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Research Article

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Optimizing *Agrobacterium*-mediated Transformations Procedures in Cotton Local Cultivars for Developing Bt Cotton in Pakistan

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ABSTRACT

Cotton is one of the world's largest fiber-producing crops. It is also known as the important cash crop of Pakistan. Cotton crop products contribute to economy as cottonseed oil, animal feed and the fiber support the textile industries, thus, playing a significant role in uplifting the economy of a country. But over time, bollworm attacks had escalated, severely reducing the yield of cotton and damaging cotton production. Bt (*Bacillus thuringiensis*) cotton is being widely used nowadays due to its enhanced resistance against cotton bollworms which are responsible for reducing crop production leading to high economic loss. It greatly affects the economy by increasing the cost of production and protection. Using resistant strains of Bt cotton not only makes it easier to combat bollworm attacks but also reduces the substantial expense issues that farmers face. Bt has many toxic proteins among which the two crystal proteins, *Cry1Ac* and *Cry2Ab*, are used to develop resistance against bollworms in cotton. This study aimed to optimize the developing transgenic callus containing *Cry1Ac* and *Cry2Ab* genes in cotton. The dual gene construct (*Cry1Ac* + *Cry2Ab*) provides more resistance to the cotton crop as compared to the single gene construct against whom the most bollworms have developed resistance. The vector was inoculated into the hypocotyl segments and the transformation process was preceded by shifting the hypocotyls at different mediums *Agrobacterium tumefaciens* strain LBA4404 was used for cotton transformation. The construct-containing vector was introduced into a standard cotton line. A molecular confirmation test was carried out utilizing PCR and gene-specific primers after the formation of the transgenic callus. As a result of the research an indigenous cotton line that expresses double Bt genes for insect resistance was developed.

Keywords: *Agrobacterium tumefaciens*, *Cry1Ac* and *Cry2Ab,* Dual gene construct, Transgenic callus

INTRODUCTION

Cotton is one of the world's largest fiber-producing crops. It is also termed white gold because it plays a vital role in up bringing the economy of a country. The cotton area under cultivation grew to 2.4 million hectares (ha) in 2023–24 from 2.1 million ha in the previous year, indicating a 13.1% rise [1]. On the other hand, its output increased to 10.2 million bales, a phenomenal 108.2 percent gain. During recent years, Punjab cultivated 4.7 million acres of cotton per year, producing only 7 million bales and 700 kg of lint each hectare [2]. Bahawalpur, Lodhran, Multan, Khanewal, Vehari, Sahiwal, Faisalabad, Rahim Yar Khan, Bahawalnagar, etc. are some of the principal cotton-growing areas in Punjab. Cotton production had been affected badly in the past due to increased attacks of bollworms which reduced the yield and economy. It is grown in hot and humid areas of Pakistan which are favorable sites for the growth of insects and pests [3].

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During the fiscal year 2015-16, the usual yield of cotton remained at 693 kg/ha, while the consumption of cotton remained at 23.80 million tons [4]. To enhance yield, a recent study emphasized role of *GhBRH1_A12* gene in Brassinosteroids signaling, influencing plant and boll size in the cotton [5].

Cotton, owing to its economic importance, has always been a top priority crop for farmers and the government. It contributes 5.1 percent of agricultural value-added [6]. The world's fourth leading cotton producer and third leading cotton user is Pakistan. Out of a total of 5 million farmers, cotton is grown by more than 1.5 million farmers on 2.91 million ha, accounting for almost 10-12% of the country's total agricultural land [7]. Over time, insect pests posed a significant threat to global cotton production, causing substantial economic losses [8]. Almost 15 insect pests attack cotton plants where American bollworm, pink bollworm, spotted bollworm, and army worm are the most destructive pests among them, aside from sucking pests [9]. A survey, conducted in India to study the impacts of transgenic cotton cultivation, estimated the socio-economic benefits of Bt cotton cultivation that could be achieved by every farmer to mitigate the rate of poverty. This was manifested by reducing the pesticide use and other chemicals that are very costly to afford by a common farmer [10]. A study reported that cultivating Bt cotton annually resulted in a high invasion of sucking insects thus making key pests of Bt cotton in China [11]. In context of fiber quality parameters, a recent study emphasized crucial role of Eriodictyol for increased growth of cotton fiber through mediating carbohydrate metabolism, manipulating phytohormone signaling, and stimulating flavonoid production [12]. Overall, the Increased yield of Bt cotton per hectare was also observed in Pakistan and China. Traditionally to control insects and pests \$8.1 billion per annum globally is spent on chemicals out of which \$2.7 billion is spent on cotton by pest management. Constant use of pesticides for controlling pest insects causes cotton plants to be more susceptible, which reduces cotton yield and harms the environment and human health. Weed infestation contributes to low cotton yield by 37%, making it one of the challenges faced by cotton farmers [13].

