Minireview Article

Development of Tomato Leaf Curl Virus Resistance: From Plant Breeding to Genome Editing-A Mini Review

Abstract

Tomato leaf curl virus (ToLCV) is a major constraint for global tomato production. ToLCV affects a variety of crops other than those in the *Solanaceae* family. ToLCV genome is compact and codes for structural and functional proteins that helps the virus to evade host mechanism. This review discusses the functions of viral genes, their interactions with each other and the host genes, and the techniques used to introduce ToLCV resistance in tomato and other crops. In addition, we have discussed the role of various methods for producing ToLCV resistant crops and the unexplored methods for producing disease resistance in plant system. Moreover, the involvement of the numerous factors responsible for increased incidence of disease, evolution, spread and diversity of begomoviruses has been discussed. We also suggested a multi-level approach required to target various critical life-cycle virus genes in a single system with multiple approaches to increase resistance and prevent mutant escape and evolution.

Key words: Begomovirus, Genome Editing, RNAi, ToLCV

Introduction

Tomato (*Solanum lycopersicum* L., 2n=2 x=24) is a common source of protective and essential nutrients such as lycopene, betacarotene, flavonoids, and vitamin C (R. K. Singh et

al., 2019; Vats et al., 2022). Tomato is the second most valuable vegetable crop after potato, with approximately 182.05 million tonnes of tomato fruit produced worldwide each year (W. Liu et al., 2022). Tomato plants are susceptible to numerous viral, bacterial and fungal diseases, such as late blight, leaf mould, spotted wilt, mosaic, and powdery mildew (W. Liu et al., 2022; Moriones et al., 2017).ToLCV infection is a significant threat to tomato cultivation because it affects leaves, flowers, and fruits, resulting in yield losses of upto 100 percent for tomatoes and 90-100 percent for chili (an important crash crop) (Chowdhury et al., 2022; Sahu & Sanan-Mishra, 2020; R. K. Singh et al., 2019). Cup-shaped curling of leaves, stunted growth, curling of lamina between veins, decreased leaf size, deformed leaflets, chlorotic and necrotic patches on leaves, vein clearing, characteristic bushy appearance, flower drop, poor fruit setting and sterility are the symptoms associated with ToLCV infection (Chowdhury et al., 2022; Sharma & Prasad, 2020).

In addition to tomato, ToLCVand its variants affects a wide variety of crops such as brinjal, chili pepper, cucurbit, okra, papaya and potato (Jeevalatha et al., 2017). There are more than 58 plant species in the ToLCV host range, that are mainly widespread in the world's tropical and subtropical regions (Sahu & Sanan-Mishra, 2020).

ToLCV belongs to the genus begomovirus, the Geminiviridae family (Caruso et al., 2023; Tripathi et al., 2018). Begomovirus is the largest group in the Geminiviridae family, comprising almost 88% of all species of Geminivirus(Cantú-Iris et al., 2019). The begomoviruses are small circular single-stranded, 2.7 kb long DNA viruses that infect plants around the world and pose a significant threat to the global food security (Jeevalatha et al., 2017; Sarkar et al., 2021). Their genomes are encapsulated (18 x 30 nm) in twin icosahedral particles. Begomoviruses have either one (monopartite) or two (bipartite, DNA A and DNA B) genomic components (Kachoie et al., 2018). Virus replication occurs in the nucleus of the host plant cell via rolling circleor recombination-dependent mechanism involving a double-

stranded replicative intermediate (Dai et al., 2022; Kachoie et al., 2018). The begomovirus are persistently, circulatively and non-propagatively vectored by the arthropod whitefly *Bemisiatabaci* (Hemiptera: Aleyrodidae) (Dai et al., 2022; Islam et al., 2018). Various attempts have been made to develop ToLCV resistant tomato and scientists has achieved limited success. To make a strategy for ToLCV resistant tomato, we should know about the structures and functions of ToLCV genomes and the strategies that have been already used to target ToLCV gene.

1. ToLCV genome structure and function

ToLCVs can be monopartite or bipartite and the majority of monopartite ToLCVs are found to be associated with betasatellites(Chowdhury et al., 2022; Gaikwad et al., 2011). Four ORFs, C1 to C4 (AC1 to AC4 for bipartite begomovirus) are encoded by the complementary sense strand, however in the subsequent part of manuscript gene names are used in reference to monopartite genome only. The C1-C4 proteins are also named as replication-associated protein (C1, Rep); the transcriptional activator protein (C2, TrAP); the replication enhancer protein (C3, REn) and the C4 protein respectively. ORFs in virion sense strand viz., V1 and V2 (AV1 and AV2 for bipartite species) encodes for coat protein (CP) and precoat protein respectively. DNA B codes for the movement protein (BC1, MP) and nuclear shuttle protein (BV1, NSP), both are essential for systemic spread and symptom expression (Chowdhury et al., 2022; Roy et al., 2019)(Figure 1).

