A RAPID ASSAY FOR GENE EXPRESSION IN COTTON CELLS TRANSFORMED WITH ONCOGENIC BINARY *AGROBACTERIUM* STRAINS

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Abstract

A simple expression assay for evaluation of gene constructs for input of traits into cotton cells (*Gossypium hirsutum* L.) using oncogenic binary *Agrobacterium* strains is presented. Seedling explants from four commercial cotton varieties, representing diverse genotypes, exhibited tumor or root formation to an equal degree in response to infection by different types of oncogenic *Agrobacterium* strains. Cotyledon or hypocotyl explants readily developed tumors (100%) within a week and the tumors doubled in fresh weight every two weeks. *A. rhizogenes* super-rooting mutant strain MT232 was highly infective on cotyledon explants. Experiments with oncogenic strains served as a basis for development of an assay using tumor-inducing binary vectors carrying the gene to be evaluated, an insecticidal Bt protoxin gene. An oncogenic binary vector containing a chimeric neomycin phosphotransferase II and a Bt protoxin gene conferred antibiotic resistance and insect resistance to Lepidopteran larvae in tumorigenic cells from cotyledon explants. The efficacy of the insecticidal protoxin gene towards the control of a Lepidopteran cotton insect pest, tobacco budworm (*Heliothis virescens*), was demonstrated in this study using oncogenic cotton cells. In a parallel study, the efficiency of this gene construct was also demonstrated using the tobacco model system against another Lepidopteran pest, tobacco hornworm (*Manduca sexta*). The time needed to conduct the experiment with cotton tumor cells was about three to four months from the time of initiation, same as the time needed for the tobacco model system. The rapidity of this assay is extremely useful in evaluation of gene constructs for input traits such as insect-, disease -, or herbicide resistance in the laboratory, especially in recalcitrant species such as cotton, where more than 15 months are needed for selection and regeneration of transgenic plants.

Introduction

Genetic transformation of cotton is a very time consuming procedure and it takes more than 15 months to produce transgenic cotton plants only to discover that the gene construct was not suitable or not working properly. For this reason, scientists often use model species such as tobacco, mustard weed or carrot to understand how the new gene might work in a test plant. Even when the gene is working correctly in a model plant system, the results could not be extrapolated to crop species such as cotton. A quick and easy method for testing new gene constructs in rapidly growing cotton tumor cells, caused by the soil-borne *Agrobacterium*, is presented here. The rapidity of the assay circumvents the time-consuming process of transforming and regenerating cotton plants and allows the researchers to test the gene construct for input traits such as insect resistance, disease resistance, and herbicide tolerance in a native cotton cell environment. Tumor cells expressing the gene of interest could be tested for efficacy within a reasonable time frame (3-4 months), similar to the period needed for testing with model plant systems, before using the gene construct for subsequent transformation and regeneration of fertile cotton plants.

Materials and Methods

The following oncogenic *Agrobacterium* strains were used in this study: Octopine type: Ach5, LBA4434; Nopaline type: C58; Agropine/mannopine type: A281 and *A. rhizogenes* MT232. The binary derivatives of these oncogenic strains were also made by introducing the plasmid vector pCIB10 (*nos-neo-nos*) by freeze-thaw method. A deletion protoxin gene containing approx. 645 amino acids of the full length *Cry*1A(*c*) gene under the control of 35S:CaMV promoter and terminator was ligated into BamH1-cleaved pCIB10. This plasmid was later introduced into Ach5 to produce oncogenic binary vector for transformation of cotton seedling explants (cotyledons and hypocotyls) or introduced into LBA4404 to produce non-oncogenic binary vector for transformation of tobacco leaf discs in a parallel experiment.

Results

Tumor Induction

Infection of tumorigenic *Agrobacterium tumefaciens* on cotton seedling cotyledon or hypocotyl explants (up to 100%) occurred with equal efficiency in all the cotton varieties (DPL90, Coker 315, Acala SJ2 and Acala 1618) tested. The average size of tumors from infected cotyledon explants after two weeks of infection was >3 mm for the strains Ach5, LBA4434 and C58. In comparison, the strain A281 produced smaller tumors (1.5 mm on an average). Solid, spherical greenish tumors on cotyledon explants were readily identifiable within a week of culture initiation. Cotyledon explants also produced prolific numbers of roots (an average of 24 roots per SJ2 explant) in response to treatment with the *A. rhizogenes* strain MT232.

Antibiotic Resistance of Tumors/Roots

Cotyledon and hypocotyl explants treated with oncogenic binary strains containing the plasmid pCIB10, which carries the marker gene that confers resistance to kanamycin, also produced tumors in equal numbers when selected first on MS basal medium and then on basal medium containing kanamycin (50 mg l^{-1}). Antibiotic-resistant (thus npt II-ELISA positive) tumors were obtained at a relatively high frequency (up to 56% of the total tumors plated) from cotyledon explants. The double selection (selection for autonomous growth and antibiotic resistance) procedure yielded no escapes although the growth of the antibiotic-resistant tumors was slightly slower compared to regular tumors. The doubling in fresh weight of tumor occurred every two or three weeks.

Similarly, treatment with binary strains of *A. rhizogenes* produced several hairy roots. Isolated root tip segments (10 mm long) were placed on MS basal medium containing 50 mg l^{-1} of kanamycin. Nearly 40% of the root tips continued to grow and elongate on antibiotic selection showing the efficient transfer of pCIB10 T-DNA.

Insect Feeding Assay to Evaluate Gene Expression

The transgenic tobacco plants, transformed with the Bt protoxin gene, were completely resistant to the *Manduca sexta* (tobacco hornworm) larvae compared to controls, which were defoliated by the caterpillars, thus demonstrating the efficiency of the Bt protoxin *in planta*. The size of the larvae increased to more than 65 mm after 14 days of feeding on control plants, whereas all the larvae stopped feeding on the first day and dropped off the transgenic plants. The toxicity of the protoxin in cotton tumor cells was also evident. *Heliothis virescens* (tobacco budworm) larvae readily consumed control tumor cells. The larvae grew rapidly in size, reaching a maximum length of 2 cm by the end of the six-day period, by which time all of the tissue provided had been eaten. Many of the larvae placed on tumor cells expressing the insecticidal protein stopped feeding and died in three to four days. Thus, the feeding behavior and the differences in larvae growth in control vs. transgenic cotton cells after four days of feeding were very similar to the results obtained from the tobacco plants, as described above.

Conclusions

Rapidly growing tumor cells or roots could be produced in all cotton species and varieties with equal efficiency. Using the tumor cells, a time saving, efficient assay for evaluating gene constructs in a native cotton cell environment for input traits is presented. Evaluation of gene constructs in cotton cells or roots could be accomplished within three to four months of initiation of experiments similar to the time frame needed with the tobacco or *Arabidopsis* model systems. We are currently using this approach to test disease resistance conferred by different antimicrobial gene constructs in cotton.

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