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# Expression Pattern of *UidA* Gene under the Control of Rice Glutelin *GluA-2* Gene Upstream Sequence in Transgenic Rice Endosperm

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**Abstract**: In order to study the expression pattern of rice glutelin endosperm specific promoter in Chinese cultivar Zhonghua 8 (*Oryza sativa* L. subsp *japonica*), *UidA* gene was fused with rice glutelin *GluA-2* gene 5′ upstream sequence 2.3 kb and 750 bp upstream respectively and transferred into rice by *Agrobacterium* mediated transformation. Southern blot indicated that *UidA* gene was integrated into the genome of transgenic plants as single copy. Northern blot demonstrated that the expression of *UidA* gene and endogenous *GluA-2* gene reached their highest level at 13 ~ 15 days and 11 ~ 13 days after pollination respectively, and then declined. Histochemical staining of immature transgenic rice seeds showed *UidA* gene was specifically expressed in endosperm and the highest level GUS expression was observed in aleurone layer. Quantitative analysis of GUS activity showed seeds GUS activity of that 2.3 kb transgenic plant was about two to three folds of those of 750 bp transgenic plant. Sequence analysis suggested that the G-box located in the – 2 170 bp (from transcription start site) may be a quantitative *cis*-element.

**Key words**: rice glutelin *GluA-2* gene; expression pattern; G-box

# 水稻谷蛋白 *GluA-2* 基因 5′ 上游序列控制下的 *UidA* 基因在转基因水稻胚乳中的表达模式

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**摘 要**: 为了研究谷蛋白胚乳特异性表达启动子在我国栽培稻品种中的表达模式, 将 *UidA* 基因分别置于水稻谷蛋白 *GluA-2* 基因 750 bp 和 2.3 kb 上游序列下游, 利用农杆菌转化法导入栽培稻品种中花 8 号并获得转基因植株。Southern blot 检测表明, *UidA* 基因已经整合到水稻基因组当中并以单拷贝存在。Northern blot 检测表明, 开花后 13 ~ 15 d 和 11 ~ 13 d, *UidA* 基因和水稻内源的 *GluA-2* 基因的表达量分别达到最高, 随后逐渐降低。对转基因植株种子的 GUS 染色表明, *UidA* 基因仅在胚乳中表达, 在糊粉层中 GUS 表达量最高。测定了 2.3 kb 和 750 bp 转基因植株种子的 GUS 活性, 结果表明前者的 GUS 活性是后者的 2 ~ 3 倍。序列分析表明, 位于 *GluA-2* 基因转录启始位点上游 2 170 bp 的 G-box 可能是一个与表达量相关的顺式调控元件。

**关键词**: 水稻谷蛋白 *GluA-2* 基因; 表达模式; G-box

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Glutelin is the major seed storage protein of rice and accounts for about 80% of the endosperm protein, which is encoded by a small multigene family and comprise at least 8 members per haploid genome[1]. These genes are classified into two subfamilies as GluA and GluB. GluA contains *GluA-1* (*Gt2*), *GluA-2* (*Gt1*), *GluA-3* (*Gt3*) and

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*GluA-4* genes. *GluB* contains *GluB-1*, *GluB-2*, *GluB-3* and *GluB-4* genes. The *GluA-4* and *GluB-4* are pseudogenes. During endosperm development, these genes are highly specifically expressed in rice endosperm. So far, several regulatory elements, such as Prolamine box [TG(t/a/c)AAA(g/t)], ACGT motif [GTACGTGC], AACA motif [AACAAACTCTATC], GCN4 motif [TG(c/a)GTCA], G-box [CACGTG] and GCAA motif [(g/c)CAAAA(A/-)TGA] responsible for endosperm-specific expression have been characterized[2~4]. Among them AACA motif and GCN4 motif are conserved in all six glutelin gene promoters. The AACA motif could suppress the expression of glutelin gene in vegetative tissue[5]. The GCN4 motif not only directs glutelin gene expressed specifically in endosperm, also positively affects promoter activity[3,5]. The prolamine box repeats many times in 5′ upstream sequence of glutelin gene, but it looks more like a negative element[6]. The ACGT motif, G-box and GCAA motif may be positive *cis*-elements.

The endosperm specific expression is a desirable characteristic for controlling the expression of foreign proteins because constitutive expression of foreign genes may interfere with the plant development, growth or reproduction in transgenic plants[7]. Several genes such as bean β-phaseoline gene, soybean ferritin gene and phytoene synthase gene were introduced into rice under the control of rice glutelin gene promoter. These genes were highly specifically expressed in rice endosperm and stably transmitted to $T_3$ generation[8~10].

