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CRISPR/Cas9-Mediated Resistance Strategies Against Tomato Leaf Curl New Delhi Virus (ToLCNDV) under Thermal Stress Conditions

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ABSTRACT

Tomato leaf curl New Delhi virus (ToLCNDV) is a bipartite begomovirus that has spread from the Indian subcontinent into South and Southeast Asia, the Mediterranean Basin and North Africa. Growers have few tools left. Insecticidal control of the whitefly vector is failing, and the introgressed Ty loci were selected against a different virus and break down at high temperature. CRISPR/Cas editing offers another route, because the virus replicates through a double-stranded DNA intermediate in the nucleus and can be cut directly. Proof of concept in tomato was published in 2025.

Two problems separate that proof of concept from a deployable line. The first is temperature. Almost every published geminivirus editing experiment ran at 22–25 °C, while the epidemics these constructs must survive occur at 32–38 °C. Temperature acts on the system through at least four channels that do not point the same way. SpCas9 cuts roughly five times more efficiently at 37 °C than at 22 °C. Salicylic-acid-dependent immunity collapses above 28 °C through thermosensitive transcription of CBP60g and SARD1. Virus-derived siRNA accumulation rises between 25 °C and 30 °C. Vector pressure climbs with warming. The second problem is sequence diversity, which we measured directly.

We assembled 88 complete DNA-A and 77 complete DNA-B sequences from GenBank spanning seven countries and eleven host genera, and scanned every NGG-flanked protospacer in both components. None of the 506 candidate sites matched all isolates exactly. Thirteen of 274 DNA-A protospacers reached 90% seed-plus-PAM

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conservation; no DNA-B protospacer did, the best reaching 83%. The non-coding intergenic and common regions, which the geminivirus editing literature recommends as the most escape-resistant targets, proved to be the least conserved parts of both components at 88.7% and 79.0% mean identity. Guide design for this virus therefore runs into a conflict between durability and coverage that has not been quantified before.

We report five DNA-A target sites above 93% exact conservation, show that no comparable DNA-B site exists in the current sequence record, and set out a three-regime thermal evaluation protocol with defined pass criteria. Constructs validated at 25 °C are tested under conditions that flatter them, and the field durability of every published geminivirus editing construct remains unknown for that reason.

Keywords: Begomovirus; Genome Editing; Guide RNA Design; Heat Stress; Plant Immunity; Resistance Durability; Sequence Conservation; Solanum Lycopersicum

Introduction

ToLCNDV was first described from tomato in India in 1995. It has since been reported from more than twenty countries, moved from solanaceous hosts into cucurbits, reached Spain in 2012 and spread across the Mediterranean Basin, and caused a recent Chinese outbreak costing about US$15 million over 650 hectares. Yield reductions in tomato and cucurbits range from severe to total depending on crop stage and cultivar. The European Food Safety Authority categorised it as a regulated pest in 2020.

Every control option is under pressure. Whitefly populations rebuild quickly in warm weather and have accumulated insecticide resistance. Screening and exclusion cost more than most growers in the affected regions can carry. Genetic resistance is thin. The Ty loci were introgressed from wild Solanum species against tomato yellow leaf curl virus rather than against ToLCNDV, they confer tolerance more often than immunity, and Koeda and Kitawaki showed in 2024 that Ty-1 fails at high temperature. In their experiments, Ty-1-bearing cultivars infected with a virulent TYLCV strain developed symptoms almost as severe as susceptible cultivars, with viral DNA content tracking symptom severity. A milder strain produced no breakdown, so the failure depends on heat combined with virulence rather than on heat alone.

CRISPR/Cas editing is attractive here for a structural reason. Geminiviruses replicate through a double-stranded DNA intermediate inside the nucleus, in the same compartment where a nuclear-localised Cas9 works, so the nuclease acts on the viral genome directly. Transgenic plants expressing Cas9 with guides against viral sequences have shown reduced accumulation and milder symptoms across several geminiviruses, and Ali and colleagues reported Cas9-mediated interference with ToLCNDV in tomato in 2025, with relative viral titres falling to 0.25 by nine days after co-infiltration.

Two things are missing from that literature. Temperature is the first. Published geminivirus editing work was done under controlled moderate conditions, and many papers do not state the temperature at all, yet this is a hot-season disease of hot regions. The second gap is quantitative: nobody has measured how much sequence

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variation a ToLCNDV guide RNA actually has to cover. The virus has diversified into several strain groups with frequent recombination, and guide design has so far proceeded from single reference genomes.

