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CONTENT

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Suppression of CLCuV in cotton (*Gossypium hirsutum* L.) using CRISPR/Cas9 system

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Abstract

When developing CRISPR/Cas9 systems for crops, it is crucial to invest time characterizing the genome editing efficiency of the CRISPR/Cas9 cassettes, especially if the transformation system is difficult or time-consuming. Cotton is an important crop for the production of fiber, oil, and biofuel. Furthermore, cotton is a heterotetraploid and targeted mutagenesis is considered to be difficult as many genes are duplicated in this complex genome. The application of CRISPR/Cas9 in cotton is severely hampered by the long and technically challenging genetic transformation process, making it imperative to maximize its efficiency. In this study, we provide a new system to evaluate and validate the efficiency of CRISPR/Cas9 cassettes in cotton using a transient expression system. By using this system, we could select the most effective CRISPR/Cas9 cassettes before the stable transformation. The resistance against virus infection and suppression of replication of the virus was assessed by PCR, RT-PCR and symptoms development on plant leaves in terms of days' post inoculation (dpi). Expression of the Cas9 and gRNA was quantified using RT-PCR. The results showed partial inhibition of CLCuV replication, lower disease symptoms and virus accumulation as compared to control plants.

Keywords: CRISPR/Cas9, CLCuV, Transient expression, Genome editing

Introduction

Cotton is one of the leading fibres, a strong source of biofuels and oil production. Increased sequence availability stressed the need for quick and cost-effective instruments to generate targeted mutations to conduct large scale cotton gene functional studies (Zhang *et al*., 2015). Targeted genome editing is an extremely useful method for basic and applied plant science, like meganucleases, zinc fnger nucleases (ZFNs), and TALENs have been available (Gaj *et al*., 2013; Zhang *et al*., 2017). However, before the advent of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/ CRISPR-associated Protein9) the technological uncertainty of this technology hindered the broadest community's acceptance by the scientific community. The inherent in CRISPR/Cas9 versatility, simplicity and high efficiency have led to the explosion of gene editing research as the preferred method of precisely generating genome changes for a wide range of plant species (Shan *et al*., 2014).

CRISPR/Cas9 comes from a microbial adaptive immune system with its principal components as Cas9, which creates double-strand breaks and a small guide RNA (sgRNA). In important crops and models such as rice, wheat, Arabidopsis, Nicotiana, sorghum, poplar, maize and tomatoes, CRISPR/Cas9 was successfully used for genetic processing (Li *et al*., 2013; Mao *et al*., 2016). In the cotton industry, a growing number of agricultural genes, mostly involved in stress resistance and fibre development, are identified and a CRISPR/Cas9 functioning system for this plant is urgently developed (Gao *et al*., 2011; Long *et al*., 2014). CRISPR/Cas9 can be used to edit the genome of cotton (Chen *et al*., 2017; Li *et al*., 2017. The comprehensive application of this technology however is limited by the long and technically complicated transformation method for this crop. In this study we have established a rapid method to validate CRISPR/Cas9 gene editing in cotton with the use of transient cotyledon expression, which can be performed within three days. The new approach has been employed successfully for a variety of purposes, including validation of sgRNAs for individual genes. The cotton stable transformation usually takes 8 to 12 months to produce T0 plant using *Agrobacterium tumefaciens*. Cotton is also a heterotetraploid and is considered difficult to target mutagenesize since several genes in the complicated genome are duplicated. The long and technically challenging genetic transformation process is hampering the application of CRISPR/Cas9 in cotton and therefore it is imperative to increase its production. In this analysis, the functionalities of the SgRNAs are validated by means of a transient expression method have been established quickly and efficiently.

Material and Method

Before being allowed to germinate at 28 ˚ C for 24 hours in the dark, cotton (*Gossypium hirsutum* L.) seeds were submerged in deionized water for 8 hours. The seedlings were cultivated in soil under 12 h/12 h light/dark conditions at 22/25◦C (night/day) after germination. For transient transformation experiments, ten-day-old

seedlings were used. For transient transformation experiments, CRISPR/Cas9 vectors were pushed into *Agrobacterium tumefaciens* (GV3101), respectively. For transient conversion, GV3101 was infiltrated using an unnecessary syringe into cotyledons of 14-day-old cotton seedlings. In the plant growth chamber at 25◦C the seedlings were incubated. Every vector has been attached to *A. tumefaciens* GV3101 and GV3101 vector strains in the selection media were grown at 28 degrees Celsius. Centrifuged and suspended in the infltrative media [10 mM magnesium chloride, 10 mM 2-(N-morpholine) ethyl sulfonic acid and 200µm acetosyringon] were collected by Agro bacterium cells. The Agrobacterium suspension was affected by tobacco leaves or cotton cotyledons following incubations at room temperature for 3 h. At least three times with over 8 leaves per test were replicated (Geo et., 2017). After an incubation of 48 hours, cotyledon infiltrations were moved into the fields for two weeks and virus symptoms appeared on the plant leaves (Gao *et al*., 2011). Genomic DNA with a DNA extraction device has been isolated from infiltrated cotyledons. Complete RNA has been isolated by means of an RNA extraction kit from transgenic cotton lines. The M-MLV Reverse Transcription Method was applied to cDNA of the first beam from 1 µg of total RNA. The following PCRs were performed. The sequences of CRISPR were downloaded from different databases and Multisequence alignment and phylogenetic analysis was done using Mega X software (Dornelas *et al*., 2000).

