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Isolation and deletion analysis of meristem specific promoter from *Sorghum bicolor* (L.) Moench.

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A meristem specific promoter of the S-phase specific gene (*cyc07)* has been cloned from *Sorghum bicolor* by polymerase chain reaction (PCR) using a proof reading DNA polymerase (Pfu polymerase). The 595 bp promoter consists of 2 repeats, a CAAT box and TATA box. The sequence was cloned in pBI 121 vector carrying the gene for β-D-glucuronidase (*GUS*) by replacing the 35S CaMV promoter. Transgenic tobacco plants were developed by *Agrobacterium*-mediated transformation and analyzed for tissue-specific expression. Incubation of kanamycin-resistant tobacco shoots with GUS substrate resulted in intense blue colouration in the shoot tips, root tips and root hairs proving that the promoter sequences were enough to induce meristem specific activity of the reporter gene. Four sequential deletions from the 5' end were carried out. The deletion analysis indicated that a 13 bp repeat I sequence was essential for regulation of meristem specific expression of the reporter gene.

Keywords: *Sorghum bicolor*, cyc07S promoter, deletion analysis

IPC Code: Int. Cl.[7] C12N15/10; 15/79

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench.] is an important crop grown in semi-arid regions of India, Africa, Australia and USA. In India, during the rainy season, the crop is heavily infested at the seedling stage by shootfly (*Atherigona soccata*), a dipteran pest. The larvae of the insects cause extensive damage to the growing seedlings by destroying the shoot apex and causing dead hearts[1]. One of the effective and alternate strategies to manage shootfly is to genetically engineer sorghum to express insecticidal proteins (δ-endotoxins) of *Bacillus thuringiensis* (Bt) and confer durable protection to the crop. Dipteran-specific genes of Bt are known[2] and can be expressed in plants after suitable modifications for plant expression[3]. Management of shootfly in transgenic sorghum by expressing the insecticidal crystal proteins in a tissue-specific manner is envisaged. A meristem-specific promoter, which expresses the transgene in the dividing cells obviates unnecessary production of the toxin in other tissues where it is not required.

In the present study, a meristem specific promoter of the *cyc07* gene was isolated from *Sorghum bicolor* by simple PCR using degenerate primers. This promoter was chosen because of small size and stable expression in the dividing cells. The promoter was sequenced and found to be identical to the promoter sequence of *Catharanthus roseus*. A search into the database revealed that the *cyc07* gene is more extensively studied than the promoter sequences. This gene shows a striking similarity in terms of amino acid sequences in a number of plants i.e. *Oryza sativa*[4], *Arabidopsis thaliana* (Accession no. AJ001342) and *Daucus carota* (Accession no. AB029635). Other genes which are homologous to the *cyc07* gene are PLC1 and PLC2 the two closely related genes found in yeast[5] which, are essential for the proliferation of yeast cells.

Materials and Methods

Plant Materials

Sorghum seeds (cv. 296B) were germinated in dark for 48 h. The young seedlings were used for total DNA isolation. Seeds of *Nicotiana tabacum* were

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germinated in the culture tubes. The leaves were used for further propagation by placing them on MS medium with 1mg/L benzylaminopurine (BAP) and 0.1 mg/L α-naphthalene acetic acid (NAA) with 3% sucrose.

Isolation of cyc07S Promoter from *S. bicolor* by PCR

Total genomic DNA was isolated from the seedlings of *S. bicolor* by the procedure described by Saghai-Maroof *et al*[6]. PCR was performed using the custom-synthesized primers (Genset). The primer was designed so as to amplify 595 bp promoter sequence of the *cyc07* gene of sorghum. Two restriction sites for the enzymes *Hin*d III and *Bam*H I with compatible nucleotides were incorporated in the forward and reverse primers respectively so as to facilitate cloning in the vectors. The primers were as follows:

