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RESEARCH ARTICLE
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Transformation of *Allium sativum* Leaf Agglutinin (*ASAL*) Gene in Cotton through Pollen Tube Method to Enhance Resistance against Whitefly

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ABSTRACT

Cotton is the most significant crop grown for fiber in more than 50 countries. China has achieved the highest productivity among the main producers, which include the USA, India, Pakistan, China, and Uzbekistan. The numerous biotic and abiotic stresses have always led to an ample loss of the crop, hence, causing a significant loss in its yield. Different biotic stresses like weeds, pathogens and pests jeopardize cotton production by 82% approximately. The significant damage causing insects include stem, leaf and foliar feeders, bollworms and sap-sucking pests. Sap-sucking pests, whiteflies, jassids, viz. and aphids are very hard to control with conventional pesticide management on account of their quick adaption and resistance development to insecticides. The *ASAL* gene has been reported in other crops like garlic and belongs to gluten family. The gene construct was mediated through *Agrobacterium* and was transformed into the bud through pollen tube transformation method. The gene construct was injected with a varied concentration ranging from 1-10µL. Transgene integration and differential expression of the gene was manifested through PCR and real-time qPCR respectively. The transformation efficiency was found to be 1.36%. The transgenic plants showed significant resistance of 72% against *Aleyrodidae*, sucking insects. The differential expression of the gene was found to be 6.8 folds with a concentration of 5µL. The enhanced resistance can decrease the loss of crop to sucking insects, hence increasing its yield.

Keywords: *Gossypium*, Gene transformation, *Agrobacterium,* Pollen tube, Sucking insect resistance

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INTRODUCTION

One of the main natural fibers in the world, cotton (*Gossypium* spp.) is grown commercially in more than 50 nations and is an important source of revenue (Razzaq et al., 2021a, b; Anwar et al., 2023). China, India, the United States, Pakistan, and Uzbekistan are the top five producers of cotton, with China having the highest production at 1,265kg/ha (Zafar et al., 2022; Ijaz et al., 2024). The 26 chromosomes present in the 45 known *Gossypium* species are divided into eight cytogenetic groups, A through G and K. Currently, there are 50 different *Gossypium* species. The remaining five are allotetraploids with 52 chromosomes and belong to a single group called AD (Zafar et al., 2023; Zafar et al., 2024). According to the International Cotton Advisory Committee (ICAC) of the USA, Pakistan is the second-largest exporter of cotton, the third-largest exporter of textiles, and the seventh-largest manufacturer of fabrics in the world. Additionally, it is the fourth-largest producer of yarn in the world. Over 60% of Pakistan's foreign income comes from the sale of cotton-related goods. (Sial et al., 2014; Zafar et al., 2021). In Pakistan, genetically modified cotton is

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generally considered to pose one of the most significant risks to cotton cultivation. The crop is attacked by insect pests that cause significant damage, resulting in an annual decrease in production of 35-45% (Masood et al., 2011; Zafar et al., 2020).

Aphids, which are sap-sucking bugs belonging to the Hemiptera order, are responsible for 13% of global crop losses (Chougule & Bonning, 2012). The commercially valued crop *Brassica juncea* suffers significant yield losses due to the presence of the mustard aphid, *Lipaphis erysimi*. Galanthus nivalis agglutinin (GNA), a mannose-binding lectin obtained from snowdrops, has been demonstrated in recent decades to be a potent biocontrol agent against hemipteran insects that feed on sap (Gatehouse et al., 1996). Many other GNA-related lectins, such as *Pinellia ternata agglutinin* (PTA), *Allium sativum leaf agglutinin* (ASAL), and *Allium cepa agglutinin* (ACA), have also been shown to be effective in transgenic plants (Dutta et al., 2005; Hossain et al., 2006).

