

# Inheritance and segregation of exogenous genes in transgenic cotton

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## Abstract

Three transgenic cotton varieties (lines) were chosen for the study of inheritance and segregation of foreign Bt (*Bacillus thuringiensis* toxin) and *tfdA* genes in cotton. The transformed cotton varieties CCRI 30 and NewCott 33B expressing the Bt *cryIA* gene, and cotton line TFD expressing the *tfdA* gene were crossed with CCRI 19, CCRI 12 and Lumian 6. The results confirm inheritance and segregation of (i) the exogenous Bt gene in transgenic CCRI 30 and NewCott 33B, governing resistance to bollworm, and (ii) the exogenous *tfdA* gene in transgenic TFD, governing resistance to the herbicide 2,4-D. Both resistance characters were governed by a single dominant nuclear gene, and were not affected by cytoplasm. Our data support the conclusion that foreign traits encoded by single genes are inherited and expressed in Mendelian fashion in cotton. Our results also indicate that a practical backcross breeding program could be used to develop cotton cultivars combining one or more resistance traits from foreign and native gene sources.

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## Introduction

Cotton is an important economic and fibre crop worldwide. In 1987 Umbeck *et al.* first obtained transgenic cotton plants. These transgenic cotton plants carried two marker genes, encoding neomycin phosphotransferase II (NPT-II) and chloramphenicol acetyltransferase (CAT), which specify resistance to the antibiotics kanamycin and chloramphenicol, respectively. Since then, much progress has been made in genetic engineering of cotton (Zhang and Zhao 1997). Many laboratories have obtained insect-resistant (Zhang and Feng 1998; Zhang and Zhang 1998) or herbicide-resistant transgenic cotton plants via *Agrobacterium*-mediated transformation (Umbeck *et al.* 1987; Rajasekaran *et al.* 1996), particle bombardment (Finer and McMullen 1990; McCabe and Martinell 1993), or pollen tube pathway (Zhang *et al.* 2000). Up to now, at least seven foreign genes have been introduced into the cotton genome. Of these, Bt (Perlak *et al.*

1990; Cousins *et al.* 1991; Xie *et al.* 1991; Jenkins *et al.* 1997; Ni *et al.* 1998), CpTI (Li *et al.* 1998a), API (Thomas *et al.* 1995) and STKI (Wang *et al.* 1998) genes provide resistance to insect pests; *tfdA* (Bayley *et al.* 1992; Lyon *et al.* 1993; Chen *et al.* 1994), AHAS (Rajasekaran *et al.* 1996) and *bar* (Keller *et al.* 1997) genes provide resistance to herbicides. Other genes, for disease resistance, drought resistance, salt resistance, cold resistance and fibre improvement, are being sought for introduction into cotton.

Cotton is among the first transformed crops to be commercialized, yet the inheritance and segregation of exogenous genes in commercial transgenic cotton varieties have not been reported. Although several recent studies have reported the inheritance and expression of some marker genes (NPT-II gene and CAT gene) and Bt *cryIA*, and environmental effects on expression of these genes (Sachs *et al.* 1998; Umbeck *et al.* 1989; Li *et al.* 1998), these studies were limited to the transgenic regenerative plants. Up to now, all of the commercial transgenic cotton varieties are not transgenic regenerative plants; they were bred by cross and/or backcross between transgenic regenerative plants and commercial varieties.

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It is very important to understand the inheritance, segregation and stability of exogenous genes for a given transgenic event. We conducted this study to plan an efficient plant breeding strategy and ensure the stability of expression in different genetic backgrounds. The objective was to characterize the inheritance, segregation and stability of two foreign genes (Bt and *tfdA*) in commercial transgenic cotton varieties.

