热带亚热带植物学报 2003, 11(2):127-131
Journal of Tropical and Subtropical Botany

水稻重复序列 RRD3 缺失体介导 *gusA* 在水稻愈伤组织中的表达

陈中健 刘 兵 王宏斌 王金发* 刘良式
(中山大学生命科学学院基因工程教育部重点实验室, 广东 广州 510275)

摘要: 将水稻中等重复序列 RRD3 及其系列缺失体克隆到植物启动子检测载体中, 通过根癌土壤杆菌介导转化水稻愈伤组织, 利用 GUS 组织化学方法检测其在水稻愈伤组织中的启动子活性。结果显示: 全长 RRD3、410 bp 及 150 bp 缺失体具有强的启动子活性, 而 700 bp、120 bp 缺失体仅有弱的启动子活性。通过与 RRD3 系列缺失体在哺乳动物 CHO 细胞中的启动子活性比较后推测: 在 RRD3 中存在两个真核生物启动子的调控元件,一个对动物细胞的启动子起正调控,但对植物细胞中的启动子起负调控作用; 另一个调控元件仅对动物细胞的启动子起负调控,而对植物细胞启动子无影响。此外在 RRD3 序列中至少存在一个与 TATA 盒相关的真核启动子核心元件, 但在动物和植物细胞中的调控方式不同。

关键词: 水稻; 植物启动子; 瞬时表达; GUS 组织化学检测
中图分类号: Q943.2 **文献标识码**: A **文章编号**: 1005-3395 (2003) 02-0127-05

Expression of *gusA* in Rice Calli Mediated by Deletants of Rice Repetitive DNA Sequence RRD3

CHEN Zhong-jian LIU Bing WANG Hong-bin WANG Jin-fa* LIU Liang-shi
(*The Key Laboratory of Gene Engineering of Ministry of Education, School of Life Sciences, Zhongshan University*, Guangzhou 510275, China)

Abstract: Rice repetitive DNA sequence RRD3 and its six serial deletants were inserted into the promoter-capture vector of plants. Thus, *gusA* gene can be used to assess the promoter activity of inserted DNA. The promoter activities of serial deletants were detected after co-culture of rice calli with *Agrobacterium* LBA4404 containing different recombinant DNA by transient GUS (beta-glucuronidase) assay. The results indicated that total length of RRD3, 410 bp and 150 bp deletants had stronger promoter activities, while the promoter activities in 700 bp and 120 bp deletants were reduced. There are some positive/negative control elements in RRD3 sequence.

Key words: Rice; Plant promoter; Transient expression; GUS histochemical assay

The kinetics of reassociation reaction indicated that over 50% of the plant genome contained many repetitive sequences presenting in different copies[1]. Recent studies indicated that many eukaryotic gene expressions were controlled by the repetitive tandom sequences in 5' untranslated regions or by the repetitive sequences in introns. The repetitive sequences played an important role in regulation of gene expression[2-5]. Ott and Hansen[6] have identified some enhancer fragments from repetitive sequences in *Arabidopsis thaliana* with GUS assay.

RRD3 was a moderately repetitive DNA sequence isolated from rice genome by reassociation kinetics and S1 enzyme digestion. After the fragment was inserted into the promoterless vector pKK175-6, the promoter activity of RRD3 was determined in *E. coli*[7]. The total length of RRD3 is 820 bp containing 4

Received: 2002-06-05 **Accepted:** 2002-12-06
Foundation item: This research was supported by Foundation of National '863' High Technology Program (BH-01- 02-02-03); Natural Science Foundation of Guangdong Province (021656); Foundation of National 'Plant Transgenic Program' (J00-A-009).
* Corresponding author

TATA boxes —the promoter characteristic sequence. Six serial deletants of RRD3 based on the TATA boxes deletion in turn were cloned into CAT (chloramphenicol acetyltransferase) vector and used to transfect the CHO (Chinese hamster ovary) cell line. By CAT analysis in transfected CHO cell line, these deletants appeared to have very different promoter activities[8].

