Northern blot analysis, 20 μg of total RNA was separated on 1.2 % agarose containing 6 % formaldehyde and transferred onto *Hybond*$^{TM}$-$N^+$ nylon membrane (*GE Healthcare*, Piscataway, NJ, USA). Loading of equal amounts of total RNA for Northern blots was determined by visualization of ethidium bromide-stained rRNA bands. Gene-specific probe for *OsTUB4* was PCR-amplfied from cDNA using 5'-TAAGGTGGCTTTTGC -3' as forward primer and T3 as a reverse primer. PCR product was purified from agarose gel (*Qiagen*, Hilden, Germany), and radio labeled using [α-$^{32}$P] dCTP (GE healthcare) and the random prime labeling system (*Rediprime*$^{TM}$ II, *GE Healthcare*). Hybridization was performed at 42 °C in an ultrasensitive hybridization buffer (*ULTRAhyb*$^{TM}$, *Ambion*, Austin, TX, USA) overnight. The blots were washed twice in 2× SSC, 0.1 % SDS at 42 °C for 5 min, and in 0.1× SSC, 0.1 % SDS at 68 °C for 15 min, and then analyzed by phosphor image program using *Typhoon 8600k* variable imager (*GE Healthcare*).

**Promoter analysis:** *OsTUB4* promoter fragment (2 000 bp) was PCR amplified from rice genomic DNA with primer pairs of 5'-CACCCTCCACAACGACATCC TGTCCAT-3' and 5'-CTTTGCACTGCAAAGGGGAAGT GGAACA-3' using *KOD plus* (*Toyobo*, Osaka, Japan) the 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 PCR product was purified from agarose gel (*Qiagen*) and cloned into *Gateway* entry vector pENTR/SD-TOPO (*Life Technologies*, Carlsbad, CA, USA) and then into binary vector pHGWFS7 through LR recombination (Karimi *et al.* 2002). The resulting vector carrying *OsTUB4* promter::GUS fusion was transformed into rice *via Agrobacterium*-mediated transformation (Komari *et al.* 1998).

GUS staining was conducted according to Jefferson (1997). Seedlings of transgenic 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 (*Wako Pure Chemical*) and 5 % methanol at 37 °C for 24 h. The reaction was stopped by adding ethanol.

**GUS activity assay:** 10-d-old *OsTUB4* promoter*::GUS* transgenic rice seedlings on MS medium were replaced to the solutions without or with 5, 10 and 50 μM $GA_3$. At 24 h after $GA_3$ treatment, plants were homogenized in extraction buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1 % sodium *n*-lauroylsarcosine and 0.1 % *Triton X-100*. To assay GUS activity, 0.15 $cm^3$ of extract was mixed with 0.2 $cm^3$ of 1 mM 4-methylumbelliferyl β-D-glucuronide in extraction buffer prewarmed to 37 °C. The mixture was incubated at 37 °C for 30 min, and 0.1 $cm^3$ mixture was mixed with 2 $cm^3$ of stop solution (0.2 M sodium carbonate). The resulting fluorescence was measured with *MTP-100F* microplate reader (*Corona Electric*, Ibaraki, Japan) at 365 (excitation) and 465 (emission) nm. Protein content was determined according to Bradford (1976) to normalize GUS activity.

**Yeast two-hybrid assay:** The *BD Matcher*™ library construction and screening kits (*Clontech*, Palo Alto, CA, USA) was used to screen for the *in vitro* interaction between OsTUB4 and proteins obtained from cDNA library prepared from rice leaf sheath including basal part of seedlings. All procedures were performed according to the manufacturer's protocol. Briefly, full-length *OsTUB4* cDNA (Catterou 2001) was fused in-frame with GAL4 DNA binding domain in the pGBKT7 vector. cDNA was cloned into pGADT7 vector encoding the GAL4 activation domain. The GAL 4 fusion constructs were used for the transformation of yeast strain AH109 and the cells were plated on synthetic medium. Screening for the protein-protein interaction events was performed according to the manufacturer's instructions.

