Plant Cell Rep (2008) 27:1587–1600
DOI 10.1007/s00299-008-0577-y

GENETIC TRANSFORMATION AND HYBRIDIZATION

Activity of the 5′ regulatory regions of the rice polyubiquitin *rubi3* gene in transgenic rice plants as analyzed by both *GUS* and *GFP* reporter genes

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Received: 5 April 2008 / Revised: 3 June 2008 / Accepted: 20 June 2008 / Published online: 18 July 2008
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Abstract Ubiquitin is an abundant protein involved in protein degradation and cell cycle control in plants and *rubi3* is a polyubiquitin gene isolated from rice (*Oryza sativa* L.). Using both *GFP* and *GUS* as reporter genes, we analyzed the expression pattern of the *rubi3* promoter as well as the effects of the *rubi3* 5′-UTR (5′ untranslated region) intron and the 5′ terminal 27 bp of the *rubi3* coding sequence on the activity of the promoter in transgenic rice plants. The *rubi3* promoter with the 5′-UTR intron was active in all the tissue and cell types examined and supported more constitutive expression of reporter genes than the maize *Ubi-1* promoter. The *rubi3* 5′-UTR intron mediated enhancement on the activity of its promoter in a tissue-specific manner but did not alter its overall expression pattern. The enhancement was particularly intense in roots, pollen grains, inner tissue of ovaries, and embryos and aleurone layers in maturing seeds. The translational fusion of the first 27 bp of the *rubi3* coding sequence to *GUS* gene further enhanced *GUS* expression directed by the *rubi3* promoter in all the tissues examined. The *rubi3* promoter should be an important addition to the arsenal of strong and constitutive promoters for monocot transformation and biotechnology.

Keywords *GFP* · *GUS* · Intron · Rice *rubi3* promoter · Transgenic plant

Abbreviations

5′-UTR	5′ Untranslated region
CaMV	Cauliflower mosaic virus
DIC	Differential interference contrast
GFP	Green fluorescent protein
GUS	β-Glucuronidase
IME	Intron-mediated enhancement of gene expression
MU	4-Methylumbelliferone
MUG	4-Methylumbelliferyl-β-D-glucoside
NOS	Nopaline synthase

Introduction

Ubiquitin is a highly conserved 76-residue protein found in all eukaryotes. It has been implicated in multiple cellular processes, including protein degradation and cell cycle control (Rechsteiner 1991; Hochstrasser 1996). Polyubiquitin genes have been isolated and characterized in a variety of plant species (Sivamani and Qu 2006). Promoter–reporter gene fusions were used in studies to examine the activity of the polyubiquitin gene promoters in transient assays or in transgenic plants. Such studies have been performed for polyubiquitin promoters isolated from sunflower (Binet et al. 1991), maize (Christensen et al. 1992; Toki et al. 1992; Gallomeagher and Irvine 1993; Wilmink et al. 1995; Gallomeagher and Irvine

Communicated by P. Ozias-Akins.

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1996; Bhattacharjee et al. 1997; Muhitch and Shatters 1998; Stoger et al. 1999; Hill-Ambroz et al. 2001; Streatfield et al. 2004), tobacco (Genschik et al. 1994), potato (Garbarino et al. 1995), sugarcane (Wei et al. 1999; Wei et al. 2003), *Arabidopsis* (Kamo et al. 2000), and rice (Wang et al. 2000; Wang and Oard 2003). Some studies suggested that the maize *Ubi-1* promoter, including its 5′-UTR intron, is one of the promoters with the highest expression in monocots (Christensen et al. 1992; Cornejo et al. 1993; Gallomeagher and Irvine 1993; Schledzewski and Mendel 1994; Wilmink et al. 1995; Wei et al. 2003; Atkinson et al. 2004). In spite of the magnitude of investigations on the expression of the plant polyubiquitin genes, their expression patterns have rarely been studied comprehensively at tissue and cellular levels in transgenic plants. Brief histochemical analyses on the activity of the important maize *Ubi-1* promoter are spread over several reports in rice (Cornejo et al. 1993; Takimoto et al. 1994) and wheat (Stoger et al. 1999; Rooke et al. 2000). Two other similar studies examined the expression pattern of the tomato *ubq1-1* promoter (Rollfinke et al. 1998) and the tobacco *Ubi.U4* promoter (Plesse et al. 2001) in a few organs of tobacco. These histochemical localization assays using GUS as the reporter indicated that the polyubiquitin gene promoters function in most but not all tissue and cell types. However, since these reports did not address the potential artifacts contingent upon the use of *GUS* as a reporter gene (Mascarenhas and Hamilton 1992), the reliability of the observed expression pattern was compromised.

A striking structural feature of plant polyubiquitin genes is that they usually contain an intron within their 5′-UTR at a conserved location, that is, immediately upstream the translation initiator codon. The 5′-UTR introns of some plant polyubiquitin genes have been reported to be able to quantitatively enhance reporter gene expression and thus are capable of intron-mediated enhancement of gene expression (IME). These genes include the *Arabidopsis UBQ3* and *UBQ10* genes (Norris et al. 1993), the tomato *ubq1-1* gene (Rollfinke et al. 1998), the tobacco *Ubi.U4* gene (Plesse et al. 2001), and the rice *RUBQ2* gene (Wang and Oard 2003). However, few studies have investigated the effects of such 5′-UTR introns on the expression pattern of plant polyubiquitin genes or characterized their IME at tissue and cell levels. This is particularly true for polyubiquitin genes of monocot origin.

The rice polyubiquitin gene *rubi3* (GenBank accession AY954394), isolated in our laboratory, encodes a pentameric polyubiquitin and its 5′ regulatory region contains an intron immediately preceding its translation initiation codon (Sivamani and Qu 2006). The 5′-UTR intron enhances gene expression at transcriptional, post-transcriptional and translational levels in rice suspension cells (Samadder et al. 2008). Using *GUS* as a reporter gene, we have shown that the *rubi3* intron differentially enhanced the activity of its promoter in leaf and root of transgenic rice plants (Lu et al. 2008). In the present study, we analyzed the expression pattern of the *rubi3* promoter and the effect of its 5′ intron on its activity in vegetative and reproductive organs of transgenic rice plants at tissue and cellular levels. As reporters for gene expression studies, GUS has the advantage of high sensitivity but is prone to produce artifacts (Mascarenhas and Hamilton 1992); and GFP can accurately reflect gene expression pattern at the cellular level but suffers from autofluorescence in certain cell types (Sheen et al. 1995). To ensure accuracy and minimize artifacts, we used both the *GUS* and *GFP* as reporters to faithfully reflect the activity of the *rubi3* promoter and the effects of its intron. In addition, we previously observed significant increase of GUS activity in leaf and root of transgenic rice plants as a result of the substitution of a translational fusion between the first 27 bp of the *rubi3* coding sequence and the *GUS* gene for a transcriptional fusion between a 20-bp *GUS* leader sequence and the *GUS* gene (termed "27-bp substitution") in a construct controlled by the intron-containing *rubi3* promoter (Lu et al. 2008). In the present study, we investigated the effects of the 27-bp substitution on the expression pattern and strength of the intron-containing *rubi3* promoter in vegetative and reproductive organs of transgenic rice plants at tissue and cellular levels. In this report, we demonstrate that the *rubi3* promoter is a more constitutive promoter than the maize *Ubi-1* promoter and it drives the expression of the two reporter genes in all the tissue and cell types examined. We show that the *rubi3* 5′-UTR intron enhances the activity of the proper *rubi3* promoter throughout vegetative and reproductive stages and the degrees of the enhancement are affected by cell and tissue type. Moreover, we reveal that the 27-bp substitution further augments *GUS* gene expression in plants to a level comparable to that of the maize *Ubi-1* promoter. For the first time, the enhancing effects of the 5′-UTR intron and the 5′ terminal coding sequence of a polyubiquitin gene are elucidated elaborately at tissue and cell levels in a monocot species. The results suggest that the *rubi3* promoter and its derivatives might have potential applications in the transformation and biotechnology of grasses including major cereal crops.