This paper addresses both. We analysed 165 complete genome components from GenBank to measure conservation across the ToLCNDV population and to find which target sites survive that variation. We then combined those results with the published temperature literature to build a model of how warming shifts the balance between nuclease activity, host immunity and viral escape, and we specify the experiment that would test it.

Genome architecture and points of intervention

A bipartite genome with an unequal division of labour

ToLCNDV carries two circular single-stranded DNA components of about 2.7 kb each (Figure 1). DNA-A holds the whole replication and expression apparatus: AC1 encodes the replication-associated protein (Rep) that initiates rolling-circle replication, AC2 the transcriptional activator (TrAP), AC3 the replication enhancer (REn), AC4 a pathogenicity and silencing-suppression determinant, and the virion-sense AV1 and AV2 frames encode the coat protein and its precursor. DNA-B holds two genes only, BV1 for the nuclear shuttle protein and BC1 for the movement protein, and it cannot replicate without Rep supplied in trans from DNA-A.

ToLCNDV retains an AV2 frame, unusual for a bipartite begomovirus and more typical of Old World monopartite viruses. Both components share a common region of several hundred nucleotides carrying the conserved TAATATTAC nonanucleotide at the tip of a stem-loop, together with the iterated Rep-binding motifs that set replication specificity. That region is non-coding, shared and functionally indispensable, which is why the editing literature has treated it as the preferred target. Section 5 shows why that recommendation does not survive contact with the sequence data.

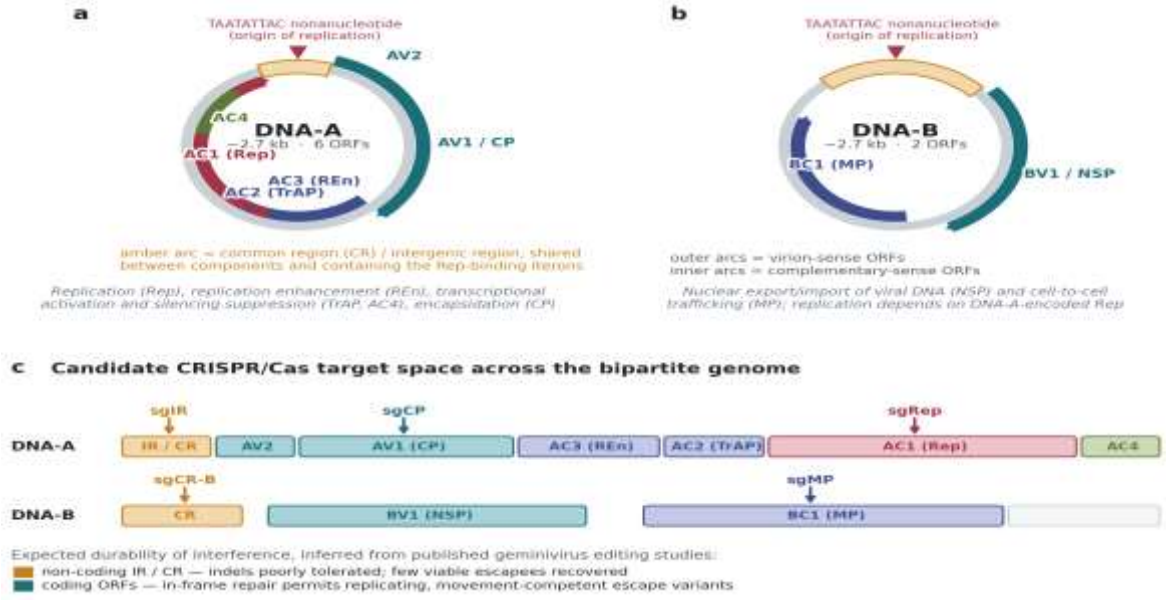


Figure 1. Genome architecture of ToLCNDV and the candidate target space for

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CRISPR/Cas intervention. (a, b) Circular maps of DNA-A and DNA-B, with virion-sense open reading frames outside the ring and complementary-sense frames inside. The amber arc marks the common region carrying the nonanucleotide origin and the Rep-binding iterons. Coordinates are representative rather than isolate-specific. (c) The same information drawn linearly, with candidate guide RNA positions marked.

Replication produces an editable double-stranded intermediate

Figure 2 traces the cycle. Viruliferous whiteflies deposit virions into phloem during feeding. The single-stranded genome is uncoated, imported into the nucleus, and converted by host polymerases into a double-stranded replicative form. Rep nicks the nonanucleotide and drives rolling-circle amplification, producing further replicative forms and progeny single strands for encapsidation. Transcription from the double-stranded template yields coat protein, silencing suppressors and movement machinery, and BV1 and BC1 carry the genome out of the nucleus and through plasmodesmata into the phloem.

