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# Cloning of Tobacco TA29 and Sesame GN13 promoters in plant expression vector for *Agrobacterium*-mediated transformation V.1

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Anirban Jyoti Debnath<sup>1</sup>, Debabrata Basu<sup>1</sup>, Samir Ranjan Sikdar<sup>1</sup>

<sup>1</sup>Division of Plant Biology, Bose Institute, Centenary Campus, P-1/12, C. I. T. Road, Scheme VII M, Kolkata – 700 054, West Bengal, India



Anirban Jyoti Debnath

Bose Institute

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**We use this protocol and it's working**

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## Abstract

The protocol describes the molecular cloning steps of two genomic upstream elements (hereafter, promoters): TA29 of tobacco (*Nicotiana tabacum* L.) and GN13 of sesame (*Sesamum indicum* L.). The TA29 is a well-known anther-specific promoter of the tobacco TA29 gene. The GN13 is the anther-specific promoter of the sesame  $\beta$ -1,3-glucanase gene. In this protocol, these promoters were PCR-amplified from the respective plants and incorporated into the plant expression vector pCAMBIA 2301 replacing its CaMV35S promoter and fusing with the GUS reporter gene. The recombinant constructs were mobilised into *Escherichia coli* strain DH5 $\alpha$  using heat shock transformation. The correctness and integrity of the inserted promoters were checked by PCR, restriction digestion and DNA sequencing. Plasmids were isolated from the positive *E. coli* clone with the highest sequence homology of the inserted promoter(s) and mobilised into *Agrobacterium tumefaciens* strain LBA4404. These transformed *A. tumefaciens* harbouring the TA29 and GN13 promoters can be used in subsequent transgenesis-mediated anther-specific GUS expression studies.

## Materials

Agar = HiMedia (Mumbai, India)  
Beef extract = HiMedia (Mumbai, India)  
Big Dye Terminator Automated Sequencer = Applied Biosystems (USA)  
DNeasy plant mini kit = QIAGEN (Germany)  
EDTA = Sigma-Aldrich (USA)  
Ethanol = Merck (Germany)  
Fluorescent tube lights = Philips, Model Champion (India)  
HCl = SRL (India)  
*HindIII* = Fermentas (USA)  
Kanamycin = Sigma-Aldrich (USA)  
KOH = HiMedia (Mumbai, India)  
LB medium = HiMedia (India)  
MgSO<sub>4</sub>·7H<sub>2</sub>O = HiMedia (Mumbai, India)  
MOPS buffer = Sigma-Aldrich (USA)  
MS medium = HiMedia (Mumbai, India)  
NAA = Sigma-Aldrich (USA)  
*NcoI* = Fermentas (USA)  
Peptone = HiMedia (Mumbai, India)  
QIAquick gel extraction kit = QIAGEN (Germany)  
QIAquick PCR purification kit = QIAGEN (USA)  
QIAGEN plasmid miniprep kit = QIAGEN (Germany)  
Recombinant *pfu* DNA polymerase kit = Fermentas (USA)  
Recombinant Taq DNA polymerase kit = Fermentas (USA)  
Rifampicin = Sigma-Aldrich (USA)  
RIN ALA = Hindustan Unilever Limited (India)  
Rubidium chloride = Sigma-Aldrich (USA)  
Sucrose = HiMedia (Mumbai, India)  
T4 DNA ligase = Promega (USA)  
Thermal cycler, Model 2720 = Applied Biosystem (USA)  
Tris-HCl = Sigma-Aldrich (USA)  
Yeast extract = HiMedia (Mumbai, India)

## PREPARATION OF CULTURE MEDIA AND STOCK SOLUTION OF NAA

- 1 All the experiments of tissue culture were carried out using the full-strength basal MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose (w/v) and 0.8% agar (w/v). Seeds were germinated in ½ MS medium (half-strength MS salts, 1.5% w/v sucrose, and 0.6% w/v agar). MS medium, sucrose and agar were purchased from HiMedia (Mumbai, India). Sterilisation of media was carried out by autoclaving at 121°C temperature with 15 psi pressure for 15 minutes. Before autoclaving, the media pH was adjusted to 5.8 using 1N HCl and 1N KOH solutions. A 1 mg/ml NAA (Sigma-Aldrich, USA) stock solution was prepared in 1N NaOH solvent, filter sterilised, and stored at 4°C in an amber bottle. NAA was added to the media before autoclaving.

