Efficient virus-mediated genome editing in cotton using the CRISPR/Cas9 system

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Plant virus-mediated sgRNA delivery and expression have great advantages; sgRNA expression can rapidly expand and accumulate along with virus replication and movement, resulting in efficient gene editing efficiency. In this study, a VIGE system based on cotton leaf crumple virus (CLCrV) was established using cotton overexpressing Cas9 (Cas9-OE) as the VIGE receptor. CLCrV-mediated VIGE could not only target and knock out the GhMAPKK2, GhCLA1 and GhPDS genes subgroup A and D genome sequences but also achieve double mutation of GhCLA1 and GhPDS genes at the same time. These results verified the effectiveness and efficiency of this system. In addition, the off-target effect assay demonstrated that the CLCrV-mediated VIGE system not only has high gene editing efficiency but also high gene editing specificity in cotton. We further explored whether the FT-sgRNA strategy could transport sgRNA to cotton apical meristem (SAM) over long distances to avoid using tissue culture to obtain stable genetic mutants. The results showed that the sgRNA fused with FT mRNA at the 5’ end could also efficiently achieve targeted editing of endogenous genes in cotton, but it was difficult to detect heritable mutant progeny. The above results showed that the CLCrV-mediated VIGE system provided an accurate and rapid validation tool for screening effective sgRNAs in cotton.

KEYWORDS
cotton, Cas9-OE, CLCrV, VIGE, FT-sgRNA, mutation

Introduction

The acquisition of genetic variant mutants is required for the identification of gene functions and the selection of new varieties of cotton. Random mutations, such as those from artificial mutagenesis and insertional mutagenesis using either T-DNA or transposons, are far from meeting the needs of scientific research and breeding in cotton. The emergence of CRISPR/Cas9 genome editing technology avoids the disadvantage of random mutation blindness and provides important technical tools for realizing precise genetic improvement of
cotton. To date, a series of cotton CRISPR/Cas gene editing systems have been established and successively upgraded (Long et al., 2018; Li B et al., 2019; Qin et al., 2020; Wang et al., 2022). However, nearly all genetic variation created through CRISPR/Cas relies on the method of tissue culture, which is time-consuming and laborious for cotton. In addition, some studies have found that sgRNA, one of the key elements of the CRISPR system, is inefficient or invalid at many genomic loci (Rehmoun and Barbara, 2015). Moreover, it is difficult to predict the activity of sgRNAs by bioinformatics methods (Xie et al., 2014; Zhao et al., 2019). Therefore, before using the CRISPR system to obtain cotton mutants, it is necessary to quickly screen out effective sgRNAs. Protoplast transient transformation (Chen et al., 2017) and Agrobacterium-mediated transient transformation of cotton leaves (Gao et al., 2017) are the two most common methods to verify sgRNA activity. However, due to low transient transformation efficiency resulting from the large size of the Cas9 gene (>4000 bp), gene editing events are difficult to detect, which leads to false negative results.

Viruses can efficiently deliver sgRNAs to achieve editing of target genes. However, nearly all current research reports have shown that these editing events can only occur in contemporary plants (Yin et al., 2015; Cody et al., 2017; Ali et al., 2019; Ellison et al., 2020; Jiang et al., 2019; Li et al., 2021; Chen et al., 2022), soybean (Luo et al., 2021) and maize (Hu et al., 2019). The application of the VIGE system in cotton and verified the accuracy of this system in screening effective sgRNAs. At the same time, an FT-sgRNA strategy to achieve heritable gene editing in cotton was explored.

Materials and methods

Plant materials and growth conditions

Cas9-OE cotton variety YZ-1 (Zhu et al., 2018) and wild-type seeds were soaked in ddH2O water for 3 days and then germinated in the dark at 28°C for 48 h. After germination, the seedlings were transplanted into nutrient soil and grown at 28°C under 12 h light/12 h dark conditions. The virus inoculation transformation experiment was performed when the two cotyledons of the cotton seedlings were fully expanded.

qRT–PCR analysis of Cas9 expression

RNA was isolated from wild-type and Cas9-OE cotton cotyledons, and cDNA was synthesized by reverse transcription. The 188 bp Cas9 gene fragment was amplified by qRT–PCR, and the GhUBQ7 gene was used as the reference gene. The primer design method is shown in Supplementary Table 1. Three technical replicates were set up for each sample. After the reaction, according to the Ct value of the Cas9 gene and the reference gene, the relative expression levels of the Cas9 gene in different plants were calculated using the 2−ΔΔCt method (Livak and Schmittgen, 2001).

