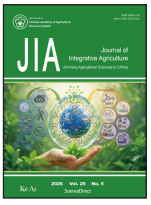
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RESEARCH ARTICLE

Application of an endogenous *pGhαGloA* promoter in the CRISPR/Cas12a system for efficient genome editing to create glandless cotton germplasm

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Highlights

- The cotton endogenous Pol II type promoter (*pGhαGloA*) can drive high transcription of crRNAs and improve editing efficiency.
- The *GhPGF* gene negatively regulates the formation of cotton pigment glands.

Abstract

An efficient genome editing tool, the CRISPR/Cas12a system, has been used in research on plant functional genomics and the improvement of agronomic traits. In this study, the CRISPR/Cas12a system was optimized by using the endogenous *pGhαGloA* promoter in cotton. With this system, crRNAs were driven by the Pol II *pGhαGloA* promoter to construct the pGhRBE3-pGhαGloA-*GhPGF* vector and carry out genetic transformation. The vector worked efficiently in all positive transgenic plants and the editing efficiencies at the crRNA1 and crRNA2 target sites were up to 93.37 and 88.24%, respectively. This system had significantly higher editing efficiency than the pGhRBE3 system with a Pol III promoter-*Ubi 6.7* promoter, indicating that the Pol II promoter is more suitable for expressing multiple sgRNAs or crRNAs than the Pol III promoter in cotton. The vector mainly generated the editing type of fragment deletion, and the deletion sizes were in the range of 3–12 bp with the editing sites spanning the 14th to 29th bases downstream of the protospacer adjacent motif (PAM). All the targeted mutation loci were stably inherited from the T_0 to T_2 generations, and three transgene-free lines with target site mutations in the *GhPGF* gene were obtained. These glandless and gossypol-free (or low content) cotton germplasms will play a key role in healthy cottonseed oil/cake production. Therefore, the CRISPR/Cas12a system driven by the *pGhαGloA* promoter can efficiently edit target genes in cotton, so it can provide a powerful tool for cotton functional genomics and genetic improvement.

Keywords: cotton, genome editing, CRISPR/Cas12a, Pol II promoter, glandless cotton, gossypol-free

1. Introduction

The identification of gene functions and the breeding of new crop varieties are inseparable from the acquisition of mutant plants. Before the advent of gene editing technology, mutant plants were mainly obtained by physical mutagenesis, chemical mutagenesis, and natural mutations (Zhang *et al.* 2017). However, compared with gene editing technology, these methods suffer from certain limitations. The emergence of genome editing technology has provided a new method for obtaining mutants.

Genome editing technology can modify the genome sequence and mutate specific genes by targeting and editing

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specific DNA segments, and it has been widely used in biological crop breeding efforts (Kamburova *et al.* 2017; Mao *et al.* 2019). Many types of genome editing systems have been applied in crops, including the zinc-finger nuclease (ZFN) genome editing system (Townsend *et al.* 2009), transcriptional activator-like nuclease (TALEN) genome editing system (Joung and Sander 2013), and the third generation clustered regularly interspaced short palindromic repeat (CRISPR) genome editing system (Jinek *et al.* 2012). After cutting a specific DNA sequence in the genome, these tools repair the genome mainly through non-homologous end-joining (NHEJ) or homology directed repair (HDR). In the repair process, a base deletion, base mutation, or base insertion occurs at the targeted sites, which leads to changes in the genome sequence and finally achieves the purpose of the targeted gene mutation (Bak *et al.* 2018).

The CRISPR/Cas9 editing system cleaves specific DNA double strands to form double strand breaks, which are mostly repaired by non-homologous end-joining repair. In most cases, small insertions or deletions of nucleic acid fragments are introduced near the DNA cleavage site. Their introduction causes a frame shift mutation of codons in the process of protein translation, which disrupts the function of the corresponding genes (Ghezraoui *et al.* 2014). Although the CRISPR/Cas9 system is widely used in plants, it has many shortcomings compared with the CRISPR/Cas12a system. CRISPR/Cas12a needs only one Cas protein to cut double-stranded DNA, but its editing process is completely different from that of CRISPR/Cas9 (Zetsche *et al.* 2015). Compared to the CRISPR/Cas9 editing system, the CRISPR/Cas12a editing system offers the advantages of using cutting sites located far from the protospacer adjacent motif (PAM) sequences and the continuous cutting of DNA, so it can potentially achieve higher editing efficiency and the deletion of larger fragments (Mahfouz *et al.* 2017). At the same time, the Cas12a protein recognizes the PAM sequence of thymine-rich 5′-TTTV-3′ thus expanding the selection range of target sites (Li B *et al.* 2019). The Cas12a protein can target dsDNA and ssDNA, and it shows both *cis*- and *trans*-cleavage activity (Kim *et al.* 2016). Cas12a also has higher specificity and a lower missing rate than Cas9 (Kleinstiver *et al.* 2016).

As a DNA sequence that initiates gene transcription, the promoter plays an important role in the regulation of gene expression. It is both recognized by and combined with RNA polymerase (Pol) to control the initiation and intensity of gene expression (Connors *et al.* 2002). Based on the ability of a promoter to recognize RNA polymerase, promoters are divided into the Pol II and Pol III types (Dharmendra *et al.* 2023). Generally, in the gene editing vector system, the *U6/U3* promoter (based on RNA polymerase III) is used to drive gRNA expression; while the *Ubi* promoter (based on RNA Pol II) and the *CaMV 35s* promoter are used for *Cas9* gene expression (Razzaq *et al.* 2019). Moreover, compared to the heterologous promoter, the homologous promoter can improve the expression level of the target gene, and it can also increase the stability and repeatability of transgene expression. In addition, a heterologous promoter such as the *CaMV 35s* promoter from viruses may be associated with biosafety uncertainty (Ren *et al.* 2019; Cao *et al.* 2022; Taway *et al.* 2023). At the same time, research has shown that a system based on the Pol III promoter is not suitable for expressing multiple crRNAs in human and plant cells, and its efficiency is low (Zhang *et al.* 2021).