Results

1 Plant materials and growth conditions

Cotton (*Gossypium hirsutum L.*) seeds were imbibed in deionized water for 8 h before being allowed to germinate at 28◦C for 24 h in the dark. Following germination, the seedlings were grown in soil at 22/25◦C (night/day) under 12 h/12 h light/dark conditions.

2 Transient transformation in cotton and *N. benthamiana*

Ten-day-old seedlings of *G. hirsutum L* were used for transient transformation experiments. CRISPR/Cas9 vectors were transferred into *Agrobacterium tumefaciens* (GV3101 and LBA4404) for transient and stable transformation experiments, respectively. For transient transformation, GV3101 was infiltrated into cotyledons of 10-day-old cotton seedlings using a needless syringe.

The seedlings were incubated in a plant growth chamber at 25◦C and after 2 days plants was shifted into field and symptoms of virus appeared with in 2 weeks after that cotyledons were harvested for genomic DNA isolation and PCR/RE analysis.

Fig. 3 Agro-infiltrated of infectious clone (*N. benthamiana*) was shows symptoms of virus after 21 days

3 PCR amplification and Expression analysis of CRISPR/Cas9 and CLCuv confirmation

Infectious clones (CLCuKV/CLCuMB) infiltrated leaves were taken at 14 dpi to detect the presence of virus through PCR and compared with symptom development. Ten out of 10 virus infected plants showed strong disease symptoms and presence of virus. Results of PCR showed that all plants accumulated CLCuKV/CLCuMB in the systemic leaves showing disease symptoms. Accumulation of virus under transient expression of Cas9 was analyzed in *N. benthamiana*. A decrease in virus symptoms, in terms of leaf curling and vein thickening, was evident due to Cas9. The results of RT-PCR analysis indicated low titer of virus (0.2-0.4 compared to 1 of control) due to Cas9 and low accumulation of the virus in systemic leaves. Co-infiltrated plants with Cas9 and CLCuKV/CLCuMB showed decrease in the virus titer up to 60-80%. Expression of gRNA-Cas9 showed suppression of virus proliferation in terms of delay in symptoms was observed through PCR, mild and attenuated symptoms, and low virus accumulation.

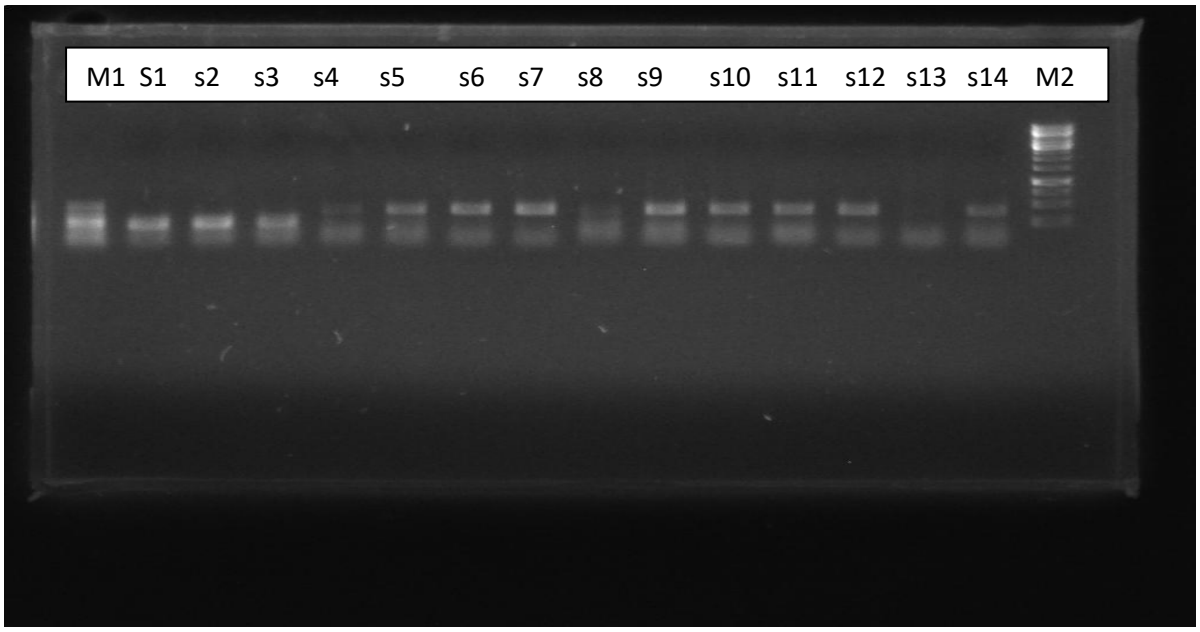

Fig. 4 M1= Ladder 50 bp and M2 = Ladder 1 Kb S show sample 1 to 14 Semi PCR confirmation of Cas9