## Materials and methods

**Plant material:** Two commercial transgenic insect-resistant cotton varieties, CCRI 30 and NewCott 33B, and one transgenic herbicide-resistant cotton line, TFD, were selected for this study. CCRI 30 and NewCott 33B carry a Bt (*Bacillus thuringiensis*) gene and express the CryIA insecticidal proteins for resistance to *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae). TFD carries the *Alcaligenes eutrophus tfdA* gene which encodes 2,4-D monooxygenase, which confers resistance to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). CCRI 30 was provided by the Cotton Research Institute of the Chinese Academy of Agricultural Sciences, NewCott 33B comes from the commercial variety bred by Monsanto and Delta and Pine Land Company. CCRI 30 was passed for wide planting after a national examination and approval in 1998. NewCott 33B was imported by the Jida Company and was permitted for planting in 1997, and passed the biosafety evaluation in 1998 by the Chinese government. TFD comes from the Cotton Research Institute in Shanxi Province, and then was purified by selfcrossing at the Cotton Research Institute. The nontransgenic varieties used, CCRI 12, CCRI 19 and Lumian 6, come from the Cotton Research Institute. All materials were purified by continuous two-generation selfcross, to ensure the purity of resistance character genes.

**Experimental design:** Transgenic and nontransgenic cotton were planted by conventional method. At luxuriant blooming, CCRI 30, NewCott 33B and TFD were selfcrossed or crossed with conventional nontransgenic cotton cv. CCRI 12, CCRI 19 and Lumian 6. More than 100 selfcrossed or crossed bolls were harvested for the next experiments.

In the next year, the selfcrossed populations of transgenic materials and the F<sub>1</sub> seeds were planted in the field, and were examined for resistance to bollworm or 2,4-D by the methods described below. At the same time, more than 50 F<sub>1</sub> plants were selfcrossed or backcrossed with recurrent parents to produce F<sub>2</sub> and BC<sub>1</sub> populations.

In the third year, the F<sub>2</sub> and BC<sub>1</sub> seeds were planted in the field, and resistance was checked by the same method. The BC<sub>1</sub> plants were backcrossed with recurrent parents to produce BC<sub>2</sub> populations.

**Identification of insect resistance:** At six-to-eight-leaf stage, three to five bollworms were placed on the shoot of all test

plants. Five days later, survival of bollworm and damage to the cotton plant were examined. Plants without live bollworms and no damage were classified as insect-resistant plants. Plants with live bollworm(s) and much damage were classified as not insect resistant.

**Identification of herbicide resistance:** The identification of resistance to 2,4-D was conducted in the field. At four-leaf stage, test plants were sprayed with 200 mg/L 2,4-D. After two weeks, growth of the plants was investigated. The plants without damage were resistant plants and the damaged plants were nonresistant.

**Data analysis:** Chi-square goodness-of-fit tests were performed on data from the F<sub>2</sub>, F<sub>3</sub>, BC<sub>1</sub> and BC<sub>2</sub> populations derived from crosses between CCRI 30, NewCott 33B or TFD and the nontransgenic conventional varieties CCRI 12, CCRI 19 or Lumian 6 to determine if the observed segregation ratios of Bt-positive or *tfdA*-positive plants to negative plants fit the expected Mendelian 3:1, 7:5 or 1:1 phenotypic ratio, respectively.

## Results and analysis

### *Inheritance and segregation of exogenous Bt gene in transgenic cotton*

After five days of exposure to bollworms, conventional cotton variety CCRI 19 was damaged badly in leaves and shoots. The leaves showed many holes and the shoots had been bitten off. The transgenic insect-resistant Bt-cotton, both CCRI 30 and NewCott 33B, grew well, and there was almost no trace of damage by bollworms.

Transgenic cotton varieties CCRI 30 and NewCott 33B were crossed with conventional cotton variety CCRI 19 to produce F<sub>1</sub> populations. The F<sub>1</sub> plants grew healthily and were not damaged by bollworms. The results (table 1) indicate that they are all insect-resistant plants. The results confirm that the insect-resistance character controlled by the exogenous Bt gene is a dominant character. It did not matter whether the transgenic insect-resistant cotton cultivars CCRI 30 and NewCott 33B were male parent or female parent in the cross with conventional cotton cultivar CCRI 19; all of the F<sub>1</sub> plants resisted bollworms (table 1). This indicates that the insect-resistance characteristic controlled by the Bt gene was not affected by cytoplasmic factors.