We have replaced the 35S promoter of Ti vector pBI121 with RRD3, then transformed the reconstructed plasmid into tobacco and rice calli by the *Agrobacterium tumefaciens* mediated method. The promoter activity of RRD3 in plants was determined by the expression of *gusA* gene in the transformed tobacco and rice calli[9]. For study of the regulatory pattern of RRD3, in this paper serial deletants of RRD3 from 5' end were inserted into the multiple cloning site of plant promoter checking vector pCAMBIA1391Z. After transformation mediated by LBA4404, the promoter activities of serial deletants in rice calli were assayed.

1 Materials and methods

Plant material *Oryza sativa* L. subsp. *Japonic* (Taipei 309).

Strain and vectors *E. coli* DH5α; *Agrobacterium tumefaciens* LBA4404; CAMBIA1391Z/1381Z; CAMBIA1301Z.

Construction of the promoter checking plasmids in plants The RRD3 fragment was isolated from pKK175-6 (Pharmacia) by *Eco*R I & *Hind* III (Takara) digestion, then inserted into pCAMBIA1381Z in forward direction. The serial deletants of RRD3 were isolated from the animal promoter checking vector pCAT-Basic (Promega) and inserted into pCAMBIA1391Z. pCAMBIA1381Z and pCAMBIA1391Z (gift from Dr. Yin Zhongchao) were designed for promoter checking in plants, there was no promoter element upstream to *gusA* gene.

Extraction, purification, digestion, ligation and transformation of the plasmids The methods described in Molecular Cloning 2nd ed [10] were used.

Transformation of *A. tumefaciens* LBA4404 by frozen-thawed method *A. tumefaciens* LBA4404 was transformed according to the method of Dityatkin[11].

Development of rice calli The mature rice seeds being stripped of seed coats were sterilized for 30 minutes in 20% (V/V) NaClO solution, then washed by sterilized water many times, cultured on MSD medium (2,4-D 40 $\mu g\ ml^{-1}$) to develop callus (26–29℃, about 2 weeks in dark). Select the embryogenic calli.

Transient GUS histochemical assay in rice calli Rice calli were co-cultivated with *A. tumefaciens* LBA4404 for 3 days. After being washed 5–10 times with sterilized water, the calli were immersed into the GUS chromogenic solution ($K_3Fe(CN)_6$ 5 mmol/L, $K_4Fe(CN)_6$ 5 mmol/L, NaH_2PO_4 100 mmol/L, X-Gluc (Amresco) 0.3 (W/V), pH 7.0), incubated at 37℃ for 2–4 hours. After chromogenic reaction rice calli were rinsed with 70% ethanol, then observed by microscopy and taken photographs. In chromogenic reaction the strict control was needed to check the background signals.

2 Results

2.1 Construction, transformation and identification of the recombinant pCAMBIA containing RRD3 and its serial deletants

RRD3 fragment and serial deletants were isolated by appropriate restriction digestion and inserted into pCAMBIA1381Z or pCAMBIA1391Z. All the target fragments located in the 5' region upstream to *gusA* gene. Then the recombinant plasmids were used to transform *E. coli* DH5α and the transformants were identified by Amp resistance. The plasmid DNA were extracted and identified by electrophoresis after RE digestion. The clones inserted in forward direction were entitled pCRRD3, pC700, pC410, pC150 and pC120 according to the length.

2.2 Transformation of *A. tumefaciens* LBA4404 with pCRRD3 and its serial deletants

A. tumefaciens LBA4404 was transformed with recombinant plasmids containing RRD3 and serial

deletants by frozen-thawed procedure. Promoterless pCAMBIA1391Z was used as the negative control in GUS histochemical assay and pCAMBIA1301Z (containing CaMV 35S) was used as the positive control. Both were transformed into *A. tumefaciens* LBA4404.