## Results

**OsTUB4 expression pattern:** *OsTUB* transcripts were previously shown to have different expression patterns in different tissues while *OsTUB4* was mainly expressed in the leaf sheath (Yoshikawa *et al.* 2003). The OsTUB4 expression pattern at the protein contents were examined using an OsTUB4- specific antibody raised against a synthetic peptide of 21 amino acid residues of the C-terminal of OsTUB4 since this region amongst eight OsTUB isotypes was not conserved. Different contents of OsTUB4 protein were observed with moderate expression in the leaf sheath including the basal part of 10-d-old seedlings, weak expression in callus and root but no detectable signal in leaf blade and mature anther (Fig. 1*A*). This localization pattern of OsTUB4 protein was quite similar to its transcript expression pattern detected previously (Yoshikawa *et al.* 2003). Next, in order to determine whether *OsTUB4* mRNA was expressed in specific tissues, *in situ* hybridization was performed. Longitudinal sections of shoots from 10-d-old seedlings were hybridized to Dig-labeled antisense RNA prepared from the 3′-UTR of *OsTUB4* cDNA, and Dig-labeled sense RNA was used as a negative control (Fig. 1*B*). Higher levels of *OsTUB4* mRNA were expressed in root primodia and the shoot apical meristem, where cells vigorously undergo division and/or elongation.

**Effect of $GA_3$ treatments on OsTUB4 expression:** In oats, GA-enhanced transcripts of total *TUB* genes and increased *TUB* expression correlated with GA-induced elongation of internode segments (Mendu and Silflow 1993). To examine whether $GA_3$ could enhance *OsTUB4* expression in rice, a Northern blot analysis was carried out using total RNA extracted from leaf sheath of 7-d-old seedlings treated with 1, 5, 10 and 50 μM $GA_3$ for 24 h. *OsTUB4* expression was enhanced dose-dependently by $GA_3$ treatment and $GA_3$ at the concentration of 10 μM was optimum (Fig. 2*A*).

To examine dynamic changes of *OsTUB4* expression as a result of $GA_3$ treatment, a Northern blot analysis was carried out using total RNA extracted from leaf sheath of 7-d-old seedlings treated with 10 μM $GA_3$ for 0, 1, 3, 6, 12, 24 and 48 h, respectively. Although there is stronger signal for control after 12 h, *OsTUB4* transcripts were clearly induced 6 h after $GA_3$ treatment and this increase was maintained at a higher level even at 48 h (Fig. 2*B*).

**OsTUB4 expression in GA-deficient mutants:** *OsTUB4* was always expressed at relatively higher levels in the leaf sheath tissue of rice seedlings. To examine whether endogenous GAs regulate *OsTUB4* expression, *OsTUB4* transcript abundance was compared in GA-deficient rice