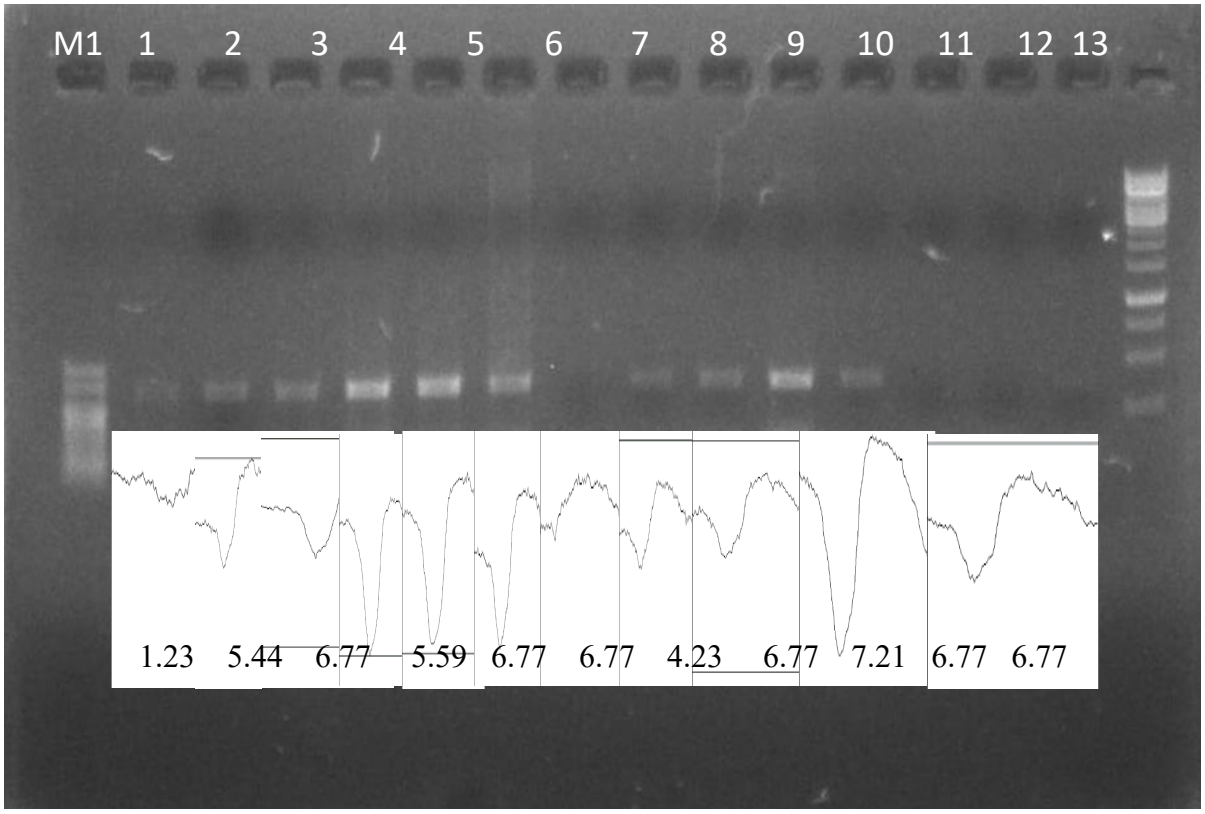

Fig. 5 RT PCR Expression results of CLCuV in Cotton (*G. hirsutum*) Leaves

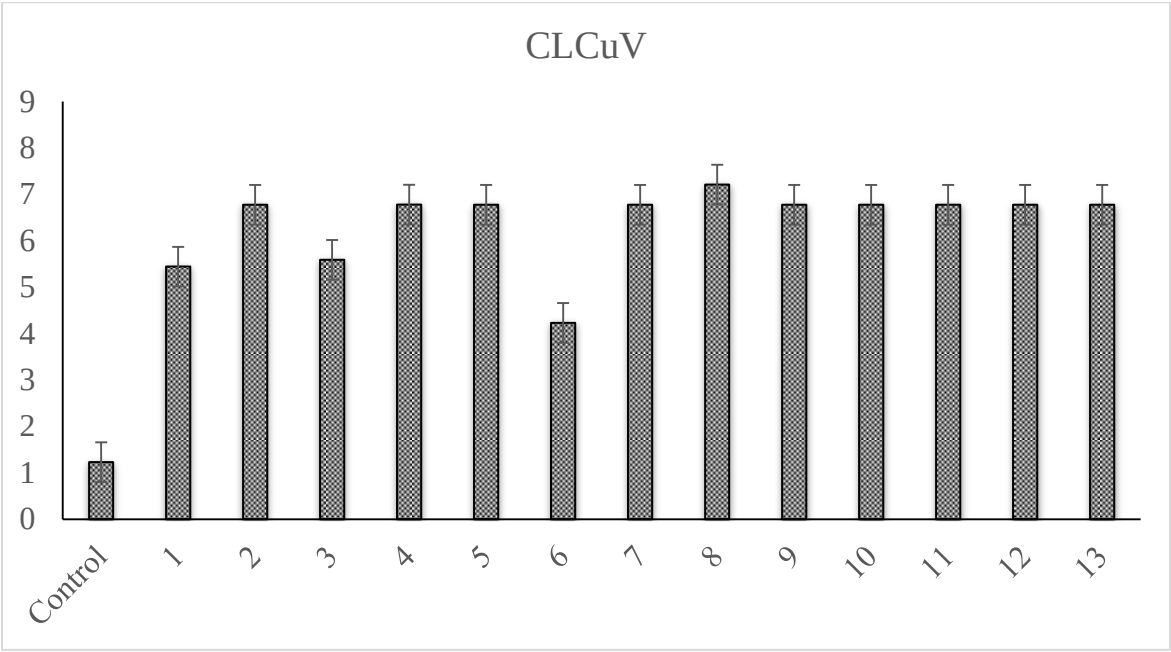

Fig.6 Image J software and PCR results for virus infection CLCuV graph bar is showing relative virus accumulation in the plants. Bar 1 to 13 are showing relative virus accumulation in the samples. Control plant show zero suppression and sample 10 shows maximum virus suppression.

