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Full Length Article

Comparative Analysis of Synthetic Rep Protein Efficacy in Transgenic Plants with Rep Mutant to Develop Resistance against TYLCV-OM Infection in Tomato

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Abstract

This research compares the effectiveness of Rep protein mutants and codon-optimized truncated Rep (Repsyn130) in transgenic tomato plants against the tomato yellow leaf curl virus-Oman (TYLCV-OM) evaluating their ability to inhibit replication and enhance plant health and productivity. Tomato yellow leaf curl disease poses substantial loss to tomato production globally, including Oman. Replication-associated genes play a very important role in the replication of TYLCV and other begomoviruses. However, synthetic proteins in particular have shown potential in reducing viral replication and symptoms in genetically modified tomato plants. Rep mutants were created through substitution mutagenesis and Repsyn130 plants were generated through Agrobacterium-mediated transformation. The study revealed that Rep mutants and Repsyn130 can decrease symptoms caused by TYLCV-OM. Repsyn130, in particular, demonstrated enhanced viral resistance. Utilizing real-time PCR analysis, it was confirmed that Repsyn130 plants displayed a decrease in viral load. This research emphasizes the potential of utilizing codon-optimized Repsyn130 to provide viral resistance and promote sustainable agricultural practices. Future research will investigate its impact on additional defense mechanisms.

Keywords: *Begomovirus*; TYLCV-OM; Rep mutant; Synthetic rep; Transgenic plants

Introduction

Tomato (*Solanum lycopersicum* Mill.) is one of the most important vegetable crops globally, serving as a primary source of income for farmers and alleviating poverty (Yan *et al.* 2018). The Tomato yellow leaf curl virus (TYLCV), has widely disseminated across various regions, including the Arabian Peninsula, and is notably economically impactful in Oman, where it severely affects tomato plants (Abdelbacki *et al.* 2010; Al-Shihi *et al.* 2017). TYLCV a member of the Begomoviruses genus family; *Geminiviridae*, is a circular, single-stranded DNA virus transmitted by whiteflies. The begomoviruses rely on their host plants for replication, transcription, and movement, and have evolved mechanisms to manipulate host cellular processes and evade plant defenses (Seal *et al.* 2006). Begomoviruses encode different proteins out of these replication-associated proteins (Rep; AC1/C1) are crucial for viral DNA replication and interact with host factors to ensure successful infection (Guerrero *et al.* 2020). A major effort to control TYLCV in the field would be using transgenic resistance (Yang *et al.* 2004). To establish plant resistance to geminivirus infection including TYLCV, a common and effective approach is generating transgenic plants that overexpressed the viral replication protein (Rep) itself or a silencing suppressor interacting with the viral genome (Gupta *et al.* 2021). A number of these strategies include overexpression and/or knockout of plant genes which showed different levels of effectiveness in resistance to different geminivirus strains.

The most extensive work by Polston's group revealed that the segment of the *rep* gene has a vital role in the gene silencing mechanism and replication. By targeting the amino acid sequence from position 71 to 215 of the Rep protein, it was demonstrated that truncation at the C-terminus affects the suppression of the virus and the replication of viral DNA, with maximum inhibition observed. A recent study in Oman revealed siRNA and micRNA mediated silencing of rep and the reduction in disease symptom severity (Ammara *et al.* 2015; Al-Roshdi *et al.* 2023). The gene expression of rep leads to the induction of various cellular pathways and thus contributes

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to the reduction of the disease symptom severity. The synthesis of viral single-stranded DNA in response to the host during the initiation of replication by encapsidating viral ssDNA is essential for TYLCV disease progression.

