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Designing and Validation of sgRNA for CRISPR/Cas12a-based Diagnosis of Tomato Leaf Curl Virus (ToLCV)

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Abstract

Tomato Leaf Curl Virus (ToLCV) significantly impacts tomato causing stunted growth, leaf curling and reduce yield. In developing a sound management strategy of ToLCV, it is important to diagnose the virus at the earliest to deploy suitable interventions. In this regard, CRISPR/Cas12a-based systems offer great promise for early and accurate detection. Cas12a, a CRISPR-associated protein, provides high specificity and sensitivity in nucleic acid detection, enabling the recognition of target DNA sequences and subsequent activation of collateral cleavage and detection of fluorescence. In the present study, genomic DNA was extracted from ToLCV-infected tomato leaves using a modified CTAB method and the target region, encompassing the coat protein and partial replicase genes (1461 bp), was successfully amplified, purified and cloned into the pTZ57R/T vector. The cloning was confirmed by blue-white screening, restriction digestion producing bands of 647 bp and 814 bp and sequencing. Further, off-target minimized sgRNAs were designed using online software, CHOP CHOP 2.0, *in vitro* synthesized and validated for their secondary structure and integrity. The cleavage efficiency of the ribonucleoprotein (RNP) complex (Cas12a + sgRNA) was tested in vitro, yielding 1331 bp and 195 bp fragments. This study aims to develop and validate a CRISPR/Cas12a-based diagnostic system for rapid and specific detection of ToLCV.

Keywords : Tomato leaf curl virus, CRISPR/Cas12a, Single guide RNA, *In-vitro* restriction

Tomato (*Solanum lycopersicum* L.) is a globally significant crop, highly valued for its rich nutritional content including vitamins C, K and folate, as well as antioxidants like lycopene and beta-carotene, which contribute to its vibrant colors (Sattar *et al*., 2024). In India, tomatoes are a cornerstone of the agricultural economy with a production of approximately 20.7 million metric tons during 2021-2022 season, accounting for 11 per cent of global output (NHB, 2022). This extensive cultivation not only supports millions of farmers but also fuels the food processing industry and export markets, enhancing the country's economic growth (NABARD, 2020 and APEDA, 2021).

Despite their economic importance, tomato production faces challenges from biotic and abiotic factors that results in significant yield losses. Biotic factors such as plant diseases caused by bacteria, fungi and viruses have a substantial impact on crop productivity and quality (Varma and Malathi, 2003). Among these, viral diseases pose a critical threat with Begomoviruses emerging as a major concern. These single-stranded DNA viruses, transmitted by whiteflies, infect diverse crops and can reduce yields by up to 50-60 per cent (Olivé and Castillo, 2023). Begomoviruses are classified as monopartite or bipartite based on their genomic structure with the latter consisting of two DNA components (Roshan *et al*., 2017). Tomato leaf

curl virus (ToLCV), a monopartite Begomovirus, severely limits tomato production in many regions, including Karnataka, India where infection rates can reach nearly 100 per cent in the summer, leading to yield losses of 27-90 per cent. ToLCV causes symptoms such as stunted growth, upward leaf curling, wrinkling and premature flower drop, significantly affecting crop productivity (Hamilton *et al*., 2015). These challenges underscore the need for sustainable disease management strategies to ensure food security and agricultural sustainability.

Accurate and timely diagnosis of plant pathogens, including Begomoviruses is essential for effective management and preventing crop damage, especially as no proven viricides are available for field use (Jeong *et al*., 2014). Traditional serological and molecular techniques have been widely employed for virus detection, but recent advancements have introduced CRISPR/Cas-based systems as highly sensitive tools. Cas12a, a class 2 type V-A RNA-guided endonuclease, can detect plant DNA viruses at attomolar concentrations with exceptional accuracy. Cas12a operates using a unique 'T'-rich PAM sequence unlike 'G' rich PAM sequence in case of Cas 9 (Bhargava *et al*., 2024) and processes crRNA autonomously, making it a powerful tool for precise diagnostics (Chen *et al*., 2018 and Srivastava *et al*., 2022).

The DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR), developed in 2018, utilizes Cas12a's collateral cleavage activity to identify DNA viruses with high specificity (Chen *et al*., 2018). This system employs a fluorescent reporter tagged with a quencher and fluorophore, emitting a signal upon cleavage. With its ability to detect viruses in low titers and under field conditions, CRISPR/Cas12a-based detection represents a novel, efficient and accessible method for managing plant viral diseases (Li *et al*., 2018 and Mao *et al*., 2022).

Optimization of single-guide RNA (sgRNA) is essential prior to employing CRISPR/Cas12a for the detection of Tomato Leaf Curl Virus (ToLCV). The objective of this study was to identify target regions within the ToLCV genome, design sgRNAs with minimal off-target potential and perform an *In-vitro* cleavage assay to validate the recognition and cleavage efficiency of the target site by the ribonucleoprotein (RNP) complex assembled using purified Cas12a protein. Additionally, the assay evaluates the integrity of the sgRNA, ensuring its functionality and reliability for potential *in vivo* applications.

Material and Methods

Collection of ToLCV Infected Tomato Samples from Field

Tomato plants exhibiting characteristic symptoms of Tomato Leaf Curl Virus (ToLCV) infection, such as upward leaf curling, stunted growth and wrinkled leaves were selected for sample collection. These symptomatic plants were carefully uprooted from an experimental field at the ICAR - Indian Institute of Horticultural Research (IIHR), Bengaluru, India. Samples were immediately placed in sterile polyethylene bags to prevent contamination and were transported to the laboratory under cold conditions using an icebox. Upon arrival, the collected samples were processed for downstream molecular and diagnostic analyses to confirm the presence of ToLCV.

Genomic DNA Extraction from the ToLCV Infected Tomato Leaves

Genomic DNA was extracted from ToLCV-infected tomato leaves using the CTAB (cetyltrimethylammonium bromide) method with slight modifications. Approximately 100 mg of infected leaf tissue was ground into a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. The powdered tissue was transferred to a microcentrifuge tube containing 500 µL of pre-warmed CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0 and 0.2% β-mercaptoethanol). The mixture was incubated at 65°C for 1 hour with intermittent vortexing to ensure proper lysis. Following incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added and the mixture was centrifuged at 12,000 rpm for 10 minutes to separate the aqueous phase. The supernatant was carefully transferred to a new tube and DNA was precipitated by adding 0.6 volumes of

ice-cold isopropanol. The tube was incubated at -20°C for 30 minutes and centrifuged at 12,000 rpm for 10 minutes to pellet the DNA. The DNA pellet was washed with 70 per cent ethanol, air-dried and resuspended in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The quality and concentration of the extracted DNA were assessed using a Nanodrop spectrophotometer and agarose gel electrophoresis. The DNA was stored at -20°C for subsequent analysis.

Target Region Selection and Polymerase Chain Reaction (PCR)

A comprehensive literature review and bioinformatics analysis were conducted to identify potential viral target region for the detection of Tomato leaf curl virus. The target region selected spanning complete coat protein region and half of replicase gene. The polymerase chain reaction (PCR) was performed to obtain complete target region using gene specific primers (Table 1). Components of PCR reaction and conditions used for the amplification of target gene are given in Table 2 and Table 3, respectively. The PCR product was subsequently purified following the manufacturer's instructions using the FavorPrep GEL/ PCR Purification Mini kit (Favorgen Biotech Corp.) and resolved on 1.2 per cent agarose gel.

