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Cloning of an ovule specific promoter from *Arabidopsis thaliana* and expression of β-glucuronidase

Vikrant Nain, Anju Verma[a], Neeraj Kumar, Priyanka Sharma[b], B Ramesh[c] & P Ananda Kumar*
National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India

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Tissue specific expression of transgenes in plant species has several advantages over constitutive expression. Identification of ovule specific promoters would be useful in genetic engineering of plants with a variety of desirable traits such as genetically engineered parthenocarpy, female sterile plants or seedless fruits. Relative inaccessibility and difficulty in harvesting adequate amounts of tissue at known developmental stages has impeded the progress in cloning of promoters involved in ovule development. In the present study an ovule specific promoter was cloned from *Arabidopsis AGL11*[Ψ] gene and used to express GUS (β-*glucuronidase*) gene in transgenic *Arabidopsis*. Histochemical staining of GUS appeared in the center of young ovary (ovules), but no detectable GUS activity was observed in vegetative plant tissues, sepals, petals and androecium. *AGL11* gene promoter can be useful to modify the developmental path of plants by expressing either plant hormones or lethal genes for agronomic purpose.

Keywords: AGAMOUS, Ovule, Transcription factor, Transgenics, Tissue specific promoter

Interest in plant promoters stems from myriad opportunities available for controlling gene expression in transgenic plants. Plant promoters have played a significant role in genetic engineering that have resulted in production of crops with value-added traits[1]. During early phase of plant genetic engineering, few promoters such as CaMV 35S were available that were used to express a wide range of traits in various plant species[2,3]. However, in order to have a minimal effect on overall growth and development of transgenic plant with novel phenotypes, it is desirable to use tissue specific promoters. This approach is dependent on availability of promoters specific enough to limit the expression of transgene to particular organ or tissue of interest.

Angiosperm ovule plays a central role in producing and sheltering the female gametophyte, which ultimately gives rise to egg cell in plant sexual reproduction[4]. Seed, the final product of the ovule, is a significant food source as well as a common means of propagation for plants. Identification of ovule specific promoters will facilitate the genetic engineering of plants with a variety of desirable traits *viz* development of seedless fruits (parthenocarpy)[5], female sterile plants with restorable fertility that can be used in breeding programs[6] and in controlling the escape of transgenes into the environment[7]. Another potential use of ovule specific promoters is in genetic manipulation for apomixis. As a result of its importance, there is a wealth of descriptive knowledge concerning ovule anatomy and morphology[8], though little is known about the molecular basis of ovule development and function.

Floral binding protein 7 (*FBP7*) gene in *Petunia hybrida* is involved in determination of ovule identity[9]. This gene is first expressed in placenta before the ovule primordia emerge with some expression in young primordia and latter in funiculus and integuments[9-11]. Phylogenetic analysis indicates that *petunia FBP7* gene share maximum amino acid sequence similarity with *AGAMOUS LIKE 11*

*Correspondent author
Phone: +91 11 25848783; Fax: +91 11 25843984
E-mail: polumetla@hotmail.com

Present address
[a]Department of Botany, University of Delhi, Delhi 110 007, India
[b]Department of Genetics and Plant Breeding, CCS University, Meerut 250 001, India
[c]National Institute of Malaria Research, 22 Sham Nath Marg, Delhi 110 054, India

[Ψ]Nomenclature of AGAMOUS LIKE 11 (AGL11) gene from *Arabidopsis thaliana* has been changed to SEEDSTICK (STK) in some of the recent publications.

(*AGL11*) gene in *Arabidopsis thaliana*[12-14]. *AGL11* is a MADS box transcription factor, also known to be involved in development of ovule[15]. Phenotypic observations of *AGL11* mutant and *in situ* hybridization to *AGL11* transcript have confirmed ovule specific expression of this gene also[16]. However, alignment of promoter sequences of the two genes do not show any significant similarity and no studies have been carried out to evaluate *AGL11* promoter activity in transgenic model systems. The present study was undertaken to clone *AGL11* gene promoter and use it to direct heterologous gene (GUS) expression, to confirm its ovule specific expression.