The transformed LBA4404 strains were identified with *gus*-PCR. Because there was no inner *gus*A gene in *A. tumefaciens*, the transformants in which *gus* fragment can be amplified must contain the foreign DNA. So we designed a pair of PCR primer in the 400–420 and 1 579–1 599 region of *gusA* gene, respectively. In transformants the 1.2 kb product was generated by *gus*-PCR while no PCR product was generated in untransformed LBA4404 (Figure 1).

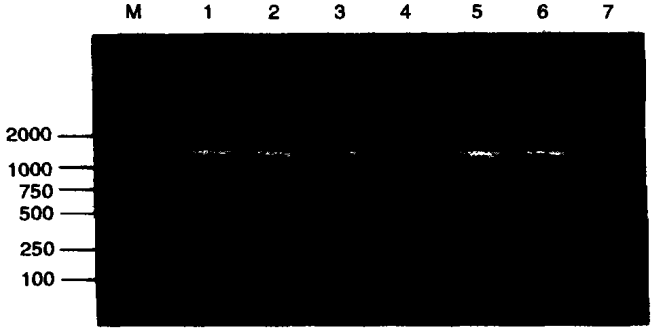

Fig. 1 Product of *gus*-PCR with LBA4404 as template

M: DL-2000 marker; 1–6: LBA4404 transformed with pC1301, pCRRD3, pC700, pC410, pC150 and pC120, respectively, as template; 7: untransformed LBA4404 as template

2.3 Transient GUS histochemical assay in rice calli

Rice calli were co-cultivated for 3 days with *A. tumefaciens* LBA4404 containing RRD3 and serial deletants, then washed by sterilized water for 5–10 times and immersed into the GUS chromogenic solution. Calli were selected randomly for GUS histochemical assay. The chromogenic activity was different in rice calli co-cultivated with *A. tumefaciens* LBA4404 containing different plasmids (GUS activity was assessed by number and intensity of the blue foci on surface of calli): Chromogenic reactions were not detected in untransformed calli or rice calli co-cultivated with LBA4404 (pCAMBIA1391Z). Strong GUS positive reactions were observed in calli co-cultivated with LBA4404 (pCAMBIA1301, pCRRD3, pC410 and pC150) while weak GUS positive reactions with pC700 and pC120 (Figure 2). These results indicated that there existed not only the core element of plant promoter but also positive/negative regulatory elements for promoter in RRD3 sequence.

3 Discussion

3.1 Comparison of promoter activity of RRD3 and serial deletants in CHO cells and in rice calli

Above results and our former report[8] indicated

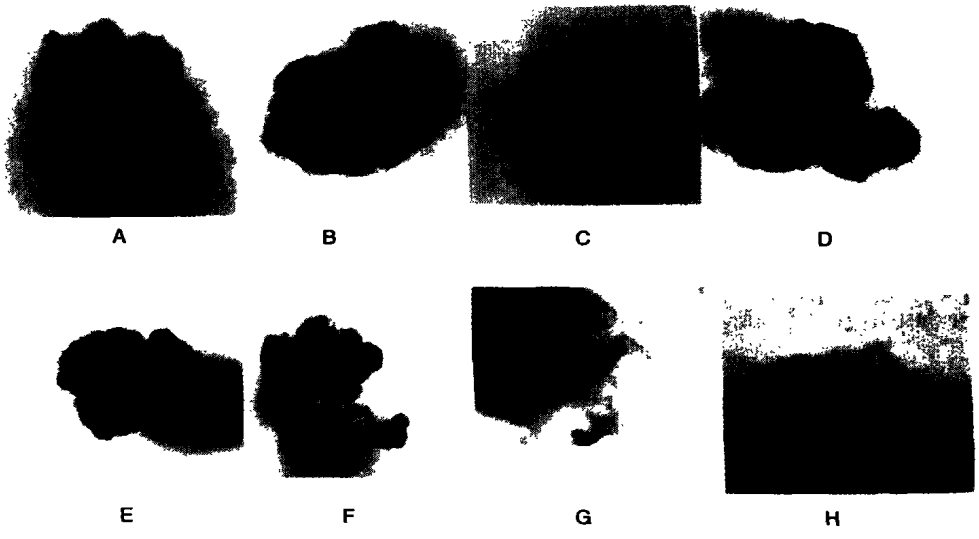

Fig. 2 Transient expression of GUS in Taipei 309 calli after co-cultivation with *A. tumefaciens* LBA4404

