Introduction of exogenous DNA into cotton via the pollen-tube pathway with GFP as a reporter

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Abstract The green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* as a vital reporter for gene expression in plants is considered to have several advantages over other reporter genes. The pBIN35S-mGFP4 plasmid DNA has been introduced into cotton embryos by the pollen-tube pathway method. A transformed seedling has been verified according to its GFP-related fluorescence and Southern blotting analysis. The results provided direct and convincing facts in cytology and molecular biology for the pollen-tube pathway method, an efficient transformation technique used in plants.

Keywords: **green fluorescent protein (GFP), pollen-tube pathway, cotton.**

THE pollen-tube pathway method, which is a transformation technique for introducing exogenous DNA into plants after self-pollination, was first developed and applied to cotton breeding by Zhou *et al.*[1]. In nature, when a pollen falls on the stigma, it germinates and a pollen tube grows through the style tissue until arriving ultimately at the ovary. A pollen tube enters an ovule at the microphyle, then the degenerated nucellus cells form a column to facilitate passage of the pollen tube. Exogenous DNA can pass through the column, a pathway of the pollen tube in the nucellus, entering the embryonic sac and transforming the eggs and zygotes which do not have normal cell walls at that time and resemble natural protoplasts. It was confirmed by the isotopic tracer technique that exogenous DNA enters embryonic sac via the pollen-tube pathway rather than the pollen tube itself[2]. Exogenous DNA had been introduced into cotton[3], rice[4,5], wheat[6] by the pollen-tube pathway technique whose transforming mechanism has not been understood clearly. It is important and necessary to continue working on the feasibility of this transforming method.

In previous works, β-glucuronidase (GUS) has been used extensively as a reporter of gene expression in plants. The GUS gene product can be localized or quantified by using histochemical techniques, but these are generally destructive tests and some plant tissues present background color interference. The green fluorescent protein (GFP) from *Aequorea victoria* can be directly visualized and therefore shares none of the above problems. It strongly fluoresces and requires no substrate or cofactors other than molecular oxygen to form the fluorescent molecule. The GFP gene as a reporter has been successfully expressed at high levels in many plants[7]. In this study we introduced exogenous DNA into cottons with the GFP gene as a reporter by the pollen-tube pathway method.

1 Materials and methods

Plasmid pBIN35S-mGFP4[8] was presented by Dr. Jim Haseloff. The wild-type GFP gene is cur-

tailed in aberrant mRNA processing in plants, and 84-nt cryptic intron is efficiently recognized and excised from transcripts of the GFP coding sequence. The mgfp4 gene is the modified version in which the intron was mutated to restore proper expression in plant cells. The A:T content was decreased between NdeI and AccI sites in the gfp coding region to remove the cryptic intron.

The DNA plasmids were extracted and purified according to the method in reference [9].

Upland cotton (*Gossypium hirsutum*) Ji-123 was grown in the field until flowering. After self-pollination for 24 h or so, exogenous DNA was injected into cotton ovaries by using the method described in ref. [1]. Some of the injected bolls were used for checking the young embryos after 20-d developing, others continued growing to produce mature seeds. The young embryos were examined using a fluorescence microscope with blue light excitation. The transformed whole plant was irradiated with a hand-held, long-wave UV lamp.

All the genomic DNA extraction procedures were carried out in eppendorves. The individual transgenic seedling was ground with SiO_2 powder by using a pestle. After extraction with 5-fold volume of a buffer containing 2% SDS, 6% PVP, 1% mercaptoethanol, equal-volume phenol was added to remove proteins. The resulting supernatant was ethanol-precipitated with mixing 1/3 volume of ammonium acetate (7.5 mol/L). The resulting nucleic acid solution was treated with RNAase for 30 min at 37℃, repurified with phenol/chloroform/isopentanol (25:24:1) twice, then routinely precipitated with ethanol to get the genomic DNA.

Southern hybridization analysis was done using DIG-labeling kit from Boehringer Mannhein Biochemicals according to its procedures.

2 Results

(ⅰ) GFP gene expression in cotton embryo. 40% of the bolls injected with exogenous DNA withered and fell a few days later because of serious injury by the micropipette. Only one young embryo in 285 injected bolls expressed GFP at high levels, presenting bright-green fluorescence (Plate Ⅰ-3) while the control showed red fluorescence (Plate Ⅰ-4) when examined under a fluorescence microscope. With ultraviolet and violet light excitation, the transformed embryo could also produce weak green fluorescence. The transformed embryo and control all produced red fluorescence when excited with green light. The background autofluorescence in cotton embryos was observed in this experiment, but it could be distinguished from the GFP fluorescence.