Materials and methods

Plasmid construction

The transformation plasmids pJLU7, pJLU2 and pJLU6, which contain the *GUS* reporter gene driven by the *rubi3*

promoter without its 5′-UTR intron, with its 5′-UTR intron, and with the 5′ terminal 27 bp of the *rubi3* coding sequence between the *rubi3* 5′-UTR intron and the *GUS* coding sequence, respectively, were constructed as described (Lu et al. 2008). To make a transformation plasmid containing the maize *Ubi-1* promoter, a 4135 bp *Hin*dIII fragment harboring the *GUS* expression cassette in pRESQ29 (Sivamani and Qu 2006) was inserted into the *Hin*dIII site in the binary vector pCAMBIA1300, generating plasmid pJLU5 (Fig. 1).

To generate constructs using *GFP* as the reporter gene, the *sGFP*(S65T) gene was amplified from the plasmid pRTL2-sGFP (Sit et al. 1998) using the FailSafe™ PCR kit (Epicentre, Madison, WI, USA) and the primers:

FsGFP 5′GACCCGGGCCATGGGATCGATGCATCAT
*Sma*I
C3′
RsGFP 5′GGAGCGAGCTCTTACTTGTACAGCTCGT
*Sac*I
CCATGC3′

Restriction sites *Sma*I and *Sac*I were added to the 5′ and 3′ ends of the *sGFP*(S65T) coding sequence, respectively, to facilitate gene construction. The PCR product was cloned into the TA cloning vector pCR® 2.1 (Invitrogen, Carlsbad, CA, USA) to generate pJLU8. A 757 bp *Sma*I and *Sac*I fragment from pJLU8 containing the *sGFP*(S65T) sequence was ligated with *Sma*I and *Sac*I-digested pPSRG30 and pRESQ4 (Sivamani and Qu 2006) to replace the *GUS* gene in these vectors, resulting in plasmids pJLU10 and pJLU11, respectively. A 1,905 bp *Eco*RI fragment from pJLU10 was inserted into the *Eco*RI site in the binary vector pCAMBIA1300. The resultant plasmid, pJLU12, contains the intron-less *rubi3* promoter, the *sGFP*(S65T) gene, and the *NOS* terminator in the pCAMBIA1300 backbone. Similarly, a 3045 bp *Eco*RI fragment from pJLU11 was ligated into *Eco*RI linearized binary vector pCAMBIA1300 to generate pJLU13, which contains the *rubi3* promoter, its 5′-UTR intron, the *sGFP*(S65T) gene, and the *NOS* terminator in the pCAMBIA1300 backbone (Fig. 1).

Gene fragments obtained by PCR were verified using sequencing analysis by the DNA Sequencing Facility at Iowa State University (Ames, IA, USA). All the plasmids were validated by restriction digestions and transient expression assays.

Rice transformation

The plasmid DNAs of pJLU7, pJLU2, pJLU6, pJLU5, pJLU12 and pJLU13 were used separately to transform the *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) harboring the plasmid pToK47 (Jin et al. 1987) to generate *Agrobacterium* strains for the transformation of the rice cultivar Taipei 309. Transformation of *A. tumefaciens* and rice as well as the subsequent maintenance of transgenic plants were conducted as previously described (Lu et al. 2008).

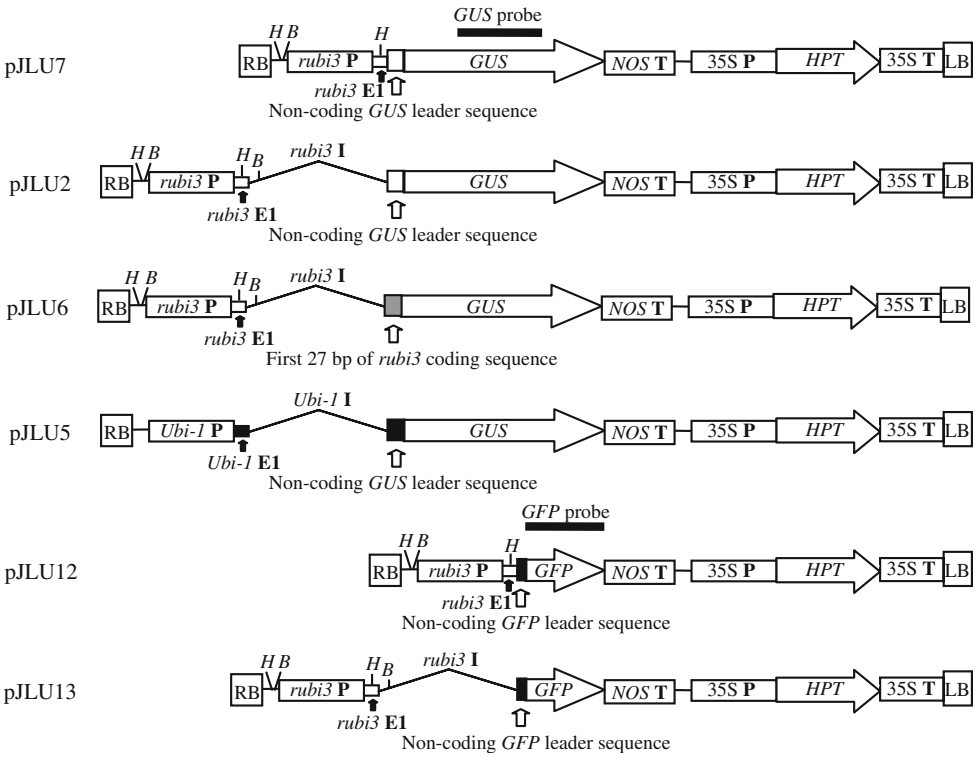


Fig. 1 Structures of the T-DNA regions of the binary plasmids used in rice transformation. All of the plasmids are based on pCAMBIA1300. *LB* left border, *RB* right border, *P* promoter, *T* terminator, *E1* exon 1, *HPT* hygromycin phosphotransferase gene,*GUS* β-glucuronidase gene, *GFP* the green fluorescent protein gene sGFP(S65T), *NOS* nopaline synthase gene, 35S CaMV 35S, *H Hin*dIII, *B Bam*HI. Probing regions used in Southern hybridization of T_0 transgenic plants are also shown

Determination of transgene copy number

The copy numbers of the *GUS* gene in the T0 transgenic plants for pJLU7, pJLU2, pJLU6 and pJLU5 were determined using quantitative real-time PCR of genomic DNA (Ingham et al. 2001) at Syngenta Biotechnology, Inc. (Research Triangle Park, NC, USA). Southern blotting was used to confirm the *GUS* gene copy number of the pJLU7, pJLU2, and pJLU6 transformants which contain a single-copy *GUS* gene according to quantitative real-time PCR (Lu et al. 2008). The copy numbers of the *sGFP*(S65T) gene in the primary transformants of pJLU12 and pJLU13 were estimated by Southern hybridization of genomic DNA (Lu et al. 2008) with a ^{32}P-labeled 768-bp PCR product amplified using FsGFP and RsGFP as primers and pRTL2-sGFP as the template (as described previously; Fig. 1)

Histochemical localization and quantification of *GUS* gene expression

Histochemical localization and fluorometric quantification of the GUS enzyme activity in transgenic plants were conducted as previously reported (Lu et al. 2008). Briefly, samples were taken from T0 transgenic and control plants at various developmental stages (six-leaf, eight-leaf, eleven-leaf, pre-boot, heading, and waxy-seed stages). For histochemical localization, plant tissues were sectioned with a VIBRATOME® Series 1000 sectioning system (Technical Products International, Inc., St. Louis, MO, USA) and/or by manual sectioning. Sections were then incubated for 16 hrs at 37°C in GUS assay buffer. Micrographs were taken after the reactions were stopped. Images for the same type of tissues were captured under identical settings. For fluorometric quantification of the GUS enzyme activity, 10 µl of diluted extracts was mixed with 130 µl MUG (4-methylumbelliferyl-β-D-glucoside) assay buffer and incubated at 37°C for 20 min. Fluorescence from the reactions was quantified using a Fluostar® instrument set at Time-Resolved Fluorescence mode (BMG Labtech, Inc., Duhram, NC, USA). 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 the same Fluostar® set at Absorbance mode. GUS activities were expressed in nmol MU (4-methylumbelliferone) released min^{-1} mg^{-1} total protein.

