Upstream sequences of rice proliferating cell nuclear antigen (PCNA) gene mediate expression of PCNA-GUS chimeric gene in meristems of transgenic tobacco plants

Shunichi Kosugi, Iwao Suzuka[1], Yuko Ohashi[2]*, Taka Murakami[2] and Yuji Arai
Institute of Applied Biochemistry, University of Tsukuba, [1]National Institute of Animal Health and [2]National Institute of Agrobiological Resources, Tsukuba Science City, Ibaraki 305, Japan

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ABSTRACT

The transgenic tobacco plants have been generated that express the E. coli β-glucuronidase (GUS) gene under control of the promoter from the rice proliferating cell nuclear antigen (PCNA, DNA polymerase auxiliary protein) gene. GUS expression detected *in situ* by staining with the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), was restricted to meristems in the organs of the transgenic tobacco plants. This expression responded to the phytohormones which promote callus formation. Furthermore, *in situ* thymidine uptake showed that the GUS expression pattern corresponded well to the active sites of DNA synthesis. Deletion analysis of the 5′ upstream sequence confined the GUS expression pattern to a fragment extending 263 bp upstream of the transcription start site of the rice PCNA gene. Thus, we have identified this fragment as a main regulatory element of the rice PCNA gene promoter.

INTRODUCTION

Proliferating cell nuclear antigen (PCNA) is identified as a DNA polymerase δ auxiliary protein (1−3), and plays an essential role in DNA replication in eukaryotes (4−8). This protein is highly homologous among eukaryotes and, thus, is conserved during evolution (9−14). In the preceding papers, we have demonstrated the presence of PCNA gene in higher plants (15) and determined the nucleotide sequences of rice PCNA gene including the 5′- and 3′-flanking regions as well as cDNA (16). Southern blot hybridization analysis indicates the presence of homologous sequence in genomic DNAs from rice, soybean and tobacco hybridized with a rat PCNA cDNA probe. The deduced rice PCNA amino acid sequence shared approximately 80% similarity with the sequence of rat PCNA cDNA, including the conservative substitutions of amino acid.

In mammalian cycling cells, PCNA protein and mRNA changes relatively little in amounts during the cell cycle (17−19). However, they are growth regulated, being very low in quiescent G_0 cells and increasing dramatically when cells are stimulated to proliferate by serum or growth factors (2, 9, 10, 20). Infection of adenovirus can also induce expression of the cellular gene for PCNA in primary baby rat kidney cells (21). Recent studies have revealed that the 5′ flanking sequence of human PCNA gene has a promoter function for directing expression of reporter genes (11, 22), and that this promoter function is transactivated by adenovirus E1 gene products (21, 22). Ottavio et al. have suggested that introns of human PCNA gene are involved in the growth regulation of the mRNA levels (23). Furthermore, they have shown that the human PCNA mRNA amounts are regulated at transcriptional and post-transcriptional levels (24).

As compared with studies on the regulation of mammalian PCNA genes, direct evidence about plant PCNA genes have not yet been elucidated. To determine whether the regulation of plant PCNA gene expression depends on DNA synthesis, we have examined the activity of promoter sequences of rice PCNA gene during development of plant organs. It was found that the 5′ flanking sequences are capable of driving the expression of a β-glucuronidase (GUS) reporter gene and that the GUS staining pattern is restricted to the rapidly dividing cells in organs. It was further found that the 5′ upstream sequences between position −263 and a translation start site of the rice PCNA gene contain sufficient sequence information to direct the meristem-specific expression of the GUS reporter gene.

MATERIALS AND METHODS

Northern blot analysis of rice poly(A)$^+$ RNA

Poly(A)$^+$ RNAs were prepared from primary shoots and root tips of 5 days-rice seedlings, and from mature leaves from adult rice plants (Japonica of *Oryza sativa* L., cv Nipponbare). Five μg each of poly(A)$^+$ RNAs were transferred to nitrocellulose membranes after electrophoresis on 1% agarose/formaldehyde gel, and hybridized with the 0.6 Kbp BalI fragment of the pCJ-1 insert as described previously (15).