Materials and Methods

Cloning of promoter-The T5L19 clone of *Arabidopsis* genomic library, having complete sequence of *AGL11* gene was obtained from *Arabidopsis* Biological Resource Centre, Ohio State University, and used in the present study.

PCR primers were designed to amplify 3.1 kb region encompassing the promoter and other regulatory sequences (first intron) of *AGL11* gene, from the published sequence of *Arabidopsis* genome (www.arabidopsis.org), forward primer: [5′-cggaattcGCTCTGCAATTTCACCTTTC-3′] and reverse primer: [5′-cgggatccCCTTCATTTTAAACATCAAAC-3′]. Lowercase letters in primers indicates extra nucleotides added to incorporate a restriction site, *Eco*RI in forward and *Bam*HI in reverse primer, respectively. PCR was carried out with *Pfu* DNA polymerase (MBI Fermentas), PCR cycle program consisted of 93°C for 3 min, followed by 30 cycles of denaturation at 93°C for 1 min, annealing at 55°C for 30 sec and extension at 72°C for 3 min, with T5L19 plasmid DNA from T5L19 used as the template for PCR. CaMV 35S promoter in pCAMBIA1301 was replaced by *AGL11* promoter to develop pAGL11::GUS construct. The 35S promoter of pCAMBIA1301 vector was removed by *Bam*HI and *Nco*I restriction and self-ligated after blunting with Klenow enzyme. The resultant pCAMBIA vector was deficient in 35S promoter and recreated *Bam*HI site. *Eco*RI and recreated *Bam*HI sites were used to clone *AGL11* promoter upstream to GUS gene, to make pAGL11::GUS construct (Fig. 1A). The pAGL11::GUS construct was mobilized to *Agrobacterium tumefaciens* strain GV3101.

Development of AGL11::GUS transgenic Arabidopsis—*A. thaliana* ecotype Columbia was used to study the expression of *AGL11* gene promoter. *Arabidopsis* was genetically transformed by floral dip method, using the protocol provided by Clough and Bent (1998)[17]. Seeds collected from *Agrobacterium* infected plants were germinated on half strength MS medium supplemented with hygromycin (10 mg/l). Hygromycin resistant putative transformants were transferred to pots for further development and molecular analysis. All the putative transformants were tested positive for integration and expression of *hpt* gene by RT-PCR. PCR primers for *hpt* gene were designed to amplify 820 bp. The plants confirmed positive for expression of *hpt* gene were analyzed for expression of GUS.

RT-PCR—Expression of *hpt* and GUS gene was assessed by RT-PCR using gene specific primers: *hpt* forward [5′-CGATTGCGTCGCATCGACCCTGCGC-3′], *hpt* reverse [5′-CGACCTGATGCAGCTCTCCGAGGGC-3′], *GUS* forward [5′-GGAAGTGATGGAGCATCAGGGCGGC-3′], and *GUS* reverse [5′- CAGCCCGGCTAACGTATCCACGCCG-3′]. Each RT-PCR reaction was conducted at a final volume of 50 μl. The master mix contained all components required for RT-PCR except for the template RNA, which was added separately. RT-PCR amplification was initiated by placing the PCR tubes in thermal cycler when temperature reached at 50°C. Following the reverse transcription at 50°C for 30 min, PCR was initiated by heating at 95°C for 15 min to activate the Hot-Start *Taq* polymerase. The reaction mixture was cycled 40 times at 94°C for 30 sec (denaturation), 55°C for 30 sec (annealing) and 72°C for 60 sec (extension), followed by final extension at 72°C for 7 min.

GUS assay—Twenty-five independent *Arabidopsis* plants carrying *AGL11::GUS* construct were analyzed. Seeds, seedlings, petals, androecium and gynoecium from the transgenic tobacco plants were analyzed for *in situ* GUS expression according to Jefferson *et al*[20]. Plant materials were stained with 2 m*M*,X-Gluc; 100 m*M*, Tris-HCl (*p*H 7.0); 50 m*M*, NaCl; 2 m*M*, potassium ferricyanide; 2 m*M*, potassium ferrocyanide and 0.1% (v/v) Triton ×-100 at 37°C overnight. After staining, the tissues were

incubated in ethanol (70%) to clear chlorophyll and were subsequently fixed in ethanol (70%).

