Structure, expression and promoter activity of two polyubiquitin genes from rice (*Oryza sativa* L.)

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Abstract

We have isolated two rice polyubiquitin genes designated as RUBQ1 and RUBQ2 by screening a Bacterial Artificial Chromosome (BAC) genomic library with a 32P-labeled ubiquitin cDNA probe. DNA sequence data revealed that both genes contained an open reading frame encoding a hexameric precursor ubiquitin and an intron immediate upstream of the initiation codon. The deduced amino acid sequences of both genes were identical to each other and to other plant ubiquitin sequences. Several putative regulatory elements such as enhancer core and heat shock consensus sequences were found in the 5'-upstream regions of both genes. Northern blot analyses using the 3'-untranslated region as gene specific probes showed that both genes were actively expressed in all rice plant tissues tested. Differential expression was observed in roots where RUBQ2 appeared to be predominantly expressed. Chimeric genes containing the 5'-upstream region including the intron of RUBQ1 or RUBQ2 and the β-glucuronidase (GUS) coding region were constructed and transferred into rice suspension cells via particle bombardment. GUS activity from constructs containing RUBQ1 and RUBQ2 promoters in rice suspension cells was ten to 15-fold greater than those using the Cauliflower Mosaic Virus 35S (CaMV 35S) promoter, and two to threefold greater than constructs with the maize polyubiquitin Ubi1 promoter. The results demonstrate the potential usefulness of the two rice polyubiquitin promoters in rice or other monocot transformation systems. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Polyubiquitin gene; Expression; Promoter activity; *Oryza sativa* L.

1. Introduction

Ubiquitin, a 76 amino acid protein, is present in all eukaryotic cells and is one of the most conserved proteins, differing in only three of 76 amino acids between higher plants and animals [1]. Ubiquitin is encoded by small multigenic families containing two types of genes: polyubiquitin and ubiquitin extension/fusion genes. The polyubiquitin gene consists of tandem repeats of a 228-bp ubiquitin coding region [2–4], while the ubiquitin extension/fusion gene consists of a single ubiquitin coding region linked to a sequence encoding ribosomal proteins either 52 or 76–81 amino acids in length [5,6]. Both types of genes are translated as a polyprotein precursor, then proteolytically processed to the ubiquitin monomers for ubiquitin function [1]. The best-characterized function of ubiquitin is its conjugation to target proteins as a recognition signal for protein degradation [7]. Ubiquitin is also involved in other cellular processes, such as ribosome biosynthesis, chromatin structure and cell cycle control [8–10].

Various ubiquitin genes have been isolated in a number of organisms. In yeast, a total of four ubiquitin genes have been characterized, one of which belongs to the polyubiquitin class [11]. In *Arabidopsis thaliana*, 14 ubiquitin genes representing the complement of the ubiquitin gene family have been isolated, five being polyubiquitin genes...
[12]. Ubiquitin extension/fusion genes in plants are expressed in meristematic tissues and their expression is significantly reduced in mature tissues [5,6,13]. The polyubiquitin genes were reported to be constitutively expressed in all plant organs tested [3,14], with an increased level in young tissues [2,15]. Differential expression of ubiquitin genes has been documented in potato tuber tissues in response to injury and hormone treatment [16], and in Nicotiana sylvestris to heavy metal treatment and pathogen infection [17]. Expression of ubiquitin genes in response to heat-shocked treatment was reported to be up-regulated [3,4], unchanged [14], or down-regulated [16]. Perturbation in the ubiquitin system of higher plants was found to elicit a hypersensitive response similar to that caused by pathogen infection [18–20].

Various promoters from ubiquitin genes have been tested for their potential use in plant transformation, including those from A. thaliana [5,21], sunflower [22], N. tabacum [23], potato [6], and maize [4]. The maize polyubiquitin Ubi1 promoter has been successfully used in maize and other monocot transformation systems [4,15,24].