Mutations impairing the nuclease activity of the Rep protein can confer resistance to begomovirus diseases in transgenic plants. Tomatoes overexpressing Rep exhibit enhanced tolerance to TYLCV compared to the original PLCT variety. Engineered variations of Rep produce transgenic tomatoes with increased tolerance to TYLCV. The Rep protein serves as a crucial factor in initiating the replication of begomovirus genomes through the rolling circle mechanism, a key step in the viral infection process of monopartite begomoviruses, and in maintaining the status of begomovirus-encoded betasatellites (Zhang *et al.* 2016). Repsyn130, a mutant form of Rep with a 390 amino acid deletion, was previously developed by (Voorburg *et al.* 2020). It has been demonstrated that providing Repsyn130 in trans alleviates the growth deficit in yeast associated with expressing Rep from its coding sequence, and it lacks nuclease activity. The experiments involving the transfection in *Arabidopsis thaliana* showed that Repsyn130 outperforms authentic Rep from three different begomoviruses. These studies demonstrated the diminished capacity of Repsyn130 to maintain the betasatellite, resulting in its significant accumulation compared to the wild-type Rep (Wang *et al.* 2017). These findings support the reasoning behind the proposed investigations: leveraging deficiencies in Rep functions to restore transgenic assays and enhance the tolerance of transgenic plants.

Transgenic plants are crops genetically modified with the introduction of one or more genes from different organisms, providing resistance to environmental conditions, pests, or diseases (Rogers and Parkes 1995). Due to their wide geographic tolerance and resistance to insects and viruses, transgenic plants can create a substantial impact on international agriculture (Christou and Twyman 2004). Hence, transgenic plants coding for Rep-mutant proteins are resistant to TYLCV-OM. Though the viral resistance was increased by replacing the Rep protein with Rep-mutant proteins, sometimes the mutant protein was not formed, and the plants could not be transformed resulting in poor viral resistance. Hence it was confirmed that the wild-type viral gene encoding Rep mutant protein is not the reason for the low resistance generation (Pramanik *et al.* 2021).

The objective of this study was to compare the efficacy of Rep mutant and synthetic Rep protein in combating TYLCV-OM in Omani tomato plants with specific focus at evaluating their ability to inhibit viral replication and enhance plant health and productivity.

Materials and Methods

Substitution mutagenesis of Rep ORF

A substitution mutation was inserted after the nucleotide sequence (nt) and the relevant amino acids in the Rep ORF were examined to synthesis Codon-optimized truncated Rep (Repsyn130. This mutation was produced using mutagenic oligonucleotides, which are intended to produce stop codons. Fig. 1 shows how two stop codons ATT and TAG, respectively, were used to replace the original codons for glutamic acid (GAG, codon number 49) and lysine (AAA, codon number 42 of Rep). TYLCm RepF: '5-CTG CAG ATT CTA ATG AAT TAT TTG GAA C-3' and TYLCm RepR: '5-CTG CAG ATA GCT TCA CGA AGA TGG GAC-3' combination of primers was utilized to generate this mutation. The stop codons in Fig. 1 are highlighted, and the PstI restriction site (CTGCAG), was used to build the Partial Tandem Repeat (PTR).

Construction of PTR

Mutagenic primers were used to construct the infectious construct. pCAM-TYLCV-OM-PTR was amplified by PCR to create a full-length genome of a Rep mutant (Zhou *et al.* 2003). The resultant fragment was then cloned in pUC19 to create pUC19-TYLCV-OMΔRep. In the binary pCAMBIA-1301 vector, the PTR for TYLCV-OM\Rep was constructed as described by Rouhibakhsh *et al.* (2011). A fragment that was liberated from pUC19-TYLCV-OMΔRep using PstI/EcoRI was ligated to form pCAM-TYLCV-OMPΔRepPE, a partial construct. Eventually, PstI linearized pCAM-TYLCV-OMPΔRepPE, releasing a monomer of TYLCV-OMΔRep from pUC19-TYLCV-OMΔRep, which was then joined end to end using PstI to create pCAM-TYLCV-OMΔRepPTR.