Since almost all motifs are located in +1 ~ −500 bp region (from transcription start site) of glutelin promoter sequence, therefore, it is enough for this fragment to direct gene specifically expressed in endosperm[3,4,6]. However, Zheng *et al*[11] reported that the expression efficiency of glutelin *GluA-2* gene was positively related with the size of glutelin gene 5′ upstream sequence. While it was deleted from 5.1 kb to 1.8 kb, the attached *UidA* gene expression decreased approximately 20-folds, while the deletion from 1.8 kb to 507 bp didn't reduce the GUA activity. These results suggested that some quantitative elements probably located between −1.8 ~ −5.1 kb *GluA-2* gene upstream sequence.

In this paper, we introduced rice glutelin *GluA-2* gene 750 bp and 2.3 kb 5′ upstream sequence *UidA* fusion genes into Chinese cultivar Zhonghua 8 (*Oryza sativa* L. subsp *japonica*). First, we hope to investigate the expression pattern of *GluA-2* gene promoter. Second, to identify whether the G-box that located between 1.8 kb and 2.3kb *GluA-2* gene 5′ upstream sequence was a quantitative *cis*-element.

# 1 Material and Methods

## 1.1 Material

*Oryza sativa* ssp. *japonica* cv. *Kinmaze* and Zhonghua 8 were grown under greenhouse condition.

## 1.2 Methods

### 1.2.1 Isolation and sequencing of *GluA-2* gene upstream sequence

The PCR primers were designed according to *GluA-2* gene 5′ upstream sequence (GenBank accession number AC021891). They are:

Forward 23 :5′ TACTATCTGAGCATTCCCCGA 3′;

Forward 75 :5′ AATGAAAGAAGATGTGGTG 3′;

Reverse :5′ ACTGAATGCTTTTTGTGAG 3′.

Forward 23 and reverse primers were used for amplifying 2.3 kb fragment, Forward 75 and reverse primers were used for amplifying 750 bp fragment. *pfu* DNA polymerase (*Pyrobest* TaKaRa Co. Ltd Dalian), which has proofreading activity, was used to enhance the fidelity of PCR amplification. The amplification was programmed in a PTC-100 thermocycler (MJ Research Inc. Watertown MA USA) at 94℃ for 5 min to allow denature of DNA, followed by 35 cycles of 94℃ for 1 min, 56℃ for 1 min, and 72℃ for 2 min. The final extension step was 10 min at 72℃.

The PCR products were cloned into pGem-Teasy vector (Promega, Madison, WI, USA) and sequenced to verify that no miscorporation had occurred in the DNA sequence during PCR amplification.

### 1.2.2 Vector constructs

The 750 bp and 2.3 kb fragments were cloned into GUS reporter gene cassette pDMC201.3 (Kindly provided by Dr. Jefferson, CSIRO Australia) cut with *Apa* Ⅰ and

*Pst* Ⅰ. At last, 2.3 kb and 750 bp fragments and attached *UidA* gene and nos terminator were assembled into binary vector PCAMBIA-1300 (Kindly provided by Dr. Jefferson, CSIRO Australia) cut with *Kpn* Ⅰ and *Xba* Ⅰ, thereby creating plasmids p23GUS1300 and p75GUS1300 (Fig. 1).

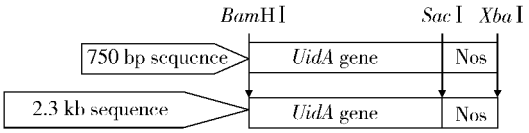


**Fig.1 Expression cassettes of p75GUS1300 and p23GUS1300**

*UidA* gene is about 2 kb, Nos terminator is about 250 bp.

1.2.3 *Agrobacterium*-mediated transformation

The *Agrobaterium* strain LBA4404 harboring P23-GUS1300 and P75GUS1300 were used to transform rice calli induced from the mature embryos of a chinese rice cv. Zhonghua 8 according to the method of Hiei *et al*.[12]. All the transgenic plants were recovered from 50 mg/L Hygromycine containing medium, the resistant plants were then transformed to soil.