Discussion

Despite cotton's global economic importance, only a few gene functional studies were reported (Gao *et al*., 2011; Li *et al*., 2015; Zhang *et al*., 2015). Due to the polypoid nature of the crop, many important agronomic and quality traits controlling fiber quality, yield or defense resistance are regulated by multiple genes or genes with multiple copies, making it difficult to perform gene functional studies. The successful application of the CRISPR/Cas9 system for crop improvement or functional analyses relies on the generation of stably transformed mutants in order to perform phenotypic characterization of homozygous stable mutants. The sequence of the target site contained in the sgRNA is a key factor affecting the overall mutagenesis efficiency of the CRISPR/Cas9 system as different sgRNAs can result in very different efficiencies when targeting the same gene (Ma *et al*., 2016). The generation of cotton mutants utilizing stable transformation is a labor-intensive and time consuming process, and therefore it is essential to select the best possible sgRNA in order to reduce the workload. Most of the sgRNA design and selection process is currently based on bioinformatics analysis (Fan *et al*., 2015). Even though bioinformatics analysis is essential to predict the specificity and theoretical efficiency of the target sites, our work provides a fast and effective method to experimentally validate candidate sgRNAs against cotton leaf curl virus.

Summary

In summary, we provide a fast and effective method to validate sgRNA mutagenesis efficiency in cotton using CRISPR/Cas9 and transient expression methods. We also provide an improved CRISPR/Cas9 cassette using against cotton leaf curl virus in cotton. Generating stable transformation of cotton is time-consuming and labor-intensive and thus the improvements should result in important savings for research groups using CRISPR/Cas9 in cotton.

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Enhancing Virus Resistance in Plants Using TALEs, TALENs, and CRISPR: A Multifaceted Approach to Combat *Begomoviruses*

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Abstract

The green revolution has reached its biological limits after delivering food security for many decades, as shown by the standstill in yield. The largest difficulty facing plant scientists is supplying the world's expanding population with the food they need. CRISPR-edited crops have enormous promise for global agriculture and food security. With fast-growing applications in academic research (functional genomics and reverse genetics) as well as practical research for creating crop varieties with new or enhanced features, CRISPR technology has emerged as a new face of genome editing. More ambitious initiatives to address issues with food security in light of the expanding global population and changing environment are underway after the successful use of gene editing technology to change basic features. CRISPR may be used in several ways to develop resistance in plants; targeting host susceptibility genes/factors, inserting resistance genes, activating host immunity, breaking host-pathogen cross-talk/interaction etc. We have used the CRISPR system against bacterial and viral diseases and found promising results. In the case of bacterial disease, bacterial leaf spot, we targeted the susceptibility gene with CRISPR to enhance plant immunity against the disease in tomato, while in the case of viral diseases (caused by begomoviruses), we targeted several viral genes to develop resistance. It was found that targeting multiple genes simultaneously may provide resistance up to 70-80%. Moreover, delay in symptoms, low virus titer and attenuated symptoms were observed on the CRISPR plants. Initially, we screened all gRNAs in the model plants; *Arabidopsis* and *Nicotiana benthamiana*. Later, we expressed selected gRNAs-CRISPR, transient and stable expression, in tomato and cotton. CRISPR technology has the potential to be used for the genetic improvement of plants to ensure food security and safety. Moreover, producing DNA-free, non-GMO plants with CRISPR is an attractive approach for breeders from all regions of the world.

Keywords: Plants Diseases, CRISPR/Cas, GMO, New breeding techniques, Climate-smart crops, Diseases Resistance, Food Security

Introduction

Genome biology and genetic engineering have long been interesting fields of research and invention. The establishment of animal gene expression in plants as well as the production of disease- and stress-resistant agricultural varieties are all results of advancements in this sector. Genetic variants that may be used in breeding programs are continually being sought by plant breeders (Bai *et al*., 2020). Targeted changes to the genome are now achievable thanks to the development of genome editing technologies. The range of genome editing applications has been increased by the adaptability of utilizing various designed proteins and nucleases to obtain precise and desired results.

Off-target effects are a critical restriction on the use of genome editing techniques. Researchers have looked at different techniques in the genome editing toolkit to overcome this issue. In comparison to other technologies like ZFNs and CRISPR/Cas systems, studies have demonstrated that TALENs, with their longer target sites, display fewer off-target effects (Chang *et al*., 2017) (Figure:1). Researchers in this area are optimistic as a result of the United States Department of Agriculture (USDA)'s (USDA) announcement that there won't be any restrictions on genome alterations requiring precise deletions. Various agricultural plants and animals have been successfully targeted using engineered nucleases (ENs) and synthetic DNA-binding proteins, with encouraging results.

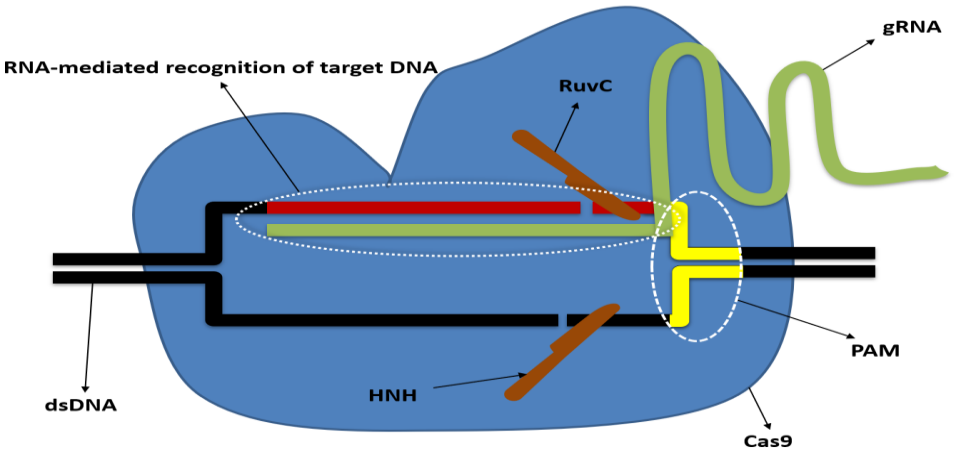

Figure:1 CRISPR Toolkit (Khan et al. 2022)