We report here two polyubiquitin genes isolated from a rice genomic library, their expression in different rice plant tissues, and their response to heat shock treatment. The possible roles of the putative regulatory elements in gene expression are discussed. Furthermore, promoters from the two polyubiquitin genes have been tested for their potential use in rice transformation. Our results showed that the two rice polyubiquitin genes were actively expressed in all tissues tested and a high level of glucuronidase (GUS) expression under control of the two rice polyubiquitin promoters was observed in transformed rice cells, indicating potential usefulness of the two rice polyubiquitin promoters in rice and other monocot transformation systems.

2. Materials and methods

2.1. Plant materials

Eight-month-old rice suspension cells (cv. Taipei 309, provided by Mr S. Yang, Department of Plant Pathology and Crop Physiology, Louisiana State University) were maintained in 200 ml flasks containing 50 ml R2 medium [25] on a gyratory shaker (80 rpm). Cultures were grown in light at 25°C and subcultured once a week. Rice plants and seedlings (cv. Cypress) were grown in the greenhouse. For heat shock treatments, the 2-week-old seedlings were immersed in pre-heated distilled water for 20 min at 42°C and allowed to recover for up to 90 min.

2.2. Isolation of genomic clones

A bacterial artificial chromosome (BAC) rice genomic library, kindly provided by Dr P. Ronald, UC Davis, consisted of 11 000 clones with an average DNA insert size of 125 kb [26]. The 1.3-kb region of sugarcane ubiquitin cDNA clone Scubi561 [27] was used as a probe for library screening. Filters were prehybridized in 5 × Denhardt’s solution, 100 μg/ml denatured sperm DNA, 6 × SSC and 0.5% SDS for 6 h at 65°C. Hybridizations were performed overnight at 65°C in the same buffer containing 32P-labeled probe. Filters were washed twice at room temperature in 2 × SSC, 0.1% SDS and then once at 65°C for 20 min in 0.2 × SSC, 0.1% SDS solution. Autoradiography was carried out at −80°C with Kodak Biomax MS film and one intensifying screen. Twenty-four positive clones were identified, and two of them, designated RUBQ1 and RUBQ2, were selected for further analysis because both clones showed strong hybridizing signals to the sugarcane ubiquitin cDNA probe (data not shown) but differed from each other based on restriction enzyme analysis.

2.3. DNA sequencing

HindIII restriction fragments encompassing RUBQ1 and RUBQ2 were cloned into pBluescript II SK + (Stratagene) for restriction mapping (Fig. 1). When digested with XhoI or BglII restriction enzymes, both clones produced several fragments hybridizing to the sugarcane ubiquitin cDNA probe. These fragments were gel-purified and subcloned into pBluescript SK II + for DNA sequencing. Double stranded DNAs were prepared using plasmid mini preparation kit (Qiagen). DNA sequencing was performed on an ALF-win automatic DNA sequencer (Pharmacia) using the ALF-express autocycle sequencing kit (Pharmacia) according to the manufacturer’s recommendations. Both strands were sequenced using M13-40 probe.
and M13-reverse or specific oligodeoxyribonucleotides as primers. The DNAsis (Hitachi, CA) software was used for data analysis. DNA sequences presented here corresponding to RUBQ1 and RUBQ2 have been entered into Genbank with accession numbers AF184279 and AF184280, respectively.

2.4. Northern and Southern bolt analyses

The 0.33 kb XhoI fragment of the RUBQ2 coding region (nucleotides 3513–3846 of RUBQ2) was used as a probe for Northern blot analysis. Gene specific probes 3'-RUBQ1 (181 bp in length) and 3'-RUBQ2 (218 bp in length) were produced by polymerase chain reaction (PCR) using primers flanking the 3'-untranslated regions of RUBQ1 and RUBQ2. Two sets of primers used to amplify 3'-RUBQ1 and 3'-RUBQ2 were 5'-GTGGCCAGTAAGTCTTACG-3' and 5'-GCAAAACGTTTGCAGACACCA-3' and 5'-AATCAGCCAGTTTGGAG-3', respectively. The PCR thermocycle parameters were 95°C/36 s, 50°C/36 s, and 72°C/84 s for 25 cycles. PCR products (3'-RUBQ1 and 3'-RUBQ2) were gel purified and 32P-labeled using the random primed DNA labeling kit (Boehringer Mannheim) for Northern and Southern blotting.

