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ORIGINAL PAPER

Gene expression enhancement mediated by the 5′ UTR intron of the rice *rubi3* gene varied remarkably among tissues in transgenic rice plants

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Abstract Introns are important sequence elements that modulate the expression of genes. Using the *GUS* reporter gene driven by the promoter of the rice (*Oryza sativa* L.) polyubiquitin *rubi3* gene, we investigated the effects of the 5′ UTR intron of the *rubi3* gene and the 5′ terminal 27 bp of the *rubi3* coding sequence on gene expression in stably transformed rice plants. While the intron enhanced *GUS* gene expression, the 27-bp fused to the *GUS* coding sequence further augmented *GUS* expression level, with both varying among different tissues. The intron elevated *GUS* gene expression mainly at mRNA accumulation level, but also stimulated enhancement at translational level. The enhancement on mRNA accumulation, as determined by realtime quantitative RT-PCR, varied remarkably with tissue type. The augmentation by the intron at translational level also differed by tissue type, but to a lesser extent. On the other hand, the 27-bp fusion further boosted GUS protein yield without affecting mRNA accumulation level, indicating stimulation at translation level, which was also affected by tissue type. The research revealed substantial variation in the magnitudes of intron-mediated enhancement of gene expression (IME) among tissues in rice plants and the importance of using transgenic plants for IME studies.

Keywords IME · Intron · *rubi3* gene promoter · Transgenic rice · Translation

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Introduction

To successfully apply plant biotechnology in agricultural production, environmental protection, and molecular farming, it is essential to understand and control transgene expression. Plants have evolved various mechanisms to regulate their gene expression at different levels, including transcriptional, post-transcriptional, translational, and post-translational. Introns and sequences flanking translation initiation codon are important *cis*-elements that plants employ to modulate the expression of their genes.

Introns have been documented to augment gene expression in various organisms. In plants, this effect has been termed intron-mediated enhancement (IME) of gene expression (Mascarenhas et al. 1990). Many plant introns are capable of IME (McElroy et al. 1990; Zhang et al. 1991; Clancy and Hannah 2002). The increase in expression caused by introns is usually associated with increased levels of mRNA accumulation (Callis et al. 1987; Rose and Last 1997; Rethmeier et al. 1997; Rose 2002; Wang et al.

2002). However, the half-lives of mRNAs from the intron-less or intron-containing genes were reported to be comparable (Rethmeier et al. 1997). Therefore, the enhanced mRNA accumulation was proposed to be due to enhanced transcription (Rose 2004). In several studies, the increase in protein accumulation caused by introns exceeded that in mRNA accumulation, supporting the notion that plant introns also elevate translation (Mascarenhas et al. 1990; Bourdon et al. 2001; Clancy and Hannah 2002; Rose 2004). Interestingly, IME studies in dicot plants were usually performed in stably transformed plants (Fu et al. 1995a, b; Chaubet-Gigot et al. 2001; Rose 2002; 2004) whereas most IME studies in monocot plants were carried out by transient expression assays in cultured cells (Callis et al. 1987; Mascarenhas et al. 1990; Rethmeier et al. 1997; Bourdon et al. 2001; Clancy and Hannah 2002; Morello et al. 2006; Sivamani and Qu 2006) with few exceptions (Jeon et al. 2000; Dugdale et al. 2001; Bourdon et al. 2004). Moreover, few studies simultaneously investigated the characteristics of IME in different tissues of plants. Thus, it was less understood whether the results from cell cultures reflected the IME at plant level.

We recently isolated a polyubiquitin gene, designated as *rubi3*, from rice and studied the effects of its 5′ regulatory region on gene expression (Sivamani and Qu 2006). The 5′-regulatory region of the *rubi3* gene is composed of an 808 bp promoter, a 67 bp non-coding exon (exon 1), and a 1,140 bp intron, which is immediately followed by an open reading frame of 1,140 bp (exon 2) encoding a pentameric polyubiquitin. In transient assays in bombarded rice suspension cells, the promoter containing the 5′ UTR intron conferred approximately 20-fold higher *GUS* gene expression than an intron-less, but otherwise identical, construct (Sivamani and Qu 2006). Using stably transformed rice suspension cells, we revealed that the enhancement in *GUS* gene expression by the *rubi3* 5′ UTR intron occurred at transcriptional, post-transcriptional, and translational levels with most of the enhancement taking place at post-transcriptional level (Samadder et al. 2008). Moreover, constructs containing various lengths of N-terminal ubiquitin coding sequence fused in-frame to the *GUS* coding sequence generated approximately 4-fold higher GUS enzyme activity in transient assays when compared with the aforementioned intron-containing construct (Sivamani and Qu 2006). In this study, we investigate the effects of the 5′ UTR intron, and a fusion construct with the first 27 bp of the *rubi3* coding sequence on *GUS* reporter gene expression in transgenic rice plants. We demonstrate that the augmentation of gene expression mediated by the 5′ UTR intron of the *rubi3* gene is remarkably affected by tissue type. We also reveal that the 27-bp fusion construct augments gene expression at translational level, and the degree of this enhancement also varies from tissue to tissue.