The cotton α-globulin A promoter is a Pol II type cotton seed-specific promoter. The β-glucuronidase gene is expressed at the torpedo stage in transgenic tobacco and *Arabidopsis thaliana*, driven by the *pGhαGloA* promoter. In cotton, the expression intensities in mature embryos and cotyledons are significantly higher than in the hypocotyl and radicle (Sunilkumar *et al.* 2002). Moreover, previous research successfully used Pol II promoters to drive multiple sgRNAs in series by tRNA Gly intervals, and an efficient cucumber multi-gene editing system was established. The authors previously speculated that this promoter can be highly expressed in the embryogenic callus during tissue culture (Ma *et al.* 2024). In addition, there are a variety of *cis*-acting elements and promoter alleles in the promoter, which are frequently associated with crop agronomic traits. Therefore, the application of genome editing at the promoter to achieve crop improvement is a very attractive strategy (Swinnen *et al.* 2016). To test this speculation, this study used the cotton endogenous Pol II promoter to drive crRNAs and construct a gene editing vector to improve the efficiency of gene editing.

Cotton (*Gossypium hirsutum* L.) is an important economic crop. Cotton fibers have the highest purity among known natural fibers and important economic value in the global textile industry (Gao *et al.* 2020). Similarly, as the third largest oilseed crop in the world, cottonseed accounts for 58–65% of the total cotton production and has high economic value. It is a source of vegetable oil, and after extraction of the oil, the residual meal or cake is used as a feed for ruminant livestock or as an organic fertilizer (Mageshwaran *et al.* 2021). However, the gossypol in cottonseed limits its utilization as animal feed to some extent, since reproductive toxicity can easily occur in animals fed with gossypol-containing feed for a long time. When the gossypol content of cottonseed is less than 0.02%, it can be used directly for human consumption or animal feed, although the presence of gossypol limits the comprehensive utilization of cottonseed. Therefore, gossypol-free or low-gossypol cottonseed can be better utilized in animal feed products, which will not only make full use of resources, but also improve the quality of animal husbandry products.

The gossypol gland of cotton is the organ for storing secondary metabolites such as gossypol. With the development of gene editing technology, the gossypol synthesis gene in cotton has been edited, and the main effect gene for the formation of cotton gland (*GhPGF*) can be knocked out to obtain cotton material without gossypol or with low gossypol (Li *et al.* 2020). Establishing a variety of different CRISPR genome editing systems in cotton is conducive to verifying functional genes, accelerating the process of combining conventional breeding with molecular breeding techniques, and cultivating cotton varieties with improved yield, quality, and resistance. The purpose of this study was to establish an efficient cotton CRISPR/Cas12a

genome editing system using the cotton endogenous Pol II *pGhαGloA* promoter. A further aim was to verify whether transgene-free plants can be obtained with target site mutations by detecting the existence of the *LbCas12a* gene in plants and the editing type of the target gene *GhPGF*. This technique provides a new scheme for gene editing in cotton.

2. Materials and methods

2.1. Cloning and analysis of the *GhαGloA* promoter

The gene *GhαGloA*, which is highly expressed in the cotton ovule, was screened based on RNA seq data, and the expression data were visually analyzed using TBtools software. The promoter sequence 1,144 bp upstream of this gene was cloned from upland cotton Jin668 by homologous cloning (Yang *et al.* 2023; Zhu *et al.* 2023). The *cis*-acting element was analyzed using the online tools PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and New PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace) (Yang *et al.* 2024).

2.2. Objective vector construction for genome editing in cotton

In this study, the pGhRBE3-Cas12a plasmid (Li B *et al.* 2019, 2020) was digested with restriction endonucleases *Hin*dIII and *Sbf*I, and the *pGhαGloA* promoter was inserted into the vector after endonuclease digestion had been terminated. After sequencing, the plasmid was named pGhRBE3-pGhαGloA. Then, two cRNAs at the 5′ end of *GhPGF* (Ghir_A12G025340, Ghir_D12G025370) were designed using the CRISPR design tool CRISPR-P v2.0 (http://cbi.hzau.edu.cn/CRISPR2/) (Yang *et al.* 2014). The tRNA-gRNA-crRNA unit was synthesized by Kingsley Biotechnology Co., Ltd. (https://www.genscript.com.cn) and cloned into the binary vector pGhRBE3-pGhαGloA. After sequencing, it was named pGhRBE3-pGhαGloA-*GhPGF*. The above genome editing vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation and screened in medium with kanamycin and rifampicin. After monoclonal detection, positive clones were isolated and stored at –80°C.

2.3. Cotton genetic transformation mediated by *A. tumefaciens*

The upland cotton cultivar Jin668 was used as the receptor material for genetic transformation (Li J Y *et al.* 2019; Zhu *et al.* 2023). Jin668 cotton seeds were obtained and the seed coat was peeled off. Seeds were soaked for 30 s in 75% alcohol, then soaked with 10% sodium hypochlorite for 10 min, and then rinsed three times with sterile water for 5–10 min each time. The sterilized seeds were placed on sterile seedling medium (5–6 seeds per bottle) and cultured in the dark at 28°C. Two days later, the radicle tip was inserted into the culture medium to trigger rapid growth. After 2–3 days of dark culture at 28°C, their hypocotyls were cut into 5–10 mm fragments as explants for *Agrobacterium*-mediated transformation. The subsequent process of tissue culture was conducted with reference to previous studies (Sun *et al.* 2018, 2024; Yu *et al.* 2023; Wang *et al.* 2024).

