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Expression enhancement of a rice polyubiquitin gene promoter

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Abstract

An 808 bp promoter from a rice polyubiquitin gene, *rubi3*, has been isolated. The *rubi3* gene contained an open reading frame of 1140 bp encoding a pentameric polyubiquitin arranged as five tandem, head-to-tail repeats of 76 aa. The 1140 bp 5′ UTR intron of the gene enhanced its promoter activity in transient expression assays by 20-fold. Translational fusion of the *GUS* reporter gene to the coding sequence of the ubiquitin monomer enhanced GUS enzyme activity in transient expression assays by 4.3-fold over the construct containing the original *rubi3* promoter (including the 5′ UTR intron) construct. The enhancing effect residing in the ubiquitin monomer coding sequence has been narrowed down to the first 9 nt coding for the first three amino acid residues of the ubiquitin protein. Mutagenesis at the third nucleotide of this 9 nt sequence still maintains the enhancing effect, but leads to translation of the native GUS protein rather than a fusion protein. The resultant 5′ regulatory sequence, consisting of the *rubi3* promoter, 5′ UTR exon and intron, and the mutated first 9 nt coding sequence, has an activity nearly 90-fold greater than the *rubi3* promoter only (without the 5′ UTR intron), and 2.2-fold greater than the maize *Ubi1* gene promoter (including its 5′ UTR intron). The newly created expression vector is expected to enhance transgene expression in monocot plants. Considering the high conservation of the polyubiquitin gene structure in higher plants, the observed enhancement in gene expression may apply to 5′ regulatory sequences of other plant polyubiquitin genes.

Abbreviations: GUS, β-glucuronidase; LUC, luciferase; MU, methylumbelliferone; MUG, 4-methyl-β-D-glucuronide; NOS, nopaline synthase; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Introduction

A high level of constitutive gene expression is usually needed for the production of important proteins for agronomical or commercial purposes in transgenic plants. To achieve this goal, we need to understand and exploit the mechanisms plants developed to regulate expression levels of their various genes during evolution. Gene expression in eukaryotes is a multi-stage process controlled at transcriptional, post-transcriptional, translational and post-translational levels (Lewin, 2000). Ubiquitin is a 76 aa protein, highly conserved in all eukaryotes and involved in selective protein degradation in cells (Callis and Vierstra, 1989). Cells have a high demand for this protein, and have evolved to have a tandem repeat sequence to code for polyubiquitin proteins, which are later cleaved by ubiquitin-specific proteases in the cells (Hondred *et al.*, 1999) to release the ubiquitin monomers. The polyubiquitin gene expression was reported to be nearly constitutive in plants with increased expression levels in young tissues (Burke *et al.*, 1988; Binet *et al.*, 1991a, b; Cornejo *et al.*, 1993; Kawalleck *et al.*, 1993).

Many promoters of plant polyubiquitin genes have been tested and reported to drive high expression of foreign genes placed under their control. Such promoters have been isolated from *Arabidopsis* (Callis *et al*., 1990), sunflower (Binet *et al*., 1991a, b), tobacco (Genschik *et al*., 1994), potato (Garbarino and Belknap, 1994), maize (Christensen and Quail, 1996), rice (Wang *et al*., 2000) and sugarcane (Wei *et al*., 2003). The high expression capacity of these promoters is usually due to an intron located within the 5′ untranslated region (UTR). The intron-mediated enhancement (IME) of gene expression is often more pronounced in monocots than in dicots (Rose and Beliakoff, 2000). Among these the maize polyubiquitin *Ubi1* promoter has been extensively and successfully used to express chimeric genes in monocot transformation studies (Streatfield *et al*., 2004).

Although the location of plant polyubiquitin introns shows a high degree of conservation (in the 5′ UTR of the gene and immediately before the translational initiation codon ATG), the size of the 5′ introns varies in different species. The rice RUBQ1 and RUBQ2 introns were found to be 782 and 962 bp, respectively (Wang *et al*., 2000). In *Arabidopsis*, the polyubiquitin genes *UBQ3*, *10* and *11* had 5′ UTR introns of sizes 375, 304, or 334 bp, respectively (Norris *et al*., 1993). The 5′ introns of the two sugarcane polyubiquitin genes *ubi4* and *ubi9* were found to be 1360 and 1374 bp, respectively (Wei *et al*., 2003), whereas the maize *Ubi1* gene has an intron of 1010 bp (Christensen *et al*., 1992).

In addition to the enhancement of polyubiquitin promoter activity by its 5′ UTR, it was shown in dicots that *GUS* and *LUC* reporter gene expression were significantly enhanced when the reporter gene coding sequences were in-frame fused to a ubiquitin monomer coding sequence in the gene constructs (Hondred *et al*., 1999). It was also shown that the ubiquitin moiety is subsequently removed by ubiquitin C-terminal hydrolases or de-ubiquitinating enzymes, a family of novel, sequence-specific proteases that release the ubiquitin monomers so that only the unmodified reporter gene products accumulated *in vivo* (Wilkinson 1997; Hondred *et al*., 1999).

In this study, we identified a new polyubiquitin gene from rice, which we designated as *rubi3*, and studied the effects of its 5′ regulatory sequence on gene expression. The *rubi3* gene has an intron of 1140 bp within its 5′ UTR and the intron enhances its own and other promoters' activities in transient assays of rice suspension cells bombarded with the constructs. In addition, similar to the results in dicot plants, we observed that the GUS enzyme activity was enhanced approximately 4-fold when the gene was fused with the *rubi3* monomer coding sequence in a construct using the *rubi3* promoter and 5′ UTR intron to drive the fusion gene. Furthermore, we have narrowed down the enhancement effects to the first 9 nt of the ubiquitin coding sequence, located at the beginning of the exon 2 and immediately following the 5′ UTR intron. Mutagenesis at the third nt of this sequence abolishes translation from the ubiquitin translation initiation codon ATG but does not affect the enhancing effect on the GUS enzyme activity and yields native protein (rather than a fusion one) from the transgene. The newly created expression vector which contains the *rubi3* promoter, 5′ UTR exon and intron, and the 9 nt mutated exon 2 sequence can be used to enhance transgene expression in monocot plants.

Materials and methods

Database used

Monsanto's draft rice genome sequence database (http://www.rice-research.org) of *japonica* cv. Nipponbare was used in the BLAST search for polyubiquitin gene sequences. The sequences for *rubi3* were identified in Monsanto's BAC contigs OSM14960 and 14965 in the database.

Plant materials

Rice seeds (cv. Nipponbare) were kindly provided by Harold E. Bockelman from the National Small Grains Collection, USDA-ARS, Aberdeen, ID 83210. Rice suspension cells derived from primary calli induced from mature seeds were maintained in 125 ml flasks containing 50 ml of AA medium (Toriyama and Hinata, 1985) on an orbital shaker (150 rpm). Cultures were grown in darkness at 25 °C and subcultured once a week by diluting 25 ml suspension culture with 25 ml of fresh AA medium.

Isolation of genomic DNA and PCR

Genomic DNA from rice leaves was isolated using a modified procedure of Dellaporta *et al*. (1983). For isolation of the *rubi3* promoter and intron sequences, 4 primers were commercially synthesized, and used in different combinations to amplify the 5′ promoter region plus intron, promoter alone, or intron alone. The names and sequences of the primers are as follows:

4 – 5′ GTCGACCACCCAACCCCATATCGA CAGAGG 3′
UR7sma – 5′ CCCGGGCTGGAAGAGGCA AGAAAGGATTGGAATTAAC 3′
2sma – 5′ CCCGGGTAACTAATCAATCAC CTCGT 3′
UR8sma – 5′ CCCGGGCTTGACGAGGCG ATTAGAGAACGC 3′

The locations of the primers in the *rubi3* genomic sequence are marked in Figure 1. Polymerase chain reactions to clone these genomic DNA fragments were performed with Failsafe™ PCR kit (Epicentre, Madison, WI, USA) using conditions recommended by the manufacturer.

