

Tissue-Preferential Expression of a Rice α -Tubulin Gene, *OsTubA1*, Mediated by the First Intron¹

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The genomic clone encoding an α -tubulin, *OsTubA1*, has been isolated from rice (*Oryza sativa* L.). The gene consists of four exons and three introns. RNA-blot analysis showed that the gene is strongly expressed in actively dividing tissues, including root tips, young leaves, and young flowers. Analysis of chimeric fusions between *OsTubA1* and β -glucuronidase (*GUS*) revealed that the intron 1 was required for high-level *GUS* expression in actively dividing tissues, corresponding with normal expression pattern of *OsTubA1*. Fusion constructs lacking the intron 1 showed more *GUS* staining in mature tissues rather than young tissues. When the intron 1 was placed at the distal region from 5'-upstream region or at the 3'-untranslated region, no enhancement of *GUS* expression was observed. Sequential deletions of the *OsTubA1* intron 1 brought about a gradual reduction of *GUS* activity in calli. These results suggest that tissue-preferential expression of the *OsTubA1* gene is mediated by the intron 1 and that it may be involved in a mechanism for an efficient RNA splicing that is position dependent.

Microtubules are the basis of morphogenetic processes that occur by the definition of the plane of cell division and the direction of cell elongation in plant cells. Microtubules are mainly composed of α - and β -tubulin, which have genes that have been conserved through evolution (Little and Seehaus, 1988). α -Tubulin genes have been characterized from several plant species including *Chlamydomonas reinhardtii* (Silflow et al., 1985), Arabidopsis (Ludwig et al., 1988; Kopczak et al., 1992), maize (Montoliu et al., 1989, 1990, 1992; Villemur et al., 1992), *Prunus amygdalus* (Stöcker et al., 1993), and rice (*Oryza sativa* L.; Qin et al., 1997). As shown previously in vertebrates, plant α -tubulin genes form a multigene family; for example, six α -tubulin genes exist in Arabidopsis, and at least seven in maize. Previous studies in Arabidopsis have shown that certain α -tubulin genes are tissue specific, whereas others are constitutively expressed in all tissues (Ludwig et al., 1988; Kim and An, 1992; Kopczak et al., 1992). In maize, two α -tubulin genes are preferentially expressed in the radicular system (Montoliu et al., 1989). It has also been reported in maize that certain α -tubulin genes are preferentially expressed in rapidly dividing tissues such as root tips rather than in more mature tissues (Joyce et al., 1992). Generally, the expression

of most α -tubulin genes in plants has been detected preferentially in actively dividing tissues.

Recent studies have shown evidence that the sequences downstream of the transcription initiation site often play important roles on gene expression in plants. The pea ferredoxin (*Fed-1*) gene requires exon sequences for light response (Dickey et al., 1994). In the pea plastocyanin (*PetE*) gene, both the coding region and the 5'-untranslated leader region have been found to be required for light regulation (Helliwell et al., 1997). A light-responsive element of the tobacco *psaDb* gene has been located within the coding region (Yamamoto et al., 1997). The *Me1* gene from *Flaveria bidentis* requires an enhancer-like element in the 3'-flanking region for high-level expression in leaves (Marshall et al., 1997). In *Sesbania rostrata*, nodule parenchyma-specific expression of *SrEnod2* gene is mediated by the 3'-untranslated region (Chen et al., 1998).

It has been reported that some introns in plant genes increase gene expression. Expression enhancements by an intron have been found in maize *Adh1* (Callis et al., 1987; Luehrsen and Walbot, 1991), *Sh1* (Vasil et al., 1989; Maas et al., 1991), and *Ubi1* (Christensen et al., 1992), rice *Act1* (McElroy et al., 1990), and the *PAT1* gene of Arabidopsis (Rose and Last, 1997). In addition to enhancement effects, cases in which an intron was required for regulated- or tissue-specific gene expression in plant genes have also been found. For example, the *PsaD* gene of the spinach plant requires an intron sequence for plastid- and light-dependent expression (Bolle et al., 1996). An intron of the *AGAMOUS* (*AG*) floral homeotic gene in Arabidopsis contains enhancer sequences for

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specific gene expression in flowers (Busch et al., 1999).

In this study, we have analyzed β -glucuronidase (GUS) expression in transgenic rice plants carrying various *OsTubA1::GUS* fusion constructs. From this analysis, we have found that actively dividing tissue-preferential expression of *OsTubA1* is controlled by the intron 1.

RESULTS

Isolation of the Rice α -Tubulin Gene *OsTubA1*

Five rice α -tubulin cDNA clones were isolated from an immature anther cDNA library (Chung et al., 1994) using the Arabidopsis α 1-tubulin gene as a probe (Ludwig et al., 1988). Because the nucleotide sequences of the 3'-untranslated regions are not conserved among α -tubulin genes, we categorized the clones into three different groups by sequence analyses of the regions. Three of the clones were identical to *OsTubA1* and the other two clones, *OsTubA2* and *OsTubA3*, out of previously published clones (Qin et al., 1997).

As the first step in examining the diversity of expression patterns among the various α -tubulin genes, we isolated a genomic clone corresponding to the *OsTubA1* cDNA clone using 3'-untranslated region as the gene-specific probe. The 4.3-kb *XbaI-PstI* fragment, containing the entire coding region along with the flanking regions, was subcloned into the pBlue-script SK(-) vector. Sequencing of the genomic clone revealed that nucleotide sequences of the gene are different from the *OsTubA1* cDNA at one site (1,102) of the 5'-untranslated region and at two sites (2,256 and 3,604) of the coding region. Consequently, instead of Ser, the 52nd and 437th amino acids of *OsTubA1* were Phe and Ala in the present clone. It is likely that the differences between the two clones may have resulted from the different cultivars used. It can be concluded that our clone is a variant genomic clone of *OsTubA1* (Qin et al., 1997).

