SEGREGATION FOR GUS GENE IN TRANSGENIC COTTON

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Abstract

The *GUS* gene is a marker gene that has been used in efforts to genetically engineer soybean, rice and cotton. Genes inserted into plants via biotechnological approaches may not always be inherited in a Mendelian fashion. Experiments were executed to determine inheritance of the *GUS* gene in cotton. Leaf and pollen assays were used to determine the presence of *GUS* in F_2 populations and strains derived from the original transformed plant. The chi-square analysis of *GUS* segregation patterns were non-significant except in F_2 populations and one DP50 progeny row. Fewer *GUS* positive plants were recovered in F_2 populations than predicted based on an expected 3:1 phenotypic ratio. We hypothesize that *GUS* positive gametes may not be as competitive as *GUS* negative types, thus resulting in fewer *GUS* positive offspring.

Introduction

People first began breeding plants when they discovered that better, or more desirable characteristics, could be obtained by crossing different varieties of the same species (Welsh, 1981). The science of genetics began to take shape with the work of an Augustinian monk, Gregor Mendel. He developed principles and analytic rules in mid-nineteenth century Czechoslovakia. His garden pea methods and analyses laid the groundwork for the genetic research performed today (Suzuki, 1991).

Agricultural plant breeding is currently used around the world, both on the small private level and the large industrial level. Breeding programs in the United States are operated through the United States Department of Agriculture, State Experiment Stations, and the private sector. Commercial breeding programs are most concerned with economics (Welsh, 1981). Delayed ripening in tomatoes, virus resistant squash, insect resistant potatoes and cotton, modified canola oils, herbicide resistant cotton, and herbicide resistant soybean are all recent examples of transgenic crops and their products (Oard, 1996). Plant breeding in cotton is currently being used to find everything from pest resistant cotton to naturally colored cotton. Pest resistant cotton is being researched because of the destruction of our environment by pesticides. The same companies that market the pyrethroid insecticides being used today are funding research towards more environmentally safe methods. At first, a pest resistant cotton could be costly to produce, but in the future, savings from fewer sprayings would accumulate for the farmer.

Knowledge of the inheritance of the target gene and a linked, selectable marker gene is generally neccessary for a successful transgenic breeding program in plants. Marker genes verify the existence of another gene in an organism. In plant breeding, marker genes are sometimes used as early indicators of the existence of a linked transgene. This is useful because certain phenotypic characteristics in plants do not appear until late in the growing season, such as cotton lint. If a breeding program was based on lint color, then success or failure would not be indicated until the harvesting is done. A marker gene could give an indication of success or failure after the first leaf appears. Breeders can also selectively pollinate for the target gene and cross in the same season, if testing for the maker gene is positive. Mendelian rules may not always apply to the inheritance of genes introduced through biotechnological approaches because of the transformation process used to insert the foreign gene.

The *GUS* gene is a marker gene that has been used in soybean, rice, and cotton. When it was used as a marker in soybeans, 90% of the plants that tested positive for *GUS* were transgenic, in that they carried the gene that *GUS* was marking (McCabe and Martinell, 1993). The *GUS* gene was also inherited through ninth generation progeny. The objective of this project was to use the *GUS* marker to design effective breeding strategies for transgenic cotton.

Methods and Materials

Germplasm lines isolated from genetically engineered cotton cultivar Deltapine 50 and crosses between the germplasm lines and other upland cultivars were used in this project. In 1996, individual cotton plants were analyzed for *GUS* activity, using the methods of McCabe and Martinell (1993). Plants expressing *GUS* were allowed to self-pollinate at Pee Dee Research and Education Center in Florence, South Carolina. Selected transgenic plants were outcrossed as a female to upland cultivars and germplasm lines to produce F_1 seed. Certain F_1 plants were self-pollinated in the winter of 1996-1997 to produce F_2 seed.

The 1997 field experiment consisted of DP50 progeny rows derived from plants self-pollinated in 1996 and F_1 and F_2 populations from the field and greenhouse, respectively.