Materials and methods

Plasmid construction

Construction of pRESQ4, pRESQ36, and pPSRG30 were previously reported (Sivamani and Qu 2006). A 4,147 bp *EcoR*I fragment from pRESQ4 was inserted into the *EcoR*I site of the binary vector pCAMBIA1300 (GenBank accession AF234296) resulting in the plasmid pJLU2 (Fig. 1). A 546 bp *BstE*II and *SnaB*I fragment from pPSRG30 was used to replace a 1,686 bp *BstE*II and *SnaB*I fragment in pJLU2 to obtain plasmid pJLU7, which would contain the *GUS* expression cassette of pPSRG30 in the pCAMBIA1300 backbone. A 672 bp *Bgl*II and *SnaB*I fragment from pRESQ36 was used to replace a 665 bp *Bgl*II and *SnaB*I fragment in pJLU2. The resultant plasmid, pJLU6, contains the same *GUS* expression construct as pRESQ36 but uses pCAMBIA1300 as the backbone. It should be noted that, in pJLU6, the 27-bp *rubi3* coding sequence replaced a 20-bp untranslated sequence immediately preceding the *GUS* coding sequence ("*GUS* leader sequence") in pJLU2. pJLU6 would be referred to as "27-bp substitution" construct hereafter.

Rice transformation

DNAs of the binary vectors pJLU2, pJLU6, pJLU7 were separately introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) harboring the plasmid pToK47, which contains an extra copy of the *virB*, *virC* and *virG* genes (Jin et al. 1987), by the freeze–thaw method (An et al. 1988). The resultant *A. tumefaciens* strains were grown on YEP medium containing 5 mg/L tetracycline, 20 mg/L rifampicin and 50 mg/L kanamycin (An et al. 1988). Before inoculation, Acetosyringone (Aldrich, Milwaukee, WI, USA) and Silwet L-77 (OSi Specialties, Inc., Danbury, CT, USA) were added into diluted cultures to the final concentrations of 100 μM and 200 μL/L, respectively, to facilitate infection. Transformation of the rice cultivar Taipei 309 with *A. tumefaciens* was performed according to Azhakanandam et al. (2000) with minor modifications. Transgenic plants were transferred to sterilized topsoil in plastic pots of 20 cm in diameter placed in trays filled with nutrient solution in a walk-in growth chamber at the North Carolina State University Phytotron. Environmental factors for rice growth were set as follows: 30°C light period temperature, 25°C dark period temperature, 11.5-h day length, ca. 575 μmol m^{-2} s^{-1} of light intensity, and ca. 50% of relative humidity. The trays were regularly replenished with a nutrient solution provided by the Phytotron (Thomas et al. 2006).

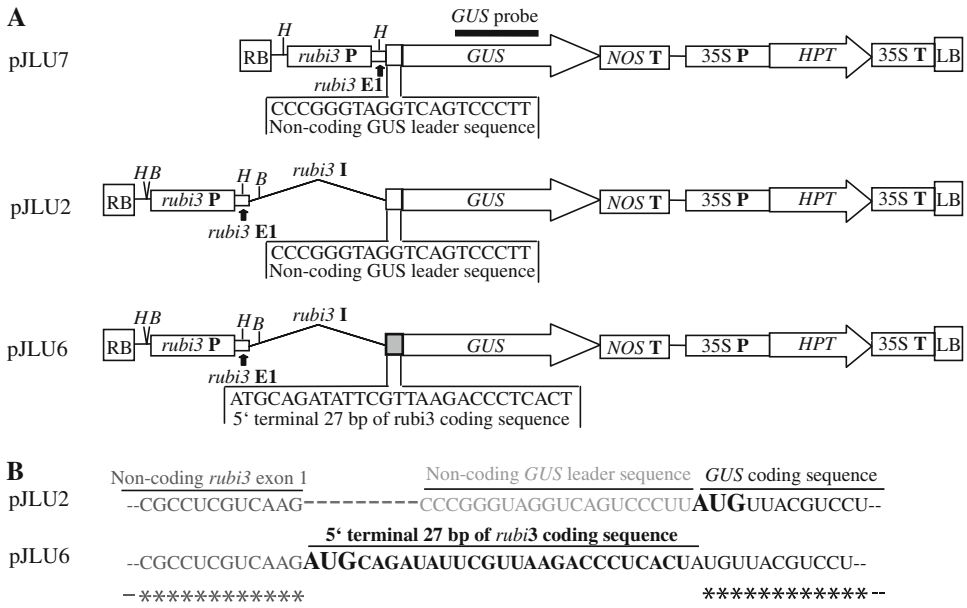

Fig. 1 **a** Structures of the T-DNA regions of the binary vectors used in rice transformation. All of the vectors are based on pCAMBIA1300. *RB*, right border; *rubi3* P, rice *rubi3* gene promoter; *rubi3* E1, rice *rubi3* gene exon 1; *rubi3* I, rice *rubi3* gene 5′ UTR intron; *GUS*, *β*-glucuronidase gene coding sequence; *NOS* T, nopaline synthase gene 3′ terminator; 35S P, CaMV 35S promoter; *HPT*, hygromycin phosphotransferase gene; 35S T, CaMV 35S terminator; *LB*, left border; *H*, *Hind*III; *B*, *Bam*HI. Probing region used in Southern blotting of T_0 transgenic plants is also shown. **b** Difference in the context sequences flanking the translation initiation codons of mature *GUS* mRNAs from pJLU2 and pJLU6. Sequence elements of different sources are indicated by different shades of color, and the translation initiation codons are in larger font sizes

Callus induction from T_0 transgenic rice plants

To study the reporter gene expression in callus tissue, young inflorescences of about 1 cm in length were taken from T_0 transgenic plants. Excised inflorescences were sterilized with the leaf sheath on in 50% Clorox® for 12 min, unwrapped from the leaf sheath, cut into about 2 mm pieces, and cultured on LS2.5 medium (Azhakanandam et al. 2000) to induce callus formation. Induced callus lines were subcultured on fresh LS2.5 medium every 2 weeks before use.