Agroinoculation of tobacco and tomato plants

Agrobacterium strain AGL1 containing pCAM-TYLCV-OMPTR and pCAM-TYLCV-OMΔRepPTR were separately introduced into 50 mL of YEB liquid medium (comprised of five g L^{-1} bacto-beef extract, one g L^{-1} bacto-yeast extract, five g L^{-1} peptone, five g L^{-1} sucrose, and 0.492 g L^{-1} MgSO4, with a pH of 7.2). This medium was supplemented with 50 mg L^{-1} kanamycin and 50 mg L^{-1} carbenicillin. The cultures were then incubated at a temperature of 28°C on a rotary shaker at 200 rpm for a duration of 48 h, until the optical density (OD600) reached a range of 0.6 to 1. Subsequently, bacterial cells were collected through centrifugation at 5000 rpm for 15 min at a temperature of 20°C. A solution of 3 mL containing 10 mM MgCl2 and 100 µM acetosyringone was added to the cells, which were then incubated at 28°C on a rotary shaker for four hours. The activated A. tumefaciens was utilized to infiltrate the experimental plants. Agroinoculation was carried out on the young leaves of *Nicotiana Benthamiana* and transgenic Repsyn130 plants by introducing the AGL1 strain containing pCAM-TYLCV-OMPTR and

pCAM-TYLCV-OMΔRepPTR into the leaf tissues of the plants using a needleless syringe at 3-4 weeks. The agroinoculated plants were maintained in a green greenhouse with temperatures set between 28–29°C and relative humidity ranging from 80 to 90%. The development of symptoms was observed for a period of 30 days and analyzed for viral DNA replication. Both wild TYLCV-OMs, Repsyn130 and Rep mutant TYLCV-OMs were inoculated onto the test plants separately.

Sequencing of mutant clones

DNA sequencing was used to confirm the mutations. Using the primer walk technique, the PCR product of the Rep mutations was cloned into the plasmid vector pUC19 and commercially sequenced in both orientations (Macrogen Inc., Seoul, Korea).

Production of wild and Rep mutant Agro-infectious clones

To explore the potential impact of a mutation in the Rep gene of TYLCV-OM on the virus's ability to infect, we generated partial repeat constructs of TYLCV-OM-[JN604484] and TYLCOMΔRepV using a binary vector. To create an infectious clone of the wild type TYLCV-OM, we extracted a 1.9 kb fragment with PstI/EcoRI from the pUC-TYLCV-OM and inserted it into a binary pCAMBIA-1301 vector (Fig. 2). The 2.75 kb monomer, obtained by cleaving pUC-TYLCV-OM with PstI, was then ligated into the partial construct digested with PstI. This resulted in the production of pCAM-TYLCV-OMPTR, which was tested for orientation by releasing a monomer of the genome using EcoRI. To create a Rep-mutant version of TYLCV-OM with a diagnostic restriction site and a denatured start codon in the Rep ORF, we employed mutagenic primers. These primers were used to extract a 0.6 kb fragment with KpnI/BamHI, which was then inserted into the binary pCAMBIA-1301 vector. The 2.75 kb monomer, obtained by digesting pUC-TYLCV-OM with KpnI, was ligated into the partial construct digested with KpnI. The resulting construct, named pCAM-TYLCV-OMΔRepPTR, was also tested for orientation by releasing a monomer of the genome using BamHI.

Tomato transformation through the utilization of the Repsyn130 gene construct

Tomato transformation was carried out by the method of Ammara *et al.* (2014), Ammara *et al.* 2020. Four transgenic lines (L23, L24, L34 and L38) were selected for the comparative analysis. It is with these lines that a detailed comparative analysis was conducted, aiming to shed light on their characteristics and potential implications for further research and development endeavors.

