

## Stacking of *cryIAb* and *Chitinase* Genes in Commercial Cotton Varieties through Crossing

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### ABSTRACT

Cotton (*Gossypium hirsutum*) is one of the most important fiber crops with a raw material value of \$5.5 billion per year worldwide. Cotton breeders with the help of conventional breeding programs have improved the cotton germplasm for resistance to pests and pathogens. However, there has been little success in producing cotton lines resistant to biotic stresses, due to limited cotton germplasm with adequate resistance to biotic stresses, especially pests and diseases. In this study, *cryIAb* (resistance to cotton bollworm) and *chitinase* (resistance to cotton Verticillium wilt) genes were transferred to Iranian commercial cotton varieties (Varamin, Khordad, Sahel, and Bakhtegan), by crossing transgenic lines containing *cryIAb* and *chi* transgenes with Iranian commercial varieties. To recover genetic background of commercial varieties, the obtained progenies were backcrossed with own commercial parent. The progenies of backcrosses were assessed by western blot and PCR analysis. Insect and fungus *in vitro* bioassay showed resistance against cotton bollworm and *Verticillium dahliae* in plants containing the stacked transgenes. According to the results, *cryIAb* and *chi* genes were successfully transferred and stacked in Iranian commercial cotton varieties. Also, plants with two transgenes and plants with one transgene were protruded to BC<sub>1</sub> and BC<sub>2</sub> generations, respectively.

**Keywords:** Cotton bollworms, Multiple resistances, Transgene, Verticillium wilt.

### INTRODUCTION

Cotton (*Gossypium hirsutum*) is an important fiber plant and inexpensive source of edible protein and oil worldwide, which grows in a wide range of soil and climate condition (Rios, 2015). Cotton breeders have improved cotton germplasm using conventional plant breeding methods (Agrawal *et al.*, 1997; McCarty *et al.*, 2004; Lukonge *et al.*, 2008; Zeng *et al.*, 2011). Although many problems have been solved in cotton cultivation by these breeding methods, further developments in cotton

breeding have been challenging due to limitations in access to germplasm with resistance to biotic stresses, especially pests and diseases (Bakhsh *et al.*, 2015). With the advent of recombinant DNA methods and genetic engineering, production of resistant plants to pests and diseases is feasible by gene transfer from unrelated organisms (Sharma and Ortiz, 2000). Therefore, with transfer of anti-insect genes, such as *cryIAb* and *cryIAC* (Katageri *et al.*, 2007; Taverniers *et al.*, 2008; Bakhsh *et al.*, 2012; Khan *et al.*, 2013; Awan *et al.*, 2015), and anti-fungal genes, such as *Chitinase* and *Glucanase* (Tohidfar *et al.*, 2005; Tohidfar

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et al., 2012; Cheng et al., 2005), into cotton plant, one can produce varieties resistant to pests and diseases.

In cotton plant, most desirable genes were initially transferred into Coker, and then to cotton cultivar(s) of interest by conventional backcrossing breeding methods (Satyavathi et al., 2002, Vinodhana et al., 2014). In addition to producing transgenic plants using a single gene, it is possible to introduce two or more transgenes into a plant and produced transgenic plants with multiple resistances to different types of stresses. These compounds are referred to as 'stacked' or 'pyramided' traits (Taverniers et al., 2008) which are preferred by most of the farmers around the world. In total, area under cultivation stacked crops in 2015 had increased 14 percent equivalent to 7.1 million hectares, from 51.4 million hectares in 2014 to 58.5 million hectares in 2015 (James, 2015). The stacked transgenes have been applied in several crops, such as rice to achieve resistance to three pests including rice leaf folder (*Cnaphalocrocis medinalis*), yellow stem borer (*Scirpophaga incertulas*) and brown plant hopper (*Nilaparvata lugens*) (Maqbool et al., 2001); and resistance to yellow stem borer (*Scirpophaga incertulas*) and bacterial blight disease (caused by *Xanthomonas oryzae* pv *oryzae*) (Datta et al., 2002). The same method was applied to tobacco to create resistance to cotton bollworm (*Helicoverpa armigera*) bacterial soft rot disease (caused by *Erwinia carotovora*) and damping-off disease (caused by *Pythium aphanidermatum*) (Senthilkumar et al., 2010); maize for resistance to insect lepidopteran species and glyphosate-based herbicides (Agapito-Tenfen et al., 2014); pepper for resistance to tobacco cutworm (*spodoptera litura*) and tolerance to drought stress (Zhu et al., 2015), etc. Currently, the most extensively used method for stacking of multiple genes in one plant is crossing between different transgenic plants containing one or more genes of interest (Zhao et al., 2015).

