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Short Communication

CRISPR/Cas9 Protection System for Chilli Leaf Curl Virus

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Abstract

Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated nuclease 9 (Cas9) targeted genome editing system has already modernized plant science research and has been used to provide resistance sources against plant viral diseases. In this study, we have used CRISPR/Cas9 technology to introduce sequence-specific targeted gene silencing in *Nicotiana benthamiana* to successfully engineer complete resistance to Chilli leaf curl virus (ChiLCuV). We proposed the multiplex type sgRNA (short guide) with CRISPR/Cas9 to increase the effective control against IR and Rep gene of DNA-A of ChiLCuV. Highly conserved regions having PAM (Protospacer Adjacent Motif) sequence were selected to design sgRNA cassette and inserted into binary vectors. This cassette was used to transform *N. benthamiana* by the leaf disc method and further transgenic plants were challenged with ChiLCuV and screened for resistance. Our results suggest that the resistance shown in *N. benthamiana* against ChiLCuV is quite stable.

Keywords: Chilli leaf curl virus, phylogenetic analysis, multiplex cassette, CRISPR/Cas9

Introduction

Chilli (*Capsicum annuum*) is a vital spice used in Indian food in fresh and dried form. Chilli belongs to the genus Capsicum, family *Solanaceae* originated from Mexico, Southern Peru, and Bolivia. Chilli leaf curl disease (ChiLCD) causes severe economic loss in the tropical and subtropical regions of India (Chattopadhyay *et al*., 2008; Senanayalee *et al*., 2012; Shingote *et al*., 2022). The typical symptoms of the disease are leaf curling, crumpling, reduced leaf size and clustering of leaves. In India, ChiLCD is associated with diverse group of begomovirus like Chilli leaf curl virus (ChiLCuV), Chilli leaf curl India virus (ChiLCINV), Chilli leaf curl Vellanad virus (ChiLCVV), Tomato leaf curl Joydebpur virus (ToLCJoV) and Tomato leaf curl New Delhi virus (ToLCNDV) (Kumar *et al*., 2011). ChiLCD has a typical begomovirus genome organization, six open reading frames (ORF); Rep, TrAP, REn, and C4 in the complementary sense; and CP and V2 genes in virion-sense (Gilbertson *et al*., 2015). The wide host range of ChiLCD poses the possibility of an outbreak in the Indian sub-continent. The strategies for controlling the begomoviruses remain lavish and inadequate due to the wide range of host and path-interaction vectors (Shihi *et al*., 2014). However, numerous site-specific nucleases have been developed for directing interference against the begomovirus genome (Ali *et al*., 2015a; Zaidi *et al*., 2016; Robertson *et al*., 2022).

The RNA-programmable prokaryotic CRISPR (Clustered Regularly Interspersed Short Palindromic Repeats)–Cas9 system allows precise and scalable genome editing or correction of mutations, transcriptional perturbation, and labeling of DNA in a multitude of cell types and organisms in the three domains of life. CRISPR/Cas9 system uses the Cas9 endonuclease and a single guide RNA (sgRNA). SgRNA is a combination of the CRISPR

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RNA (crRNA) with trans-activating crRNAs (tracrRNA) and is responsible for recognizing the target DNA. The specificity of Cas9 depends on homology between the 20 nucleotides of the sgRNA and the genomic target as well as the recognition of a protospacer adjacent motif (PAM) on the non-target DNA strand. Therefore, CRISPR/Cas9 system can be easily reprogrammed with ~20 bp sgRNA molecules to target any DNA sequence (Barrangou and Marraffini 2014; Aouida *et al*., 2015). With its unprecedented efficiency and stunning ease of use, CRISPR/Cas9 has been implemented in a multitude of plant species and can be applied to all transformable plant species. To maximize the efficiency of genome editing in plants transient or stable expression of Cas9 and the sgRNA is required (Ali *et al*., 2015b). Decrypting the process of delivery of these two components of the CRISPR/Cas9 machinery, and their respective PAM sequences enables convenient genome editing. Earlier CRISPR/Cas9 was employed by using a single gene of Geminivirus which limits the result. Here, we proposed the multiplex type sgRNA with CRISPR/Cas9 for maximizing the effective control against ChiLCD (Fig. 1).

Materials and Methods

Designing of expression vector with CRISPR/Cas9 system

Full-length sequences of DNA-A of ChiLCuV isolates were retrieved from the NCBI database and were aligned with our ChiLCuV isolates (OQ107574) by using the online software CRISPR-Multitargeter (http://www.multicrispr.net/). Consensus sequences were identified as a potential target for gRNA through CRISPR-Multitargeter. The highly conserved regions with PAM sequence (Protospacer Adjacent Motif end sequence as underlined below) were selected for designing sgRNA cassette (Fig. 1). The two selected sequences (about 20 nt each) from the ChiLCuV genome were TCCGCATTAATATTACCGGATGG (IR: Intergeneric Region) and AATTGGGTCCTGGATTGCAGAGG (Rep: Replication protein gene region). The IR is the most common in all the geminiviral species; similarly, the Rep region tends to be highly conserved among the geminivirus community, hence it was selected and justified. The designed sgRNA was screened by using GenCrisprs gRNA screening kit (USA) similar to the earlier study by Iqbal *et al*. (2016). Further, sgRNA target sequences trailed by DR (Direct Repeat sequence) were inserted into the binary vector pCAMBIA 1300 T-DNA region as per the protocol devised by Xing *et al*. (2014) for multiplex genome editing (Fig. 1). After the gRNAs were chosen, PAM sequences were removed, and adaptors were inserted into the 5′ (ATTG-) and 3′ (-CAAA) ends of the gRNAs. To create double-stranded gRNAs, overlapping forward and reverse primers were created and subsequently de-phosphorylated using the enzyme T4 Polynucleotide Kinase (New England Biolabs, Ipswich, MA, USA). Each gRNA was ligated into a pHSE401 CRISPR vector (supplied by ADDGENE) and then transferred to *E. coli* (Secgin *et al*., 2021). The modified single colonies were chosen by cultivating them for two days at 28°C on solid selective LB media containing 50 mg/L kanamycin, 50 mg/L gentamicin, and 10 mg/L rifampicin. Sanger sequencing was used to confirm the construct following colony PCR.