2.4. Target site mutation analysis

The genomic DNA extraction kit for polysaccharides and polyphenols (purchased from Beijing Tiangen Biochemical Technology Co., Ltd., Beijing, China) was used to extract DNA from both the T_0 generation cotton regenerated plants and wild type controls. Specific primers, designed according to the LbCas12a and crRNAs sequences, were used to assess whether the regenerated plants were positive. The target site was amplified with specific primers, and the amplified PCR sequence was ligated into the pGEMT-Easy vector with T4 DNA ligase (purchased from Beijing Quanshijin Biotechnology Co., Ltd.) (Appendix A). The ligated product was transformed into *Escherichia coli* receptive Top10 and the positive clone was sequenced by Sanger sequencing to assess whether the crRNAs were edited.

2.5. High-throughput deep sequencing (barcode design)

For mutant plants, a pair of primers with barcodes were designed, and each primer added six different bases to the barcode label of a single plant sample. Each barcode label was added to the 5′ end of both the forward and reverse primers to amplify the target sequence. Corresponding bar code primers were used for the PCR amplification of independent samples, and PCR products were mixed in the same amounts and purified using a purification kit (OMEGA, D2500-02, Georgia, USA). The purified samples were sent to Novogene Company (Beijing, China) for the second generation sequencing, and the results were further analyzed.

3. Results

3.1. Cloning and identification of the *pGhαGloA* promoter in cotton

The RNA-seq sequencing data were used to visually analyze the expression of the cotton α-globulin A gene (Ghi_A09G13901) in various tissues. The results showed that the expression of the *GhαGloA* gene was highest during cotton ovule development (Fig. 1-A). Therefore, its promoter was cloned (Fig. 1-B), and the *cis*-acting elements of the sequence were analyzed using the PlantCARE and New PLACE tools (Fig. 1-C; Appendix B). The results showed that the promoter contains basic core elements such as a TATA-BOX and CAAT-BOX. It also contained hormone responsive elements, such as an abscisic acid response element, indicating that the pGhαGloA promoter may be involved in regulating both the growth and development of cotton.

3.2. Construction of the pGhRBE3-pGhαGloA-*GhPGF* gene editing vector

Based on the CRISPR/Cas12a genome editing system, the cotton genome editing vector pGhRBE3-Cas12a was modified and the *pGhU6.7* promoter was replaced with the cotton endogenous promoter *pGhαGloA*. The resulting vector was named pGhRBE3-pGhαGloA (Fig. 2-A). The synthesized tRNA-gRNA-crRNA fragments containing two crRNAs of the *GhPGF* gene were ligated to the pGhRBE3-pGhαGloA vector by infusion. After PCR detection and sequencing, these fragments were named pGhRBE3-pGhαGloA-*GhPGF* (Fig. 2-B).