Vector construction

Construction of sgRNA expression vector: Truncated AtU6-26 (331 bp) (Feng et al., 2013) and AtU6 (79 bp) (Nekrasov et al., 2013) promoters were used to drive sgRNA expression. The 20 bp guide RNA (Supplementary Table 1) was synthesized according to the method in the literature (Lei et al., 2021), and sequencing was performed to verify the correctness of the sgRNA containing the target sequence. The AtU6-26:sgRNA fragments with target sequences were recovered by SpeI and PacI digestion and assembled on CLCrV-A. In addition, an intact editing vector (AtU6-26: GhMAPKKK2-sgRNA-Cas9-p1300) targeting the knockout GhMAPKKK2 gene was constructed to transform wild-type cotton.

Construction of multiple gene editing vector: The GhCLAI and GhPDS genes were used as targets, and two different AtU6-
26 (331 bp) and AtU6 (79 bp) promoters were used to drive sgRNA expression to minimize the probability of gene silencing caused by using the same promoter. The CLCrV-AtU6::GhPDS-sgRNA-AtU6-26::GhCLA1-sgRNA1 vector was constructed by enzyme digestion and ligation.

Construction of the FT-sgRNA expression vector: The AtU6-26::GhCLA1-sgRNA1 and AtU6-26::GhPDS-sgRNA plasmids was used as the acceptor template, and the Arabidopsis FT gene (528 bp) was used as the donor plasmid template. Then, 102 bp FT was fused to the 5' ends of GhCLA1-sgRNA1 and GhPDS-sgRNA by transfer PCR (Erijman et al., 2014). The primer design is shown in Supplementary Table 1. Sequencing was used to verify whether the FT-sgRNA vectors were correct.

Transient transformation of Cas9-OE cotton with CLCrV-sgRNA

For virus inoculation and transient expression, AtU6-26::GhMAPKKK2-sgRNA-Cas9-p1300 and different CLCrV-sgRNA expression vectors and CLCrV-B were transformed into Agrobacterium tumefaciens strain GV3101. The Agrobacterium cultures were inoculated in 15 ml of LB medium (containing 50 μg/ml Rif and 50 μg/ml Kan) at 28°C and 180 rpm and grown overnight at OD600 to approximately 1.6-1.8. The Agrobacterium cultures were harvested and resuspended in transformation solution (10 mM MgCl₂, 10 mM MES and 200 μM acetosyringone), adjusted to an OD600 of approximately 1.0, and incubated at room temperature for 3-4 h in the dark. For virus inoculation, CLCrV-B and derivatives of CLCrV-A were mixed 1:1 in equal proportions. The transformation solution was injected into Cas9-OE cotton leaves with a 1 mL syringe. In addition, AtU6-26::GhMAPKKK2-sgRNA-Cas9-p1300 Agrobacterium transformants were inoculated into wild-type cotton.

Mutation detection

To detect whether the target gene was mutated, genomic DNA was extracted from cotton leaves inoculated with AtU6-26::GhMAPKKK2-sgRNA-Cas9-p1300 and different CLCrV-A derivatives. A PCR/RE assay (Gao et al., 2017) was used to detect the mutation of the target site, and PCR amplified a genomic fragment containing the target site and appropriate restriction endonuclease site. The amplification primers are shown in Supplementary Table 1. The PCR product was digested with restriction enzymes at the target site, while undigested PCR amplicons were cloned into a Blunt Zero cloning vector and sequenced. Finally, the plants with the mutant phenotype were selected, and Hi-TOM high-throughput sequencing was used to detect the gene editing efficiency of the mutant individual plants. The amplification primers are shown in Supplementary Table 1.