Comparing *OsTubA1* with the other cDNA clones revealed that the gene consisted of four exons and three introns in the coding region (accession no. AF 182523). The first intron is 946 bp long and located between codons 31 and 32. The 86-bp second intron and the 108-bp third intron are located at codon 110 and between codons 233 and 234, respectively. The number and position of these introns were identical to those of previously identified α -tubulin genes, such as Arabidopsis *TUA2* (Kopczak et al., 1992) and *Zea mays Tub α 1* and *Tub α 2* (Montolieu et al., 1989). All three introns of *OsTubA1* present the consensus 5'-GT and 3'-AG recognition signals as reported in the literature (Hanley and Schuler, 1988).

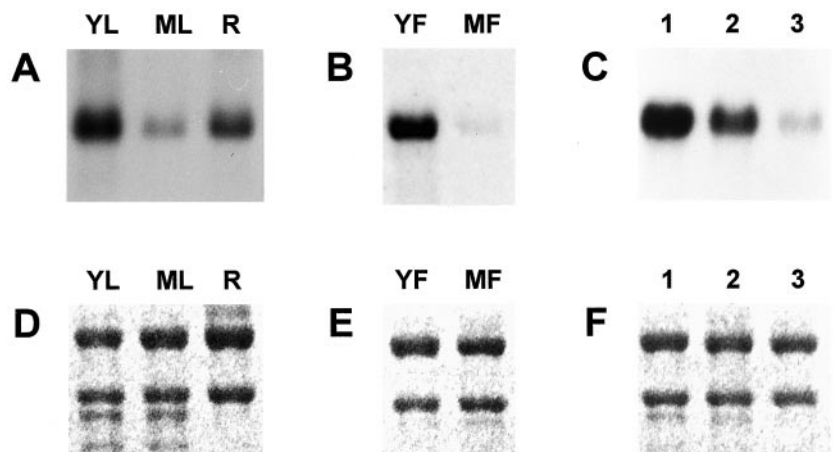
Expression of *OsTubA1* in Rice

To examine the expression pattern of *OsTubA1*, RNA gel-blot analysis was carried out using various tissues of rice (Fig. 1). The result showed that *OsTubA1* RNA was distributed abundantly in roots and leaves of 7-d-old seedlings and flowers in the early to mature pollen stage and was distributed poorly in mature leaves and flowers at the heading stage (Fig. 1, A and B). This suggests that *OsTubA1* may be expressed mainly in rapidly dividing tissues. To confirm the premise, further experiments were carried out using total RNA isolated from manually dissected samples of dividing, elongating, and maturing cells of 7-d-old seedling roots (Fig. 1C). RNA gel-blot analysis demonstrated that *OsTubA1* RNA was accumulated mainly at root tips, supporting that the gene is expressed preferentially in actively dividing tissues: root tips, young leaves, and flowers of the meiotic to mitotic pollen stage during rice growth and development.

Effect of Intragenic Region on *GUS* Expression in Transgenic Rice Plants

The spatial expression pattern of *OsTubA1* was investigated using *GUS* reporter gene fusions. Two

Figure 1. RNA gel-blot analyses of the *OsTubA1* gene. A, Expression of *OsTubA1* mRNA in young leaves (YL) of 7-d-old seedlings, mature leaves (ML) of flowering plants, and roots (R) of 7-d-old seedlings. B, Expression of *OsTubA1* mRNA in young flowers (YF) at less than 10-cm panicle size and mature flowers (MF) at the heading stage. C, Expression of *OsTubA1* mRNA in roots dissected in dividing (1), elongating (2), and maturing zones (3) of 7-d-old roots. Each 10- μ g portion of total RNAs isolated from various tissues was blotted onto a nylon membrane and hybridized to the 32 P-labeled *OsTubA1* gene-specific probe. D to F, Ethidium bromide staining of 25S and 17S rRNAs demonstrating equal amounts of RNA loading in A to C, respectively.



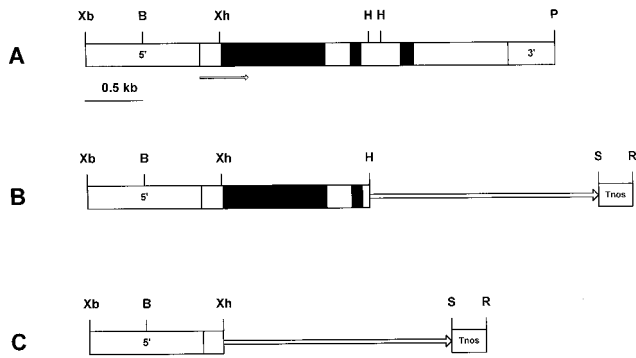


Figure 2. Restriction map of the *OsTubA1* gene and construction of the chimeric plasmid between the *OsTubA1* promoter and *GUS* gene. A, Restriction map of the *OsTubA1* gene. The coding regions and introns are represented by white and black bars, respectively. The promoter (5') and terminator (3') regions are shown. The arrow indicates direction of transcription. B, Diagram of pGA2135 carrying upstream sequences, exon 1 and 2, intron 1 and 2, a portion of exon 3 of *OsTubA1*. The *Hind*III site of the exon 3 of the gene is fused to the *GUS* gene. C, Diagram of pGA2136 carrying upstream sequences and a portion of exon 1 of *OsTubA1*. The *Xho*I site of the exon 1 of the gene is fused to *GUS*. *Tnos* signifies the *nopaline synthase* (*nos*) terminator. The *GUS* coding region is indicated by a solid line with an arrow. Letters represent restriction enzyme sites: B, *Bam*HI; H, *Hind*III; P, *Pst*I; R, *Eco*RI; S, *Sac*I; Xb, *Xba*I; Xh, *Xho*I.