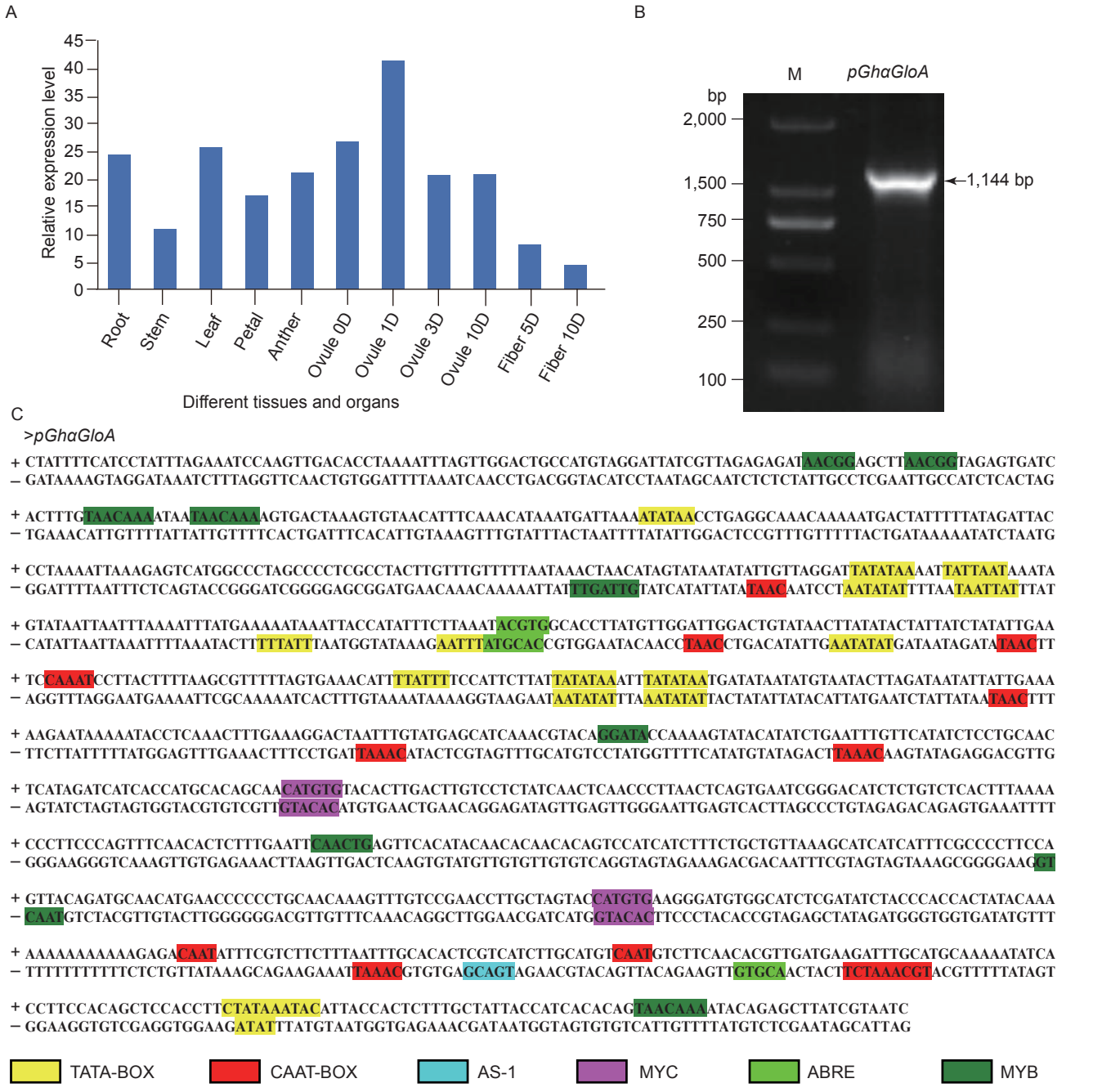


Fig. 1 The *GhαGloA* gene and its promoter. A, expression of the *GhαGloA* gene in different tissues. B, cloning of the *pGhαGloA* promoter. C, *cis*-acting elements of the *pGhαGloA* promoter.

3.3. Genetic transformation mediated by *A. tumefaciens* and tissue culture of cotton

The pGhRBE3-pGhαGloA-*GhPGF* vector was transferred into the upland cotton cultivar Jin668 by *A. tumefaciens*-mediated hypocotyl transformation. A total of 24 positive plants of the T_0 generation transformed with the pGhRBE3-pGhαGloA-*GhPGF* vector were obtained by tissue culture (Fig. 3; Appendix C), and these progeny plants showed a glandless phenotype (Fig. 3). Then, the gene editing efficiency of the vector in cotton was tested. Usually, only plants with an editing efficiency greater than 30% will show the phenotype.

3.4. Barcode detection in cotton plants edited by the pGhRBE3-pGhαGloA-*GhPGF* system

The DNA of 24 individual T_0 generation mutant plants was extracted, the target sites of these plants were analyzed by Barcode sequencing, and individual plants with an editing efficiency exceeding 1% were defined as edited. The results showed that the target sites of the 24 positive plants were all edited successfully, so the editing efficiency was 100%. However, the editing efficiency of crRNAs in each plant differed. The two target sites of 16 T_0 plants were edited, in which only crRNA1 was edited but crRNA2 was not edited, so the efficiency of simultaneous editing of both target sites was

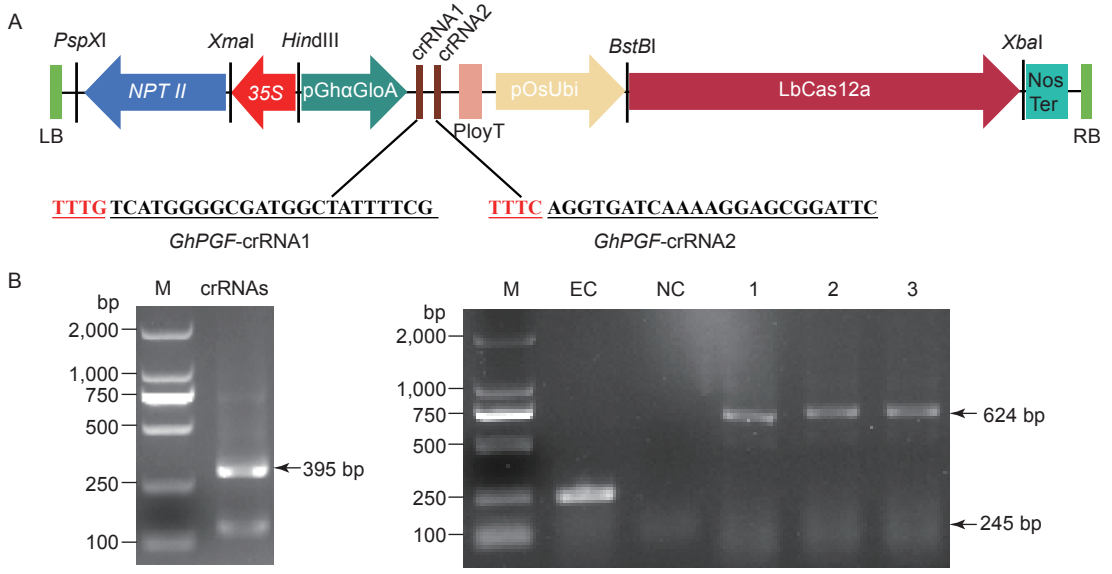


Fig. 2 Construction of the pGhRBE3-pGhαGloA-*GhPGF* genome editing vector. A, schematic diagram of the *GhPGF* genome editing vector. B, acquisition of the *GhPGF* genome editing vectors. M, DL2000 marker; crRNAs, fragment of tRNA-cRNA1-tRNA-cRNA2; EC, empty carrier control; NC, negative control; 1–3, positive strains.