Constructions of gene fusions

The 2028 bp 5′ flanking sequence in a 5.3 Kbp HindIII insert of a rice PCNA genomic clone pCJ-1 (15) used in the present work was completely sequenced [(16), see accession number X54046 from the EMBL Data Base]. 5′ deletion mutants were

* To whom correspondence should be addressed

prepared by polymerase chain reaction (PCR) using Gene Amp^R kit (Perkin Elmer Cetus) to make an accurate junction of the PCNA 5′ upstream sequence with the reporter gene, GUS coding sequence (25). The native and three 5′ deleted fragments contained 2005 bp, 558 bp, 263 bp or 82 bp of the regions upstream from the transcriptional start site and 68 bp of the 5′ untranslated leader sequence of the rice PCNA gene, respectively. These fragments that harbored Hind site at 5′ endpoints and BamHI site at 3′ endpoints were cloned in pUC18, and checked by sequencing. To produce a clone containing the native 5′ upstream region, a 1.58 Kbp HindIII-XbaI fragment of 5′ upstream region was excised from the pCJ-1 and replaced with a HindIII-XbaI fragment in a 5′ deletion clone containing a 558 bp of the 5′ upstream region. The native and deleted fragments with 5′ endpoints at −2028, −558, −263 and −82 were cloned in the binary vector pBI101.1 (25) to fuse the GUS coding sequence, and the resultant constructs were designated PCB2K, PCBΔ1, PCBΔ2 and PCBΔ3, respectively (Figure 1). The cauliflower mosaic virus 35S RNA promoter (CaMV35S) -GUS construct (pBI121) was purchased from Clontech Lab.Inc. as a control for constitutive expression.

Transformation of tobacco plants

The chimeric PCNA-GUS fusions in the binary vector were mobilized into *Agrobacterium tumefaciens* strain LBA4404 by a triparental mating using E.coli JM109 harboring the mobilization plasmid pRK2013 (26). Tobacco (*Nicotiana tabacum* cv Samsun NN) leaf discs were transformed according to Horsch et al.(27). Kanamycin-resistant plants were grown in a temperature-controlled greenhouse.

In vitro culture of leaf disc from transgenic tobacco plants

Leaf discs (7 mm in diameter) were cut out from detached leaves of transgenic tobacco plants after sterilization with 2% calcium hypochlorite. The discs were cultured with shaking in the liquid medium composed of 1/2 MS basal salt (28) and 0.1% sucrose under light at 27°C in the presence or absence of the phytohormones for callus induction. The mixture of hormones consisted of 1 μg/ml of naphthaleneacetic acid (NAA) as auxin and 0.1 μg/ml of benzyladenine (BA) as cytokinin.

Fluorometrical GUS assay

GUS assay with protein extracts of mature and immature leaves from transgenic plants was carried out using the fluorometric assay procedure described by Jefferson et al. (25), except that the reaction mixture contained 20% methanol to suppress an endogenous GUS-like activity (29). The protein contents of extracts were measured as described by Bradford (30).

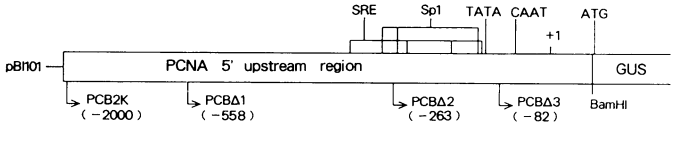

Figure 1. Schematic representation of rice PCNA promoter-GUS fusions. Bent arrows indicate PCNA promoter length with respect to the transcription start site. ATG indicates the translation start site of GUS reporter gene. Consensus DNA motifs are represented as follows [CAAT, CAAT box; TATA, TATA-like box (TATTAA); Sp1, GC box (Sp1 binding site); SRE, serum-regulated element of heat shock protein (HSP70) (31)].

Histochemical GUS assay

Histochemical assay for GUS was performed by a modification of the method by Jefferson et al.(25). Intact root tips and seedlings were directly reacted in 50 mM phosphate buffer (pH 7.0) containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) and 20% methanol at 37°C for 1, 3 or 6 hr. Apical shoots were embeded in 5% agar, and then longitudinally cut into sections (100 μm in thickness) in a small water bath with a microslicer DTK-1000 (D.S.K. Dosaka EM Co.Ltd., Kyoto, Japan). The sections were vacuum-infiltrated and incubated in the GUS reaction mixture. Histochemical GUS reaction with X-gluc produced the indigogenic blue precipitation.