## PLANT MATERIAL, SEED CULTURE, AND CLONAL PROPAGATION

- 2 The seeds of sesame (*Sesamum indicum* L.) cultivar JK-1 and tobacco (*Nicotiana tabacum* L.) cultivar SR1 were collected from the National Bureau of Plant Genetic Resources, Pusa, New Delhi, India. Healthy and uniform seeds were washed with 50% commercial bleach (RIN ALA, Hindustan Unilever Limited, India) containing 4% chlorine (v/v) for 5 min. Surface sterilisation of the cleaned seeds was executed with 0.1% (w/v) aqueous mercuric chloride solution for 2.5 min followed by 70% ethanol for 10 s. Subsequently, surface-sterilised seeds were washed four times with sterile distilled water, transferred to Petri dishes having 25 ml sterilised ½ MS medium, and incubated at 28±2°C in the dark for germination. The germinated seedlings were used to establish the clonally propagating population.

Clonal propagation of sesame was achieved by apical shoot tip culture. The sesame clonal population was established and maintained by root induction of apical shoot tips from germinated seedlings in MS medium supplemented with 2.69 µM of NAA, and subsequent transfer of rooted explants in fresh basal MS medium. Clonal propagation of tobacco was established and maintained by apical and nodal sub-culture in fresh basal MS medium once a month.

All the cultures were incubated in the culture room at 25±2°C under the 16/8 photoperiod. The sesame and tobacco cultures had irradiances of 35 µmole/m<sup>2</sup>/s photosynthetic photon flux density and 70 µmole/m<sup>2</sup>/s photosynthetic photon flux density, respectively, using daylight fluorescent tube lights (Philips Champion, India). The clonal populations were refreshed in the same medium every four weeks.

## PCR CYCLING PARAMETERS

- 3 In every PCR experiment hereafter, the reaction was carried out in 25 µl of the reaction mixture. It contained 2.5 µl of 10x Taq buffer, 3 mM of MgCl<sub>2</sub>, 0.2 mM of each of the dNTPs, 1.25 units of Taq DNA polymerase (Fermentas recombinant Taq DNA polymerase kit, USA) and 0.2 mM of each of the primer. The PCR amplification was carried out in a thermal cycler (2720 Thermal Cycler, Applied Biosystem, USA). The thermal cycling parameter was as follows: initial DNA denaturation at 95°C for 2 minutes, then 35 cycles of denaturation at 95°C for 30 seconds, annealing for 45 seconds (annealing temperature is primer-dependent, and is mentioned in the particular experiment), and extension at 72°C for 1 minute. A final extension was carried out at 72°C for 7 minutes, after that the sample was kept on hold for infinity time at 4°C. Every parameter remained the same in all the cases unless otherwise mentioned.

## CLONING OF UPSTREAM REGIONS OF TOBACCO *TA29* AND SESAME *GN13* IN pCAMBIA 2301 PLANT EXPRESSION VECTOR

- 4 The tobacco *TA29* gene with its upstream region has already been sequenced (Seurinck et al. 1990) and the sequence is available from NCBI (accession no. X52283). Furthermore, the upstream element of the  $\beta$ -1,3-glucanase homologue of sesame was also available from NCBI (accession no. KT246471). Hereafter, the upstream elements of *TA29* and  $\beta$ -1,3-glucanase sesame homologue genes are called *TA29* and *GN13*, respectively. The upstream elements sized 871-bp of *TA29* gene and 793-bp of *GN13* gene were cloned into the plant expression vector pCAMBIA 2301 replacing its CaMV35S promoter, fusing with GUS reporter gene and mobilised into *Agrobacterium tumefaciens* LBA4404.