Forward Primer:
5′- C**CCAAG**AAAAAATCGATTTTCT-3′

Reverse Primer:
5′-GCG**GGATCC**AATGCATATAAAACCCTAGG-3′

PCR was performed in 0.2 mL thin-walled tubes. PCR carried out in 50 µL of reaction mixture contained components at following concentrations: 50 m*M* KCl, 10 m*M* Tris-HCl (*p*H 9.0 at 25°C), 0.1% Triton X-100, 1.5 m*M* $MgCl_2$, 100 m*M* each of dATP, dCTP, dGTP, dTTP, 0.5 m*M* of each primer, 1U *Pfu* DNA polymerase (Stratagene) and 30 ng plant genomic DNA. PCR cycling conditions were as follows: an initial denaturation at 94°C for 2 min followed by 29 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1 min. This was followed by a final extension step of 3 min at 72°C. The PCR product was gel purified and restricted with the enzymes *Hin*d III and *Bam*H I. The restricted PCR fragment was cloned in pBluescript SK^+ (Stratagene) vector at *Hin*d III and *Bam*H I sites and transformed in competent *Escherichia coli* DH5α cells[7]. The positive colonies were picked up and the plasmid (pBlue cyc07S) was isolated. The 595 bp *Hin*d III and *Bam*H I fragment was restricted and subcloned in pBI 121 (Promega) by replacing the CaMV 35S promoter (800 bp).

The ligation product was transformed into *E. coli* DH5α competent cells. Positive transformants were further screened by the direct colony PCR method. The pBI 121 plasmid, containing amplified PCR product, was named pBI cyc07S (Fig. 1) and was subsequently used to transform *Agrobacterium* strain LBA4404.

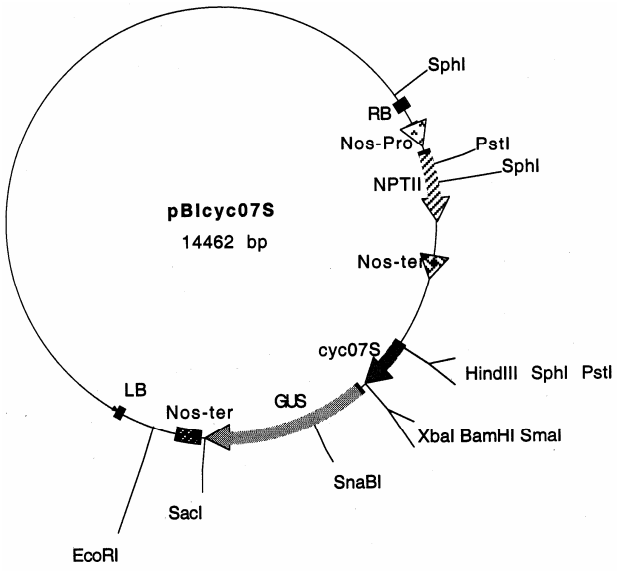

Fig. 1—*Agrobacterium* transformation vector pBIcyc07S carrying the 589 bp 5' meristem specific promoter by replacing the CaMV promoter tagged to the *GUS* gene.

Construction of cyc07S Promoter Deletions

In order to define the region of the cyc07 promoter necessary for meristem-specific activity, deletions were made with Exonuclease III, and truncated promoters were placed upstream of the reporter gene β-glucuronidase (*GUS*). The unidirectional deletion derivatives were constructed by the procedure developed by Henikoff[8]. Exonuclease III was used to specifically digest insert DNA from protruding or blunt end restriction site at different time periods. The pBlue cyc07S plasmid was cleaved at unique site *Kpn* I and *Hin*d III site to linearise the plasmid. The *Kpn* I site resists the deletions by exonuclease III and is used as blocking site. This was then subjected to digestion by exonuclease III to generate a series of deletions on the 5' end of the promoter. The products from different time periods of incubation were end filled with Klenow fragment of DNA polymerase I in the presence of all four nucleotides to create blunt ended DNA and subsequently digested with *Bam*H I. The digestion was run on the agarose gel and fragments of less than 595 bp were gel purified. The different deletion fragments were inserted individually into pBI 121, which was first cut with *Hin*d III and filled with Klenow fragment of DNA polymerase I and then cut with *Bam*H I so as to generate one blunt site and one *Bam*H I site to allow the ligation of the