Autoradiography of [^{3}H]thymidine uptake into tobacco leaf discs

Mature leaf discs from the PCB2K-6 plant were pre-cultured for 2 days in the presence or absence of the phytohormones (1 μg/ml of NAA and 0.1 μg/ml of BA). The cultured leaf discs were incubated in a MS liquid culture medium containig [^{3}H]thymidine (370 KBq/ml) in a 3.5 cm plastic schale at 27°C for 30 min with shaking at 80 rpm. The [^{3}H]thymidine-incorporated leaf discs were fixed in ethanol-acetic acid (3:1) solution, and after several washings with the fixing solution, they were immersed in EN^{3}HANCE (Dupont/NEN Research Products) for 3 hr to be impregnated. After a treatment of the discs with 5% acetic acid for 1 hr, the discs were dried under vacuum and the autoradiogram was taken at −70°C by 36-hr exposure to Fuji AIF RX X-ray film.

RESULTS

Expression of PCNA mRNA in rice organs

To study the level of PCNA mRNA, poly(A)$^+$ mRNA from rice organs (root tips, primary shoots and mature leaves) was isolated, electrophoresed, transfered onto nitrocellulose filters and hybridized with rice genomic probe. A single species of PCNA mRNA was detected in poly(A)$^+$ RNA fractions from root tips and primary shoots, whereas no hybridization was observed in that from mature leaves (Figure 2), indicating that rice PCNA mRNA was specifically expressed in rapidly cell-dividing organs of rice plants.

Figure 2. Northern blot analysis of rice PCNA mRNA. Poly(A)$^+$ RNA (5 μg) from root tips (lane 1), primary shoots (lane 2) and mature leaves (lane 3) of Japonica rice were subjected to RNA gel blot analysis with radiolabeled 0.6-Kbp BalI fragment of the pCJ-1 insert.

Fluorometric analysis of PCNA promoter activity in transgenic tobacco plants

Having detected mRNA transcripts in rapidly cell-dividing organs, it was of interest to determine whether the 5′ flanking sequences of the PCNA gene containes sequence information necessary for the regulation of gene expression. To address this question, we constructed the chemeric genes by positioning the GUS reporter gene containing the nopaline synthase (NOS) terminator under the control of a series of 5′-to-3′ deletions of the rice PCNA upstream region previously sequenced (16). These constructs contain 2028 (PCB2K, undeleted), 558 (PCBΔ 1), 263 (PCBΔ2) or 82 bp (PCBΔ3) of the 5′ flanking sequence and 68 bp of the additional 5′ untranslated leader sequence fused to the GUS gene (Figure 1). After subcloning into binary vector pBI101, these constructs were transfered to tobacco leaf discs via Agrobactrium-mediated transformation, and then kanamycin-resistant plants were selected. We obtained six to eight transformants for each construct. As a control containing a strong constitutive plant promoter fused to the same reporter gene, we also transformed the tobacco plants with the CaMV35S promoter-GUS gene fusion. The GUS activity was measured directly after punching leaf discs from fully expanded mature and immature leaves (Figure 3). The PCB2K plants carrying full length of the 5′ sequence generated an appreciable level of the GUS activity in immature leaves, indicating the promoter mediating transcription of the GUS gene. Although deletion of the 5′ upstream sequences gradually reduced the GUS activity of the immature leaves, the PCBΔ2 plants carrying 263 bp still retained the GUS activity corresponding to about 14% of the activity of the PCB2K plants. However, when the deletion was extended to a position −82 (PCBΔ3), the activity dropped to a background level. In a separate experiment, a similar pattern was found in transient assay of the GUS activity when tobacco mesophyll protoplasts were transfected with the transgenes. Thus, we have tentatively localized by the 5′ deletion analysis a shorter promoter region which includes only 263 bp upstream of the transcriptional start point. In contrast to the GUS activity in immature leaves, only low levels of the GUS activity were observed in mature leaves; even for the PCB2K plants, their activities exhibited only 5% of that of immature leaves. Contrary to this, with the 35S-GUS chimeric gene, the mature leaves gave rise to an about 3-fold higher activity as compared with the immature leaves. This contrast reflects a salient feature of the rice PCNA gene promoter.