5'-tta----- agt ccc atc ttc gtg aag ctc tct gca gat tct aat gaa ttt ttt gga agt ggg -----tgc cat-3' (V-strand)

3'-aat----- tca ggg tag aag cac ttc gag aga cgt cta aga tta ctt aaa aaa cct tca ccc-----acg gta-5' (Wild Rep)

3'-aat----- tca ggg tag aag cac ttc gat aga cgt cta aga tta ctt aat aaa cct tca ccc-----acg gta-5' (Mutant Rep

Fig. 1: The sequence of V-strand, wild Rep, and mutant Rep of TYLCV-OM

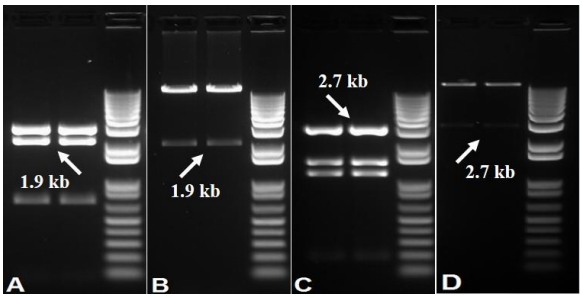

Fig. 2: Construction of infectious clones of both wild and Rep-mutant TYLCV-OM proceeded as follows: (A) The TYLCV-OM sequence of 1.9 kb was extracted using the PstI/EcoRI enzymes. (B) The same 1.9kb TYLCV-OM sequence was obtained from the pCAMBIA-1301 vector. (C) The complete genome of TYLCV-OM was obtained by releasing it from the pUC 19 vector. (D) The full genome of TYLCV-OM was also extracted from the pCAMBIA-1301 vector, employing the PstI enzyme

Results

Analysis of the infectivity of wild, Rep mutant and Repsyn130 construct against TYLCV-OM in *N. benthamiana*

N. benthamiana susceptible plants were exposed to wild-type, Rep mutant, and Repsyn130 TYLCV-OM via Agrobacterium-mediated inoculation. Four transgenic lines, designated as L23, L24, L34 and L38, were used for comparison with the wild-type and Rep mutant constructs. The wild-type TYLCV-OM displayed visible infection symptoms between 10-15 days post inoculation (dpi) on the *N. benthamiana* plants (Fig. 3; Table 1). However, it developed typical initial symptoms of infection 20-25 dpi on tomato plants such as upward curling of leaflet margins, decreased leaflet area, yellowing of young leaves, and overall stunting. On the other hand, Rep mutant TYLCV-OM failed to develop symptoms, either on *N. benthamiana* plants or on tomato plants (Fig. 4; Table 2).

The replication and movement of TYLCV-OM throughout the system were subsequently confirmed through PCR analysis using primers specifically designed to amplify the Core-CP of begomoviruses. Notably, the plants inoculated with the wild-type TYLCV-OM displayed the expected 650 bp band, while no amplification was observed with the same primers in the samples inoculated with Rep mutants and Repsyn130 agroinfectious constructs. These findings indicated that Rep mutant and Repsyn130 TYLCV-OM cannot replicate

Table 1: Agroinoculation of tobacco plants with Rep mutants and Repsyn130 at days after inoculations

Plant line tested	Inoculum[a]	Infectivity (plants symptomatic/plants inoculated)	Plant symptoms[a] @ 7 Days after Inoculation	Plant symptoms[a] @ 14 Days after Inoculation	Plant symptoms[a] @ 21 Days after Inoculation
Nb*	Mock inoculated	0/l0	NS	NS	NS
Nb*	Un-inoculated	0/10	NS	NS	NS
Nb*-1	TYLCOMV	10/10	SS	SS	SS
Rep-mutant-1	TYLCOMV	10/10	NS	MS	MS
Rep-mutant-2	TYLCOMV	10/10	NS	MS	MS
Rep_{syn130}	TYLCOMV	10/10	NS	VMS	VMS

*Non-transformed *N. benthamiana*

[a]Plants were either not inoculated, inoculated with *Agrobacterium* cultures harboring the empty pGreen0029 vector (mock), or inoculated with cultures harboring infectious constructs for TYLCOMV

[b]*N. benthamiana* physically observed symptoms were classified as no symptoms (NS), very mild symptoms (VMS), mild symptoms (MS), and severe symptoms (SS), The inoculation results of TYLCOMV indicated at 7 days after inoculation, 14 days after inoculation and 21 days after inoculation