Total RNA from different tissues of rice plants and heat-shocked versus control seedlings (cv. Cypress) were isolated with phenol/SDS extraction and LiCl precipitation [28]. Total RNA (20 μg per lane) were electrophoretically separated in 1.0% agarose–formaldehyde gels and blotted onto nylon membranes (Hybond-N, Amersham) [28]. Filters were prehybridized for 6 h at 50°C in 5× SSC, 5× Denhards, 50 μg/ml salmon sperm, and 50% formamide. Hybridization was performed overnight using the same conditions with addition of 32P-labeled probe (specific activity of 1 × 10⁶ cpm/ml). Filters were washed twice at room temperature in 2× SSC and 0.1% SDS for 20 min followed by two 20 min washes at 50°C in 0.2× SSC and 0.1% SDS. Autoradiography was carried out with Kodak Biomax MS film for 6 h. For Southern blots, DNAs were digested with restriction enzymes, fractionated by 1.0% agarose gel electrophoresis, and transferred onto nylon membranes (Hybond-N, Amersham) according to the Molecular Cloning Manual [29]. Hybridization and subsequent washing conditions were the same as described for the BAC library screening.

2.5. Chimeric gene constructions

A 2.2 kb HindIII/EcoRI fragment containing the GUS coding region and nopaline synthase (NOS) terminator was removed from pBI101 (Clontech) and ligated into the corresponding site of pUC18, generating pRGL110. Promoter regions to be tested were inserted into the polylinker of pRGL110 as a translational fusion to the GUS coding sequence.

For expression of the GUS gene under control of the rice ubiquitin promoters, the 1.7 kb SalI/XhoI and 2.8 kb PstI/XhoI fragments, each containing the 5'-upstream region and the intron of RUBQ1 and RUBQ2, respectively, were ligated into the SalI site (compatible with XhoI site) of the pRGL110, creating pRGL111-1 and pRGL112-1 (Fig. 6). Constructs pRGL111-2 and
pRGL112-2 are the same as pRGL111-1 and pRGL112-1, except for inclusion of an additional 2.7 kb HindIII/SalI or 3.3 kb HindIII/PstI fragment at the 5'-upstream region of RUBQ1 and RUBQ2, respectively (Fig. 6). All constructs encoded a fusion protein at the N-terminus, consisting of a portion (15 amino acids) of ubiquitin followed by linker sequence of 11 amino acids upstream of the GUS protein.

For construction of the chimeric gene containing the RUBQ2 promoter and the Luciferase (LUC) coding region, the SV 40 terminator in vector pGL3 (Promega, Madison, WI) was replaced with the NOS terminator, creating pGL3-1. The RUBQ2 promoter, contained in a 2.8 kb HindIII/BamHI fragment from pRGL112-1, was blunt-ended by treating with T4 DNA polymerase (New England Biolab) and ligated into the blunt-ended HindIII site of the pGL3-1. The resulting construct designated as pRUBQ2-LUC (Fig. 6) encoded a fusion protein with 29 amino acids at the N-terminus upstream of the LUC protein.

Two controls were used for rice transformation. The plasmid pAHC25, containing a maize polyubiquitin Ubi1 promoter-GUS cassette, was kindly provided by Dr P. Quail (Plant Gene Expression Center, USDA, Albany, CA). Plasmid pBI121, containing a CaMV 35S promoter-GUS cassette, was obtained from Clontech (Palo Alto, CA).