RT-PCR

RT-PCRs were performed to obtain cDNA fragments and to identify the exact location of the putative *rubi3* intron. Total RNA from cv. Nipponbare leaves was isolated as described (Sivamani *et al*., 1999). About 2 μg of total RNA was reverse transcribed with the AMV reverse transcriptase (Promega, Madison, WI, USA) using 40 pmol of primer 5′ ACACGATGATATGACAGACGAGC 3′, which is complementary to a region in the 3′ UTR of the *rubi3* gene. The reverse transcription (RT) reaction was performed for 1 h at 42 °C. PCR was carried out with *Taq* DNA polymerase (Eppendorf, Westbury, NY, USA), using 5 μl of RT reaction as template, 40 pmol each of the above primer and primer 5′CGAATCGACCGAAGGGGAGG 3′, derived from a sequence located in the 5′ UTR region of the *rubi3* gene. The PCR product of ca. 1.3 kb was cloned in the TA cloning vector pCR2.1™ (Invitrogen, Carlsbad, CA, USA) to obtain clone pRESQ30.

Identification of transcription initiation site

Transcription initiation site of the *rubi3* gene was identified using the GeneRacer™ kit (Invitrogen), which specifically targets 5′ capped mRNA to yield full-length cDNA information. Total RNA from cv. Nipponbare leaves was extracted using the RNeasy Mini™ Kit (Qiagen, Valencia, CA, USA). About 2 μg of total RNA was used for reverse transcription. The resultant cDNA molecules were amplified with the kit, and cloned in pCR2.1™. Three independently amplified clones were subjected to sequence analysis.

DNA sequencing

The PCR products were gel-purified and cloned in the TA cloning vector pCR2.1™. Plasmid DNAs were prepared using a standard plasmid mini preparation method. DNA sequencing was performed at the DNA sequencing facility of Iowa

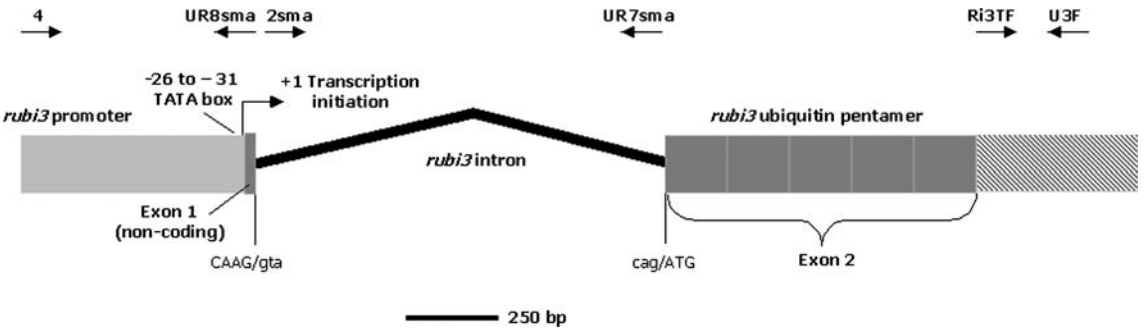

Figure 1. Structural organization of the rice *rubi3* gene. The *rubi3* gene contains 2 exons separated by a 5′ UTR intron of 1140 bp. The non-coding exon 1 in the 5′ UTR contains 67 bp, and the exon 2 has an open reading frame of 1140 bp encoding a pentameric polyubiquitin. The transcriptional initiation site is represented by an arrow at nt +1. A putative TATA box sequence is located between nt −31 and −26. The sequences surrounding the 5′ and 3′ splicing sites of the 5′ UTR intron are indicated. The locations of the six primers used in PCR amplification of the *rubi3* gene fragments for the study are also marked.

State University (Ames, IA, USA) or by MWG Biotech (High Point, NC, USA).

Chimeric gene constructs

The constructs used in the experiment are summarized in Figure 2.

The clone pRESQ21 was made by ligating a 2 kb PCR product obtained using primers 4 and UR7sma in a pCR2.1™ vector using a TA cloning kit. After checking the sequence and orientation, the 2 kb *Spe*I/*Sma*I fragment was cloned into the *Xba*I/*Sma*I site of pRESQ8 [a pUC119 based vector containing the *GUS* gene (Jefferson *et al*., 1986) and NOS terminator] to create pRESQ4 that contains the *rubi3* promoter, 5′ UTR exon 1 and intron, the *GUS* gene (including a 20 bp leader sequence coming from pAHC25 (Christensen and Quail, 1996)), and the NOS terminator.

The clone pPSRG30 was created to contain the *rubi3* promoter, the *GUS* gene and the NOS terminator (Samadder *et al*., in preparation), and would have the identical mRNA sequence as pRESQ4 does after the intron is spliced.

A 4.2 kb *Hin*dIII fragment containing the maize *Ubi1* promoter-*GUS* construct from pAHC25 (Christensen and Quail, 1996) was ligated in the *Hin*dIII site of pUC119 to create pRESQ29 so the vector's size would be comparable to others tested in the experiments.

Construction of the chimeric rubi3-GUS *fusion vectors*

Standard cloning techniques, and the three-primer PCR technique (Yon and Fried, 1989) were used to make the *rubi3-GUS* fusion gene constructs. The NOS terminator is used for all the constructs. The following two 'outer oligos' were used in combination with a 'linking-oligo', and with pRESQ8 and pRESQ30 DNA as templates, to make the fusion constructs:

Outer-oligo1: 5′ GTATTGTATCTGGCTCTT TGCC 3′
Outer-oligo2: 5′ AGTAAGACTGCTTTTTCT TGCC 3′

Translational fusion between the first monomer of the *rubi3* gene and the *GUS* coding sequence (pRESQ38) was made using a 'linking-oligo' with the following sequence: 5′ CTACAGGACGTAA CATGCCTCCACGAAGGCGGA 3′.

Translational fusion of the first 9 aa coding sequence of the *rubi3* gene with the *GUS* coding sequence (pRESQ36) was made using a 'linking-oligo' with the following sequence: 5′ GGGGTTT CTACAGGACGTAACATAGTGAGGGTCTT AACGAATATCTGCATCTGGAAGA 3′.

Translational fusion of the first 3 aa coding sequence of the *rubi3* gene with *GUS* (pRESQ42) was made using a 'linking-oligo' with the following sequence: 5′ GGGGTTTCTACAGGACGTAAC ATTATCTGCATCTGGAAGA 3′.

Construction of the mutated rubi3-GUS *fusion vectors*

Site-directed mutagenesis of the third nt G in the in-frame fusion of the first 9 nt of the *rubi3* coding sequence (ATGCAGATA) with the *GUS* coding sequence was performed using the vector pRESQ42 by the three-primer PCR technique as described above. In the resultant vectors, the G was mutated to A, T, or C yielding pRESQ46 (G to A), pRESQ47 (G to T), and pRESQ48 (G to C), respectively (Figure 2). The mutagenesis was confirmed by sequence analysis.

Transient GUS *and* LUC *assays*

All the plasmids used for transformation experiments were purified using the Qiagen® plasmid midi kit (Qiagen) according to manufacturer's procedure.

Particle bombardment of various *GUS* gene constructs on suspension rice cells was employed to evaluate the expression levels of the constructs using the Bio-Rad PDS-1000 He biolistic particle delivery system (Bio-Rad, Hercules, CA, USA). Since the size of the vectors is not the same, to obtain more precise results, bombardment was performed using the same molar quantities of the plasmid DNA in each treatment. To minimize the variability from shot to shot, the firefly luciferase gene (*LUC*) was used as an internal control to normalize the GUS data (Leckie *et al*., 1994).