Although the gene stacking approach to create multiple resistances is not novel, no

reports have described stacking *cry* and *chitinase* genes for resistance to cotton bollworms (*Heliothis armigera*) and Verticillium wilt disease (caused by *Verticillium dahliae*) in cotton plant. The purpose of this study was to stack *cry1Ab* and *Chitinase* genes in Iranian commercial cotton varieties by sexual crossing method and retrieving genetic background of commercial varieties using backcrossing method.

## MATERIALS AND METHODS

### Plant Material

Two stable transgenic Bt and chi lines that presented a desirable level of resistance expression for a few generations, non-transgenic cotton 'Coker 312' variety (as control) and four Iranian commercial cotton varieties (Varamin, Khordad, Sahel and Bakhtegan) were used in this study. Transgenic cotton lines were the Bt line that carried the *cry1Ab* gene in the genetic background of Coker 312 for resistance to *Heliothis armigera* pest (Tohidfar et al., 2008) and the chi line carrying *chitinase* gene in the genetic background of Coker 312 for resistance to Verticillium wilt disease (Tohidfar et al., 2005; Tohidfar et al., 2012). All seeds were provided by the Agricultural Biotechnology Research Institute of Iran, Karaj.

The cultivation of seeds was done in 10 days intervals, so that the crossing was performed in different periods. After the confirmation of transgenic events in cultivated transgenic seedlings by PCR analysis, verified transgenic seedlings were grown to flowering stage in greenhouse with 14 hours light period at 25-30°C temperature.

### Cross Pollination

At the flowering stage, suitable buds were emasculated manually. After maturity of

stigma and anthers, artificial pollinations were performed and the flowers were covered with a clean cotton bag. Pollination date and parents' names were recorded on labels. Bolls were maintained on the maternal plants until formation and maturation of seeds (Figure 1).

In all crosses, commercial varieties were used as recurrent parents and transgenic lines as donor parents. In order to reduce flower abscission, peduncles were treated by 100 ppm of gibberellic acid for 7 days after crosses.

Backcrossing was performed with commercial varieties in order to produce commercial plants containing single transgene. In each generation, the presence of transgene was evaluated by Polymerase Chain Reaction (PCR) analysis.

Also, all of these processes were done the same for introducing two genes i.e. *cryIAb* and *Chitinase*, into genetic background of the commercial varieties.

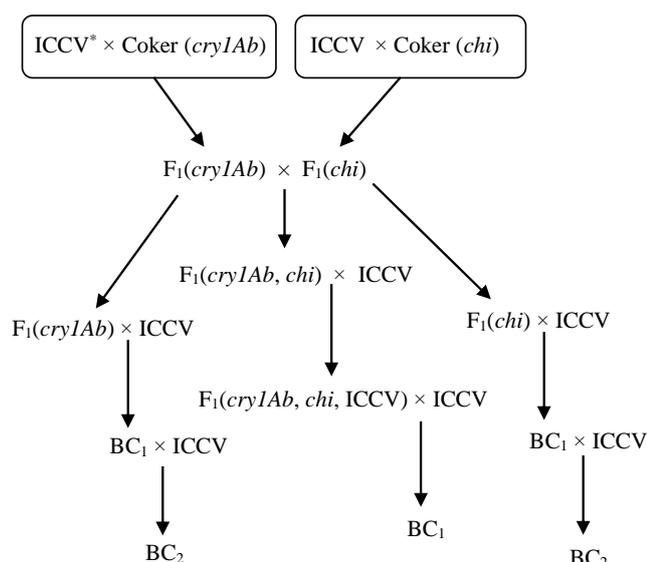
of  $F_1$ ,  $BC_1$  and  $BC_2$  transgenic and non-transgenic plants using the modified Cetyl-Trimethyl Ammonium Bromide (CTAB) method as described by Doyle (1991).

The PCR analysis was carried out in a 20  $\mu$ L reaction volume containing, 2  $\mu$ L of PCR buffer (10X), 2  $\mu$ L  $MgCl_2$  (25 mM), 1  $\mu$ L of dNTPs (10 mM), 1  $\mu$ L of each forward and reverse primers (10 pmol), 1  $\mu$ L template DNA (50–100 ng) and 0.2  $\mu$ L (1 U) *Taq* DNA polymerase. The specific primers for detection of *cryIAb* and *chi* transgenes are shown in Table 1.