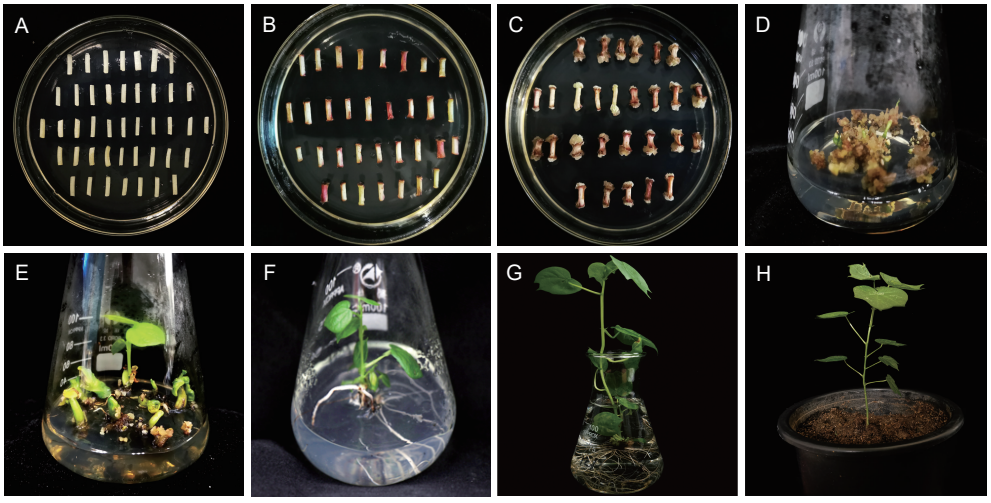


Fig. 3 Process of cotton genetic transformation and somatic embryogenesis. A, co-culture stage. B and C, callus growth stage. D and E, embryogenic callus growth stage. F, rooting growth stage. G and H, regenerated plant growth stage.

66.7% (Table 1).

The editing efficiency of the crRNA1 target in a single plant ranged from 0.96–93.37% and the editing efficiency of the crRNA2 target in a single plant ranged from 0–88.24% (Fig. 4-A; Appendix D). According to the order of vector construction and editing, we first edited crRNA1 and then edited crRNA2. In all crRNA2 edited plants, crRNA1 was also edited. Therefore, according to this set of experimental data, we speculate that the *pGhαGloA* promoter has a better effect on the first target site of the vector. This phenomenon will be explained in more detail below. An analysis of the type of editing site in the vector showed that the editing type was mainly fragment deletion, which is consistent with the editing characteristics of the CRISPR/Cas12a system. The sizes of deleted DNA fragments varied from 1 to 30 bp, mainly concentrated in the 3–12 bp range (Fig. 4-B; Appendix E). The deletion was mainly located between the 14th and 29th bases in the downstream of the PAM sequence (Fig. 4-C; Appendix F). This method achieved a good genome editing

Table 1 Summary of multiplexing data for two crRNAs in cotton by the pGhRBE3-pGhαGloA vector

Construct	Targeted locus	Tested T_0 lines	Mutated T_0 lines (no., percentage)	Two crRNA mutation lines (no., percentage)
pGhRBE3-pGhαGloA-*GhPGF*	*GhPGF*-crRNA1	24	24 (100%)	16 (66.7%)
	GhPGF-crRNA2	24	16 (66.7%)	