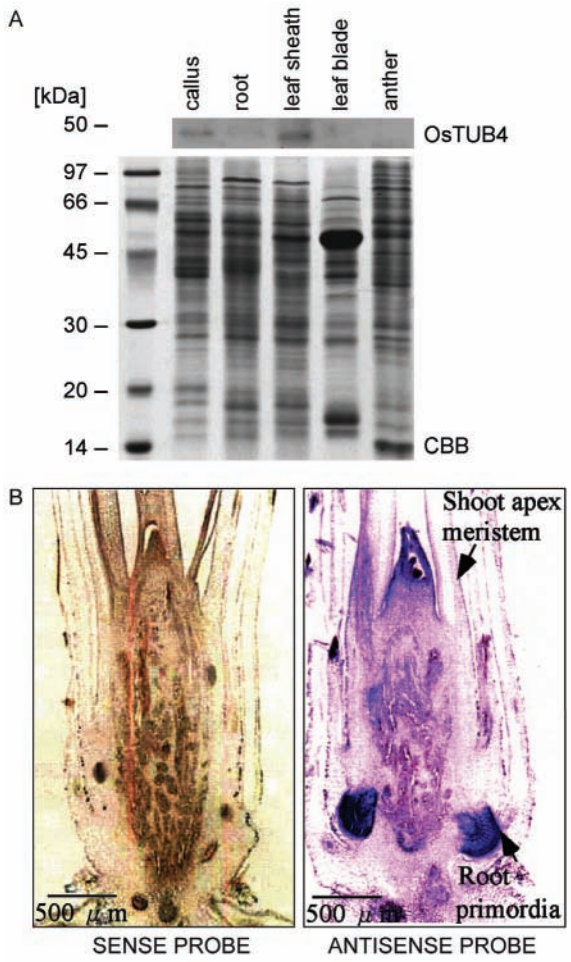


Fig. 1. Tissue specific expression of OsTUB4 protein and *OsTUB4* mRNA localization. Crude proteins were extracted from callus, or from root, leaf sheath and leaf blade of 1-week-old seedlings, or from mature anther. Western blot was carried out using anti-OsTUB4 antibody, and CBB stained proteins were shown to verify equal loading (*A*). Longitudinal sections of shoot from 10-d-old seedlings were hybridized to Dig-labeled antisense RNA prepared from 3'UTR of *OsTUB4* cDNA (*B right*). Dig-labeled sense RNA was used as a negative control (*B left*).

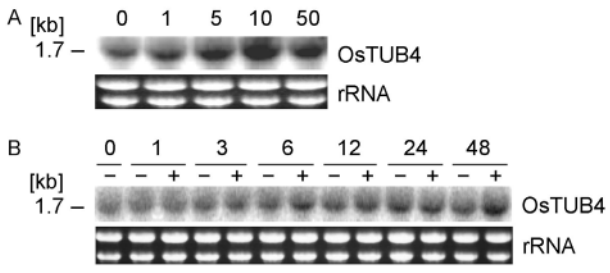


Fig. 2. Dose dependent and time-course changes in *OsTUB4* expression in rice seedlings after $GA_3$ treatment. 1-week-old seedlings were treated with 0, 1, 5, 10 and 50 μM $GA_3$ for 24 h (*A*) or with 10 μM $GA_3$ for 0, 1, 3, 6, 12, 24 and 48 h (*B*). Total RNAs (20 μg per lane) were probed with specific probe prepared from 3'UTR of *OsTUB4* cDNA. rRNA stained with ethidium bromide was used as loading control.

mutants, Tanginbozu, Shojyotamanishiki, Akibarewaisei and their respective WT Ginbozu, Tamanishiki and Akibare (Fig. 3). Tanginbozu and Shojyotamanishiki are semi-dwarf and Akibarewaisei is a severe dwarf mutant (Fig. 3*A*). Northern blot analyses were carried out using total RNAs extracted from leaf sheaths of one-week-old seedlings. Compared to their respective wild types, *OsTUB4* transcript abundance in Tanginbozu and Shojyotamanishiki was low, and *OsTUB4* transcript levels in Akibarewaisei was very low (Fig. 3*B*). This result indicated that *OsTUB4* expression was regulated by endogenous GA.

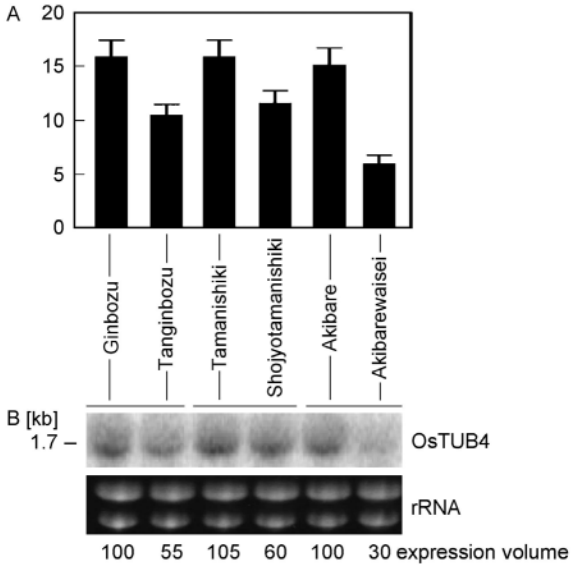


Fig. 3. Comparison of *OsTUB4* transcript in GA deficient rice mutants and their wild types. $GA_3$ deficient rice mutants Tanginbozu, Shojyotamanishiki, Akibarewaisei and their wild type Ginbozu, Tamanishiki and Akibare were used (*A*). Total RNAs was extracted from leaf sheath of one-week-old seedlings and probed with *OsTUB4* specific probe (*B*). rRNA stained with ethidium bromide was used as loading control.