The genome editing toolbox's variety has increased, opening up a wider range of possibilities. In comparison to currently used techniques like RNA interference (RNAi), genome editing has shown to be a technology that is more accurate and effective. By inserting deletions or insertions in the target DNA, it enables gene silencing at the DNA level. In comparison to alternative methods like

RNAi, TILLING, or other mutagens, the mutations produced by genome editing tools are more predictable and provide particular results (Shafique *et al*., 2021). Furthermore, using effector domains like TALEs, ZFs, and dCas alone or in conjunction with genome editing tools, precise control of gene expression is possible. Additionally, it has been noted that after the transformation of genome editing reagents, successive generations of transgenic plants may be produced by segregation without these proteins, providing the possibility of transgene-free plants.

Genome editing has a promising future, and scientists and researchers are hopeful about it. The biological sciences often use these methods to generate desired genetic alterations in both plants and animals. The use of genome editing methods in the genetic engineering of cotton creates new opportunities for the study of functional genomics. These methods may be used to learn more about intricate metabolic processes involving several genes. Opportunities exist to enhance the quality of cotton seeds and fiber thanks to genome editing techniques. Targeted gene modifications work well, as shown by reports on genetic engineering in cotton. A constitutive, powerful, and inducible exogenous promoter may be employed to replace an endogenous promoter when the CRISPR/Cas system is used in conjunction with the nickase enzyme for gene repair and replacement. This method lowers the chance of a foreign gene insertion in the host plant and allows for precise regulation of an indigenous gene's expression.

Another useful characteristic made possible by genome editing methods is gene pyramiding or stacking. It may aid in overcoming the segregation of advantageous genes, enabling the stacking of genes linked to disease resistance, insect resistance, herbicide resistance, improved production, and improved quality (Malik *et al*.,2023). Additionally, epigenetic markers associated with flowering, stress tolerance, and fiber quality may be changed by genome editing tools including ZFs, TALEs, and dCas9 with numerous effector domains. The genome editing toolkit, in conclusion, provides beneficial methods to solve challenges and enhance cotton growth, fiber quality, and production.

Researchers have used TALEs, TALENs, and CRISPR for a variety of tactics to fight begomovirus infections. One strategy is modifying TALEs or TALENs to bind and block crucial viral genes, hence preventing infection. This method has the potential to lessen viral multiplication and the severity of symptoms in infected plants. The CRISPR-Cas system has also been used to specifically target and damage viral genomes. To stop viral DNA from replicating, Cas9 may cleave and disable it by creating sgRNAs that are complementary to conserved viral sequences. Additionally, in order to directly destroy viral RNA transcripts, researchers have investigated the creation of RNA-targeting CRISPR-Cas systems, such as CRISPR-Cas13 (Cohn *et al*., 2016). To increase plant viral resistance, it is essential to identify relevant target genes. Begomoviruses often encode proteins that block host defences or alter host elements to promote infection. Targeting these viral

genes may thereby prevent infection and reduce viral dissemination. In order to improve plant resistance, host genes that are involved in viral replication, migration, or symptom development might be targeted. Researchers can efficiently and effectively impart resistance to begomovirus infections by carefully altering these genes using TALEs, TALENs, or CRISPR (Figure 2).

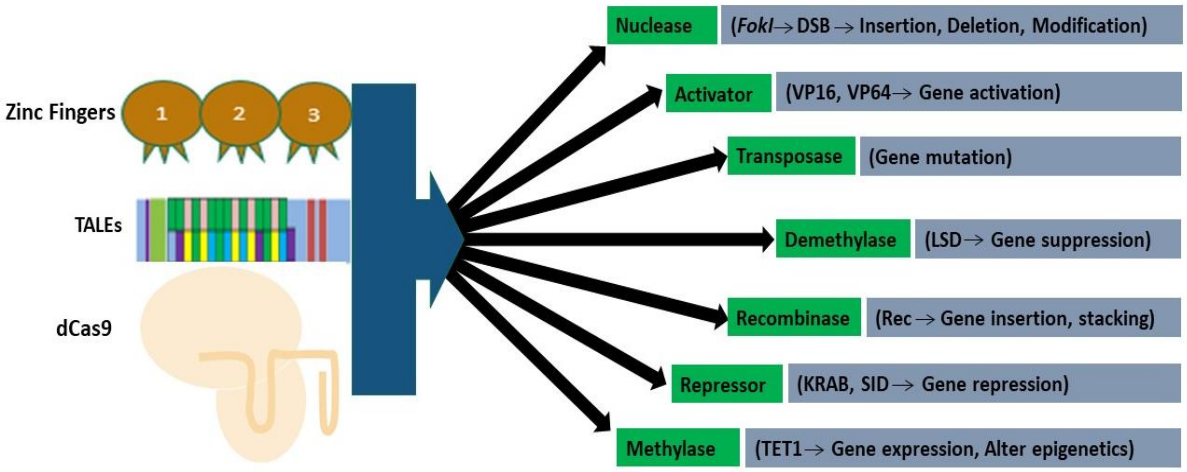

Figure 2: Using various effector domains with genome editing tools; ZFNs, TALENs and CRISPR for several purposes (Khan et al. 2021)