Results and Discussion

Sequence analysis—FBP7 gene from *P. hybrida* and *AGL11* gene from *A. thaliana* share 83% similarity in amino acid sequence, expression pattern and function[12-14]. However, promoter sequences of these two genes did not show significant sequence similarity. Possible reason of this difference in nucleotide sequences might be that the regulatory sequences are mostly small (4-10 base pair) and during evolution specific arrangement of these conserved regulatory elements might have changed. General alignment methods like Clustal W cannot align these small patches of conserved sequences in a background of highly dissimilar sequences. MEME is a method of choice to find out conserved sequences in the background of evolutionary diverse species[19]. Results of twenty, 6-10 nucleotide long conserved sites detected in FBP7 and AGL11 promoters have been summarized in Fig. 1D. Database assisted sequence analysis of these conserved sequences with PLACE[20], CARE[21] and Matinspector[22] did not show any known transcription factor binding sites.

*Cloning of promoter—*In *AGL11* gene, first exon (400 bp) remains untranslated and the first intron is 1.2 kb in length. Thus, it has 1.6 kb region between transcription and translation start site. This 1.6 kb untranslated region in genomic DNA may have some regulatory sequences responsible for ovule specific expression of this gene. This assumption gets additional support from the studies of regulatory regions of *AGAMOUS* gene, also a MADS box transcription factor that is required to specify the identity of stamens and carpels, that arise in the third and fourth whorls, respectively[23]. The first intron of *AGAMOUS* has been found necessary for tissue specific expression by negatively inhibiting the expression in vegetative tissue[24]. On the basis of these evidences, the first intron was used along with the 1.5 kb upstream promoter region to ensure the ovule specific expression of *AGL11* promoter.

*GUS expression analysis—*Expression of GUS gene was analyzed by histochemical staining with X-gluc. Plants at vegetative stage of development and