deletion fragments in the right orientation resulting in generating the cyc07S promoter-GUS-nos cassette in pBI 121 vector. The nucleotide sequence was determined by the dideoxy chain termination method[9]. Mutants possessing various 5' deletions were examined and chosen after sequencing and were further used for the transformation of *Agrobacterium.*

Production of Transgenic Plants

The binary vectors (pBI 12I) carrying various chimeric cyc07S-*GUS* gene were used for transforming strain LBA 4404 by freeze-thaw method[10]. Tobacco (*Nicotiana tabacum* var. Petit Havana SR2) leaf discs were infected and co-cultivated with *Agrobacterium* and transformed shoots were selected on 250 mg/L kanamycin. The shoots were separated and placed on MS medium with 0.5 mg/L of IBA for rooting. The plantlets with roots were transferred to small pots containing sand and agropeat in 3:1 ratio and covered with polythene bags. The plants were allowed to grow in the culture room for 20-25 days after which they were slowly exposed to the ambient environment. The plants were later shifted to the glass house.

Histochemical GUS Assay

Histochemical staining of GUS was performed as described by Jefferson *et al*[11]. Transformed plants growing on kanamycin were fixed in freshly prepared 10 m*M* MES (*p*H 7.0), containing 0.3% (w/v) formaldehyde and 0.3 *M* mannitol for 1 h and vacuum infiltrated and incubated for 5 min at room temperature. Tissues were washed with 50 m*M* sodium phosphate buffer (*p*H 7.0) and incubated in the buffer containing the chromogenic substrate 5-bromo-4-chloro 3-indolyl-β-D glucuronidase (x-gluc) overnight. They were then transferred to 70% (v/v) ethanol to remove the chlorophyll for detailed observation. The GUS stained materials were mounted on the slide and examined under the stereomicroscope.

Results and Discussion

Confirmation of Transformation

To check for the cyc07 promoter in the transformed tobacco plant, total genomic DNA from leaf was extracted for genomic Southern blot analysis. As shown in Fig. 2, the 595 bp promoter sequence from cyc07S hybridized with single 2.7 Kb fragment of *Hin*d III-*Eco*R I-cut genomic DNA. Similarly, a single band corresponding to 4.7 Kb was detected when genomic DNA was digested with *Hin*d III and a 4.4 Kb fragment was detected with *Eco*R I cut genomic DNA.

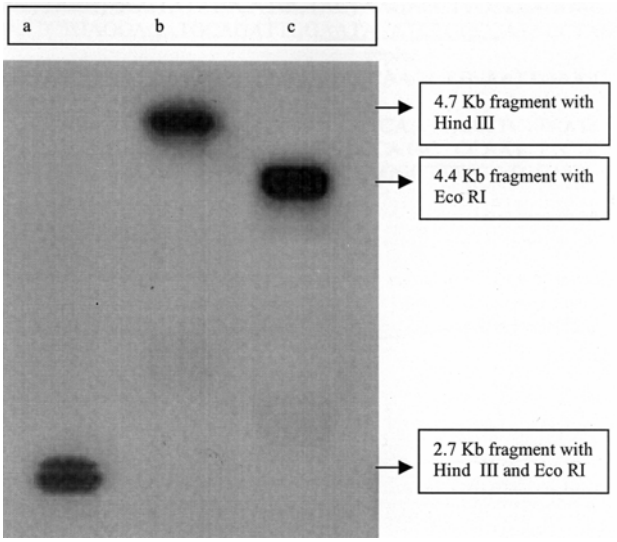

Fig. 2—Southern analysis of 595 bp promoter sequence from cyc07S hybridized with single 2.7Kb fragment (a) of *Hin*d III-*Eco*R I-cut genomic DNA. A single band corresponding to 4.4Kb (c) was detected when genomic DNA was digested with *Eco*R I and a 4.7Kb fragment (b) was detected with *Hin*d III cut genomic DNA.

