35S 启动子甲基化引起棉花转基因沉默

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摘要：用带 PBI121 质粒的农杆菌 LBA4404 菌株转化泗棉 3 号胚性愈伤组织，获得的再生植株进一步对 *gus* 和 *npt* Ⅱ基因进行 PCR 跟踪检测，共得到 97 株阳性转基因植株。GUS 组织化学检测发现，97 株转基因幼苗中有 10 株 GUS 检测阴性，嫁接后，只成活一株。利用来源于同一愈伤系的 GUS 检测阳性植株作为对照，对这一 GUS 检测阴性的植株进行 *gus* 基因沉默机理研究。Southern 分析表明，该 GUS 检测阴性植株与 GUS 检测阳性植株有相同拷贝数。GUS 组织化学检测和 RT-PCR 分析显示，*gus* 基因在 GUS 检测阴性植株中没有表达，而 *npt* Ⅱ基因在这两株转基因棉花中都表达。用限制性内切酶-PCR 法分析 35S 启动子区甲基化发现：GUS 检测阴性植株 35S 启动子区 TATA box 的 HapII/MspI 酶切位点发生甲基化，而 GUS 检测阳性植株该位点没有甲基化。以上研究表明，这株 *gus* 沉默的转基因棉花可能是由于其 35S 启动子区甲基化引起的。

关键词：甲基化；转基因沉默；转基因棉花；35S 启动子
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Transgene Silencing Caused by 35S Promoter Methylation in Upland Cotton (*Gossypium hirsutum*)

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Abstract: In this report, we found that transgenes were inactived during the transformation of cotton mediated by *Agrobacterium*, and we studied the mechanism of inactivation. Cotton embryogenic calli (EC) were transformed by *Agrobacterium* strain LBA4404 possessing the pBI121 binary vector. Ninety-seven transgenic plantlets were identified by PCR amplification for *gus* reporter gene and *npt-II* selection marker gene. Among the 97 transgenic plantlets, 10 (about 10%) *gus* inactive individuals were detected. After grafting the transgenic plantlets in the greenhouse, only one GUS-negative plant survived. In an effort to study the silencing mechanism of the GUS-negative transgenic plant, a GUS-positive plant generated from a single calli line was chosen for comparisons. Southern analysis revealed that the two transgenic plants possessed the same insertion copy numbers, and they had the same transformation event. GUS assay and RT-PCR analysis indicated that *gus* was silenced in the GUS-negative plant but it was expressed in the GUS-positive plant; while RT-RCR detection showed that the *npt-II* gene expressed in both transgenic plants. Restriction endonuclease-PCR analysis of methylation in the 35S promoter was conducted by using of the methylation-sensitive enzymes *HapII*/*MspI*. The results demonstrated that the *HapII* site in the TATA box of the 35S promoter region was methylated in the *gus* inactive transgenic plant and not in the *gus* active plant, which indicated

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that methylation of the 35S promoter caused *gus* silencing in transgenic cotton plants. This study represents the first report of transgenic silencing mechanisms in cotton transformation mediated by *Agrobacterium*.

Key words: methylation; transgene silencing; transgenic cotton; 35S promoter
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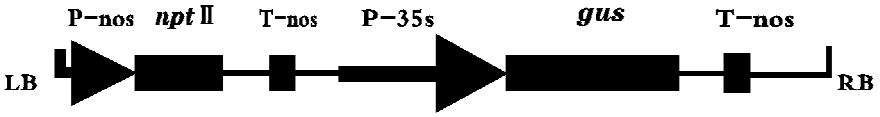
Plant biotechnology is a vital tool used to improve agronomic traits and creates novel variation in crops. Transgenic silencing has been widely reported in crops such as rice[1,2], maize[3], wheat[4], soybean[5], and tobacco[6]. Due to preliminary efforts supporting its use in plant genetic engineering, gene-silencing mechanisms are being actively investigated. Transgenic silencing results from either transcriptional transgene silencing (TGS), which requires homology in the promoter region, and post-transcriptional gene silencing (PTGS), which requires homology in transcribed regions[5,7]. Both TGS and PTGS are associated with DNA methylation. TGS is often associated with methylation in the promoter regions of the transgene[8-10], and PTGS is associated with DNA methylation in transcribed regions[11,12]. TGS and PTGS have been identified as a cellular defense mechanisms against invasive genes.