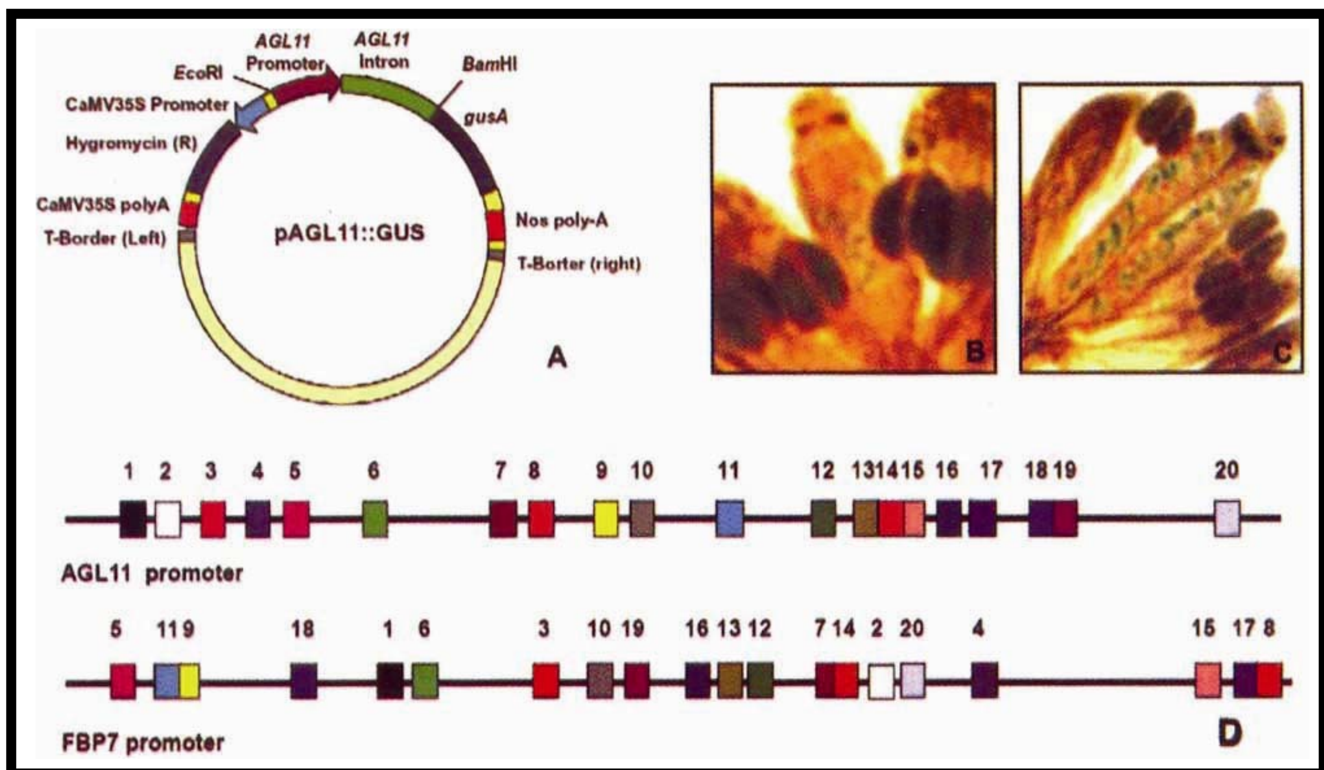

Fig. 1—(A)-vector map of pAGL11::GUS construct; (B)-GUS staining in wild type *Arabidopsis*; (C)- GUS staining in AGL11::GUS transgenic *Arabidopsis* showing promoter activity restricted to ovules and (D)- Location of conserved motif among 495 bp of AGL11 and FBP7 promoters as predicted by MEME.

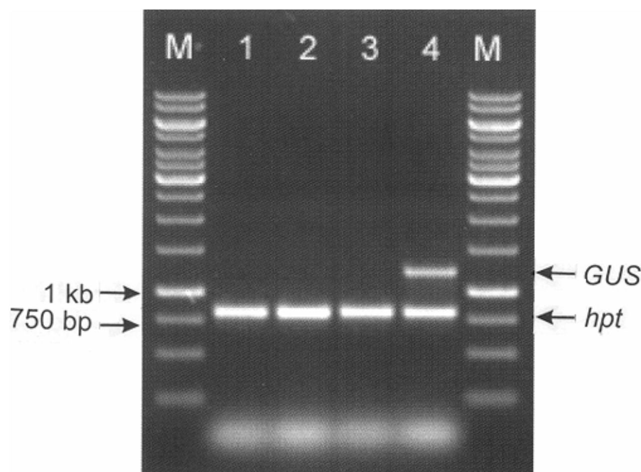

Fig. 2—RT-PCR analysis for presence of GUS transcripts in different *Arabidopsis* plant tissues [1. Roots, 2. Leaves, 3. Stem, 4. Inflorescence, M. 1kb DNA ladder (MBI)]. GUS transcript was detected only in the inflorescence while *hpt* transcript is present in all plant tissues.

flowers at different developmental stages were analyzed for GUS expression. No GUS staining was observed in any of the tissues during the vegetative stage of plant development. GUS staining appeared in the center of young ovary (ovules). The sepals, petals and androecium did not exhibit GUS expression indicating the ovule specific activity of the promoter (Fig. 1C).

Expression of GUS in different plant tissues was further analyzed by RT-PCR. RNA expression data indicated that there was no detectable GUS mRNA present in any other plant part except the inflorescence (Fig. 2). This high level of tissue specific expression might have evolved, because the *AGL11* gene belongs to MADS box family of transcription factors, involved in ovule development. Being a transcription factor, even a low level expression of this gene in other plant tissue may disturb the gene expression pattern in that tissue, disturbing the plant development. The regulatory sequences involved in ovule specific expression of *AGL11* gene were used in expression of GUS in this study, leading to high level of ovule specific expression.

The present study confirmed that 1.5 kb of *AGL11* promoter with its first intron could drive the tissue specific expression of GUS reporter gene in *Arabidopsis*. *AGL11* and *FBP7* promoter sequences confer ovule specific gene expression although they do not share sequence similarity. This promoter can be used to modify the developmental path of plants by expressing either plant hormones or lethal genes.

References

1 James C, *Global status of commercialized biotech/GM crops: ISAAA briefs* (ISAAA Ithaca, NY) 35, 2006.

2 Brisson N, Paszkowski J, Penswick J, Gronenborn B, Potrykus I & Hohn T, Expression of a bacterial gene in plants using a viral vector, *Nature*, 310 (1984) 511.