Sequencing of cyc07S Promoter from *S. bicolor*

The cyc07S promoter cloned in pBluescript was sequenced using T3 and T7 primers. The 595 bp sequence of the promoter contained a TATA and a CAAT box at positions -28 and -68, respectively. Two direct repeats, each having 13-nucleotide sequence, were present. The repeat I (AATATTGAT(G)GTAG) was present at positions-371 and -344. Repeat II (AATATATCTCGCC) was present at positions -200 and -235 (Fig. 3). These 2 repeats were unique to this promoter and not found in any other promoters. When the sequence of this promoter was compared to the other related promoters, i.e. histone genes, no similarity was found in terms of sequences and various motifs present which influence the meristem specific activity.

Construction of Deletions of cyc07S Promoter

Four clones having deletions of 45, 129, 257, and 389 bp each from the 5' end obtained in pBluescript were sequenced. These fragments were relocated into pBI121 for transformation into *A. tumefaciens.*

Activity of cyc07S Promoter and its Deletions in Transgenic Tobacco Plants

Transgenic tobacco plants containing the pBIcyc07S constructs were tested for GUS activity after two months of plantlet formation. In the stably transformed lines, the *GUS* gene displayed a distinct

Fig. 3—Nucleotide sequence of Sorghum cyc07S promoter

blue colouration in the meristematic tissues i.e. the shoot apex and root apex and root hairs (Fig. 4a-d). The GUS activity was not seen in the mature explants i.e. stem and the leaves. It indicated that 595 bp of the promoter sequence carried the information for the promoter to specific for dividing cells.

The GUS activity in the shoot and root meristems was also checked in the plants carrying the deletions in the promoter sequences. In the first deletion of 45 bp and the second deletion of 129 bp resulting in a 550 bp and 466 bp sequence, respectively of the promoter (Fig. 5), enclosing the two repeats I and II, the CAAT and TATA box controlling the *GUS* gene showed the same pattern of blue colour accumulation in shoot tips, root tips and root hairs, which was seen previously with the complete promoter sequence. In the third deletion of 257 bp resulting in 338 bp of the promoter sequence and lacked the 13 bp Ist repeat sequence did not show any GUS activity in the explants tested. It shows that this 13 bp sequence is important in controlling the meristem-specific activity. The fourth deletions of 389bp resulting in 206 bp of the promoter enclosing repeats II sequences, the CAAT box and the TATA box, the blue colourations in any of the meristems was never observed in any of the explants checked for the activity.

In this communication, the promoter of the *cyc07* gene from *S. bicolor* is characterized in transgenic tobacco plants. In the first step, function of the cyc07S promoter was analyzed by monitoring its meristem-specific expression in transgenic tobacco plantlets. It was revealed that 595 bp of the 5′ flanking sequence of the *cyc07* gene was enough to confer the meristem-specific expression of the gene encoding β-glucuronidase (GUS) in transgenic tobacco. In the second step, unidirectional deletion from the 5′ end of this promoter was carried out. Sequential deletion of 45, 129, 257 and 389 bp from the 5′ end revealed that 460 bp of the promoter encompassing the repeat I, repeat II, CAAT and TATA box were enough to induce the meristem-specific expression. The removal of 129 bp from the 5′ end of the promoter, which included the repeat I sequence resulted in no expression of the reporter gene in the meristematic tissue.