Cultivation of Bt transgenic cotton plants has reduced both the cost of pesticide applications and exposure to pesticides[13]. Cottonseed oleic acid content has been increased by transforming *fad* two into transgenic cotton plants[14]. Recent research reported that transgenic cotton over-producing sucrose phosphate synthase (SPS) could improve fiber quality under controlled environmental conditions[15]. Although genetic engineering in cotton has been widely used in pest management, oil content of seeds and improvement of fiber quality applications; gene silencing in transgenic cotton has not previously been reported. In this study, transgenic *gus* silencing cotton plants were obtained by transformed embryogenic calli mediated via *Agrobacterium*. We found that *gus* silencing in a transgenic cotton plant was caused likely by the methylation of the 35S promoter region.

1 Materials and Methods

1.1 Materials

1.1.1 *Bacterium strain and plasmid*. *Agrobacterium* strain LBA1404 harboring the binary vector pBI121 plasmid was used for transformation in this research (Fig. 1).



LB: left border repeat; P-nos: nopaline synthase gene promoter; npt-II: neomycin phosphortransferase II gene; P-35s: CaMV 35S promoter; gus: β-Glucuronidase gene; RB: right border repeat.

Fig. 1 Structure of T-DNA region of the plasmid vector pBI12

1.1.2 *Plant materials*. Cottonseeds of the Simian 3 cultivar (a major cotton cultivar from the 1990s and originating in the Yangtze River cotton growing region of China with no Coker in its pedigree) were delinted with concentrated sulphuric acid and washed 3-4 times with water. Seeds were surface sterilized with 70% ethanol, subsequently sterilized for 3～4 h with 30% (W/V) hydrogen peroxide (H_2O_2), followed by a wash of 2-3 times with sterilized distilled water. The sterile seeds were submerged in sterilized distilled water for 18～24 h at 28 °C; decoated and inoculated in half-strength MS medium[16] for germination in the dark at 28 °C for 3 days; and transferred to a light culture (under a 14:10 h (day:night) photoperiod) at 28 °C for 3 days. Hypocotyls from aseptic seedlings were cut into 5～7 mm segments and inoculated in

MSB_1 medium [MSB (MS basal salts, B5 vitamins[17]) supplemented with 3% (W/V) glucose, 0.1 mg·L^{-1} 2,4-D plus 0.1 mg·L^{-1} KT, and solidified with 0.25% (W/V) Phytagel (Sigma, St. Louis, USA)] for callus induction. Calli were subcultured in MSB_2 medium [MSB medium supplemented with 1.9 g·L^{-1} KNO3, 3% (W/V) glucose, and 0.25% (W/V) Phytagel] for proliferation and somatic embryogenesis. After 2～3 subculture applications, light yellow, loose and fine grainy embryogenic calli (EC) were removed and maintained in the same medium by subculturing every three weeks.

1.2 Methods

1.2.1 *Transformation and plant regeneration.* *Agrobacterium* strain LBA4404 possessing the pBI121 plasmid vector was grown in LB liquid medium (Tryptone 5 g·L^{-1}, NaCl 10 g·L^{-1}, Yeast Extract 5 g·L^{-1}) supplemented with 50 mg·L^{-1} Kanamycin and 10 mg L^{-1} Rifampicin for 24 h at 28℃. The bacteria were resuspended in liquid MSB_1 medium and the standard OD600 was adjusted to 0.3～0.5 (OD600 = 1 is equivalent to 1×10^{11} cell per liter). EC were inoculated with the *Agrobacterium* suspension, held at room temperature for 20 min, and subsequently blotted dry on sterile filter papers. The calli were dispersed and co-cultured on MSB_1 medium placed on filter paper in the dark for 48 h. Then EC were selected three times on the selection medium: MSB_2 medium supplemented with 100 mg·L^{-1} Km and 500 mg·L^{-1} cefotaxime to identify kanamycin(Km)-resistant lines. Km-resistant lines were transferred to the differentiation medium [MS basal salts plus B5 vitamins, NH_4NO_3 eliminated, and supplemented with 0.5 g·L^{-1} asparagine, 1.0 g·L^{-1} glutamine, 1.9 g·L^{-1} KNO_3, 3% (W/V) glucose, 0.25% (W/V) Phytagel] placed on sterilized filter papers for embryo maturation and plant regeneration. After tested by PCR and GUS histochemical analysis, the transgenic plantlets with 3 to 4 leaves were grafted to the Simian 3 untransformed plants.