Visualization of green fluorescent protein in transgenic plants

Visualization of GFP expressed in transgenic plants was conducted at the Cell and Molecular Imaging Facility at North Carolina State University. Fresh samples were taken from various developmental stages as described in the "Results". For each type of tissue, the settings of the microscopic system were adjusted so that the fluorescence signals from samples should not saturate, and, in most cases, the signal from control should be at the background level. If the control tissue showed strong autofluorescence, the settings were adjusted to only ensure that the fluorescence signals from samples should not saturate. Images for the same type of tissues were taken using the same settings.

At high magnification, GFP was visualized with a Leica TCS SP confocal laser scanning system attached to an inverted Leica DM IRBE microscope (Leica Microsystems, Wetzlar, Germany). Samples were sectioned with a VIBRATOME® Series 1000 sectioning system (Technical Products International, St Louis, MO, USA) and/or manually. Specimens were mounted on microscopic slides immediately following sectioning and before examination on the microscope with a 20× NA 0.7 dry lens or a 40× NA 1.25 oil immersion lens. GFP was excited with an argon laser at 488 nm and emission was collected from 500 to 553 nm. Differential Interference Contrast (DIC) images were recorded simultaneously and compared with the fluorescence images to locate the cells or tissues that generate fluorescence.

At low magnification, GFP was visualized using a Leica MZ FLIII dissecting microscope coupled with a Hamamatsu (Bridgewater, NJ, USA) Cooled Color CCD Camera (C5810) connected to a computer system. The GFP 3 filter set (excitation 470 ± 20 nm, dichroic 495 nm, emission 525 ± 25 nm) was used for imaging. The light source used was an HBO 100 W mercury lamp. A bright field image was taken for each fluorescent image to identify the cells or tissues that fluoresce by comparison of the two images.

Statistical analyses

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

Results

Generation and analysis of transgenic rice plants

In order to study the regulation of gene expression by the rice *rubi3* gene promoter, 5′-UTR intron, and sequences flanking the translation initiation codon, six binary transformation plasmids, namely pJLU7, pJLU2, pJLU6, pJLU5, pJLU12, and pJLU13 were generated for *Agrobacterium*-mediated transformation of the rice cultivar Taipei 309. As shown in Fig. 1, pJLU7 and pJLU2 contain

the *GUS* gene under the control of the *rubi3* promoter, without or with its 5′-UTR intron, respectively; pJLU6 has the first 27 bp of the *rubi3* coding sequence in place of the 20 bp *GUS* leader sequence in pJLU2; pJLU5 contains the maize *Ubi-1* promoter, its 5′-UTR intron, and the *GUS* gene; pJLU12 and pJLU13 contain the *sGFP*(S65T) gene driven by the *rubi3* promoter, without or with its 5′-UTR intron, respectively.

To minimize the effects of transgene copy number and DNA rearrangement on transgene expression (Hobbs et al. 1993; Jorgensen et al. 1996; Wang and Waterhouse 2000; Butaye et al. 2005), we chose *Agrobacterium*-mediated transformation because it produces predominantly simple integration patterns with defined borders (Hansen and Chilton 1999), and we selected transgenic plants with a single-copy transgene for gene expression analysis. Primary transformants for pJLU7, pJLU2, and pJLU6 were analyzed preliminarily using real-time PCR and further by Southern blotting to screen for transgenic events with a single copy *GUS* gene insertion (Lu et al. 2008). All of the transformants containing a single copy of the *GUS* gene as assayed by real-time PCR were confirmed by Southern analysis, indicating the criteria used in real-time PCR analysis were stringent enough to identify single-copy transgenic plants. Therefore, T0 plants of pJLU5 were screened for single-copy *GUS* gene plants directly using real-time PCR. Eventually, 6, 7, 7, and 10 independent, single-copy transformants were identified for pJLU7, pJLU2, pJLU6 and pJLU5, respectively. The T0 transgenic plants of pJLU12 and pJLU13 were directly analyzed by Southern blot hybridization, whereby 4 and 3 single-copy-transgene plants were identified, respectively (Fig. 2). Only transgenic plants with a single-copy transgene were used in further studies.

Five independent, single-copy T0 transgenic plants were randomly chosen for each of the pJLU7, pJLU2, and pJLU6 constructs to characterize their *GUS* expression pattern by histochemical assays. Since only 3 or 4 single-copy transgenic plants were obtained from the *GFP* constructs, all of these T0 transformants were used in the subsequent studies. Because of transgene segregation and recombination in the pollen grains and seeds produced by the T0 plants, sample number was increased for seeds and pollen grains for all the plants studied. At least 16 seeds and dozens of pollen grains collected from each of the T0 sample plants were used to observe the expression patterns of the reporter genes. Individual plants transformed with the same constructs showed considerable variation in the expression intensity of reporter genes (Lu et al. 2008), but no alteration of their expression patterns was observed. Presented in this report are the expression patterns of plants with medium expression intensities among the plants transgenic of the same constructs.

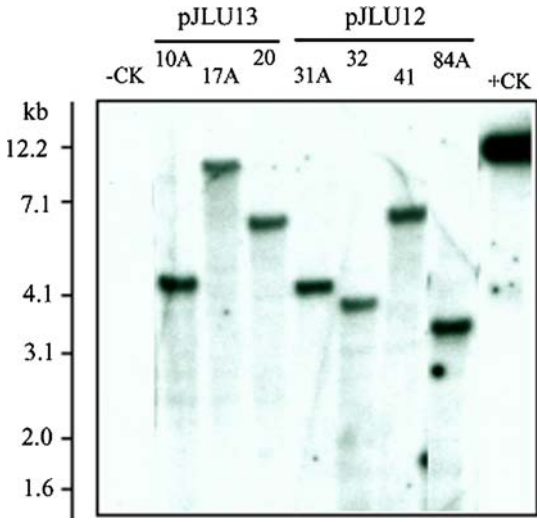


Fig. 2 Southern hybridization analysis of T0 transgenic plants transformed with the *GFP* constructs. Ten micrograms of genomic DNAs from leaf extracts were digested with *Hin*dIII, size-resolved by electrophoresis, and the blot was hybridized with ^{32}P-labeled *GFP* probe depicted in Fig. 1. Results of the single-copy transgenic plants are presented here. *–CK* Negative control, genomic DNA from non-transformed rice cultivar Taipei 309; *+CK* positive control, 140 pg of pJLU13 plasmid DNA mixed with 10 μg of genomic DNA from Taipei 309. Molecular weight markers are shown on the left

The activity of the *rubi3* promoter in transgenic rice plants

The *rubi3* promoter, including its 5′-UTR intron, was able to drive reporter gene expression in all the tissues and cell types of the transgenic rice plants examined, but the expression levels were not uniform among the tissues. Columns 3 and 6 of Fig. 3 and columns 2 and 5 of Fig. 4 illustrate its activity when controlling the expression of the *GFP* and *GUS* genes, respectively. A combined analysis of the expression pattern of the two reporter genes provided more reliable information regarding the activity of the promoter.