Cloning and Transformation of Target Gene of ToLCV into pTZ57R/T TA Cloning Vector

The target gene of Tomato Leaf Curl Virus (ToLCV) was ligated into the pTZ57R/T TA cloning vector (Thermo Scientific, USA, Catalog Number #K1213) using T4 DNA ligase. The ligation reaction was set up in a 10 µL volume containing 1X ligase buffer, 50 ng of pTZ57R/T vector, equimolar amounts of purified DNA fragment and 1U of T4 DNA ligase. The reaction was incubated at 22°C for 1 hour to ensure efficient ligation. The ligation product was transformed into competent *Escherichia coli DH5α* cells using the heat-shock method. Transformed cells were plated onto LB agar plates containing 100 µg/mL ampicillin, 40 µg/mL X-gal and 0.1 mM IPTG for blue-white screening. The plates were incubated at 37°C overnight and white colonies, indicating successful cloning of the insert were selected for further analysis. Selected colonies were grown in LB broth supplemented with 100 µg/mL ampicillin and plasmid DNA was extracted using a miniprep kit (Thermo Scientific, USA, Catalog

TABLE 2

PCR components for amplification of ToLCV target gene

Reagents	Volume (µl)	Final Concentration
Nuclease free water	12.8	-
10X LA PCR buffer (Mg+2 free)	2.5	1X
dNTPs mix (2.5 mM)	4	0.4mM
$MgCl_2$ (25 mM)	2.5	2.5mM
ToLCV genomic DNA	1	100ng
ToLCV Forward Primer	1	0.2 µM
ToLCV Reverse Primer	1	0.2 µM
TaKaRa Taq	0.2	1 unit/ µl
Total volume	25	

TABLE 1

List of gene specific primers and sgRNA used in the present study

Primers	Sequences (5 - 3)
ToLCV Forward primer	GCTCCCTGAATGTTCGGATGGA
ToLCV Reverse primer	GCGTACACAGGATTAGAGGCATGAG
ToLCV gRNA FP	GAAATTAATACGACTCACTATAGGTAATTTCTACTAAGTGTAGATA CAATGAGCCTAGCACTGCTACTG
ToLCV gRNA RP	CAGTAGCAGTGCTAGGCTCATTGTATCTACACTTAGTAGAAATTACC TATAGTGAGTCGTATTAATTTC

TABLE 3
PCR conditions for ToLCV target gene amplification

Steps	Temperature (°C)	Duration	Cycles
Initial Denaturation	95	2 minutes	1
Denaturation	98	10 seconds	
Annealing	56	10 seconds	35
Extension	68	45 seconds	
Final extension	68	10 minutes	1
Store	4	∞	

Number #K0503). The presence of the target gene was confirmed by restriction digestion followed by sequencing.

Designing and Synthesis of Off-Target Minimized sgRNAs

The CHOPCHOP tool (https://chopchop.rc.fas.harvard.edu) was used to design sgRNAs targeting specific ToLCV genes for Cas12a-based detection, selecting sequences with high on-target scores, minimal off-target effects based on genome variants (Table 1), optimal GC content (40-60%) and reduced secondary structure interference while ensuring stringent mismatch tolerance. The secondary structures of the sgRNAs were validated using the RNA fold web server. Each sgRNA was synthesized with a forward oligonucleotide containing a T7 promoter, a 5' handle and a 20-24 bp sgRNA sequence, while reverse primers were complementary. The sgRNAs was annealed separately in a buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 8.0) by heating at 95°C and cooling to 25°C. The resulting dsDNA templates were purified using the FavorPrep GEL/PCR Purification Mini kit for *in vitro* transcription.

***In-vitro* Transcription**

In-vitro transcription of sgRNA for ToLCV detection using Cas12a was performed in a 150 µL reaction containing 5x transcription buffer, 2 µg purified dsDNA template, 10 mM NTP mix (2 mM final), RiboLock™ RNase Inhibitor (50 U) and T7 RNA polymerase (30 U), incubated at 37°C for 16 hours. The reaction underwent DNase treatment to remove template DNA, followed by purification using the Monarch RNA Clean-Up Kit. The sgRNA was quantified with a NanoDrop, verified for integrity by 2 per cent agarose per cent gel electrophoresis, diluted to 300 ng/µL in RNase-free water and stored at -80°C.