Off-target analysis of CLCrV-mediated VIGE systems

Based on the designed GhCLA1-sgRNA1 and GhPDS-sgRNA sequences, potential off-target sites were identified using CRISPR-GE (Xie et al., 2017) (http://skl.scau.edu.cn/offtarget/) online software. Each potential off-target sequence contained 3-4 bp of mismatched bases, and these potential off-target sequences were used to search the upland cotton database (https://www.cottongen.org/) for the corresponding homologous sequence of each predicted off-target sequence. The primers (Supplementary Table 1) were designed to detect the off-target rate, and the fragment covering the off-target sequence was amplified by PCR. The off-target rate was detected by Hi-TOM high-throughput sequencing.

Results

Detection of Cas9 gene expression in cotton Cas9-OE plants

In the VIGE system, high expression of Cas9 is the primary key factor in obtaining gene editing efficiency (Liu et al., 2017; Cho et al., 2018). qRT-PCR analysis showed that the Cas9 gene was stably expressed in different Cas9-OE cotton plants (Zhu et al., 2018) with only minor differences in expression (Figure 1). Cas9-OE transgenic lines with higher Cas9 expression were selected for further VIGE analysis.

Development of efficient methods for validating sgRNA activity in cotton

CLCrV is a cotton DNA virus whose infection is not affected by coat protein deficiency (Tuttle et al., 2012) and can carry 800 bp of exogenous DNA segments for gene silencing (Gu et al., 2014). In this study, the CLCrV coat protein gene was replaced with a sgRNA expression cassette. To investigate the efficiency of the CLCrV-mediated VIGE system in cotton, the accuracy of two methods to verify sgRNA activity, Agrobacterium-mediated transient transformation of intact gene editing vectors with Cas9 and sgRNA (hereafter referred to as ATTI) and CLCrV-mediated transient transformation of the sgRNA expression cassette, were tested. GhMAPKKK2 (Li et al., 2022) was used as a target, and AtU6-26::GhMAPKKK2-sgRNA-Cas9-p1300 and CLCrV-AtU6-26::GhMAPKKK2-sgRNA were inoculated into the leaves of wild-type and Cas9-OE cotton plants, respectively. The mutation detection results showed that all the PCR products of the GhMAPKKK2 gene inoculated with AtU6-26::GhMAPKKK2-sgRNA-Cas9-p1300 plants were completely digested, and
FIGURE 1
qRT–PCR detection of Cas9 expression. Different letters indicate significant differences among different treatments at the 0.05 probability level.

FIGURE 2
Detection of gene editing effects in two transient transformation modes. (A) Detection of AtU6-26::GhMAPKK2-sgRNA-Cas9-p1300 targeted mutations. Wild-type served as a control, and 1-10 were plant numbers. The gel image shows PCR products of the GhMAPKK2 gene and digested PCR products with NdeI. (B, C) Detection of CLCrV-AtU6-26::GhMAPKK2-sgRNA targeted mutations. (B) Wild-type served as a control, and 1-4 were plant numbers. The gel image shows PCR products of the GhMAPKK2 gene and digested PCR products with NdeI. (C) The undigested PCR products lacking the NdeI site (due to the presence of a mutation) that were subsequently purified, cloned, and analyzed by sequencing. The green color indicates the PAM sequence. The NdeI restriction site on the target sequence is underlined in blue. M indicates the mutation sequence. Deletions are shown as red dashes.
almost no amplification products remained (Figure 2A). In contrast, incomplete digestion of the GhMAPKKK2 gene PCR product was detected in Cas9-OE plants inoculated with CLCrV-AtU6-26::GhMAPKKK2-sgRNA (Figure 2B). Further sequencing results showed that different types of base deletion mutations appeared in the GhMAPKKK2 gene (Figure 2C). The results showed that the editing of cotton endogenous genes of subgroups A and D could be achieved efficiently by using the CLCrV delivery sgRNA strategy, which verified the feasibility of sgRNA, but ATTI did not. These results indicated that the CLCrV-mediated VIGE system verified sgRNA activity accurately and avoided false negative results.