1.2.4 Southern blot

Genomic DNA was isolated from leaves of putative transgenic plants and untransformed plants according to CTAB method[13]. After digested with both *Bam*HⅠ and *Sac* Ⅰ and *Bam*HⅠ only, the DNA fragments were electrophoresed in 0.8% agarose gel and blotted onto Hybond $N^+$ membrane (Amersham Buckinghamshire, England) according to manufacture described. The 2 kb *UidA* gene was isolated by digestion of pDMC201.3 plasmid DNA with *Bam*HⅠ and *Sac* Ⅰ. The 2 kb *UidA* gene and Nos terminator was isolated by digestion of pDMC201.3 plasmid DNA with *Bam*HⅠ and *Xba* Ⅰ. Both fragments were labeled with $^{32}$P-dCTP by the random priming method (Primer Glabeling system Promega) and used as probes. Hybridization and washing were carried out as standard procedure.

1.2.5 Northern blot

Total RNA fraction was isolated from seeds of one p75GUS1300 transgenic plant. The seeds of different developmental stages (6, 9, 11, 13, 15, 17, 20 days after pollination) were frozen in liquid nitrogen and cut longitudinally. One half was treated with X-Gluc (Sigma Aldrich, USA) to identify GUS expression seeds, the other half of GUS expressing seeds was used to extract RNA. The extraction buffer was 0.1 mol/L Tris-Cl (pH 9.0), 0.1 mol/L NaCl, 1% SDS. After exfraction the supernatant was extracted by phenol/chloroform, ethanol precipitation and reprecipitated by 5 mol/L LiCl. Total RNA (20 μg) was resolved by electrophoresized on 1.2% agarose-formaldehyde gel and blotted onto Hybond-$N^+$ membrane according to manufacture described. Hybridization and washings were carried out under strigent condition as Sambrook *et al*[14] described. The 2 kb *UidA* gene and nos terminator, 1.7 kb rice glutelin *GluA-2* gene cDNA fragment were labeled with $^{32}$P-dCTP by the random priming method (Primer G labeling system, Promega) as probes.

1.2.6 Quantitative analysis of GUS activity

Samples of leaf, root and halves of 1 ~ 2 differently developmental stage GUS expression seeds were stored at −80℃ prior to extraction for quantitative GUS analysis. All tissues were ground in a microfuge tube with 55 μL extraction buffer (0.1 mol/L $KH_2PO_4$ (pH7.8), 1 mmol/L EDTA and 7 mmol/L β-mercaptoethanol) with a glass pestle. Following spinning down of the cell debris by centrifugation at 15 000 r/min for 15 min at 4℃, 5 μL of supernatant was used for assaying GUS activities. The fluorometric method was according to Jefferson *et al*[15] described and average values of GUS activities were recorded.

1.2.7 Histological GUS staining

The rice seeds were cut longitudinally and immersed in X-Gluc solution [0.1 mol/L $Na_3PO_4$ (pH 7.0), 0.5 mmol/L $K_3Fe(CN)_6$, 0.5 mmol/L $K_4Fe(CN)_6$, 10 mmol/L EDTA, 0.5 mg/mL X-Gluc] at 37℃ for 2 ~ 3 h. Then fixed by 75% ethanol.

## 2 Results

### 2.1 Southern blot and analysis of progeny of self pollinated transgenic plants

To examine the integration pattern of transgene, DNA was extracted from leaf of transgenic plants and Southern blot analysis was carried out. A mixture of *Bam*HⅠ and *Sac* Ⅰ and *Bam*HⅠ were used to digest genomic DNA

separately, the results indicated that the transgene was fully integrated into genome as single copy (Fig. 2). The GUS staining analysis of the seeds of transgenic plants showed the inheritance of progeny of self pollinated transgenic plants according to typical mendelian seperation with a 3:1 ratio (Table 1). This result confirmed the conclusion obtained by Southern blot also.

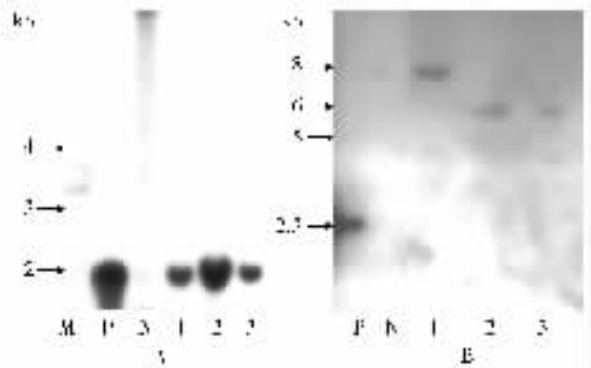

**Fig.2 Southern blot of transgenic plant**

A: Leaf DNA (30 μg) was digested by *Bam*H Ⅰ and *Sac* Ⅰ and hybridized with 2.0 kb *UidA* gene coding sequence; B: DNA digested by *Bam*H Ⅰ and hybridized with 2.0 kb *UidA* gene and nos terminator. M: 1 kb ladder; P: plasmid; N: negative control; 1: p23GUS1300 transgenic plant 2,3: p75GUS1300 transgenic plant.