The expression pattern of the *rubi3* promoter in roots and leaves was examined in plants at the six-leaf stage. In root tips, the strongest expression of *GFP* was found in the border region of the procambium, the protoderm, and the apical meristem. The expression in the root cap and inside the procambium was medium, and the expression in the cortex was relatively weak (Fig. 3c). In young roots, the *rubi3* promoter conferred the strongest expression in several layers of cells surrounding the endodermis and the pericycle (Fig. 4e). Expression was strong in the exodermis, the sclerenchymatous layer and the epidermis including root hairs (Fig. 4b, e). Expression was also strong in the central region of the vascular cylinder (Fig. 4e) mainly due to the strong expression in the parenchyma cells in the phloem and xylem (data not shown). The central region of the cortex showed relatively weak

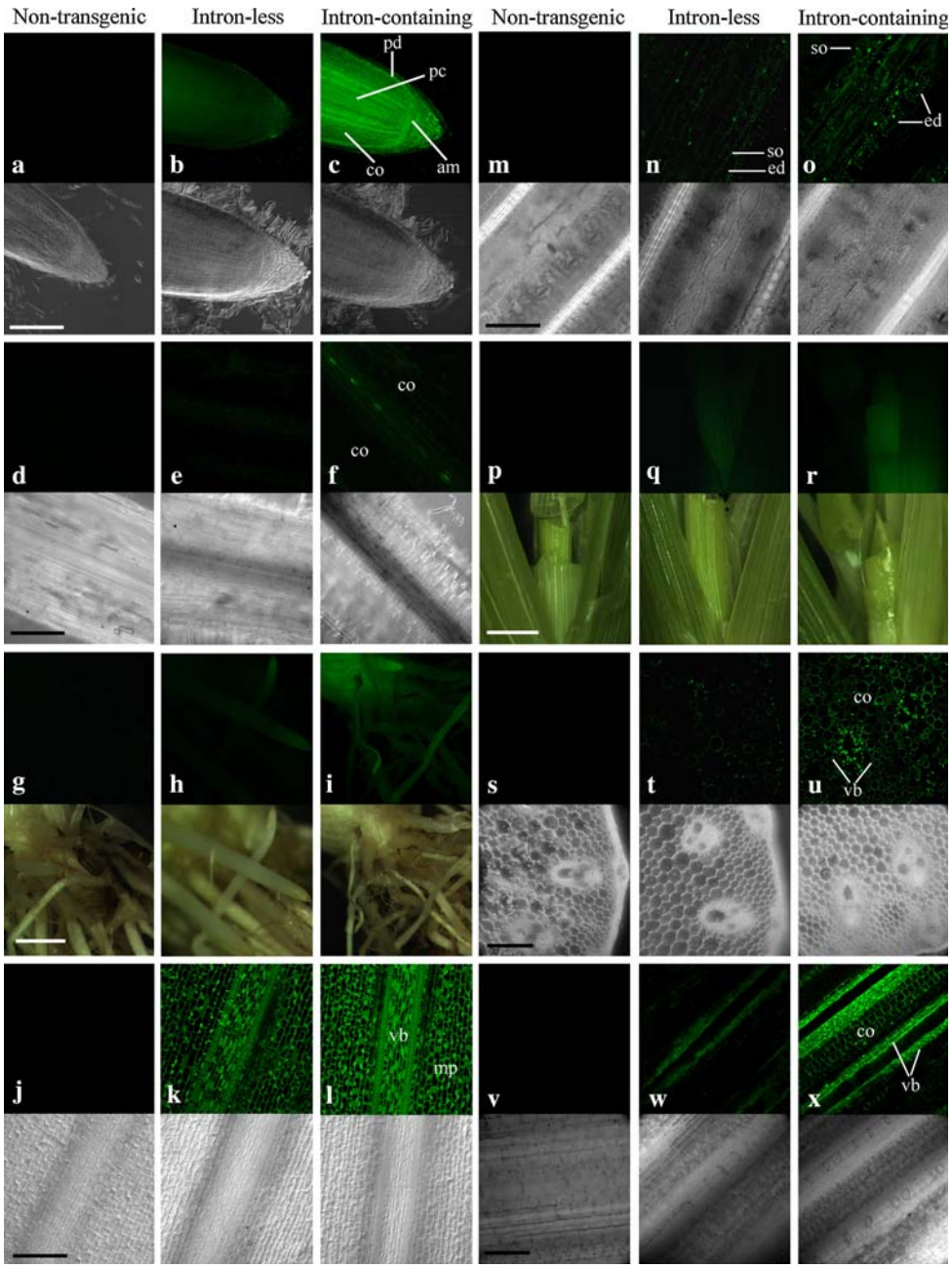
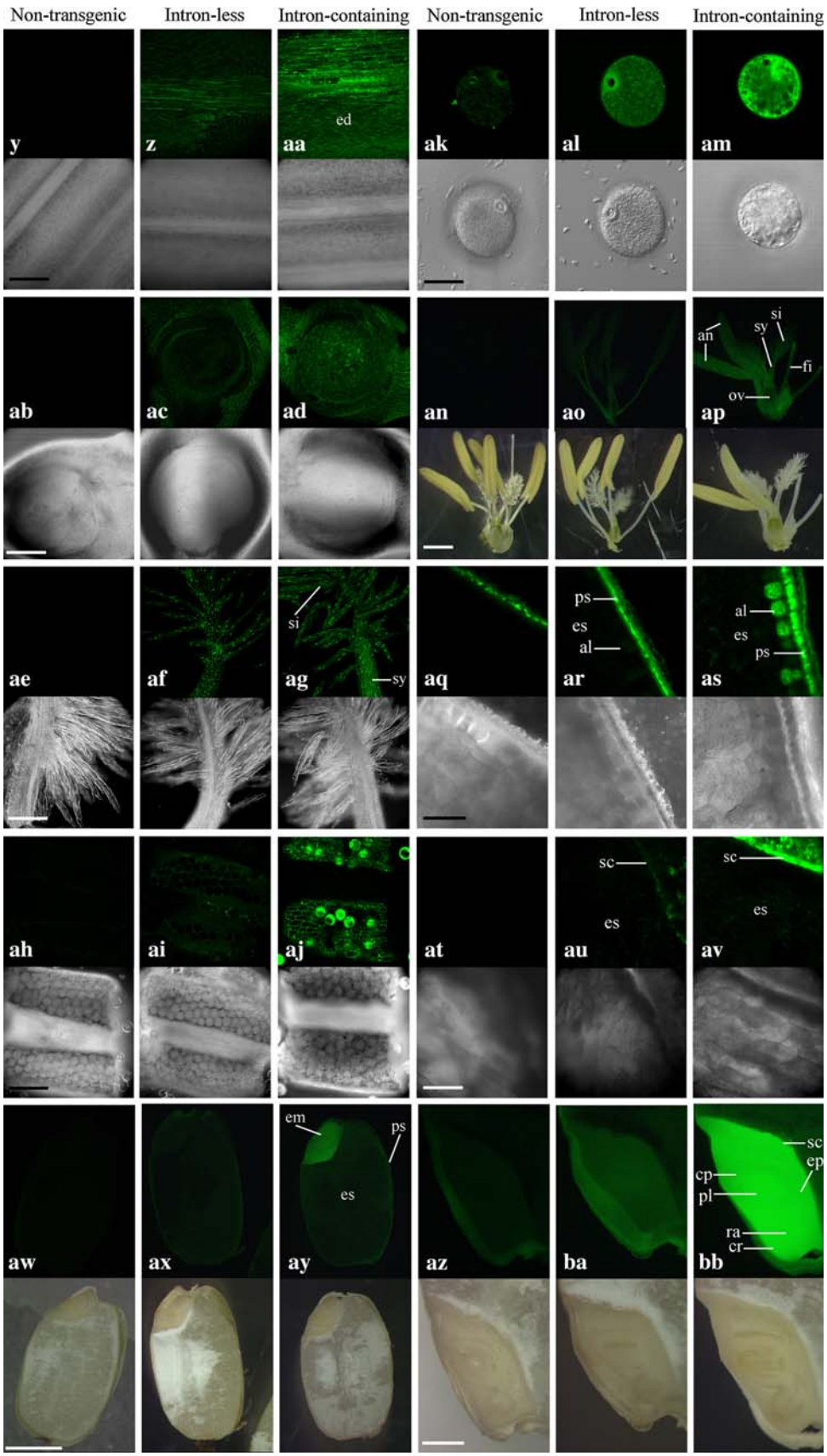