A variety of insects from the orders Coleoptera, Diptera, Lepidoptera, and Homoptera are susceptible to the insecticidal properties of lectins, which bind to the carbohydrates found in leaves. The mannose-binding homodimeric protein *ASAL* is hostile to the cowpea aphid (*Aphis craccivora*), which is one of the numerous sucking pests in the homopteran order. For instance, cultivars of chickpeas with lower trichome densities are more prone to aphid infestation than cultivars with higher densities. However, several insecticides that are still widely used in India are no longer effective against these aphids. The expression of the *ASAL* gene enhances garlic's resistance to sap-sucking pests. (Chakraborti et al., 2009). Genetically modified chickpea plants, created by incorporating the *ASAL* gene, exhibit resistance to sucking pests. It has been asserted that the ability to tolerate whiteflies is necessary for combating plant diseases. Utilizing site-specific promoters in transgenic chickpeas has enhanced the technique for tissue-specific expression of the *ASAL* gene (Chakraborti et al., 2009). Recent research has shown that the protein Tma12, which was isolated from the edible fern *Tectaria macrodonta* (Fee) C., has significant potential. Chr., possesses insecticidal qualities that are effective against the whitefly (*Bemisia tabaci*). Transgenic cotton lines that produced Tma12 at less than 0.01% of the total soluble leaf protein were shown to be resistant to whitefly infestation without significantly reducing yield in controlled field trials (Shukla et al., 2016).

Sucking insect pests, such as aphids, jassids, whiteflies, and mealybugs, pose a serious threat to commercially important crops. Phloem is attacked and consumed by pest insects that feed on its sap. Phloem sap contains high concentrations of all necessary substances, including proteins, sugars, and amino acids (Kehr, 2006). Hemiptera, Pseudococcidae, mealybug (*Phenococcus solenopsis*) is a serious pest feeding on phloem. Several important agricultural crops, including cotton, tomatoes, okra, tobacco, and tomatoes, are now seriously threatened by it. It was in Pakistan in 2005 that mealybugs were first discovered on cotton and other major crops (Muhammad, 2007). Numerous plants have demonstrated the efficacy of *ASAL* technology, including rice, mustard, and tobacco. (Yarasi et al., 2008). Plant lectins are highly affinitous proteins that bind glycolipids, glycans of glycoproteins, or polysaccharides (Goldstein & Hayes, 1978).

Plant lectins may have the ability to deter sap-eating insects. Most likely, when lectins come into contact with the intestinal glycoproteins of insects and other predators, they limit the absorption of nutrients (Czalpa & Lang, 1990; Keresa et al., 2008). Certain insect species can allow a number of plant lectins to attach to their midgut surface, pass through their epithelium, and enter their hemolymph (Macedo et al., 2015; Yarasi et al., 2008). Transgenic rice expressing *Allium sativum* leaf agglutinin (*ASAL*) exhibits high-level resistance against major sap-sucking pests. When compared to other lectins, the mannose-binding lectin ASAL from *Allium sativum*, which kills the cowpea aphid (*Aphis craccivora*), was found to be quite effective (Chakraborti et al., 2009). The resistance of the green leaf hopper (GLH), brown plant hopper (BPH), and white-backed plant hopper (WBPH) was assessed using the *Allium sativum leaf agglutinin* (ASAL) lectin gene derived from garlic plants (Yarasi et al., 2008). The expression of the *ASAL* gene also reduced the population of sap-feeding mustard aphids (*Lipaphis erysimi*) (Dutta et al., 2005). As several studies reported the efficiency of *ASAL* gene against insect attack in different crops, the transformation of *ASAL* gene in cotton may produce significant resistance that would lead to increase in cotton production.

MATERIALS & METHODS

Source of Gene

The sequences of *ASAL* genes were retrieved from NCBI and synthesized further from Bio Basic Canada.

Cassette Preparation

After codon optimization, the CaMV35S promoter was used to control the gene, and a Nos terminator was introduced as shown in Fig. 1. Primer3 software version 3 was used to create the primers.