Preparation of Ribonucleoprotein (RNP) Complex of Lb-Cas12a and *In-vitro* Restriction Assay

The *In-vitro* restriction assay was conducted to confirm target recognition and cleavage by the RNP complex. A PCR-amplified target gene containing the sgRNA site served as the template. Additionally, target gene templates underwent restriction digestion with the NdeI enzyme, to check its integrity. Further, *in vitro* restriction assay was carried out with the reaction, comprising 50 nM template DNA, 100 nM sgRNA, 50 nM purified Cas12a and 1X NEBuffer r2.1, involved pre-incubating the sgRNA by gradual cooling from 65°C to 25°C. Cas12a and buffer were then added, followed by incubation at 25°C for 10 minutes. The template DNA was introduced and the mixture was incubated at 37°C for 30 minutes. Following incubation, the samples were treated with RNase at 37°C for 15 minutes, followed by Proteinase K treatment under the same conditions and the digested products were resolved via 2 per cent agarose gel electrophoresis.

RESULTS AND DISCUSSION

Collection of ToLCV Infected Tomato Samples from Field

Tomato plants exhibiting typical symptoms of Tomato Leaf Curl Virus (ToLCV) infection, including upward

Fig. 1 : ToLCV infected tomato

leaf curling, stunted growth and wrinkled leaves were collected from the experimental fields of ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru, India (Fig. 1). The observed symptoms, including stunting, chlorotic mottling and leaf curling were consistent with those reported by Navot *et al*., 1992 in field-infected tomatoes.

Genomic DNA Extraction from the ToLCV Infected Tomato Leaves and its Quantification

High-quality genomic DNA was successfully extracted from ToLCV-infected tomato leaves using a modified CTAB method. The extracted DNA showed a concentration range of from 850–1600 ng/µL and a purity ratio (A260/A280) between 1.8–2.0, as determined by a Nanodrop spectrophotometer (Table 4). Agarose gel electrophoresis confirmed the integrity of the DNA with clear, intact bands observed without significant degradation (Plate 1). The extracted DNA was suitable for downstream molecular analyses.

Table 4

Nanodrop spectrophotometer readings for ToLCV genomic DNA

Sample	Concentration (ng /µl)	Purity (260/280)
ToLCV 1	856	1.88
ToLCV 2	976	1.93
ToLCV 3	1046	1.98
ToLCV 4	1567	1.82

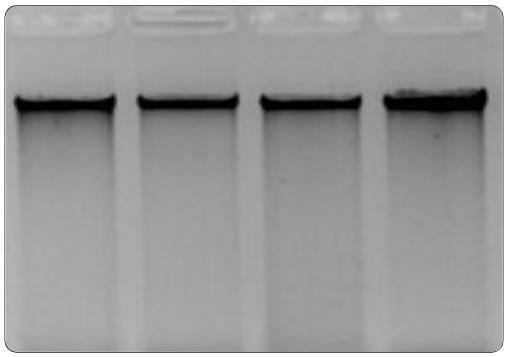

Plate 1 : Total genomic DNA from ToLCV

Target Region Selection and Polymerase Chain Reaction (PCR)

The target region spanning 1461 bp in the the complete coat protein gene and a partial replicase gene of Tomato Leaf Curl Virus (ToLCV) was successfully amplified by PCR using gene-specific primers. The amplified product was visualized as a distinct band on a 1.2 per cent agarose gel, confirming successful amplification (Plate 2). The PCR product was purified