translational fusions, pGA2135 and pGA2136, of *OsTubA1* to the *GUS* gene were made to test the influence of intragenic sequences on gene expression (Fig. 2). The only difference between the two constructs was the length of the included intragenic region. pGA2136 contained only the first 27 codons, whereas

pGA2135 included all of exon 1 and 2, intron 1 and 2, and 15 codons of exon 3. The fusions were introduced into rice by *Agrobacterium*-mediated transformation, and more than 10 lines of each construct were analyzed by histochemical *GUS* staining.

In pGA2135 plants, intense *GUS* staining was observed from three different parts: actively growing tips of the primary roots, lateral roots at the initiation stage, and actively growing lateral roots (Fig. 3, A–C). This coincided with the distribution pattern of the *OsTubA1* RNA in wild-type rice (Fig. 1C). In contrast to the staining pattern of pGA2135, plants transformed with pGA2136 showed more *GUS* staining in elongation and maturation parts rather than in root tips (Fig. 3, E–G). To determine whether the differences in the *GUS* staining patterns also appeared in leaves, we stained the shoots of 4- to 6-week-old plants. Histochemical analysis of cross-sectioned tissues of actively growing shoots revealed that the inner young leaves were preferentially *GUS*-stained in pGA2135 (Fig. 3D). However, in pGA2136 lines, the outer mature leaves were stained as strongly as the inner young ones (Fig. 3H). To determine whether the *GUS* staining patterns correlated with levels of the *GUS* RNA, RNA gel-blot analysis was carried out using total RNA isolated from inner young and outer mature leaf tissues (Fig. 4). The results exhibited that in pGA2135 lines, the *GUS* RNA level was much higher in young leaves than in mature leaves, whereas in pGA2136 lines there was no significant difference between them. These results demonstrated that the 1.3-kb intragenic region of *Os-*

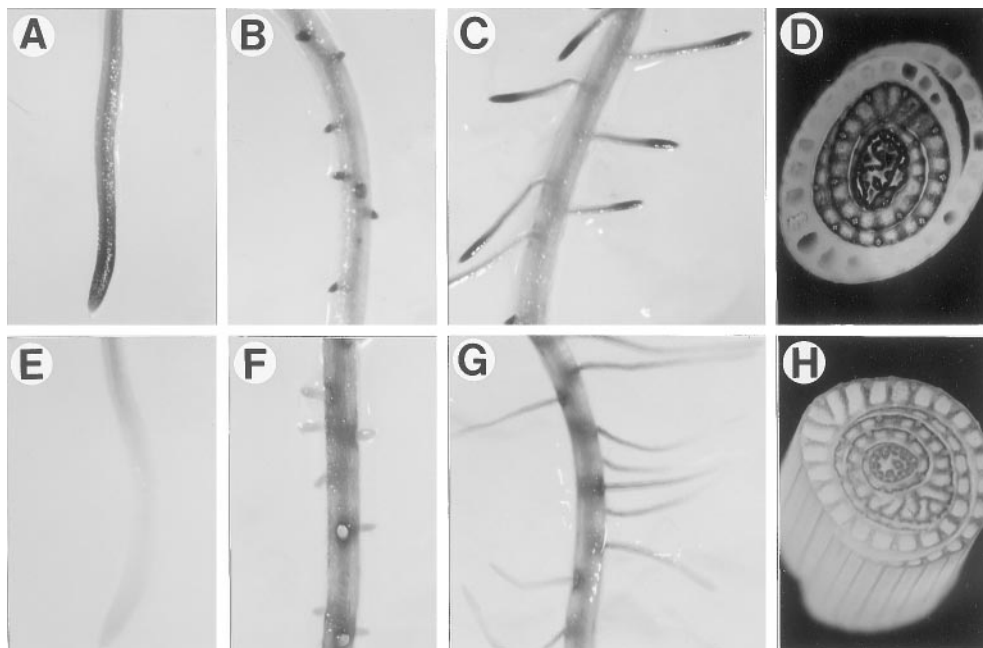


Figure 3. Histochemical localization of *GUS* activity directed by *OsTubA1* promoter::*GUS* fusions in transgenic rice. A to C, Transgenic roots carrying pGA2135. D, Transgenic shoots carrying pGA2135. E to G, Transgenic roots carrying pGA2136. H, Transgenic shoots carrying pGA2136. All reactions were stopped after 12 h.