PCR amplification profiles were as follow: initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C (*cryIAb* and *chi* genes) for 1 minute, extension at 72°C for 1 minute, and the final extension at 72°C for 5 minute. The amplified fragments were separated using 1.0% (w/v) agarose gel electrophoresis in 1X TAE buffer and detected by Ethidium bromide staining.

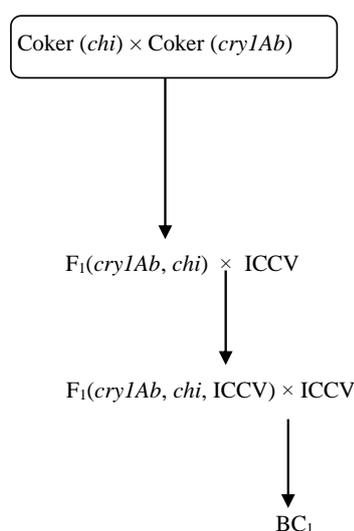
### Polymerase Chain Reaction (PCR) Analysis

Genomic DNA was extracted from leaves



### Western Blot Analysis

Total protein was extracted from leaves of  $BC_1$  transgenic (containing single and



**Figure 1.** Flowchart of crossing processes for producing transgenic plants and stacked plants. ICCV\* = Iranian Commercial Cotton Varieties.

**Table 1.** Primer sequences and length of amplified fragments in PCR and Duplex PCR.

Primer name	Primer sequences (5' to 3')	PCR product size (bp)
<i>cry1Ab</i>	F- CGCAAAGGGTATCAAGGTATCT	822
	R-CACGGACGGCTTCAATGGA	
<i>chi</i>	F-GAGTGGTGTGGATGCTGTTG	910
	FGCCATAACCGACTCCAAGCA	

stacking transgenes) and non-transgenic plants and western blot analysis was done according to Tohidfar *et al.* (2005, 2008).

### *In vitro* Assay for Antifungal Activity

Evaluation of resistance to *Verticillium* wilt was performed for BC<sub>1</sub> transgenic (stacked plants) and non-transgenic plants using non-defoliating strain (T<sub>1</sub>) of *Verticillium dahliae* as described by Tohidfar *et al.* (2005).

### Insect Bioassay

Insect bioassay was carried out as described by Tohidfar *et al.* (2008). It was conducted using the Neonate larvae of cotton bollworms (*Heliothis armigera*) and cut-leaves of BC<sub>1</sub> stacked plants in petri dish. Degree of leaf damage and insect mortality rates were measured using the method of Gallic *et al.* (1988) and was estimated as follow:

$$\text{Mortality\%} = \frac{\text{Number of dead larvae}}{\text{Total number of larvae}} \times 100$$

Also, degree of leaf damage was calculated using the method of Tohidfar *et*

*al.* (2008) (Table 2).

## RESULTS

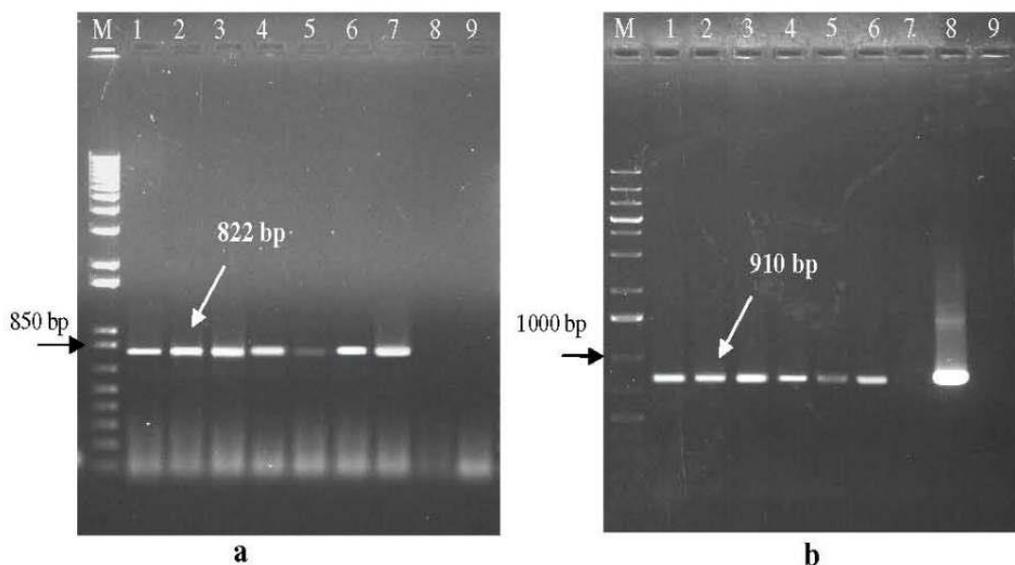
### Identification of Introgressed Transgenic Plants with *chi* and *cry1Ab* Genes