Two features of this cycle matter for editing. The replicative form is a legitimate nuclease substrate sitting in the same compartment as a nuclear Cas9. And the cycle is amplificatory rather than integrative, with no viral reservoir in the host genome, so a nuclease destroying replicative forms faster than Rep generates them can clear an infection rather than suppress it. RNA interference cannot do that; it lowers viral load while leaving the template intact.

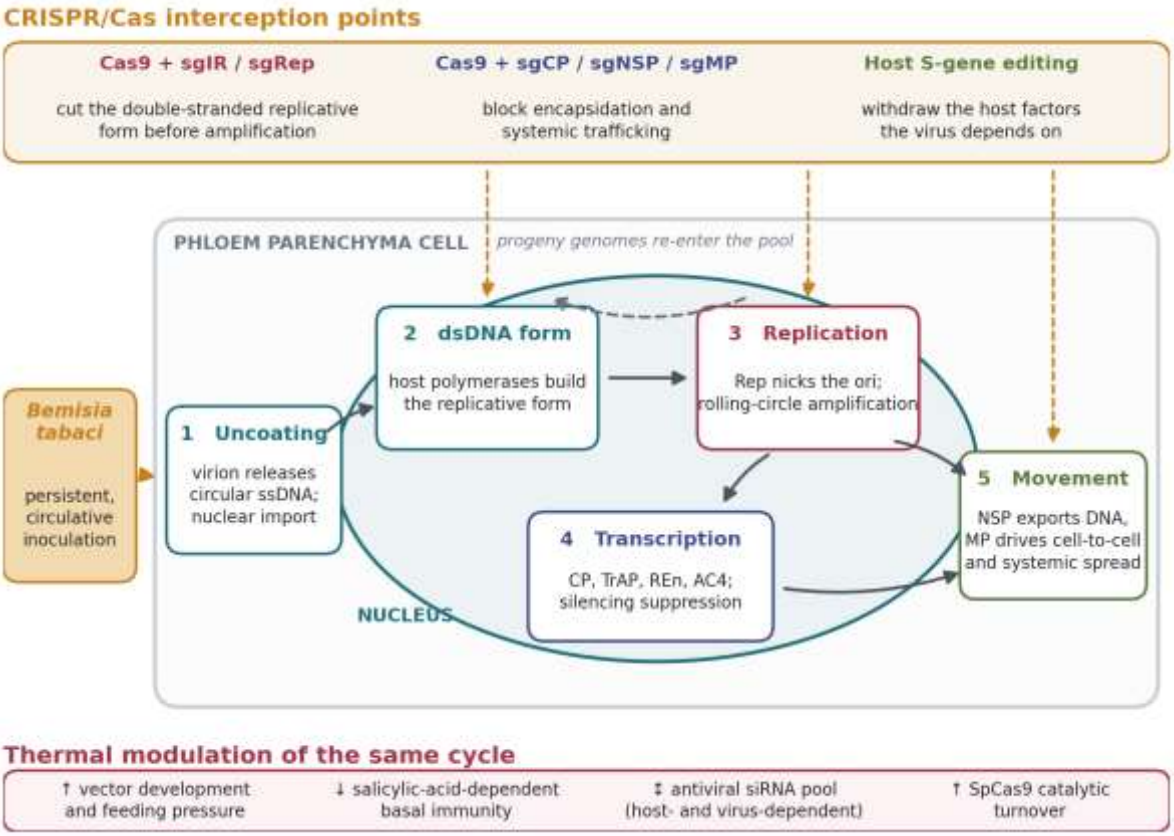


Figure 2. The ToLCNDV infection cycle, the points at which CRISPR/Cas can

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intervene, and the components of the cycle that temperature modulates. Numbered stages run from whitefly inoculation through nuclear import, replicative-form synthesis, rolling-circle amplification, transcription and systemic movement. The upper band shows where each editing strategy acts. The lower band lists the parts of the same cycle that shift with canopy temperature.