Fig. 3: Non-transgenic *N. benthamiana* plants inoculated either with TYLCV-OM or co-inoculated with TYLCV-OM and Repsyn130 or Rep-mutant 1 or Rep-mutant 2 via agro-inoculation

N. benthamiana plants a healthy control (A). *N. benthamiana* plants inoculated with TYLCV-OM (B) or co-inoculated with TYLCV-OM and Repsyn130 (C). Similarly, non-transgenic *N. benthamiana* plants were co-inoculated with TYLCV-OM and Rep-mutant 1 (D) and Rep-mutant 2 (E), respectively. All plant photographs were taken at 21 days post-inoculation (dpi)

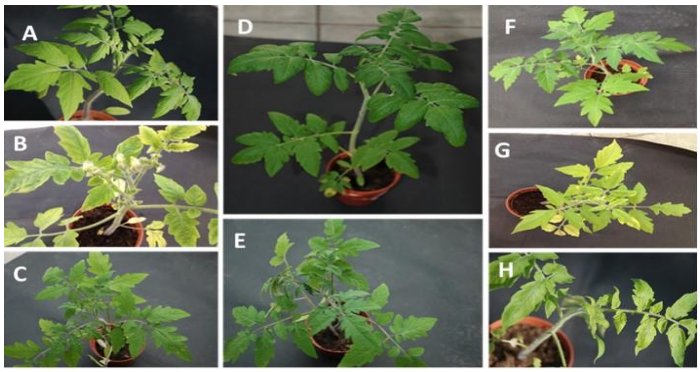

Fig. 4: The images illustrate the phenotypic symptoms observed in Repsyn130 transgenic *Pusa ruby* plants inoculated via agro-inoculation, compared to control plants

In Picture A the non-transgenic *Pusa ruby* plants and in picture B non-transgenic *Pusa ruby* plants inoculated with TYLCV-OM. The plants shown in pictures C, D, E, and F are L-23, L-24, L-34, and L-38 Repsyn130 transgenic plants, respectively, inoculated with TYLCV-OM. The images in panel G depict non-transgenic *Pusa ruby* plants co-inoculated with TYLCV-OM/Rep-mutant 1, while the images in panel H depict non-transgenic *Pusa ruby* plants co-inoculated with TYLCV-OM/Rep-mutant 2. Plant images were taken at 21 dpi

and spread in the plant system, thus providing further evidence for the role of the Rep protein in TYLCV-OM infection dynamics. Additionally, the absence of infection symptoms in the Rep mutant further supported the notion that the Rep protein is crucial for the development of TYLCV-OM-related symptoms.

All tested plant lines, including Rep mutants and Repsyn130 transgenic plants, displayed 100% infectivity when inoculated with TYLCV-OM, as evidenced by the presence of symptomatic plants at all-time points (Fig. 4). At 7 dpi, all plant lines exhibited severe symptoms (SS) caused by TYLCV-OM infection, indicating a rapid onset of disease. However, 14 and 21 days after inoculation, distinctions in symptom severity were noted among the plant lines. The control plant line Nb*-1 exhibited severe symptoms, Rep mutants exhibited a shift from mild symptoms (MS) at 14 days to severe symptoms (SS) at 21 days (Fig. 4; Table 1). In contrast, Repsyn130 transgenic plants showed very mild symptoms (VMS) at both time points, indicating a delayed and less severe progression of the disease compared to the control and Rep mutant plants (Table 2).