Virion sense and complementary sense reading frames (ORFs) are separatedby the non-coding intergenic region (IR). In both DNA molecules, the IR region is conserved and can be folded into a possible loop structure. IR contains cis-acting regulatory elements for gene expression and possesses a hairpin structure with a conserved nonanucleotide sequence (TAATATTAC) (Borah et al., 2016; Caruso et al., 2023; Zaidi et al., 2017). IR also has small

repeat sequences known as 'iterons' which are sequence-specific binding sites for Rep to ensure that the replication of another virus or non-cognate virus could not be initiated by Rep (Jyothsna, Haq, Singh, et al., 2013; Yang et al., 2019).The IR is the key source of the small-interfering RNA referred to as vsRNA which targets the lncRNA and thus weakens plant immunity (Yang et al., 2019). Within IR, there is a common region (CR) sequence shared by bipartite begomoviruses' cognate DNA A and DNA B. CR is required to ensure the Rep mediated initiation of virion strand replication for both DNA A and DNA B (Borah et al., 2016; Caruso et al., 2023; Zaidi et al., 2016).

1.1 Functional Genes / Gene functions of ToLCV

Geminiviruses are dependent on host machinery to replicate them by triggering the genes necessary for transition to the S phase of the cell cycle (Z. Ali et al., 2015; Jeevalatha et al., 2017). Geminiviruses affects host gene expression by altering the signal transduction pathway via association with host protein kinases. Viral proteins have many functions because of their compact genome (Fondong, 2019; Jeevalatha et al., 2017)(Figure 2). Rep, REn and TrAP are referred to as early genes as they have role in viral replication while CP, MP and NSP are regarded as late genes as they are needed at the last stage of infection of ToLCV life cycle for encapsidation and systemic spread (Chowdhury et al., 2022; Shimada-Beltrán & Rivera-Bustamante, 2007).

ToLCV life cycle early genes

The Rep protein is responsible for rolling circle amplification (Roy et al., 2019). TrAP stimulates the transcription of viral DNA and suppresses the silencing route of the host RNAi (Roy et al., 2019; Sarkar et al., 2021). TrAP is expressed early in the infection process, which then prompts the expression of late genes (CP and DNA B encoded genes) (Cantú-Iris et al., 2019).REn protein plays role in enhancing viral DNA replication. REn is also responsible for

viral DNA accumulation in host cells (Padidam et al., 1996; Roy et al., 2019; Tashkandi et al., 2017). C4 is the smallest ORF and required for viral DNA accumulation in host cells (Tashkandi et al., 2017). C4 induces tumor growth and hyperplasia in infected plant cells thus referred as oncogenes to plant cells (Fondong, 2019). V2 encoded pre-coat protein suppresses both host transcriptional gene silencing (TGS) and post transcriptional gene silencing (PTGS). V2 inhibits host-specific RDR1 (RNA-dependent RNA polymerase1), necessary for the generation and amplification of virus-specific siRNAs and enhanced methylation of viral promoters during plant antiviral response (S. Basu et al., 2018).

ToLCV life cycle late gene

AV1/V1 (CP-Coat protein) is expressed during the last phase of viral life cycle and is transcriptionally regulated by TrAP(Cantú-Iris et al., 2019). CP is responsible for the encapsulation of genome into virion particles, transmission of insects, long-distance movement and vector identification (Roy et al., 2019; Tashkandi et al., 2017). MP is the major symptom determining factor in bipartite begomoviruses(Jyothsna, Haq, Singh, et al., 2013). Amino acid residue glutamate at the 19^{th} position in the N-terminus of MP is responsible for mechanical transmission of ToLCNDV(Lee et al., 2020).MP also facilitates the release of viruses from phloem tissue and interacts with the other cellular proteins to induce DNA B mediated yellow mottling disease symptom (Jyothsna, Haq, Singh, et al., 2013). Since, MP is involved in the mechanical transfer of begomoviruses, have become a target gene for developing strategies to control spread of ToLCV(Jeevalatha et al., 2017). Nuclear shuttle protein is vital for systemic spread, mechanical transmission and symptom expression(Lee et al., 2020).

1.2. ToLCV associated with satellite DNA

The vast majority of monopartite begomoviruses are associated with ssDNA (half the size of helper viruses, 1.3 kb) referred to as alphasatellites, betasatellites and deltasatellites(Zaidi et al., 2016). Alphasatellites earlier regarded as DNA-1 are molecules of ~1.4 kb in size. Rep or A-rich regions are known to be hotspots of recombination between diverse alphasatellites(Vinoth Kumar et al., 2017).Alphasatellites are able to replicate autonomously in permissive plant cells, so they are not strictly satellites; however they rely on the helper begomovirus for movement within plants (Vinoth Kumar et al., 2017; Zaidi et al., 2016).