To further test the reliability of the CLCrV-mediated VIGE system for validating sgRNA activity and whether a mutant phenotype can be observed, the GhCLA1 and GhPDS genes, whose mutant leaves were albino, were used as targets.

Three target loci were determined (Supplementary Table 1), referring to the sequences of the GhCLA1 and GhPDS genes. The leaves of some plants inoculated with GhPDS-sgRNA, GhCLA1-sgRNA1, and GhCLA1-sgRNA2 showed a yellow spot phenotype, while the controls with empty vectors did not (Figure 3A). The mutation detection results showed that there were base deletions, insertions, and substitutions in the GhCLA1 and GhPDS genes (Figures 3B–D and Supplementary Figures 1–3). The results indicated that CLCrV-mediated VIGE is a good system for identifying the effectiveness of sgRNA and gene function. In addition, we selected some plants with albino phenotypes to detect the mutation efficiency. The Hi-TOM high-throughput sequencing results showed that the mutation efficiencies of GhCLA1-sgRNA1, GhCLA1-sgRNA2 and GhPDS-sgRNA were 30.01-51.14%, 16.85-42.46%, and 25.74-52.68%, respectively.
CLCrV-mediated multiple gene editing in cotton

The CRISPR/Cas9 system can simultaneously cause mutations in multiple genes (Doll et al., 2019; Zeng et al., 2020), which may prove extremely valuable for crops such as cotton, for which transformation is time-consuming and laborious. To verify the multiplex editing capability of the CLCrV-mediated VIGE system in cotton, the GhCLA1 and GhPDS genes described above were used as targets. The DNA segments of AtU6-26::GhCLA1-sgRNA1 and AtU6::GhPDS-sgRNA were connected in series into the CLCrV-A vector (Figure 4A). Mutation detection results showed that a single gene mutation could be detected in most samples. In contrast, double mutations of both GhCLA1 and GhPDS could be detected in only a few samples (Figure 4B). Hi-TOM high-throughput sequencing results showed that the double mutation efficiencies of GhCLA1-sgRNA1 and GhPDS-sgRNA were 8.02% and 25.73%, respectively. The results indicated that the CLCrV-mediated VIGE system could deliver multiple sgRNAs at the same time to enable multigene editing.

Off-target analysis of CLCrV-mediated VIGE systems

The occurrence of off-target effects is a key issue in the application of CRISPR/Cas9 gene editing technology in plant functional genomics research and molecular breeding (Li JY, et al., 2019; Wang et al., 2021). To verify whether there was an off-target effect of the CLCrV-mediated VIGE system, five potential off-target sites were retrieved from the cotton database based on GhCLA1-sgRNA1 and GhPDS-sgRNA (Figure 5). The off-target rate was identified in the genomic DNA of leaves inoculated with GhCLA1-sgRNA1 and GhPDS-sgRNA. The high-throughput sequencing results showed that no off-target phenomenon was found in the five predicted potential off-target sites (Table 1), indicating that the CLCrV-mediated VIGE system in cotton not...
only has high gene editing efficiency but also has high gene editing specificity.

**Gene editing in cotton using the FT-sgRNA strategy**

Referring to the FT-sgRNA strategy that could achieve heritable gene editing progeny in *Nicotiana benthamiana* and *Arabidopsis* (Ellison et al., 2020; Lei et al., 2021), it was further verified whether this strategy could work in cotton. *GhCLA1* and *GhPDS* were used as targets, and the FT (102 bp) gene was fused to the 5’ end of *GhPDS*-sgRNA and *GhCLA1*-sgRNA1, respectively (Figure 6A). The two fusion expression vectors were transformed into Cas9-OE cotton leaves. Mutation detection results showed that the PCR products of the *GhPDS* gene from eleven plants transformed with *FT*-GhPDS-sgRNA were incompletely digested, and three of them (#1, #9 and #22) were selected for mutation detection. Deletions or insertions were detected in the *GhPDS* gene (Figure 6B and Supplementary Figure 4). For the plants transformed with *FT*-GhCLA1-sgRNA1, PCR products from five plants were incompletely digested, and the genotypes of three plants (#4, #11 and #18) were deletions, insertions and substitutions of bases in the *GhCLA1* gene (Figure 6C and Supplementary Figure 5). The above results showed that fusion of 102 bp FT mRNA to the 5’ end of sgRNA could also effectively achieve gene editing in cotton. High-throughput sequencing results showed that the editing efficiency of FT-sgRNA for *GhCLA1* and *GhPDS* genes was 23.98-50.07% and 28.26-55.43%, indicating that there was no significant difference between unmodified sgRNA and FT-sgRNA with editing efficiency.