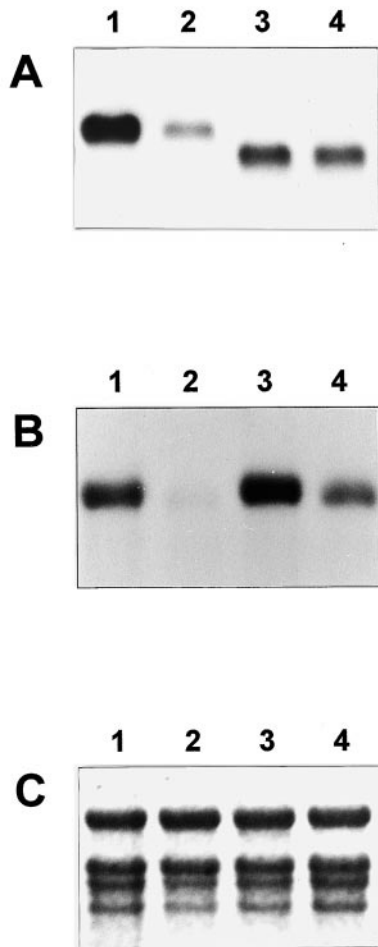


Figure 4. RNA gel-blot analyses in shoots of transgenic plants. A, Ten-microgram portions of total RNAs isolated from a total of three independent transgenic shoots were hybridized with the *GUS* gene. Samples 1 and 2 indicate inner young and outer mature leaves, respectively, of 4- to 6-d-old transgenic plants carrying pGA2135, and samples 3 and 4 indicate inner young and outer mature leaves, respectively, of pGA2136 transgenic plants at the same stage. Due to an additional coding sequence of approximately 0.3-kb, the transcripts in lanes 1 and 2 are larger than those in lanes 3 and 4. B, The filter was washed and reprobed with an *OsTubA1* gene-specific probe. C, Ethidium-bromide staining of 25S and 17S rRNAs demonstrating equal amounts of RNA loading.

TubA1, which was included in the pGA2135 construct, confers young tissue-preferential gene expression.

Effect of the Intron 1 on *GUS* Expression in Transgenic Rice Plants

The 1.3-kb intragenic region, which was essential for normal expression of the *OsTubA1* gene, contained exon 1, exon 2, intron 1, intron 2, and a portion of exon 3. To determine the region that specified the young tissue-preferential expression, we constructed various *OsTubA1::GUS* fusion molecules that contained different portions of the intragenic region (Fig. 5).

Because calli are groups of actively dividing cells, we compared the relative *GUS* expression levels of the different constructs in transformed calli (Fig. 5). The results showed that pGA2135 provided *GUS* activity 18 times greater compared with that of pGA2136. A similar result was observed with the constructs pGA1822 and pGA1876, which carried a shorter promoter fragment (491 bp; 555–1,045), although overall activity was slightly reduced compared with the longer promoter (1,045 bp; 1–1,045).

Since introns have been known to enhance gene expression, the roles of the three introns of the *OsTubA1* gene were examined by placing each intron between the *OsTubA1* promoter and *GUS* reporter. The highest *GUS* activity was recorded for pGA1902 in which the intron 1 was inserted between the *OsTubA1* promoter and the *GUS* coding sequence. The *GUS* activity driven by the *OsTubA1* promoter and intron 1 was 34-fold higher than that by the *OsTubA1* promoter alone (pGA1899), which displayed a low level of *GUS* expression. Intron 2 (pGA2116) and intron 3 (pGA2117) also increased the expression level to some extent, which were approximately 20% level of that achieved by intron 1. The construct, pGA1876, that contained both intron 1 and intron 2 did not show higher activity compared with the construct carrying the intron 1 alone. The result indicates that the combined presence of intron 1 and intron 2 provided neither synergistic nor additive. Our results agree with the previous reports that the first intron can significantly enhance gene expression in monocots (Callis et al., 1987; Vasil et al., 1989; McElroy et al., 1990).

The enhancing effects of the introns were studied in transgenic roots by histochemical *GUS* analyses (Fig. 6). In pGA1902 containing the *OsTubA1* intron 1, *GUS* staining was mainly detected at actively dividing cells such as in the tips of primary and secondary roots (Fig. 6, A and B). Unlike the case, in pGA1899, which contained no introns, *GUS* staining was observed mainly in elongating and maturing regions of roots and was hardly detectable in root tips, which are rich in dividing cells (Fig. 6, C and D). To determine whether the intron 2 and intron 3 of *OsTubA1* would exhibit the tissue-preferential enhancer function observed from the intron 1, transgenic roots of the pGA2116 and pGA2117 lines carrying the intron 2 and intron 3, respectively, were analyzed by histochemical *GUS* staining. The results showed that their *GUS*-staining patterns were similar to those of pGA1899 (Fig. 6, E–H), indicating that the growing cell-preferential expression is particular to intron 1 of *OsTubA1*.

It was observed in pGA1945 that placing the first intron under control of the heterogeneous 35S promoter resulted in a 70-fold increase in *GUS* expression level over that of pGA1875, the 35S promoter-*GUS* fusion without the intron 1 (Fig. 5). This result further supports that *OsTubA1* intron 1 can signifi-