**Table 1.** Resistance of Bt-cotton F<sub>1</sub> populations to bollworm.

Cross	Resistant	Susceptible	Total
NewCott 33B × CCRI 19	89	0	89
CCRI 19 × NewCott 33B	107	0	107
CCRI 30 × CCRI 19	63	0	63
CCRI 19 × CCRI 30	97	0	97

**Table 2.** Segregation of backcross and F<sub>2</sub> populations for resistance to bollworm.

Cross	Total	Resistant	Susceptible	Ratio	Test ratio	$\chi^2$	P
NewCott 33B × CCRI 19 F <sub>2</sub>	243	183	60	3.05:1	3:1	0.012	0.50 ~ 0.95
CCRI 19 × NewCott 33B F <sub>2</sub>	357	266	91	2.92:1	3:1	0.031	0.50 ~ 0.95
CCRI 30 × CCRI 19 F <sub>2</sub>	277	208	69	3.01:1	3:1	0.001	0.95 ~ 0.99
CCRI 19 × CCRI 30 F <sub>2</sub>	433	326	107	3.05:1	3:1	0.019	0.50 ~ 0.95
(NewCott 33B × CCRI 19) × NewCott 33B BC <sub>1</sub>	58	58	0				
(CCRI 19 × NewCott 33B) × NewCott 33B BC <sub>1</sub>	67	67	0				
(CCRI 30 × CCRI 19) × CCRI 30 BC <sub>1</sub>	63	63	0				
(CCRI 19 × CCRI 30) × CCRI 30 BC <sub>1</sub>	63	63	0				
(NewCott 33B × CCRI 19) × CCRI 19 BC <sub>1</sub>	140	69	71	0.97:1	1:1	0.029	0.50 ~ 0.95
(CCRI 19 × NewCott 33B) × CCRI 19 BC <sub>1</sub>	204	102	102	1:1	1:1	0	>0.99
(CCRI 30 × CCRI 19) × CCRI 19 BC <sub>1</sub>	265	135	130	1.04:1	1:1	0.094	0.50 ~ 0.95
(CCRI 19 × CCRI 30) × CCRI 19 BC <sub>1</sub>	237	118	119	0.99:1	1:1	0.004	0.95

To determine how many Bt gene copies were present in the cotton genome, F<sub>1</sub> plants of Bt-cotton and conventional cotton were selfcrossed to produce F<sub>2</sub> seeds and backcrossed with recurrent parent cultivars to produce BC<sub>1</sub> seeds. The phenotypic segregation ratios of the insect-resistance character and Chi-square values are presented in table 2. Phenotypic segregation ratios in F<sub>2</sub> populations are 2.92–3.05 : 1, and the Chi-square test indicated that segregation in F<sub>2</sub> populations fits the Mendelian 3:1 monogenic ratio. The results indicate that the insect-resistance characteristic conferred by the Bt gene is controlled by one pair of dominant genes. Phenotypic segregation ratios in BC<sub>1</sub> and their Chi-square goodness-of-fit tests also support this conclusion (table 2). In addition, the test results of BC<sub>1</sub> populations also indicate that the insect-resistance character provided by the Bt gene was not affected by cytoplasmic factors.

**Inheritance and segregation of exogenous *tfdA* gene in transgenic cotton**

Cotton is highly sensitive to 2,4-D. Even low concentrations of 2,4-D make cotton leaves to twist and fall off, cause great damage to the crop, and result in yield loss. Use of 2,4-D is prohibited in cotton fields and adjacent fields. Genetically engineered broadleaf herbicide protection for 2,4-D in cotton was obtained by incorporating an *Alcaligenes eutrophus* gene encoding 2,4-D monooxygenase (*tfdA*). Transformants carrying *tfdA* exhibited 50-fold to 100-fold more tolerance to 2,4-D compared with nontransformed plants (Bayley *et al.* 1992; Chen *et al.* 1994). We have bred transgenic cotton carrying the *tfdA* gene, and obtained elite transgenic 2,4-D-resistant cotton line TFD. Field examination indicated that TFD is highly resistant to 2,4-D. TFD plants could grow well when 1000 mg/L of 2,4-D was sprayed on their leaves. In contrast, 50 mg/L of 2,4-D could greatly damage nontransgenic cotton leaves and plants.