(ⅱ) GFP gene expression in the transformed cotton seedling. The mature transformed seeds were germinated until real leaves developed. One transformant was found in 56 seedlings with a hand-held long-wave UV source. The transformed seedling (Plate Ⅰ-2(b)) and the control (Plate Ⅰ-2(a)) looked alike in appearance except their height under visible light, but obvious differences were observed during irradiation with a long-wave UV lamp. The transformant regenerated green fluorescence (Plate Ⅰ-1(b)), especially brighter at leaf stalks than other parts, while no fluorescence was found with the control (Plate Ⅰ-1(a)). The green fluorescence of GFP was visualized in leaf veins of the transformed seedling. The leaves produced orange-yellow fluorescence because of both chlorophyll and GFP existing (Plate 1-5), while the untransformed leaves gave rise to red fluorescence (Plate Ⅰ-6).

(ⅲ) Southern blotting analysis of the transformed plant. To confirm the results above, Southern hybridization was carried out on the GFP-expressed seedling. The results are shown in Fig. 1. The genomic DNA of the transformed seedling shows a positive hybridization signal in contrast to that of the control plant.

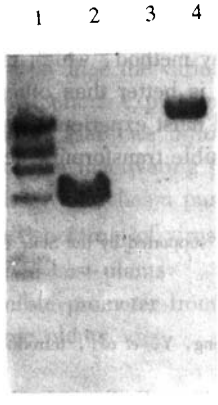

Fig. 1. Southern blotting result of the transformed seedling. 1, DNA fragments of λ-DNA treated with HindⅢ; 2, plasmid DNA with GFP gene; 3, genomic DNA of the control plant; 4, genomic DNA of the transformed plant.

3 Discussion

The pollen-tube pathway method, which was advanced by Chinese scientists, has been confirmed in a few of crops. Since the mutated offsprings were obtained by the method with exogenous total DNA, and its transforming mechanism was not clear, the method has not been accepted extensively by all researchers, and therefore its application and development have been halted. The results of molecular hybridization based on separation and extraction *in vitro* provided evidence for transformants obtained by the pollen-tube pathway method, but they could not display the situation of exogenous genes expression in living cells. In addition, transformed tissues or plants can be identified histochemically with GUS gene as a reporter, but this is generally a destructive test which neither is suitable for assaying primary transformants and following the time course of gene expression in living plants, nor serves as a means of rapidly screening segregating populations of seedlings. The GFP shares none of these problems and has a few advantages as a gene maker or reporter: (ⅰ) The fluorescence emission of GFP does not require a cofactor or a substrate, direct visualization of gene expression in individual cells is therefore possible without cell lysis, subsequent biochemical analysis and tissue distortion caused by fixation and staining, etc. (ⅱ) The GFP produces a green fluorescence which can be easily distinguished from the background autofluorescence of plants through regulating light intensity and changing excitation wavelength. (ⅲ) GFP reports transformed results accurately instead of false positive signals as GUS does because of no inner GFP existing in plants.

The GFP gene as a reporter has many priorities over the GUS gene. The potential use of GFP in plants has drawn wide attention and interest. In this note, the GFP gene was transformed into cotton by the pollen-tube pathway method. Its expression was detected both in the young embryo and seedling. Southern hybridization results suggested that the GFP gene had integrated into the cotton genome. All these provided direct and trustworthy evidence for the feasibility of genetic transformation by the pollen-tube pathway method.

A major use of GFP would be as a replacement for GUS gene and in the cotton research of cell and gene engineering because of its high expression in cotton. The GFP-expressed tissues could be monitored using their fluorescence *in vivo*, avoiding any need for destructive testing.

Although GFP-expressed transformants have been obtained successfully by other transformation methods, some results suggested that high levels of GFP were toxic to some plant cells, or inhibited their dividing and regenerating[10]. In our experiments, the transformed young embryo and seedling of cotton were found to be smaller than the controls obviously. These results showed that GFP could also be toxic to cotton cells.

Transformed plantlets were obtained in low ratio in this experiment because we lacked experience about GFP detecting at the beginning. In fact, previous experiments demonstrated that the pollen-tube pathway method had high transformation efficiency in cotton, wheat and rice. The embryonic sac's egg could be considered as a protoplast allowing easy entry of exogenous DNA following the pollen-tube pathway[11].

The pollen-tube pathway method, which is worth advocating and spreading because of its easy manipulation and convenience, is better than other direct transformation methods with which the recipients are limited by genetypes and must experience tissue culture and regeneration. Its transformation efficiency can be improved if more feasible transforming schemes are designed to fit the individual character of flowers of various crops.

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