Materials and methods

Sequence retrieval and panel assembly

Complete ToLCNDV genome components were retrieved from the NCBI Nucleotide database on 31 August 2026 through the Entrez E-utilities programmatic interface. The query restricted results to the ToLCNDV taxon, to lengths between 2,500 and 2,800 nt, and to records whose titles indicated a complete genome or complete sequence; 600 records were returned. Records were assigned to DNA-A or DNA-B from the segment annotation in the record title, then length-filtered to 2,680–2,760 nt for DNA-A and 2,600–2,720 nt for DNA-B to exclude partial and misannotated entries. Duplicate isolate designations were collapsed, retaining the longest record per isolate. GenBank flat files were parsed with Biopython to extract sequence, country of origin, host and collection date, and records containing ambiguity codes were discarded. The final panel held 88 DNA-A and 77 DNA-B sequences from seven countries and eleven host genera (Table 1).

Coordinate normalisation

Geminivirus genomes are circular, and GenBank entries conventionally begin at the Rep nick site, so the TAATATTAC nonanucleotide spans the end-to-start junction instead of appearing in the linear sequence. Each record was therefore searched in its doubled form and in its reverse complement, then rotated so that position 1 is the first base 3′ of the nick. All coordinates reported here follow that convention.

Conservation analysis

For each component an Indian isolate of modal length served as the reference: PX996129.2 for DNA-A at 2,739 nt and OR678743.1 for DNA-B at 2,693 nt. Every panel member was aligned to its reference using the Biopython PairwiseAligner in global mode with match +1, mismatch −1, gap opening −6 and gap extension −0.4. Aligned blocks were projected back onto reference coordinates to give a per-position identity vector, and values were averaged across the panel. The landscapes in Figure 4 use a 60-nt sliding window computed on the circularly padded vector, so positions near the origin are not artefactually depressed. Region boundaries for the per-region means came from the CDS features of each reference record, adjusted for the rotation offset.

Protospacer scan

Every 20-nt sequence followed by an NGG protospacer-adjacent motif was enumerated on both strands of each reference, working on the doubled sequence so that sites spanning the origin were included. Low-complexity candidates containing fewer than three distinct bases were discarded. Each candidate was scored twice against the panel: by exact match of the full 20-nt protospacer, and by match of the

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12-nt PAM-proximal seed together with the GG dinucleotide, since mismatches inside the seed abolish Cas9 cleavage far more reliably than distal ones. Both searches ran against the doubled sequence and the reverse complement of every isolate. The scan produced 274 candidates in DNA-A and 232 in DNA-B.

Diversity structure

Pairwise distances were computed with an alignment-free 12-mer Jaccard method and converted to Mash distances, which avoids the cost of all-against-all global alignment while tracking nucleotide divergence closely at this level of similarity. Principal coordinates analysis was performed on the resulting matrix by double centring and eigendecomposition. Hierarchical clustering used average linkage (UPGMA).

Software

Analyses were carried out in Python 3.12 with Biopython for sequence handling and pairwise alignment, NumPy 2.4 for numerical work, SciPy 1.17 for distance and clustering routines, and Matplotlib 3.10 for figures. No proprietary software was used. Analysis scripts and the full accession list are available from the corresponding author.

Table 1. Composition of the ToLCNDV sequence panel. Assembled from GenBank on 31 August 2026. Host genera are those recorded in the source annotation.

Attribute	DNA-A	DNA-B	Notes
Complete sequences analysed	88	77	After length filtering and removal of duplicate isolate designations
Reference accession	PX996129.2	OR678743.1	Indian isolates of modal length for each component
Reference length (nt)	2,739	2,693	Numbered from the Rep nick site
Countries represented	7	4	India, China, France, Pakistan, Bangladesh, Indonesia, Malaysia
Mean identity to reference	92.5%	84.3%	DNA-B is substantially more variable, consistent with its distinct evolutionary history
Mean pairwise divergence	6.2%	6.7%	Mash distance on 12-mers; maxima 14.2% and 19.6%
NGG protospacers scanned	274	232	Both strands, low-complexity sites excluded

Results of the sequence analysis

The panel is structured by geography, not by host

Principal coordinates analysis of the DNA-A distance matrix separates the panel

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along one dominant axis carrying 58% of the variation (Figure 3a). The 24 French isolates form a tight cluster at one extreme, consistent with the Mediterranean ToLCNDV-ES lineage that entered Spain in 2012 and has since spread through southern Europe on cucurbits. Chinese and Indian isolates occupy the opposite side of the axis, with the Chinese sequences forming a compact group and the Indian sequences spread much more widely. Pakistani isolates fall among the Indian ones, which is what a shared Indus-plain epidemic would predict.

Host of origin does not organise the data in the same way. Melon, zucchini and Luffa account for most sequences, and isolates from the same host genus appear on both sides of the first coordinate. The UPGMA tree (Figure 3d) shows the same picture: clades correspond to countries, not to crops. For guide design this matters because it means a construct built on European sequences will not automatically cover South Asian isolates, and vice versa.

