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RESEARCH ARTICLE

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# Establishment of transgenic cotton lines with high efficiency via pollen-tube pathway



**Abstract** The transformation method via pollen-tube pathway has great potential roles in crop molecular breeding. In this paper, the effects of genotype, exogenous DNA concentration injected, and the flower positions in plant on cotton genetic transformation mediated by pollen-tube pathway were evaluated. It was found that there were no obvious differences on the boll setting rate and transformation efficiency between the cultivars 33B and 99B. However, the DNA concentrations and the flower positions largely affected the transformation efficiency. The putative transgenic plants derived from boll seeds injected with binary expression vector pCAMBIA3301 DNA were all to be PCR positive. On the other hand, the expression levels of reporter gene *Gus* varied dramatically, including high, middle, and nontranscripts detected. Both the GUS activity of the transgenic plants and the intensity of histochemical GUS staining in the representative transgenic plants were in accordance to those of the transcripts of reporter gene *Gus*. The selection of high quality and suitable concentration of exogenous DNA and the injection of the DNA in basal flowers at the middle fruit branches are important factors for improving the cotton transformation efficiency. The transformation method via pollen-tube pathway established in this study has provided an efficient genetic transformation technique in cotton breeding.

**Keywords** cotton (*Gossypium hirsutum* L.), pollen-tube pathway, genetic transformation, molecular identification

## 1 Introduction

In angiosperms species, such as cotton, maize, and wheat, the double fertilization process is indispensable for the new seed formation, in which one sperm nucleus unites with the egg to form the diploid zygote, from which the embryo develops. The other sperm unites with the two nuclei located in a single cell at the center of the embryo sac to develop the endosperm (Faure and Dumas, 2001). During the process, the pollen grain first adheres to the stigma of the carpel (female reproductive structure) and grows a pollen tube that penetrates the ovum through a tiny pore called a micropyle. The two sperm cells are then released into the ovary through this tube. One of the two sperm cells fertilizes the egg cell, forming a diploid zygote or embryo, also called the ovule. The other sperm cell fuses with two haploid polar nuclei in the center of the embryo sac, and the resulting triploid (3n) cell divides through mitosis and forms the endosperm, a nutrient-rich tissue inside the seed (Lord and Russell, 2002; Weterings and Russell, 2004).

Microscope observation revealed that in the process of pollen tube extending, some cells of nucelli began to degenerate and became a pollen-tube pathway through that the pollen tube could enter embryo sac by nucelli. The pathway was larger than the pollen tube, and then, between pollen tube formation and closing, heterologous DNA could enter the embryo sac and integrate into the zygote cell and the forepart embryo cells (Russell, 1993).

The pollen-tube transformation approach was put forward based on the hypothesis of Zhou et al. (1979), in which it is assumed that the heterologous DNA segment injected at certain time could be transferred into the ovary by following the nucellus pathway. In the past several years, there were some successful reports on transformation via pollen-tube pathway approach in various plant species, such as cotton (Li et al., 1999; Li et al., 2000; Li et al., 2005; Liu et al., 2007), wheat (Chong and Tan, 1995; Geng et al., 2006), rice (Luo and Wu, 1988; Xie et al., 1991), and soybean (Lei et al., 1992; Wang et al., 2004), suggesting that this transgenic approach could be a promising genetic transformation technique based on its simplicity, easy manipulation, and undependable genetic background.

To date, though some cotton transformation events

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based on the pollen-tube pathway approach have been reported (Li et al., 2000; Liu et al., 2007), and several transgenic cotton cultivars integrated with pest-resistant genes have been released (Li et al., 1999; Li et al., 2000), there are still some shortcomings in the pollen-tube pathway transformation in cotton, such as low transformation efficiency. Therefore, it is necessary to further improve the cotton transformation frequency. In this study, the authors evaluated the effects of genotype, concentration of the injected target DNA, and flower position in plants on transformation efficiency in cotton. Based on the study, the transformation system via pollen-tube pathway with high efficiency in cotton was established.

## 2 Materials and methods

### 2.1 Experimental materials

The cotton cultivars 33B and 99B were used for the transgene receptors. The tested cotton cultivars were grown at the Experimental Station of Agricultural University of Hebei, Baoding.