Fig. 3 Expression of GFP in single-copy transgenic rice plants. *Columns 1* and *4* non-transgenic rice cultivar Taipei 309, *columns 2* and *5*, plants transformed with the intron-less pJLU12, *columns 3* and *6* plants transgenic of the intron-containing pJLU13. GFP images on the same rows of the first three columns and the second three columns were captured under the same settings, with differential interference contrast or bright field images taken at the same time. Sampling stages: **a–r** six-leaf stage, **s–aa** eleven-leaf stage, **ab–ap** heading stage, **aq–bb** waxy stage of seed development. **a–c** Root tips, *bar* 150 μm; **d–f** longitudinal sections of mature roots, *bar* 150 μm; **g–i** root systems of seedlings, *bar* 2 mm; **j–l** longitudinal sections of un-emerged, young leaves, *bar* 150 μm; **m–o** surfaces of fully emerged leaves, *bar* 150 μm; **p**–r transverse views of seedlings, *bar* 2 mm; **s–u** cross sections of stems, *bar* 150 μm; **v–x** longitudinal sections of stems, *bar* 150 μm; **y–aa** stem surfaces, *bar* 150 μm; **ab–ad** longitudinal sections of ovaries, *bar* 150 μm; **ae–ag** stigmas and styles, *bar* 150 μm; **ah–aj** pollen sacs, *bar* 150 μm; **ak–am** pollens, *bar* 150 μm; **an–ap** flowers with glumes removed, *bar* 1 mm; **aq–as** endosperms, pericarps and seed coats, *bar* 50 μm; **at–av** scutella and endosperms, *bar* 150 μm; **aw–ay** longitudinal sections of seeds, *bar* 2 mm, **az–bb** longitudinal sections of embryos, *bar* 0.5 mm. *al* Aleurone layer, *am* apical meristem, *an* anther, *co* cortex, *cp* coleoptile, *cr* coleorhiza, *ed* epidermal cell, *em* embryo, *ep* epiblast, *es* endosperm, *fi* filament, *mp* mesophyll cells, *ov* ovary, *pc* procambium, *pd* protoderm, *pl* plumule, *ps* pericarp and seed coat, *ra* radicle, *sc* scutellum, *si* stigma, *so* stomata, *sy* style, *vb* vascular bundle

Fig. 3 continued



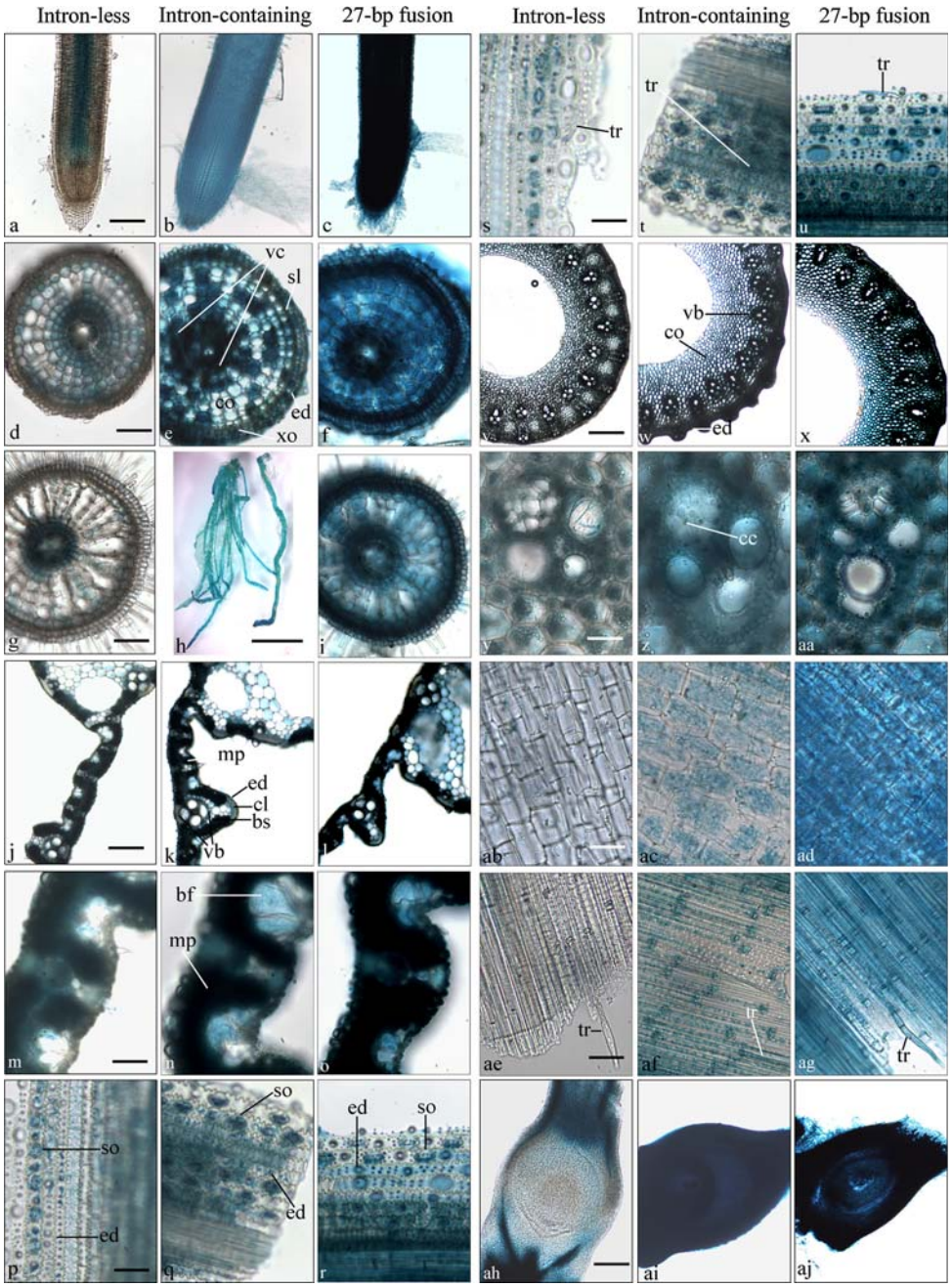
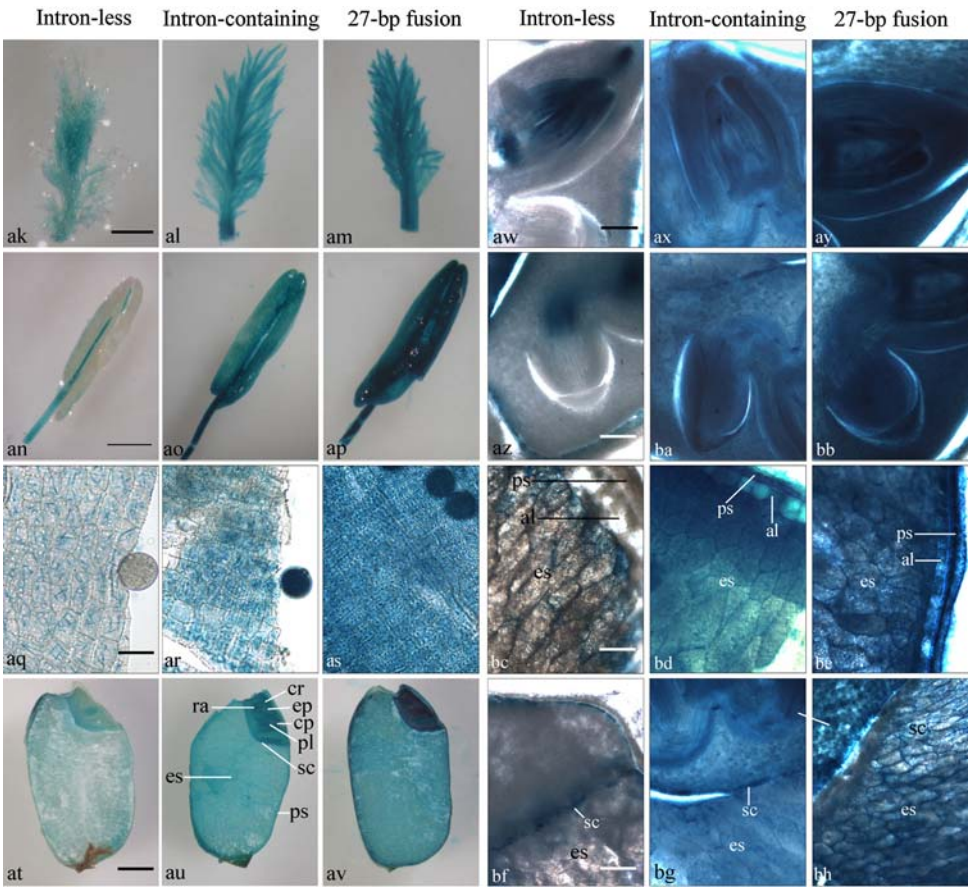