**Table 1 Mendelian inheritance of progeny of self pollinated transgenic plant**

| Transgenic plants | GUS positive | | GUS negative | | $\chi^{2}$ b) |
|---|---|---|---|---|---|
| | Recorded | Expected a) | Recorded | Expected | |
| p23GUS1300/1 | 14 | 13.5 | 4 | 4.5 | 0.074 |
| p75GUS1300/3 | 34 | 31.5 | 8 | 10.5 | 0.79 |

a) Expected numbers were calculated assuming a 3:1 segregation; b) Significant values: $\chi^{2}\ 0.05 = 3.84$.

## 2.2 Temporal and spatial expression analysis of *UidA* gene

For understanding the temporal expression pattern of *UidA* gene directly, RNA was collected from differently developmental seeds of one p75GUS1300 transgenic plant and hybridized with *UidA* gene and glutelin *GluA-2* gene cDNA sequence. The resultant Northern blot analysis was shown in Fig. 3, which indicated that the temporal expression pattern of *UidA* gene and endogenous *GluA-2* gene were distinct. At 6 days after pollination (DAP) the transcripts for *UidA* gene and *GluA-2* gene were detected. At 15 DAP, *UidA* gene transcripts reached a maximum level and then declined during subsequent periods of seed development. For the endogenous *GluA-2* gene transcripts, at 11-13 DAP, attained a maximum level and then declined.

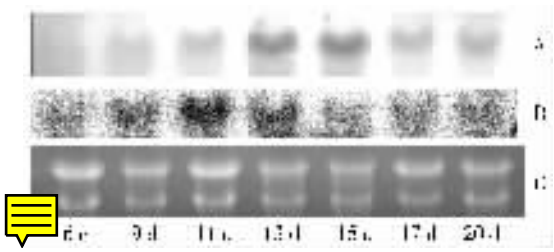

**Fig.3 Temporal expression patterns of *UidA* and *GluA-2* gene mRNA during seed development**

A: Total RNA sample 20 μg obtained from developing rice seeds at 6,9,11,13,15,17,20 DAP and probed with $^{32}$P labeled *UidA* gene under strigent hybridization and washing condition; B: Total RNA probed with $^{32}$P labeled *GluA-2* gene cDNA sequence; C: RNA of seeds of p75GUS1300 transgenic plant.

Similar results were obtained by GUS quantitative analysis. At 17 DAP, both p75GUS1300 and p23GUS1300 construct transgenic plants seeds GUS activities were reached maximum level and rapidly decline through 20 DAP. Although the expression level was different by 2 ~ 3 folds between two constructs, no difference in GUS activity of roots was observed between two constructs transgenic plants. However, p23GUS1300 construct transgenic plants leaves GUS activities were lower than those of p75GUS1300 construct (Fig. 4). This result was in good agreement with former reports that the vegetative tissues GUS activities of transgenic plants were negatively correlated with the size of glute gene promoter[5,11,16]. This

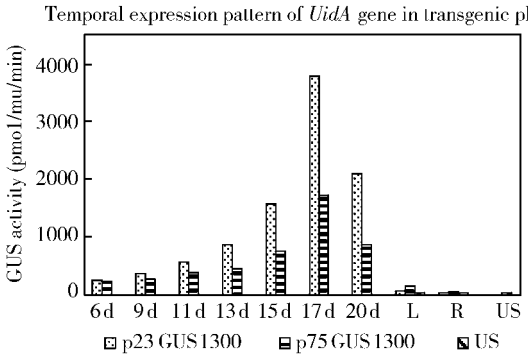


**Fig.4 GUS activity of different seed developmental stage and different tissues of p23GUS1300 and p75GUS1300 construct transgenic plants**

From left to right mean transgenic rice seed of 6,9,11,13,15,17,20 DAP, L, R leaf and root of transgenic plant; US: seeds of untransformed plant.

maybe, due to some regulatory elements such as prolamine box, suppress glutelin gene expressed in vegetative tissues located between 750 bp and 2.3 kb *GluA-2* gene upstream sequence. Fig.5 showed *UidA* gene specifically expressed in endosperm tissue of both two constructs transgenic plants. At 6 DAP the staining was first observed in the aleurone layer and then became denser. No staining was observed in embryo or any other tissues . Similar results were obtained in the seeds of p23GUS1300 transgenic plants (Data not shown).