The virus's effective repair mechanism or tolerance for mutations might explain why it is unable to lower viral infection by targeting the rep gene. The results of this study show that employing TALEs and TALENs to suppress CLCuVs and other begomoviruses is feasible. These results broaden antiviral approaches and provide new avenues for research in the field (Binyameen *et al*., 2021). Tomato (*Solanum lycopersicum*) is commonly regarded as an important and extensively produced fruit vegetable. While many bacterial infections impact plants, bacterial leaf spot (BLS) is a major threat to agricultural productivity, especially in tomato plants. The prevalent pathogen *X. gardneri* causes BLS in tomato plants (Gimenez Ibanez *et al*., 2017). Through the activity of Transcription Activator-Like Effectors (TALEs), this pathogen causes spot lesions on tomato fruit. TALEs are virulence factors that bind to Effector Binding Elements (EBEs) in the promoter regions of tomato plants' bHLH3 susceptibility gene, triggering its expression. The unique sequence of TALEs' tandem repeat sections determines their DNA binding selectivity, which may vary significantly amongst TALEs.

The *AvrHah1* gene encodes a TALE responsible for producing water-soaked disease lesions on tomato fruits and foliage, eventually leading to bacterial spot disease in *X. gardneri*. The *X. gardneri* transcriptome study indicated control of the transcription factor bHLH3, which included anticipated *EBEs* for *AvrHah1* (Dong *et al*.,

2019). Potential guide RNAs (gRNAs) based on the EBEs inside the *bHLH3* gene area were created to decrease the expression of the bHLH3 gene. Through the agrobacterium transformation approach, these gRNAs were subsequently employed to create specific alterations in the gene.

The results indicated that the CRISPR/Cas9 system was an efficient technique for decreasing *bHLH3* gene expression. These genetic changes in tomato plants increased resistance to bacterial leaf spot disease (Gimenez Ibanez *et al*., 2017). The work effectively strengthened the plant's innate defense systems, eventually leading to higher resistance to bacterial leaf spot disease, by using CRISPR/Cas9 technology to target and change particular genes linked with susceptibility to infections.

Conclusion

The development of TALEs, TALENs, and CRISPR technologies has made it possible to fight begomovirus infections in plants with effective techniques. The precise and focused alterations provided by these genetic engineering techniques may improve plants' resistance to viral infections. The potential advantages of adopting these methods to battle begomoviruses are enormous, even if there are still difficulties, such as off-target consequences and regulatory issues. It is quite likely that further study and development in this area will help to secure the world's food supply and lessen the financial toll that viral infections have on agriculture.

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Potential and Opportunities of Wild Relatives of *Gossypium*

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Abstract

Cotton, belonging to genus *Gossypium*, is one among the important fiber crop across the globe. The *Gossypium* genus has more than 54 species consisting of both wild and domesticated, where 47 of them are diploid, and seven are allotetraploid in nature. Owing to its yield potential and spinnable fiber, *G. hirsutum* being the most adapted and cultivated allotetraploid species. But high intensity selection pressure has narrowed down the genotypic variation in *G. hirsutum*, and it became vulnerable to several biotic and abiotic stresses. There is a dire need to exploit and utilize the existing genetic diversity among wild species of *Gossypium* and to transfer into the local *hirsutum* cultivars to cope with changing climatic trends and biotic stresses.

Keywords: *Gossypium*, Wild species, Genetic potential, Genetic diversity, Improved traits

Introduction

The *Gossypium* genus includes more than 54 species, both wild and domesticated, among them 47 are diploid and seven are allotetraploid (Gallagher *et al.*, 2017). These species represent eight different genomes, denoted A to G and K. These genomes are from South Mexico, South America (Peru), the Indian subcontinent, Australia and South Africa. *Gossypium hirsutum* (cultivated species, allotetraploid, AD_1), *G. barbadense* (cultivated specie, allotetraploid, AD_2), *G. arboreum* (cultivated diploid, A_2), *and G. herbaceum* (cultivated diploid, A_1) are four cotton species that were brought into cultivation due to their spinnable fiber. The

putative parents of *G. hirsutum* and *G. barbadense* are linked to genome D from (*G. raimondii*) and the A genome from (*G. arboreum* or *G. herbaceum*) (Wendel *et al*, 1989). *Gossypium* species possess substantial variation in morphological characters, ranging from perennial herbivorous to tiny trees with a huge variety of vegetative and reproductive characteristics (Wendel and Cronn, 2003). Changing trends towards agriculture sustainability requires that new cotton cultivars must be developed with broad genetic base having tolerance to temperature extremes, low precipitation, salinity, resistance to novel biotypes of pests and diseases (Hize *et al*, 2017). The wild cotton species are the potential source of biotic (insects, diseases) and abiotic (salinity, cold, drought, and heat) stress resistance traits (Yik and Birchfield, 1984, Narayanan and Singh, 1994). This review focuses on the potential characteristics of wild *Gossypium* species examined and reported over the past years that can be introgressed into cultivated species of cotton through conventional breeding or with the use of molecular approaches to develop high yielding, climate smart cotton varieties.