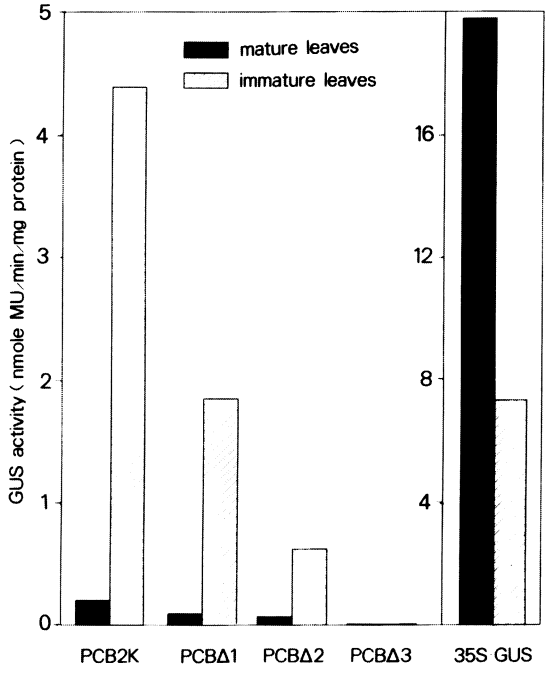

Figure 3. GUS activities in mature and immature leaves of PCNA-GUS transgenic tobacco plants. Fully expanded mature leaves (>200 mm in length) and immature leaves (10−20 mm in length) were excised from five independent transgenic tobacco plants carrying the PCB2K (2028 bp), PCBΔ1 (558 bp), PCBΔ2 (268 bp) or PCBΔ3 (82 bp) transgenes, and three independent plants carrying the 35S-GUS transgene as a positive control. The GUS activities in extracts of the leaves were fluorometrically measured as in the text and are given in nmoles MU (4-methylumbelliferone)/min/mg of extracted protein. The data indicate the average from three independent plants.

Induction of the GUS activity by phytohormone treatment

If the GUS activity is coordinated to cell proliferation, one would expect that the GUS activity responds to phytohormones which facilitate callus formation. To examine this possibility, the GUS activity was measured after 4 days of the incubation of mature leaf discs in the presence or absence of phytohormones (NAA plus BA). As shown in Table 1, the transgenic plants (PCB2K, PCBΔ1 and PCBΔ2) did not induced the GUS activity in the absence of the phytohormones. Treatment with the phytohormones, on the other hand, displayed a significant induction of the GUS activity, although extending the deletion to positions −558 (PCBΔ1) and −263 (PCBΔ2) led to 70% and 80% decrease in the GUS activity, respectively, compared with that of the native 5′ upstream sequence (PCB2K). However,the

Table 1. Induction of GUS activity by hormone treatments in PCNA-GUS transgenic plants.

Transgenic plants	Number of plants tested	GUS activity(pmole MU/min/mg protein)			Induction ratios
		without culture	4-days culture − Hormone	4-days culture + Hormone	
PCB2K	4	110	333	3755	11
PCBΔ1	4	107	139	1142	8.2
PCBΔ2	4	33	72	718	9.9
PCBΔ3	2	3.4	19	13	0.7
35SGUS	2	8777	14535	12061	0.8

Mature leaf discs (7 mm in diameter) were cut from independent plants and then cultured in MS liquid medium containing 0.1% sucrose under light at 27°C with shaking for 4 days, in the presence or absence of the phytohormones (1 μg/ml of NAA and 0.1 μg/ml of BA). After the culture, GUS assay was carried out as described in the text. The induction ratios are expressed as values of the hormone-induced activities devided by the hormone-less activities.