It is very important to research the inheritance and segregation of exogenous *tfdA* gene in transgenic cotton for breeding and application of transgenic 2,4-D-resistant cotton cultivars. We found that when TFD was crossed

**Table 3.** Resistance of *tfdA*-cotton F<sub>1</sub> progeny to 2,4-D.

Cross	Resistant	Susceptible	Total
TFD × CCRI 19	36	0	36
CCRI 19 × TFD	30	0	30
TFD × CCRI 12	27	0	27
CCRI 12 × TFD	32	0	32
TFD × Lumian 6	53	0	53
Lumian 6 × TFD	28	0	28

with CCRI 12, CCRI 19 or Lumian 6, irrespective of whether TFD was the male or female parent, all of the F<sub>1</sub> plants resisted 2,4-D (table 3). The result confirms that the herbicide-resistance characteristic provided by the exogenous *tfdA* gene is a dominant phenotype and is not affected by cytoplasmic factors.

Segregation for resistance was observed in F<sub>2</sub> populations of crosses of TFD and the conventional cultivars, and some of the F<sub>2</sub> plants were highly resistant to 2,4-D and others were not (table 4). The phenotypic segregation ratios (resistant : susceptible) were in the range 2.79–3.54 : 1. By the Chi-square test, the results fit the theoretical Mendelian segregation ratio of 3:1. This indicates that the herbicide-resistance character provided by the exogenous *tfdA* gene is a dominant character and is controlled by a pair of genes. It also indicates that one copy of *tfdA* was inserted in the cotton genome when cotton explants were transformed by the *Agrobacterium*-mediated method.

Segregation for resistance in the F<sub>3</sub> populations derived from TFD and CCRI 19 is also shown in table 4. The results indicate that segregation in the F<sub>3</sub> populations generally fit the Mendelian 7:5 monogenic ratio. These results support the conclusions from the F<sub>2</sub> populations.

The segregation ratios of BC<sub>1</sub> populations (table 5) also confirm that the herbicide-resistance character is governed by a single dominant nuclear gene pair. When conventional cultivars CCRI 12, CCRI 19 or Lumian 6 were the recurrent parent, the resistance segregation ratios in BC<sub>1</sub> populations were 0.84–1.26 : 1, which fit the Mendelian 1:1 monogenic ratio. When TFD was used as the recurrent parent, all BC<sub>1</sub> plants were herbicide resistant.

**Table 4.** Segregation of F<sub>2</sub> and F<sub>3</sub> populations for resistance to 2,4-D.

Cross	Total	Resistant	Susceptible	Ratio	Test ratio	$\chi^2$	<i>P</i>
TFD × CCRI 19 F <sub>2</sub>	125	92	33	2.79:1	3:1	0.131	0.50 ~ 0.95
CCRI 19 × TFD F <sub>2</sub>	118	92	26	3.54:1	3:1	0.554	0.30 ~ 0.50
TFD × CCRI 12 F <sub>2</sub>	131	99	32	3.09:1	3:1	0.023	0.50 ~ 0.95
CCRI 12 × TFD F <sub>2</sub>	131	96	35	2.74:1	3:1	0.206	0.50 ~ 0.95
TFD × Lumian 6 F <sub>2</sub>	127	96	31	3.10:1	3:1	0.024	0.50 ~ 0.95
Lumian 6 × TFD F <sub>2</sub>	98	73	25	2.92:1	3:1	0.014	0.50 ~ 0.95
TFD × CCRI 19 F <sub>3</sub>	128	78	50	7.80:5	7:5	0.356	0.50 ~ 0.95
CCRI 19 × TFD F <sub>3</sub>	109	67	42	7.98:5	7:5	0.442	0.50 ~ 0.95