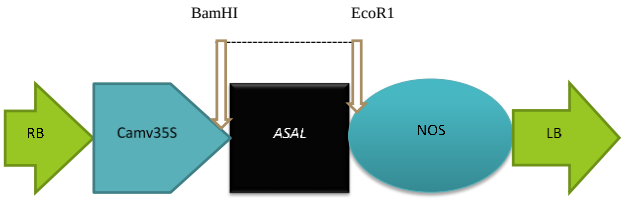

Fig. 1: Synthesis of *ASAL* gene cassette

Gene Amplification in a Gene Cassette

The gene cassette was received in the puc57 vector. The gene was located using full-length, gene-specific primers F (5'-ATG GGT CCT ACT ACT TCA TCT CCT-3') R (5'-TCA AGC AGC ACC GGT GCC AAC CTT-3') that targeted the gene cassette. The reaction mixture was prepared by combining 1U of Taq DNA polymerase, 150M of each dNTP, 0.5 l of each forward and reverse primer at a final concentration of 0.25M, and 100ng of template DNA with 1X assay buffer (10mM pH 9.0 Tris-HCl, 50mM KCl, 1.5mM MgCl2, and 0.01% gelatin). The initial denaturation step lasted for 3min

at 95°C, followed by a denaturation step at 95°C for 45s, and an annealing step at 65.4°C for 45s. The extension time lasted for 10min at 72°C. The desired gene band was observed on a 1% agarose gel.

Gene Cloning into the Plant Expression Vector pCAMBIA2300

The purified *ASAL* gene and a plant expression vector pCAMBIA2300 were digested with *EcoR1* and *BamH1*. The digested products were ligated using fast ligation kit. The purified ligated product was cloned into *Agrobacterium tumefaciens* using the liquid nitrogen method. The YEP agar plates containing a drug Kanamycin (50mg/L) were used for the selection of the positive clones. The positive clones were PCR amplified.

Pollen Tube Transformation

During the flowering season, flowers were selected that would bloom the next day. To ensure self-pollination and prevent cross-pollination, closed the tip of the bud with nail polish. To prevent damage to the ovaries, carefully removed the petals and stamens after the flowers have bloomed. The microinjector was cleaned, and a 10µL construct was drawn. The ovary was vertically perforated with the microinjector, approximately 5mm below its top. After carefully removing the microinjector from the ovary, a gradual injection of 5-10µL of the construct was performed (Fig. 2; Table 1). The entire vegetative section of the branch was cut after the injection was completed. Throughout the entire harvest season, individual cotton bolls were collected. The seeds from each boll were removed and placed in the pots. Cotton seedlings were planted in the field after germination. Once the bolls matured, the first-generation (T_1) plants were transferred to the field for second-generation (T_2) plants.

Fig. 2: Pollen tube transformation in cotton.

DNA Extraction and PCR Identification of *ASAL* Gene

First-generation plants' second leaves were cut off, kept cold to prevent wilting, and used as a sample for gene analysis. The leaf sample was combined with 700µL of buffer A in a pestle and mortar before being ground using liquid nitrogen. A 2 mL Eppendorf tube was used to separate the sample after the mixture was blended with a 500µL concentration of buffer B. Prior to spending 30min at 65°C in the incubator, the substance was thoroughly vortexed. The material was then placed in a fresh Eppendorf tube after centrifuging it to extract the supernatant. The waste was discarded after being incubated for 10min at 11,000rpm. Next, 500µL of CIA was added to the Eppendorf tube, which was then centrifuged for another 10min at 11,000rpm. Following the meticulous collection of the supernatant, 500µL of chloroform-isoamyl alcohol (CIA) was introduced into the mixture. To aid in the separation process, the mixture was centrifuged for approximately 10min at 11,000rpm. Subsequently, the supernatant was mixed with 300µL of ice-cold isopropanol and allowed to incubate at -20°C for 20min. The mixture was then centrifuged for 15min at 14°C and 13,500rpm. After discarding the supernatant, the pellet was washed in 400-500µL of 70% ethanol before being centrifuged for two to 3min. The pellet was dissolved in 40L of autoclave water after drying for 2min. At a final concentration of 5µL, RNase was added to the DNA. After brief centrifugation, kept at 37°C for 30min or overnight. A more comprehensive evaluation of the DNA quality was observed through agarose gel.