Fig. 4 Expression of GUS in single-copy transgenic rice plants. *Columns 1* and *4* plants transformed with the intron-less pJLU7; *columns 2* and *5* plants transgenic of the intron-containing pJLU2; *columns 3* and *6* plants transformed with pJLU6, which contains the 27-bp fusion. GUS images on the same rows of the first three columns and the second three columns were captured using the same settings. Sampling stages: **a–u** six-leaf stage, **v–ag** eleven-leaf stage, **ah–as** heading stage, **at–bh** waxy stage of seed development. **a–c** Root tips, *bar* 200 μm; **d–f** cross sections of root tips at elongation zone, *bar* 100 μm; **g** and **i** cross sections of mature roots, *bar* 100 μm; **h** root system of a seedling, *bar* 3 mm; **j–l** cross sections of leaf blades, *bar* 200 μm; **m–o** close-ups of partial views in **j–l**, *bar* 50 μm; **p–r** leaf surfaces, *bar* 50 μm; **s–u** leaf surfaces, *bar* 50 μm; **v–x**, cross sections of stems, *bar* 0.5 mm; **y–aa**, vascular bundles of stems, *bar* 50 μm; **ab–ad** cortexes of stems, *bar* 50 μm; **ae–ag** epidermis of stems, *bar* 50 μm; **ah–aj** longitudinal sections of ovaries, *bar* 200 μm; **ak–am** stigmas and styles, *bar* 200 μm; **an–ap** anthers and filaments, *bar* 0.5 mm; **aq–as**, pollens and pollen sac walls, *bar* 50 μm; **at–av** longitudinal sections of seeds, *bar* 2 mm; **aw–ay**, plumules and coleoptiles, *bar* 200 μm; **az–bb** radicles and coleorhizae, *bar* 200 μm; **bc–be** pericarps and endosperms, *bar* 100 μm; **bf–bh** scutella and endosperms, *bar* 200 μm. *al* Aleurone layer, *an* anther, *bf* bulliform cells, *bs* vascular bundle sheath, *cc* companion cell, *cl* collenchyma cell, *co* cortex, *cp* coleoptile, *cr* coleorhiza, *ed* epidermis, *ep* epiblast, *es* endosperm, *fi* filament, *mp* mesophyll cells, *pl* plumule, *ps* pericarp and seed coat, *ra* radicle, *sc* scutellum, *sl* sclerenchymatous layer, *so* stomata, *tr* trichome, *vb* vascular bundle, *vc* vascular cylinder, *xd* exodermis

Fig. 4 continued



expression (Figs. 4e, 3f). Expression was stronger towards the tip region of the roots than in the upper region, and in young roots than in older roots (Figs. 3c, f, i; 4b, e, h).

In leaves, the highest expression was observed in the mesophyll cells (Fig. 4k, n). Expression in the vascular bundle sheath, bundle sheath extension, bulliform cells, stomata, epidermis, trichomes and parenchyma cells in the vascular bundle was also high (Figs. 3l, o; 4k, n, q, t). But, GUS staining was not as intense in the collenchyma cells interior to the epidermis (Fig. 4k). Figure 3r is a view through the whorled leaves of the main stem of a plant at the six-leaf stage, which shows clearly that stronger GFP fluorescence is associated with the inner whorls that correspond to younger leaves. To understand the effect of leaf age on *GUS* gene expression, we examined the GUS activity levels of fully emerged leaves at consecutive positions along the pJLU2 plants at the eight-leaf stage (Fig. 5). GUS enzyme activity varied highly significantly with leaf position ($P < 0.0001$), with younger leaves showing higher GUS activity.

The activity of the promoter in stems was studied in plants at the 11-leaf stage. The vascular bundles exhibited the most intense GUS and GFP reporter activities mainly due to their high expression in the tissues surrounding the xylem vessels and phloem elements as well as in the companion cells within the phloem (Figs. 3u, x; 4w, z). Expression in the epidermis tissue and trichome were strong as well (Figs. 3aa; 4w, af). The cortex tissue showed

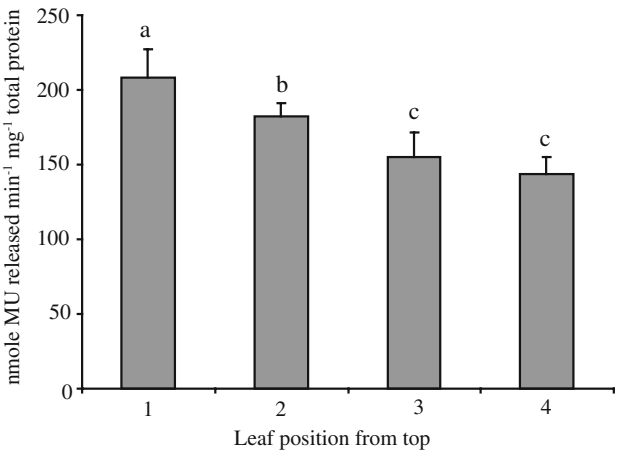


Fig. 5 GUS enzyme activity in leaves at consecutive positions along T0 transgenic rice plants of the plasmid pJLU2. Four fully emerged leaves were taken from the top of eight-leaf stage plants. Total protein extracts were incubated with MUG reaction buffer for 20 min, and GUS activities were presented as nmoles of MU released min^{-1} mg^{-1} total protein. Means and their standard errors are shown. Means labeled with the same letter are not significantly different according to Tukey's Studentized Range (HSD) Test at $\alpha = 0.05$

less intense expression than other tissues in both the *GFP* and *GUS* plants (Figs. 3u, x; 4w, ac).