**Response of OsTUB4 promoter::GUS to exogenous $GA_3$:** Based on rice genomic DNA sequence information, a 2000 bp fragment flanking the 5′ side of the *OsTUB4* transcription initiation site was PCR-amplified and cloned into a binary vector pHGWFS7 (Fig. 4*A*). The resulting plasmid carrying *OsTUB4* promoter*::GUS* fusion was transformed into rice *via Agrobacterium*- mediated transformation. Strong GUS activity as reflected by GUS staining, was observed in the leaf sheath including the basal part of rice seedlings and weaker staining in root was also observed (Fig. 4*B*). Whole seedlings of 35S CaMV promoter::GUS were used as a positive control (Fig. 4*B*), and no staining was observed in promoter-less GUS construct used as negative control (Fig. 4*B*). This result was consistent with that of *OsTUB4* transcript and protein expression analyses.

Next, *OsTUB4* promoter*::GUS* transgenic seedlings grown on MS solid medium were treated with 5, 10 and 50 μM $GA_3$ for 24 h, and $GA_3$-induced GUS activity was

examined. Exogenous $GA_3$ induced GUS activity dose dependently in the range of $GA_3$ concentrations examined (Fig. 4*C*). The GUS activity assays confirmed the responsiveness of *OsTUB4* to $GA_3$ and also show that the 2000 bp *OsTUB4* promoter fragment was sufficient to confer $GA_3$- enhanced reporter gene expression.