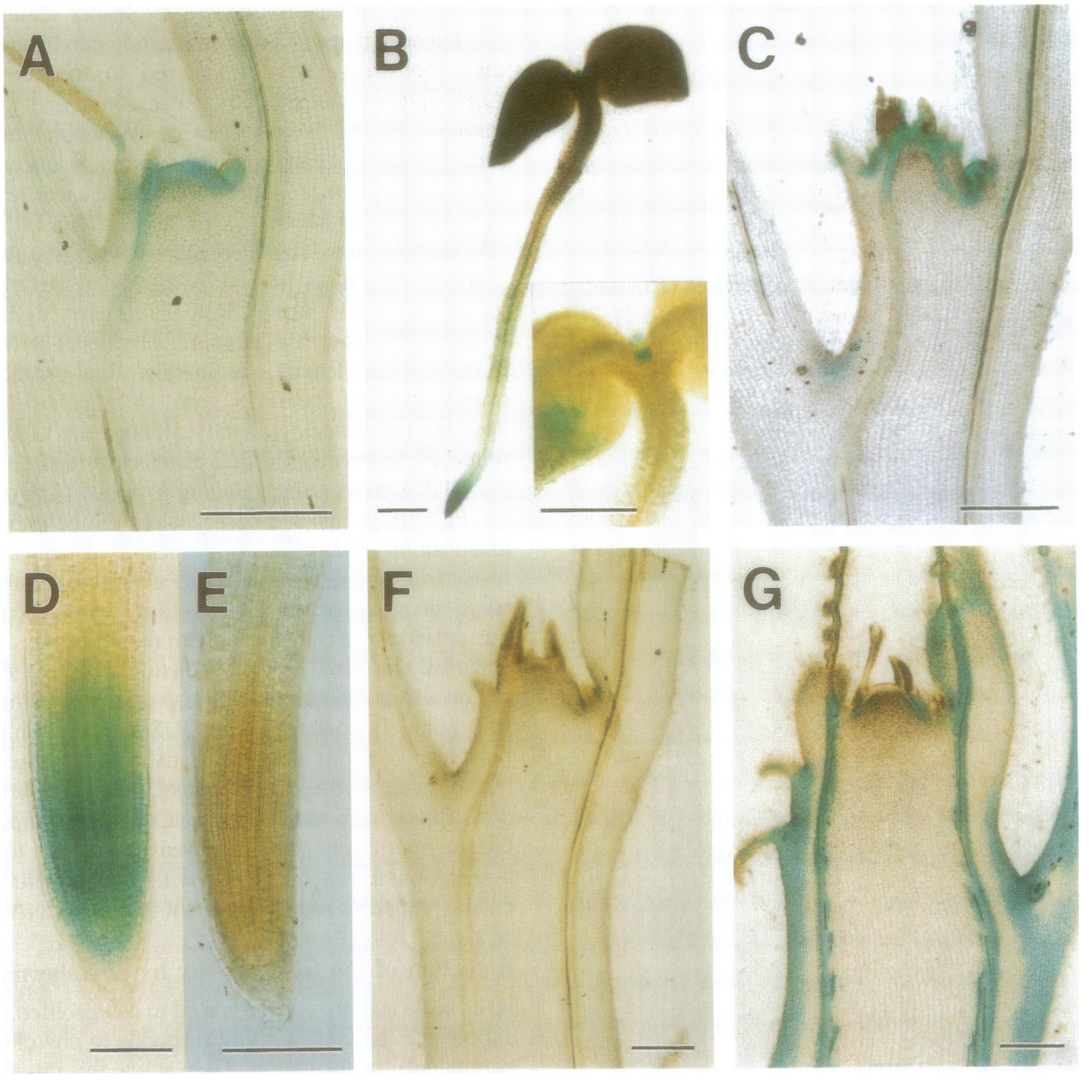

Figure 4. Histochemical localization of GUS activity in the PCNA-GUS transgenic tobacco plants. Longitudinal sections of shoot apex, intact root tips and a seedling from transgenic plants were reacted in the reaction mixture containng 1 mM X-Gluc. The reactions were carried out at 37°C for 1 hr (A, C, G), for 3 hr (D, E, F) or for 6 hr (B). A, shoot from the PCB2K plant; B, seedling from the PCB2K plant; C, shoot from the PCBΔ2 plant; D, root from the PCBΔ2 plant; E, root from the PCBΔ3 plant; F, shoot from the PCBΔ3 plant; G, shoot from the 35S-GUS plant. Size bars indicate 800 μm (A, B, C, F, G) and 200 μm (D, E).