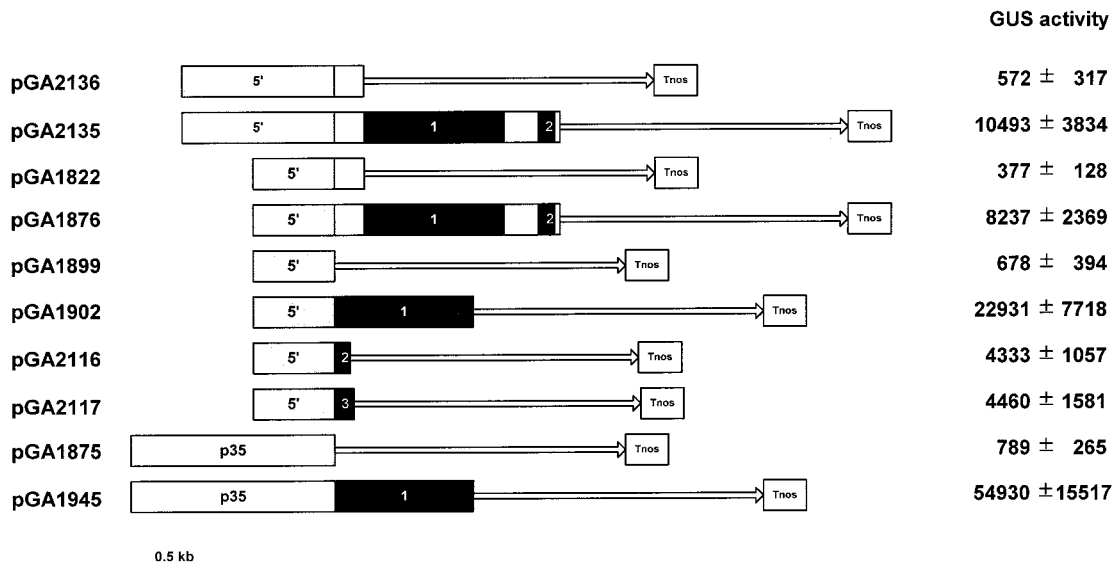


Figure 5. Structures of the tested GUS fusion constructs and GUS activity in the calli carrying them. Numbers 1, 2, and 3 in black bars indicate introns 1, 2, and 3 of *OsTubA1*, respectively. Gray bars represent the 5'-upstream sequence of *OsTubA1*, and white bars indicate the p35 cauliflower mosaic virus 35S promoter. Exons of *OsTubA1* are shown in white bars, and the *GUS* coding region is shown with a solid line with an arrow. Tnos signifies the *nos* terminator. GUS values are expressed as pmol 4-MU min⁻¹ mg⁻¹ soluble protein.

cantly enhance gene expression in actively growing cells.

Possible Mechanism of the Growing Cell-Preferential Gene Expression by the *OsTubA1* Intron 1

To study the mechanism of enhancing activity of the *OsTubA1* intron 1 on gene expression, we constructed additional *GUS* fusion molecules and examined GUS activities in transformed rice calli (Fig. 7). Placing the intron 1 upstream of the *OsTubA1* promoter (pGA2054) or downstream of the *GUS* coding sequence (pGA2119) did not enhance the gene expression, suggesting that the increased gene expression was position-dependent and was not due to an enhancer-like activity present in the intron 1.

To further understand a possible mechanism of the intron-1-mediated enhancement, we constructed six deletion mutants, pGA2127-1 to -6, within the intron-1 sequences and the effects were examined in transgenic calli (Fig. 7). The deletions of the intron-1 sequence resulted in a reduction of GUS activities by approximately 25% to 60%. The largest deletion construct pGA2127-6, which carried 291-bp intron sequence, still retained a significant level of the GUS activity. Further deletion of the 5' or 3' region of the intron 1 resulted in severe loss of the GUS activity (data not shown), indicating that the remaining sequences of the intron 1 in pGA2127-6 contained the legitimate splicing sequences, including donor, acceptor, and branch sites. The GUS activity analysis in roots and leaves of pGA2127-1 to -6 lines exhibited

nearly identical expression patterns to those of transgenic lines, pGA2135, pGA1876, and pGA1902, with the entire intron 1 (data not shown). These results suggest that the primary effect of the *OsTubA1* intron 1 on gene expression is probably mediated by RNA splicing.

DISCUSSION

Regulatory elements for tissue-specific expression have often been found in an intron rather than in the 5'-flanking region of a gene. The leader intron of potato *Sus4* was required for high-level tuber expression and Suc inducibility (Fu et al., 1995). The spinach *PsaD* gene required its intron for correct plastid- and light-dependent regulation (Bolle et al., 1996). Recently it has been reported that the large second intron of *AG* contains LEAFY-responsive enhancer sites, which are required for proper spatial and temporal expression of *AG* in *Arabidopsis* (Busch et al., 1999). In the present study, we report that the first intron of the rice *OsTubA1* gene has a strong enhancing effect in actively growing cells such as calli, root tips, and young leaves.

Through histochemical GUS analysis of transgenic rice plants carrying various *OsTubA1*::*GUS* fusions, we have shown that the transcribed region of the gene is required for a normal expression of *OsTubA1* in rice. Further analysis of each intron revealed that the intron 1 played an important role in tissue-preferential expression of the *OsTubA1* gene. Transgenic lines with the intron 1 gave much more intense