Whether CLCrV-mediated VIGE can achieve gene editing in germ cells of cotton and obtain mutant seeds for target genes remains to be determined. The plants inoculated with unmodified sgRNA and FT-sgRNA continued to grow for 50-60 days, and the phenotype of incomplete albinism of plant leaves continued until the reproductive growth stage (Figure 7). Seeds were harvested from these plants and sown. The mutant phenotype was observed after 15-20 days. However, no obvious phenotypes were observed in any progeny of plants inoculated with unmodified sgRNA (M1, n=158) or inoculated with FT-sgRNA (M1, n=109). The high-throughput sequencing analysis of all M1 generation plants showed that only different base substitution types of *GhPDS* and *GhCLA1* genes in the target sequence were detected, the frequency was less than 3% similar to the control, and no mutation types of base insertion and deletion were detected (Supplementary Figure 6). We speculate that these variations may be caused by random mismatches during PCR amplification rather than the result of gene editing.

**Discussion**

Whole-genome sequencing of cotton has been completed. However, the function of cotton genes and the biological
FIGURE 6
FT-sgRNA targeted mutagenesis of GhPDS and GhCLA1 in cotton. (A) Truncated FT RNA was fused to the 5' end of GhPDS-sgRNA and GhCLA1-sgRNA. (B) Detection of FT-GhPDS-sgRNA targeted mutations. Wild-type served as a control, and 1-23 were plant numbers. The gel image shows the digested PCR products of the GhPDS gene with BfaI, and the undigested PCR products lacking the BfaI site (due to the presence of a mutation) were subsequently purified, cloned, and analyzed by sequencing. (C) Detection of FT-GhCLA1-sgRNA targeted mutations. Wild-type served as a control, and 1-23 were plant numbers. The gel image shows the digested PCR products of the GhCLA1 gene with PstI, and the undigested PCR products lacking the PstI site (due to the presence of a mutation) were subsequently purified, cloned, and analyzed by sequencing. Green indicates the PAM sequence. The restriction site on the target sequence is underlined in blue. M indicates the mutation sequence. Insertions are denoted with red capital letters. Deletions are shown as red dashes. Substitutions are denoted with red lowercase letters.
significance of all DNA elements in the whole genome are still poorly understood. Mutants from target site mutations in cotton are required for the above research, but they are not yet available. The emergence of CRISPR/Cas gene editing technology provides a powerful reverse genetics tool for cotton functional genomics research. Although cotton CRISPR/Cas gene editing systems have been established and a few gene-edited cotton plants have been obtained with the system, it is inconceivable to build gene mutant libraries of cotton and achieve precise genetic improvement at the genome-wide level. Therefore, it is worth establishing a gene editing system with high efficiency and easy operation in cotton.

Plant viral vectors are ideal tools for the delivery and transient expression of exogenous genes in plants and are often used for gene silencing and gene editing in plants. The CRISPR/Cas system is large (usually >5 kb); therefore, it is difficult to use viruses to deliver the system into plant cells by virus infection (Yin et al., 2015; Cody et al., 2017; Ali et al., 2018; Jiang et al., 2019), except for negative-strand RNA sonchus yellow net rhabdovirus (SYNV) (Ma et al., 2020). CLCrV is a DNA virus whose genome has been modified to establish the VIGS system in upland cotton (Gu et al., 2014; Zhang et al., 2016), however, it is still unknown whether CLCrV can also be used as a sgRNA delivery tool for targeted editing of endogenous genes in cotton. Although the CLGv vector is not suitable for expressing the Cas9 gene, it is sufficient to express short sgRNAs. Therefore, Cas9-OE cotton plants were used as VIGE receptors, and sgRNA was delivered by CLCrV, which can accumulate and spread rapidly in cotton. For GhMAPKK2-sgRNA, GhCLA1-sgRNA and GhPDS-sgRNA, mutation analysis results demonstrated that this system could efficiently edit endogenous genes in subgenomes A and D of cotton (Figures 2, 3). Furthermore, double mutation of GhPDS and GhCLA1 genes could be achieved by expressing multiple sgRNAs simultaneously in cotton (Figure 4), which provides a favorable tool for studying the functions of gene families and signaling pathways in cotton. For potential off-target sites, no gene editing events were detected. Our experiments demonstrated that the CLCrV-mediated VIGE system not only has high gene editing efficiency but also high gene editing specificity in cotton (Figure 5 and Table 1).