The presence of *cry1Ab* gene in BT lines and *chi* gene in *chi* lines was confirmed by PCR analysis and verified lines were used in crosses with Iranian commercial cotton varieties. In each generation, seedlings containing transgene(s) were confirmed by PCR analysis and used in the next crossing. Stacking of *cry1Ab* and *chi* genes was performed successfully in each four Iranian commercial cotton varieties (Varamin, Khordad, Sahel and Bakhtegan). The BC<sub>1</sub> and BC<sub>2</sub> generations were protruded to restore genetic background (70 %) of Iranian commercial cotton varieties and continued until BC<sub>6</sub> or BC<sub>7</sub> to retrieve complete genetic background of Iranian commercial cotton varieties.

Also, PCR analysis was conducted to verify the presence of expected genes in transgenic plants and progenies obtained

**Table 2.** Grading the extent of leaf damage.

The extent of leaf damage	Degree of leaf damage
Little or no damage (0-10%)	0
Mildly damaged (10-20%)	1
Moderately damaged (20-40%)	2
Severely damaged (40-70%)	3
Completely destroyed (70-100%)	4



**Figure 2.** (a) Detection of *cryIAb* gene with amplification of 822 bp fragment in BC<sub>1</sub> generation of stacked plants. (Lane M): Molecular weight marker 1 kb Plus DNA Ladder (Invitrogen BM211-01), (Lanes 1–6): Putative stacked plants (1: Khordad, 2: Varamin, 3: Varamin, 4: Khordad, 5: Khordad and 6: Varamin), (Lane 7): Positive control (pBI121-BCH plasmid), lane 8: non-transgenic plant (Coker 312), (Lane 9): Negative control (without template DNA). (b) Detection of *chi* gene with amplification of 910 bp fragment in BC<sub>1</sub> generation of stacked plants. (Lane M): Molecular weight marker 1 kb Plus DNA Ladder Thermo Scientific SM1343, (Lanes 1–6): Putative stacked plants (1: Khordad, 2: Varamin, 3: Varamin, 4: Khordad, 5: Khordad and 6: Varamin), (Lane 7): Non-transgenic plant (Coker 312), (Lane 8): Positive control (pBI121-*cryIAb* plasmid), (Lane 9): Negative control (without template DNA).

from the crosses (F<sub>1</sub>, BC<sub>1</sub> and BC<sub>2</sub> generations). The PCR analysis amplified 910 and 822 bp fragments corresponding to *chi* and *cryIAb* genes, respectively (Figure 2).

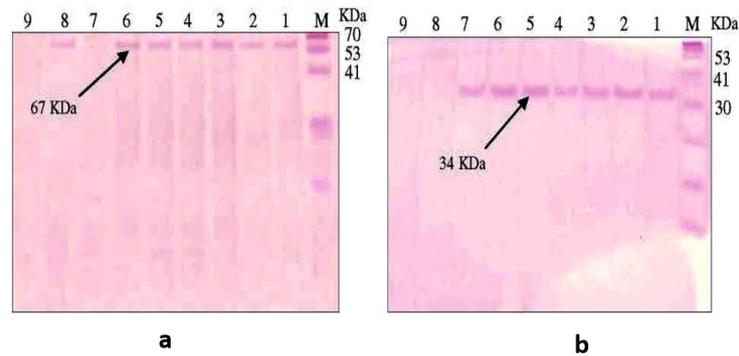
#### Western Blot Analysis

To demonstrate the expression of *cryIAb* and *chi* genes in BC stacked plants (containing 67 and 34 KDa bands, respectively), all of *cryIAb*- and *chi*-positive hybrids (selected based on PCR analysis) were subjected to western blot analysis (Figure 3). The expected bands were observed in positive hybrids, while these bands were not observed in non-transgenic plants. The results confirmed the expression of *cryIAb* and *chi* proteins in backcross

generations of each four stacked varieties. Therefore, the presence and expression of *cryIAb* and *chi* genes were confirmed by both PCR and western analysis.