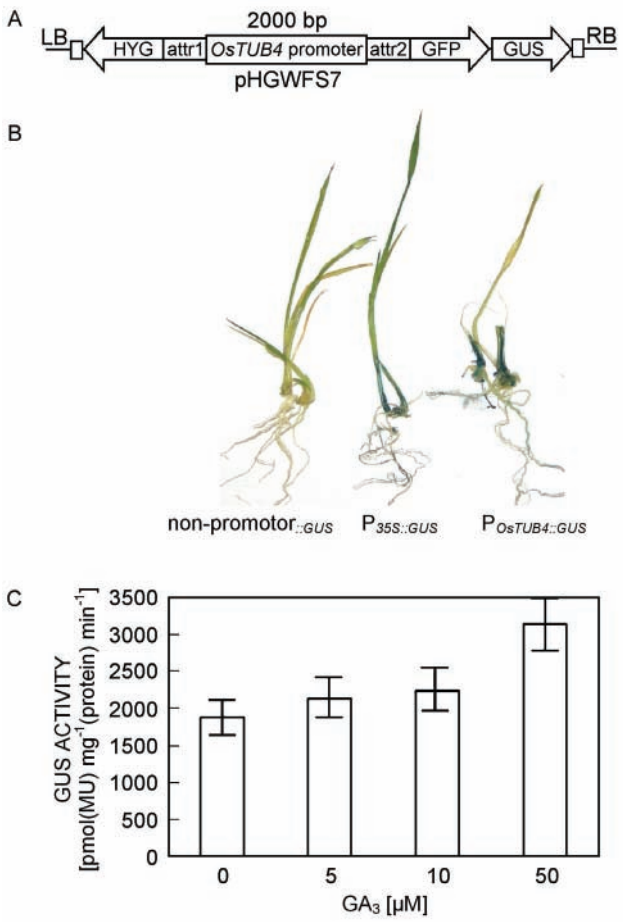


Fig. 4. *OsTUB4* promoter::GUS analysis. *A* - Two thousand base pairs of 5' *OsTUB4* genomic sequence was amplified by PCR from genomic DNA and cloned into binary vector pHGWFS7. *B* - The expression pattern of GUS staining. 35SCaMV promoter::GUS and promoter-less GUS construct was used as positive and negative control respectively. One-week-old seedlings of transgenic plants were incubated in GUS staining buffer. Reporter GUS expression in response to $GA_3$ treatment was measured. *C* - Dose dependency of GUS activity. 10-d-old seedlings were treated with 0, 5, 10 and 50 μM $GA_3$ for 24 h. GUS activity was measured by fluorescent method. Values are the means of 4 plants ± SE, and the experiment was repeated twice.

**Screening for OsTUB4 interacting proteins using yeast two-hybrid analysis:** A full-length *OsTUB4* cDNA fused with the DNA-binding domain of GAL4 was expressed from the yeast plasmid GBKT7. The cDNA library from rice leaf sheath including basal part of seedlings, where OsTUB4 was mainly expressed, was screened for proteins that interacted with OsTUB4. A total of 13 positive cDNA clones were obtained, sequenced and subjected to homology search. Seven cDNA clones out of 13 positively interacting cDNAs showed an identical nucleotide sequence that is represented in the rice full-length cDNA database (AK119301). A *BLAST* search for this cDNA in the rice genomic sequence database gave a perfect match with a sequence encoded by a BAC clone (AP005908) located on chromosome 7. The full-length cDNA has an ORF of 1090 amino acid residues with unknown function; it was therefore designated OsTUB4intP1. OsTUB4intP1 was predicted as a nuclear-localized protein by PSORT (Nakai and Kanehisa 1991) with 70 % possibility. Two cDNA clones out of 13 positively interacting cDNAs showed an identical nucleotide sequence also present in the rice full-length cDNA database (AK103688). The protein encoded by this cDNA is designated OsTUB4intP2 and contains a tetratricopeptide repeat (TPR) that functions as a protein-protein interaction domain (Das *et al.* 1998).

The effect of different plant hormones on expression of OsTUB4 and the genes of its interacting proteins was examined by treatment of leaf sheaths with 10 μM $GA_3$, 1 μM BL, 5 μM BA, 5 μM 2,4-D and 5 μM ABA for 24 h (Fig. 5). $GA_3$ specifically up-regulated the expression of OsTUB4 and the genes of its interacting proteins, but there was little effect of the other plant hormones on their expression.

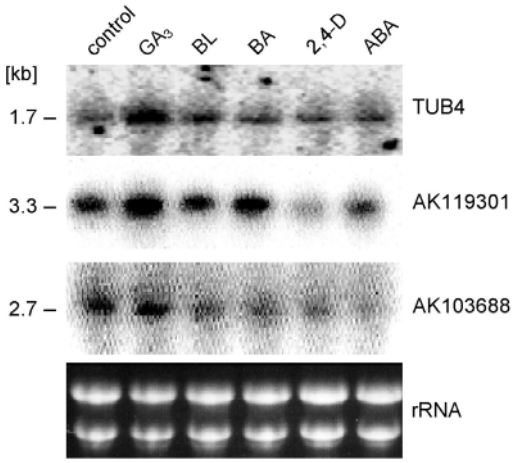


Fig. 5. Hormonal regulated expression of OsTUB4 and its interacting proteins. Rice leaf sheath segments were treated without or with 10 μM $GA_3$, 1 μM BL, 5 μM BA, 5 μM 2,4-D and 5 μM ABA for 24 h. Total RNA was extracted from leaf sheath segment of 1-week-old seedling and probed with the OsTUB4, AK119301 (OsTUB4intP1) or AK103688 (OsTUB4intP2). rRNA stained with ethidium bromide was used as loading control.