### 2.2 Target DNA for transformation

The binary expression vector pCAMBIA3301 plasmid was used to fuse the target DNA for transformation. The plasmid was isolated from an *E. coli* strain DH5α, harboring the vector using TIANprep mini plasmid isolation kit (Tiangen Company, China). After the plasmid isolation, the DNA quality and quantity were identified by agarose electrophoresis and spectrophotometric analysis.

### 2.3 DNA injection concentrations and flowers selected for exogenous DNA injection

The concentrations for transformation were set at three levels, including 100 $\mu g \cdot mL^{-1}$, 200 $\mu g \cdot mL^{-1}$, and 400 $\mu g \cdot mL^{-1}$, with 5 μL for each ovary. The normally growing flowers located at the basal positions of the second fruit branch to tenth fruit branch, which were divided into three groups, were used for exogenous DNA injection. The ovaries for injection of target DNA was 10–20 hours after pollination, based on the observation of the coloration of flower corolla. For DNA injection, the target DNA was first adjusted to the expected concentration and then separately injected into the center of the ovary by a syringe. After injection, the flower stalk was smeared with 40 $\mu L \cdot L^{-1}$ gibberellin ($GA_3$) to decrease the abscission rate. For each cultivar, total ovaries of 1068 from 33B and 1066 from 99B were selected for injection of the target DNA, at different flower positions and with different DNA concentrations.

### 2.4 Calculation of boll setting rate of the DNA injected ovaries

At maturity, the bolls injected with the target DNA were harvested and the boll seeds were separated. The boll setting rate of the DNA injected ovaries was calculated by dividing the harvested bolls to the ovaries injected with the target DNA.

### 2.5 Calculation of the transformation efficiency

Three seeds from each boll after two months of harvest were sown in a plastic pot filled with sand. For the normal seed germination and seedling growth, the Hoagland nutrient solution was regularly irrigated twice a week. At the fully expanding stage of the third leaf, the plants whether or not to be transformed were detected by smearing 5 $mg \cdot L^{-1}$ PPT on the third leaf surface. Seven days later, the plants with leaf-smeared PPT, and those showing normal growth were classified into the putative transgenotype. The transformation efficiency was calculated by dividing the transformed plants to the ovaries injected with the target DNA.

### 2.6 Molecular identification of the transformed seeds

At the fully expanding stage of the third leaf, nine transgenic plants of 33B and 99B (five from 33B and four from 99B, respectively) showing PPT-resistance were randomly selected for further molecular identification. For polymerase chain reaction (PCR) analysis of the transformed plants, the genome DNA of the plants was isolated by cetyltrimethyle ammonium bromide (CTAB) method (Doyle and Doyle, 1989). The primers for amplifying the reporter gene (beta-glucuronidase gene, *Gus*) fragment (639 bp) were as follows: 5′-gacattggcgtgaccaaggaaa (forward) and 5′-cagtccgtcgttcaccagttcc (reverse). The PCR thermal reaction conditions were at 94°C for 4 min, then followed by 35 cycles of template denaturation at 95°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s. A final extension step at 72°C for 10 min was added for improvement of the PCR products.

For the detection of *Gus* expression level in the transgenic plants, semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. First, the total RNA of ten plants including nine above transformed plants, and the control (untransformed 33B) was separately isolated by CTAB-LiCl precipitation method (Giordani et al., 2000). The mRNAs in the total RNA were reversely transcribed into cDNAs by AMV reverse transcriptase (TaKaRa) following the suggestion of the manufacturer. The PCR, with the transcribed cDNA as the template, was performed by using the same primers and thermal reaction cycle parameters as those in PCR analysis of the transgenic plants mentioned above.

2.7 Assay of the GUS activities

The third leaf seedlings were also assayed for the beta-glucuronidase (GUS) activity. For that, about 2 g leaf samples were grinded into powder in liquid nitrogen and measured by following the description of Xiao et al. (2005).