Similar to other plant viruses, such as PEBV (Ali et al., 2018), TMV (Cody et al., 2017), BNYVV (Jiang et al., 2019), BSMV (Hu et al., 2019), and CaLCuV (Yin et al., 2015)-mediated VIGE systems, the CLCrV-mediated VIGE system could efficiently achieve targeted editing of endogenous genes in Arabidopsis (Lei et al., 2021) and cotton. However, these editing events can only occur in contemporary plants, and it is difficult to obtain heritable gene-edited offspring. Although the strategy of fusing sgRNA with FT (Ellison et al., 2020; Lei et al., 2021) can avoid the use of tissue culture to obtain heritable gene-edited plants, it currently only works in Nicotiana benthamiana and Arabidopsis, while its application on cotton has not been reported. In this study, we continued to investigate whether the FT-sgRNA strategy could generate heritable gene-edited offspring in cotton based on the CLCrV-mediated VIGE system. Similar to the results in Arabidopsis (Lei et al., 2021), sgRNAs fused with FT mRNA at its 5’ end could also efficiently achieve targeted gene editing in cotton (Figure 6). However, mutations in target sites were not detected in M1 progeny seedlings from the
parental plants infected with \textit{FT}-sgRNA and unmodified sgRNA. Therefore, we speculate that CLCrV virus or \textit{FT}-sgRNA cannot diffuse into cotton germ cells to achieve heritable editing, or even if it can enter the germ cells, the efficiency of mutation is very low, indicating that CLCrV does not belong to the virus type that can colonize the SAM or \textit{FT} could not transport sgRNA into the SAM of cotton. Recently, it was shown that transgenic \textit{Nicotiana attenuata} with the expression of Cas9 driven by the germ cell-specific RPS5A promoter was used as the VIGE receptor, and TRV-mediated VIGE was used to obtain low-efficiency heritable gene editing in the offspring (Oh and Kim, 2021). Even though the heritability of this approach is low, it provides another strategy for the targeted creation of mutant materials in plants that are difficult to genetically transform without going through a tissue culture process.

The above results showed that the CLCrV-mediated VIGE system can effectively achieve targeted editing of cotton endogenous genes but cannot or at least is difficult to achieve heritable gene editing. BSMV has been found to be a plant virus that can colonize SAM and achieve heritable gene editing (Lin and Langenberg, 1985; Li et al., 2021); it is of interest to determine whether there is a certain type of virus that can colonize SAM for heritable gene editing in cotton. This requires extensive screening of the various types of viruses that can enter the SAM of cotton. If not, other strategies will need to be explored to achieve heritable editing. Our study provides an accurate and rapid validation tool for screening effective sgRNAs in cotton.

Author contributions

JL and XL designed the experiments. JL performed most of the experiments and analyzed the data, other authors assisted in experiments. JL and XL wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Xinjiang Uygur Autonomous Region Major Science and Technology Project (2021A02001-3).

Acknowledgments

We thank Professor Xueping Zhou from Zhejiang University for providing the CLCrV-A and CLCrV-B vectors. We thank Professor Jie Sun from Shihezi University for providing the Cas9-OE cotton seeds.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement

The data presented in the study are deposited in the JL repository, accession number DOI: 10.3389/fpls.2022.1032799. Email: kylejianfeng@163.com.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1032799/full#supplementary-material
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