All media described were adjusted to pH 6.5 prior to autoclaving, and antibiotics were filter-sterilized.

1.2.2 *DNA extraction and PCR analysis.* Genomic DNA was extracted from regenerated cotton plantlets according to the CTAB extraction protocol[18]. PCR was subsequently conducted to detect both *npt*-II and *gus*. A 0.75-kb fragment of *npt*-II was amplified using the forward primer F: 5-GAGGCTATTCGGCTATGACTG-3 and reverse primer R: 5-TAGAAGGCGATGCGCTGCGA-3. The PCR conditions were: 95℃ for 5 min, followed by 30 cycles of 94 ℃ for 30 s, 55℃ for 1 min, and 72℃ for 1 min, and a final extension at 72℃ for 10 min. A 1.2-kb fragment of the *gus* gene was amplified using the forward primer F: 5-GGTGGGAAAGCGCGTTACAAG-3 and reverse primer R: 5-GTTTACGCGTTGCTTCCGCCA-3. The PCR conditions were: 95 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 30 s, 61 ℃ for 1 min, and 72℃ for 1 min, and a final extension at 72 ℃ for 10 min.

1.2.3 *Histochemical GUS assay.* Following PCR, both *npt*-II and *gus* positive plants were analyzed by GUS histochemical assay. GUS analysis was conducted by incubating the leaves overnight in 20 μL GUS reaction buffer containing 50 mmol·L^{-1} sodium phosphate (pH 7.0), 5 mmol·L^{-1} $K_4Fe(CN)_6$, 5 mmol·L^{-1} $K_3Fe(CN)_6$, 10 mmol·L^{-1} EDTA, 0.1% TritonX-10, and 1 mmol·L^{-1} X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) at 37℃[19]. The assay buffer was decanted and the leaves were bleached with 70% alcohol and photographed.

1.2.4 *Southern blot analysis.* For Southern analysis, 40 μg of genomic DNA was extracted from the transgenic plants and digested with EcoRI, which cuts the T-DNA at a unique site. Digested genomic DNA was fractionated on a 0.8% agarose gel and blotted onto a nylon membrane. Hybridization was performed with the *npt*-II coding region probe. Standard procedures for Southern blot analysis and probe labeling were conducted using DIG DNA Labeling and Detection Kit1 (Roche, Germany).

1.2.5 *RT-PCR analysis.* Total RNA was extracted from the leaves of transgenic plants according to the CTAB extraction protocol[20].

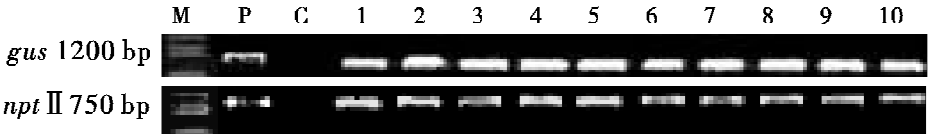
RNA was digested with 0.5 μg RNase-free DNaseI for 30 min at 37℃. cDNA synthesis was conducted with oligo-dT primers. PCR amplification for *npt-II* cDNA or *gus* cDNA was performed using both the primers and the PCR procedures described above. EF1α cDNA, as a constitutive expression control, was amplified with primers F: 5-AGACCACCAAGTACTACTGCAC-3 and R: 5-CCACCAATCTTGTACACATCC-3.

1.2.6 *Restriction Endonucleases -PCR analysis of methylation of 35S promoter region.* Approximately 1μg of genomic DNA was digested overnight at 37℃ with two different methylation sensitive endonucleases, *Hap*II and *Msp*I. Digested genomic DNA was amplified for the 35S promoter with primers F: 5-ATGGTTAGA GAGGCTTACGC-3, R: 5-CACATCAATCCACTTGCTTT-3. The PCR conditions were: 95 ℃ for 2 min, followed by 30 cycles of 95℃ for 45 s, 55℃ for 30 s, and 72℃ for 1 min, and a final extension at 72℃ for 10 min. The expected amplification production was 697 bp containing one *Hap*II/*Msp*I recognition site.