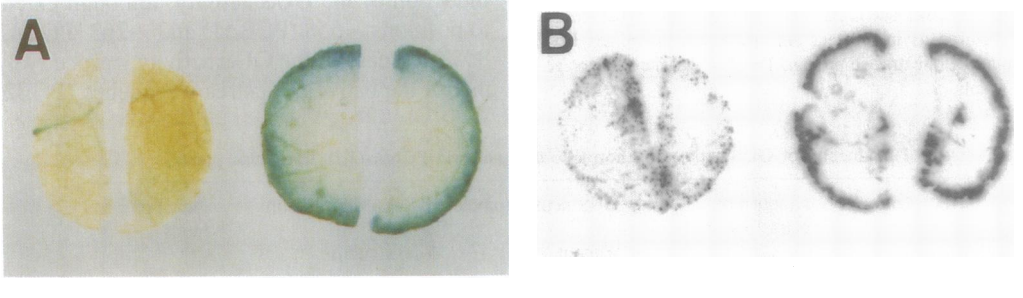

Figure 5. Histological patterns of PCNA-GUS expression and [^{3}H]thymidine uptake in cultured leaf discs. Mature leaf discs from the PCB2K-6 plant were cultured for 2 days in the presence or absence of the phytohormones. Just at end of the culture, a half circle was made by diagonal cutting in the cultured discs. Histological localization for GUS and [^{3}H]thymidine uptake was determined as described in 'Materials and Methods'. A, right, GUS staining (with hormones); A,left, GUS staining (without hormones); B, right, thymidine uptake (with hormones); B, left, thymidine uptake (without hormones).

inducibility as expressed by the induction ratios between 8 to 10 fold decreased only slightly among these three constructs. By contrast, the 35S-GUS plants constitutively expressed the GUS activity regardless of the presence or absence of the hormones. In addition, when the deletion was extended from a position −263 to −82 (PCBΔ3), a complete loss of the hormonal inducibility is observed. Therefore, the GUS activity responds to the hormones under control of the 263 bp 5′ upstream region.

Visualization of specificity of PCNA promoters by histochemical analysis

To confirm the results described in the preceding sections, we examined the spatial patterns of the PCNA promoter activity caused by the PCNA-GUS transgenes in transgenic organs by histochemical analysis of GUS activity *in situ* using the chromogenic substrate X-Gluc for GUS reaction (Figure 4). The blue indigo color indicates the presence of GUS activity. In PCB2K plants, GUS staining pattern was restricted to the apical meristematic region in shoots (Figure 4A). A similar staining pattern was obtained both in the growing point of the root after 5 days seedling (Figure 4B) and in a premordia between the hypocotiles (insert in Figure 4B). Interestingly, in PCBΔ2 plants, the GUS staining pattern retained along the apical of shoot (Figure 4C) and the growing point of root (Figure 4D), while a root cap and a vascular bundle were not stained. In contrast, no GUS activity was detected in root tips nor in shoots from the PCBΔ3 plants (Figure 4E and 4F). Also, in the CaMV35S-GUS plants, the GUS expression was not detectable in the apical meristematic region of shoot (Figure 4G). Instead, it appeared to a cell layer surrounding the vascular system. These results indicate that the PCNA-GUS transgenes are restrictively expressed in the meristems of the organs, and suggest that the restricted patterns of the GUS expression emerge from regulatory information in the 263 bp 5′ upstream sequence.

Relationship between GUS activity and DNA synthesis

In order to establish a topographical relationship between the expression of the GUS activity and occurrence of DNA synthesis, we examined histochemical patterns of the GUS activity and autoradiogram of [^{3}H]thymidine uptake on the cultured leaf discs.