Determination of transgene copy number

The copy numbers of the *GUS* gene in the primary transformants were determined from genomic DNA using quantitative realtime PCR (Ingham et al. 2001) at Syngenta Biotechnology, Inc. (Research Triangle Park, NC, USA), and further confirmed by Southern analysis.

Genomic DNA was extracted from about 1 g leaves of transgenic plants as described (Dong and Qu 2005). DNA from a non-transgenic plant was used as the negative control. From each sample, 10 μg of DNA was digested with *Hind*III or *Bam*HI overnight. The positive control was 85 pg of pRESQ4 plasmid DNA mixed with 10 μg of DNA from a non-transgenic plant digested with the same enzyme. Restricted DNAs were separated on 1% (w/v) agarose gels and blotted to Hybond-N+® nylon membrane (Amersham Biosciences Corp., Piscataway, NJ, USA) with upward capillary transfer mediated by 0.4 M NaOH according to the manufacturer's instructions. The genomic DNA blots were probed with a 798 bp fragment of the *GUS* coding sequence. The probe DNA was generated using PCR with the primers:

GUS-1F: 5′CAACGAACTGAACTGGCAGA3′ and
GUS-1R: 5′TTTTTGTCACGCGCTATCAG3′.

PCR products were labeled with [α-^{32}P]dCTP (Amersham) using the Primer-It II® random primer labeling kit (Stratagene, Cedar Creek, TX, USA) according to the manufacturer's instructions. Hybridization and washing of the blots were performed according to the instructions (Amersham). Washed membranes were exposed to Kodak BioMax MS film (Eastman Kodak, Rochester, NY, USA).

Realtime quantitative RT-PCR

The steady-state level of *GUS* mRNA in primary transformants was evaluated by realtime quantitative RT-PCR according to Samadder et al. (2008) with the following modifications. Leaf and root samples were taken from plants at the same developmental stage. The morphological markers used for this stage were that the flag leaf was still enclosed in the second leaf with its collar about 4 cm below that of the second leaf when the young panicle on main

stem was about 1 cm in length. Callus samples were taken from callus lines induced from the transgenic plants. During RNA extraction, the isolated RNA was digested on-column with the RNase-free DNase Set (Qiagen, Valencia, CA, USA) to remove possible DNA contamination. Reverse transcription was performed in 20 μL reactions containing 1 μg total RNA. The realtime quantitative RT-PCR was performed in 25 μL reactions containing 1.2 ng/μL cDNA, 50 nM each primer and 12.5 μL Power SYBR® Green PCR Master Mix. The thermal cycling parameters were: 50°C for 1 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. The reactions were performed in four replicates.

Histochemical localization of *GUS* gene expression

Samples were taken from T_0 transgenic and control plants to identify the cells or tissues expressing the *GUS* gene. Leaf and root samples were collected at early panicle development stage, using the same morphological markers as those used in sampling for realtime quantitative RT-PCR. Plant tissues were sectioned with a VIBRATOME® Series 1000 sectioning system (Technical Products International, St Louis, MO, USA) and/or by manual sectioning. Sections were then incubated overnight for 16 h at 37°C in GUS assay buffer (Jefferson et al. 1987). The reaction was stopped by replacing the GUS assay buffer with 70% ethanol. Green tissues were further incubated in 70% ethanol for 5 h at 37°C with changing of the 70% ethanol every hour to remove the chlorophyll. Sections were kept in 70% ethanol at 4°C before micrographs were taken. Low magnification images were generated and recorded using a Nikon SMZ-U dissecting microscope coupled with a Nikon E995 digital camera (Nikon Instruments, Melville, NY, USA). For photography at high magnification, specimens were mounted on microscopic slides, and were examined using a Nikon Eclipse E400 microscope. Images were recorded with a Qimaging® Micropublisher 5.0 RTV color CCD camera (Burnaby, BC, Canada). Images for the same type of tissues were taken under identical settings.

Determination of GUS enzyme activity

Fluorometric quantification of GUS enzyme activity in transgenic plants was performed according to the method described by Gallagher (1992) with minor modifications. Leaf and root samples were collected at early panicle development stage, using the same morphological markers as described above. Total protein concentration was determined with the Bio-Rad Protein Assay reagent according to the microtiter plate protocol recommended by the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). Absorbance of the binding reaction was measured using a Fluostar® instrument (BMG Labtech, Duhram, NC, USA) set at Absorbance mode. For MUG (4-methylumbelliferyl-β-D-glucoside) assay reaction, 10 μL of each of diluted extracts was mixed with 130 μL MUG assay buffer and incubated at 37°C for 20 min. Fluorescence from MUG assay reactions was quantified with the same Fluostar® but set at Time-Resolved Fluorescence mode. GUS activities were expressed in nanomoles MU (4-methylumbelliferone) released per minute per milligram total protein. Three replicate assays were performed for each sample. The MU (for standard curve) and MUG were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Quantification of GUS protein by ELISA

GUS protein levels in the leaves of plants transgenic of pJLU2 and pJLU6 were determined using ELISA according to Sivamani et al. (2000) with minor modifications. Leaf samples were collected at early panicle development stage using the same morphological markers as previously mentioned. Anti-β-glucuronidase rabbit IgG fraction (Molecular Probes, Eugene, OR, USA) was used as the primary antibody and the 1-Step™ PNPP method (Pierce Biotechnology, Rockford, IL, USA) was used for colorimetric measurement of absorbance at 405 nm.