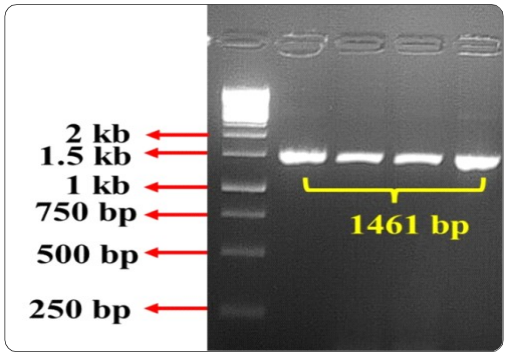

Plate 2 : PCR amplification of target genes of ToLCV

using the FavorPrep GEL/PCR Purification Mini kit, yielding high-quality DNA suitable for downstream applications.

Cloning and Transformation of Target Gene of ToLCV into pTZ57R/T TA Cloning Vector

The target gene of Tomato Leaf Curl Virus (ToLCV) was successfully ligated into the pTZ57R/T TA cloning vector (Fig. 2) and transformed into *Escherichia coli* DH5ị cells. Blue-white screening on LB agar plates with ampicillin, X-gal and IPTG identified white colonies, indicating successful cloning of the target gene (Plate 3). The blue-white selection of the colony allowed recombinants to be identified from non-recombinants (Pradhan *et al*., 2023). The extracted plasmid DNA was of high quality and suitable for subsequent analyses. Restriction digestion with EcoRI and Hind III produced fragments of 1461 bp and 2886 bp (Plate 4), confirming the presence and correct size of the insert. Sequencing further verified the accuracy and orientation of the cloned target gene. The use of the pTZ57R/T vector and Sanger sequencing aligns with protocols described by Saunders *et al*. (2000), enabling the assembly of complete genomic sequences using tools like BioEdit.

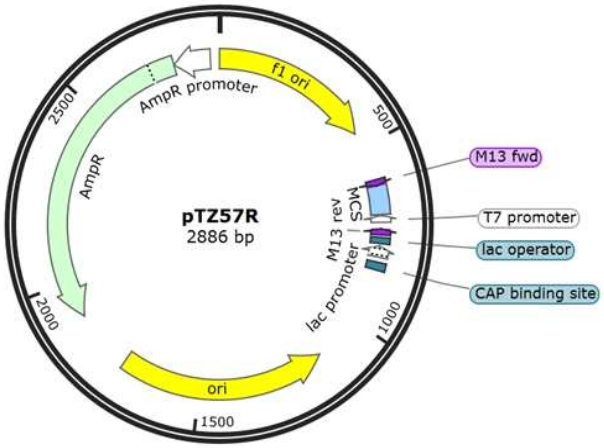

Fig. 2 : Vector map of pTZ57R/T TA cloning vector

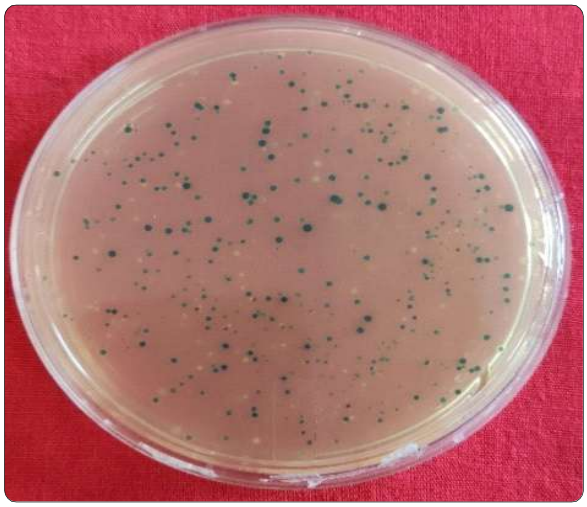

Plate 3 : Blue-white screening of the *E.coli* colony to identify the transformants. Blue colour colony indicating the non- transformants and white colour colony indicating the transformants