## Discussion

In eukaryotes, tubulins are important components of microtubules that are involved in cell division, cell motility and cell morphogenesis (Goddard *et al.* 1994). *TUA* and *TUB* genes are the principal components of microtubules and are encoded by dispersed families of related genes in higher eukaryotes (Luduena 1998). Eight *OsTUB* isotypes were identified in the rice genome and the transcripts of these Os*TUB* isotypes showed different expression patterns while *OsTUB4* was primarily expressed in leaf sheaths (Yoshikawa *et al.* 2003). In the present study, *OsTUB4* was selected for further characterization. The localization pattern of OsTUB4 protein, as revealed by Western blot analysis, was quite similar to its transcript expression pattern detected previously (Yoshikawa *et al.* 2003) (Fig. 1). *OsTUB4* transcript was increased by $GA_3$ dose- and time-dependently (Fig. 2). *OsTUB4* transcript abundance in GA-deficient mutants was less than that of wild type rice (Fig. 3). An *OsTUB4* promoter*::GUS* assay also confirmed the responsiveness of *OsTUB4* to exogenous $GA_3$ (Fig. 4), suggesting that *OsTUB4* expression is regulated by $GA_3$, although no known GA response element was found within the 2000 bp promoter region.

Tubulins play a crucial role in plant development and their expression and post-transcriptional modification are tightly regulated. Although the eight *OsTUBs* are highly conserved at the amino acid level, their expression showed differential and tissue-specific patterns (Yoshikawa *et al.* 2003). *OsTUB4* at both the transcriptional and translational levels was primarily expressed in leaf sheaths, indicating its possible special role in regulating the growth of the leaf sheath. GA is an important factor in determining the heights of plants including monocot rice. Leaf sheaths of 2-week-old rice seedling treated with 5 μM $GA_3$ for 24 h elongated by almost 100 % (Shen *et al.* 2003). It has been demonstrated that GA treatment could stabilize micro- tubules in maize suspension cells (Huang and Lloyd 1999). GA-enhanced transcript abundance of total *TUB* genes correlated with GA-induced elongation of oat internode segments (Mendu and Silflow 1993). Furthermore, Gallardo *et al.* (2002) reported that α-2,4 tubulin and β-2 tubulin in *Arabidopsis* appeared to depend on the action of GAs, and an increase in this protein was noted for the wild-type seeds but not for the GA-deficient mutant, *ga1* seeds in incubated for 1 d on water. Our study and previous report indicated that GAs appeared to be involved in GA-regulated leaf sheath growth, directly or indirectly, in controlling the tubulin.

Plant microtubules are highly dynamic and their stability depends on the activity of various microtubule-associated proteins (MAPs). Several MAPs have been identified, including 65kDa MAPs, MAP190 and MOR1 (Hussey *et al.* 2002). Many other proteins are also known to bind to microtubes but do not function as MAPs. These proteins are often called microtubule-interacting proteins (MIPs) (Mendu ans Silflow 1993), and likely bind to microtubules to regulate their activities such as their subcellular localization or accumulation at a specific location within a cell (Chuong *et al.* 2004). Li *et al.* (2007) reported that pull-down and co-sedimentation experiments demonstrated that AtMAP65-1 bound to tubulin dimers, at a molar ratio of 1:1, and cross-linking experiments showed that AtMAP65-1 bound to tubulin dimers by interacting with α-tubulin of the tubulin heterodimer. Furthermore, Dhonukshe *et al.* (2006) reported that *Arabidopsis* tubulin folding cofactor β interacted with α-tubulin *in vivo* and its overexpression reduced the number of microtubules.

A large-scale proteomics project identified 122 proteins purified by tubulin affinity chromatography (Chuong *et al.* 2004). This large number of potentially interacting proteins suggests that tubulins fulfill numerous roles besides coordinating cell division and morphogenesis. Two OsTUB4 interacting proteins were identified using a yeast two-hybrid assay in the present study and the expression of both was up-regulated by $GA_3$ treatment (Fig. 5). Further characterization of these proteins will provide information about their function and the special function of OsTUB4 as well. *OsTUB4* may be involved in GA-regulated growth processes. Identification of OsTUB4 interacting proteins provides clues for investigating the special functions of OsTUB4 microtubules other than coordinating cell division and morphogenesis.

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