Transgenic cotton, having Cry protein, has brought up a huge revolution in controlling pests without harming nature, humans, and other living organisms [14]. It has provided farmers with many benefits by lowering the use of pesticides, a healthy environment, high yield preservation, beneficial insects, low production cost, and high profits to farmers. Transgenic cotton provides us with high cotton yield and sustainability [15]. But, with time the success ratio of transgenic cotton started decreasing when the pests developed a resistance mechanism against the Cry toxins produced by the Bt crops [16]. To obtain maximum yield of cotton, there is a great need to control pests and for this, it is important that the plant must produce toxins at a specific level, which is lethal to the targeted arthropods, and it should not survive above this target level. The toxic material must be produced by all parts of the plant and most importantly during the time of pest attack. It is also reported that the Bt toxins produced by the plant vary in their levels of production not only in the genotypes [17] but also in the different tissues and parts of the plant, and it decreases gradually as the plant ages [18]. The main concern of using Bt cotton was its increased resistance against lepidopterans and a huge reduction in the use of other chemicals and fertilizers [19].

Genetic transformation procedures are labor-intensive and time-consuming along with sophisticated lab environment and skill requirements. In this context, cotton varieties exhibiting efficient genetic transformation potential and adaptations could save time, money, and manpower [20]. Insect-resistant and weed-tolerant varieties of cotton being commercialized worldwide are mainly the result of Agrobacterium-mediated transformations. Although there are several other methods used to transfer genes such as particle bombardment and micro-injection, no other method success has been reported abundantly yet. In Pakistan, cultivation of Bt cotton started in 2010 with the approval of the eight Bt cotton varieties and one hybrid, currently grown on a total of 5.9 million acres, or almost 75% of the total cotton acre of the country [21, 22]. The main reason for Bt cotton adaptation is higher yield, resistance against bollworms, and reduced production cost [23, 24].

The disadvantage of Bt is the development of insect resistance and pests that are not effectively controlled by the cry toxins. The crucial one is insect resistance and five insect species reported which have developed resistance against Bt toxins [25-29]. This problem could be minimized by stacking another gene like *Vip3A*, i.e., vegetative expressed toxin [30]. When *Cry1Ac* + *Cry2Ab* were inserted into the cotton plant, the resistance against lepidopterans was increased. A synergistic relation had been reported between *Cry1Ac* and *Cry2Ab* against insects. So, a cotton variety with both *Cry1Ac* and *Cry2Ab,* genes will show a better resistance against lepidopterans as suggested in the study. For gene transformation in cotton, Coker varieties have been reported to efficiently respond to tissue culture and gene transformation procedures [31]. In Pakistan, all cotton GMO products are being imported and fewer transgenic syntheses are reported to be produced locally. This study aimed at optimizing agrobacterium-mediated gene transformation

procedures in local cotton cultivars to facilitate the production of Bt cotton using regional cultivars locally.

MATERIALS AND METHODS

The seeds of four cotton varieties Coker312, Cyto124, CIM554, and MNH786 were thankfully provided by Central Cotton Research Institute (CCRI) and Cotton Research Institute (CRI), Multan. The whole experiment was conducted at Quarantine Cotton Biotechnology Laboratory, Institute of Plant Breeding and Biotechnology, MNS-university of Agriculture Multan, Pakistan under controlled conditions as per procedures.