DNA β satellites (DNA β) are half the size of begomoviruses (1,350 nt) and encode a single gene on complementary sense strand that codes for 118 amino acid protein known as βC1 (Zaidi et al., 2016). Betasatellites are functionally identical to DNA B of bipartite begomoviruses(Eini et al., 2017). βC1 suppresses host RNA interference and may be involved in programming infected cells to produce more hostile environments for begomovirus replication (Jyothsna, Haq, Jayaprakash, et al., 2013; Zaidi et al., 2016).β satellite is dependent on the helper begomovirus for encapsidation, insect transmission, replication, and movement within the host plant (Sattar et al., 2019). By interacting with Asymmetric Leaf 1 (AS1), β satellite increases the accumulation of the helper virus and may exacerbate symptoms in some host plant species (Mandal et al., 2015; Zaidi et al., 2016).

Except for the nona-nucleotide sequence, betasatellite does not show any sequence identity with the helper begomovirus(Sattar et al., 2019). Often DNA β satellites are found associated with bipartite begomoviruses that cause tomato leaf curl disease, e.g. DNA β associated with ToLCNDV(Gaikwad et al., 2011; Jyothsna, Haq, Singh, et al., 2013). It has been experimentally shown that DNA A can be maintained if it is associated with betasatellite in the absence of DNA B as the βC1 protein enables the transport of ToLCNDV DNA A from the nucleus (Jyothsna, Haq, Singh, et al., 2013).

Deltasatellites have a role in reducing the severity of symptoms and are capable of trans-replication by helper begomovirus(Sattar et al., 2019). Tomato leaf curl deltasatellite (ToLCV-sat) was the first satellite detected in plant viruses. All deltasatellites have similar characteristics; they have an A-rich sequence, a nonanucleotide TAATATTAC stem loop structure, and a secondary stem-loop structure (Akmal et al., 2017).

1.3. PLANT –PATHOGEN INTERACTION: Molecular Mechanism

Plant defense is a complex phenomenon that involves cross-talk between signaling molecules and various defense proteins (Chandan et al., 2019; Hanley-Bowdoin et al., 2013). Plant cytoplasmic and nuclear degradation machinery, such as proteases, ubiquitin 26S proteasome systems, and autophagy pathways, target all viral proteins differently (Gorovits et al., 2016). Viruses hijack or interfere with these host defense mechanisms for their own multiplication and systemic transmission throughout the host plant (Moshe et al., 2016).Viruses confront host degradation by developing small or medium-sized aggregates such that they are less vulnerable to the host degradation mechanism (Gorovits et al., 2016).Cellular heat shock proteins (HSPs), HSP70 typically accumulate in virus-infected plants. Many plant viruses recruit HSP70 to help with viral protein synthesis, folding, and localization, to regulate viral replication, and to disrupt the host's antiviral defense (Jeevalatha et al., 2017).

In plants, Armadillo repeat family proteins (ARM) have multiple regulatory functions, including stress response, development, growth and resistance to pathogens (Mandal et al., 2018). SlARM18 virus induced gene silencing (VIGS) in a ToLCNDV resistant cultivar resulted in a susceptible variety (Mandal et al., 2018). This clearly indicates that Armadillo repeated family proteins are the focus of begomoviruses. The CTR1 (Constitutive Triple Response) gene is a negative regulator of ethylene signaling cascade (Chandan et al., 2019).

For example, induced expression of LeCTR1 is observed in ToLCV infected tomato plants (Chandan et al., 2019). The host factor SlSnRK1 (*Solanum lycopersicum* Sucrose Non-fermenting 1-related Kinase) may directly inhibit betasatellite mediated symptoms and replication of viruses by phosphorylating βC1 protein (Mandal et al., 2015).

Viral proteins inhibit host post transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) and methylation machinery for their effective transmission, replication and spread. AC4 / C4 interacts with CLAVATA1 receptor kinase that functions in the maintenance of meristem (Fondong, 2019) and host AGO4 and influences viral genome methylation of cytosine (Vinutha et al., 2018). In infected plant cells, Rep contributes to perinuclear chloroplast clustering (Ding et al., 2019). ToLCV-Australia virus C4 gene interacts with SHAGGY HOST KINASE (SlSk) and SlSGS3 host for PTGS (Dai et al., 2022; Mandal et al., 2015).

In BSCTV (Beet Severe Curly Top Virus) C4 protein interacts with CLAVATA1 (CLV1) receptor kinase, thus inhibiting co-operative interaction between CLV1 and WUSCHEL resulting in leaf curling and vein swelling symptoms (Fondong, 2019; Gómez et al., 2019). Moreover, the importance of long non-coding RNAs should not be neglected as they play an immense role in plant immunity against viruses (Yang et al., 2019).