### 4.1 Amplification of the upstream fragments from genomic DNA

According to the kit-specified protocol, the total genomic DNAs of clonally propagated 4-week-old tobacco and sesame leaf tissues were isolated by the DNeasy plant mini kit (QIAGEN, Germany). The upstream elements of both genes were isolated by using PCR sequence-specific primers. The *TA29* primer sequences were 5'-GATCAAGCTTCTTTTGGTTAGCGAATGC-3' as the forward (*TA29-F*) and 5'-AGTCCCATGGTTTAGCTAATTTC-3' as the reverse primer (*TA29-R*) (annealing temperature 46°C). The *GN13* primer sequences were 5'-GATCAAGCTTATCCGGCCCGGG-3' for the forward (*GN13-F*) and 5'-ATGCCCATGGGATCATGAGCTCG-3' for the reverse primer (*GN13-R*) (annealing temperature 52°C). Both primer sets contained adapter sequences for the recognition site of *Hind*III and *Nco*I to facilitate downstream directional cloning. The PCR was done in 100 µl of the reaction mixture using five units of *pfu* DNA polymerase

(Fermentas recombinant *pfu* DNA polymerase kit, USA) with two minutes of extension; other parameters remained the same as mentioned earlier. The PCR-isolated fragments were purified using the QIAquick PCR purification kit (QIAGEN, USA) using the kit-specified protocol.

## 4.2 Construction of recombinant plant expression vectors

The PCR-isolated upstream element fragments and the pCAMBIA 2301 vector were digested with HindIII and NcoI (Fermentas, USA) in separate sets to cut open the ligation sites and to linearise the vector, respectively. The restriction digestion was carried out in 100 µl reaction volume using the protocol specified by Fermentas. By this process, the CaMV35S promoter fragment of the vector was also isolated and discarded. Both restriction-digested PCR-isolated upstream element and the linearised vector were gel purified using the QIAquick gel extraction kit (QIAGEN, Germany) using the kit-specified protocol.

The restriction digested and purified upstream element fragment of both genes was ligated by T4 DNA ligase (Promega, USA) in the same restriction site of the linearised vector by fusion with the GUS reporter gene. Promega-specified protocol was followed for ligation. The recombinant plasmid vector constructs containing TA29 and GN13 were named TA29::GUS and GN13::GUS, respectively.

## 4.3 Transformation of the recombinant vectors in *Escherichia coli*

### 4.3.1. Preparation of competent cells

An overnight *Escherichia coli* strain DH5α culture was prepared by inoculating a single colony in 10 ml LB broth (HiMedia, India). From that overnight grown culture, one ml was used to inoculate 50 ml of fresh LB broth medium and was cultured at 37°C with 180 rpm shaking for 2-3 hours or till the OD<sub>600nm</sub> reached up to 0.3-0.4. Cells were then harvested from that culture by centrifugation at 5000 rpm for five minutes at 4°C and subsequently re-suspended in 25 ml of solution-I (MOPS buffer and rubidium chloride, both 10 mM, pH 7.0, Sigma-Aldrich, USA) by gentle tapping. It was then centrifuged at 5000 rpm for five minutes at 4°C, discarded the supernatant, the pellet was re-suspended in 25 ml of solution-II (MOPS buffer, rubidium chloride, both 10 mM, pH 6.5, 75 mM CaCl<sub>2</sub>·2H<sub>2</sub>O) and kept on ice for 45 minutes. Then the cells were centrifuged at 5000 rpm for another five minutes at 4°C, and the supernatant was discarded. Finally, the pellet was re-suspended in 2.5 ml of solution-III (8.5 ml solution-II + 1.5 ml glycerol), distributed in 200 µl aliquots in pre-chilled tubes, frozen in liquid nitrogen and stored at -80°C.

#### 4.3.2. Transformation of *E. coli* by recombinant vectors

The recombinant vectors were then mobilised into *E. coli* by using the heat shock method of transformation. About 50-100 ng of the recombinant plasmid DNAs were mixed with the slowly thawed (on ice) competent cells of *E. coli* and incubated in ice for 30 minutes. After the incubation period, a heat shock at 42°C was applied to the bacterial culture for 90 seconds, followed by the quick transfer on the ice, and subsequent ice incubation for five minutes. The putatively transformed bacterial cells were recovered by culturing in 1 ml of LB broth without selection pressure for 1 hour. The recovered cells were plated in LB medium supplemented with 1% agar (w/v) and 50 mg/ml kanamycin (Sigma-Aldrich, USA) to select the putatively transformed bacteria. Overnight-grown kanamycin-resistant *E. coli* colonies were assayed for the presence of the respective cloned upstream fragments.