Mean pairwise divergence is 6.2% for DNA-A and 6.7% for DNA-B, with maxima of 14.2% and 19.6%. At a 12-mer clustering threshold of 2% the panel resolves into 28 DNA-A and 22 DNA-B groups. Any single reference genome therefore represents a small fraction of the diversity a deployed construct would meet.

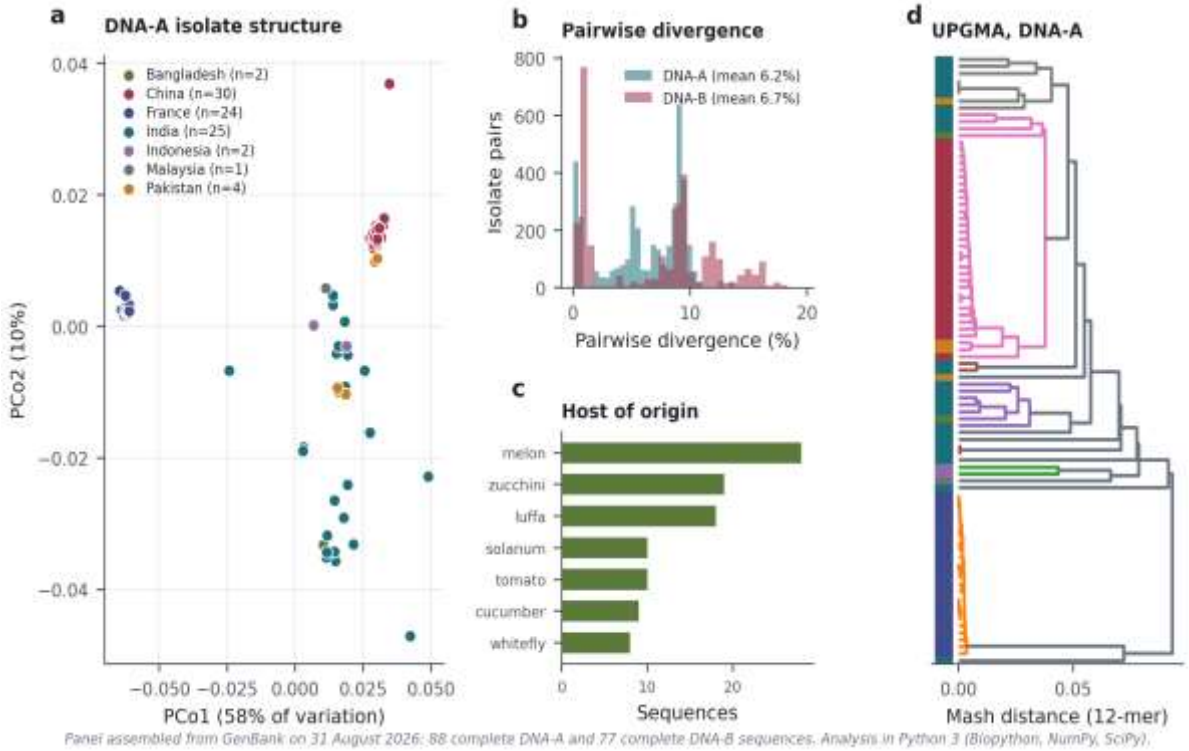


Figure 3. Diversity structure of the ToLCNDV sequence panel. (a) Principal coordinates analysis of DNA-A Mash distances, coloured by country of origin. (b) Distribution of pairwise divergence within each component. (c) Host genera represented across both components. (d) UPGMA tree of DNA-A with a country colour strip at the leaves. The first coordinate separates Mediterranean isolates from Asian isolates and carries 58% of the variation.