Rice calli co-cultivated with A: LBA4404 (pC1301); B: LBA4404 (pCRRD3); C: LBA4404 (pC700); D: LBA4404(pC410); E: LBA4404 (pC150); F: LBA4404 (pC120); G: LBA4404 (pC1391Z); H: Untransformed rice callus

that there were promoter elements for plants and promoter elements for animals in RRD3 fragment, but the regulatory pattern of the promoter was different in animal cells compared with that in plant cells (Table 1).

Table 1 Comparison of promoter activities of RRD3 and serial deletants in different host cells

Length of DNA fragment (bp)	Contained TATA box	Promoter activity	
		CHO	Rice calli
820	1,2,3,4	+++++	+++++
700	1,2,3,4	++++	++
410	3,4	-	+++++
150	4	+++++	+++++
120	-	++	++

Total RRD3 (820 bp) had strong promoter activity in CHO cells and in rice calli, but promoter activities of serial deletants of RRD3 were very different in CHO cells and in rice calli. After 100 bp fragment from 5' end was deleted, the promoter activity of deletant in CHO cells was weakened a little while promoter activity in rice calli was weakened significantly. This result indicated that there was a positive regulatory element in the 100 bp region, but its regulatory efficiency in animals differed from that in plants. Interestingly, after 410 bp fragment (containing the second TATA box) was deleted, the promoter activity in CHO cells was depressed, but the activity in rice calli was retrieved. The conclusion showed that there existed an negative regulatory element for plant promoter in the 100–410 bp region from 5' end, deletion of this region caused retrieval of the activity of plant promoter; but in CHO cells this region had positive regulatory efficiency to its downstream element and the downstream element's activity was lost after deletion of this region. After the 670 bp region from 5' end (containing 1, 2, 3 TATA boxes) was deleted, the promoter activity of deletant was still strong in rice calli and the promoter activity in CHO cells was retrieved. This indicated that the forth TATA box was related to the promoter activity in animal cells and plant cells while an negatively regulatory element existed in 411 - 670 bp region depressed the activity of animal promoter.

Two primary conclusions can be made. First, two regulatory elements of eukaryotic promoter presented in RRD3 sequence. The element in 100–410 bp region was positive to animal promoter but was negative to plant promoter; another element in 411–670 bp region was negative only to animal promoter. Second, there was at least one promoter related to TATA box (the forth TATA box) in RRD3 sequence, but the regulatory pattern of the promoter in animal cells seemed to be different from that in plant cells. Further serial deletion of RRD3 from 3' end will help analyze the relationship between promoter activity and other TATA boxes.

3.2 GenBank analysis of RRD3 sequence

Result from GenBank Blast search indicated that about 554–820 bp region of RRD3 was homologous to conjectured intron of some sequences published in GenBank.

Table 2 Comparison of homology between RRD3 and sequences in GenBank

Region of RRD3	Homologous sequence*	Homology (%)
554 - 820 bp	Conjectured intron of P0434D08	96%
554 - 820 bp	Conjectured intron of BI804283	100%
554 - 820 bp	Conjectured intron of BI801267	99%
554 - 820 bp	Conjectured intron of BI804243	99%
554 - 820 bp	Conjectured intron of BI803865	99%

* All homologous sequences were derived from rice (*Oryza sativa*)

The promoter activity of eukaryotic intron was firstly obtained in animal cells[12]. The corresponding paper about plant intron was published only recently: The DNA construct containing only first intron and first exon of maize *Ubiquitin* gene could promote the expression of *gusA* gene in scutella of transgenic wheat. After it was combined with promoter of maize *Ubiquitin* gene, this construct could enhance the expression of *gusA* gene significantly[13]. In our results, this DNA fragment derived from rice moderately repetitive sequence also has promoter activity in rice calli. Perhaps this sequence can function as promoter when it exists as intron in rice genome. If the inference is right, the unknown regulated gene and regulatory mechanism will be an interesting topic for study.