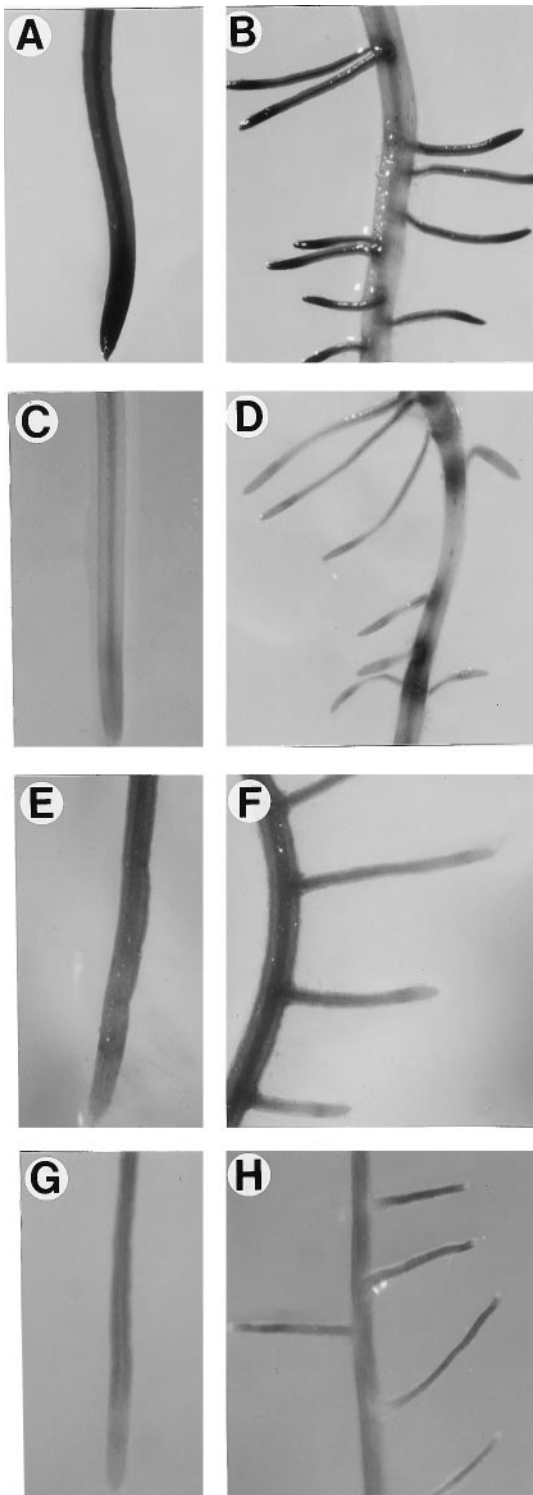


Figure 6. Histochemical localization of GUS activity directed by various *OsTubA1::GUS* fusions in transgenic roots. A and B, pGA1902. C and D, pGA1899. E and F, pGA2116. G and H, pGA2117. All reactions were stopped after 12 h.

GUS staining in actively dividing cells such as calli and root tips, whereas transgenic lines with only the intron 2 or intron 3 exhibited major GUS activities in

mature regions instead. The enhancer effects were also observed when the intron 1 was placed under the heterogeneous 35S promoter.

It is unlikely that a transcriptional enhancer element is present in the intron 1, since placing the intron-1 sequence in the 5'-upstream region of the *OsTubA1* promoter or the 3'-downstream region of the *GUS* coding region did not result in any enhancing effect. Position-dependent effects of an intron on gene expression have also been observed in maize *Adh1* and *Sh1* and rice *Act1* and *triosephosphate isomerase (tpi)* genes (Mascarenhas et al., 1990; McElroy et al., 1990; Maas et al., 1991; Snowden et al., 1996). Deletion mutagenesis studies have indicated that a majority of the intron-1 sequence could be deleted without interfering with the tissue-preferential enhancer activity. This result is in agreement with previous reports in which modification of an intron by deletions or substitutions did not significantly affect enhancement effects of maize *Adh1* or human factor IX gene expression (Luehrsen and Walbot, 1991, 1994; Kurachi et al., 1995).

Our results suggest that the enhancer activity of the *OsTubA1* intron 1 in actively growing tissues may be controlled at a post-transcriptional level, probably by the mediation of tissue-preferential RNA splicing or other RNA-processing events. In higher plants, genes encoding the major subunits of the spliceosome uridylate-rich small nuclear ribonucleoprotein particles consist of a large number of sequence variants. It was shown that the uridylate-rich small nuclear RNA variants exhibit developmental differences in their pattern of expression (Hanley and Schuler, 1991). Thus, it has been suspected that compositionally or functionally distinct uridylate-rich small nuclear ribonucleoprotein particles may affect developmental gene expression patterns (for review, see Simpson and Filipowicz, 1996). In our experiments, unspliced transcripts of the gene were not observed in RNA gel-blot and RT-PCR experiments in all tissues tested (Fig. 1 and 4; data not shown). This indicates that unspliced transcripts of the gene might be rapidly degraded in vivo as suggested by Mascarenhas et al. (1990). Therefore, it can be suspected that tissue-preferential accumulation of the mature *OsTubA1* mRNA might depend upon amount of the intron-1-associated splicing factors. We also do not exclude the possibility that other RNA processing events like polyadenylation and nucleocytoplasmic transport might be involved in an increase of the steady-state level of the *OsTubA1* mRNA. It was reported in mammals that intron enhancement resulted from an increase in the efficiency of 3'-end processing (Huang and Gorman, 1990). The regions near the 5'- or 3'-splicing site might retain regulatory sites for RNA splicing or other processing-associated factors, which are probably abundant in actively dividing tissues.

Enhancer effects have been found from the introns of several monocot genes, such as *Adh1*, *Sh1*, and *Bz1*