In flowers of the transformants at heading stage, strong expression of both reporter genes was observed in ovary, style, stigma, anther and filament (Figs. 3ad, ag, aj, ap; 4ai, al, ao, ar). The expression in the vascular tissues of the filament, anther, style and stigma was particularly strong. The strongest expression was found in pollen grains (Figs. 3aj, am, ap; 4ar). Non-transgenic pollen grains showed autofluorescence (Fig. 3ak), but the fluorescence from the *GFP* expressed in the transformants was much stronger than the autofluorescence (Fig. 3am), demonstrating intense activity of the *rubi3* promoter in the pollen grains. In addition, the inner side of the pollen sac showed very weak autofluorescence (Fig. 3ah), which did not interfere with the strong fluorescence from the GFP in that tissue (Fig. 3aj).

In waxy-stage seeds, the expression of both the *GUS* and *GFP* genes in the embryos was the most prominent, with the expression in the pericarp, seed coat, and aleurone layer being strong, and that in the endosperm weak (Figs. 3as, av, ay, bb; 4au, ax, ba, bd, bg). Inside the embryos, the strongest expression was found in the plumule, radicle, coleoptile and coleorhiza (Figs. 3bb; 4au, ax, ba). Expression in the scutellum was also strong but that in the epiblast was less intense (Figs. 3bb; 4au, bg). It was not clear whether the light GUS staining on the surface of the endosperm section was an artifact (Fig. 4au, bd, bg). However, the expression of the *GFP* in the endosperm provided more reliable evidence to show that the *rubi3* promoter did have expression in the endosperm (Fig. 3ay). The pericarp and seed coat showed considerable autofluorescence (Fig. 3aq), but the autofluorescence was weaker when compared to the fluorescence from the expressed GFP (Fig. 3as). The activity of the *rubi3* promoter in pericarp and seed coat was further supported by the expression of *GUS* in these tissues (Fig. 4au, bd).

The ability of the *rubi3* promoter to drive *GFP* and *GUS* expression in all the tissues and cell types examined in the transgenic plants indicates that it is a *bona fide* constitutive promoter.

The effects of the 5′-UTR intron on the activity of the *rubi3* promoter

Columns 2 and 5 of Fig. 3 and columns 1 and 4 of Fig. 4 illustrate the expression of the *GFP* and *GUS* genes driven by the intron-less *rubi3* promoter (constructs pJLU12 and pJLU7, respectively). Compared with the expression conferred by the *rubi3* promoter containing its 5′-UTR intron (pJLU13 in columns 3 and 6 of Fig. 3 and pJLU2 in columns 2 and 5 of Fig. 4), the expression conditioned by the intron-less *rubi3* promoter was weaker in expression intensity but similar in expression pattern, that is, the 5′-UTR intron enhanced the expression level of the *rubi3* promoter but did not alter its overall expression pattern. However, the degrees of the enhancement varied with tissue types.

In root tips and young roots, the expression of both the *GFP* and *GUS* driven by the intron-containing *rubi3* promoter is much stronger than that conferred by the intron-less promoter (Figs. 3b, c, e, f; 4a, b, d, e). Such drastic enhancement in reporter gene expression mediated by the *rubi3* 5′-UTR intron was also observed in embryos (Figs. 3ba, bb; 4at, au, aw, ax, az, ba), aleurone layers (Figs. 3ar, as; 4bc, bd), and the inner tissue of ovaries (Figs. 3ac, ad; 4ah, ai).

The "basal" expression level of the intron-less *rubi3* promoter was relatively high in leaves (Figs. 3k, n; 4j, m), stigma (Figs. 3af; 4ak), and vascular bundles (Figs. 3k, w; 4j, y, an), while the enhancement by the 5′-UTR intron was relatively low in these tissues, as reflected in Figs. 3l, o, x, ag, and 4 k, n, al, ao.

This tissue-specific enhancement in expression level by the *rubi3* 5′-UTR intron was also observed in anther and pollen grains. In plants containing the intron-less *rubi3* promoter, *GFP* and *GUS* expression in pollen grains was weaker than that in the anther tissue, while in plants harboring the intron-containing *rubi3* promoter, pollen grains had much higher expression than the anther tissue (Figs. 3aj, ai; 4ar, aq).

The effects of the 27-bp substitution on the GUS activity directed by the intron-containing *rubi3* promoter

In leaf and root of transgenic plants, the 27-bp substitution significantly elevated GUS activity conferred by the intron-containing *rubi3* promoter (Lu et al. 2008). In the present study, we investigated the effects of the 27-bp substitution in various tissues and cells of transgenic plants. As shown in Fig. 4, the GUS staining intensity of the pJLU6 plants (columns 3 and 6) was generally higher than that of the pJLU2 plants (columns 2 and 5), indicating that the 27-bp substitution enhanced the expression level of the *GUS* gene throughout all tissues and organs and at all developmental stages examined. However, the 27-bp substitution did not alter the expression pattern of the intron-containing *rubi3* promoter in transgenic plants.

To evaluate the enhancing effect of the 27-bp substitution, the GUS activities in the leaves and roots of pJLU6 plants were compared with that of pJLU5, which contained the maize *Ubi-1* promoter driving the expression of the *GUS* gene. Arithmetically, the pJLU6 plants generated higher GUS activity than the pJLU5 plants, but their difference was not statistically significant with the plant

Table 1 GUS activity in leaves and roots of T0 transgenic rice plants of pJLU6 and pJLU5

	pJLU6	pJLU5	*P*-value
Leaf[a]	254 ± 4[b]	198 ± 29[b]	0.28
Root[a]	467 ± 47[b]	465 ± 87[b]	0.98
Sample size	7	10	

[a] Leaf samples were taken from emerging flag leaves at pre-boot stage and root samples were taken simultaneously

[b] Means and their standard errors are shown

numbers used in the test (Table 1). Thus, we concluded that the pJLU6 and pJLU5 plants had comparable levels of GUS enzyme activity in leaves and roots, suggesting that the 27-bp substitution substantially augmented the GUS activity directed by the intron-containing *rubi3* promoter.

Discussion

It is essential to understand and control transgene expression in plant transformation and biotechnology. While promoters play a pivotal role in controlling gene expression, introns and sequences surrounding translation initiation codons are important *cis*-elements that modulate the expression of genes. Using both *GFP* and *GUS* as reporter genes, we have characterized the expression pattern of the rice *rubi3* promoter and the effects of its 5′-UTR intron on its expression in transgenic rice plants. We have also investigated the effects of the 5′ terminal 27 bp of the *rubi3* coding sequence on the expression of the *rubi3* promoter.

The *β*-glucuronidase encoded by *GUS* gene is often used as a reporter because of its high sensitivity and convenience in assays. But when used as an indicator for expression pattern, the reporter could produce artifacts caused by the diffusion of the soluble indoxyl product into a neighboring tissue and the formation of the insoluble indigo by oxidation and dimerization in that tissue (Mascarenhas and Hamilton 1992). Moreover, histochemical GUS staining would not take place in tissues that the substrate is unable to penetrate, causing false negative results for gene expression in those tissues. On the other hand, GFP as a reporter can be visualized directly in living plant cells, eliminating the need of a substrate and the problems associated with it. However, some plant tissues produce autofluorescence that may confound the fluorescence from the expressed GFP, rendering it unreliable to report the expression pattern in those tissues (Sheen et al. 1995). In this study, by using both *GFP* and *GUS* as reporter genes and comparing our observations with the two reporters, we were able to reliably analyze the expression pattern of the *rubi3* promoter and the effects of its 5′-UTR intron.