Table 1: Genetic transformation of the gene in pollen tubes of cotton variety FBG666

Exp. No.	Time (AM)	Concentration (µl)	Buds injected	Bolls survived	Detected as positive	Transformation Efficiency (%)
1		1	240	13	1	0.41
2		2	280	17	2	0.71
3		3	230	14	1	0.4
4		4	225	12	2	0.89
5		5	220	13	3	1.36
6		6	190	6	2	1.05
7	7-11	7	240	17	3	1.25
8		8	215	14	2	0.93
9		9	220	12	1	0.45
10		10	260	16	1	0.38

RNA Extraction and qRT-PCR

Transgenic cotton leaves were treated with an Agilent kit to extract RNA (Agilent Technologies, Santa Clara, CA, Cat # 5185-6000). Using a Nano-Drop 2000 spectrophotometer, the amount of RNA in a sample was measured inng/L at 260 and 280 nm. The entire RNA was treated with DNase to generate cDNA using a first-strand cDNA synthesis kit (Thermo Scientific, Cat. #K1632) and was subsequently stored at -20°C. A quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in triplicate using Maxima SYBER Green/ROX (Thermo Scientific, Cat #K0221). A total of 20µL of Maxima SYBER Green/ROX qPCR Master Mix (2x), The reaction mixture was prepared with 1µL of cDNA (50ng/µL) and 5µL of forward and reverse primers (10pmol each). Primer sets for the enzyme glycerol-3-phosphate dehydrogenase (*GAPDH*) were employed as an internal control for normalization in order to assess the relative expression.

Insect Bioassay

The efficacy of transgenic and non-transgenic cotton containing the *ASAL* gene was tested against whitefly. The upper fully expanded positive leaves of the plant were taken and exposed to whitefly. The mortality rate was calculated by the following formulae;

$$\% \text{ Mortality} = \text{No. of dead Larvae} / \text{Total No. of Larvae} \times 100$$

RESULTS

Detection of *ASAL* Gene

Restriction digestion was used to extract the gene cassette carrying the *ASAL* gene from the PUC57 vector. A 546bp product from the *ASAL* gene was detected (Fig. 3).

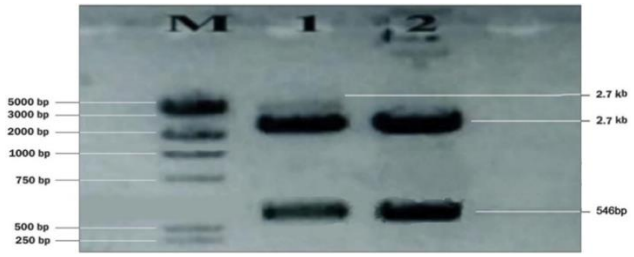

Fig. 3: Gene detection (*ASAL*) through restriction digestion; Lane M: Molecular weight markers; Lane 1-2: lower band shows gene (*ASAL*) size (546bp) and upper bands contain PUC57 (2.7kb) vector.

Gene Amplification

Full-length primers were used to amplify the gene through PCR. The finished product shows PCR bands by using a gel illuminator. The *ASAL* gene size was found to be 546bp as shown in Fig. 4.

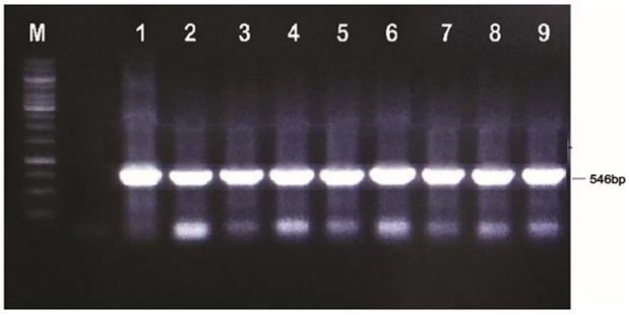

Fig. 4: Purified detection of gene (*ASAL*) through PCR; Lane M: shows molecular weight markers and 1-9 lane contain gene (*ASAL*).