2.8 Histochemical staining of the transgenic plants

At the third leaf expansion stage, the third leaf was cut off using a scissor and put into a staining solution with the following components: 250 mg·L$^{-1}$ 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt (X-gluc), 100 mmol·L$^{-1}$ phosphate buffer (pH 7.0), 0.1% Triton X-100, and 1.0 mol·L$^{-1}$ EDTA. The staining biochemical reaction was performed at 37°C for 16 h. The stained leaves were then destained by transferring them in 70% ethanol for about seven days. The histochemical staining results were recorded by a digital camera.

## 3 Results

3.1 The cassette of exogenous DNA for transformation and quality identification of the target DNA

The binary expression vector pCAMBIA3301 plasmids were used for transforming the cotton via pollen-tube pathway. The cassette of pCAMBIA3301 was described in Fig. 1 (a). In this cassette, the selection gene and the reporter gene were *bar* and beta-glucuronidase (*Gus*), respectively, which were all driven by the cauliflower mosaic virus 35S (CaMV35S) promoter. In the meantime, the 35S polyA and NOS polyA inserted to the downstream of the genes were separately used for the polyA tailing of *bar* and *Gus*. The plasmids of pCAMBIA3301 were isolated by TIANpreo Mini kit (Tiangen, China). The isolated plasmids were identified in quality based on agarose electrophoresis before the DNA injection (Fig. 1 (b)). Based on spectrophotometric analysis, the ratio value 1.86 of $OD_{260}$ to $OD_{280}$ was consistent with the agarose electrophoreses results, indicating that the plasmids were pure enough for the use in injection.

3.2 Boll setting rates of the DNA-injected ovaries

The ovaries injected with the DNA and the boll setting numbers in the tested cultivars 33B and 99B under the conditions of various DNA injection concentrations and flower positions in plant are listed in Table 1. As for the DNA injection concentrations, on the average, there was no significant difference in the boll setting efficiencies between the two cultivars; however, the boll setting efficiency was dramatically affected by the flower position in plant. The flowers at fruit branches 5–7 could easily set the boll after exogenous DNA injection, compared with those at the lower fruit branches 2–4 and at the upper fruit branches 8–10. Therefore, it plays a key role for the improvement of genetic transformation efficiency via pollen-tube pathway by selecting a suitable target DNA concentration and the flower position in plant.

3.3 Transformation efficiencies in the treatments

The seedlings originated from the DNA injected ovaries whether it was to be transformed or not to were identified by smearing 5 mg·mL$^{-1}$ PPT on the young leaf surface in plants. The PPT-resistant plants and the transformation efficiencies (the percentage of PPT-resistant plants to the total ovaries injected with DNA) in cultivars 33B and 99B under the conditions of various DNA injection concentrations and flower positions in plant are listed in Table 2, showing that the transformation frequencies changed from 1.04% to 3.63%, much higher than those reported