Approximately 200 mg rice suspension cells (cv. Nipponbare) were evenly distributed over the surface of a 15 mm Whatman No. 2 filter paper in a 100×15 mm petri dish for particle bombard-

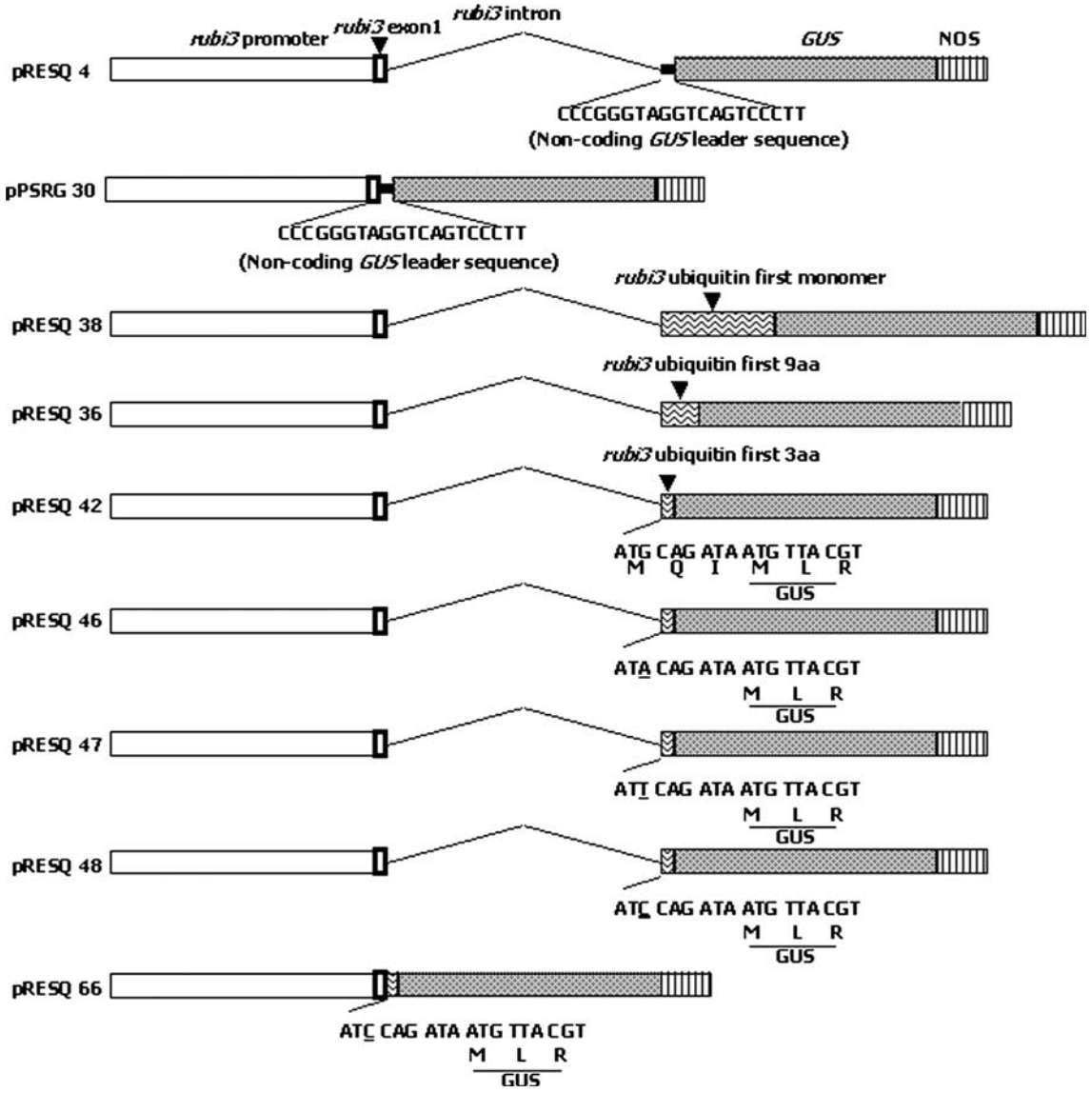

Figure 2. Schematic diagrams of the *GUS* expression cassettes based on the *rubi3* regulatory sequences. All the constructs use NOS terminator. Salient features of these constructs are as follows: pRESQ4 – the *rubi3* promoter, its 5′ UTR exon and intron to drive the *GUS* gene. pPSRG30 – identical to pRESQ4 except that it does not contain the *rubi3* 5′ UTR intron. Both the pRESQ4 and pPSRG30 cassettes contain a 20 bp stretch of non-coding leader sequence of the *GUS* gene as shown. pRESQ38 – the *rubi3* promoter, its 5′ UTR exon and intron, and the first monomer coding sequence of the *rubi3* gene translationally fused with the *GUS* coding sequence. pRESQ36 – the *rubi3* promoter, its 5′ UTR exon and intron, and the first 9 aa coding sequence (27 nt) of the *rubi3* gene translationally fused with the *GUS* coding sequence. pRESQ42 – the *rubi3* promoter, its 5′ UTR exon and intron, and the first 3 aa coding sequence (9 nt) of the *rubi3* gene translationally fused with the *GUS* coding sequence. pRESQ46, 47 and 48 – identical to pRESQ42 except that the third nucleotide G in the first 9 nt *rubi3* coding sequence was mutated to A (pRESQ46), T (pRESQ47) or C (pRESQ48). pRESQ66 – identical to the construct pRESQ48 except that it does not contain the *rubi3* 5′ UTR intron.

ment. Plasmids were coated onto gold particles (1.0 μm in diameter) and introduced into suspension cells via particle bombardment according to the method described by Sivamani *et al.* (1996). Equimolar amounts of each construct containing the chimeric *GUS* gene were delivered to the suspension cells with four replications. To normalize the transient assay data, the plasmid

pJD313 (J. Sheen, personal communication) containing the *LUC* gene under the control of CaMV 35S promoter and the maize *Adh1* intron 1 was mixed in a 1:1 ratio with the test plasmid constructs. After 24 h incubation at 25 °C, the bombarded cells from each filter were transferred into a microfuge tube and homogenized with an equal volume of 2× GUS extraction buffer (Leckie *et al*., 1994). Cell homogenates were centrifuged at 10 000×*g* for 5 min at 4 °C, and the supernatant was collected and assayed. The protein concentration of the supernatant was determined using the Bio-Rad protein assay kit (Bio-Rad).

For GUS enzyme assay, 50 μl of the supernatant were assayed according to the method described by Gallagher (1992) using a FLUO-star BMG fluorometer (BMG Lab Technologies, Durham, NC, USA). GUS activities of the three replicates were analyzed, and expressed as pmole MU released $min^{-1}mg^{-1}$ total protein. Simultaneously, histochemical GUS assays (Jefferson *et al*., 1987) were performed on the fourth replicate to visualize the transient *GUS* gene expression. For luciferase activity measurement, 25 μl of the supernatant were assayed with a luciferase assay system (Promega) and quantified using a LUMIstar BMG luminometer (BMG Lab Technologies). Normalization of the GUS data was performed as in the following: In an experiment, the treatment with the highest LUC activity was used as the reference, GUS activities in other treatments were adjusted proportionally based on the ratio of the LUC activities in a treatment and in the reference. Non-transformed cells were used as controls.

Stable transformation of rice calli

Co-transformation of the rice suspension cells (cv. Nipponbare or Taipei 309) with pRESQ38 plasmid constructs mixed with pMON410 (Rogers *et al*., 1987) were performed using particle bombardment as described by Sivamani *et al*. (1996). The calli were first selected on AA solid medium containing 2.5 g/l phytagel and 30 mg/l hygromycin B (A.G. Scientific, San Diego, CA, USA) and later maintained in liquid cultures as described. Another transgenic rice suspension line (line P13, cv. Taipei 309), containing the *GUS* expression cassette from pRESQ4, used in the experiments was obtained by *Agrobacterium*-mediated transformation, and was determined to have a single *GUS* transgene copy (Lu *et al*., unpublished).

Heat shock treatment and RNA analysis

Four-day-old etiolated rice seedlings (cv. Nipponbare) were heat shocked for 0, 1, 2, or 3 h continuously at 42 °C in darkness. Total RNA from the treated seedlings was isolated using Trizol® reagent (Invitrogen) according to manufacturer's procedure. About 5 μg of total RNA was separated in a 1% MOPS–Formaldehyde agarose gel (Sivamani *et al*., 1999). After electrophoresis, RNA was blotted to the Hybond-N+® nylon membrane (Amersham Biosciences Corp., Piscataway, NJ, USA). Because of the presence of other polyubiquitin genes in rice, which are highly homologous to the *rubi3* gene, the gel blot was probed with a PCR-amplified 3′ UTR sequence of the *rubi3* gene, which is divergent from other rice polyubiquitin genes. The 287 bp PCR product was generated using the primers Ri3TF: 5′GAGC TCGCGTGTGCTGTGGTGCAGCATTGGAC TTCATTAT3′ and U3F: 5′AACCAAACCAACA TCGACACC3′ (Figure 1). DNA probes labeled with [^{32}P]dCTP were prepared using the Prime-It II® random priming kit (Stratagene, Cedar Creek, TX, USA) according to manufacturer's protocol. Hybridization and washing of filters were performed using procedures provided by Amersham. Washed filters were exposed to BioMax MS film (Eastman Kodak, Rochester, NY, USA). After exposure, the blot was stripped and re-probed with the PCR-amplified rice *Act1* gene coding sequence as an internal control.