#### *In vitro* Assay for Anti-Fungal Activity

*In vitro* anti-fungal activity assay was conducted against *Verticillium dahliae* in stacked Iranian commercial cotton varieties. According to the results, the growth of fungal mycelium was inhibited by BC<sub>1</sub> leaf protein extracts of each four stacked varieties (Varamin, Khordad, Sahel & Bakhtegan) containing *cryIAb* and *chi* genes (Data not shown) (Figure 3). Fungal growth was reduced around the sample wells containing leaf protein extracts of stacked plants. This inhibitory effect was

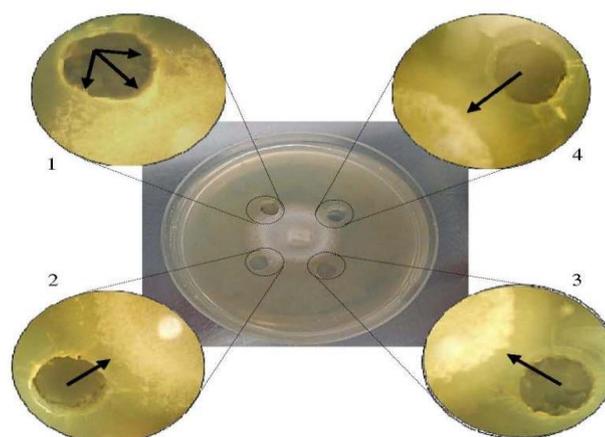


**Figure 3.** Western blot assays of *cryIAb* and *chi* gene expression in stacked plants ( $BC_1$  generations). (a) *cryIAb* gene expression. (b) *chi* gene expression. (Lane M): Molecular mass marker (Prestained protein ladder, 10-170 KDa); (Lane 1-8): Stacked plants (1: Khordad, 2: Varamin, 3: Sahel, 4: Bakhtegan, 5: Khordad, 6: Varamin, 7: Sahel and 8: Bakhtegan), (Lane 9): Non-transgenic plant (Coker 312).

proportional to the concentration of Chitinase extract used in plate bioassay. Therefore, the wells (3 & 4) containing 75 and 100  $\mu$ g leaf protein extracts had the most inhibitory effect. However, fungal growth had continued inside the well 1 that contained control (non-transgenic leaf protein extracts) (Figure 4). Among these four stacked varieties, highest anti-fungal activity against *Verticillium dahliae* was observed in stacked Sahel variety. Finally, bioassay examination of *V. dahliae* revealed the adverse effects of leaf protein extracts of stacked plants on fungal mycelia growth.

### Insect Bioassay

Insect bioassay was carried out on  $BC_1$  generation of plants by using cotton bollworm (*Heliothis armigera*) larvae. After being infected for 7 days, it was revealed that the larvae were dead or did not grow. But, the larvae that fed on non-transgenic control leaves (Coker 312) were mostly alive and well-developed (Table 3). The



**Figure 4.** *In vitro* anti-fungal activity assay against *V. dahliae* based on the leaf protein extracts of stacked plants containing *cryIAb* and *chi* genes. 1: Leaf protein extracts of control plant (Coker 312) and 2, 3, and 4: Concentrations of 50, 75, and 100  $\mu$ g leaf protein extracts of transgenic plant.

**Table 3.** Larvae mortality rates and leaf damage degrees in insect bioassay.

Stacked plants (In BC <sub>1</sub> generation)	Mortality (%)	Leaf damage
Stacked 'Varamin' variety	93.3	0.33
Stacked 'Khordad' variety	80	0.66
Stacked 'Sahel' variety	73.3	1.0
Stacked 'Bakhtegan' variety	86.6	0.33
Control plant (Coker 312)	20	2.66

mortality of cotton bollworm larvae ranged from 73.3 to 93.3% in stacked plants. As shown in Figure 5, the degrees of leaf damage of stacked plants were unnoticeable compared to the control non-transgenic plant leaves (Coker 312). Among these four stacked plants, highest resistance to cotton bollworm larvae related to 'Varamin stacked plant' variety.



**Figure 5.** Insect bioassay of cotton bollworm on BC<sub>1</sub> generation leaves of stacked plants containing *cryIAb* and *chi* genes. Leaf damage after 7 days incubated with larvae. (a) Stacked plant, (b) Control plant or non-transgenic plant (Coker 312).