With rapid development of plant molecular

biology, more and more genes were determined by gene cloning technology such as map clone, many of which were related to agronomic traits. Current research works are mainly to recombine these genes with proper vector and transfer them into plants for the improvement of agronomic traits. Among all above problems, how to choose an appropriate transformation vector with high efficient or tissue-specific promoter is a key procedure. Therefore isolation and identification of promoter elements for plants will play a significant role in transgenic research.

In this paper the promoter activities of rice repetitive sequence RRD3 and its serial deletants are assayed qualitatively. Further quantitative analysis and site-directed mutation will contribute to determination of regulatory elements in RRD3.

References

[1] Walbot V. Rapid genomic change in higher plants [J]. Annu Rev Plant Physiol, 1985, 36:367–396.

[2] Doolittle W F, Saphieza C. Selfish genes, the phenotype paradigm and genome evolution [J]. Nature, 1980, 284:601–603.

[3] Dawid S, Barenkamp S J, St Geme J W. Variation in expression of the *Haemophilus influenzae* HMW adhesins: a prokaryotic system reminiscent of eukaryotes [J]. Proc Natl Acad Sci USA, 1999, 96 (2):1077–1082.

[4] Glew M D, Baseggio N, Markham P F, et al. Expression of the pMGA genes of *Mycoplasma gallisepticum* is controlled by variation in the GAA trinucleotide repeat lengths within the 5' noncoding regions [J]. Infect Immun, 1998, 66(12):5833–5841.

[5] Kenyon J R, Craig I W. Ahalysis of the 5'regulatory region of the human Norrie's disease gene: evidence that a nontranslated CT dinucleotide repeat in exon one has a role in controlling expression [J]. Gene, 1999, 227(2):181–188.

[6] Ott R W, Hansen L K. Repeated sequences from the *Arabidopsis thaliana* genome function as enhancers in transgenic tobacco [J]. Mol Gen Genet, 1996, 252:563–571.

[7] Huang W, Long Y, Xu L P, et al. Studies on rice repetitive DNA sequence pRRD3 [J]. Acta Bot Sin, 1998, 40:216–222. (in Chinese)

[8] Wang J F, He H Q, Li Y K, et al. The promoter activity of RRD3 in CHO and 3T3 cell lines [J]. Chin J Biochem Mol Biol, 2000, 16 (6):732–736. (in Chinese)

[9] Wang J F, Li Y K, He H Q, et al. Promoter function of a rice repetitive DNA sequence RRD3 in transgenic plants [J]. Acta Bot Sin, 2000, 42(10):1057–1061. (in Chinese)

[10] Sambrook J, Fritsch E F, Maniatis T. Molecular Cloning [M]. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.

[11] Dityakin S Y, Lisovskaya K V, Panzhava N N, et al. Frozen-thawed bacteria as recipients of isolated coliphage DNA [J]. Biochim Biophys Acta (Amst.), 1972, 281:319–323.

[12] Raimond J, Rouleux F, Monsigny M, et al. The second intron of the human galectin-3 gene has a strong promoter activity down-regulated by p53 [J]. FEBS Lett, 1995, 363 (1–2):165–169.

[13] Salgueiro S, Pignocchi C, Parry M A. Intron mediated *gusA* expression in tritordeum and wheat resulting from particle bombardment [J]. Plant Mol Biol, 2000, 42(4):615–622.