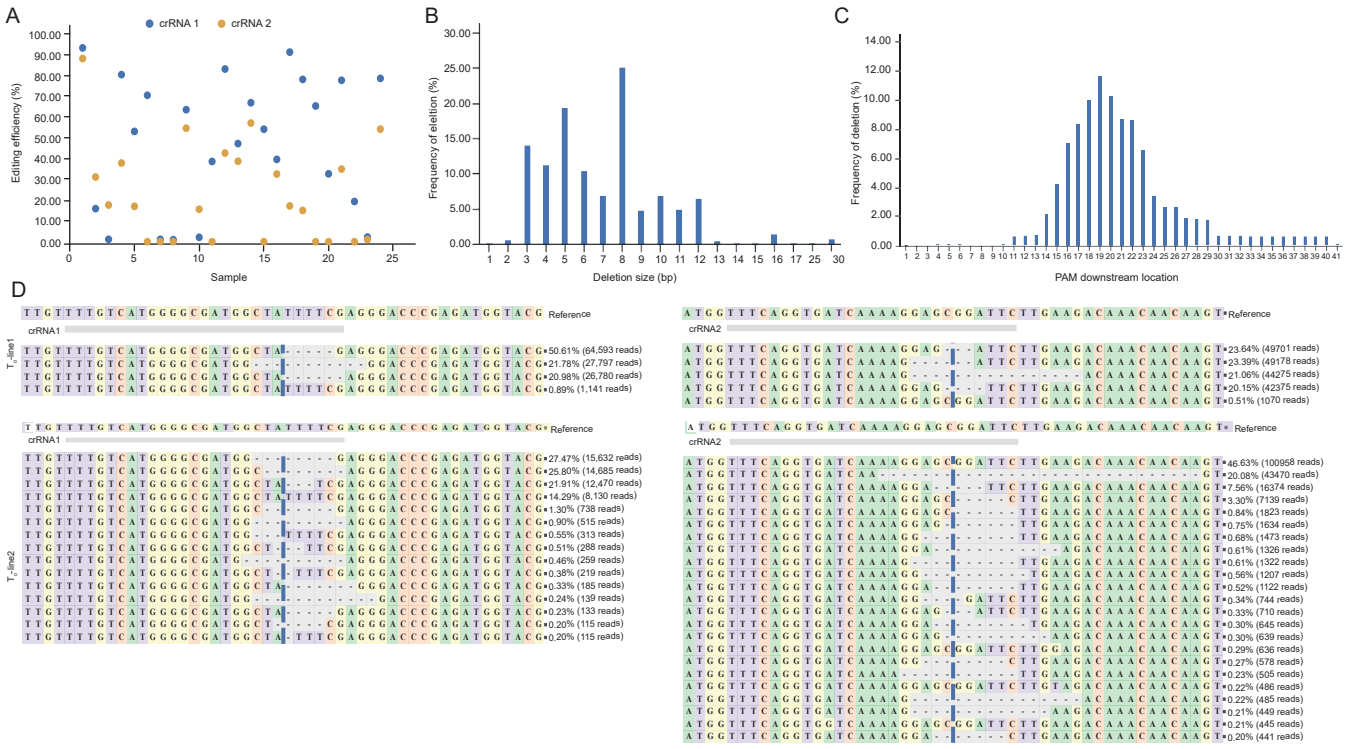


Fig. 4 Editing efficiency of pGhRBE3-pGhαGloA-*GhPGF* in plants. A, editing efficiency of crRNAs in different samples. B, ratios of different DNA deletion sizes. C, frequency of DNA deletions at different locations downstream of protospacer adjacent motif (PAM). D, editing efficiency at target sites in T_0-line1 and T_0-line2 plants.

effect in cotton. The T_0-line1 (with crRNA1 editing efficiency of 93.37% and crRNA2 editing efficiency of 88.24%) and T_0-line2 (with crRNA1 editing efficiency of 80.48% and crRNA2 editing efficiency of 37.91%) were randomly selected. Two plants with high editing efficiency were selected as the materials for subsequent genetic analyses (Fig. 4-D).

3.5. Phenotypic identification of T_1 generation plants with *GhPGF* knockout mutants

To assess whether the edited target site can be stably inherited from the T_0 generation to the T_1 generation, eight progeny plants of the T_0-line1 were selected for Sanger detection and phenotypic observation (Fig. 5; Appendix G). The results showed that the target sites of these eight T_1 plants had been completely edited. At the same time, these plants showed the glandless phenotype in the seed, petal, flower bud, stamen, cotton boll, leaf, and stalk (Fig. 5). This result shows that the editing of the target gene is heritable. To ensure stability in the progeny of gene edited plants, it is necessary to screen the progeny lines for transgene-free genome editing so that plants carrying the correct mutations can be stably passed on to the next generation.

3.6. Screening of transgene-free *pgf* mutants

In this study, transgenic materials without the glandular phenotype were obtained using the pGhRBE3-pGhαGloA gene editing vector based on the CRISPR/Cas12a system. To obtain homozygous non-glandular transgene free lines (without T-DNA residues), lines in which the *GhPGF* gene had been completely edited were backcrossed and the harvested T_2 lines were used for PCR detection to screen for homozygous progeny materials without DNA residues (Fig. 6-A). Among them, the PCR results of eight progeny plants using LbCas12a-specific primers showed that no LbCas12a fragment was present in T_2-line1, T_2-line4, or T_2-line7, which were presumed to be DNA-free progeny materials (Fig. 6). At the same time, the editing of the *GhPGF* gene target site was verified in three plants, as detected by Sanger sequencing. In these three plants, 5, 8 and 8 bp fragments were deleted in crRNA1, while fragment deletions of 6, 10 and 0 bp were found in crRNA2 (Fig. 6-B). Therefore, three homozygous lines without T-DNA residues and a glandless phenotype were obtained after identification. These lines can provide experimental materials for future variety breeding efforts and serve as important germplasm resources for the cultivation of glandless cotton varieties. This result also shows that the gene mutation produced by the CRISPR/Cas12a gene editing system driven by *pGhαGloA* in cotton can be stably passed on to the offspring plants.

4. Discussion

4.1. Advantages and further optimization of the CRISPR/Cas 12a system

The CRISPR/Cas9 system has been widely used in the life sciences, while the CRISPR/Cas12a system has been introduced more gradually (Lin *et al.* 2021; Wang *et al.* 2022, 2024). In rice, the *OsD18* promoter was edited by CRISPR/