Agrobacterium transformation

The recombinant plasmids were obtained from *E. coli* using the Bacterial Plasmid Isolation Kit (FavorGen, Wien, Austria) and electroporated into *Agrobacterium tumefaciens* (LBA4404) in accordance with the Seçgin *et al*. (2021) methodology. Agrobacterium cells were grown on selection media before being cultured in liquid selective media as single colonies until the bacterial density reached OD600 0.8. *Agrobacterium tumefaciens* harboring a binary plasmid with gRNA and hygromycin phosphor transferase gene (hpt) as plant selection marker was driven from Cauliflower mosaic virus (35S CaMV) promoter and NOS-terminator. This cassette was used to transform *N. benthamiana* by the leaf disc method as described by Horsch *et al*. (1985).

Results and Discussion

The transgenic plants along with the control were used for various molecular analyses for the presence of gRNA. The transgenic Nicotiana lines were evaluated by challenging ChilCV with and without the satellite molecules under greenhouse conditions. The non-transgenic plant/control showed leaf curling just after 16 days post-agro-infection (dpa) whereas the transgenic plant harboring gRNA was symptomless (Fig. 1).

This was further confirmed by PCR using region-specific primers. Further, a northern blot for the concerned RNA population was also performed to confirm the resistance (data not shown). For the detection of sgRNA Digoxin-labeled ChiLCuV-specific probe was used. The silencing activity was confirmed by validating the levels of sgRNAs supported by strong signals in the northern blot technique. Plant growth conditions and the production

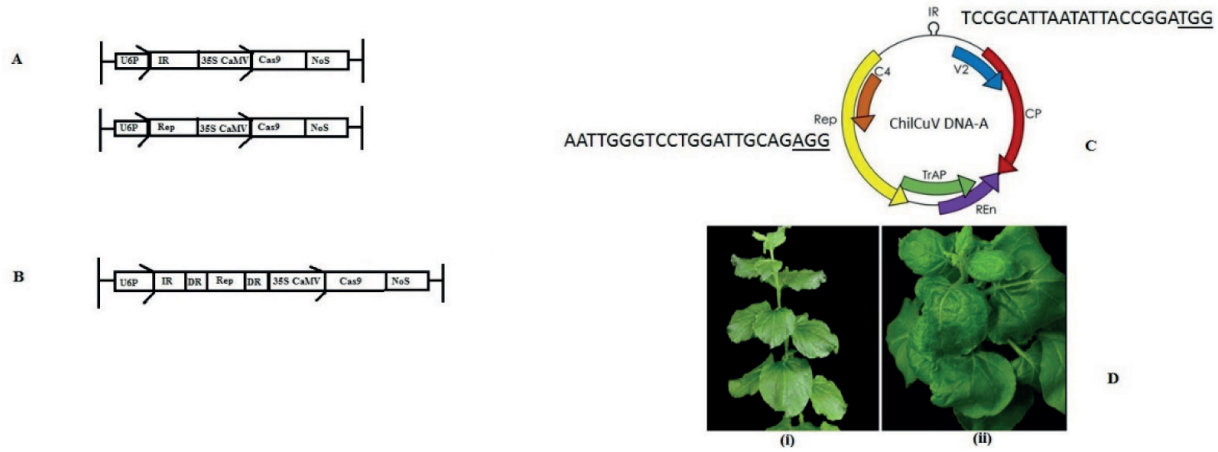

Fig. 1. CRISPR/Cas9 system used against the viral pathogen. (A) Cassette for IR and Rep of ChiLCuV. (B) Multiplex cassette of the sgRNAs expressed with common U6P. (C) Region utilize for developing CRISPR/Cas9 cassette. (D) (i) Transgenic *N. benthamiana* plant expressing CRISPR/Cas9 showed resistance against viral complex and (ii) negative control plant showed typical ChiLCV symptoms.

of transgenic plants were performed following previous protocols as described by Yin *et al*. (2019).

We accomplished complete interference of DNA virus in the current study as the Cas9 protein was expressed along with sgRNA using the same vector. CRISPR/Cas9 was initially defined as an immune system for prokaryotes; in addition, our results justify the adaptability of CRISPR/Cas9 in the eukaryote cell system as a protection/defensive tool against attacking DNA viruses. Such an immune system could be of great utility for other plant species to bestow resistance to invading DNA viruses. Further, the transfer of foreign construct DNA comprising of cas9 and sgRNA into the plant genome, its stability characterization, and regeneration of tissue cultured raised plant; seek considerable time and skilled labor/expertise. Such plant transformation and regeneration tasks vary from species to species. Just 20 nucleotides of edited sgRNA are sufficient to confer resistance in the host plant; CRISPR/Cas9 has been renowned as a strong genome editing tool with wide applications in medical as well as agriculture fields (Ma *et al*., 2015).

Conclusions

With the already existing RNA interference technology, CRISPR technology is much more beneficial in showing specificity. RNA interference is primarily effective to the viruses residing in the cytosol, whereas CRISPR/Cas9 system has an extra advantage over it by showing strong gene silencing suppressor activity even in the host nuclei as employed less by the former technology. This reason placed CRISPR technology ahead of RNA interference technology to fight against DNA/RNA viruses and even the viroids for plant security.

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