In another experiment, cells of a transgenic rice suspension line, P13, harboring the pRESQ4 expression cassette were grown in liquid medium in darkness at 25 °C in an orbital shaker with speed set at 150 rpm. Heat shock treatment was performed at 42 °C. Suspension cell aliquots were withdrawn at intervals of 0, 1, 2, and 3 h during the treatment. The aliquots were filtered through a Whatman No. 1 filter paper disk and the cells collected in a microfuge and stored in liquid nitrogen. Total RNA from these cells was isolated using Trizol® reagent. RNA (20 μg per sample) was size-separated on a 1% agarose gel, transferred onto the Hybond-N+® membrane, and probed with the *GUS* coding sequence, rice *Act1* coding

sequence, and the 3′ UTR of the *rubi3* gene sequentially as described above.

Protein extraction and Western blot analysis

Soluble protein extraction from rice suspension cells was performed using a procedure described by Yu *et al*., (1991). The total protein (15 μg/lane) was separated by SDS-PAGE (10%) (Laemmli, 1970) using the Mini-PROTEAN®II electrophoresis cell (Bio-Rad). The separated proteins were electrotransferred onto a nitrocellulose membrane (Bio-Rad) using the Tris–glycine–methanol buffer system in a Mini Trans-Blot® electrophoretic transfer cell apparatus (Bio-Rad). Manufacturer's instructions were followed. The GUS polyclonal antibodies were purchased from Invitrogen. Immunoblot analysis was performed using a SuperSignal® West Femto Western blotting kit (Pierce Biotechnology, Rockford, IL, USA).

Results

Sequence and structure analysis of the rice rubi3 *gene*

The coding sequence and an approximately 2 kb sequence upstream the coding sequence were studied in details. The structure of the *rubi3* gene is presented in Figure 1. The *rubi3* gene promoter, exon 1, 5′ UTR intron, and the coding sequences have been deposited in GenBank with an accession number of AY954394. A database search found that the *rubi3* gene is located in chromosome 4 of rice (Feng *et al*., 2002).

The sequence data revealed that the *rubi3* gene contains an open reading frame of 1140 bp encoding a pentameric polyubiquitin (Figure 1). The deduced amino acid sequence of the five tandem, head-to-tail repeats of 76 aa ubiquitin monomer from the *rubi3* was identical to the ubiquitin sequences in maize and *A. thaliana*. The coding sequence of *rubi3* gene showed high homology to polyubiquitin genes from *Arabidopsis* (Burke *et al*., 1988; Callis *et al*., 1995), sunflower (Binet *et al*., 1991a, b) potato (Garbarino and Belknap, 1994), tomato (Hoffman *et al*., 1991), *N. tabacum* (Genschik *et al*., 1994), sugarcane (Wei *et al*., 2003), and yeast (Ozkaynak *et al*., 1987). For example, the coding sequence of *rubi3* gene showed 86% identity to the rice RUBQ1 (GenBank Accession NO. AF184279) and RUBQ2 (GenBank Accession No. AF184280), 84% to the maize *Ubi1* gene (GenBank Accession No. S94464), and 79% and 80% to a polyubiquitin gene of *A. Thaliana* (GenBank Accession No. U84968) and sunflower (GenBank Accession No. X57004), respectively.

The 5′ terminal cDNA sequence obtained from the experiments using the GeneRacer system indicates that the *rubi3* transcription initiates at an A, which is 1207 nt upstream from the translational initiation site of the gene. The sequence surrounding this site reads as CGCATAG (transcription initiation site underlined), compared to the reported consensus sequence of YTCATCA of plant genes (Joshi, 1987). A putative TATA box sequence (TATATA) was found 31 bp upstream from the determined transcription initiation site, similar to the TATA position of other plant genes (Joshi, 1987).

There is an intron located at the 5′ UTR in the *rubi3* gene, as verified by comparison of the *rubi3* cDNA sequence with its genomic sequence. No intron is found within the *rubi3* coding sequence. The intron was located immediately before the translation initiation codon of the *rubi3* gene (Figure 1). The location and the sequences CAAG/gta at the 5′ splice site and cag/ATG at the 3′ splice site are invariant for all plant polyubiquitin genes characterized to date (Binet *et al*., 1991a, b; Christensen *et al*., 1992; Kawalleck *et al*., 1993; Norris *et al*., 1993). The 1140 bp *rubi3* intron is AT-rich (61%), a character of plant introns. Sequence comparison with other published plant polyubiquitin introns did not reveal any significant homology. Together with the determined transcription initiation site, the results indicated that the *rubi3* gene has a 67 bp 5′ UTR exon 1.

At the beginning, a 1309 bp (nt 1–1309 in AY954394) and an 808 bp fragments (nt 502–1309 in AY954394) were isolated as the *rubi3* promoter regions for the study. Preliminary experiments using the two fragments, together with the 5′ UTR intron, indicated that the 808 bp fragment had slightly higher GUS expression, and thus this fragment was used as the *rubi3* promoter for further studies. No sequence homology with other plant polyubiquitin gene promoters was observed. A unique feature of the *rubi3* promoter is the presence of a GC-rich block between nt −460 and −676, containing 180 G + C out of a 217 bp stretch

(82.9%). Such a GC-rich block is neither present in the maize *Ubi1* promoter (Christensen and Quail, 1996), nor in the promoters of the *RUBQ1* and *RUBQ2*, the other two rice polyubiquitin genes reported (Wang *et al*., 2000). An enhancer core consensus sequence 5′-GGTGTGG AAA(or TTT)G-3′ (Khoury and Gruss, 1983; Weiher *et al*., 1983; Hood *et al*., 1993) identified in the promoters of RUBQ1 and RUBQ2 (Wang *et al*., 2000), was not observed within the *rubi3* promoter sequence.

A heat shock element consensus sequence CNNGAANNTTCNNG (Pelham, 1982) is usually present in plant polyubiquitin gene promoters (Wang *et al*., 2000; Streatfield *et al*., 2004) in close proximity to the TATA box. Such a sequence was not observed in the *rubi3* promoter sequence. However, a strong enhancement of the *rubi3* transcripts was evidenced when the rice seedlings or suspension cells were subjected to a continuous heat shock at 42 °C for 0, 1, 2, and 3 h (Figure 3A and B). Meanwhile, the *Act1* transcript levels showed a decline in association with the heat exposure in both cases. Surprisingly, the transcript level of the *GUS* gene, under control of the *rubi3* promoter and 5′ UTR intron, did not rise in the transgenic suspension line, as did the native *rubi3* transcript level, but decreased during the course of the heat shock treatment (Figure 3B).

Enhancer core elements with sequences of GTTGTGGTTGG and GTTGTGGTTTG were found in the two other rice polyubiquitin promoters and were suggested to be responsible for enhancement of gene expression (Wang *et al*., 2000). A similar sequence GCCGTGGTTTG in the maize *adh1* promoter was defined as an anaerobic responsive element (ARE) (Olive *et al*., 1990). The maize *adh1* promoter was most active in anaerobically induced root tissues (Kyozuka *et al*., 1991). Such a sequence was not present in the *rubi3* promoter region.

5′ UTR intron of the rubi3 *enhances activities of its own promoter and other gene promoters in rice suspension cells*

In transient expression assays to evaluate the 5′ UTR intron effect on the *rubi3* promoter activity, a 20-fold enhancement of *GUS* expression was observed for the construct with the *rubi3* intron

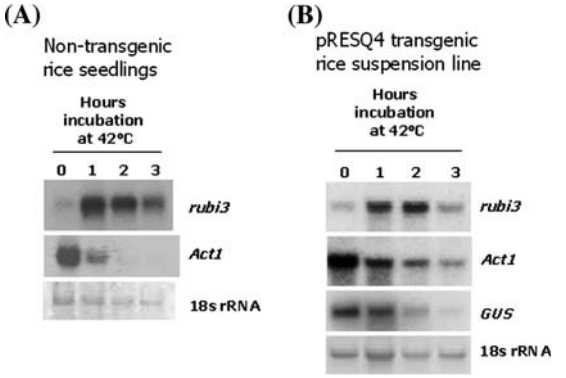

Figure 3. Northern hybridization to show the effects of the heat shock treatment on the endogenous *rubi3* gene and the *GUS* transgene expression. (A) *rubi3* transcript accumulation in etiolated 4-day-old rice seedlings (cv. Nipponbare) during the heat shock treatment. Total RNA from the 42 °C treated seedlings was isolated at various time points (0–3 h) post-treatment, separated on a 1% agarose gel, and the blot was probed with the 3′ UTR sequence of the *rubi3* gene. The RNA blot was stripped and re-probed with the rice *Act1* coding sequence. The ethidium bromide-stained 18s rRNA is shown in the bottom panel as a loading control. (B) A similar heat shock treatment on a rice suspension line (P13, cv. Taipei 309) transformed with the pRESQ4 expression cassette. The RNA levels of the *rubi3*, *Act1*, and the *GUS* gene during the treatment are shown in separate panels with the ethidium bromide-stained 18S rRNA as a loading control.