Leaf Assays

Two methods were used in obtaining leaf assay results. The first required collection of the leaves from each plant, in scintillation vial boxes, and transporting them into the laboratory. The boxes contained 100 holes for the leaves to be placed in. At the beginning of each plot a stake was

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placed denoting the genotype of the plants following it. An entire leaf was collected from each plant and taken into the laboratory, where a .5 cm^2 size segment of each leaf was placed into one well of a 48-well tissue culture plate. The top of the plate was then labeled with the corresponding plant numbers. Approximately 500 µL of buffered substrate was pipetted into each well. The *GUS* buffered substrate had to be stored at 4°C when not in use. The plates were incubated at 37°C overnight. The following morning, the plates were removed from the incubator and observed for a color change. A sample containing no blue tint indicated a negative *GUS* result. A slight or pale blue to a darker blue indicated a positive GUS plant (McCabe and Martinell, 1993).

The second method did not require bringing the leaves into the lab and it greatly reduced a chance of confusing the plant numbers. A pipette was used to place 250 µL of the buffer into 1.5-ml micro-centrifuge tubes. These tubes had to be refrigerated if they were not used within 2 hr, in order to keep the buffer cool. In the field, a piece of each plant was clipped off and placed into the tube. Then, the tube was attached directly to the plant. The leaf segments incubated over-night on the plant, and they were observed in the field the next morning.

Pollen Assays

In order to prevent contamination with pollen from other plants, the pollen had to be taken from self-pollinated flowers. Every afternoon, unopened flowers on *GUS* positive plants were paper clipped at the tips. They were paper clipped at the tips to prevent pollination by an outside vector, but also in a way to prevent harming the female part on the inside of the flower. The flower was tagged and a leaf of the plant was spray painted. The next morning, the flowers were collected. If the flower had opened, it was discarded. If the flower was still closed in a way that prevented outside pollination, it was collected and labeled in accordance to the plant number. In the lab, the pollen was dislodged from the flowers by tapping onto weigh paper. The pollen was then placed into one well of a 24-well tissue culture plate. The goal was to get between 300 and 600 grains into each well. It was very important to make sure that the pollen only got into one of the wells, so as to prevent mixing, and in turn, contamination. Approximately 250 µL of buffered substrate was placed into each well. Each plate was then swirled gently to disperse the pollen throughout the well, and then incubated at 37°C for 2 hr. Afterwards, they were observed under a microscope. Grains that were stained any gradation of blue were compared to the unstained grains, and a ratio was obtained. The percent of blue pollen was recorded. A lack of blue color denoted a homozygous negative flower. A fifty-percent ratio denoted a heterozygous plant. Eighty to one hundred percent stain denoted a homozygous positive plant.

Chi-square goodness-of-fit tests to known Mendelian ratios were used to analyze the results of the *GUS* assays. The equation for chi-square is $X^2=\Sigma[(O-E)^2/E]$. One degree of freedom and a 95% level of significance was used for all tests.

Results

The ratios of the expected GUS expressive to the non-expressive plants evaluated in the 1997 DP 50 progeny rows (Table 1) were based on the inferred genotypes of the 1996 parent plants. Genotypes of the 1996 parent plants were inferred from GUS expression in pollen. Based on a visual estimate, plants producing about 50% GUS positive pollen were assumed to be heterozygous at the GUS locus, while plants producing mostly GUS positive pollen were assumed to be homozygous. GUS positive F1 plants selfed to produce F2 populations were assumed to be heterozygous at the GUS locus. Our objective was to test these assumptions regarding the GUS genotype of DP 50 plants and F1s, because transformation may affect inheritance of the inserted gene. Particle bombardment transformation can result in multiple insertions in the genome, which could lead to unexpected segregation patterns. Additionally, the presence of the foreign gene may affect the fitness of the male and female gametes that could also affect GUS segregation patterns.