2.6. Transient GUS and LUC assays

Approximately 200 mg rice suspension cells (Taipei 309) were evenly distributed over the surface of a 70 mm Whatman no. 2 filter paper in a 100 × 15 mm² petri dish for particle bombardment. Plasmids were precipitated onto gold particles (1.0 μm in diameter, Biorad) and introduced into suspension cells via particle bombardment according to the method described by Christou et al. [30]. Two independent experiments were conducted as follows: Equimolar amounts of each of six constructs containing the chimeric GUS gene were co-transferred with pRUBQ2-LUC into suspension cells with four replications. The pRUBQ2-LUC served as internal control and inclusion of an internal control was reported to improve the reliability of transient expression studies by controlling the variations among replications [31]. After 48 h incubation of treated suspension cells at 25°C, cells were transferred into a 50 ml centrifuge tube and homogenized with 300 μl 1 × reporter assay buffer (Promega). Cell homogenates were centrifuged twice at 10 000 × g for 10 min at 4°C, then the supernatant was transferred to 1.5 ml eppendorf tube and stored at −80°C until use for both GUS and LUC assays. The means of GUS/LUC relative activity were tested for significance with Tukey’s test using SAS version 6.12.

For the GUS enzyme assay, 20 μl of supernatant were assayed according to the method previously described by Jefferson et al. [32] in a TKO 100 fluorimeter (Hoefer Co.). GUS activity was expressed as pmol MU (4-methylumbelliferone)/min. For the LUC enzyme assay, 20 μl of supernatant were assayed in a scintillation analyzer (Packard) using a Luciferase assay system kit (Promega). Measurements began 2 min after mixing the 20 μl supernatant and the luciferase assay reagent, which took 12 s for each sample. The LUC activity was expressed as the number of counts per minute (CPM). Measurements based on light emission were standardized with defined amounts of purified recombinant luciferase (Promega) (1 light unit (L.U.) is equivalent to 2.8 × 10⁵ CPM).

3. Results

3.1. Sequence and structure of RUBQ1 and RUBQ2

DNA sequences of the 5'-flanking region including the first exon, an intron and the first 48 nucleotides of the ubiquitin-coding regions of RUBQ1 and RUBQ2 are presented in Fig. 2A and B, respectively.

Both genes contained an open reading frame of 1371 bp, arranged as six tandem, head to tail repeats of 228 bp, encoding a hexameric precursor ubiquitin (Fig. 1). The deduced amino acid sequence of the 12 ubiquitin monomers from both genes was identical to each other and other ubiquitin sequences in maize [4] and A. thaliana [2]. The last (sixth) monomer of both RUBQ1 and RUBQ2 contained a glutamine extension. The extension of one to several amino acid residues at the C-terminal of the last ubiquitin monomer is a common feature of the polyubiquitin genes [1].

The coding sequences of RUBQ1 and RUBQ2 genes showed 88% identity to each other, and they
showed high homology to other ubiquitin genes from *Arabidopsis* [2,12], sunflower [3], potato [6], tomato [13], *N. tobackum* [23], and sugarcane [27] and yeast [11]. For example, the coding sequences of RUBQ1 and RUBQ2 genes showed 88% identity to the maize polyubiquitin gene (Genbank accession no. S94464), 81% to the *A. thaliana* ubiquitin gene (accession no. U84968), and 82% to the sunflower ubiquitin gene (accession no. X57004). The coding sequence of RUBQ1 showed 99% identity to the rice polyubiquitin cDNA clone Rub1 [33] and Rubq1 [34], suggesting the genomic clone RUBQ1 reported here corresponded to these two previously isolated cDNA clones.