(pRESQ4) over the one without the intron (pPSRG30) (Figure 4) suggesting a strong expression enhancement by the intron. Moreover, when the *rubi3* intron was placed behind the rice *Act1* promoter (McElroy *et al*., 1990) (without its own 5′ UTR intron) or a rice histone *H3A* promoter (Sivamani and Qu, unpublished data), it enhanced those promoter activities by 8-to 9-fold (Figure 4). These results suggest that the *rubi3* intron enhances the activity of its own or ectopic promoters in rice cells when being placed at 5′ UTR.

Further enhancement of GUS activity by translational fusion with ubiquitin monomer coding sequence

Although the *rubi3* promoter with its 5′ UTR intron has a relatively high activity, the strength of this promoter is about half that of the maize *Ubi1* promoter (Figure 5). To explore ways to further enhance the *rubi3* promoter activity, we

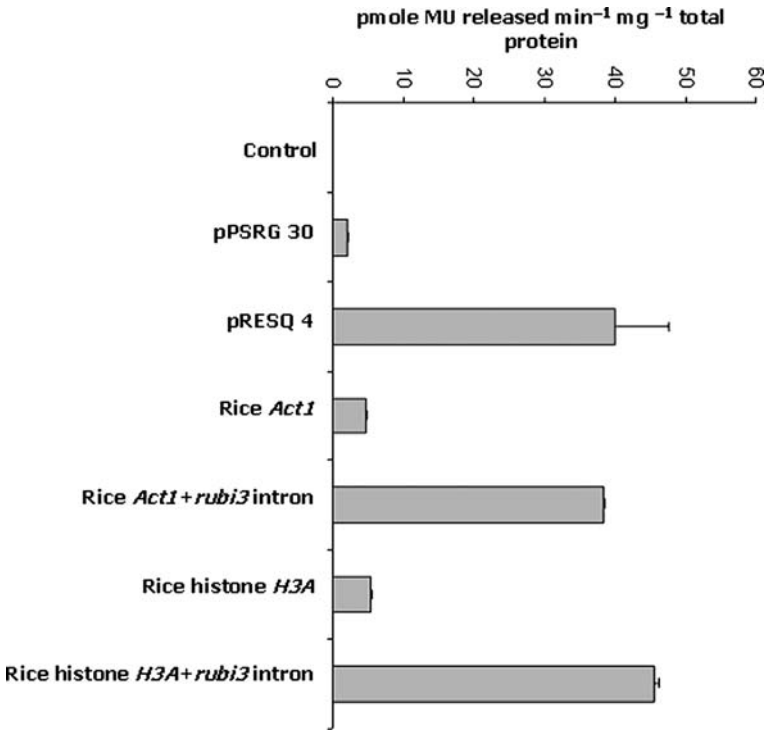

Figure 4. Enhancement effect of the *rubi3* 5′UTR intron on activities of the rice *rubi3*, *Act1* and *H3A* gene promoters. Transient MUG assays showing GUS activities in rice suspension cells transformed with individual constructs using the biolistic bombardment. Each construct was co-transformed with pJD313 containing a *LUC* expressing cassette, which served as an internal control. The GUS activities were normalized with the LUC activity and presented as pmoles of MU released min^{-1} mg^{-1} total protein. The non-transformed cells were used as a control.

tested an in-frame fusion of the ubiquitin monomer coding sequence to the *GUS* reporter gene. Gene construct pRESQ38 was made to contain the *rubi3* promoter, exon 1, 5′ UTR intron, and the ubiquitin monomer coding sequence fused in frame with the *GUS* coding sequence. Our results showed that the monomer ubiquitin-fusion construct enhanced the transient *GUS* expression by about 4-fold when compared to a construct without the monomer sequence (pRESQ4, Figure 5). Furthermore, immunoblot analysis of the transgenic rice callus lines showed that the GUS proteins expressed from both constructs migrated to the same position on SDS-PAGE, suggesting that the monomer ubiquitin was likely cleaved from the fusion protein to yield the native GUS protein (Figure 6) as observed in dicots (Hondred *et al*., 1999). This implicates that a proteolytic cleavage mechanism, similar to the one in dicots, may also exist in monocot plants.

The in-frame fusion of the first 9 nt of ubiquitin coding sequence is sufficient for the expression enhancement by the monomer fusion

To further explore which element in the ubiquitin coding sequence is responsible for the enhanced GUS activity, two more constructs were made to include the first 9 and 3 aa coding sequence of the *rubi3* gene, respectively, fused in frame with the *GUS* coding sequence. They were designated as pRESQ36 and pRESQ42. The constructs would yield GUS proteins with first 9 or 3 aa residues of the ubiquitin monomer fused at its N-terminus, respectively. The resultant GUS activities from these two constructs were similar to the one observed from pRESQ38 (full-length monomer fusion) (Figure 5), indicating that the first 9 nt of the ubiquitin coding sequence is sufficient to account for the enhanced GUS activity. The transient GUS activities of these modified constructs in rice suspension cells are about 2.2-fold of

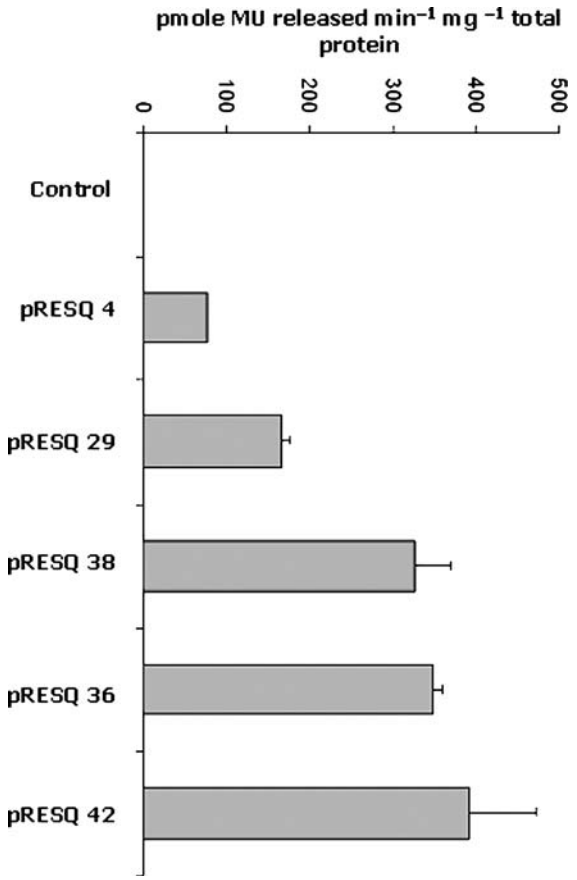

Figure 5. Comparison of the role of the *rubi3* coding sequence in *GUS* gene expression. Constructs containing various lengths of the *rubi3* coding sequence translationally fused to the *GUS* coding sequence (pRESQ36, 38, 42) were co-transformed with pJD313, a *LUC* construct served as an internal control. The results of pRESQ4 (using the *rubi3* promoter and its 5′ UTR intron) and pRESQ29 (using the maize *Ubi1* promoter and its 5′ UTR intron) are also shown for comparison. The normalized values of the GUS activity are presented in the chart as pmoles of MU released min^{-1} mg^{-1} total protein.

the one from the maize *Ubi1* promoter-*GUS* construct (pRESQ29, Figure 5).