Vector construct: A double gene (*Cry1Ac* + *Cry2Ab*) construct in (Fig. 1) was thankfully provided by the Agricultural Biotechnology Research Institute (ABRI) at AARI, Faisalabad (NCBI Accession IDs: KX622797 and KX622798). A further process was followed by its transformation in *E. coli* and then it was spread on the LB agar plates containing kanamycin. After confirmation of the clone, Maxi Prep was done to transfer the construct to the *A. Tumefaciens* strain LBA4404 for transformation in cotton is mentioned in Table 1. *Agrobacterium tumefaciens* strain LBA4404 containing the two genes (*Cry1Ac* + *Cry2Ab*) construct will be used to carry out cotton transformation.

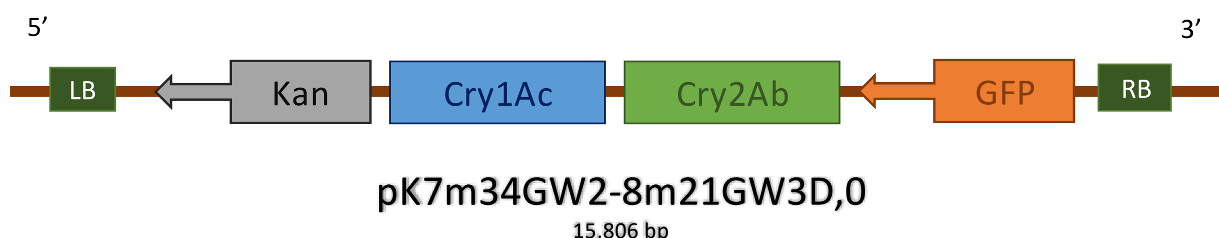

Figure 1. Graphical view of the vector construct.

Table 1. The sequences of forward and reverse primers that were used for detection of *Cry1Ac* and *Cry2Ab*

Sr. No	Primers	Primer's Sequence
1.	*Cry1Ac*	F:TTCTCTTCAACGGTTCTGTCAT
2.	*Cry1Ac*	R:ACTGGAATGAACTCGAATCTGT
3.	*Cry2Ab*	F:CCAGGCTCCATGACATGCTCGA
4.	*Cry2Ab*	R:TCGTTGGCGGCGTAGATGTTGT

Sowing and root stretching: The seeds of cotton were grown in a flask containing sowing media, dehusking of cotton seeds was done, and transferring the seeds to the sowing media under controlled conditions. The seeds were sterilized in 0.1% $HgCl_2$ solution and then washed with distilled H_2O. Seeds were transferred to the filter paper for drying and the flask safety cover was removed, seeds were transferred with the help of forceps into the flask containing sowing media. After sowing all the flasks were kept in the germinator at 28ºC under dark. After 48 hours, root stretching of all seeds in all flasks was done then the flaks were again transferred to the germinator at 28ºC under the dark.

Media Preparation: Non-selective and selective media, MGL, LB, and IBA media were prepared as per procedures previously reported [32] along with modifications. Non-selective media were prepared by adding peptone, yeast extract, and distilled water. Selective media was prepared to inhibit unwanted microbial growth. LB media was prepared using tryptone, yeast extract, sodium chloride, agar and distilled water. IBA media was prepared with indole-butyric acid, supplemented with nutrients and agar.

Vector inoculum preparation: About 4-5 days after root stretching, the plants were grown from cotton seedlings. Before starting the process of cutting the vector was cultured 24h prior and 20µl kanamycin and 20µl spectinomycin in case of selective media. Eppendorf of the cultured vector was prepared, covered, and placed in a shaker for 24h at 28ºC.

Hypocotyls cutting and inoculation: All the plants were taken out from the conical flasks with the help of preheated (later cooled for aseptic tissue handling) forceps and placed one by one on the petri plate containing sterilized filter papers. The roots and leaves

were cut and removed using a sharp sterilized blade. The elongated hypocotyls were kept on the petri plate and chopped into fine segments of 6-8 mm length pieces, injury sites on both ends thus facilitating agrobacterium inoculation. The vector placed in the shaker was taken out and poured on the double-cut hypocotyls. Later these were air-dried enough then they were shifted on 2, 4 D medium-nonselective media (no bubble in it). These petri plates were placed in the dark at 21ºC for 48h.

Callus formation: After 48h the transformed hypocotyls were shifted to the selective medium (containing antibiotics) almost 6-9 segments of hypocotyls were spread on the selective medium in proper alignment. Then each petri plate was covered with parafilm and placed at 28ºC under light for callus formation. The hypocotyls were moved to fresh media plates after 3-4 weeks intervals. After 2 months the callus was shifted to the IBA medium under controlled conditions. After this, the callus was shifted to the maturation medium.