## DISCUSSION

Stacked plants carrying more than one transgene offer wider economic benefit in complex agricultural conditions. However, gene stacking in plants is difficult in terms of creating transgenic plants with different favorable traits (Halpin, 2005). Although genetic engineering is an efficient method for gene transfer, it is dependent on tissue culture. Therefore, most of the time the genes will be introduced to regenerative varieties, which will be crossed to the commercial cultivars for the purpose of transgene introgression.

Despite, valuable successes in producing transgenic cotton, there are serious limitations in this context (Wang *et al.*, 2012). The most important limitations is low regeneration rate, that is dependent on the genotype (Ma *et al.*, 2013), type of explants, culture medium, temperature, light intensity, callus production and somaclonal variations (Smith *et al.*, 1990). *In vitro* regeneration rates are very low for Iranian cotton varieties due to the lack of efficient and optimized regeneration protocols. Therefore, in this attempt, we used the conventional plant breeding methods including sexual crossing between transgenic lines with origin of 'Coker' variety (which is amenable for gene transformation and *in vitro* regeneration) and Iranian commercial varieties to transfer our favorable genes (*cryIAb* and *chi*). Then, progenies were backcrossed with own recurrent commercial parent to retrieve



genetic background of commercial varieties. Finally, the results obtained from PCR and western blot analysis showed successful transfer and expression of transgenes (*cryIAb* and *chi*) in progenies. To complete retrieval of genetic background of commercial parents, backcrossing was continued until BC6 or BC7 generations. Finally, these stacked commercial varieties will be grown for field-testing and seed production.

Although transgenic plants with multiple resistances against biotic and abiotic stresses are very valuable and essential for sustainable agriculture, there is no report about stacking resistance genes against bollworm and Verticillium wilt in cotton. In this study, regardless of the genetic position, resistance to insect pest was improved in stacked Sahel and Varamin varieties. The effective resistance may be due to the similarity of genetic background of Coker with Sahel and Varamin varieties, because these varieties were obtained from Coker (Vafaie Tabar and Tajik Khavé, 2013).

The expression levels of transferred genes were different between Iranian commercial varieties, which can be due to genetic background effect and several internal and external factors (Rao *et al.*, 2011; Bakhsh *et al.*, 2012). Also, stability of transgenes is variable among transgenic plants, crop growth stage, and in different generations (Khan *et al.*, 2013).

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## انباشت ژن‌های *cryIAb* و کیتیناز در ارقام پنبه تجاری از طریق تلاقی

س. میرزایی، س. دژستان، و م. توحیدفر

### چکیده

پنبه (*Gossypium hirsutum*) یک گیاه بسیار مهم الیافی با ارزش مواد خام معادل ۵/۵ میلیارد دلار در سال در سراسر جهان است. اصلاح گران پنبه با کمک برنامه‌های اصلاح نباتات سنتی، ژرم پلاسم پنبه را برای مقاومت به آفات و عوامل بیماریزا بهبود بخشیده‌اند. با وجود این، موفقیت‌های محدودی برای تولید لاین‌های پنبه مقاوم به تنش‌های زیستی به دست آمده است که این امر ناشی از ژرم پلاسم پنبه با مقاومت کافی به تنش‌های زیستی به خصوص آفات و بیماری‌ها می‌باشد. در این تحقیق، ژن‌های *cryIAb* (مقاومت در برابر آفت کرم غوزه پنبه) و کیتیناز (مقاومت در برابر پژمردگی ورتیسلیومی پنبه) به ارقام تجاری پنبه ایران (ورامین، خرداد، ساحل و بختگان)، از طریق تلاقی لاین‌های تراریخته حاوی تراژن‌های *cryIAb* و *chi* با ارقام تجاری ایرانی منتقل شدند. برای بازیابی زمینه ژنتیکی ارقام تجاری، نتاج حاصل با والد تجاری خود تلاقی برگشتی داده شدند. نتاج حاصل از تلاقی برگشتی با آنالیز PCR و لکه‌گذاری وسترن ارزیابی شدند. آزمایشات زیست‌سنجی در شرایط آزمایشگاهی علیه قارچ و آفات حشره ای در گیاهان حاوی تراژن‌های انباشت‌شده، مقاومت در برابر آفت کرم غوزه پنبه و بیماری پژمردگی ورتیسلیومی پنبه را نشان دادند. با توجه به نتایج، ژن‌های *cryIAb* و *chi* با موفقیت به ارقام تجاری پنبه ایرانی منتقل و انباشت شدند. همچنین، گیاهان با دو تراژن و گیاهان با یک تراژن، به ترتیب تانسل‌های BC<sub>1</sub> و BC<sub>2</sub> تولید شدند.