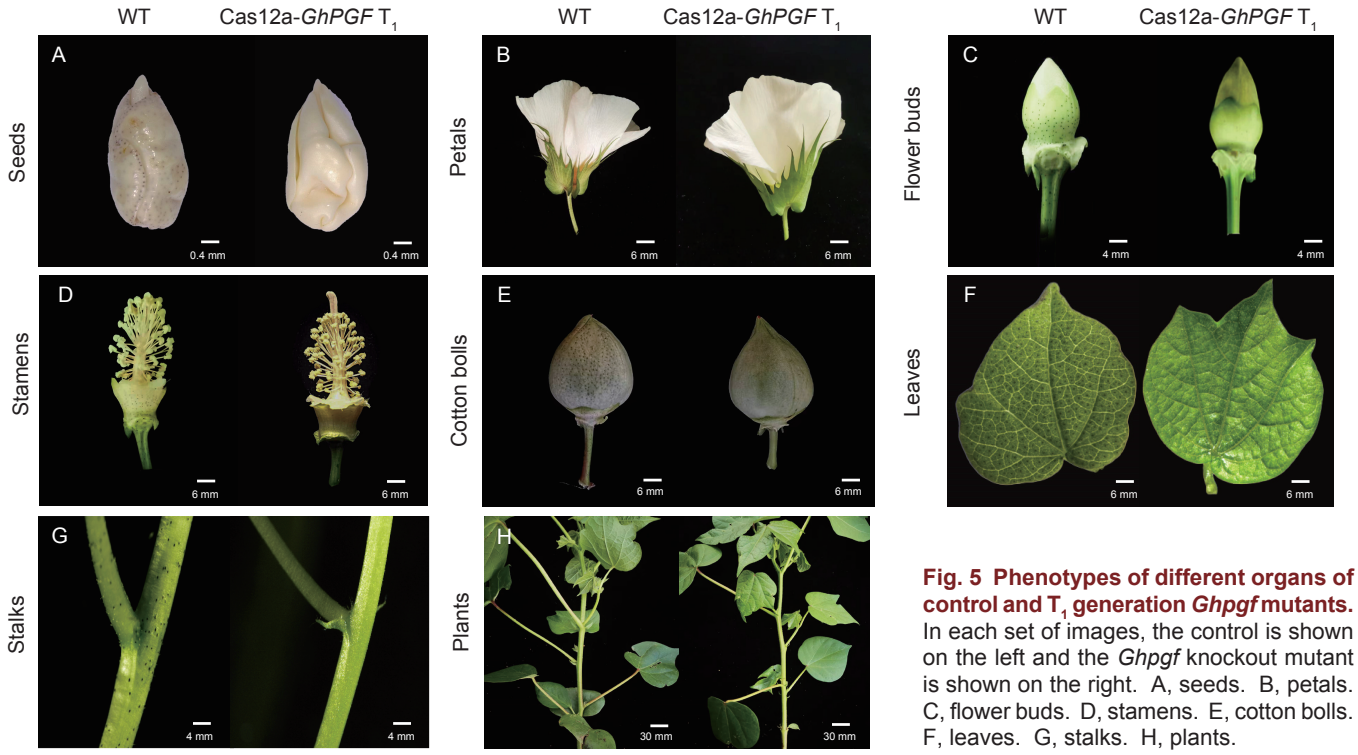


Fig. 5 Phenotypes of different organs of control and T_1 generation *Ghpgf* mutants. In each set of images, the control is shown on the left and the *Ghpgf* knockout mutant is shown on the right. A, seeds. B, petals. C, flower buds. D, stamens. E, cotton bolls. F, leaves. G, stalks. H, plants.

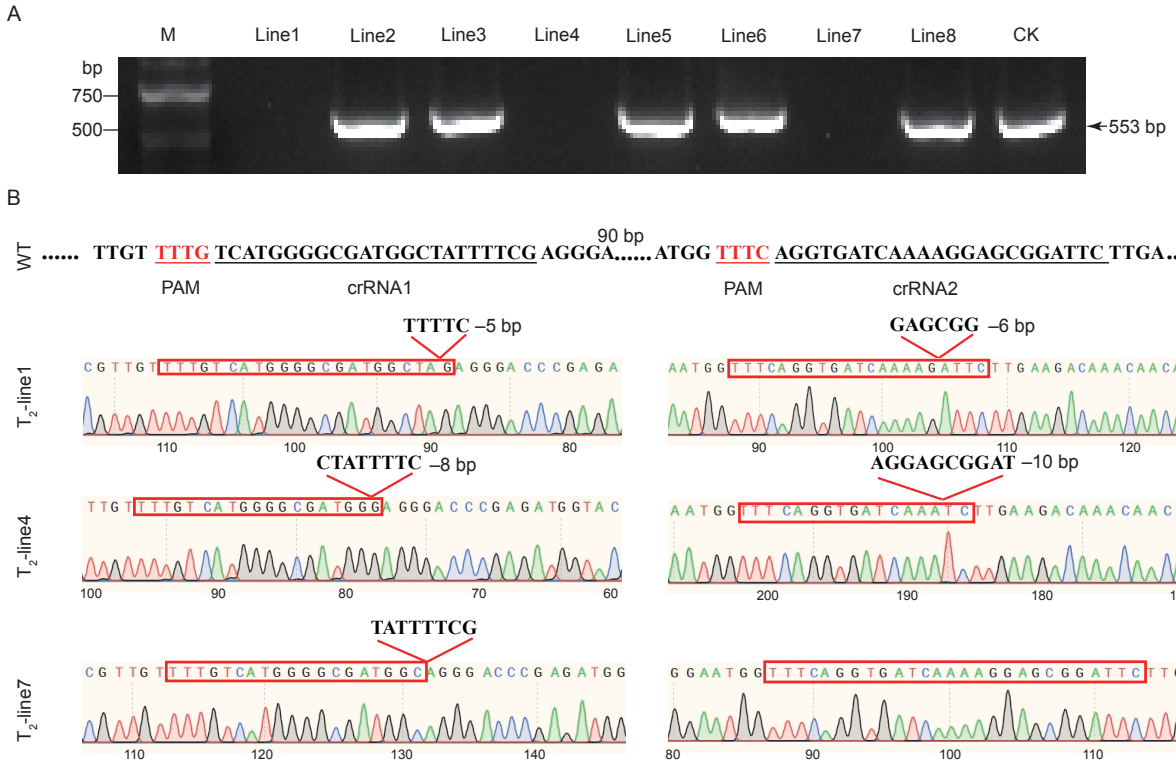


Fig. 6 Identification of transgenic and glandless T_2-generation cotton plants. A, transgene-free plant PCR assay. M, DL2000 marker; line1, line4 and line7, transgene-free plants; line2, line3, line5, line6, and line8, transgenic plants containing the pGhRBE3-pGhαGloA-*GhPGF* vector; CK, plasmid of the pGhRBE3-pGhαGloA-*GhPGF* vector. B, detection of editing in transgene-free plants (T_2-line1, T_2-line4, and T_2-line7).