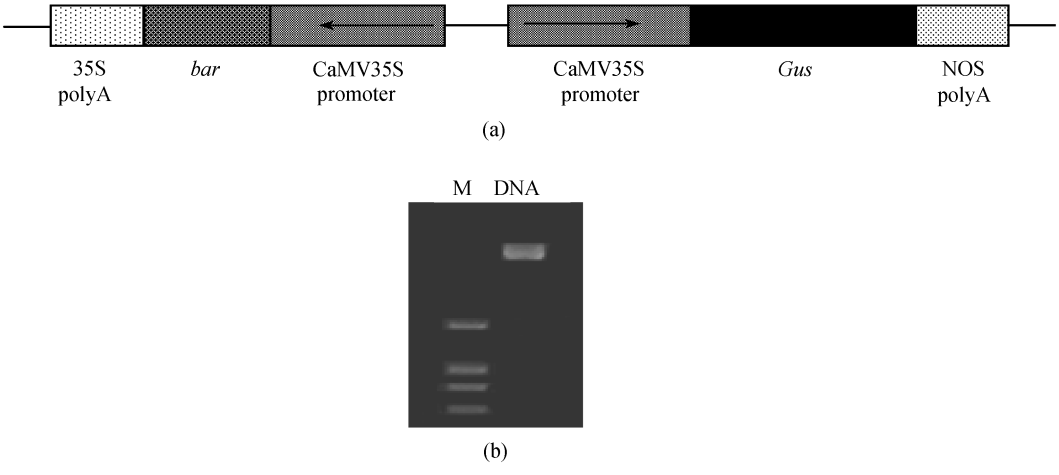


**Fig. 1** The cassette of pCAMBIA3301 (a) and the plasmids identification based on agarose electrophoresis (b)

**Table 1** The boll setting rates of the DNA injected ovaries in the tested cultivars

| DNA concentration | cultivar | fruit branch 2–4 | | | fruit branch 5–7 | | | fruit branch 8–10 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | injected ovaries | set bolls | setting rate/% | injected ovaries | set bolls | setting rate/% | injected ovaries | set bolls | setting rate/% |
| 100 μg·mL$^{-1}$ | 33B | 116 | 30 | 25.86 | 132 | 62 | 46.97 | 126 | 48 | 38.10 |
| | 99B | 96 | 32 | 33.33 | 132 | 56 | 42.42 | 112 | 42 | 37.50 |
| | average | 106 | 31 | 29.60 | 132 | 59 | 44.70 | 119 | 45 | 37.80 |
| 200 μg·mL$^{-1}$ | 33B | 126 | 34 | 26.98 | 120 | 54 | 45.00 | 118 | 40 | 33.90 |
| | 99B | 112 | 32 | 28.57 | 126 | 56 | 44.44 | 144 | 46 | 31.94 |
| | average | 109 | 33 | 27.78 | 123 | 55 | 44.72 | 131 | 43 | 32.92 |
| 400 μg·mL$^{-1}$ | 33B | 124 | 38 | 30.64 | 110 | 48 | 43.64 | 96 | 32 | 33.33 |
| | 99B | 110 | 24 | 21.82 | 128 | 58 | 45.31 | 106 | 32 | 30.19 |
| | average | 117 | 31 | 26.23 | 119 | 53 | 44.48 | 101 | 32 | 31.76 |

**Table 2** The PPT-resistant plants derived from the seeds whose ovaries were injected exogenous DNA and the transformation efficiencies in the tested cultivars

| DNA concentration | cultivar | fruit branch 2–4 | | fruit branch 5–7 | | fruit branch 8–10 | |
|---|---|---|---|---|---|---|---|
| | | PPT-resistant bolls | transformation rate/% | PPT-resistant bolls | transformation rate/% | PPT-resistant bolls | transformation rate/% |
| 100 μg·mL$^{-1}$ | 33B | 2 | 1.72 | 3 | 2.27 | 2 | 1.59 |
| | 99B | 1 | 1.04 | 3 | 2.27 | 2 | 1.79 |
| | average | 1.5 | 1.38 | 3 | 2.27 | 3 | 1.69 |
| 200 μg·mL$^{-1}$ | 33B | 2 | 1.59 | 3 | 2.50 | 2 | 1.69 |
| | 99B | 2 | 1.79 | 3 | 2.38 | 3 | 2.08 |
| | average | 2 | 1.69 | 3 | 2.44 | 2.5 | 1.89 |
| 400 μg·mL$^{-1}$ | 33B | 3 | 2.42 | 4 | 3.63 | 2 | 2.08 |
| | 99B | 2 | 1.82 | 4 | 3.13 | 3 | 2.83 |
| | average | 2.5 | 2.12 | 4 | 3.38 | 2.5 | 2.46 |

previously in cotton (Li et al., 1999). Similar to the boll setting rate, the transformation efficiency was also higher in ovaries from fruit branches 5–7 than those from fruit branches 2–4 and 8–10. In the meantime, the DNA injection concentrations also had obvious implications on the transformation efficiency, which showed an increasing tendency with the increase of the DNA concentration in the tested regime.