2. EVOLUTION AND DISTRIBUTION OF BEGOMOVIRUSES

Modern farming techniques have increased the prevalence of plant viruses, particularly in monoculture vegetable crops (Leke et al., 2015). The emergence of new begomovirus-satellite complex could result from interactions between geographically diverse begomovirus and non-cognate DNA satellites (Sattar et al., 2019).

Viruses can be spread over long distances through commodities carrying viruliferous *B. tabaci*, by virus infected seeds, trade of infected plants (for planting), parts of infected plants (e.g. cut flowers) and possibly by infected seeds (Bragard et al., 2020). Changes in insect vector population, crop cultivation pattern system and the climate pattern also increase disease incidence and diversity. For instance: ToLCNDV virus was transmitted to potatoes from infected sponge gourds and tomatoes due to overlapping planting seasons and early planting of potatoes, which exposed them to high temperatures and whitefly populations (Jeevalatha et al., 2017). ToLCV variants can be mechanically transmitted through sap of infected plants, and through propagation of infected scions and cuttings used for vegetative propagation and grafting (Bragard et al., 2020).

EVOLUTION of VIRAL STRAINS

Bipartite begomoviruses are thought to have evolved from monopartite viruses through the acquisition of DNA B (Li et al., 2019). The dynamic evolution of begomovirus disease is mainly due to recombination, capture of components, gene flow, natural selection and mutation (Vinoth Kumar et al., 2017; Zaidi et al., 2016). New viral variants are often more pathogenic and may exhibit a wider host range compared to existing pathogens (Lee et al., 2020). It has been documented that ToLCV-CP interacts with *Bemisiatabaci's* vitellogenin, this interaction is essential for the entry of the virus into the whitefly ovary and hence contributes to transovarial transmission of begomoviruses leading to the development and global spread of certain begomoviruses(Wei et al., 2017). Transovarial transmission of begomoviruses means that at least two generations of white flies can be sustained without the need for infected hosts and raises the risk of almost invisible virulent eggs laid by flies on plants and plant parts that can be transported to long distances by human activities and may lead to an outbreak of viral disease in the field or an area (Wei et al., 2017).

Moreover, begomoviruses display an opportunistic relationship with various betasatellites, depending on the host it infects, which could lead to a permanent relationship and thus lead to a different disease scenario (Jyothsna, Haq, Singh, et al., 2013; Sattar et al., 2019). For example: Resistance by breeding in cotton against cotton leaf curl disease (CLCuD) was broken in 2001 by begomovirus associated with a particular satellite, the cotton leaf curl Multan betasatellite (CLCuMB) associated with the recombinant begomovirus, Cotton leaf curl Kokhran virus strain Burewala (CLCuKoV-Bur) (Hassan et al., 2017; Zaidi et al., 2016). To contain the virus evolution and spread various methodologies and techniques have been developed(Table 1).

3. Strategies for development of virus resistant plants varieties

3.1. Marker assisted breeding and gene pyramiding

Marker assisted breeding using simple sequence replication (SSR) markers and molecular labelling of resistance genes has been used to improve disease resistance (R. K. Singh et al., 2015). Five resistance genes (Ty-1, Ty-2, Ty-3, Ty-4 and Ty-5) have been identified in tomatoes using these markers (Sharma et al., 2022; R. K. Singh et al., 2015). Resistance genes Ty-1 and Ty-3 encodes for RDRs that amplify the siRNA signal, improving resistance to disease (Sharma et al., 2022; Yang et al., 2019). Tomato cultivar H-88-78-1 resistant to ToLCV resulted in over two decades of breeding for resistance against ToLCV in India (R. K. Singh et al., 2015). Also introgession of tomato lines carrying Ty-2 and Ty-3 genes resulted in disease resistance against ToLCD (Prasanna et al., 2015).

3.2. Transgenesis

Plants that have artificially introduced integrated genetic material from a foreign source are known as transgenic (Steinwand & Ronald, 2020). In the early stage of adoption of transgenic technology, the viral coat protein was constitutively expressed. It was assumed that CP mediated resistance works by preventing an early infection event that prevents virion uncoating, thereby preventing systemic spread through the plant's vasculature. ToLCV CP gene was cloned into an expression vector and co-cultured with Agrobacterium containing ToLCV-CP construct into Pusa Ruby cotyledon leaf explants, resulting in transgenic plants with varying degrees of resistance (Raj et al., 2005).

3.3. Antisense technology

A main strategy for the production of disease resistant plants is considered to be the silencing of the negative regulators involved in plant defense. For example, the Tobacco Rattle Virus (TRV) virus-induced gene silencing technique to silence the LeCTR1 (*Lycopersicon esculentum* Constitutive Triple Response) gene produced increased resistance to ToLCV infection (Chandan et al., 2019). It was observed that the degree of gene silencing corresponds to the length of the dsRNA of the viral target gene when sense and antisense forms of the conserved sequence of viral AC4 (21nt-200nt) genes were targeted against TYLCV (Praveen et al., 2010). Possibly because multiple siRNAs will be produced and amplified to improve target gene silencing.