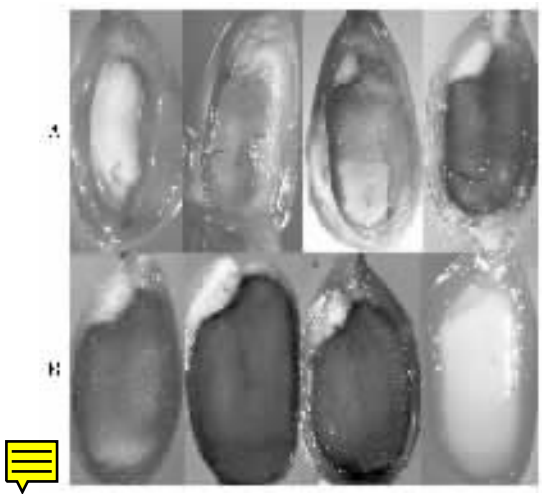

**Fig.5 Histological staining of cross-section seeds of p75GUS1300 transgenic rice plant**

A :From left to right mean transgenic rice seeds of 6,9,11,13, DAP ;B :From left to right mean transgenic rice seeds of 15,17, 20 DAP, untransformed rice seed.

## 3 Discussion

The regulatory elements related to endosperm specific expression were almost all located between + 1 ~ - 500 bp region and the fragment can direct the endosperm specific expression[11,17] . Whereas the quantitative cis-regulatory elements located in the distal region were not characterized well. The G-box existed in six rice glutelin promoters and rice globulin promoters were characterized as common *cis*-regulatory element, which can cooperate with GCN4, AACA or ACGT motif as essential *cis*-element directing quantitatively high seed-specific expression in dicot transformation system[2] . In monocot system, the rice bZIP transcriptional activator RITA-1 was highly expressed in aleurone cells of rice endosperm and bound to A-, C- and G-boxes[18] . Another rice bZIP-type protein, REB, was also bound to rice α-globulin promoter through a G-box[19] . Takaiwa *et al*[20] found that three motifs (AACA, GCN4, G-box like) were indispensable for *GluB-1* gene highly efficient expression in endosperm. These proofs suggested that the G-box may be a binding site of transactivators, such as $O_2$ or $O_2$ like factor.

Zheng *et al*[11] compared the GUS activities of 5.1 kb, 1.8 kb and 507 bp *GluA-2* gene upstream sequence GUS fusion gene transgenic plants. Almost no difference in GUS activity was found between 1.8 kb and 507 bp construct transgenic plants, however, the GUS activity of 5.1 kb construc is about 20 folds of 1.8 kb construct. We analysed the *GluA-2* gene 5′ upstream sequence using OMIGA program (Oxford Molecular Ltd.). No *cis*-regulatory elements that have been characterized in glutelin genes was found between - 336 bp to - 2 164 bp from transcription start site except the prolamine box. Early report have demonstrated that the mutation of prolamine box in *GluB-1* 245 bp promoter caused *UidA* gene expression efficiency to grow 4 folds and no positive correlation was shown between the prolamine box and glutelin gene expression level[6,20] . This may explain why the same GUS activities were obtained in 1.8 kb and 507 bp *GluA-2/GUS* fusion gene transgenic plants. In region - 2 209 ~ - 2 164 bp, a prolamine box and a G-box were found. Since G-box was a common cis-regulatory element as described above combing with the result of higher GUS activities obtained in p23GUS1300 construct transgenic plants, hence, a primary conclusion came that the G-box located in - 2 141 bp of *GluA-2* gene 5′ upstream sequence may be a key quantitative *cis*-element for the expression of *UidA* gene. Of course, it needs further proof by gain and loss of function experiments.

Takaiwa F *et al*[17] used tobacco to analyse the expression pattern of *GluA-2* gene. Compared with theirs, similar results in our experiments were observed. GUS activities of the transgenic plants seeds of rice and tobacco both reached a highest level at 17 DAP and gradually declined later. But until 12 DAP, the GUS activities were just detected in tobacco seeds. This meant the expression pattern of glutelin gene in dicots system might be not comparable with that seen in monocots system. This result was

in agreement with previous studies also.

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