Genetic Transformation of Cotton through Pollen Tube Method

About 1µL construct was injected in 240 buds at various time intervals between 7:00 A.M.-11:00 A.M. Out of the 13 surviving buds, 1 was detected as positive, giving us a transformation efficiency of 0.41%. On the second day of transformation, 280 buds were injected with 2µL construct, out of which 17 survived, and 2 were positively detected. Hence, the transformation efficiency was calculated to be 0.71%. A total of 230 buds were injected with 3µL construct on the 3rd day, 14 of them survived, and 1 was detected positive. So, the transformation efficiency was 0.4%. On the 4th day, 12 bolls, out of 225 injected with 4µL construct were survived, and 2 were detected positive. As a result, the transformation efficiency was 0.89%. A concentration of 5µL construct was injected in 220 buds on the 5th day. The 13 surviving bolls were tested and 3 were detected as positive, therefore, the transformation efficiency was 1.36%.

The next day, 190 buds were injected with 6µL construct, out of which 6 survived and 2 were positively detected. The transformation efficiency was 1.05%. On the 7th day of transformation, 7µL construct was injected in 240 buds. The surviving bolls were tested and 3 were found positive. So, the transformation efficiency was 1.25%. An 8µL concentration of the construct was injected on the 8th day, in 215 buds. Out of these 14 bolls survived and 2 were detected as positive, providing us with a transformation efficiency of 0.93%. About 9µL concentration of construct was injected on the 9th day in 220 buds. The 12 surviving buds were tested and 1 was positively detected. The transformation efficiency was 0.45%. On the final day, 260 buds were injected with 10µL construct. The surviving 16 bolls and 1 was detected as positive. Hence, the transformation efficiency was 0.38% (Fig. 5 and 6).

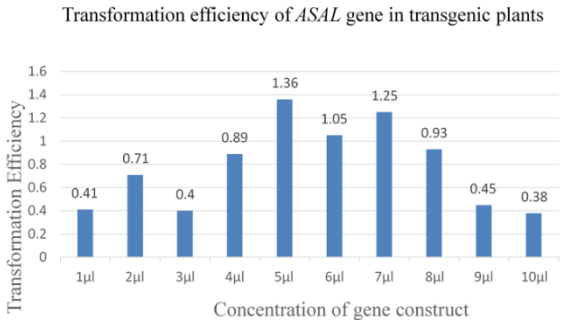

Fig. 5: Transformation efficiency of *ASAL* gene in transgenic plants

Gene Confirmation by using Short-length Primers through PCR

Amplification of gene samples, taken from the leaves, was performed through PCR. The amplified product was in turn with a 220bp size as shown in Fig. 7.

Gene Detection by using Full-length Primers through PCR

Amplification of gene samples, obtained from leaves, was done via PCR. The PCR product was found to be 546bp size as shown in Fig. 8.

Quantitative Expression Analysis of Gene through qRT-PCR

The quantitative expression analysis of the gene was carried out through qRT-PCR. The cotton transgene expression as compared to non-transgenic cotton was 3.7, 4.3, 3.5, 4.9, 6.8, 5.9, 6.4, 5.4, 3.9 and 3.2 folds. The transgenic plants injected with 5µL concentration showed highest level of expression at 6.8 folds followed by 6.4 and 5.9 folds at 7 and 6µL concentrations respectively (Fig. 9).

Insect Bioassay

The plant containing *ASAL* gene showed significant resistance to whitefly whereas non-transgenic plants were highly susceptible to whitefly. The mortality ratio was found to be 71% as shown in Fig. 10.

DISCUSSION

Cotton (*Gossypium hirsutum* L.) is the first plant species to be used for the pollen tube transformation (PTT) procedure, which involves introducing exogenous DNA into the plant embryo (Zhou et al., 1983). Using the pollen-tube technique and the gramineous expression vector pGU4ABBar, the *Cryla* gene, a synthetic insecticidal crystal

Fig. 6: Schematic flow diagram of pollen tube transformation.

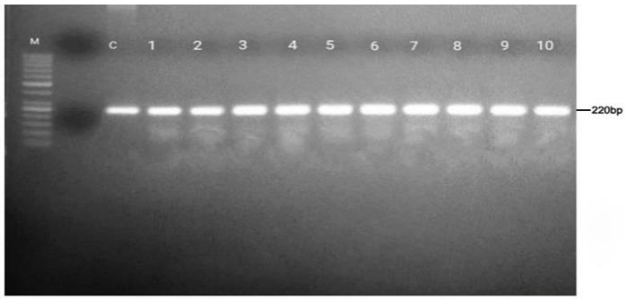

Fig. 7: Confirmation of *ASAL* gene through PCR; Lane M: Molecular weight markers; Lane C: shows control; Lane 1-10: transgenic plants containing *ASAL* gene.