Genomic DNA extraction and confirmation: When the callus was developed then their samples were taken from the petri plates in the eppendroff with the help of forceps and were placed quickly in the ice containing bucket. DNA was extracted from callus grown hypocotyls. Agarose gel was prepared by using the 1% solution since the gel concentration depends on the size of the DNA fragment that needs to be separated. The volume of the buffer was kept at 1/3 of the flask capacity. Gel was exposed to UV light to observe bands, or a gel documentation system was used to serve this purpose of visualizing fluorescent bands of DNA.

RESULTS

In this study, four local cotton varieties having good agronomic traits were used for comparing the regeneration efficiency, which included the callus status, callus induction, and proliferation rate as well. The protocol for development of callus from cotton plant seedlings has been developed to induce callus from four varieties in several months. Four cotton varieties were used to check the efficiency of transformed callus induction on different mediums.

Table 2. Ingredients, pH and durations of different Mediums used in this study

Medium	Ingredients	pH	Duration
Seedling media	Distilled water 1L, 15g glucose, 3.2g Gelzan, Macro 50mL	6.1-6.2	7 days
2,4-D media (non-selective)	dH_2O 500ml, Macro (25ml), Micro (5mL), Glucose (15g), Gelzan (1.7g), Iron (5ml), Inositol (5mL), Ammonium nitrate (5ml), L. Glycine (0.5ml), Organic B_5 (0.5ml) Kinetin (100 µl), $MgCl_2.6H_2O$ (0.5ml), 2,4 D (0.5ml)	5.85-5.95	48hrs
2,4-D media (selective)	dH_2O 500ml, Macro (25ml), Micro (5ml), Glucose (15g), Gelzan (1.7g), Iron (5ml), Inositol (5ml), Ammonium nitrate (5ml), L. Glycine (0.5ml), Organic B_5 (0.5ml) Kinetin (100 µl), $MgCl_2.6H_2O$ (0.5ml), 2,4 D (0.5ml), Cefotaxime (500 µl), kanamycin (500 µl), Spectromycin (500 µl)	5.85-5.95	20 days
IBA media (Selective)	dH_2O 500ml, Macro (25ml), Micro (5ml), Glucose (15g), Gelzan (1.7g), Iron (5ml), Inositol (5ml), Ammonium nitrate (5ml), L. Glycine (0.5ml), Organic B_5 (0.5ml) Kinetin (100 µl), $MgCl_2.6H_2O$ (0.5ml), IBA (0.5ml), Cefotaxime (500 µl), kanamycin (500 µl), Spectromycin (500 µl)	5.85-5.95	20-25 days

Maturation media	dH_2O 500ml, Macro (25ml), Micro (5ml), Glucose (15g), Gelzan (1.7g), Iron (5ml), Inositol (5ml), Ammonium nitrate (5ml), L. Glycine (0.5ml), Organic B_5 (0.5ml) Kinetin (100 µl), $MgCl_2.6H_2O$ (0.5ml), IBA (0.5ml), Glutamine (0.5g), Asparagine (0.25g), Cefotaxime (500 µl), kanamycin (500 µl), Spectromycin (500 µl).	6.1-6.2	20-25 days

Five types of mediums were used to induce callus formation in the research study their respective pH is indicated in (Table 2). The four cotton varieties Coker312, Cyto124, CIM554, and MNH786 cultivars were used for transformed callus formation among which only two showed better response for induced callus formation (Table 3). Coker and CIM554 showed the best responses by this protocol and onto the mentioned mediums while Cyto124 and MNH786 varieties showed poor response and their germination was very slow as compared to the Coker and CIM554 variety of cotton cultivars mentioned in (Table 3).