The gene *cyc07* is expressed specifically at the S-phase during the cell cycle. This gene consists of seven exons separated by six introns and has 595 bp promoter upstream of the *cyc07* gene. The expression of this promoter has been studied in transgenic *Arabidopsis* plants[12]. On the basis of the published sequence of the cyc07 promoter, the present investigation was planned with the purpose of isolating the promoter from *S. bicolor* and constructing a promoter cassette to drive the *GUS* gene in the meristematic tissue. The basic idea of isolating the promoter from sorghum was to the expression of an insecticidal protein gene in the shoot apex of the plant so as to target the shootfly larvae. A chimeric gene consisting of this promoter and Bt-cry-gene could be used to express the insectidal protein only in the target tissue i.e. dividing cells of the shoot tip.

Several cell cycle dependent and meristem-specific genes have earlier been studied. The S-phase genes, which are the cell cycle genes, include histone genes[13] and the *PCNA* genes[14]. The other meristem specific genes are the *ERECTA* gene, *PROTODERMAL FACTOR1* (*PDF I*) and *MERISTEM LAYER I* (*ATML I*). The promoters of these genes direct expression in a meristem-specific manner of the reporter genes[14,15].

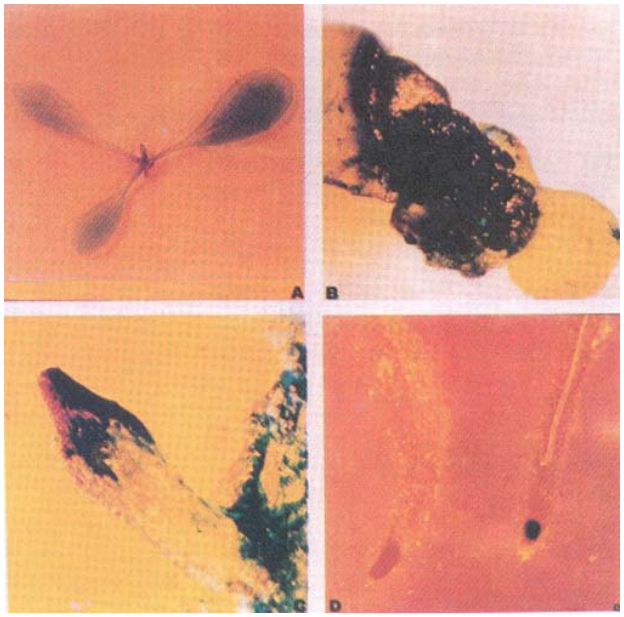

Fig. 4—Histochemical localization of GUS activity in transgenic *Nicotiana* plants. Transgenic tobacco plants transformed with cyc07S were analyzed for GUS activity. (a) Shoot apex showing GUS expression (b) Leaf whorl in the shoot apex showing intense blue color. (c) Root hair (d) Root tip showing GUS activity in the actively dividing region. No activity is seen in the root cap.