Table 2: Comparison of symptom development in transgenic tomato Repsyn130 plants and control tomato Rep mutants following agroinoculation

Plant line tested	Inoculum[a]	Infectivity (plants symptomatic/plants inoculated)	Plant symptoms[b] @ 7 Days after Inoculation	Plant symptoms[b] @ 14 Days after Inoculation	Plant symptoms[b] @ 21 Days after Inoculation
Nt*	Mock inoculated	0/l0	NS	NS	NS
Nt*	Un-inoculated	0/10	NS	NS	NS
Nt*-1	TYLCOMV	10/10	SS	SS	SS
Rep-mutant-1	TYLCOMV	10/10	NS	MS	MS
Rep-mutant-2	TYLCOMV	10/10	NS	MS	MS
L-23	TYLCOMV	10/10	NS	NS	NS
L-24	TYLCOMV	10/10	NS	NS	NS
L-34	TYLCOMV	10/10	NS	NS	VMS
L-38	TYLCOMV	10/10	NS	NS	NS

*Non-transformed *Pusa Ruby* tomato plants

[a]Plants were either not inoculated, inoculated with *Agrobacterium* cultures harboring the empty pGreen0029 vector (mock), or inoculated with cultures harboring infectious constructs for TYLCOMV

[b] *N. benthamiana* physically observed symptoms were classified as no symptoms (NS), very mild symptoms (VMS), mild symptoms (MS), and severe symptoms (SS), The inoculation results of TYLCOMV indicated at 7 days after inoculation, 14 days after inoculation and 21 days after inoculation

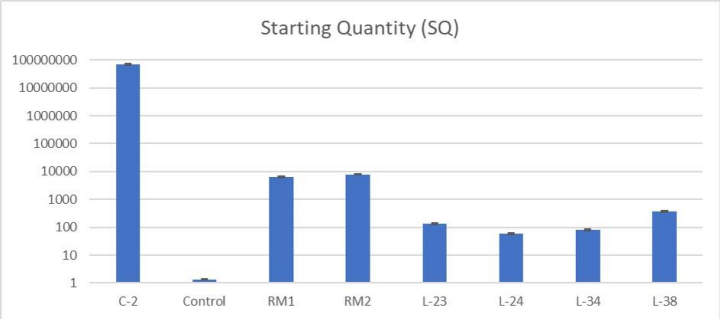

Fig. 5: Real-time quantitative polymerase chain reaction (PCR) was utilized to quantify the viral DNA concentration in both transgenic and non-transgenic tomato plants following TYLCV-OM infection

The concentration of viral DNA is displayed through the presence of blue bars, while the vertical axis represents the number of virus particles ranging from 0 to 100,000,000. The first two bars depict the control wild-type plants that were inoculated with TYLCV-OM, while the third bar represents a healthy wild-type control plant used for comparison purposes. The analysis involved the inclusion of two Rep Mutants, known as RM1 and RM2, in addition to three transgenic lines 23, 24, and 34. From each line, two representative plants were selected for each virus infection. The bars depict the mean of three replicates, with the error bars representing the standard deviation

Virus quantification in transgenic lines via RT-qPCR

The RT-PCR results revealed the quantification of DNA copies for various samples (Fig. 5). It is evident that C-1 and C-2 represented two distinct control samples, both having a high abundance of viral copies. On the other hand, the "control" sample exhibited a significantly lower copy number, indicating a different experimental condition or treatment. The RM1 and RM2 samples, potentially being Rep mutants, exhibited a higher number of copies in comparison to the control, implying an increase in the target DNA (Fig. 5). In contrast, T-23, T-24 and T-34 samples displayed relatively few copies, suggesting a distinct response compared to the control and Rep mutants. This indicated variations in gene expression or DNA content. These outcomes provided valuable insights into the relative presence of the target DNA in the tested samples, aiding in the comprehension of genetic or molecular differences between the samples under investigation. Further analysis and interpretation of these findings yielded valuable information concerning the experimental conditions and the biological significance of the observed disparities.

Discussion

The present study highlights the effectiveness of Rep mutants and the codon-optimized truncated Rep (Repsyn130) in mitigating the symptoms and viral load associated with TYLCV-OM infection in transgenic plants. Our findings reveal that Repsyn130 confers a significantly higher level of resistance to TYLCV-OM compared to both the wild-type and Rep mutant constructs. This observation is supported by previous research, which demonstrated that modifications in replication-associated proteins can alter virus-host interactions and enhance plant resistance (Ammara *et al.* 2017).