Reservoir of biotic stress tolerance

Insect tolerance

Cotton plant inherits different morphological and biochemical features such as leaf hair, leaf shape, leaf thickness, leaf nectar less, waxy layer, frego-bracts and polyphenol chemicals that imparts wide ranging tolerance to insects. Different concentrations of trichrome (pubescence) are reported on leaves and stem. Hairy traits classified as smooth (no trichrome), hirsute (moderate density), and pilose (high density), while smooth is found in majority of contemporary cotton varieties (Wright *et al.*, 1999). High phenolic content cotton plants exhibited a decreased incidence of jassid. Low tannin and high phenol levels in species namely, *G. anomalum* (B1), *G. armourianum* (D2), *G. raimondii* (D5), *G. davidsonii* (D3), and *G. thurberi* (D1) make them much vulnerable to jassid (Shinde *et al.*, 2014). Similarly, thrips and whitefly are among the most harmful sucking insects of cotton. *G. tomentosum* with pilose trait is reported to be resistant to thrips amongst five allotetraploid cotton species followed by *G. mustelinum, G. barbadense*, and *G. darwinii*, and *G. hirsutum*. Among the diploid species *G. herbaceum, G. arboreum* and *G. somalense* (E_2) are resistant sources against whitefly and thrips and bollworms (Zhang *et al.*, 2013). *Gossypium longicalyx* and *G. arboretum* are being used to introduce the resistance genes against the nematodes. *G. herbacuem, G. aridum* and *G. armourianum* served as bridge species for this introgression in cultivated cotton (Sacks and Robinson, 2009; Robinson *et al.*, 2007; Shim *et al.*, 2018). Insect resistant traits, ranging from morphological and structural to biochemical and metabolic, may be utilized from the wild *Gossypium* species to the cultivated genotypes to broaden the resistance and enhance crop production.

Reservoir of disease tolerance

Cotton plant faces severe disease problems including bacterial, fungal and viral. Bacterial blight caused by *Xanthomonas* spp. is the major bacterial disease that causes losses in yield of seed

cotton. *Gossypium* species belonging to A genome are reported to be immune for stated diseases. Successful introgression of B6 bacterial blight resistance gene from *G. arboreum* into *G. barbadense* has been reported (Zafar *et al.*, 2009). *G. capitis-viridis* (B_3), *G. sturtianum* (C_1), *G. lobatum* (D_7), *G. trilobum* (D_8), *G. australe* (G_2) are reported to be potential source of resistance against *Fusarium* and *Verticillium* wilts (Wang *et al.*, 2016, Xs *et al.*, 2012). Cotton leaf curl virus is one of the major viral diseases of cotton that hampers the plant growth and reduces yield. Since none of the varieties of *G. hirsutum* has demonstrated the resistance against CLCuV, and wild antecedents were used to transfer resistance into *G. hirsutum* through traditional backcrossing where *G. arboreum* was used as a donor (Ahmad *et al.*, 2011; Feng *et al.*, 2021). Another attempt resulted in the development of CLCuV tolerant variety "MNH-786" at Cotton Research Institute Multan, Pakistan by interspecific hybridization between *G. hirsutum* and *G. stocksii* (Nazeer *et al.*, 2014). Disease tolerance is a complex mechanism based on host-pathogen interaction and cross-talk. Transfer of disease resistance traits from the wild relatives of *Gossypium* may increase resistance in the cultivated species against bacterial, fungal and viral diseases.

Cotton improvement through interspecific hybridization

Wild species provides base for innovative genetic variation in cultivated crops that has been extinct during adaptation. In plant breeding, wild species of crops are a source of healthier choice due to heritable genetic variation and selective pressures that has resulted the biotic and abiotic resistant plants. (Alonso-Blanco *et al.*, 2004). Wild antecedents are normally present in peripheral territories with severe climatic challenges. Without any artificial selection, these wild relatives changed according to environment that allow them to survive under harsh environments. Wild germplasm are source of allete alleles that can be exploited to expand desirable trait expression in upland cotton (Keerio *et al.*, 2018). Crop failures due to low genetic diversity in different crops have been reported throughout the agriculture history. *Gossypium* wild germplasm possessing incredible amount of genetic variation can be used in hybridization program to have innovative gene blends that can perform better under different ecological zones.

The germplasm developed through interspecific hybridization have provided the base for introgression for desirable traits. Interspecific hybridization has been used for incorporating beneficial traits of wild species to the cultivated ones by Cytogenetics Section of Central Cotton Research Institute, Multan. In screening against cotton leaf curl virus through petiole grafting, 30 *Gossypium* species were evaluated for CLCuV tolerance. Among the studied species only eight diploid species viz*: G. herbaceum, G. arboreum, G. anomalum, G. captis-viridis, G. gossypioides, G. laxum, G. stocksii, G. areysianum, G. somalense and G. longicalyx* exhibited resistance to Burewala stain of cotton leaf curl virus. Two varieties, CIM-608 and Cyto-124 were developed through interspecific hybridization (Anjum *et al.*, 2014). Despite many achievements in interspecific

hybridization, a large portion of variation remains unexploited in this genus. Description of genetic basis of desirable traits is a mandatory in application of genetic diversity. Recent genomic technologies cover the sequencing and genotyping platforms will provide the platform for findings of novel gene.