A putative intron was identified immediately upstream of the ATG initiation codon for the
RUBQ1 and RUBQ2 genes (Figs. 1 and 2) based on the consensus sequences CAAG/gtac at the splice 5′ site and cag/ATG at the splice 3′ site, which are invariant for all plant polyubiquitin genes characterized to date [3,4,14,21]. The position of the putative intron for RUBQ1 was verified when sequence of RUBQ1 was compared to that of corresponding cDNA clones rub1 [33] and Rubq1 [34]. In contrast to the coding region, the RUBQ1 intron varied in length (782 bp) compared to the RUBQ2 intron (962 bp) and showed only 52% identity with each other.

The 5′-upstream regions of both genes showed 53% identity to each other. Putative regulatory elements were found in the 5′-upstream regions of both genes. For example, a putative TATA box was observed at position 811 for RUBQ1 gene and position 1712 for RUBQ2 gene (Fig. 2A and B). A heat shock sequence similar to the *Drosophila* consensus sequence 5′-CTGGAATnTTCTAGA [35] was found twice at positions 630 (182 bases upstream of TATA box) and 652 (160 bases upstream of TATA box) for RUBQ1, and once at position 1586 (126 bases upstream of the TATA box) for RUBQ2. It is interesting to note that an enhancer core consensus sequence 5′-GGTGTG-AAA(or TTT)G-3′ [36,37] was present at position 286 (526 bp upstream of the TATA box) in the RUBQ1 and at position 1019 (694 bases upstream of the TATA box) in the RUBQ2 (Fig. 2A and B).

3.2. Ubiquitin is encoded by a small gene family in rice

Southern blot analysis showed that multiple restriction fragments from rice genomic DNA hybridized to the ubiquitin cDNA clone Scubi561 (Fig. 3A). Five major hybridization bands (14, 11, 8.3, 2.4 and 2.2 kb) were observed for DNA digested with HindIII enzyme and a comparative number of major bands were also found for DNAs digested with EcoRI or XbaI (Fig. 3A). These major bands appeared to represent fragments containing polyubiquitin genes and two of them (14 and 8.3 kb) were confirmed to contain RUBQ1 and RUBQ2 genes, respectively (see below). In addition to the major bands, several faint bands were observed, which may reflect fragments carrying the ubiquitin extension/fusion genes with a single repeat of ubiquitin monomer. These results indicate ubiquitin is encoded by a small gene family in rice, though the exact number of the ubiquitin genes remains to be determined.

A simple hybridization pattern was generated when the same filter was sequentially hybridized by gene specific probe 3′-RUBQ1 and 3′-RUBQ2 (Fig. 3B and C). The 3′-RUBQ1 probe hybridized to a 14 kb fragment in HindIII-digested genomic DNA, and the specific fragments in EcoRI or XbaI-digested genomic DNA were also recognized by the 3′-RUBQ1 probe (Fig. 3B). Similarly, the 3′-RUBQ2 probe hybridized to a single 8.3 kb fragment in HindIII digested genomic DNA, and it also recognized a single specific fragment in EcoRI or XbaI-digested genomic DNA (Fig. 3C). The results suggest that RUBQ1 and RUBQ2 exist in the rice genome at a single locus and demonstrate the utility of 3′-RUBQ1 and 3′-RUBQ2 as gene-specific probes.

3.3. Expression of RUBQ1 and RUBQ2 in rice tissues

Total RNA isolated from different rice plant organs (cv. Cypress) was analyzed by Northern blot analysis using the ubiquitin coding region of RUBQ2 and gene specific regions 3′-RUBQ1 and 3′-RUBQ2 as hybridization probes (Fig. 4). Hybridization with the ubiquitin coding region (RUBQ2 coding) probe detected one predominant band with an estimated size of 1.9 kb. This 1.9 kb transcript were present in all tissues tested, which
Fig. 4. Northern blot analysis of ubiquitin expression in rice tissues. Total RNA (20 μg per lane) was prepared from old leaves (ol), full-developed leaves (fl), young leaves (yl), panicle (p), roots (r) and shoot apex (sa). The filter was sequentially hybridized with three probes: ubiquitin coding region of RUBQ2, gene specific probes 3′-RUBQ1 and 3′-RUBQ2. Finally, a 24-base antisense oligonucleotide to rice 18S rRNA was labeled with [γ-32P] ATP (Amersham) and T4 polynucleotide kinase (NE Biolabs) and used as an internal control.