Statistical analysis

Linear regression and *t* test were performed using the software package SAS® 9.1 (SAS Institute, Cary, NC, USA).

Results

Generation and analysis of stably transformed rice plants

In order to study the regulation of gene expression in the rice *rubi3* gene by its 5′ UTR intron and the context sequences of its translation initiation codon, three binary vectors, namely pJLU2, 7, and 6, were generated based on previously reported plasmids pRESQ4, pPSRG30, and pRESQ36 (Sivamani and Qu 2006), respectively, for *Agrobacterium tumefaciens*-mediated transformation of the rice cultivar Taipei 309. As shown in Fig. 1a, pJLU2 and pJLU7 contain a *GUS* gene under the control of the *rubi3* promoter, with or without its 5′ UTR intron. pJLU6 is identical to pJLU2 except that the 20 bp *GUS* "leader sequence" in pJLU2 was replaced with the first 27 bp of the *rubi3* coding sequence, which forms an in-frame fusion to the *GUS* coding sequence, in pJLU6.

Agrobacterium-mediated transformation was chosen because it produces predominantly simple integration patterns with defined borders (Hansen and Chilton 1999),

which reduces the effects of transgene copy number and DNA rearrangement on transgene expression. Seventy-eight independent transformants were generated from the three constructs, with at least 22 transformation events from each construct. To minimize the confounding effects of transgene copy number on gene expression (Hobbs et al. 1993; Jorgensen et al. 1996; Wang and Waterhouse 2000; Butaye et al. 2005), T_0 transformants were analyzed first using realtime PCR (data not shown) and further by Southern blotting (Fig. 2) to screen for transgenic events with a single transgene insertion. Eventually, six or seven independent transformants each with a single copy of the *GUS* transgene were identified for each of the constructs. These T_0 transgenic plants and the calli induced from their young inflorescences were used for further studies.

Enhancement in GUS enzyme activity varied with tissues

Histochemical assays were performed to visualize *GUS* expression in T_0 transgenic plants from various constructs. GUS staining was very weak in roots and calli of pJLU7 plants, greatly enhanced in pJLU2 plants, and further elevated in pJLU6 plants (Fig. 3). However, the GUS staining was already intense in leaves of pJLU7 plants and the enhancement by pJLU2 and pJLU6 was thus less remarkable in leaf tissues (Fig. 3b, c).

To quantitatively evaluate the effects of these constructs on *GUS* gene expression, MUG assays were performed with leaf, root and callus tissues of the T_0 transgenic plants (Fig. 4a). Despite variation in GUS activity among individual plants from the same gene construct, which was most

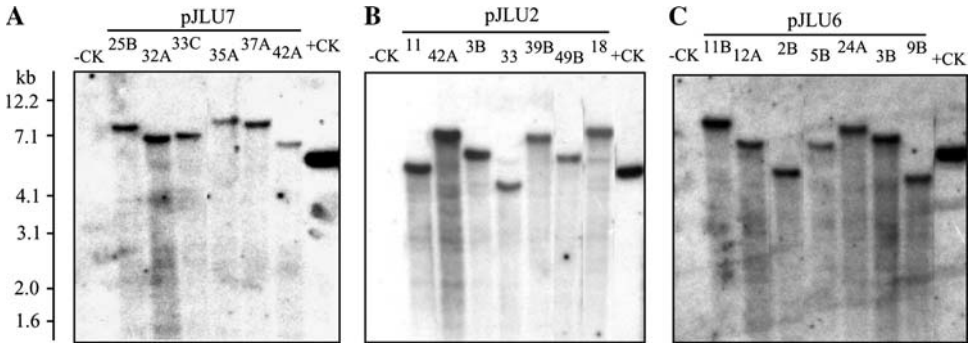

Fig. 2 Southern hybridization analysis of T_0 transgenic plants. Ten micrograms of genomic DNAs from leaf extracts were digested with *Bam*HI (for pJLU6 transformants) or *Hind*III (for other transformants), and hybridized with ^{32}P-labeled *GUS* probe depicted in Fig. 1. **a** Transgenic plants from pJLU7; **b** those from pJLU2; **c** those from pJLU6; *−CK*, negative control; *+CK*, positive control