3 Potenza C, Aleman L & Sengupta-Gopalan C, Targeting transgene expression in research, agricultural and environmental applications: Promoters used in plant transformation, *In Vitro Cell Dev Biol Plant*, 40 (2004) 1.

4 Bouman, Ovule, in *Embryology of Angiosperms* edited by B. M. Johri. (Springer-Verlag, Berlin, Germany) 1984, 123.

5 Rotino G L, Elena P, Zottini M, Sommer H & Spena A, Genetic engineering of parthenocarpic plants, *Nature Biotechnol*, 15 (1997) 1398.

6 Mariani C, Leemans J, De G W, Plants modified with barstar for fertility restoration. *US Patent 5689041* (1995).

7 Dellaporta S L & Moreno M A, Methods and compositions to reduce or eliminate transmission of a transgene, *US Patent 6743968* (2001).

8 Huang B Q & Russell S D, Female germ unit: Organization, isolation, and function. *Int Rev Cytol*, 140 (1992) 233.

9 Angenent G C, Franken J, Busscher M, Van Dijken A, Van Went J L, Dons H J M & Van Tunen A J, A novel class of MADS box genes is involved in ovule development in petunia, *Plant Cell*, 7 (1995) 1569.

10 Cheng X F, Wittich P E, Kieft H, Angenent G, Xu Han X & van Lammeren A A M, Temporal and spatial expression of MADS box genes, FBP7 and FBP11, during initiation and early development of ovules in wild type and mutant Petunia hybrida, *Plant Biol*, 2 (2000) 693.

11 Colombo L, Franken J, Van der Krol A R, Wittich P E, Dons H J M & Angenent, G C, Down regulation of ovule-specific MADS box genes from petunia results in maternally controlled defects in seed development, *Plant Cell*, 9 (1997) 703.

12 Theissen G, Kim J T & Saedler H, Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes, *J Mol Evol*, 43 (1996) 484.

13 Theissen G, Becker A, Di Rosa A, Kanno A, Kim J T, Muenster T, Winter K U & Saedler H, A short history of MADS-box genes in plants, *Plant Mol Biol*, 42 (2000) 115.

14 Immink R G, Ferrario S, Busscher-Lange J, Kooiker M, Busscher M & Angenent G C, Analysis of the petunia MADS-box transcription factor family, *Mol Genet Genom*, 268 (2003) 598.

15 Rounsley S D, Ditta G S & Yanofsky M F, Diverse roles for MADS box genes in *Arabidopsis* development, *Plant Cell*, 7 (1995) 1259.

16 Pinyopich A, Ditta D S, Savidge B, Liljeren S J, Bauman E, Wisman E & Yanofsky M F, Assessing the redundancy of MADS-Box genes during carpel and ovule development, *Nature*, 424 (2003) 85.

17 Clough S J & Bent A, Floral dip. A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, *Plant J*, 16 (1998) 735.

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

19 Bailey T L & Elkan C, Unsupervised learning of multiple motifs in biopolymers using Expectation Maximization, *Mach Learn*, 21 (1995) 51.

20 Higo K, Ugawa Y, Iwamoto M & Korenaga T, Plant *cis*-acting regulatory DNA elements (PLACE) database, *Nucleic Acids Res*, 27 (1999) 297.

21 Rombauts S, Dehais P, Van Montagu M & Rouze P, Plant CARE, a plant cis-acting regulatory element database, *Nucleic Acids Res*, 27 (1999) 295.

22 Quandt K, Frech K, Karas H, Wingender E & Werner T, MatInd & MatInspector-New fast and versatile tools for detection of consensus matches in nucleotide sequence data, *Nucleic Acids Res*, 23 (1995) 4878.

23 Yanofsky M F, Floral meristems to floral organs: Genes controlling early events in *Arabidopsis* flower development, *Annu Rev Plant Physiol Plant Mol Biol*, 46 (1995) 167.

24 Sieburth L E & Meyerowitz E M, Molecular dissection of the AGAMOUS control region shows that essential *cis*-elements for spatial regulation are located intragenically, *Plant Cell*, 9 (1997) 355.