Sources of abiotic stress tolerance

Changing climatic conditions *i.e.* extreme temperature, drought spells, erratic rainfalls and saline soils are limiting cotton productivity severely. Cotton plant shows its sensitivity for these stresses in the form of shedding of fruiting bodies namely, squares, flowers and bolls ultimately reduce the yield. The reservoir of existing genetic diversity among *Gossypium* species can be used for introgression of abiotic resistant genes in cultivated species. *G. davidsonii, G. klotzschianum* and *G. aridum* from D genome have been explored as the potential source of salinity tolerance (Wei *et al.*, 2017). Fan *et al*. (2015) found 109 WRKY genes in *G. aridum* for salinity tolerance through transcriptome analysis. Whereas *G. tomentosum, G. herbaceum and G. darwinii* have also been reported as the potential source of heat and drought tolerance (Shim *et al.*, 2018). Abiotic stress tolerance is a much-needed trait to develop climate resilient cotton varieties for sustainable production in the changing climatic conditions.

Potential sources of fiber traits

Improved fiber traits are the main concern for the breeders and cotton textile industry as well. Diploid and allotetraploid species of cotton exhibits a great range of diversity of fiber traits like fiber fineness, fiber length and fiber strength. *G. barbadense, G. mustelinum* and *G. tomentosum* are the prominent among allotetraploid species that are good donors of genes for traits associated with fiber quality (Wang *et al.*, 2016). Significant negative association among lint yield and lint quality was masked by crossing *G. hirsutum* with *G. thurberi*, *G. arboreum* and *G. barbadense* (Culp *et al.*, 1979; Zhang *et al.*, 2016). Acala variety was developed through interspecific crosses between *G. arboreum*, *G. barbadense* , *G. hirsutum*, and *G. thurberi* for improvement of fiber quality (Zhang *et al.*, 2005; Saha *et al.*, 2006; Wang *et al.*, 2011; Lu *et al.*, 2017).

Wild *Gossypium* Reservoirs maintained at Cotton Research Institute Multan Pakistan

Cotton Research Institute Multan, Pakistan is engaged in delivering solutions of CLCuV and many other problems of cotton to nation's farmer community through development of climate resilient cotton varieties. As recognizing the importance of wild species of cotton, the institute has preserved and maintained the wild *Gossypium* species that are effectively being used for introgression breeding for many useful traits. In this context a set of wild species of cotton listed below was screened for CLCuV tolerance by scientists of CRI Multan and the results were as depicted in the table below. On the basis of the screening results, CLCuV tolerance from *G. arboreum* was successfully transferred into *G. hirsutum* through interspecific hybridization between *G. hirsutum* cv CRSM-38 (2n = 4x = AADD =

52) ×*G. arboreum* cv 15-Mollisoni (2n = 2x = AA = 26). After repeated backcrossing and cytological studies finally a CLCuV tolerant *G. hirsutum* variety "MNH-1050" was developed which have been approved during 2021 in 55th meeting of Punjab Seed Council for general cultivation all over Punjab, Pakistan (Fig-1) (Nazeer W *et al*., 2014). All the listed below species are maintained and being effectively used in interspecific breeding program for CLCuV tolerance and fiber traits improvement (Table-1). *G. stocksii* have been found an effective source for fiber strength related gene and is being used in breeding for fiber improvement in cotton which is also a main concern of the textile industry.

Fig-1: Field view of MNH-1050 (CLCuV Tolerant Variety)

Table-1: Screening of Wild species of *Gossypium* maintained at CRI, Multan, Pakistan against CLCuV

Sr. No	Name of Species	Genome	Origin	CLCV Response
1.	*G. arboreum*	A2	Indus valley, Madagascar	Resistant
2.	*G. anomalum*	B1	Northern & Southern Africa	Susceptible
3.	*G. capit is viridis*	B3	Cape Verde Island	//
4.	*G. nadewarense*	C1	Eastern Australia	//

Sr. No	Name of Species	Genome	Origin	CLCV Response
5.	*G. robinsonii*	C2	Western Australia	//
6.	*G. thurberi*	D1	Sonora, Mexico; Arizona	//
7.	*G. harkansii*	D2-2	Baja California, Mexico	//
8.	*G. davidsonii*	D3-d	Sonora and Baja California, Mexico	//
9.	*G. klotzschianum*	D3-k	Galapagos Island	//
10.	*G. gossypioides*	D6	Oaxaca, Mexico	//
11.	*G. laxum*	D9	Guerrero, Mexico	//
12.	*G. stocksii*	E1	Arabia, Pakistan	Resistant
13.	*G. somalense*	E2	Somalia, Africa	//
14.	*G. longicalyx*	F1	North eastern Africa	//
15.	*G. bickii*	G1	North central Australia	Susceptible
16.	*G. australe*	G2	Northern Australia	//
17.	*G. nelsonii*	G3	Central Australia	//
18.	*G. marchanti*	K	North western Australia	Under test
19.	*G. costulatum*	K	Western Australia	//
20.	*G. hirsutum*	AD1	New World Cultigen	Susceptible
21.	*G. barbadense*	AD2	New World Cultigen	//
22.	*G.tomentosum*	AD3	Hawaiian Island	//
23.	*G. mustelinum*	AD4	Brazil	//
24.	*G. darwinii*	AD5	Galapagos Island	//

Conclusion

Cotton being the most important and profitable industrial crops faces multiple biotic and abiotic challenges. To cope with these challenges, narrow genetic base of cultivated cotton genotypes is a bottleneck in cotton improvement through breeding. Re-sequencing, gene mapping, OMICS and other advanced techniques should be utilized to characterize the wild species for various valuable traits. Wild species are a potential resource which may be used to broaden gene pool for breeding, disease and insect resistance. More emphasis should be given to the fiber quality according to the demand of the textile industry. Moreover, organic cotton production, which solely depends on the

acquired traits of the plants, cannot be flourished without utilization of natural genetic resource for crop improvement.

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