Mutagenesis at the third nt of the first 9 nt of the ubiquitin coding sequence still maintains the enhancing effect but would yield native protein of the transgene

Although the first 9 nt of the ubiquitin coding sequence fusion are sufficient for transgene expression enhancement, this approach would add three amino acid residues to the N-terminus of the protein one would express in transgenic plants,

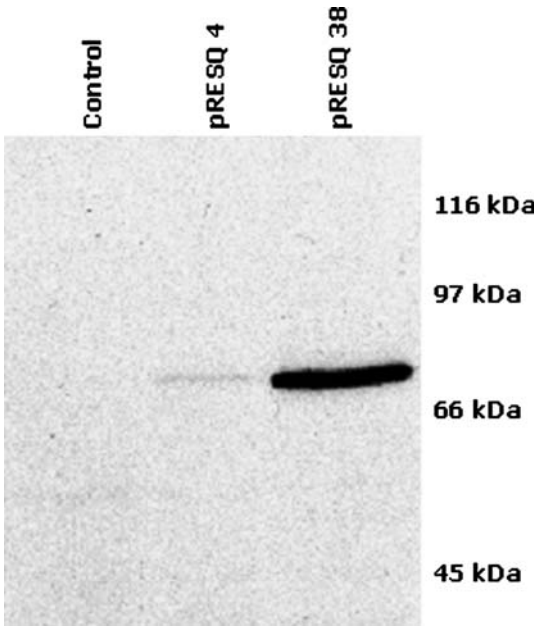

Figure 6. Expression of the GUS protein from constructs pRESQ4 and pRESQ38 in stably transformed rice cell lines by immunoblot analysis. Total protein was extracted from non-transformed rice suspension cells (as a control), a suspension line transformed with the pRESQ4 expressing the native *GUS* gene, and a suspension line transformed with the pRESQ38, which has an in-frame fusion of the *rubi3* monomer and the GUS coding sequences. Total protein extracts (15 μg/lane) were fractionated by SDS-PAGE, blotted to a nitrocellulose membrane, and probed with the GUS-specific antibody as described in 'Materials and methods'.

and may undesirably affect the transgenic protein activity in some cases. To address this potential problem, we mutagenized the third nucleotide G to abolish the translation from the ubiquitin ATG, such that the translation would start from the authentic ATG of the transgene. Gene constructs were made based on the construct pRESQ42 to mutate the G to A, T, or C, respectively, resulting in three new constructs: pRESQ46, 47 and 48. Transient expression assays showed that G to A mutation slightly reduced the GUS activity whereas G to T or C mutations increased GUS activities by about 30% (Figure 7) To investigate whether this 9 nt sequence by itself (not associated with the intron) has any enhancing effect on transgene expression, a gene construct, pRESQ66, was made by fusing the mutated (G to C) 9 nt sequence to the *GUS* coding sequence under the control of the intron-less *rubi3* promoter (Figure 2). Transient *GUS* expression assays revealed that the construct pRESQ66 did not

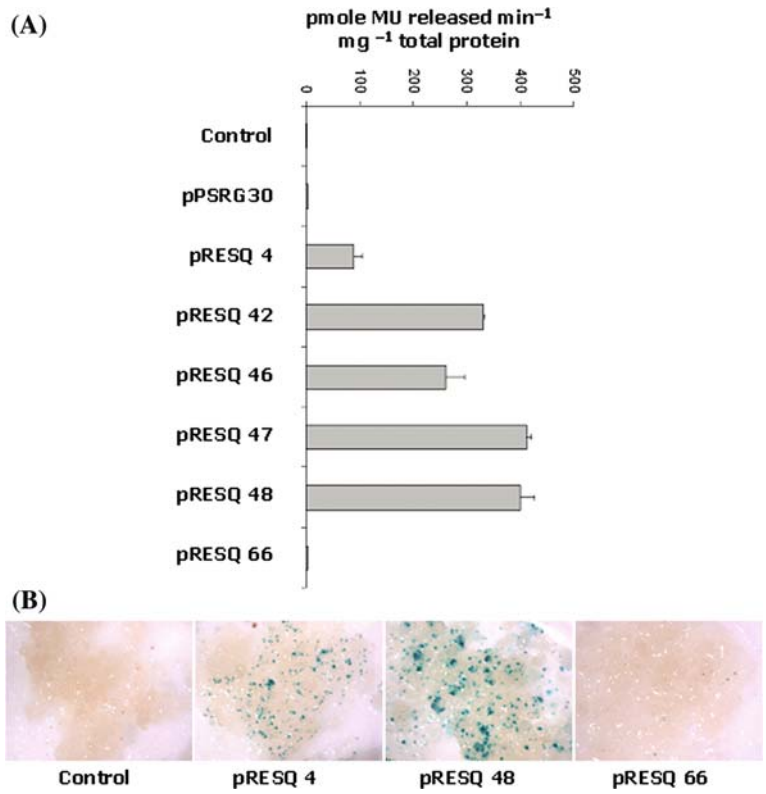

Figure 7. Effects of the first 9 nt of the *rubi3* coding sequence and its mutated forms on GUS gene expression. Vectors pRESQ42, 46, 47, and 48 contain the 5′ UTR intron and the first 9 nt (non-mutated, or with the third nt G mutated to A, T, or C, respectively) of the *rubi3* coding sequence fused to the *GUS* coding sequence. Vector pRESQ66 is identical to pRESQ48 except that it does not have the intron. Each construct was co-transformed with pJD313, a *LUC* construct served as an internal control. After normalization with the LUC activity, the GUS activities are presented in panel (A) as pmoles of MU released min^{-1} mg^{-1} total protein. Results of pPSRG30, pRESQ4, and the non-transformed control are also presented for comparison. Typical results from histochemical assays of *GUS* transient expression using vectors pRESQ4, 48 or 66 are presented in panel (B).

have an enhancing effect when compared to the intron-less pPSRG30 construct (Figure 7), suggesting that the observed 9 nt enhancing effect be intron-dependent.

In summary, we have revealed that the first 9 nt sequence of the *rubi3* coding sequence is sufficient to enhance gene expression when they are located immediately downstream the 5′ UTR intron. The enhancing effect is intron-dependent and can tolerate mutagenesis at the third nt G. The most active construct, pRESQ48, containing *rubi3* promoter, 5′ UTR exon 1 and intron, and the mutated 9 nt (ATCCAGATA) yielded GUS activity 5-fold higher than the intron-containing construct pRESQ4, about 90-fold higher than the intron-less pPSRG 30, and 2.2-fold greater of the maize *Ubi1* promoter construct pRESQ29 in transient gene expression assays in rice suspension cells.

Discussion

We report the isolation and characterization of a new polyubiquitin gene promoter from the rice genome. Although the coding sequence of the *rubi3* gene shows high homology to other plant polyubiquitin genes, the 5′ regulatory region of this gene which includes the promoter, 5′ UTR exon 1 and intron does not have any significant homology with the published promoter or intron sequences of other plant polyubiquitin genes.

Heat-shock elements (HSEs) have been reported in the upstream regions of polyubiquitin genes in maize (Christensen *et al.*, 1992), rice (Wang *et al.*, 2000), sunflower (Binet *et al.*, 1991a, b) and tobacco (Genschik *et al.*, 1994), and the heat shock enhancement of polyubiquitin genes observed (Christensen *et al.*, 1992; Wang *et al.*, 2000). Streatfield *et al.* (2004) observed heat

shock induced up-regulation of the *GUS* transgene expression driven by maize and teosinte polyubiquitin regulatory sequences in the developing embryos of transgenic maize. They also revealed that the 3′ element of the overlapping maize *Ubi1* promoter heat shock elements is required to mediate the heat shock response. It was reported that more than two interrupted arrangements of GAA/TTC blocks included in the heat shock consensus sequence are needed in a functional heat shock regulatory element (Amin *et al*., 1988; Hua and Lis., 1988). In the isolated *rubi3* promoter sequence, neither a typical heat shock consensus (CNGAANNTTCNG) nor multiple interrupted GAA/TTC blocks were present. However, similar to other polyubiquitin promoters (Christensen and Quail, 1989; Wang *et al*., 2000), there was a strong induction of the *rubi3* transcripts when the rice seedlings or transgenic suspension cells were subjected to the heat shock treatment (Figure 3A and B). Surprisingly, the GUS transcripts, regulated by the isolated *rubi3* promoter (with its 5′ UTR intron) in the transgenic rice suspension, failed to increase by the heat shock treatment. A possible explanation to the phenomenon is that the heat induction of the *rubi3* gene is controlled by distant regulatory element(s), rather than elements within the promoter region we tested in our assay, and may be position dependent. It has been well documented that enhancers, locus control regions (LCRs), and insulators (or boundary elements) regulate gene expression from a distance (Brown, 2003). The LCRs of murine and human β-globin loci span up to 130 kb on their chromosomes (Grosveld *et al*., 1987). Although the data cannot be directly compared due to the limit of the assay, it is noticeable in Figure 3 that the basic expression level of the endogenous *rubi3* gene is very low and the expression is highly induced by the heat shock treatment whereas the basic GUS expression level driven by the isolated promoter fragment is relatively high. Thus the data suggest the activity of the isolated *rubi3* promoter may not completely represent that of the fully functional endogenous one.