It has been observed that when fragements of V2 gene (sequences closest to promoter) of CLCuKoV-Bu were transformed into *Nicotiana benthamiana* in antisense orientation, the resultant transgenic plants were resistant to CLCuKoV-Bu, ToLCNDV and Pedilanthus leaf curl virus (PeLCV) begomoviruses(I. Ali et al., 2019). This finding is consistent with the notion that RNA interference is a homology-dependent phenomenon.

3.4. RNAi mediated disease resistance

Andrew Fire and Craig C. Mello shared the 2006 Nobel Prize in Medicine for their work on RNA interference (RNAi) in worm *Caenorhabditis elegans* published in 1998 (Xu & Wang, 2015). RNAi, triggered by double stranded RNA (dsRNA), is a post-transcriptional, sequence specific gene silencing mechanism in eukaryotes. The endogenous mechanism is exploited by RNAi to knockout genes (J. Liu et al., 2023; Yin et al., 2015). Double stranded RNA is first cleaved into small double-stranded fragments of~ 20 nt siRNAs with 3' overhang and a recessed 5' phosphate on each strand by the enzyme DICER when it enters the cell (Koeppe et al., 2023; Xu & Wang, 2015). Each double stranded siRNA is then segregated into the guide strand and the passenger strand. These guide strands are then embedded into the RNA-induced silencing (RISC) complex (Lisitskaya et al., 2018). This complex recognises and cleaves complementary targets via the endonuclease activity of RISC-associated Ago proteins (Koeppe et al., 2023; Mohr et al., 2014). To ease the process of designing there are some popular siRNA design tools are: siDirect, Genscript, siDESIGNCenter, Asi designer, BLOCK-iT RNAi Designer, RNAi codex, RNAi explorer, Oligowalk and RNA wizard (Fakhr & Zare, 2016).

Hairpin RNAi mediated strategy has been used to silence the overlapping AC1 / AC4 region (identified as host RNAi suppressor) of ToLCV resulting in a stable transgenesis (Ramesh et al., 2007). Single phasiRNA (phased siRNA) designed to target the AC4 and AC2 regions of ToLCNDV and Tomato leaf curl Gujarat virus (ToLCGV) with equal efficiency as both viruses show 96 per cent sequence identity (A. Singh et al., 2019).

3.5. Artificial microRNAs (amiR)

Overexpressing plant microRNAs (miRNAs) with high sequencing complementarity to virus ORFs has been an effective approach to disease resistance. According to some studies, overexpression of host miRNAs have no negative effects on the morphology or growth of

transformed plants (Akmal et al., 2017). The multiplication and spread of geminiviruses in host plants can be reduced by artificial microRNAs (amiR). AmiRNAs have been engineered to target viral genes that are successfully immune to antiviral drugs (Shweta et al., 2018). ToLCNDV highly resistant tomatoes were generated using artificial micro RNAs against AV1/AV2 overlapping region (Vu et al., 2013). Artificial amiR has also been designed to target the AC1 ATP binding domain in tomatoes that confers resistance to ToLCV(Sharma & Prasad, 2020). Tomato lines developed immunity against the ToLCNDV following the suppression of AC2 or AC4 genes by amiRNA(Shweta et al., 2018).

CONSTRAINTS IN THE DEVELOPMENT OF VIRUS RESISTANT PLANTS

Insecticides are used to control B. tabaciin various crops and is often the primary means of control. However, in addition to adverse effects on human health and the environment, the frequent use of the same types of insecticides can contribute to resistance selection in insects and may also decrease the number of beneficial insects that are natural enemies of white flies (Leke et al., 2015). Moreover, very few resistance genes (R genes) have been identified, restricting the use of breeding for resistance to viruses. It is difficult to breed resistance genes because there is often a correlation between the locus of resistance and genes associated with poor fruit quality (Z. Ali et al., 2015). Conventional breeding strategies are time-consuming, costly, labor-intensive and often unsuccessful, especially when viral variants are rapidly mutating or evolving (Z. Ali et al., 2019). By conventional breeding, a number of ToLCV resistant tomato varieties have been produced, but they have not been able to produce a stable resistant variety against ToLCV(R. K. Singh et al., 2019). The RNAi phenomenon involves

the cleavage of dsRNA by Dicer like proteins to produce siRNA, which then guides the silencing of the target sequence by the RNA-induced silencing complex (RISC). However, different siRNA products may be produced which may target other endogenous RNAs that produce OTEs (Sharma & Prasad, 2020). Moreover, the host RNAi suppression genes are present in the virus (Mehta et al., 2019; Vinoth Kumar et al., 2017). An efficient, stable and robust technique is required to generate biotic and abiotic stress resistant varieties. This could be achieved by using various genome editing techniques like Zinc Finger Nucleases (ZFNs), Transcription Activator-like Effector Nuclease (TALENs) and Clustered regularly interspaced short palindromic repeats and associated proteins (CRISPR/Cas). Among them CRISPR/Cas9 is the most efficient and popular technique for crop improvement.