2 Results

2.1 Development of transgenic gus silencing plantlets

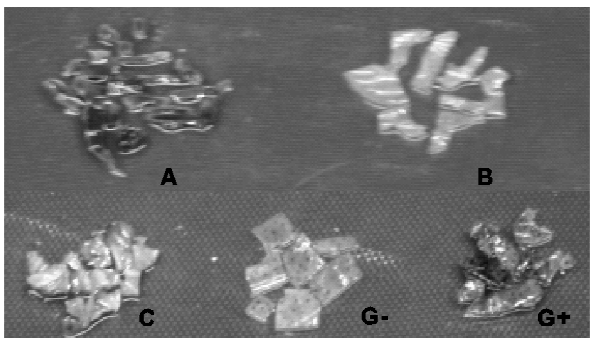
EC transformation by *Agrobacterium* strain LBA4404 resulted in 97 transgenic plantlets with both *gus* and *npt*-II. Figure 2 shows positive transgenic plantlets exhibiting both *gus* and npt-II.



M: molecular weight markers; P: positive control pBI121; C: Simian 3 untransformed control plant; Lane 1-10: 10 positive transgenic plantlets exhibiting both *gus* and *npt*-II.

Fig. 2　PCR analysis of regeneration plantlets to test for *npt*-II and gus

Results of histochemical GUS analysis from the leaves of the 97 transgenic plantlets revealed both GUS-positive and GUS-negative samples (Fig. 3). Leaves stained blue in the GUS analysis showed that the *gus* gene was successfully integrated into the genomic DNA of the plantlets (Fig. 3 A). When the leaves did not stain, this indicated that *gus* was not active and silenced (Fig. 3 B). In total, 10 transgenic plantlets showed gus silencing, and the remaining 87 plantlets were GUS-positive. These transgenic plantlets were grafted to the Simian 3 recipient plants, however, only one GUS-negative expressional plant survived. One GUS-negative plant (G-) and a GUS-positive plant (G+), both generated from a single calli line in order to eliminate the influence of genetic variation, were chosen to gain further insight into the gene silencing mechanism. Leaves of G- and G+ were further assayed by GUS staining following a period of maturation in the greenhouse. The results revealed that the *gus* gene lacked expression (Fig. 3 G-) in G- plants and demonstrated stable expression in the G+ plant (Fig. 3 G+). The study suggested G- was a *gus* silenced transgenic cotton plant.



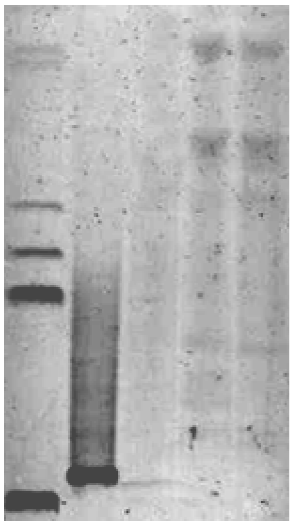
A, B: leaves of transgenic cotton plantlets tested by PCR; C: non-transformed control plant; G- and G+: leaves of negative and positive plants assayed by GUS analysis.

Fig. 3　GUS Histochemical assays of transgenic plants

2.2 Southern blot analysis

Transgenic copy number of G- and G+ ana-

lyzed by Southern hybridization showed that two copies of *npt*-II reside in the G- and G+ plants (Fig. 4), which confirmed the two lines possessed the same transformation event.



Lane M: Marker 3 the molecular size standard; lane P: plasmid pBI121; lane C: non-transformed plant control; lane G- and G+: GUS-negative transgenic plant and GUS-positive transgenic plant.

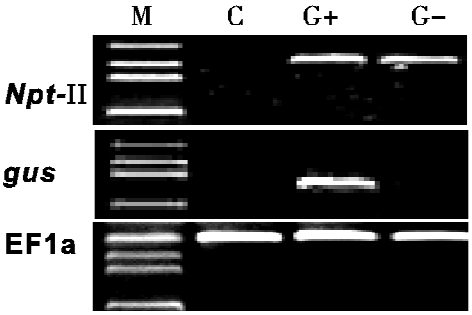
Fig. 4 Southern blot analysis of transgenic plants regenerated from a single Km-resistant callus line. Genomic DNA from the non-transformed control plant, G- and G+ transgenic plants and plasmid DNA were digested with EcoRI and hybridized with the *npt*-II probe

2.3 RT-PCR analysis

Results of transcriptional level expression of *npt*-II and *gus* in the G- and G+ transgenic plants showed stable expression of *npt*-II in both G- and G+ plants, while the *gus* gene only expressed in G+. Consistent with the GUS assays of G- and G+ plants, no transcript was detected in the G- transgenic samples. These observations suggested that GUS assay results of this transgenic *gus* silenced plant were due to the absence of *gus* transcripts. Also, *gus* silencing did not affect the activity of *npt*-II. This result is noteworthy because the selection marker gene *npt*-II and the reporter gene *gus* were in the transgenic locus spanning a 5.5 kb region.