Our data supports the hypothesis of unusual inheritance of the GUS gene. None of the F2 populations segregated in a Mendelian fashion, in that significantly fewer GUS positive progeny were recovered than expected based on segregation for a single dominant gene (Table 2). We do not know if the segregation for GUS reflects the effects of multiple loci, or altered fitness of gametes containing the GUS gene. In a similar fashion, we found unusual segregation for GUS in the DP 50 progeny rows. Three DP 50 progeny rows (M16 6 op, M16 12 op, and M16 21x) contained one or two GUS negative plants (Tables 1,2), that were not predicted based on the apparent homozygous GUS genotype of the parent plant (data not shown). We also found one DP 50 progeny row (M16 73x) that did not produce an expected 3:1 ratio of GUS positive to GUS negative plants.

Discussion

The purpose of this project was to provide the plant breeder with some direction for future research in agricultural plant breeding. Our data shows that transgenic populations may not always follow established segregation rules. We found that apparently heterozygous plants produced significantly fewer GUS positive progeny than expected. Additionally, apparently homozygous plants produced some GUS negative progeny. Thus, transgenic breeding efforts may require larger population sizes relative to non-transgenic efforts for the breeder to have the opportunity to select for characteristics other than the presence of a transgene.

This experiment provides results that can inform the breeder of the presence of the transgene, provide ratios that test the fitness of the *GUS* gene, test the accuracy and reliability of *GUS*, and aid in the planning of the next step in the breeding program.

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Table 1. Populations of transgenic cotton assayed for *GUS* expression. The first set of characters (M17...) denotes a genetic line. The second set of numbers indicates the 1996 plant number that was crossed with a cultivar to produce the F_1 . It was allowed to self-pollinate in the greenhouse during the winter of 1996-1997. The x or the o.p. was attached to the last seven DP50 populations to indicate self-pollinated or open-pollinated respectively. These ratios were gathered using known Mendelian ratios and the genotypes of the parent plants.

Populations of Transgenic Cotton	
Population	Ratio of Expected Positive to Negative
M17 30/MD51 F_2	3:1
M16 11/SG501 F_2	3:1
M18 12/SG501 F_2	3:1
M18 12/SG501 F_2	3:1
M16 8/93054 F_2	3:1
DP50 Progeny Rows	
M16 1x	1:0
M16 6op	1:0
M16 12x	1:0
M16 12op	1:0
M16 21x	1:0
M16 73x	1:1

Table 2. Raw data collected from the leaf assays comparing the number of plants expressing *GUS* to the number not expressing *GUS*. The number of plants expected to express *GUS* is also compared to the number of *GUS* expressive collected plants.

Leaf Assay Data		
Population	Number Positive Expected/Observed	Number Negative Expected/Observed
M17 30/MD51 F_2	80 / 47	27 / 60
M16 11/SG501 F_2	83 / 68	28 / 43
M18 12/SG501 F_2	90 / 46	30 / 75
M18 12/SG501 F_2	94 / 54	31 / 71
M16 8/93054 F_2	123 / 57	41 / 107
DP50 Progeny Rows		
M16 1x	15 / 15	0 / 0
M16 6op	42 / 41	0 / 1
M16 12x	11 / 11	0 / 0
M16 12op	49 / 47	0 / 2
M16 21x	21 / 20	0 / 1
M16 73x	240 / 195	241 / 286

Table 3. Chi square analysis values and probabilities for different genotypes in transgenic cotton. The results are not significant unless the probability is equal to or less than 5 %.

Chi square Analysis		
Population	Chi-Square Value	Probability
M17 30/MD51 F_2	53.95	<0.05
M16 11/SG501 F_2	10.75	<0.05
M18 12/SG501 F_2	89.01	<0.05
M18 12/SG501 F_2	68.63	<0.05
M16 8/93054 F_2	117.13	<0.05
DP50 Progeny Rows		
M16 1x	0	>0.05
M16 6op	.02	>0.05
M16 12x	0	>0.05
M16 12op	.08	>0.05
M16 21x	.05	>0.05
M16 73x	17.22	<0.05