Fig. 5. Northern blot analysis of rice ubiquitin expression in response to heat shock treatment. Two-week-old seedlings of rice variety Cypress were heat-shocked at 42°C for 20 min. Total RNA (20 μg per lane) was prepared from unshocked rice seedlings (lane 1), or heat-shocked seedlings allowed to recover for 0 min (lane 2), 30 min (lane 3) and 90 min (lane 4). The filter was sequentially hybridized with ubiquitin coding region of RUBQ2, gene specific probe 3′-RUBQ1 and 3′-RUBQ2. The filter was then probed with a 24-base antisense oligonucleotide to rice 18S rRNA.

Gene specific probes 3′-RUBQ1 and 3′-RUBQ2 were used to assay for gene specific expression. As expected, the gene specific probes specifically hybridized to the 1.9 kb transcript with active expression of both RUBQ1 and RUBQ2 in all tissues tested (Fig. 4). Differential expression of the two genes was observed in roots, where expression of RUBQ2 was substantially higher than that of RUBQ1.

3.4. Expression of RUBQ1 and RUBQ2 in response to heat shock

The effect of heat shock on expression of ubiquitin RNA was examined by Northern blot analysis using the ubiquitin coding region (RUBQ2-coding) as a probe. The level of 1.9 kb transcript was increased immediately after 20 min heat shock treatment at 42°C and accumulated over a 90-min period (Fig. 5). To test whether the increased abundance was due to higher transcript levels of RUBQ1 or RUBQ2 or both genes, Northern blots of heat shocked RNAs were hybridized with gene specific probes 3′-RUBQ1 and 3′-RUBQ2. The results shown in Fig. 5 indicate expression of both genes was up-regulated in response to heat shock, which was consistent with the heat-shock sequence located in the 5′-upstream regions of both genes.

3.5. Transient expression of rice ubiquitin promoters in rice suspension cells

Fig. 6 shows schematic features of chimeric genes containing rice ubiquitin promoters. These constructs were co-transferred with pRUBQ2-LUC into rice suspension cells via particle bombardment. Both GUS (pmol MU/min) and LUC (light unit) activities were determined and their relative values are shown in Table 1. Results across two independent experiments were consistent though the overall GUS/LUC relative activities were greater in experiment 2 than in experiment 1.

As shown in Table 1, constructs pRGL111-1 and pRGL111-2 produced similar GUS/LUC relative activities in both experiments, which suggested that the additional 5′-flanking region (2.7 kb) in pRGL111-2 did not contribute to the promoter activity of RUBQ1. However, construct pRGL112-2 showed a reduced GUS/LUC relative activity compared to that produced by construct pRGL112-1. GUS/LUC relative activities produced by pRGL112-2 and pRGL112-1 were 2.58 and 3.82 in experiment 1, and 11.17 and 16.00 in experiment 2. These values were statistically differ-
ent in both experiments, indicating the additional 5'-flanking region (3.3 kb) in pRGL112-2 may reduce transformation efficiency or contain negative regulatory elements. Though means of GUS/LUC relative activity were greater in experiment 2 than in experiment 1, the fold stimulation values related to pBI121 (35S-GUS) were relative constant across two experiments, being 11.4 (pRGL112-2) and 16.9 (pRGL112-1) in experiment 1, and 11.7 (pRGL112-2) and 16.8 (pRGL112-1) in experiment 2. Activity of the RUBQ2 promoter was ~1.5 times that of the RUBQ1 promoter in rice suspension cells when means of GUS/LUC relative activity produced by pRGL111-1 and pRGL112-1 were compared.