The punched leaf discs from the PCB2K plants were cultured in the presence or absence of the phytohormones for 2 days. During the culture, hormone-responded cell proliferation was induced around the punched edge of the discs. To exclude a possibility that the GUS staining and thymidine uptake may be due to artificial permeation of the GUS substrate or [^{3}H]thymidine by the punched wounds around the discs, a half circle was made by diagonal cutting of the cultured discs just before the GUS staining and [^{3}H]thymidine uptake were performed. The GUS activity was clearly localized around the round edge of the half circles only in the presence of the hormones (Figure 5A, right). Simultaneously, when *in situ* thymidine uptake was measured in the presence of the hormones, a strong signals were observed predominantly along to the round edge of the half discs (Figure 5B, right). Evidently, the GUS expression driven by the promoter histologically corresponded to the sites of DNA synthesis. This indicates a close relationship between the expression of PCNA gene and DNA synthesis and cell proliferation. This relationship appears not to be artificial because staining did not appear in the linear edge of the half discs which had been made just before GUS staining or [^{3}H]thymidine uptake and, further, in the absence of the hormones (Figure 5A and 5B, left).

DISCUSSION

In the present paper, we analyze the promoter activity of the 5′ flanking sequence of the rice PCNA gene in order to understand the regulatory mode of the PCNA gene expression and elucidate the cis-acting region necessary for this regulation, by using transgenic tobacco plants transformed with the PCNA-GUS gene fusions. *In situ* analysis of the GUS activity reveals that the rice promoter has the ability to direct the GUS expression specificaly in the meristematic sites of the plant organs. Moreover, we find that GUS expression responds to the phytohormones which facilitate cell proliferation and topographically correlates with the sites of DNA synthesis. The GUS expression pattern observed also parallels the observation that rice PCNA mRNA appears in rapidly cell-dividing organs of rice. Although we can not exclude some bias in the observed patterns of GUS activity owing to differences between position effects or copy number of the transgenes and the expression levels of the activity, the expression patterns we observed are clearly distinct from those observed in transgenic plants containing the CaMV35S-GUS transgene.

From these results, we concluded that the patterns of the GUS activity in the PCNA-GUS transgenic plants are mediated by sequence elements contained in the rice PCNA promoter. We further confirms this conclusion by the deletion analysis of the 5′ flanking region of the rice PCNA gene, and show that the 263 bp 5′ upstream sequence and the 5′ untranslated leader sequence provide the sufficient regulatory information to express the transgenes specificaly in meristem cells.

We have previously reported that the 263 bp 5′ upstream fragment also contains several DNA motifs similar to cis-acting elements present in many eukaryotic genes (16). At present, however, the exact DNA elements that specify the PCNA gene activation can not be identified. Since the removal of the region between a position −263 and −83 has the critical effect on the expression of the reporter gene, it will be of interest to determine if and which of these DNA motifs contribute to the regulatory expression of the reporter gene. On the other hand, we can not rule out that further upstream sequences include additional elements to enhance the promoter activity since removal of the sequence upstream a position −263 also result in a considerable decrease of the level of the GUS expression. In addition, the introns or the 3′ flanking sequence of the rice PCNA gene may somewhat influence the PCNA expression, since it has been reported that removal of the intron 4 of human PCNA gene leads to the high level of PCNA mRNA acumulation in serum-deprived cells (23).

In mammalian cells, stimulation of quiescent cells by serum or growth factors causes the marked increase of PCNA mRNA, accompanied by G_0 to S phase transition of cell cycle (2, 9, 10, 20). The growth factor-responsive expression of human PCNA gene requires elements within an about 300 bp region immediately upstream of the transcriptional start site of human PCNA gene, which are sufficient for full promoter activity in mammalian cultured cells (11, 22, 24). Our data also show that the rice PCNA promoter can direct the expression specifically in the site of DNA synthesis in plant organs. Thus, it appears that there is a considerable similarity between animal and plant systems in the regulation of PCNA gene expression, which is also supported by the high degree of conservation of PCNA structure (9−14) and function (4−8).

Since the plant PCNA gene is restrictely expressed in meristems, it will serve as an excellent probe for molecular and histological approaches in a variety of higher plant development processes. The presence of *cis*-acting region will also permit us to identify *trans*-acting factors involved in the phytohormone-regulated expression of the rice PCNA gene.

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