### 3.4 Molecular identification of transgenic cotton lines integrated with the exogenous DNA

Nine putative transgenic plants from the 33B and 99B (Lines 1–5 from 33B, and lines 6–9 from 99B), based on PPT smearing identification, were selected for further analysis. The genome DNA isolated from the transgenic plants and the wild type 33B (CK) are shown in Fig. 2 (a). PCR analysis indicated that all the nine putative transgenic plants were positive (Fig. 2 (b)). In the meantime, the total RNA of the putative transgenic plants and CK were extracted (Fig. 2(c)), and further semiquantitative RT-PCR analysis was performed to detect the expression level of the target gene *Gus* in the transgenic plants. It was shown that the *Gus* transcripts in the putative transgenic plants changed largely, with higher levels in Lines 1, 8, and 9, middle levels in Lines 2, 4, 6, and 7, and no transcripts of the target gene detected in Lines 3 and 5. These results suggested that the leaf-smearing PPT method is reliable in identifying the transgenic events in cotton via pollen-tube pathway approach.

### 3.5 GUS activities assay in the transgenic cotton lines

The GUS activities in the wild type 33B (CK) and the nine transgenic plants (Lines 1–9) were assayed based on the fluorescent spectrophotometric analysis method. It was found that the GUS activities in CK and the transgenic plants were in accordance to those of the transcripts of the reporter gene *Gus*. The higher GUS activities were assayed in Lines 1, 8, and 9, middle GUS activities were found in Lines 2, 4, 6, and 7, and negligible GUS activities were detected in Lines 3 and 5 (Fig. 3).

### 3.6 Histochemical GUS staining of the represent transgenic cotton plants

The leaves of three transgenic plants with different *Gus* transcripts, including Lines 7, 1, and 8, were selected for

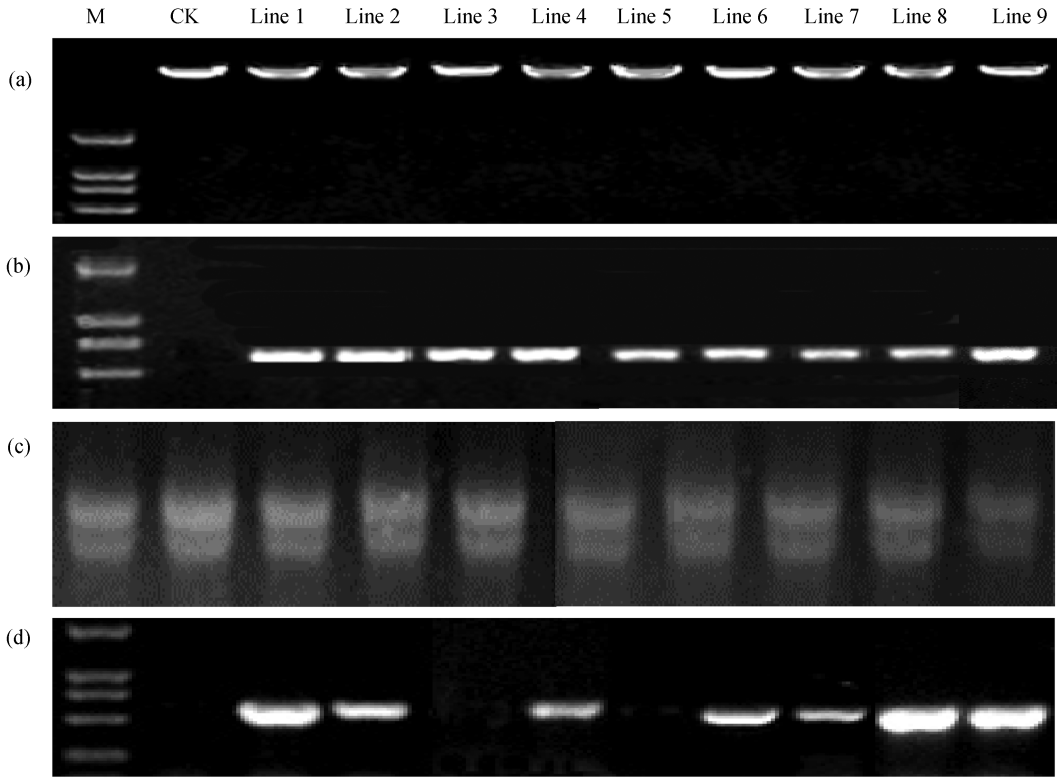


**Fig. 2** Molecular identification of the putative transgenic plants

Note: (a) represents the genome DNA isolated from the wild type of 33B (CK) and the nine putative transgenic plants; (b) represents the PCR analysis of the putative transgenic plants by using the specific primers for reporter gene *Gus*; (c) represents the total RNA isolated from the wild type of 33B (CK) and the nine putative transgenic plants; (d) represents the RT-PCR analysis to detect the expression levels of the reporter gene *Gus* in the putative transgenic plants.