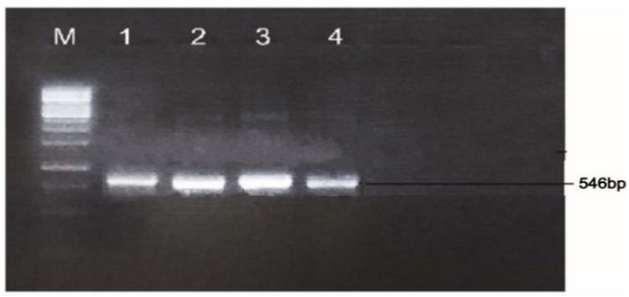

Fig. 8: Confirmation of *ASAL* gene by using full-length primers through PCR; Lane M: Molecular weight markers; Lane 1-4: Transgenic plants containing gene (*ASAL*).

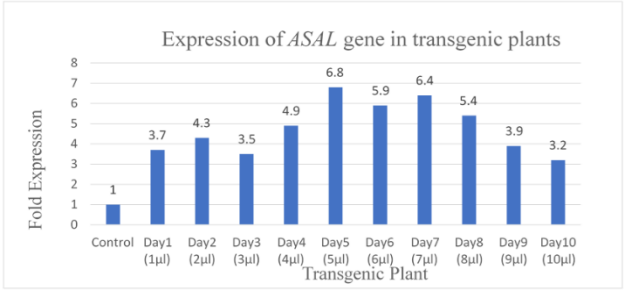

Fig. 9: Quantitative expression analysis of gene through qRT-PCR

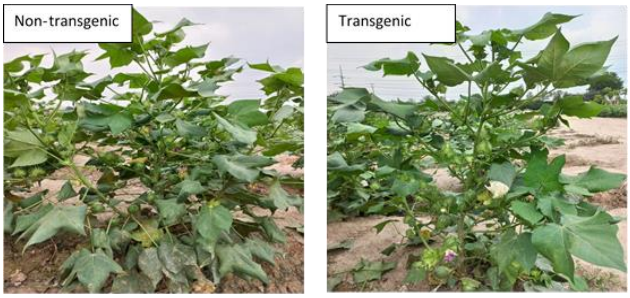

Fig. 10: a) non-transgenic plant b) transgenic plant containing *ASAL* gene

protein gene from *Bacillus thuringiensis* was inserted into the wheat cultivars Xinong 2208 and Xinong 132. The *CryIa* gene was isolated from 27 transgenic plants using PCR and Southern blotting techniques. The protein was expressed in the transgenic plants, as confirmed by Western blot analysis. The frequency of transition varied between 1.13 and 1.21% (Hou et al., 2003a). For gramineous cells, the expression vectors pGU4AGBar and pGBIU4AGBar were used. The *gna* gene, a synthetic agglutinin gene from Galanthus species, was used in the pollen tube technique to genetically modify the winter wheat cultivars Xinong 2208 and Xinong 132. The PCR and Southern blotting assays revealed that the *gna* gene was expressed in a total of 20 transgenic plants. A western blot analysis revealed that the transgenic plants expressed the target protein. The actual transition frequency ranged from 0.28% to 0.84% (Hou et al., 2003b).

Regardless of the type of agriculture, whether it is solid or liquid pollination, a low boll set (3-5%) was produced. Even though cotton stigma sensitivity was low during the evening hours and the pollen was not treated with pollen germination media, pollination in the morning resulted in efficient fertilization, leading to boll set ranging from 21 to 28.5%. Using the gene constructs for pCAMBIA AC, *cry1 Ia5, cry1 Aa3*, and *cry1 F*. A total of 5619 flowers were exposed to an agrobacterial culture. The ball set ranges from 23.1 to 29.9%. The low frequency of boll emergence was caused by both insufficient pollination and insect damage. Solium selection of these kanamycin-resistant cotton transformants resulted in the development of 521 kanamycin-resistant T1 generation plants. These plants produced 5,692 healthy seeds, which were then passed on to the T2 generation. Out of these seeds, only 1199 actually germinated. The plants growing in the transgenic greenhouse underwent thorough in Solium kanamycin screening. The gene integration tests revealed that only seven of the plants had positive PCR results. The efficiency of the change was 0.30 percent (Mogali et al., 2013; Razzaq et al., 2023a).