Higher levels of GUS expression were observed from rice suspension cells transformed with con-

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Table 1
Expression of chimeric GUS genes transferred into rice suspension cells via particle bombardment

<table>
<thead>
<tr>
<th>Transferred DNA</th>
<th>Construct</th>
<th>Experiment no. 1</th>
<th>Fold</th>
<th>Experiment no. 2</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRGL112-1</td>
<td>GUS/LUC ratios</td>
<td>3.82 ± 0.82^b</td>
<td>16.9</td>
<td>16.00 ± 3.26^b</td>
<td>16.8</td>
</tr>
<tr>
<td>pRGL112-2</td>
<td></td>
<td>2.58 ± 0.26^c</td>
<td>11.4</td>
<td>11.17 ± 2.38^c</td>
<td>11.7</td>
</tr>
<tr>
<td>pRGL111-1</td>
<td></td>
<td>2.86 ± 0.14^d</td>
<td>12.7</td>
<td>9.92 ± 2.28^e</td>
<td>10.4</td>
</tr>
<tr>
<td>pRGL111-2</td>
<td></td>
<td>2.84 ± 0.56^d</td>
<td>12.5</td>
<td>8.45 ± 2.25^e</td>
<td>8.9</td>
</tr>
<tr>
<td>pAHC25 (CK)</td>
<td></td>
<td>0.79 ± 0.06^d</td>
<td>3.5</td>
<td>4.23 ± 0.35^e</td>
<td>4.4</td>
</tr>
<tr>
<td>pBI121 (CK)</td>
<td></td>
<td>0.23 ± 0.05^e</td>
<td>1</td>
<td>0.95 ± 0.30^c</td>
<td>1</td>
</tr>
</tbody>
</table>

^a Each construct was co-transferred with pRUBQ2-LUC which served as internal control (see Section 2). The means and standard deviation of GUS/LUC ratios from four replicates are shown for two independent experiments. Data with different superscript letters (b–e) are significantly different from each other at the 5% level based on Tukey’s test.

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Fig. 6. Schematic representation of constructs containing rice ubiquitin promoter-GUS chimeric genes than those using the CaMV 35S or maize ubi1 promoter (Table 1). GUS activity from constructs pRGL111-1 and pRGL112-1 in rice suspension cells was ten to 15-fold greater than the construct pBI121 (CaMV 35S-GUS), and two to threefold greater than pAHC25 (maize Ubi1-GUS), indicating the potential usefulness of the two rice polyubiquitin promoters in rice or other monocot transformation systems.

4. Discussion

We report here the isolation and characterization of two polyubiquitin genes from rice. Though the 5'-upstream regions of the two rice polyubiquitin genes showed only 53% sequence identity to each other, they consisted of the same putative regulatory elements, such as enhancer core and heat shock consensus elements. The enhancer core elements found in the 5'-upstream regions of RUBQ1 and RUBQ2 are GGTGTGGTTG and GGTGTGTTTG, respectively, which are 9/11 and 10/11 identical to the enhancer core consensus element GGTGTGGAAA(orTTT)G found in animal systems [36,37]. A similar sequence GC-CGTGGTTTG was found in the maize adh1 promoter, located within the region defined as an anaerobic responsive element (ARE) [38], showing
9/11 identical to the enhancer core consensus element. The maize adh1 promoter was most active in anaerobically induced root tissues [39], and a substitution mutation in the enhancer core element in the maize adh1 promoter contributed to a reduced anaerobic response in roots of transgenic rice plants [40]. We speculate that the enhancer core element found in the two rice polyubiquitin genes could be also related to gene expression in roots. Furthermore, functions of the enhancer core element may not be restricted to roots. For example, part of the enhancer core element (TGTG-GTT) defined as a pollen box (PB) motif is present in the promoters of potato genes Lat52, Lat56, and Lat59, and a GG doublet in the PB core is critical for expression in pollen [41]. GUS expression was also observed in the pollen of transgenic rice plants transformed with constructs containing the chimeric RUBQ1 or RUBQ2 promoter-GUS genes (Wang and Oard, unpublished data). It is likely that the enhancer core element in the two rice polyubiquitin genes serves as a global enhancement of gene expression, though its exact function(s) in plant genes remains to be determined.