#### 4.3.3. Assay of the kanamycin-resistant bacteria for the presence of the cloned fragment

Kanamycin-resistant colonies were assayed by PCR, restriction digestion and DNA sequencing. The colony PCR was carried out among the putative clones to detect the presence of 871-bp and 793-bp fragments of TA29 and GN13, respectively, using the respective primers. PCR-positive putative clones were inoculated in fresh LB medium supplemented with 1% agar (w/v). Plasmids were isolated from the overnight grown clones using the QIAGEN plasmid miniprep kit (QIAGEN, Germany) according to the kit-specified protocol.

The isolated plasmids from the putative clones were subjected to restriction digestion with *Hind*III and *Nco*I to check the presence of the right size and integrity of the cloned fragment, i.e. 871-bp for TA29 and 793-bp for GN13. The restriction digestion was carried out in 30 µl of reaction volume according to the manufacturer-specified protocol.

Plasmids were isolated from the PCR and restriction-positive putative clones, and further assayed by DNA sequencing for the presence of the cloned DNA fragments. For sequencing, 5'-CAGCTATGACCATGATTACGAATTC-3' and 5'-TCAGTTTAAAGAAAGATCAAAGCTC-3' were used as TA29 and GN13 primers (annealing temperature 50°C), respectively. Using the manufacturer-supplied protocol, the plasmids were sequenced in an automated sequencer (Big Dye Terminator Automated Sequencer, Applied Biosystems, USA). It confirmed the cloning process. It confirmed the cloning process.

Among the confirmed *E. coli* clones, the clone(s) with the highest sequence homology with TA29/GN13 were selected for the downstream works. Plasmids were isolated

from the chosen *E. coli* clones using the QIAGEN plasmid miniprep kit according to the kit-specified protocol and used for the downstream transformation of *A. tumefaciens*.

#### 4.4 Transformation of the recombinant vector in *Agrobacterium tumefaciens*

##### 4.4.1. Preparation of competent cells

A single colony of disarmed *A. tumefaciens* strain LBA4404 was used to inoculate 10 ml of YEB medium (1 g/l yeast extract, 5 g/l peptone, 5 g/l beef extract, 5 g/l sucrose, and 0.49 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O) supplemented with 50 mg/ml rifampicin (Sigma-Aldrich, USA). It was subsequently cultured at 28°C for 48 hours with 160 rpm shaking. From that saturated culture of *A. tumefaciens*, 2 ml was used to inoculate 50 ml of fresh YEB medium and was further incubated at 28°C with 160 rpm of shaking for 6-8 hours or until the OD<sub>600nm</sub> reached 0.8-1.0. Cells were then harvested by centrifugation at 8000 rpm for 10 minutes at 4°C, and the pellet was subsequently re-suspended in 5 ml of 10:1 TE buffer (10 mM Tris-HCl, pH 7.5 + 1 mM EDTA) by gentle tapping. Further centrifugation was carried out at 8000 rpm for 10 minutes at 4°C, and the supernatant was discarded. The pellet was subsequently re-suspended in fresh 1 ml of YEB medium supplemented with 50 mg/ml rifampicin, distributed in the aliquots of 300 µl in pre-chilled tubes, frozen in liquid nitrogen and stored at -80°C.

##### 4.4.2. Transformation of *A. tumefaciens* by recombinant vectors

The isolated recombinant plasmids from the confirmed *E. coli* clones were used to transfect the *A. tumefaciens* strain (Holsters et al. 1978). In summary, 0.5-1.0 µg of the recombinant plasmids were mixed with the slowly thawed (on ice) competent *A. tumefaciens* culture and were incubated in ice for five minutes. It was then snap-chilled in liquid nitrogen for five minutes, quickly transferred to a water bath of 37°C, and kept there for five minutes. The transformed cells were recovered in an overnight culture in YEB medium supplemented with 50 mg/ml rifampicin. The recovered cells were plated in YEB medium solidified with 1% agar (w/v) in the presence of 50 mg/ml rifampicin and 50 mg/ml kanamycin to select positive clones. The presence of the upstream cloned fragment was checked by colony PCR for the respective insert(s) using TA29 or GN13 primers. One randomly selected *A. tumefaciens* clone harbouring TA29::GUS or GN13::GUS was the final product(s) of this protocol.



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