All 18,645 seeds derived from the pollen-tube route transformation were germinated and tested using herbicide. To account for potential variation in BAR gene expression among the transformants, the effects of the herbicide on the seedlings were classified into two categories: partially resistant and susceptible. After the third round of selection, most of the seedlings were found to be susceptible to the herbicide Basta. Roughly 0.648% of the total, or 121 plants, continued to exhibit Basta resistance. Of them, there were 82 brown cotton plants and 39 green cotton plants. With a disease score of 2, 15 (15.39%) of the 121 Basta-resistant plants showed moderate to high levels of wilt resistance. The LB-5-8 and ZB-1-49 lines showed strong resistance to Verticillium wilt because of the overexpression of *GAFP*, according to pathogenicity tests, in vitro antifungal trials, and molecular research. By PCR testing on their offspring, these transgenic lines of colored cotton demonstrated that the 387bp GAFP transgene had been successfully incorporated into their genomes. Transgene GAFP (387bp) was amplified in R2 plants. The two transgenic cotton lines' R2 generations displayed illness scores of 0 or 1, but the non-transgenic colored cotton line Xin-Cai 1# displayed a disease index of 55.56 and a disease score of up to 3. Although there were significant differences in disease resistance among the lines, both transgenic plant lines maintained their resistance to illness in the R3 generation. Several of the progeny lines continued to exhibit enhanced resistance levels to Verticillium wilt, as indicated by the disease index. The pathogenicity of the *V. dahliae* population was demonstrated by the disease index. The sickness index for the control group was 51.08, while for the non-control group it was 30.88. With a disease index score of 20, the tested lines demonstrated a high level of resistance (Wang et al., 2004).

The enzyme chalcone synthase-A (*CHSA*) is responsible for synthesizing all flavonoids. The color of flowers varies depending on their species. The expression vectors pBI121 and pWM101, which contain the CaMV35S promoter and are oriented in the forward direction, were used to clone the *CHSA* gene from the flower petals of petunia (*Petunia hybrida*). For germ line transformation of *Cyclamen persicum*, the pollen-tube method was employed. Approximately 4,400 seeds were collected. Among these were genetically modified flowering plants. On eight white-flowered plants, the petals occasionally turn yellow or light yellow. In very rare cases, the entire petal may change its color. Half of the petals of three white-blossoming plants, and even the entire bloom, turned pink (Zhao et al., 2005; Razzaq et al., 2023b). In our study, the transformation of the *ASAL* gene in cotton was conducted via the pollen tube method. The gene was transformed into self-pollinated buds with a microinjector. The transformation efficiency of the gene through this method was found to be 1.36%. The percentage mortality of whitefly after the transformation of *ASAL* gene was 71.42%.

Conclusion

Genetic engineering is a breeding tool that allows the introduction of several foreign genes into a plant at a time. Cotton is widely grown all around the globe for its economic value. It is extensively used at an industrial scale for the production of apparel. Cotton is selectively bred to be white for commercial uses. Insects and weeds pose great harm to cotton production as they lower the yield and quality of the crop by damaging it. Among these insects, whitefly causes higher yield reduction of cotton by sucking its sap. The transgenic cotton containing *Allium sativum* leaf agglutinin (*ASAL*), derived from garlic, showed 71.42% resistance to whitefly.

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Author Contributions

ZH, TF and FQ wrote the initial draft of the manuscript. AA, SS and SI provided the space and helped to conduct the experiment. RT SAB and HM Conceptualization, writing—review and editing; MY, AS, and FQ data curation and helped in statistical analysis. AR, MHU, GR, AI, ZA and MMZ helped in writing-review and editing, and AR, AA and MMZ reviewed and supervised the experiment. The final approval was given by AR and MMZ.

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