3.6. CRISPR/Cas9 induced gene silencing

The discovery of CRISPR/Cas technique has revolutionized the genome editing field for crop improvement. It is possible to build sgRNAs against any sequence followed by the adjacent protospacer motif (PAM). CRISPR/Cas systems are an adaptive immune system that confers resistance to phages and invasive nucleic acids in archeae and bacteria (F. Zhang et al., 2023). The CRISPR/Cas9 system is a type II system composed of mature CRISPR RNA (crRNA) and trans activating crRNA (tracrRNA) derived from *Streptococcus pyogenes.*The Cas9 endonuclease is directed by this gRNA (tracrRNA with crRNA) to bind and then cleave the specific target sequence(U. Basu et al., 2023; F. Zhang et al., 2023). SpCas9 'DNA targeting is based on the 20-nucleotide long spacer and the PAM 5’-NGG (N- represents any nucleotide (Pickar-oliver et al., 2020; Prajapati, 2021). At the target site, Cas9 produces DSBs that can be repaired by error-prone NHEJ or donor-dependent HDR pathway. CRISPR/Cas was primarily used in plants for gene knockout or replacement, but it is now

used for transcriptional regulation due to its specificity and simple design procedure (Shrestha et al., 2018). For example, for transcriptional regulation, dCas9 is used. In the Cas9 RuvC catalytic domain, the aspartate-to-alanine (D10A) mutation induces dCas9 or Cas9 nickase (Cas9n) to cleave target DNA to generate single stranded breaks (Hou et al., 2023; Ijaz et al., 2023). To increase target gene activation or repression, dCas9 was combined with activation domains (VP16, VP64) or repression domains (kruppel-associated box) (Jeong et al., 2023; Shrestha et al., 2018). Various CRISPR/Cas systems for cleaving the target sequence have been described. Both linear and circular single-stranded DNA molecules (ssDNA) can be attacked by CRISPR/Cas12a or Cpf1 proteins (Hillary & Ceasar, 2023). CRISPR/Cas13 is an RNA guided ribonuclease used against RNA viruses (Wang et al., 2023). Some popular CRISPR design tools are: CRISPR design, E-Crisp, CRISPR-P, Cas-OFFinder, CROP-IT, CRISPOR, CRISPick, RGEN Cas Designer and CHOPCHOP (Hanna & Doench, 2020; X. Zhang et al., 2015).

Transmission of Cas9 protein-gRNA ribonucleoproteins (RNPs) to hosts rather than plasmids encoding these genes is the current attraction for crop improvement (Woo et al., 2015). CRISPR / Cas9 could lead to the evolution of escape mutants if sgRNA is designed for coding regions, such a system generates Geminivirus mutants that could escape the CRISPR/Cas9 system (Mehta et al., 2019; Roy et al., 2019). For example, when ACMV (African Cassava Mosaic virus) was targeted using CRISPR / Cas9 overlapping coding sequences of AC2 and AC3, it resulted in the emergence of resistant CRISPR / Cas9 virus (Mehta et al., 2019). However, this can be solved by plant-virus genome editing based on multiplexed gRNA (Roy et al., 2019).

The successful multiplexed gRNA used to simultaneously target the overlapping regions of V1/V2 and C1/C4 against Chili leaf curl virus (ChiLCV) resulted in the highest level of inhibition of viruses without the formation of escape mutants (Roy et al., 2019). Three

separate sgRNAs designed for targeting CP, RCR II motif of TYLCV Rep and IR and observed that there was a substantial reduction in viral load for sgRNA targeting IR (Z. Ali et al., 2015). Single IR gRNA transferred to Cas9-over expressing (Cas-OE) *Nicotiana benthamiana* lines, resulting plants showed multiple virus resistance including BCTV, TYLCV and *Merremai mosaic virus*(Wu et al., 2008). CRISPR / Cas9 mediated resistance can be inherited over a multiple generation as seen in transgenic tobacco and tomato plants carrying sgRNA for CP and Rep for TYLCV (Tashkandi et al., 2017). CRISPR/Cas9 is able to simultaneously target multiple sequences of viruses (Z. Ali et al., 2015). This technique is applicable to all plant species and it can target multiple viruses (Mehta et al., 2019; F. Zhang et al., 2023). Moreover, using new sgRNAs, new evolved viral variants can be targeted and can provide broad, durable resistance against viruses (Z. Ali et al., 2015).