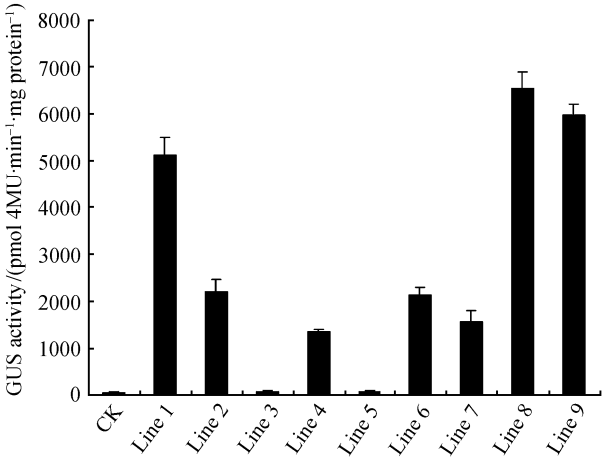


**Fig. 3** The GUS activities assayed in the wild type of 33B (CK) and the nine transgenic plants

histochemical GUS staining analysis to detect the expression patterns of the reporter gene in plants. The staining results of leaves and petioles for the three transgenic lines are shown in Fig. 4. It was found that different staining intensities were observed among the plants, showing the similar pattern with those transcripts detected mentioned above. In this study, the staining patterns in the leaves and petioles were not uniform, with a relatively strong staining around the vascular region in the leaves and the cut positions in the petioles. Extra staining for other transgenic plants with the detected *Gus* transcripts also showed these patterns. We suggested that the uneven GUS staining in the leaves and the petioles were mainly due to the epidermal structure of the cotton leaf. The relative thickness of cuticle on the epidermis and compact structure prevent the diffusion of the substrate in the staining solution to the mesophyll cells of the leaf and the inner layer cells of the petiole, resulting in low staining or nonstaining at most parts of leaves and the petioles. The strong staining at the cut positions in the petioles of Lines 7, 1, and 8 was consistent with the above conjecture.

## 4 Discussion

During the fertilization process in cotton, the mature pollen bourgeoned on the stigma, where it germinated, formed the pollen tube, and entered the ovule (Faure and Dumas, 2001). The mechanism of the pollen tube pathway