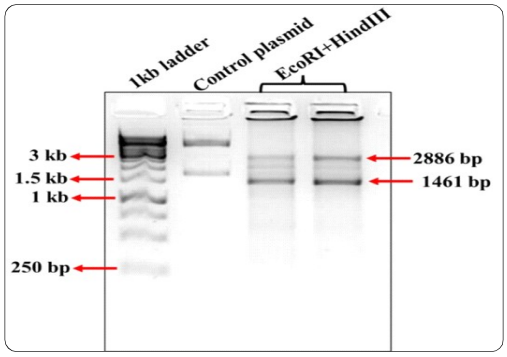

Plate 4 : Confirmation of target gene in the pTZ57R/T TA vector through restriction digestion

Designing, Synthesis of Off-Target Minimized sgRNAs and *In-vitro* Transcription

Off-target minimized sgRNAs targeting specific ToLCV genes was successfully designed using CHOPCHOP (Montague *et al*., 2014) and validated for secondary structure using RNAfold (Fig. 3). The synthesized sgRNA was annealed and dsDNA

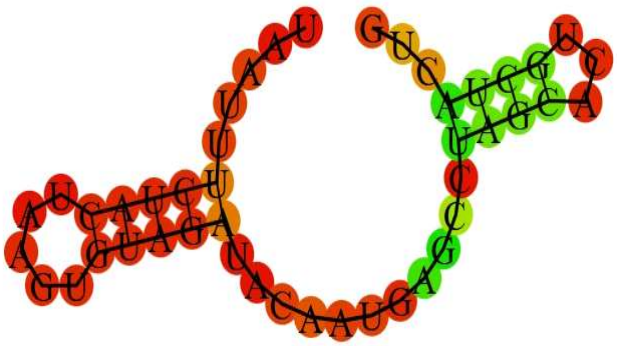

Fig. 3 : Secondary structure of ToLCV gRNA

template was purified for *in vitro* transcription. The transcription reactions yielded high-quality sgRNA, quantified at 1256 ng/µL with A260/A280 ratios of 2.03. Integrity was confirmed on a 2 per cent agarose gel, showing sharp, distinct band (Plate 5). The sgRNA was diluted to 300 ng/µL and stored at -80°C for downstream applications.

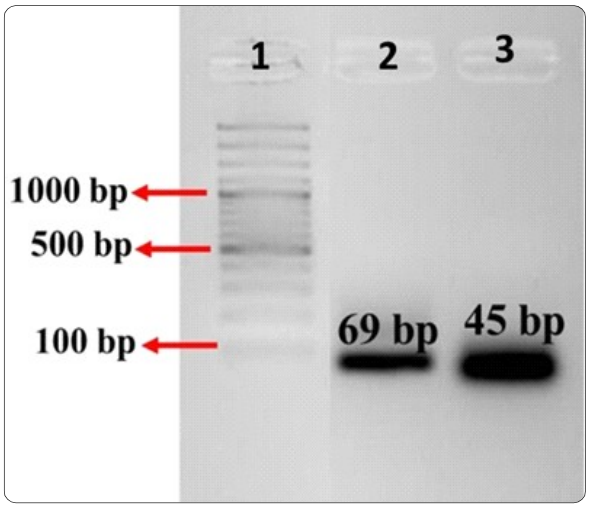

Plate 5 : Gel image of *In-vitro* transcription of ToLCVsgRNA. Lane 1 - 100bp DNA ladder, Lane 2 - Control template, Lane 3 - ToLCV sgRNA

Preparation of Ribonucleoprotein (RNP) Complex of Cas12a and *In-vitro* Restriction Assay

The functionality of the purified Cas12a protein was successfully validated through an *in vitro* restriction assay using ribonucleoprotein (RNP) complexes formed with sgRNA specific to the ToLCV genome (ToLCVgRNA). The target DNA, a PCR-amplified 1461 bp fragment was first verified for integrity through NdeI restriction digestion, yielding expected bands of 647 bp and 814 bp on a 2 per cent agarose gel (Plate 6). Subsequently, the RNP complexes were incubated with the validated DNA template under optimal conditions for Cas12a-mediated cleavage. The assay produced distinct cleavage bands of 1331 bp + 195 bp for ToLCVgRNA, confirming specific recognition and efficient cleavage by the RNP complexes (Plate 7).