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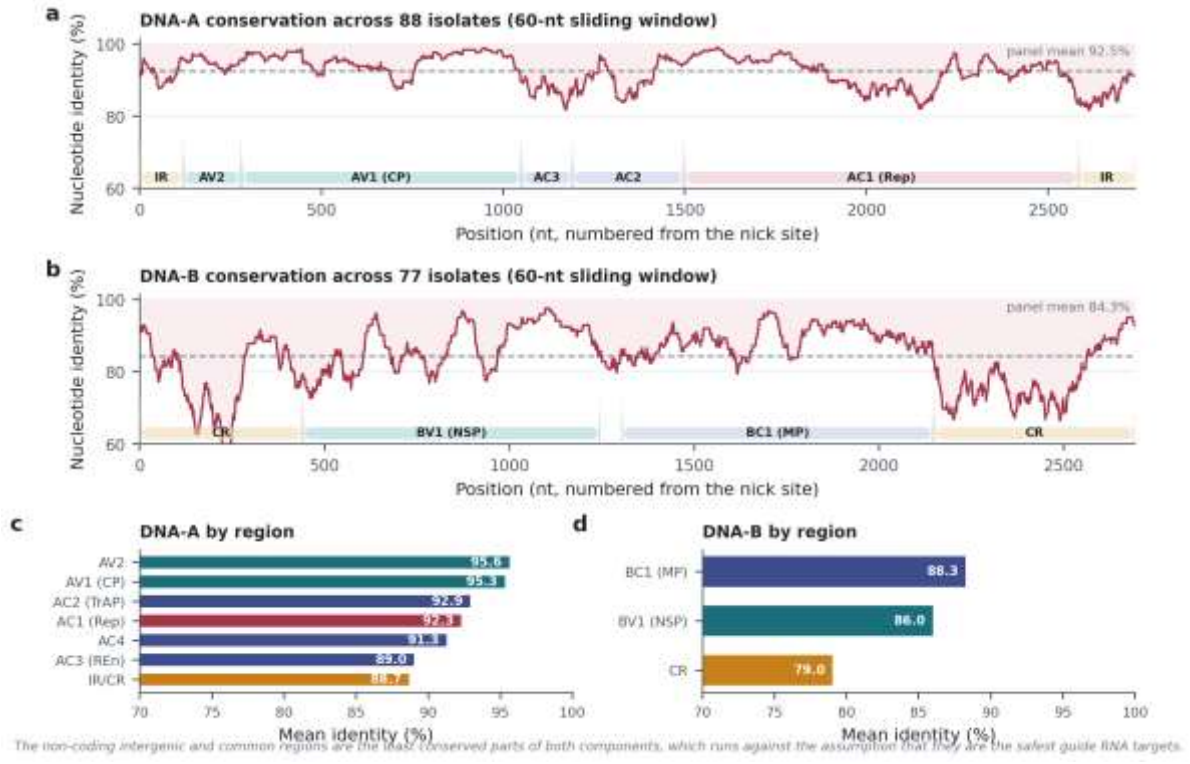
The non-coding regions are the least conserved, not the most

Mean identity to the reference is 92.5% for DNA-A and 84.3% for DNA-B across the panel. Figure 4 resolves that figure along the genome. The DNA-A profile is uneven, with conserved plateaus above 95% through most of AV1 and troughs below 85% in AC3 and in parts of Rep. DNA-B is more variable throughout and drops below 75% across long stretches of the common region.

Ranked by region, DNA-A conservation runs AV2 at 95.6%, AV1 at 95.3%, AC2 at 92.9%, AC1 at 92.3%, AC4 at 91.3%, AC3 at 89.0% and the intergenic region last at 88.7%. In DNA-B the order is BC1 at 88.3%, BV1 at 86.0% and the common region last at 79.0%. The non-coding region is the least conserved part of both components.

This result cuts against the standing recommendation in the field. Ali and colleagues showed that guides directed at the intergenic region produce fewer viable escape variants than guides directed at coding sequences, because the region has no reading frame to preserve and carries elements that cannot tolerate disruption. That finding concerns what happens after a cut. It says nothing about whether a guide will recognise its target in the first place. Our data show that the same region is where isolates differ most, so an intergenic guide designed on one isolate is the guide least likely to match another. The durability advantage and the coverage penalty pull in opposite directions, and both have to be priced into a design.

The likely explanation is that the constraint on the common region is structural rather than sequence-level. What Rep requires is a stem-loop that folds and iterons it can bind, and both tolerate compensatory substitution. Iteron sequences differ between begomovirus species precisely because they are a specificity determinant. A region can therefore be functionally indispensable and still be poorly conserved at the nucleotide level, which is exactly the combination that defeats a fixed 20-nt guide.



The non-coding intergenic and common regions are the least conserved parts of both components, which runs against the assumption that they are the safest guide RNA targets.

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Figure 4. Nucleotide conservation across the ToLCNDV genome. (a, b) Mean identity to the reference in a 60-nt sliding window computed on circularly padded data, with the open reading frame track below each profile. (c, d) Mean identity by region, ranked. The intergenic and common regions sit at the bottom of both rankings, at 88.7% for DNA-A and 79.0% for DNA-B.