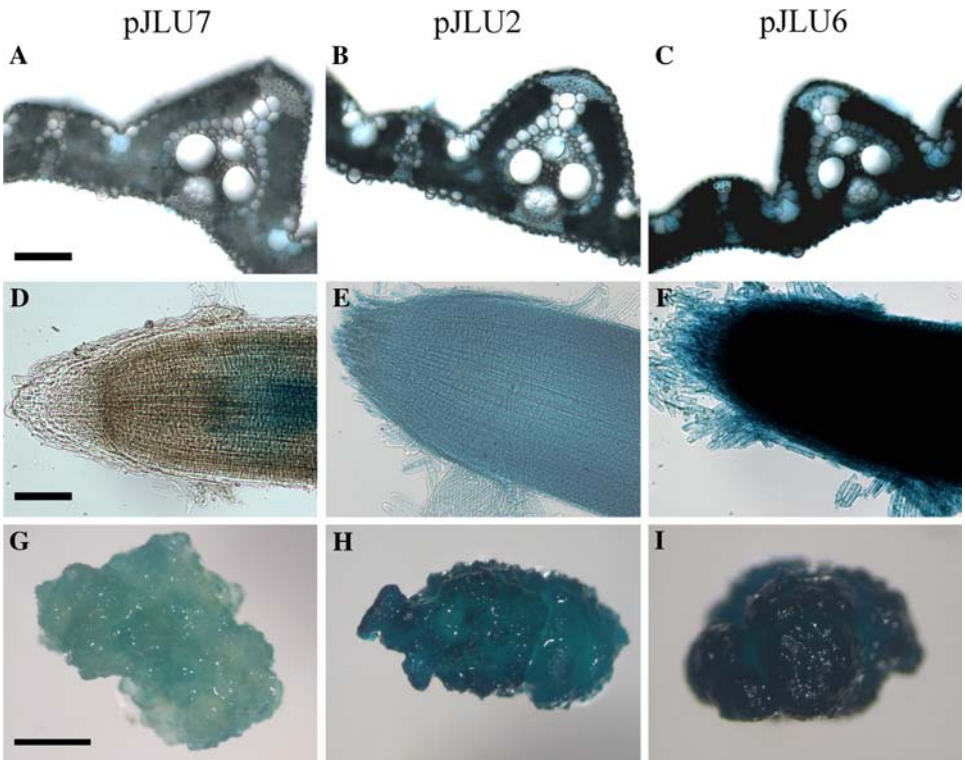

Fig. 3 Histochemical assays of *GUS* expression in leaf and root of T_0 transgenic rice plants, and in callus induced from young inflorescences of those plants. *First column*, plants transformed with pJLU7; *second column*, plants transgenic of pJLU2; *third column*, plants transformed with pJLU6. Images on the same rows were captured using the same settings. **A–c** cross sections of leaf blades, *bar* 100 μm; **d–f** root tips, *bar* 100 μm; **g–i** calli, *bar* 1 mm

likely a result of position effect of transgene insertion (Dean et al. 1988; Peach and Velten 1991), significant differences in GUS activity were observed among plants transformed with different constructs. Consistent with the histochemical assays, GUS activities among the tissues in pJLU2 plants showed less variation with differences within 2.3-fold, whereas the GUS activity of the intron-less pJLU7 varied considerably with tissues, with its activity in leaves being 6.2-fold higher than in roots, and 27.6-fold greater than in calli. When comparing plants from pJLU2 and pJLU7, the *rubi3* 5′ UTR intron enhanced GUS activity, on average, by 3.3-fold in leaf ($P = 0.037$), 26.5-fold in root ($P = 0.001$), and 51.1-fold in callus tissue ($P = 0.007$). These results suggest that the magnitude of enhancement on *GUS* gene expression mediated by the *rubi3* 5′ UTR intron was affected by tissue type and was inversely related to the GUS activity level of the intronless pJLU7 in different tissues.

Moreover, pJLU6 plants showed further elevated GUS activity when compared to pJLU2 plants. The average enhancement was 2.1-fold in leaf ($P = 0.02$), 3.0-fold in root ($P = 2.4 \times 10^{-4}$), and 4.5-fold in callus ($P = 2.3 \times 10^{-7}$). Accordingly, the construct enhanced GUS staining more in calli and roots than in leaves (Fig. 3). Therefore, the extent of elevation in GUS activity by the 27-bp substitution was also affected by tissue type.

