EVALUATION OF NOVEL TRANSFORMATION SYSTEMS FOR COTTON

Ping Song, Phat M. Dang and Randy D. Allen
Plant Molecular Biology Laboratory
Department of Biological Sciences, Plant and Soil Sciences
Texas Tech University
Lubbock, TX

Abstract

The ability to "transform" cotton by the introduction of foreign genes into its genome has initiated a slow revolution in cotton improvement. The opportunity to use genes from virtually any source allows researchers to develop plants with a wide range of useful traits. One limiting factor in the application of this technology is the relative difficulty and expense of the most widely used transformation systems. Several research groups are working to develop more tractable transformation protocols. We have attempted to adapt a procedure that is widely used for transformation of *Arabidopsis thaliana* for use with cotton. This method involves that direct infiltration of *Agrobacterium* cells into developing flowers. Using this method, we have obtained several putatively transformed cotton plants that are resistant to the herbicide Basta™. Preliminary molecular analysis indicates that these plants contain the foreign gene construct. Offspring from these plants also contain the foreign DNA and are herbicide resistant. Though not conclusive, these results indicate that, with further development, the direct flower infiltration transformation method could be a valuable tool for genetic engineering of cotton.

Introduction

The introduction of foreign genes into cotton (transformation) has the potential to provide incredible benefit to the cotton industry by expanding the pool of genes that are available for cotton improvement. No longer are cotton breeders limited to genes already present in the cotton genome or in the genomes of closely related species. Now, breeders can tap the unlimited genetic resource of recombinant DNA technology to develop cotton plants that can seemingly do the impossible. The transfer of the polyhydroxybutyrate genes from bacteria into cotton to produce plastic within the cotton fiber (John, 1996) is one of the most dramatic recent examples of this technology.

The necessary technologies for cotton transformation were developed during the early 1980's and the pieces of the puzzle were put together by Dr. Norma Trolinder's group who used the method to develop transgenic cotton plants that were resistant to 2,4-D (Bayley et al, 1992). This transformation method involves the inoculation of explants with *Agrobacterium tumefaciens* that contain a binary plasmid that carries the gene of interest along with an *nptII* gene to provide resistance to the antibiotic kanamycin (kan^R). Kan^R callus is propagated and used to develop embryogenic suspension cultures that will form somatic embryos. The somatic embryos can be induced to germinate and grow into small plants that can eventually be transferred to soil. This procedure has many advantages including efficient selection of transformed, kan^R cells and regeneration from single cells, ensuring that the plants that are regenerated are not chimeric. In our hands, most of the transgenic plants produced by this procedure express the gene of interest. However, the method is time consuming and expensive and, since the tissue is in culture for an extended period, a small but significant fraction of the plants recovered have somaclonal mutations that can lead to sterility and stunted growth.

The most serious problem with the somatic embryo regeneration system is that the ability to form somatic embryos is determined in cotton by a complex genetic system so that relatively few cotton varieties can be efficiently transformed. This genotype dependence of regeneration limits the applicability of this system.

To overcome the problems associated with the somatic embryogenesis system, several research groups have developed alternative transformation methods. These include particle bombardment of apical meristems (Koonce and Trolinder, 1996) and *Agrobacterium* inoculation of apical or axillary buds (Hemphill and Chapman, personal communication). Although progress has been relatively slow, recent reports indicate that these methods can be used to successfully introduce foreign genes into cotton tissues and to recover transgenic plants. Since the plants develop directly from meristematic tissues, the genetic restrictions on somatic embryogenesis do not apply and most, if not all cotton genotypes can be transformed.

Unlike the somatic embryogenesis procedure in which regeneration occurs from single cells, these approaches depend on the transformation of cells within meristematic tissues. Therefore, the plants that are produced are most often chimeric. Since only some of the tissues actually contain and express the foreign gene, it is necessary to identify and select the transgenic sectors. These sectors may or may not include the cell layers that produce the gametes so the transgenes may or may not be inherited.

Recently a very simple method for the transformation of *Arabidopsis thaliana* has been reported (cf. Bent et al., 1994). Using this method, flowering *Arabidopsis* plants are dipped into dilute *Agrobacterium* suspensions and a vacuum is applied. The *Agrobacterium* cells are drawn into the developing flowers and are able to transform the immature embryos. Germination of mature seeds on kanamycin

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containing media allows for the selection of those few seeds that have been transformed. The frequency of transformed seeds is reported to be between 1 and 5%. We have attempted to adapt the *Arabidopsis* flower infiltration transformation method for use in cotton.

Materials and Methods

Gene constructs A binary plant transformation vector containing a chimeric gene consisting of the *Bar* gene that provides resistance to the phosphenothricine (eg. Basta™) class of herbicides. Transcriptional control was provided by the nopaline synthase (nos) promoter and the nos 3' terminator and polyadenylation sequence. This vector was introduced into the disarmed *Agrobacterium tumefaciens* strain LBA4404. Cultures were grown at 28°C and diluted to an appropriate concentration before flower infiltration treatment.