The distinct delay and reduction in symptom severity observed in Repsyn130 transgenic plants underscore the potential of codon-optimized proteins in providing robust resistance against TYLCV-OM. The presence of very mild symptoms (VMS) at both 14 and 21 dpi in these plants contrasts with the rapid progression of severe symptoms in control and Rep mutant lines, suggesting that Repsyn130 may interfere with the virus's replication or movement within the plant system. This aligns with the study by Ammara *et al.* (2020), which reported that specific

mutations in viral replication proteins could significantly impede viral replication, thereby reducing symptom severity and viral titers. The PCR results further substantiate the role of Repsyn130 in impeding viral replication, as indicated by the absence of viral DNA amplification in plants inoculated with Repsyn130 constructs. The decrease in viral load observed in Repsyn130 plants through RT-qPCR analysis provides additional evidence for the efficacy of this synthetic protein in combating TYLCV-OM infection. This outcome is consistent with findings from Singh *et al.* (2019), who reported that engineered resistance strategies, such as the use of codon-optimized proteins, can lead to substantial reductions in viral titers in genetically modified plants. However, the variability in resistance levels observed among different Rep mutant lines highlights the need for further refinement of these genetic constructs. The gradual shift from mild to severe symptoms in Rep mutant plants suggests that while these constructs may offer some level of resistance, they are not as effective as the Repsyn130 variant. Borrelli *et al.* (2018) emphasized the importance of optimizing genetic modifications to achieve consistent and durable resistance, particularly in the face of evolving viral challenges.

The study also raises important considerations regarding the stability of these resistance traits across different growing conditions and tomato genotypes. Future research should focus on evaluating the performance of Repsyn130 and Rep mutants in diverse environmental settings and across various tomato cultivars. Additionally, extending this research to other crops susceptible to begomoviruses, as suggested by Mathur *et al.* (2017), could provide valuable insights into the broader applicability of these genetic modifications in agricultural practices. While the potential benefits of genetically modified crops, such as those expressing Repsyn130, are evident, ethical and ecological concerns must be addressed. The possibility of gene transfer to non-target species and the long-term impact on biodiversity warrant careful consideration. Regulatory frameworks and rigorous risk assessments are essential to ensure that the deployment of genetically modified crops does not lead to unintended consequences (Mathur *et al.* 2017).

Conclusion

This study underscores the potential of Rep protein mutants and the Repsyn130 variant in combatting TYLCV-OM in transgenic tomato plants. The genetic engineering techniques employed in this research demonstrate targeted alteration of plant genomes to enhance disease resistance, offering a promising avenue for reducing reliance on chemical pesticides. Both Rep mutants and Repsyn130 effectively alleviate TYLCV-OM symptoms and reduce viral quantities, with Repsyn130 showing notably improved resistance levels. This study serves as a starting point for understanding how optimized codon synthetic Rep regulates viral gene expression, potentially leading to the development of defense mechanisms against begomoviral disease in tomato plants. Notably, this landmark study aimed at identifying Repsyn130, which could target key genes in the genetic makeup of TYLCV-OM. Further investigation is needed to assess the stability of these resistance traits across different growing conditions and tomato genotypes. Research on other crops affected by similar viruses could provide insights into the wider effectiveness of these genetic modifications.

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Author Contributions

UA conducted all experiments as part of her PhD research. JK and MSS conceptualized the idea and formulated the design for the codon-optimized synthetic Rep. UA, MS and AMA collectively analyzed the data and contributed to the writing and finalized the paper.

Conflicts of Interest

Not applicable for this study.

Data Availability

The previously available genome sequences of TYLCV-OM (Accession No. DQ644565) and ToLCB-OM (Accession No. HE800544) were utilized in this study.

Ethics Approval

This study does not involve any human or animal testing and is therefore not applicable.

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