Cas12a technology, and successfully produced variation in the quantitative characters (Zhou *et al*. 2023). In sheep, researchers have established a potentially efficient and low-cost method for the genotyping of *FecB*^{B} mutations based on CRISPR/Cas12a and RPA techniques, and this approach is expected to improve the efficiency of any livestock breeding

program with single nucleotide mutations (Wu *et al.* 2026). Compared with the Cas9 protein, the Cas12a protein has a lower molecular weight, so it can more easily enter the genome for cleavage (Zetsche *et al.* 2015). When cutting the target sequence, the CRISPR/Cas12a system will form sticky ends with 4 or 5 nt gaps, which make it easier to improve the editing efficiency (Manghwar *et al.* 2019). Most members of the Cas12 family can recognize T-rich PAM sites, while promoters are rich in AT regions, which makes the CRISPR/Cas12 system a good tool for promoter editing (Altae-Tran *et al.* 2021). However, studies in many organisms have also found that the Pol II promoter is more suitable for multiple sgRNA or crRNA transcription than the Pol III promoter (Tang *et al.* 2019). In addition, the use of constitutive Pol II promoters can achieve a higher genome editing efficiency (Čermák *et al.* 2017; Mikami *et al.* 2017). Therefore, in this study, the Pol II promoter was used to drive the expression of crRNAs in upland cotton.

The RNA-seq data showed that the *pGhαGloA* promoter was dominant during ovule development, suggesting that it could drive the binding of crRNAs and Cas protein more efficiently in tissue culture, thus ultimately improving the editing efficiency. The promoter of *pGhU6.7* in the pGhRBE3-Cas12a vector was replaced with the *pGhαGloA* promoter, two crRNAs of the *GhPGF* gene were connected using the structure of tRNA-gRNA-crRNA, and a new cotton gene editing vector was obtained. As mentioned above, the editing type of this vector in the cotton genome was mainly fragment deletion; however, the efficiency of obtaining positive plants by the pGhRBE3 vector was 87% (Li B *et al.* 2019). When using the pGhRBE3-pGhαGloA vector, the efficiency of obtaining positive plants can reach 100%. At the same time, the editing efficiency of the pGhRBE3 vector in individual plants is up to 94.12% (Li B *et al.* 2019), while that of the pGhRBE3-pGhαGloA vector in individual plants is up to 93.37%, and the editing efficiency of individual plants is similar. More importantly, the editing efficiency of the second crRNA in positive plants containing the pGhRBE3-pGhαGloA vector can reach 88.24%, which exceeds the 67.6% for the pGhRBE3 vector on the second crRNA of the target gene, indicating that the Pol II promoter is more suitable for expressing multiple crRNAs. Therefore, the pGhRBE3-pGhαGloA vector has higher application value in cotton genome editing.

Cotton (*Gossypium hirsutum* L.) is a heterotetraploid crop with a complex genome. Most genes have multiple copies in the At and Dt subgenomes. When editing target genes in the cotton genome, the homologous genes of cotton in the At and Dt subgroups need to be edited simultaneously to obtain completely homozygous gene editing materials (Wang *et al.* 2020). In this study, the editing efficiency of the pGhRBE3-pGhαGloA vector in individual plants of the T_0 generation was up to 93.37%. Fully edited materials can be obtained in the T_0 generation, and these materials can be stably passed on to the next generation. These results show that the pGhRBE3-pGhαGloA genome editing vector has high editing efficiency in allotetraploid cotton. In addition, the editing efficiency of base editing in cotton is currently lower than that of gene knockout. This is especially true for the cytosine single base editing (CBE) system in which the mutation of C/G to T/A, the highest editing efficiency in cotton, is only 57.78% (Qin *et al.* 2020). Therefore, the advantage of the *pGhaGloA* promoter can be used in base editing systems to improve efficiency in the future.

4.2. Gossypol can reduce the application value of cotton

Cotton is both a food crop and a fiber crop with important economic value (Radhika *et al.* 2015). The main application of cotton as a food crop is the use of cottonseed as food. Cottonseed is rich in protein, fat, carbohydrates, vitamins, and minerals (Bello and Aladesuru 2015; Zubair *et al.* 2021). Cottonseed oil from upland cotton has high protein and fiber contents; therefore, it has great potential in the food, animal husbandry, and other industries (Chuang *et al.* 2021; Kumar *et al.* 2021; Salimath *et al.* 2021). However, gossypol is a toxic substance that is harmful to monogastric animals and abundant in cottonseed, which seriously limits the comprehensive utilization of cotton byproducts (Nagalakshmi *et al.* 2007). Studies have found that the gossypol content of cotton plants is closely related to the presence of pigment glands. Plants with pigment glands contain about 34 g of gossypol per kg of cottonseed; which is significantly higher than the <20 g of gossypol per kg of cottonseed in plants without pigment glands (Wilson and Smith 1976; Rogers *et al.* 2002; Cai *et al.* 2010). In recent years, research has shown that the gossypol content can be evaluated according to the area of cotton pigment glands (Xu *et al.* 2023). In this study, the pGhRBE3-pGhαGloA gene editing vector was used to knock out the *GhPGF* gene and produce glandless cotton materials, thus creating low-gossypol or gossypol-free germplasm resources for cotton production.

In this study, an efficient and accurate CRISPR/Cas12a genome editing system was established that utilizes the Pol II promoter in cotton. The editing efficiency was as high as 100%, and the efficiency of simultaneous editing of two crRNAs reached 66.7%. At the same time, transgene-free glandless cotton materials were obtained, providing new germplasm resources for cotton production. This developed system can promote cotton functional genomics research and molecular breeding and provide technical support for precision genome editing in cotton.

5. Conclusion

In this study, the construction of a cotton endogenous Pol II type promoter (*pGhαGloA*) into CRISPR/Cas12a-mediated genome editing vectors to drive the high transcription of crRNAs was able to improve the editing efficiency in cotton, especially for the editing of multiple crRNAs. Meanwhile, transgene-free genome editing progeny materials were obtained, which will play a key role in healthy cottonseed oil/cake production.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that we did not use AI in the preparation and writing of this manuscript.

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