Flower Infiltration Flowering Cotton and *Arabidopsis* plants differ dramatically is size and conformation. Arabidopsis is an annual that bolts to produce dozens of flowers within a short period of time. Therefore, one vacuum infiltration treatment can be expected to affect a large number of flowers at a wide variety of developmental stages. Cotton plants are perennial and flowering occurs over a protracted period; therefore, it is necessary to treat flowers on an individual basis as they mature. Infiltration of *Agrobacterium* into cotton flowers most often leads to floral abortion. We have developed a device for the effective infiltration of *Agrobacterium* into maturing cotton flowers at an appropriate time and we have identified procedures to prevent abortion of treated flowers. Mature seeds were collected from mature treated bolls, delinted and planted.

Plant Selection Plants were sprayed to run off with a solution containing the appropriate concentration of Basta™ and wetting agents. Surviving plants were resprayed approximately two weeks later. Plants that survived the second spray treatment were grown to maturity.

Purification of DNA and RNA from surviving plants was carried out according to Song et al. (1997) and polymerase chain reaction (PCR) analysis was carried out using primers that are specific for the *Bar* sequences.

Results

Several hundred seeds were obtained from *Agrobacterium* infiltration of several dozen cotton flowers. These seeds were delinted and planted and the resulting plants were sprayed with Basta™. In each experiment, more than 90% of the plants died outright within one week of the first herbicide treatment. To eliminate those plants that did not receive an adequate dose, a second herbicide treatment was applied to the surviving plants two weeks after the initial treatment. Those plants that survived the second herbicide treatment (1 to 2% of the seeds tested) were then analyzed by PCR for the presence of the *Bar* gene (Figure 1). Using reverse transcriptase-PCR (RT-PCR) analysis, amplified bands were seen from reactions using cDNA templates from two putative transgenic plants but not from untransformed plants. The identity of the amplified bands was confirmed by hybridization with a ^{32}P labeled *Bar* probe. Analysis of genomic DNA by PCR also showed specifically amplified bands from samples of putative transgenic plants and not from untransformed plants.

These herbicide selected T_0 plants were allowed to mature, flower and set seed. Mature seeds were collected and planted and young T_1 plants were sprayed two times with Basta™. Although the sample size was too small to determine segregation frequencies, the T_1 plants could be classified as herbicide sensitive, partially resistant and strongly resistant. Analysis of DNA samples from several of the strongly herbicide resistant T_1 plants by PCR indicated the presence of the *Bar* gene construct in these plants (data not shown).

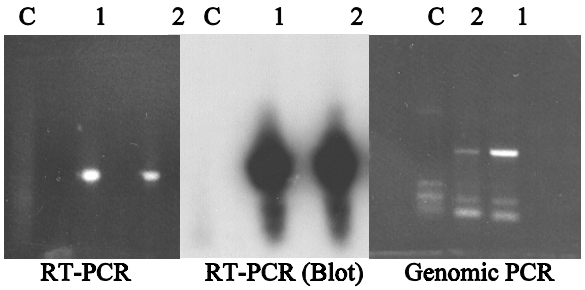

Figure 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) and genomic PCR analysis of putative T_0 transgenic cotton plants developed through the flower infiltration method. Samples from untransformed control plants (C) and two independent herbicide resistant plants (1 and 2) are shown. The identity of the products amplified by RT-PCR was confirmed by hybridization with a ^{32}P labeled *Bar*-specific probe.

Discussion

Application of gene transfer technology to the improvement of cotton is limited, in part by the inefficiency of current transformation methodologies. Successful adaptation of the flower infiltration transformation, that is widely used for transformation of *Arabidopsis*, to cotton would provide a significant benefits over current procedures. The method is technically simple, requires little in the way of specialized equipment and is very inexpensive. In addition, since the transformation occurs within the flowers, there is no tissue culture and, therefore, no somaclonal mutagenesis. All of the resulting plants can be expected to be fertile and, since there is no regeneration, we anticipate that the method can be used to transform most, if not all cotton varieties.

Our results, though not conclusive, provide substantial evidence that such a system is feasible. We have recovered plants with large Basta™-resistant sectors and these tissues were shown by PCR analysis to contain and express the *Bar* gene construct. Analysis of T_1 plants shows that both the

gene construct and the Basta™ resistance phenotype are efficiently inherited. However, it will be necessary to perform additional molecular analyses, including genomic Southern blot hybridizations, and compete the genetic analysis of the inheritance of the transgene inserts in the putative transgenic plants before the system is fully demonstrated. In addition, we anticipate that many technical improvements to both the infiltration process and the selection of plants with transgenic sectors will need to be made before this methodology can be widely adopted.

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