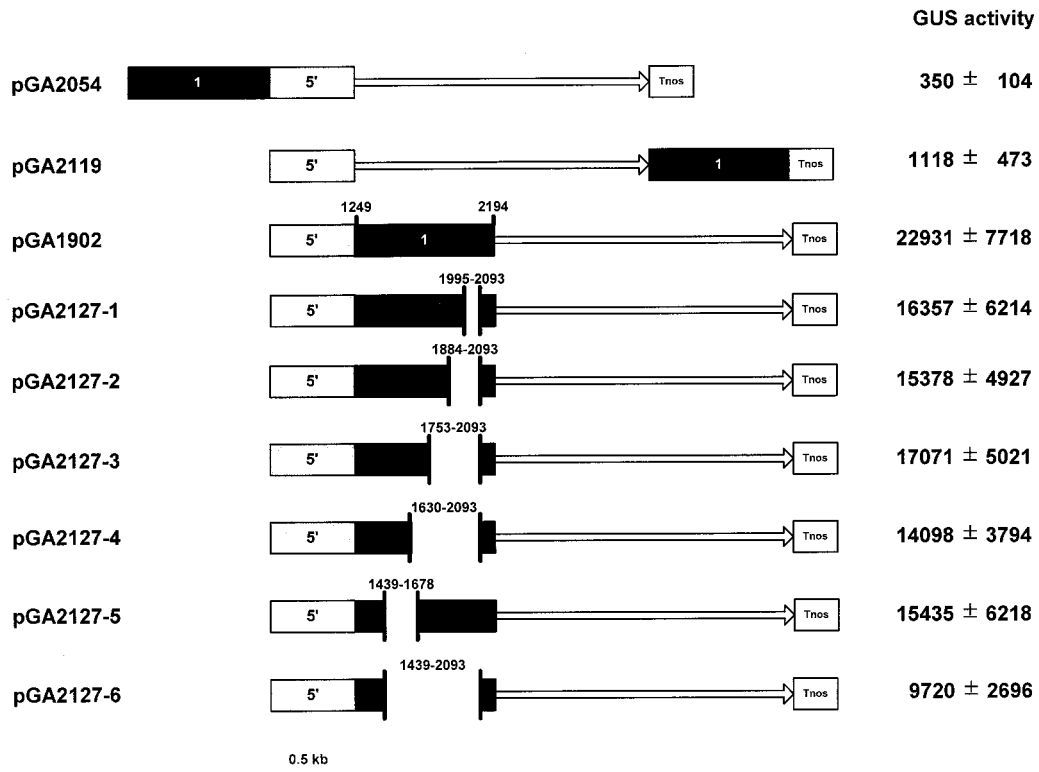


Figure 7. Diagram of the tested GUS fusion constructs and GUS activity in the calli carrying them. The number 1 contained in a black bar indicates the *OsTubA1* intron 1 (nucleotides 1,249–2,194), and gray bars represent the 5'-upstream sequence of *OsTubA1*. The numbers in truncated black bars represent the positions of deleted intron-1 fragment. The *GUS* coding region is indicated with a solid line with an arrow. Tnos signifies the *nos* terminator. GUS values are expressed as pmol 4-MU min⁻¹ mg⁻¹ soluble protein.

of maize and *Act1* and *tpi* of rice (Callis et al., 1987; Oard et al., 1989; Vasil et al., 1989; McElroy et al., 1990; Maas et al., 1991; Xu et al., 1994). Expression of the rice *Act1* gene requires intron 1 to direct constitutive GUS expression in transgenic rice, reflecting an ubiquitous requirement for cytoskeletal components in all plant cell types (Zhang et al., 1991). In the rice *tpi* gene, the intron 1 was necessary for GUS expression in transgenic rice (Xu et al., 1994). Taken together with the present study, these results suggest that the intron-mediated enhancement of reporter gene expression observed in undifferentiated cells may be due to temporal and/or spatial regulation of gene expression. It would be worthwhile to examine whether these introns also mediate normal expression of their genes in plants.

The *OsTubA1* gene is closely related to members of the plant α -tubulin subfamily I, which consists of maize *Tuba1* and *Tuba2*, and Arabidopsis *TUA2* and *TUA4*. The maize *Tuba1* promoter is active mainly in root tips and pollen in transgenic tobacco plants (Rigau et al., 1993; Uribe et al., 1998), and the promoter activity of the Arabidopsis *TUA2* gene has also been shown to be the highest in tissues with actively dividing cells (Carpenter et al., 1993). The previous studies have demonstrated that promoter regions alone were sufficient to induce the tissue-preferential

expression of the genes. These findings differ from the expression pattern of the rice *OsTubA1* gene, which appears to be controlled mainly by a post-transcriptional mechanism. The results of this study contribute to the understanding of the cellular mechanisms that regulate the expression of individual members of rice α -tubulin gene families.

MATERIALS AND METHODS

Bacterial Strains

The *Escherichia coli* strain JM83 (*ara*, *leu*, *lac*, *gal*, *str*) was used as the recipient for routine cloning experiments. *Agrobacterium tumefaciens* LBA4404 containing the Ach5 chromosomal background and a disabled helper-Ti plasmid pAL4404 was used for transformation of rice (*Oryza sativa* L.) (Hoekema et al., 1983).

Screening of cDNA and Genomic Libraries

The cDNA library made with mRNA from immature anthers of rice cv Nakdong (Chung et al., 1994) was screened with the [α -³²P]dCTP Arabidopsis α 1-tubulin gene as a probe (Ludwig et al., 1988). A genomic library constructed in the bacteriophage λ -DASH vector with partial *Sau*3AI-digested DNA of rice cv IR36 (kindly provided

by Steve Kay) was used for isolation of a genomic clone as described by Sambrook et al. (1989). Hybridizations were carried out at 55°C in 6× SSC, 5× Denhardt's reagent, 0.5% (w/v) SDS, and 100 mg L⁻¹ denatured salmon sperm DNA. The final two washes were done in 2 × SSC and 0.1% (w/v) SDS at 45°C for 30 min each. Phage DNA was prepared according to Chisholm (1989). The DNA sequence of a gene was determined by the dideoxynucleotide chain termination method using double-stranded plasmid DNA templates (Sanger et al., 1977).

RNA Gel-Blot Analysis

Total RNA was isolated from leaves, roots, and flowers of rice plants by the RNA isolation kit (TRI reagent, Molecular Research Center, Cincinnati). A 10-μg portion of total RNA was fractionated on a 1.3% (w/v) agarose gel as described previously (Kang et al., 1998). After RNA transfer onto a nylon membrane, the blot was hybridized in a solution containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% (w/v) BSA, and 7% (w/v) SDS for 20 h at 60°C (Church and Gilbert, 1984). After hybridization, the blot was washed twice with a solution containing 0.2× SSPE and 0.1% (w/v) SDS for 5 min at room temperature followed by two washes of the same solution at 60°C for 15 min.