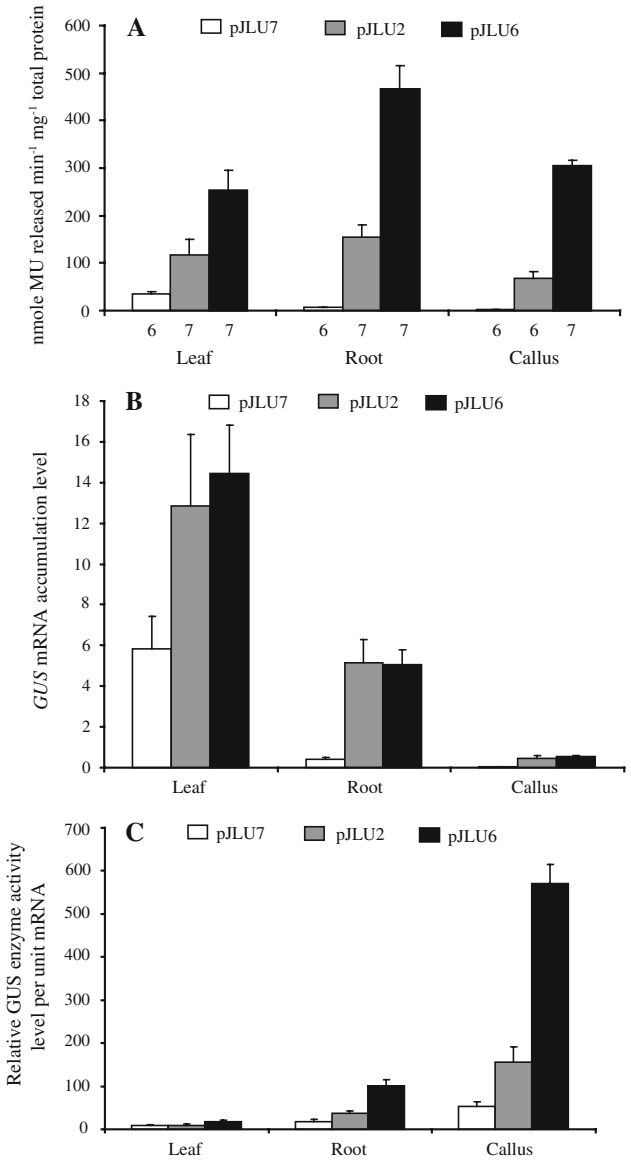

Fig. 4 Expression of the *GUS* gene in single-copy T_0 transgenic rice plants of pJLU7, pJLU2, and pJLU6. Assays were made with leaf and root tissues of the transformants, and calli derived from those plants. The same set of plants was used for all the assays, and the numbers of plants used are indicated in **a** under the columns corresponding to their respective constructs. Means and their standard errors are shown. **a** GUS enzyme activity, which is presented as nanomoles of MU released per minute per milligram total protein; **b** Steady state *GUS* mRNA accumulation. *GUS* mRNA levels were measured using realtime quantitative RT-PCR and presented in arbitrary relative unit; **c** Relative GUS enzyme activity per unit steady state mRNA. GUS enzyme activity was divided by its corresponding mRNA level to obtain the relative GUS enzyme activity per unit of *GUS* mRNA to represent the translational yield of the mRNA species

Enhancement in *GUS* mRNA accumulation

RNA blotting and realtime quantitative RT-PCR were performed subsequently to study the *GUS* mRNA accumulation in leaf, root and callus tissues of the T_0 transgenic plants for pJLU7, pJLU2 and pJLU6. In Northern blotting, the two constructs containing the *rubi3* intron (pJLU2 and pJLU6) showed clear bands for all the three tissues and unspliced *GUS* RNA band was not detected, but the intronless construct (pJLU7) had very faint or undetectable bands in root and callus tissues, indicating very low expression levels in the two tissues (data not shown). Since accurate quantitation of mRNA levels in such cases was difficult by Northern hybridization, we switched to realtime quantitative RT-PCR to determine the steady-state *GUS* mRNA accumulation levels (Fig. 4b). The results of the realtime quantitative RT-PCR were consistent with the Northern analysis. *GUS* mRNA levels among the tissues in pJLU2 plants showed substantial variation, while *GUS* mRNA accumulation of the intronless pJLU7 in different tissues varied even more dramatically with its level in leaves being 15-fold higher than in roots, and 212-fold greater than in calli. Compared with pJLU7 plants, the *GUS* mRNA accumulation in pJLU2 plants was 2.2-fold more in leaf ($P = 0.10$), 12.8-fold higher in root ($P = 0.006$), and 17.0-fold greater in callus ($P = 0.02$). The data revealed a great variation in *GUS* mRNA accumulation among the tissues in the intronless pJLU7 plants, and indicated that the magnitude of enhancement in mRNA accumulation by the *rubi3* intron varied by tissue type and was inversely related to the mRNA level of the pJLU7 plants in different tissues. Conversely, pJLU6 and pJLU2 plants did not differ significantly

in *GUS* mRNA accumulation in leaf ($P = 0.72$), root ($P = 0.96$), and callus tissue ($P = 0.60$), indicating that the 27-bp substitution did not affect *GUS* mRNA accumulation.

Translational enhancement mediated by the *rubi3* intron and the 27-bp substitution was also affected by tissue type

Plants of pJLU2 and pJLU7 would produce identical *GUS* mRNA (after splicing in pJLU2 plants) and protein, and thus are directly comparable. As noted above, the *rubi3* intron elevated the GUS enzyme activity by 3.3-fold in leaf, 26.5-fold in root, and 51.1-fold in callus tissue (Fig. 4a), but the enhancement in mRNA accumulation was 2.2-fold in leaf, 12.8-fold in root, and 17.0-fold in callus (Fig. 4b). The discrepancy between the magnitudes of augmentation in GUS enzyme activity and the levels of mRNA accumulation in the tissues suggests an enhancement at translational level that increased the GUS protein quantity generated from per unit mRNA molecule. As shown in Fig. 4c, the *rubi3* intron boosted the GUS enzyme activity per unit mRNA in pJLU2 plants over that of the pJLU7 plants by 1.1-fold in leaf, 2.0-fold in root, and 2.9-fold in callus. Statistically, the translational enhancement is not significant in leaf ($P = 0.66$), but significant in root ($P = 0.026$) and callus ($P = 0.028$), suggesting that the translational enhancement by the intron was affected by tissue type as well.