Table 3. List of cotton genotypes studied in the research

Varieties	Names	Source
1.	CIM554	CCRI Multan
2.	Coker312	CCRI Multan
3.	Cyto124	CCRI Multan
4.	MNH786	CCRI Multan

For each variety, a minimum of three plates with replications were inoculated where at least 28 hypocotyls pieces were contained in each plate. All 4 varieties with each variety having 3 replications showing the number of total flasks which were presently having inoculated callus. The remaining calluses are the ones that were left and saved at the end from contamination. Three plates were inoculated with each containing 14 hypocotyl segments also showing the black edges. After 15 days CIM554 genotype showed a better response compared to other varieties (Fig. 2). Our results showed that CIM554 showed sprouted or swelled edges around 73%. This performance was significantly higher than the traditionally used Coker312 which showed about 67% of callus induction on IBA media. The variety 'MNH786' exhibited the lowest callus induction potential (63%).

Since minute differences were observed for callus induction response among the four cultivars, the efficiency of sprouted hypocotyls was further tested for 20-25 days on IBA (selective) media. They aided in the estimation of the regeneration efficiency of callus induced hypocotyls on IBA (selective) media. Our results showed that calli from CIM554 showed a remarkably higher response (85%) showing tremendous regeneration efficiency while traditionally used Coker312 (66%) was left behind by 19% (Fig. 3). The other two varieties did not show significant improvement in regeneration response (63% & 65%) even after 20-25 days prolonged culture of sprouted induced calli in IBA (selective) media.

To determine whether *agrobacterium* facilitated the integration of *Cry1Ac* and *Cry2Ab* genes in induced and mature calli, DNA was extracted from callus-containing hypocotyls of CIM554. Samples of transgenic callus were collected carefully for DNA extraction and DNA was extracted in triplicates.

After performing PCR, gel electrophoresis was performed to visualize the bands. Our results showed that sample 1 & 3 was detected for both Cry1Ac and Cry2Ab, thus confirming the presence of dual gene construct (*Cry1Ac* and *Cry2Ab*) in the CIM554 callus which was induced through optimized culturing procedures (Fig 4).

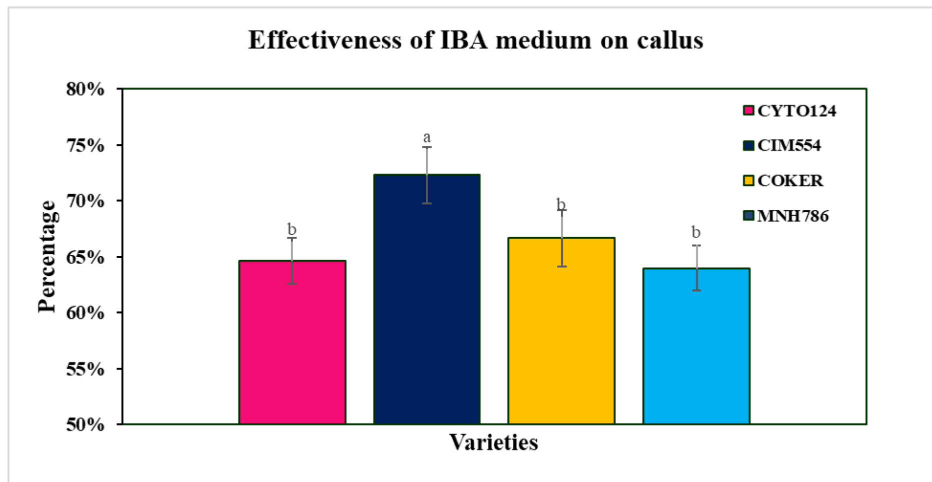

Figure 2. Evaluation of effectiveness of IBA medium for inducing callus growth in hypocotyls of four cotton varieties. The lowercased letter denote significance after *post-hoc* analysis.