Reporter genes translationally fused with the monomer ubiquitin coding sequence have been shown to enhance the expression by a few fold (Hondred *et al*., 1999) and the fusion protein is precisely processed by ubiquitin-specific proteases in dicots to release the fused protein moieties in free forms (Wilkinson, 1997). Our results indicate that such an enhancement in gene expression by fusion with the monomer ubiquitin coding sequence and, likely, the ubiquitin-fusion protein cleavage mechanism also exists in monocots. However, in the reported dicot case, the monomer fusion enhancement is intron-independent as evidenced by the gene construct having a CaMV 35S promoter and no intron within the expression cassette. In our experiments, although we did not test a construct having a monomer fusion without the 5′ UTR intron, results from the 9 nt fusion construct without the intron (pRESQ66) suggest that the observed enhancing effect is intron-dependent, and thus the mechanism behind it may be different from the one reported in dicots.

We have narrowed down the monomer ubiquitin-fusion enhancement to a 9 nt stretch at the 5′ terminus of the ubiquitin coding sequence and demonstrated that this 9 nt sequence is sufficient for the observed enhancement by the ubiquitin monomer fusion. The addition of the three amino acid residues to the N-terminus of the GUS protein is unlikely to affect the GUS activity, as demonstrated by the constructs pRESQ46, 47 and 48, in which a mutation at the third nt G would yield the native GUS protein, yet the high level of GUS activities still maintained (Figure 5). The 9 nt exonic sequence identified in the experiments is different from an exonic enhancer from the maize *Sh1* gene reported by Clancy *et al*., (1994) since it does not have an enhancing effect by its own in a construct without the *rubi3* intron (pRESQ66, which is otherwise identical to pRESQ48), indicating the dependency of the enhancement on the intron (and probably at the specific location), while the *Sh1* exonic enhancer works without the *Sh1* intron 1 (Clancy *et al*., 1994).

Analysis of flanking sequences at the translation initiation codon of the various constructs does not support the hypothesis that the enhanced GUS activity was due to sequences at this region optimal for translation. Kozak identified an optimal mRNA sequence of ACCAUGG for translation in eukaryotes (Kozak, 1986). Joshi *et al*. (1997) proposed a sequence of c(a/c)(A/G)(A/C)cAUGGCG as optimal for translation in monocot plants. In our constructs with GUS gene fused to the ubiquitin coding sequences in pRESQ36, 38 and 42, the sequence around the translation initiation codon is UCAAGAUGCAG. For constructs with mutated 9 nt in front of the GUS

coding sequence (pRESQ46, 47, 48, and 66), the corresponding sequence is AGAUAAUGUUA. Both sequences are quite divergent from the proposed 'optimal' sequences. Moreover, pRESQ66 had sequence identical to the other three in this group at the region but failed to show any enhancement effect.

In animal systems, purine (AG) rich exonic splicing enhancers (ESEs) have been identified and are reported to be recognized by specialized regulatory splicing factors (SR proteins) for efficient intron splicing (Lorkovic *et al*., 2000). Such an element was also reported in plants (McCullough and Schuler, 1997). We do not think the observed *rubi3* 9 nt enhancing element is an ESE for the following reasons: (1) Little non-spliced *GUS* transcript was detected in stably transformed callus lines using the pRESQ4 construct (Samadder *et al*., in preparation), indicating a good splicing efficiency for the construct. Thus the observed near 5-fold increase by the 9 nt enhancing element could not be attributed to the enhanced splicing. (2) Although the 9 nt sequence is AG rich (6 out of 9), it does not match the binding sequences of the major SR proteins, and the ESE Finder web (http://rulai.cshl.edu/tools/ESE/) cannot recognize this 9 nt as a potential ESE.

It seems we have observed a new kind of exonic enhancing gene structure arrangement, whose effect depends on the presence of an intron. However, the enhancing effect is not due to an ESE, and actually is substantially stronger than that of the ESEs. It has been shown that intron-mediated enhancement in animal cells is transcriptional, post-transcriptional, and translational (Nott *et al*., 2003), and an exon junction complex (EJC) plays an important role in the enhancement (Wiegand *et al*., 2003). Although EJC has not been reported in plants, several homologues of the EJC components have been identified in *A. thaliana* (Pendle *et al*., 2005). It remains to be seen whether the observed enhancing effect by the *rubi3* coding sequence as reported here is related to a potential plant EJC. In addition, considering the highly conserved organization of the polyubiquitin genes in higher plants, in which the exon 2 coding sequence immediately follows the 5′ UTR intron, the observed enhancement by the *rubi3* 9 nt coding sequence may represent a mechanism common to all polyubiquitin gene expression in higher plants.

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References

Amin, J., Ananthan, J. and Voellmy, R. 1988. key features of heat-shock regulatory elements. Mol. Cell. Biol. 8: 3761–3769.

Binet, M.N., Lepetit, M., Weil, J.H. and Tessier, L.H. 1991a. Analysis of a sunflower polyubiquitin promoter by transient expression. Plant Sci. 79: 87–94.

Binet, M.N., Weil, J.H. and Tessier, L.H. 1991b. Structure and Expression of Sunflower Ubiquitin Genes. Plant Mol. Biol. 17: 395–407.

Brownm, K.E. 2003. Chromatin folding and gene expression: new tools to reveal the spatial organization of genes. 11: 423–433.

Burke, T.J., Callis, J. and Vierstra, R.D. 1988. Characterization of a polyubiquitin gene from *Arabidopsis-thaliana*. Mol. Gen. Genet. 213: 435–443.

Callis, J., Raasch, J.A. and Vierstra, R.D. 1990. Ubiquitin extension proteins of *Arabidopsis-thaliana* – structure, localization, and expression of their promoters in transgenic tobacco. J. Biol. Chem. 265: 12486–12493.

Callis, J., Carpenter, T., Sun, C.W. and Vierstra, R.D. 1995. Structure and evolution of genes encoding polyubiquitin and ubiquitin-like proteins in *Arabidopsis-thaliana* ecotype Columbia. Genetics 139: 921–939.

Callis, J. and Vierstra, R.D. 1989. Ubiquitin and ubiquitin genes in higher plants. Plant Mol. Cell. Biol. 6: 1–30.

Christensen, A.H. and Quail, P.H. 1989. Sequence-analysis and transcriptional regulation by heat-shock of polyubiquitin transcripts from maize. Plant Mol. Biol. 12: 619–632.

Christensen, A.H. and Quail, P.H. 1996. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res. 5: 213–218.

Christensen, A.H., Sharrock, R.A. and Quail, P.H. 1992. Maize polyubiquitin genes – structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol. Biol. 18: 675–689.

Clancy, M., Vasil, V., Hannah, L.C. and Vasil, I.K. 1994. Maize *shrunken-1* intron and exon regions increase gene-expression in maize protoplasts. Plant Sci. 98: 151–161.

Cornejo, M.J., Luth, D., Blankenship, K.M., Anderson, O.D. and Blechl, A.E. 1993. Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol. Biol. 23: 567–581.

Dellaporta, S.L, Wood, J and Hicks, J.B 1983. A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 14: 19–21.

Feng, Q., Zhang, Y.J., Hao, P., Wang, S.Y., Fu, G., Huang, Y.C., Li, Y., Zhu, J.J., Liu, Y.L., Hu, X., Jia, P.X., Zhang, Y., Zhao, Q., Ying, K., Yu, S.L., Tang, Y.S., Weng, Q.J., Zhang, L., Lu, Y., Mu, J., Lu, Y.Q., Zhang, L.S., Yu, Z., Fan, D.L., Liu, X.H., Lu, T.T., Li, C., Wu, Y.R., Sun, T.G., Lei, H.Y., Li, T., Hu, H., Guan, J.P., Wu, M., Zhang, R.Q., Zhou, B., Chen, Z.H., Chen, L., Jin, Z.Q., Wang, R., Yin, H.F., Cai, Z., Ren, S.X., Lv, G., Gu, W.Y., Zhu, G.F., Tu, Y.F., Jia, J., Zhang, Y., Chen, J., Kang, H., Chen, X.Y., Shao, C.Y., Sun, Y., Hu, Q.P., Zhang, X.L, Zhang, W., Wang, L.J., Ding, C.W., Sheng, H.H., Gu, J.L, Chen, S.T., Ni, L., Zhu, F.H., Chen, W., Lan, L.F., Lai, Y., Cheng, Z.K., Gu., M.H., Jiang, J.M., Li, J.Y., Hong, G.F., Xue, Y.B. and Han, B. 2002. Sequence and analysis of rice chromosome 4. Nature 420: 316–320.