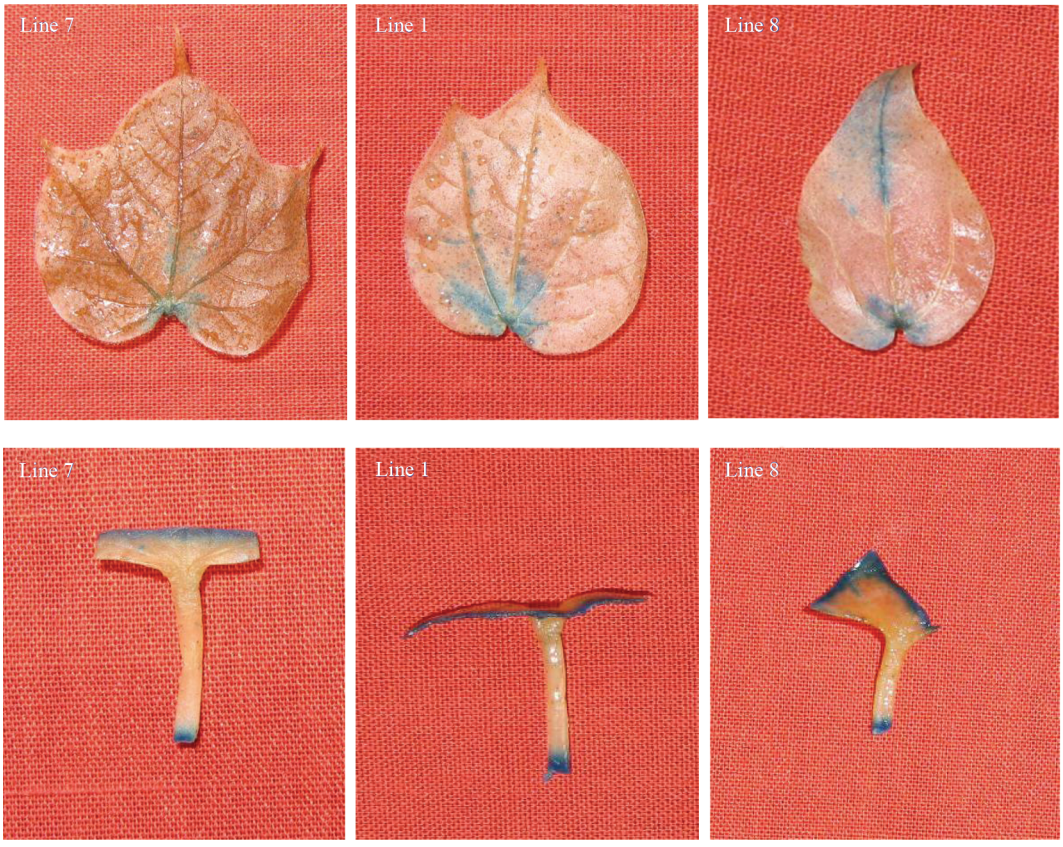


**Fig. 4** The GUS staining results of leaves and petioles in three representative transgenic plants with different *Gus* transcripts

transformation was once confirmed by isotope tracer method. Gong et al. (1988) reported that the labeled $^{3}$H-DNA at the self pollination could be found in some cotton embryos after 24 h. In addition, it was observed that micropyle had been at a close state before the pollen tube arrived, and when the pollen tube arrived at ovule, the micropyle opened. Experiments confirmed that the nucelli pathway of pollen tube after self pollination was the only pathway that heterologous DNA entered the embryo from micropyle (Song et al., 2006).

Though several researches on cotton transformation via pollen-tube pathway had been reported (Li et al., 1999; Li et al., 2000; Li et al., 2005; Liu et al., 2007), the transformation efficiency was usually relative low, ranging from 0.5% to 1%, if calculated from the setting bolls transgenically to the ovaries injected with the exogenous DNA. This drawback limited the wide application for cotton genetic transformation. We suggested that the low transformation efficiencies may be due to three reasons. First, the DNA quality was not good enough and the DNA concentration injected was unsuitable. The exogenous DNA without high quality usually was covalently combined with other molecules, such as proteins and polysaccharide, which could slow down the movement of the injected DNA in the pollen tube and decrease the integration rate of the exogenous DNA into the chromosome of the fertilized zygote. Second, high molecular weight of DNA for injection resulted in inconvenient transportation via the pollen tube, which could also affect its insertion frequency into the fertilized zygote. Thus, for the transformation of cotton via pollen-tube pathway approach, the high quality and suitable length of the exogenous DNA should be considered in advance. Third, the plants in high plant densities used for genetic transformation by pollen-tube pathway could drop the boll setting rate, due to the nutrient competition among the reproductive organs. In this case, the aggravated nutrient status for the reproductive organs that were injected with the exogenous DNA would result in abscission at the flower stage or early boll setting stage. In our study, the injected ovaries at fruit branches 5–7 had a higher boll setting rate, which may be resulted from these position flowers to have strong competition capability for nutrients. Therefore, the planting density should be low, and the reproductive organs at suitable fruit branches should be selected so as to improve the transformation efficiency.

There were reports on cotton genetic transformation showing the transformation frequency to be dependent of genotypes (Saeesd et al., 1997; Wang et al., 2006). In our study, the two exogenous DNA receptor cultivars 33B and 99B showed a similar pattern responding to the DNA concentrations and the flower positions on boll setting rate

and transformation efficiency. Therefore, the pollen-tube transformation pathway approach has an extra advantage of genotype independence, except for its simplicity, and easy manipulation. The suitable transformation system with high efficiency established in this study can be used as a powerful tool in future cotton genetic improvement.

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