No protospacer is universally conserved, and DNA-B has no good target at all

Of 506 NGG-flanked protospacers scanned across both components, none matched every isolate in the panel exactly (Figure 5). In DNA-A, 8 of 274 candidates exceeded 95% exact conservation and 13 reached 90% seed-plus-PAM conservation. In DNA-B, none of the 232 candidates reached 90% on either measure; the best seed conservation was 83.1% and the best exact conservation 9.1%.

Table 2 lists the strongest candidates. The best DNA-A site sits at position 2306 on the complementary strand inside AC1 (Rep), with 98.9% exact and 98.9% seed conservation and 40% GC. A second site at 1432 spans the AC2 region with 95.5% exact and 100% seed conservation. Two sites within AV1 exceed 93% exact conservation. All of the top candidates fall inside coding sequences, which is the opposite of what the escape literature would recommend.

The DNA-B result is the more consequential one. A bipartite virus is not cleared by a construct that only cuts DNA-A, and DNA-B replication depends on Rep supplied from DNA-A, so the two components move together in an epidemic. Yet the current sequence record contains no DNA-B site that a single guide could cover across the panel. The best available option, at position 1075 in BV1, would fail to recognise its target in roughly one isolate in six even allowing seed-only matching. Any DNA-B coverage will therefore need either two or three redundant guides against the same region, or guides selected against a defined regional isolate set rather than against the species as a whole.

Table 2. Highest-ranking candidate protospacers identified by the panel scan. Positions are numbered from the Rep nick site on the reference for each component. Exact conservation is the proportion of panel isolates carrying the full 20-nt protospacer on either strand; seed conservation uses the 12-nt PAM-proximal window plus the GG dinucleotide. Host off-target screening against the tomato genome has not been performed and is required before any of these are used.

Comp.	Position	Region	Protospacer (5′→3′)	PAM	Exact	Seed	GC
DNA-A	2306 (−)	AC1 (Rep)	CATTTCCATCCGAACATTCA	GGG	98.9%	98.9%	40%
DNA-A	1432 (−)	AC2 (TrAP)	GATCACGGATTCACGCACAG	GGG	95.5%	100%	55%
DNA-A	435 (−)	AV1 (CP)	CTATACATTCTGTACATTCT	GGG	94.3%	93.2%	30%
DNA-A	377 (−)	AV1 (CP)	CTTTTGTGACGCGGGCAATG	GGG	93.2%	97.7%	55%

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Comp.	Position	Region	Protospacer (5′→3′)	PAM	Exact	Seed	GC
DNA-A	1605 (−)	AC1 (Rep)	AAATGCAGCACTGAAGCAGT	GGG	54.5%	95.5%	45%
DNA-B	1075 (−)	BV1 (NSP)	CCCAAGTTGTGTAAGTTCTT	GGG	2.6%	83.1%	40%
DNA-B	1509 (−)	BC1 (MP)	AATAGGTCCGAACGAATCAT	GGG	9.1%	71.4%	40%
DNA-B	1729 (−)	BC1 (MP)	TGCACTTCTGTAAGATGCTA	GGG	6.5%	68.8%	40%