**Table 5.** Segregation of BC<sub>1</sub> populations for resistance to 2,4-D.

Cross	Total	Resistant	Susceptible	Ratio	Test ratio	$\chi^2$	<i>P</i>
(TFD × CCRI 19) × CCRI 19 BC <sub>1</sub>	97	48	49	0.98:1	1:1	0.010	0.50 ~ 0.95
(CCRI 19 × TFD) × CCRI 19 BC <sub>1</sub>	86	48	38	1.26:1	1:1	1.163	0.20 ~ 0.30
(TFD × CCRI 12) × CCRI 12 BC <sub>1</sub>	88	44	44	1:1	1:1	0	>0.99
(CCRI 12 × TFD) × CCRI 12 BC <sub>1</sub>	101	47	54	0.87:1	1:1	0.485	0.30 ~ 0.50
(TFD × Lumian 6) × Lumian 6 BC <sub>1</sub>	76	39	37	1.05:1	1:1	0.053	0.30 ~ 0.50
(Lumian 6 × TFD) × Lumian 6 BC <sub>1</sub>	59	27	32	0.84:1	1:1	0.424	0.50 ~ 0.95
(TFD × CCRI 19) × TFD BC <sub>1</sub>	62	62	0				
(CCRI 19 × TFD) × TFD BC <sub>1</sub>	31	31	0				
(TFD × CCRI 12) × TFD BC <sub>1</sub>	59	59	0				
(CCRI 12 × TFD) × TFD BC <sub>1</sub>	58	58	0				

## Discussion

This study was undertaken to determine the inheritance and segregation of two exogenous genes (Bt insecticidal protein gene and *tfdA* herbicide resistance gene) in commercial cotton cultivars. Knowledge of the inheritance and segregation pattern of the inserted gene would provide the opportunity to develop transgenic germplasm, strategies to maximize the efficiency of developing improved germplasm, and the ability to breed fine varieties carrying these foreign resistance genes.

The Bt and TfdA phenotypes segregated as simple dominant Mendelian traits. This is consistent with the earlier reports by Perlak *et al.* (1990) and Sachs *et al.* (1998). These two exogenous genes can thus be inherited steadily into progeny, which indicates that we can obtain new fine varieties via crossing and/or backcrossing conventional varieties with the transgenic variety.

Non-Mendelian segregation of exogenous Bt gene was observed in F<sub>2</sub> populations derived from transgenic regenerative plants and conventional cotton lines (Sachs *et al.* 1998). This inheritance pattern was not observed in commercial transgenic cotton cultivars. The reason may be that some of the exogenous genes were inserted into non-nuclear genomes during transformation. These genes could be lost in crosses with commercial cotton cultivars.

Transformation is considered to be a random event, with each inserted gene at a unique location (Kohel *et al.* 2000). Thus, each transgenic plant is the result of a separate insertion event, and this event could translate to different degrees of success in attempts at backcross improvement, depending on associated linkages (Kohel *et al.* 2000). The

number and sites of gene insertions into the plant genome have been found to influence gene expression in transgenic regenerative cotton plants. Several researchers have found differences in injury by tobacco budworm (*Heliothis virescens*) and in the behaviour, growth and survival of the budworm (Benedict *et al.* 1992, 1996). These differences were attributed to positional effects on Bt gene expression and/or somaclonal variation (Benedict *et al.* 1996).

## Conclusions

Our results have demonstrated the Mendelian inheritance of exogenous genes in cotton. Exogenous Bt and *tfdA* genes can be steadily inherited and expressed in commercial transgenic cotton cultivars, free of cytoplasmic effects. The insect-resistance and herbicide-resistance characters are governed by a single dominant nuclear Bt or *tfdA* gene pair, respectively. Classical cross and backcross can introgress the exogenous resistance genes of the transformant into conventional cotton varieties, and one can breed new cotton varieties carrying desired characters such as insect resistance, disease resistance and so on.

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