Construction of Binary Vectors

The plasmid pGA2135 was made by fusing the 2.6-kb *XbaI-HindIII* fragment (nucleotides 1–2,557) of *OsTubA1*, containing the 5'-upstream region, exon 1 and 2, intron 1 and 2, and a portion of exon 3, to *GUS*. The plasmid pGA2136 was produced by connecting the 1.2-kb *XbaI-XhoI* fragment (nucleotides 1–1,231) of *OsTubA1*, containing the 5'-upstream region and a portion of exon 1, to the *GUS* coding region. In both constructs, *OsTubA1* and *GUS* existed in the same reading frame, generating translational fusions. The plasmids pGA1822 and pGA1876 were produced from pGA2136 and pGA2135, respectively, by deletion of the 0.55-kb *XbaI-BamHI* fragment (nucleotides 1–554) at the distal region of the *OsTubA1* promoter.

To construct transcriptional fusions between the promoter and *GUS*, a *HindIII* site was generated immediately upstream of ATG codon by PCR using the oligomer 5'-GAAGCTTGGCGGAATGGGTT-3' as a reverse primer, the T3 primer (5'-AATTAACCCCTCACTAAAGGG-3') as a forward primer, and the genomic fragment cloned in pBlue-script SK(-) (Stratagene, La Jolla, CA) as a template. The amplified 0.6-kb fragment of *OsTubA1* promoter was fused to the *GUS* gene through the *HindIII* site to make pGA1899.

Three plasmids, pGA1902, pGA2116, and pGA2117, each with a DNA fragment carrying intron 1, 2, or 3 of *OsTubA1*, respectively, inserted in the *HindIII* site of pGA1899 were constructed to examine the effect of three introns of *OsTubA1* on gene expression. For construction of pGA1902, *HindIII* sites were generated at both ends of the fragment containing the intron 1 by PCR, using 5'-GAAGCTTGCATCCAGGTACG-3' and 5'-GAAGCTTCAGCCTGA-

AAGGA-3' (underlined sequences are the exon sequences flanking intron 1) as primers. pGA2116 was constructed by attaching a *HindIII* site to the 5' end and a *XbaI* site to the 3' end of the fragment containing intron 2 of *OsTubA1* by PCR using primers, 5'-TCAAGCTTGTGGTCACTACAC-CAGTGAG-3' and 5'-GTTTCTAGACAATCTCCTTGCCAA-CTG-3' (underlined sequences are the exon sequences flanking intron 2). Similarly, pGA2117 was constructed by adding *HindIII* and *XbaI* sites at the 5' and 3' ends of the DNA fragment carrying the intron 3 by PCR using primers, 5'-TCAAGCCTGTGTCTCAGGTACAGACTTG-3' and 5'-GTTCTAGAGGGATGAGATAACCTGCAC-3' (underlined sequences are the exon sequences flanking intron 3). To test the effect of *OsTubA1* intron 1 on a heterogeneous promoter, the intron-1 fragment was inserted between the 35S promoter and *GUS*, making pGA1945. Plasmid pGA1875, which contains a fusion between the 35S promoter and *GUS*, was used as a control.

To determine whether the *OsTubA1* intron 1 acts as an enhancer-like element, pGA2054 and pGA2119 were constructed. The plasmid pGA2054 was constructed by inserting the internal region of the intron 1, which lacks both donor and acceptor sites, into the 5'-distal region of the *OsTubA1* promoter in a forward orientation. The internal region of intron 1 was generated by PCR using oligomers 5'-GGAAGCTTGGCGTCCCCTCTCCCTCA-3' and 5'-GCGTCGACAAAATACATGTTAGCG-3'. The plasmid pGA2119 was constructed by inserting the DNA fragment containing the intron 1 after the *GUS* coding region. The PCR fragment was produced using 5'-GGAGCTCGCATC-CAGGTACGGATCCGC-3' and 5'-GGGTACCATCAGCCT-GAAAGGACAAA-3' as primers.

The cis-acting regulatory elements of the *OsTubA1* intron 1 were studied by the construction of the truncated intron 1 from pGA1902. These plasmids, pGA2127-1 to -6, have deletion of the nucleotides 1,995 through 2,093, 1,884 through 2,093, 1,753 through 2,093, 1,630 through 2,093, 1,439 through 1,678, and 1,439 through 2,093 from the *OsTubA1* intron 1 (nucleotides 1,249–2,194), respectively.

All constructs contain the *nos* terminator after the *GUS* gene and the 35S::*hygromycin phosphotransferase* gene for a plant selectable marker.

Agrobacterium-Mediated Rice Transformation

A japonica rice variety, Dongjin, was used for transformation by the *Agrobacterium*-mediated cocultivation method as described previously (Jeon et al., 1999; Lee et al., 1999).

GUS Assays

Fluorometric analysis was performed as described by Jefferson (1987) using 4-methylumbelliferyl-β-glucuronide. GUS values were expressed as picomole 4-methylumbelliferone (4-MU) min⁻¹ mg⁻¹ soluble protein. Ten independent callus lines cultured for 2 weeks on 2N6 media (N6 medium, 2 mg L⁻¹ 2,4-D, 30 g L⁻¹ Suc, 1 g L⁻¹ casamino acids, 2 g L⁻¹ phytagel, pH 5.6–5.7) were used in

the quantitative analysis (Lee et al., 1999). Histochemical GUS staining was performed according to Dai et al. (1996) except for addition of 20% (v/v) methanol in the staining solution. The reaction was stopped after 12 h, and samples were stored in 75% (v/v) ethanol.

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