FUTURE DIRECTIONS

History has encountered several pandemics caused by begomoviruses in several crops like cotton, tomato. It is important that any resistance strategy built against plant viruses be designed to be sustainable in the field and not broken down in the face of a viral attack and, preferably, provide defense against different virus species or at least the full range of variants or strains of disease-causing species. We have seen that ToLCV has adopted several methods to manipulate host genes for successful infection and systemic spread, so we also need to formulate a strategy for targeting different virus genes with multiple techniques in a single system to successfully block each stage of the virus life cycle for enhanced disease resistance. These multiple approaches could include siRNA-based cleavage, viral promoter methylation, Cas13-based cleavage, Cas9-based IR targeting, and other regions.

Virus genes methylate host defense genes, thereby compromising the host defense mechanism. In order to improve the plant immune system, TALEN based engineered methylase or CRISPR-based engineered methylase could be used to methylate the virus promoter. TALEN-based engineered methylase has an advantage over CRISPR-based engineered methylase because of its small size, it is ideal and will have less transcriptional and translational load and more specificity. Unexplored technologies such as siRNA sponge and target mimics may be used to target the IR region of the virus because IR is a generation site for vsRNAs that silences the host lncRNA SlLNR1 gene, which plays a regulatory role in disease resistance and leaf morphology. Virus evolution can be contained by controlling the spread of contaminated plant parts and tools through cross border trade. For crop improvement and to contain virus evolution and spread the emphasis should be on silencing of critical genes involved in important stages of the virus life cycle using different techniques to develop a successful, fully resistant tomato variety with no risk of developing escape mutants, and resistant to a wide range of viral diseases.

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Cas9-mediated Genome Engineering.

Table 1: Techniques used to produce TLCV resistant tomato crop

S.No.	Target Gene	Technique Used	Observations	References
1.	LeCTR1	Virus induced gene silencing	Enhanced resistance to TLCV and Downy Mildew resistance	(Chandan et al., 2019)
2.	TLCV-CP	Agrobacterium mediated transformation	Transgenic Pusa Ruby plants were resistant under both greenhouse and filed conditions	(Raj et al., 2005)

3.	Ty-2 and Ty-3	Marker assisted selection and introgression	Resistant to ToLCNDV and ToLCBV	(Prasanna et al., 2015)
4.	TLCV betasatellite	Agrobacterium mediated transformation	Betasatellite/split barnase construct used	(Pakniat-Jahromy et al., 2010)
5.	TLCV-V2	Antisense RNA mediated silencing	Plant growth and development was not affected	(Ali et al., 2019)
6.	TLCV-AC4	RNAi	Aberrant root development and off-target effects observed	(Praveen et al., 2010)
7.	TLCV-AV1 and overlapping regions of AV1 and AV2 (amiR-AV1-1)	Artificial microRNAs (amiRs)	amiR-AV1-1 were highly tolerant to TLCV	(Vu et al., 2013)
8.	TLCV-AC2 and TLCV-AC4	RNAi	68% tomato plants resistant to both ToLCGV and TLCV	(Singh et al., 2019)
9.	TLCV-IR and overlapping region of AC1 and AC4	RNAi	IR silencing was more effective	(Ramesh et al., 2007)
10.	TLCV-AC1	artificial	higher yield observed in	(Sharma & Prasad,

		microRNAs	transgenic plants	2020)
11.	TLCV-C2	RNA silencing (C2 hairpin)	delayed symptoms	(Bian et al., 2006)
12.	TLCV-AC1 and AV1	RNAi	in-silico analysis	(Saxena et al., 2011)

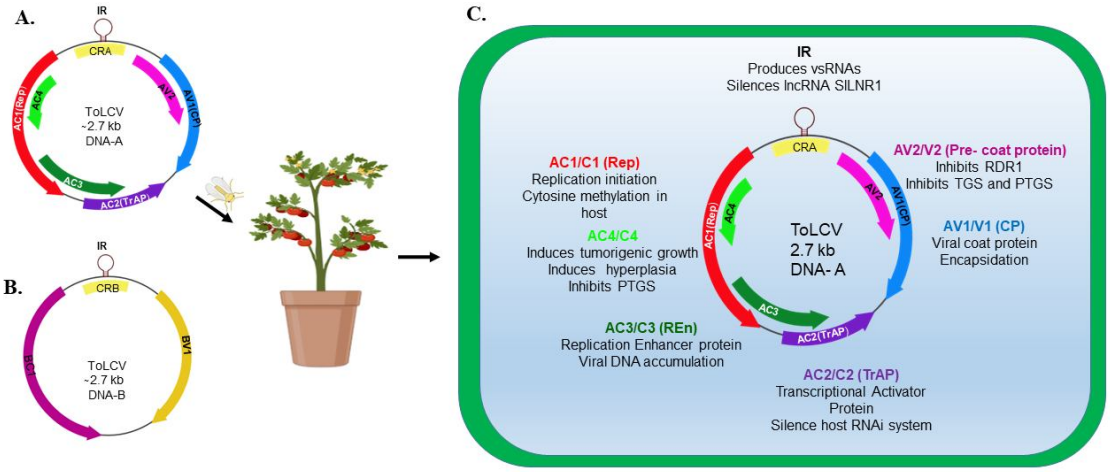

Figure 1:A. ToLCVs can be monopartite (only DNA A) or bipartite (Both DNA A and B). Four ORFs, C1 to C4 (AC1 to AC4 for bipartite begomovirus) are encoded by the complementary sense strand. The C1/AC1 encodes for replication-associated protein (Rep); C2/AC2 encodes for the transcriptional activator protein (TrAP); C3/AC3 encodes for the replication enhancer protein (REn) and the C4/AC4 protein induces tumorigenic growth