We have demonstrated that the intron-containing *rubi3* promoter directed constitutive expression of both reporter genes and its expression level was not uniform among various tissues. It appeared that the activity of the promoter was affected by cell division, differentiation and developmental stages. The promoter conferred stronger GFP fluorescence in younger roots than in older roots (Fig. 3i). In the same root, both GUS staining and GFP fluorescence diminished distal from the root tips, and the expression transited from strong to weak along the cell division, elongation and differentiation regions in the root tips (Figs. 3i; 4h). In the whorled leaves of young plants, the inner whorls, which correspond to younger leaves, showed stronger GFP fluorescence (Fig. 3r). Similarly, in waxy-stage seeds, *GFP* and *GUS* expressed much stronger in embryos than in endosperms (Figs. 3ay, av; 4au). Similar phenomena were observed with the maize polyubiquitin *Ubi-1* promoter expressed in rice (Cornejo et al. 1993; Takimoto et al. 1994) and wheat (Rooke et al. 2000), and with the tobacco polyubiquitin *Ubi.U4* promoter in tobacco (Plesse et al. 2001). In addition to its high expression in young tissues, we also observed strong activity of the *rubi3* promoter in some fully differentiated, metabolically active tissues, such as the mesophyll cells, the stomata, and the bulliform cells of leaves (Figs. 4k, n, q, t; 3o) as well as the scutellum and the aleurone layer of maturing seeds (Figs. 3as, av; 4bd, bg). Moreover, the rice *rubi3* promoter had strong expression in vascular tissues, which was evident in the differentiating vascular tissues of root tip (Fig. 3c), the vascular tissues of roots (Figs. 3f; 4e), leaves (Figs. 3l; 4k), stems (Figs. 3u, x; 4w, z), flower parts (Fig. 4ai, al, ao), maturing embryos (Fig 4au, ax, ba), as well as lemma and palea (data not shown). Some plant polyubiquitin gene promoters were previously found to confer strong *GUS* expression in vascular tissues (Cornejo et al. 1993; Takimoto et al. 1994; Rollfinke et al. 1998; Rooke et al. 2000; Plesse et al. 2001). Since vascular tissue functions in fluid conduction and thus may facilitate penetration of the GUS assay buffer within the tissue, one may argue that the intense GUS staining in vascular tissues observed in those studies could be caused by easy penetration of the GUS substrate rather than strong expression of the promoters. However, by using both the *GFP* and *GUS* as reporter genes, this study unambiguously demonstrated the strong expression of the *rubi3* promoter in vascular tissues. The spatio-temporal expression pattern of polyubiquitin genes could be due to the physiological functions of the ubiquitin protein in plants (Bachmair et al. 1990; King et al. 1996; Woffenden et al. 1998).

In this report, we also revealed that the 5′-UTR intron of the *rubi3* gene enhanced the expression level of the

reporter genes in all the tissues and cell types examined, but the degrees of the augmentation varied by tissue and cell types. The intron brought about greater increase in GUS staining and GFP fluorescence in roots than in leaves (Figs. 3a-r; 4a–d, j, k, m, n). This observation is consistent with our previous quantification results using MUG assay, where the intron augmented reporter GUS activity in stable transformants by more than 20 fold in roots but only approximately three fold in leaves (Lu et al. 2008). Therefore, the relative intensity of the histochemical images obtained in this study reflected the expression level of reporter genes in different tissue and cell types. The present study furthered our previous observation by examining the IME mediated by the *rubi3* 5′-UTR intron throughout plant organ, tissue and cell types at various developmental stages and revealed more tissues supporting differential magnitudes of IME. The additional tissues that supported higher IME by the *rubi3* intron included embryos (Figs. 3ba, bb; 4at, au, aw, ax, az, ba), aleurone layers (Figs. 3as, ar; 4bc, bd), pollen grains (Figs. 3am, al; 4ar, aq), and the inner tissue of ovaries (Figs. 3ad, ac; 4ai, ah); and the additional tissues that had lower IME included stigma (Figs. 3af, ag; 4ak, al), sac walls (Figs. 3ai, aj; 4aq, ar), and the outer tissue of ovaries (Figs. 3ad, ac; 4ai, ah). Moreover, the "basal" expression levels of the intron-less *rubi3* promoter were relatively low in tissues with higher IME and relatively high in tissues with lower IME. The mechanisms for our above observations with IME are yet to be elucidated. Although tissue dependence of IME was reported in dicots (Fu et al. 1995a, b; Chaubet-Gigot et al. 2001; Plesse et al. 2001), this is the first time the tissue-specificity of IME is revealed at tissue and cell levels in a monocot species.

Moreover, we showed that the 27-bp substitution further augmented *GUS* gene expression directed by the intron-containing *rubi3* promoter in all the tissues examined but did not alter the expression pattern. Analysis indicated that the fusion of the 27 bp elevated the translational efficiency of the *GUS* mRNA without altering the specific activity of the GUS enzyme (Lu et al. 2008). Therefore, this gene construction approach could possibly be applied to other genes or promoters to enhance their expression.

Promoters that drive strong, constitutive expression are typically used in plant transformation to produce high levels of transgene products. The maize *Ubi-1* promoter falls into this category of promoters and has been the most extensively used promoter in monocot transformation (Weeks et al. 1993; Wilmink et al. 1995; Bhattacharjee et al. 1997; Hill-Ambroz et al. 2001; Atkinson et al. 2004). But the promoter is considered near-constitutive since it did not express in certain cell types. For example, GUS activity driven by the promoter was not detected in the somatic tissues of the anthers in transgenic rice (Cornejo et al. 1993) and wheat (Rooke et al. 2000). In contrast, the *rubi3* promoter and its derivatives (the intron-less, intron-containing, and the intron-containing promoter with the 27-bp fusion) were active in those tissues (Figs. 3ai, aj, ao, ap; 4an, ao, ap, aq, ar, as) in addition to all other tissues and cell types examined in transgenic rice plants. Therefore, the *rubi3* promoter and its derivatives were able to drive more constitutive expression than the maize *Ubi-1* promoter. As mentioned above, the intron-containing *rubi3* promoter with the 27-bp fusion was as strong as, if not stronger than, the maize *Ubi-1* promoter. These features make it a good candidate for monocot transformation and biotechnological applications which require high and constitutive expression of transgenes. In practice, the availability of a spectrum of promoters that differ in their expression level is useful for tailoring transgene expression. Correspondingly, the various derivatives of the *rubi3* promoter, which differ in their expression level, might find their use in this aspect. The expression pattern and strength of the *rubi3* promoter and its derivatives, as documented at organ, tissue and cell levels in this study, would be helpful in these potential applications.

Acknowledgments We thank Drs. N. S. Allen, E. Johannes, and J. Xu for their technical assistance with the imaging work at CMIF, NCSU, the Plant Analysis Group at Syngenta Biotechnology, Inc., for assaying the transgene copy number using realtime PCR, Dr. K. Azhakanandam for technical assistance in rice transformation, as well as Drs. J. Thomas, J. Shurtleff, and C. Saravitz, and J. Edwards for assistance in using the North Carolina State University Phytotron facility for plant maintenance. We also wish to thank Dr. T. Sit for providing the plasmid pRTL2-sGFP and Dr. P. Samadder for the plasmid pPSRG30. We are grateful to Drs. G. Allen, N. S. Allen, R. Dewey, and J. Nicholson for helpful discussions on the project. This work was supported in part by grants from the Center for Turfgrass Environmental Research and Education, North Carolina State University, The Consortium for Plant Biotechnology Research, Inc., and Syngenta Biotechnology, Inc.

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