These results demonstrate the activity of the purified Cas12a protein and the effectiveness of the designed sgRNAs in targeting the ToLCV genome. Based on

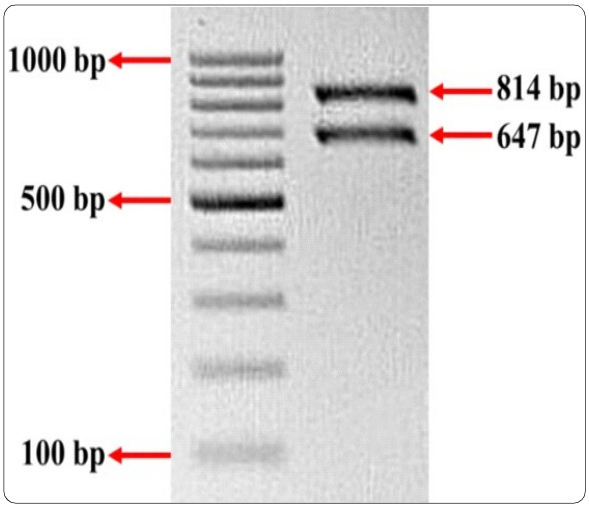

Plate 6 : Confirmation of target gene through restriction digestion

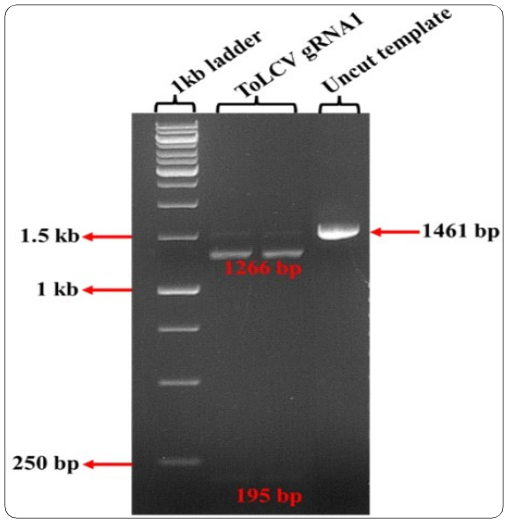

Plate 7 : *In-vitro* cleavage assays using Cas12a

their performance in generating specific cleavage products, ToLCVgRNA was selected for further diagnostic studies. CRISPR-based detection is faster, more sensitive and highly specific compared to PCR and ELISA, requiring no thermal cycling or antibodies, making it ideal for rapid and portable diagnostics. This assay demonstrated the reliability of Cas12a-sg RNA complexes for ToLCV detection and diagnostics and can be adapted for field use by integrating with a lateral flow assay, enabling rapid, instrument-free detection with visual readouts for on-site diagnostics and surveillance.

The present study employed advanced molecular and CRISPR/Cas12a-based diagnostics to detect Tomato Leaf Curl Virus (ToLCV), a major threat to tomato production. Field samples showing ToLCV symptoms were confirmed via PCR amplification of a 1461 bp target region. The target gene was cloned into the pTZ57R/T vector, verified through sequencing and restriction digestion. Off-target minimized sgRNAs were designed, synthesized and validated for high specificity. Ribonucleoprotein complexes of Lb-Cas12a and sgRNAs demonstrated precise cleavage of ToLCV DNA *in vitro*. These results establish the effectiveness of CRISPR/Cas12a as a rapid, sensitive and field-adaptable diagnostic tool for ToLCV detection and management.

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