Gallagher, S.R. 1992. GUS protocols: Using the GUS Gene as a Reporter of Gene Expression Quantitation of GUS Activity by Fluorometry . Academic press Inc, New York, pp. 47–59.

Garbarino, J.E. and Belknap, W.R. 1994. Isolation of a ubiquitin-ribosomal protein gene (Ubi3) from potato and expression of its promoter in transgenic plants. Plant Mol. Biol. 24: 119–127.

Genschik, P., Marbach, J., Uze, M., Feuerman, M., Plesse, B. and Fleck, J. 1994. Structure and promoter activity of a stress and developmentally-regulated polyubiquitin-encoding gene of *Nicotiana tabacum*. Gene 148: 195–202.

Grosveld, F., Vanassendelft, G.B., Greaves, D.R. and Kollias, G. 1987. Position-independent, high-level expression of the human beta-globin gene in transgenic mice. 51: 975–985.

Hoffman, N.E., Ko, K., Milkowski, D. and Pichersky, E. 1991. Isolation and characterization of tomato cDNA and genomic clones encoding the ubiquitin gene ubi3. Plant mol. Biol. 17: 1189–1201.

Hondred, D., Walker, J.M., Mathews, D.E. and Vierstra, R.D. 1999. Use of ubiquitin fusions to augment protein expression in transgenic plants. Plant Physiol. 119: 713–724.

Hood, E.E., Gelvin, S.B., Melchers, L.S. and Hoekema, A. 1993. New agrobacterium helper plasmids for gene-transfer to plants. Transgenic Res. 2: 208–218.

Hua, X. and Lis, J.T. 1988. Germline transformation used to define key features of heat-shock response elements. Science 239: 1139–1142.

Jefferson, R.A., Burgess, S.M. and Hirsh, D. 1986. Beta-glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. USA 83: 8447–8451.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. Beta-glucuronidase (Gus) as a sensitive and versatile gene fusion marker in plants. J. Cell. Biochem. 57–57.

Joshi, C.P. 1987. An inspection of the domain between putative tata box and translation start site in 79 plant genes. Nucl. Acids Res. 15: 6643–6653.

Joshi, C.P., Zhou, H., Huang, X.Q. and Chiang, V.L. 1997. Context sequences of translation initiation codon in plants. Plant Mol. Biol. 35: 993–1001.

Kawalleck, P., Somssich, I.E., Feldbrugge, M., Hahlbrock, K. and Weisshaar, B. 1993. Polyubiquitin gene expression and structural properties of the ubi4–2 gene in *Petroselium crispum*. Plant Mol. Biol. 21: 673–684.

Khoury, G. and Gruss, P. 1983. Enhancer elements. Cell 33: 313–314.

Kozak, M. 1986. Point mutations define a sequence flanking the aug initiator codon that modulates translation by eukaryotic ribosomes. Cell 44: 283–292.

Kyozuka, J., Fujimoto, H., Izawa, T. and Shimamoto, K. 1991. Anaerobic induction and tissue-specific expression of maize adh1 promoter in transgenic rice plants and their progeny. Mol. Gen. Genet. 228: 40–48.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature 227: 680–&.

Leckie, F., Devoto, A. and Delorenzo, G. 1994. Normalization of gus by luciferase activity from the same cell extract reduces transformation variability. Biotechniques 17: 52–&.

Lewin, B. 2000. Genes VII. Oxford University Press Inc, New York.

Lorkovic, Z.J., Kirk, D.A.W., Lambermon, M.H.L. and Filipowicz, W. 2000. Pre-mRNA splicing in higher plants. Trends Plant Sci. 5: 160–167.

McCullough, A.J. and Schuler, M.A. 1997. Intronic and exonic sequences modulate 5′ splice site selection in plant nuclei. Nucleic Acids Res. 25: 1071–1077.

McElroy, D., Zhang, W.G., Cao, J. and Wu, R. 1990. Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2: 163–171.

Norris, S.R., Meyer, S.E. and Callis, J. 1993. The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. Plant Mol. Biol. 21: 895–906.

Nott, A., Muslin, S.H. and Moore, M.J. 2003. A quantitative analysis of intron effects on mammalian gene expression. RNA 9: 607–617.

Olive, M.R., Walker, J.C., Singh, K., Dennis, E.S. and Peacock, W.J. 1990. Functional-properties of the anaerobic responsive element of the maize Adh1 gene. Plant Mol. Biol. 15: 593–604.

Ozkaynak, E., Finley, D., Solomon, M.J. and Varshvasky, A. 1987. The yeast ubiquitin genes: a family of natural gene fusions. EMBO J. 6: 1427–1439.

Pelham, H.R.H. 1982. A regulatory upstream promoter element in *Drosophila* Hsp70 heat-shock gene. Cell: 517–528.

Pendle, A.F., Clark, G.P., Boon, R., Lewandowska, D., Lam, Y.W., Andersen, J., Mann, M., Lamond, A.I., Brown, J.W.S. and Shaw, P.J. 2005. Proteomic analysis of the *Arabidopsis* nucleolus suggests novel nucleolar functions. Mol. Biol. Cell. 16: 260–269.

Rogers, S.G., Klee, H.J., Horsch, R.B. and Fraley, R.T. 1987. Improved vectors for plant transformation – expression cassette vectors and new selectable markers. Meth. Enzymol. 153: 253–277.

Rose, A.B. and Beliakoff, J.A. 2000. Intron-mediated enhancement of gene expression independent of unique intron sequences and splicing. Plant Physiol. 22: 535–542.

Sivamani, E., Shen, P., Opalka, N., Beachy, R.N. and Fauquet, C.M. 1996. Selection of large quantities of embryogenic calli from indica rice seeds for production of fertile transgenic plants using the biolistic method. Plant Cell Rep. 15: 322–327.

Sivamani, E., Huet, H., Shen, P., Ong, C.A., de Kochko, A., Fauquet, C. and Beachy, R.N. 1999. Rice plant (*Oryza sativa* L.) containing Rice tungro spherical virus (RTSV) coat protein transgenes are resistant to virus infection. Mol. Breed. 5: 177–185.

Streatfield, S.J., Magallanes-Lundback, M.E., Beifuss, K.K., Brooks, C.A., Harkey, R.L., Love, R.T., Bray, J., Howard,

J.A., Jilka, J.M. and Hood, E.E. 2004. Analysis of the maize polyubiquitin-1 promoter heat shock elements and generation of promoter variants with modified expression characteristics. Transgenic Res. 13: 299–312.

Toriyama, K. and Hinata, K. 1985. Cell-suspension and protoplast culture in rice. Plant Sci. 41: 179–183.

Wang, J.L., Jiang, J.D. and Oard, J.H. 2000. Structure, expression and promoter activity of two polyubiquitin genes from rice (*Oryza sativa* L.). Plant Sci. 156: 201–211.

Wei, H.R., Wang, M.L., Moore, P.H. and Albert, H.H. 2003. Comparative expression analysis of two sugarcane polyubiquitin promoters and flanking sequences in transgenic plants. J. Plant Physiol. 160: 1241–1251.

Weiher, H., Konig, M. and Gruss, P. 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science 219: 626–631.

Wiegand, H.L., Lu, S.H. and Cullen, B.R. 2003. Exon junction complexes mediate the enhancing effect of splicing on mRNA expression. Proc. Natl. Acad. Sci. USA 100: 11327–11332.

Wilkinson, K.D. 1997. Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. FASEB J. 11: 1245–1256.

Yon, J. and Fried, M. 1989. Precise Gene Fusion by PCR. Nucl. Acids Res. 17: 4895–4895.

Yu, S.M., Kuo, Y.H., Sheu, G., Sheu, Y.J. and Liu, L.F. 1991. Metabolic derepression of alpha-amylase gene-expression in suspension-cultured cells of rice. J. Biol. Chem. 266: 21131–21137.