respectively. ORFs in virion sense strand viz., V1 and V2 (AV1 and AV2 for bipartite species) encodes for coat protein (CP) and precoat protein respectively. Virion sense and complementary sense reading frames (ORFs) are separated by the non-coding intergenic region (IR). Within IR, there is a common region (CR) sequence shared by bipartite begomoviruses' cognate DNA A and DNA B. **B**. The DNA-B encodes the movement protein (BC1, MP) and nuclear shuttle protein (BV1, NSP). Whenever ToLCV infected whitefly feeds on plant sap, these viruses are transferred to healthy plant leading to a disease cycle. Whitefly Bemisiatabaci vectors the virus into plants during feeding on sap. This virus enters into the plant cell and hijack plant machinery for its replication and systemic spread. **C. Functions of viral genes:** Rep, REn and TrAP are referred to as early genes as they have role in replication while CP, MP and NSP are regarded as late genes as they are needed at the later stage of infection. The Rep protein is responsible for rolling circle amplification and host cytosine methylation. TrAP stimulates the transcription of viral DNA and suppresses the silencing route of the host RNAi. REn protein plays role in enhancing viral DNA replication. REn is also responsible for viral DNA accumulation in host cells. C4 induces tumor growth and hyperplasia in infected plant cells. V2 protein suppresses both host transcriptional gene silencing and PTGS and inhibits host RDR1. CP is responsible for the encapsulation of genome into geminate particles.

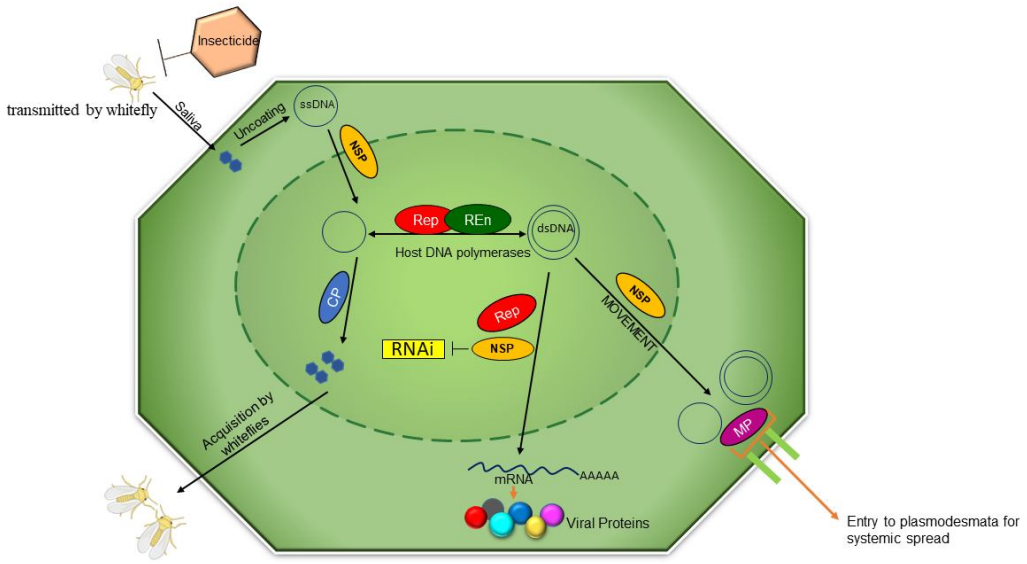

Figure 2. Life cycle of Geminivirus: Whitefly carrying the geminate particles upon uncoating releases the single stranded DNA (ssDNA) into the plant cytoplasm while feeding on plant sap. The ssDNA gets converted into double stranded DNA (dsDNA) using the host cell machinery. The viral genes are transcribed into their respective mRNAs in the nucleus with the help of nuclear protein. With the help of NSP, transcribed RNAs are exported to the cytoplasm for translation into their respective proteins. The transcribed RNAs are exported to cytoplasm with the help of NSP for translation into their respective proteins. The viral DNA can enter nearby cells via the plasmodesmatal channels with the help of movement protein, allowing for systemic infection and spread. CP encapsulates the ssDNA, which can re-enter the whitefly when it feeds on the infected plant, thereby initiating the subsequent cycle.