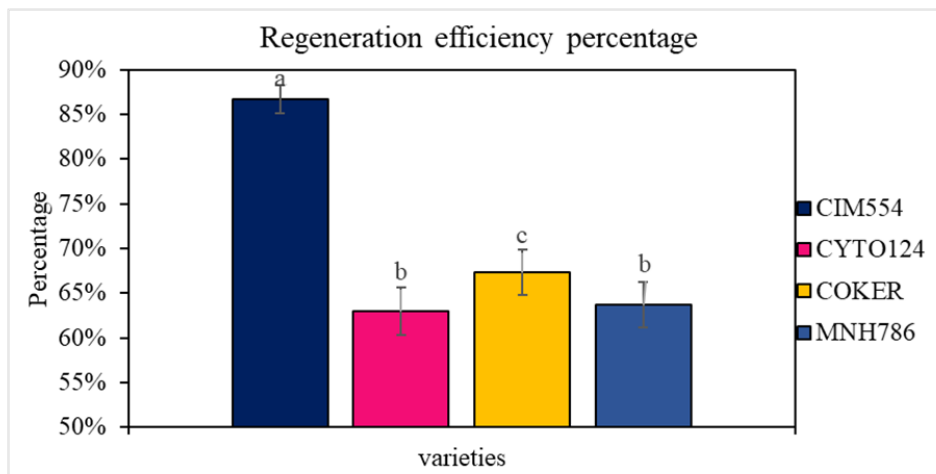

Figure 3. Regeneration Efficiency response in terms of percentage was estimated through prolonged culturing of calli under IBA (selective) media for 20-25 days. The lowercased letter denote significance after post-hoc analysis.

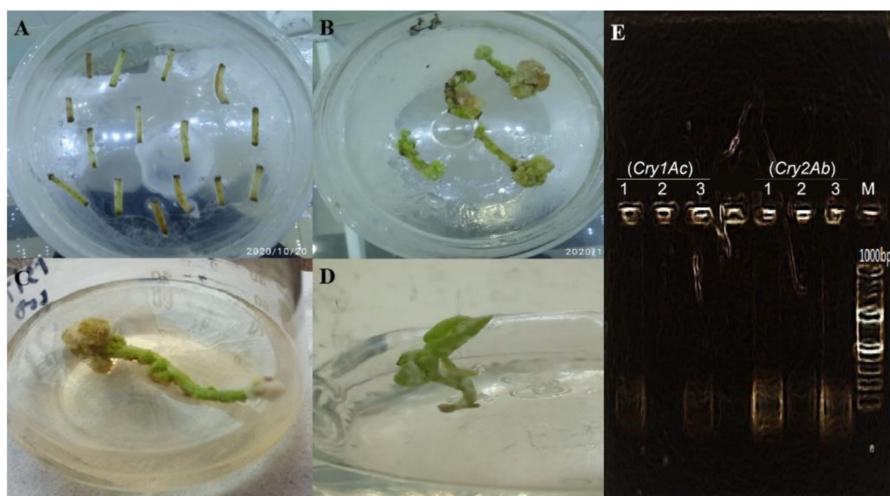

Figure 4. (A) Inoculated hypocotyls arranged on 2,4 D medium. (B) Callus with swelled edges and white and yellow granular appearing on 2,4-D media (C) Inoculated hypocotyls showing response after prolonged culturing on IBA medium (D) Plantlets developed on maturation media (E) Transgene (*Cry1Ac* + *Cry2Ab*) confirmation through PCR results using specific primers.

DISCUSSION

Cotton is a cash crop and is also said to be white gold providing an important role in economy of a country. But a major threat hyped the world when different kind of insects and pests attacked cotton thus bringing a huge economic loss. To overcome the situation scientists developed different trades of cotton including Bollgard-I, Bollgard-II, and Bollgard-III which could provide resistance against a huge number of insects and pests, especially lepidopterans. These GMO products are being imported and introduced in Pakistan. The Bollgard-II was introduced to cope the situation which contains the dual gene construct *Cry1Ac* and *Cry2Ab*. This study was carried out to provide increased resistance against the lepidopteran insects which will be very helpful in the future to reduce the loss of cotton.

Globally, the cotton variety Coker312 has been used to develop transgenic cotton. In Pakistan, Coker312 is not well adapted to the environment. It did not thrive well, not producing sufficient flowering and seeds, and compromised seed multiplication. Moreover, Coker312 did not perform well in arid to semi-arid environments, hence hybridization of its transgenic form with local cotton cultivars requires intensive efforts. Owing to poor morphological features put forth a demand of extensive breeding work on hybridized local transgenic genotypes to ensure success in variety development and commercializing aspects. Thus, developing transgenic cotton using Coker312 is challenging.

Regeneration rates may vary when compared to other Coker varieties, which highlights the need for tailored breeding programs to optimize genotype adaptation and its commercial success. To protect cotton production and economic stability, a study emphasizes the difficulty and significance of creating resilient transgenic cotton types that can flourish in a variety of environmental circumstances [33].

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