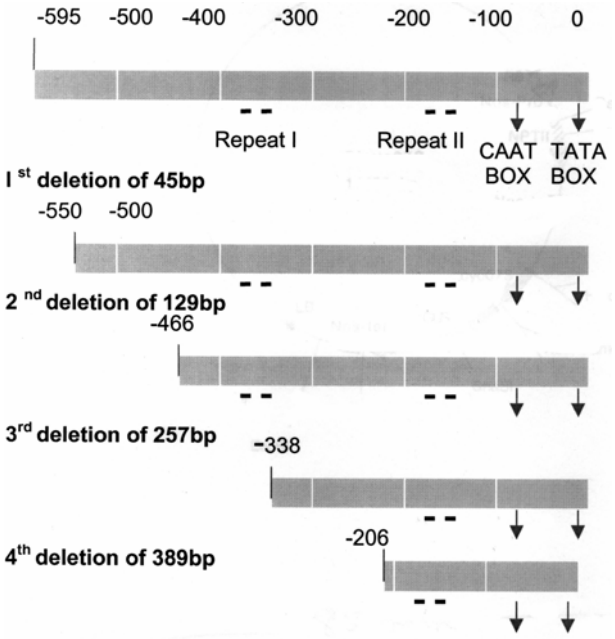

Fig. 5–Deletion analysis of cyc07S promoter

The *cyc07* gene and the histone gene[13] are known as S-phase specific genes and the promoter of these genes target the expression in the actively cycling cells. The protein of the *cyc07* gene has a large number of basic amino acids as the histone protein gene; however, no significant similarity in the protein sequence has been found. In the promoter of the histone gene a number of motifs have been reported[16], however, none of these motifs is present in the cyc07S promoter, nor do they show any similarity to the two repeats present. In the PCNA promoter[17] also no similarity with the consensus has also been found.

Other meristem-specific promoters, which have been studied for their targeted expressions include the *ERECTA* gene promoter. Yokayama *et al*[18] studied the promoter of this gene from *Arabidopsis* and fused it with β-glucuronidase (*GUS*) gene. The expression of the reporter gene was weak in the shoot apical meristem in the early plant development but increased with transition from vegetative to reproductive growth phase.

In the earlier studies[12], the promoter activity of the 5′ flanking sequence of the *cyc07* gene was analyzed. A 595 bp 5′ flanking region and three exons of this gene from *Catharanthus roseus* were fused in frame with *GUS* reporter gene so as to produce a hybrid protein that consists of the N-terminal 22 amino acids of *cyc07* gene fused with the GUS protein. High GUS activity was found in roots and in the shoot apex of the transgenic *Arabidopsis* plants.

In the present study, the authors have used only the promoter sequence without any exons or introns. The GUS assay reveals that the introns are not essential for the expression of the gene. However, it remains to be seen if these introns can enhance the expression of the genes.

The structural domains of plant promoters are identified by deletion analysis. Several meristem and S-phase promoters when studied revealed that there are internal sequences in the promoter, which are essential for the specificity of the promoter. In the ERECTA promoter deletion studies carried out from the 5′ and internal regions of this promoter showed that the essential cis-regulating elements govern the spatial and temporal specific expression and these are located close to the transcriptional initiation site[18]. Similar studies carried out in *Arabidopsis thaliana PROTODERMAL FACTOR1* (*PDF1*) gene, which is exclusively expressed in the L1 layer of shoot apices and the protoderm of organ primordia showed that the minimum region necessary to confer L1-specific expression of PDF1 is confined to a 260 bp fragment upstream of the transcription start site. The L1 box,

which is an 8 bp motif in this region, was found conserved between promoter regions of all the L1-specific genes[19]. Sessions *et al*[20] reported that L1-specific regulatory sequences of the *MERISTEM LAYER 1* (*ATML1*) promoter of *Arabidopsis thaliana* were sufficient to direct foreign gene expression in a layer-specific manner.

In the cyc07S promoter two direct repeat sequences of 13 nucleotides each were found in the 595 bp promoter sequences. These repeat sequences are not seen in any of the S-phase or the meristems specific promoters and are specific to the cyc07 promoter. Repeat I, when deleted, resulted in no expression of the meristematic tissue thus proving that it is an important factor in directing the meristem-specific activity and these could be similar to the L1 box of the PDF1 promoter. However, no internal deletions were carried out and the role of the second repeat remains to be seen.

Conclusion

The cyc07S promoter is similar in *Catharanthus roseus* and *Sorghum bicolor*. This promoter, when expressed in tobacco tagged to the gene encoding β-glucuronidase, showed expressions in the meristematic tissues viz shoot apex, root apex and root hairs. The deletion analysis revealed that the repeat I present was important in contributing to the gene regulation.

Acknowledgement

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References

1 Young W R & Teetes G L, Sorghum entomology, *Annu Rev Entomol*, 22 (1977) 193-218.

2 Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J *et al*, *Bacillus thuringiensis* and its pesticidal crystal proteins, *Microbiol Mol Biol Rev*, 62 (1998) 775-806.