Expression of both rice polyubiquitin genes are induced in response to heat shock treatment, which is consistent with heat shock elements found in the 5'-upstream regions of both rice polyubiquitin genes. Heat-shock elements have also been reported in the 5'-upstream regions of polyubiquitin genes from maize [4], sunflower [3] and N. tabacum [23]. The consensus heat shock element in Drosophila was defined as CTGGAATNTTCTAG [35]. Amin et al. [42] demonstrated that presence of a single module of an inverted repeat (nGAAAn) was sufficient for heat shock induction, and deviations from the consensus sequence were tolerant if larger arrays of the repeats occurred. The heat shock element in the RUBQ2 fits the single module of an inverted repeat (nGAAAn), while deviations from consensus sequence occur in the two heat shock elements found in the RUBQ1, which are similar to those in the maize polyubiquitin gene [4]. Little is known about the position effect of the heat shock element on the magnitude of response to heat shock treatment. The heat shock element is closer to the TATA box in the RUBQ2 than those in the RUBQ1, but comparison of the position effect is not possible since variation occurs in the sequences and the copy number of the heat shock elements found in the promoter regions.

Activity of the RUBQ2 promoter was ~1.5 times that of the RUBQ1 promoter in driving GUS expression in rice suspension cells. In addition, the RUBQ2 promoter was also shown to be more efficient than the RUBQ1 promoter in directing reporter gene expression in transgenic rice plants (Wang and Oard, unpublished data). Several factors may contribute to the enhanced expression of the RUBQ2 promoter. First, the RUBQ2 promoter contains 1 kb longer 5'-upstream region than did the RUBQ1 promoter. Second, sequence variations between the 5'-upstream regions including the introns of the two rice polyubiquitin genes may contribute to differences in gene expression levels. In comparison, promoter regions of three polyubiquitin genes from A. thaliana were reported to be quantitatively similar in driving transient GUS expression in A. thaliana leaves [21]. Their differential expression could be revealed in transgenic plants since differential expression of A. thaliana polyubiquitin genes were shown by Northern blot analysis [2]. Alternatively, the additional 5'-upstream regions or 3'-untranslational regions that were not included in the expression cassette were determinants of the differential expression [21].

The CaMV 35S promoter is widely used in both monocot and dicot transformation systems [43], while the maize Ubi1-promoter was shown to be more efficient than the CaMV 35S promoter in many monocot transformation systems including rice [15,24]. In this experiment, the transient GUS expression showed that constructs containing RUBQ1 and RUBQ2 promoters produced ten to 15-fold greater GUS activity than pBI121 (CaMV 35S-GUS), and two to threefold greater than pAHC25 (maize Ubi1-GUS), indicating the utility of the 5'-flanking regions of the rice ubiquitin genes. However, we do not rule out other factors that could contribute to the differences observed in our transient GUS assay. For examples, contribution of the rice ubiquitin intron to gene expression needs to be quantified, since introns have been shown to stimulate reporter gene activity in monocots [44]. In additions, the rice ubiquitin promoters were used in translational fusions to the GUS gene while the maize polyubiquitin ubil promoter was used in a transcriptional fusion to the GUS gene in construct pAHC25 [24].
direct comparisons of the rice ubiquitin promoters vs CaMV 35S or maize ubi1 promoters in rice and other plant transformation systems are needed to provide more information about their relative efficiency, the rice ubiquitin promoters reported here should be useful in plant transformation studies.

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