As mentioned previously, the 27-bp substitution did not significantly alter the *GUS* mRNA accumulation in transgenic plants, but, it significantly increased the GUS activity in various tissues. As shown in Fig. 4c, the substitution boosted the GUS enzyme activity per unit mRNA by 1.8-fold ($P = 0.049$) in leaf, 2.7-fold ($P = 0.003$) in root, and 3.6-fold ($P = 1.6 \times 10^{-5}$) in callus when compared to pJLU2 plants. The pJLU6 construct produces a protein with the first nine amino acids (9 AA) of the *rubi3* ubiquitin fused to the authentic GUS protein. Thus the elevated GUS enzyme activity could arise from two possible ways: (1) increase in the catalytic activity per molar unit of GUS protein and/or extension in the half-life of GUS protein due to the fusion, or (2) increase in the GUS protein yield due to enhanced translation. The first possibility can virtually be excluded according to a recent result from our group: When the ATG of the 5′ terminal 27 bp of the *rubi3* coding sequence in pRESQ36 (from which pJLU6 was derived. See "Materials and methods") was mutated to ATC to generate the construct pRESQ85, in which the translation of the *GUS* gene would start from the authentic *GUS* initiator ATG codon and yield the native GUS protein, the two constructs had comparable GUS activity in transient expression assays in rice cells (E. Sivamani, unpublished data), indicating that the fusion of the nine AA did not increase specific enzyme activity, nor extend half-life of the protein.

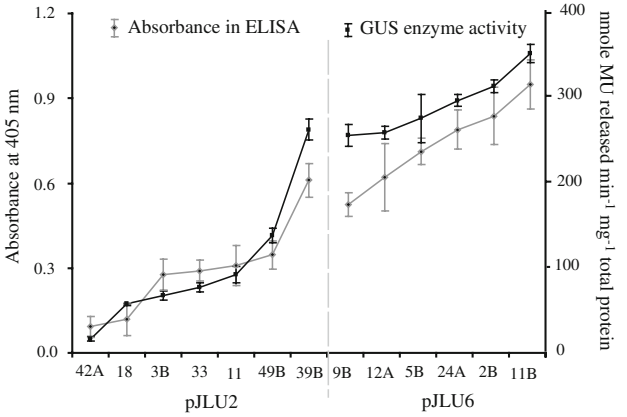

Fig. 5 Correlation between GUS enzyme activity determined by MUG assays and absorbance at 405 nm measured in ELISA for GUS protein in leaves of T_0 transgenic rice plants from pJLU2 (*left panel*) and pJLU6 (*right panel*). Plant identities are labeled on the horizontal axis. *Dark curves*, GUS enzyme activity in MUG assays; *gray curves*, absorbance in ELISA

This conclusion was further corroborated by quantitation of GUS protein in transgenic plants using ELISA. For both pJLU2 and pJLU6 plants, GUS enzyme activity level was highly correlated to the GUS protein quantity in individual plants ($R^2 = 0.916$ and $P = 7.1 \times 10^{-4}$ for pJLU2 plants; $R^2 = 0.927$ and $P = 2.0 \times 10^{-3}$ for pJLU6 plants), suggesting that the 27-bp substitution enhanced translation and thus yielded more GUS protein (Fig. 5).

Figure 6 is a summary of the analyzed stimulatory effects of the *rubi3* intron and the 27-bp substitution on *GUS* gene expression. Comparison of pJLU2 and pJLU7 plants revealed that IME by the *rubi3* intron took place mainly at the pre-translational level, where the *rubi3* intron significantly or highly significantly boosted steady state *GUS* mRNA level in leaf, root, and callus in a tissue-specific manner. The *rubi3* intron also elevated the GUS enzyme yield per unit mRNA molecule at the translational

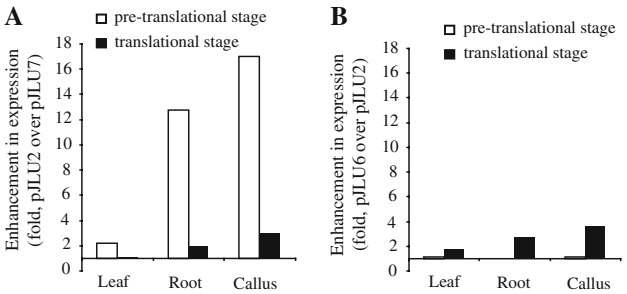

Fig. 6 Contribution of pre-translational and translational enhancement of GUS enzyme activity in leaf and root of T_0 transgenic rice plants, and in callus derived from those plants. **a** The effects of the *rubi3* 5′ UTR intron; **b** the effects of the 27-bp substitution. **a** and **b** were drawn on the same scale to show the relative strength of the effects. A value at one indicates no enhancement

level, where the enhancement was affected by tissue type as well (Figs. 4b, c, 6a). The *GUS* mRNA accumulation levels were similar between pJLU6 and pJLU2 plants, but pJLU6 plants produced significantly higher amount of protein per unit *GUS* mRNA. This translational enhancement caused by the 27-bp substitution was also affected by tissues (Figs. 4b, c, 6b).