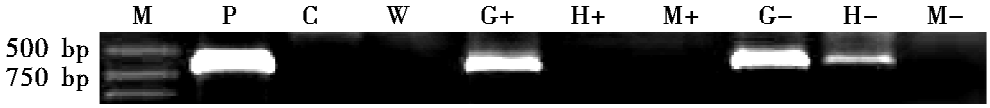
2.4 Methylation analysis of 35S promoter region

A methylation-sensitive enzyme *Hap*II/*Msp*I recognition site (C/CGG) in 515 bp of the 35S promoter region was revealed. In addition, the site was located in a TATA box of the promoter. An expected amplification product of 697 bp was produced (Fig. 6 G+ and G-) which indicated the 35S promoter region was integrated and stable in both G+ and G- plants. The results also showed that genomic DNA of G+ digested with *Hap*II and *Msp*I (Fig. 6 H+ and M+) lacked a PCR amplification product. These results suggested that the *Hap*II/*Msp*I site in the 35S promoter of the G+ transgenic plant was not methylated. However, the target fragment was detected from the genomic DNA of G- digested with *Hap*II (Fig. 6 H-). The absence of an amplified fragment from the genomic DNA of G- digested with *Msp*I (Fig. 6 M-) was observed. This indicated that the second C site of CCGG recognized by *Hap*II/*Msp*I enzyme in the TATA box of the 35S promoter was methylated in the G- plant. These results suggested that *gus* inactivation in this transgenic cotton plant was caused by cytosine methylation in the TATA box of the 35S promoter.



M: molecular weight marker; C: non-transformed plant; G+ and G-: GUS-positive transgenic plant and GUS-negative transgenic plant, respectively.

Fig. 5 RT-PCR analyses of transgenic plants



M: molecular weight marker; P: plasmid DNA; C: non-transformed plant; W: water; G+: GUS-positive transgenic plant; H+ and M+: genomic DNA of G+ digested with *Hap*II and *Msp*I, respectively; G-: GUS-negative transgenic plant; H- and M-: genomic DNA of G- digested with *Hap*II and *Msp*I, respectively.

Fig. 6 Methylation analysis of 35S promoter

3 Discussion

Bellucci et al[3] reported that methylation of the 35 promoter resulted in transcriptional gene silencing in maize. Chromatin remodeling of the promoter region appeared to be an integral part of the transcription activation process[21]. The methylation of the TATA box in the 35S promoter could result in no gus transcripts in this transgene silencing cotton plant. It is likely that methylation interferes with transcription factor binding or by changing the arrangement of chromatin. These two transgenic plants held two copies and transgene copy number is negative correlated with the transgene activity[22-23]. So, we speculated that homology in 35S promoter region probably induced the methylation of 35S promoter region by making particular rearrangement or DNA-DNA pairing.

The cotton transformation protocol, which uses embryogenic calli as explants via *Agrobacterium*, was a more efficient method because it shortened the transformation period, reduced labor requirements, and resulted in low copy number[24-25]. Transgene inactivity in transgenic cotton plants occurred with a frequency of about 10%, and transgenic plants regenerated from the same calli line had different active expressions. This observation indicated that transgenic silencing operated independently within the same transformation event. It is also notable that *npt*-II was widely used as the selection marker in the plant transformation, so any transformant with inactive *npt*-II was eliminated through the selection culture. But, the activity of *npt*-II was not affected by *gus* silencing although they were adjacent to each other in the transgenic locus and separated by an approximately 700 bp vector DNA sequence. Therefore, target gene silencing can exist in the Km-resistant transgenic plants, which reduces the reliability of transgenic approaches for genetic improvement of crops. The mechanism of gene silencing is complex, and there is no efficient method to eliminate or predict its impact on plant genetic engineering. The regeneration of more transgenic plants is a strategy with the potential to improve the reliability of transgenic approaches for genetic enhancement. Meanwhile, it is advised to choose transgenic plants with a single insertion copy to avoid transgene inactivation resulting from co-suppression by multi-copy number.

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