3 Kumar P A, Sharma R P & Malik V S, Insecticidal proteins of *Bacillus thuringiensis*, *Adv Appl Microbiol* , 42 (1996) 1-43.

4 Kidou S, Umeda M, Tsuge T & Uchimiya H, Isolation and characterization of rice cDNA similar to the S-phase-specific *cyc07* gene, *Plant Mol Biol*, 24 (1994) 545-547.

5 Ito M, Yasui A & Komamine A, A gene family homologus to S-phase specific gene in higher plants is essential for cell proliferation in *Saccharomyces cerevisiae*, *FEBS Lett*, 301(1992) 29-33.

6 Saghai-Maroof M A, Soliman K M, Jorgenson R A & Allard R W, Ribosomal DNA spacer length polymorphism in barley, *Proc Natl Acad Sci USA*, 81 (1984) 8014-8018.

7 Chung C T & Miller R H, A rapid and convenient method for the preparation and storage of compenent bacterial cell, *Nucleic Acids Res*, 25 (1988) 3580.

8 Henikoff S, Sequence analysis by electronic mail server, *Trends Biochem Sci*, 18 (1993) 267-268.

9 Sambrook J, Fritsch Z F & Maniatis T, *Molecular cloning: A laboratory manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) 1989.

10 Hofgen R & Willmitzer L, Storage of competent cells for *Agrobacterium* transformation, *Nucleic Acids Res*, 16 (1990) 9877.

11 Jefferson R A, Kavanagh T A & Bevan M W, GUS Fusion: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants, *EMBO J*, 6 (1987) 3901-3907.

12 Ito M, Sato T, Fakuda H & Komamine A, Meristem-specific gene expression directed by promoter of the S-Phase gene, cyc07 in transgenic *Arabidopsis*, *Plant Mol Biol*, 24 (1994) 863-878.

13 Lepetit M, Ehling M, Chaubet N & Gigot C, A plant histone gene promoter can direct both replication-dependent and-independent gene expression in transgenic plants, *Mol Gen Genet*, 231 (1992) 276-285.

14 Kodama H, Ito M, Ohishi N, Suzuka I & Komamine A, Molecular cloning of the gene for plant proliferating-cell Nuclear antigen and expression of this gene during the cell cycle in synchronized cultures of *Catharanthus roseus* cells, *Eur J Biochem*, 197 (1991) 495-503.

15 Atanassova R, Flenet M, Gigot C & Chaubet N, Functional analysis of the promoter region of maize (*Zea Mays* L.) H3 histone gene in transgenic *Arabidopsis thaliana*, *Plant Mol Biol*, 37 (1998) 275-285.

16 Chaubet N, Flenet M, Clement B, Brignon P & Gigot C, Identification of Cis-elements regulating the expression of an *Arabidopsis* histone H4 gene, *Plant J*, 10 (1996) 425-435.

17 Kosugi S & Ohashi Y, E2F sites that can interact with E2f proteins cloned from rice are required for meristematic tissue-specific expression of rice and tobacco proliferating cell nuclear antigen promoters, *Plant J*, 29 (2002) 45-59.

18 Yokoyama R, Takahashu T, Kato A, Torii K U & Kodema Y, The *Arabidopsis* ERECTA gene is expressed in shoot apical meristem and organ primordial, *Plant J*, 15 (1998) 301-310.

19 Abe M, Takahashi T & Komeda Y, Identification of a Cis-regulatory element for L1 layer-specific gene expression, which is targeted by an L1-specific homeodomain protein, *Plant J*, 26 (2001) 487-494.

20 Sessions A, Weigel D & Yanofsky M F, The *Arabidopsis thaliana* MERISTEM LAYER 1 promoter specifies epidermal expression in meristems and young primordial, *Plant J*, 20 (1999) 259-263.