Discussion

Plants employ various mechanisms to regulate the expression of their genes, among which introns play an important role. We have observed that the magnitude of enhancement on gene expression mediated by the 5′ UTR intron of the rice *rubi3* gene varied substantially with tissue type: the increase in GUS enzyme activity was 3.3-fold in leaf, 26.5-fold in root, and 51.1-fold in callus tissue (Fig. 4a). A related and unexpected result from the experiments was the great difference in the activity of the intronless *rubi3* promoter (pJLU7) among the tissues. We observed that the enhancement by the intron was inversely related to the basal (intron-less) levels of expression in the tissues (i.e. IME was higher in tissues that had lower basal expression), and the remarkable difference in the expression levels of the intronless *rubi3* promoter among the tissues was more or less compensated by the IME. Thus, GUS enzyme activity differed by only 2.3-fold among the tissues of pJLU2 (intron-containing) plants. It was observed that IME was negatively related to the strength of the promoter (Simpson and Filipowicz 1996). Since the strength of the intronless *rubi3* promoter appeared to vary among the tissues, this poorly understood phenomenon might have at least partially contributed to the observed tissue specificity of IME.

Tissue-dependent IME was reported in transgenic dicot plants. In transgenic *Arabidopsis thaliana* plants, the IME effect by the 5′ UTR introns of the two replacement histone *H3* genes was shown to be affected by both the promoters and the plant tissues (Chaubet-Gigot et al. 2001). However, the reported results were compromised by analyzing a mixed T_1 population consisting of both homozygous and hemizygous plants, and by the absence of information on transgene copy number and RNA expression analysis. In monocots, Gallie and Young (1994) demonstrated that the intron 1 of the maize *Adh1* gene differentially stimulated the *LUC* gene expression in transient assays using protoplasts or cells derived from various tissues of maize (*Zea mays*). In the present study, by analyzing IME in single-copy transgenic rice plants, we observed that the enhancement in *GUS* reporter gene expression mediated by the 5′ UTR intron of the rice *rubi3* gene varied a great deal with tissue type. Moreover, through the quantitation of *GUS* mRNA accumulation, we were able to further the previous studies by breaking down the enhancement mediated by the *rubi3* 5′-UTR intron into two levels, a pre-translational level and a translational level, and revealed that the effects of the intron at both levels depended on tissue type (Fig. 6).

Since pJLU6 and pJLU2 plants shared a similar level of accumulated *GUS* mRNA, the further enhancement in GUS activity observed in pJLU6 plants was apparently translational. We correlated the GUS enzyme activity and the protein quantity in the two groups of plants to demonstrate that the elevated GUS enzyme activity in pJLU6 plants could be attributed to the increased protein level. In addition, we revealed that the magnitude of the translational enhancement varied with tissue type as well. This is the first report on the tissue-specificity of translational enhancement mediated by sequences surrounding the translation initiation sites in plants. The context sequences flanking the translation initiation codon of an mRNA affect its translational efficiency (Kozak 2002). As judged by the consensus sequences for this region (Joshi et al. 1997; Kozak 1999; Sawant et al. 1999), the sequence around the translation initiation site in pJLU6, after intron splicing, is more favorable for translation (Fig. 1b), which could, at least partially, contribute to the enhanced translational yield of pJLU6 plants as compared to pJLU2 plants although other possible causes cannot be ruled out.

The observed tissue-specificity of IME has important implications for IME studies in plants. First, it reveals that the scale of IME differs among callus, root, leaf, and possibly other tissues. Therefore, it is important to examine various tissues when studying IME. Second, it questions the long-standing notion that the magnitude of IME in monocots is usually greater than that in dicots (Koziel et al. 1996; Simpson and Filipowicz 1996; Clancy and Hannah 2002). The notion might not have been formed on comparable bases concerning the distinct tissues mostly used in the previous studies on IME in monocots and dicots. While IME in dicots was often investigated with leaf tissue and ranged from 2 to 20-fold (Norris et al. 1993; Genschik et al. 1994; Rose 2002, 2004), the reported IME in monocots was usually observed in cells of callus origin, with magnitudes between 20 and 100-fold (Callis et al. 1987; Rethmeier et al. 1997; Bourdon et al. 2001; Clancy and Hannah 2002). In this report, we revealed a great difference in IME between the two types of tissue in transgenic rice plants, i.e., 3.3-fold in leaf versus 51.1-fold in callus. Moreover, studies suggested that some dicot introns could also mediate high magnitudes of IME: the 5′ UTR intron of the tomato (*Lycopersicon esculentum* Mill.) *ubq1−1* gene increased *GUS* reporter gene activity in the leaf tissue of transgenic tobacco (*Nicotiana tabacum* L.) plants by 40-fold (Rollfinke et al. 1998) and the 5′ UTR introns of the two *Arabidopsis* replacement histone H3 genes elevated GUS activity in roots of transgenic *Arabidopsis* plants by

70-fold (Chaubet-Gigot et al. 2001). These findings prompt us to wonder whether IME and the mechanisms behind it in the two major classes of the angiosperm plants are as different as suggested (Clancy and Hannah 2002). More studies using monocot transgenic plants will help answer the question. Exchanging introns between the two classes of plants could also provide more insights on the issue. It is known that monocot introns are not well spliced in dicots (Simpson and Filipowicz 1996). This may hinder the proper testing of monocot introns in dicot plants. However, since dicot introns can be well spliced in monocots (Simpson and Filipowicz 1996), studying IME of dicot introns in transgenic monocot plants would be feasible to help reveal the similarity and difference in IME between the two classes of angiosperm plants.

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