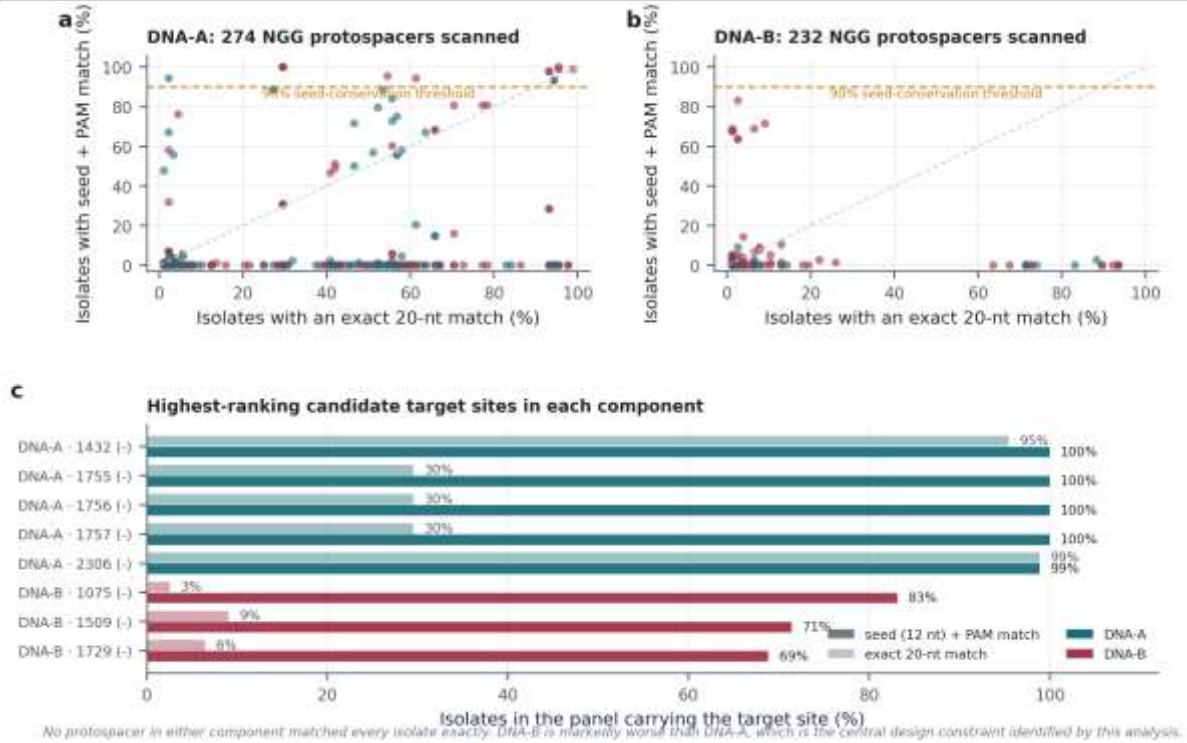


Figure 5. Conservation of candidate CRISPR target sites across the panel. (a, b) Every NGG-flanked protospacer in each component, plotted as exact 20-nt conservation against seed-plus-PAM conservation. Points above the diagonal tolerate PAM-distal mismatches. (c) The strongest candidates in each component. DNA-B has no site approaching the conservation of the best DNA-A targets, which constrains any strategy that intends to clear both components.

Thermal stress reshapes the host side of the interaction

Warmth dismantles salicylic-acid-dependent immunity

The clearest thermal effect on plant defence concerns salicylic acid. Kim and colleagues found that at 28 °C, a few degrees above standard growth temperature, Arabidopsis stops producing salicylic acid on pathogen challenge. Phytochrome B and EARLY FLOWERING 3, the canonical thermomorphogenesis regulators, are not responsible. Instead, formation of GUANYLATE BINDING PROTEIN-LIKE 3

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defence-activated condensates falls at the higher temperature, which impairs recruitment of GBPL3 and salicylic-acid-associated Mediator subunits to the CBP60g and SARD1 promoters. Those genes encode master immune transcription factors controlling ICS1, EDS5 and PBS3, so their suppression takes down salicylic-acid biosynthesis and with it both basal resistance and effector-triggered immunity.

Two aspects matter here. The threshold is low: 28 °C is an ordinary afternoon in a Punjab tomato field and sits well inside the range where ToLCNDV epidemics develop. And the loss is reversible by genetic means, since overexpression of CBP60g restores salicylic-acid biosynthesis and disease resistance at elevated temperature. That makes it a regulatory failure rather than a thermodynamic ceiling. Rossi and colleagues later showed that natural accessions differ in how strongly warmth suppresses their immunity, and that this variation does not depend on bHLH059, the thermosensitive regulator that operates at non-stress temperatures. Thermal immune resilience therefore looks heritable and selectable. For our purposes the point is narrower: a Cas9 construct deployed in a hot field works inside a plant whose baseline immunity is far weaker than the one it was validated in.

Antiviral silencing moves the other way

Chellappan and colleagues quantified virus-derived small interfering RNAs across five cassava geminivirus species in cassava and Nicotiana benthamiana. Raising the temperature from 25 °C to 30 °C increased geminivirus-induced silencing, and newly developed leaves showed milder symptoms. Viruses that normally show no recovery phenotype behaved like recovery-type viruses at the higher temperature. For recovery-type viruses the siRNA increase was moderate and the distribution along the genome did not change; for non-recovery-type viruses the increase was much larger. The authors noted that mechanisms besides silencing may also limit geminivirus accumulation at high temperature.

Between about 25 °C and 30 °C, then, the plant loses salicylic-acid immunity while gaining silencing capacity. The two curves cross somewhere inside that interval, and where they cross depends on host genotype, virus isolate, and whether the warming is sustained or intermittent. Above roughly 34 °C the silencing benefit appears to fade while the immune deficit remains. Susceptibility is therefore not a monotonic function of temperature, and a two-temperature experiment can land on either side of the inflection without the investigators knowing which.

The virus interferes with the host heat-stress response

A fourth interaction runs from virus back to host physiology. TYLCV infection weakens the heat-stress response of tomato grown at high temperature, with down-regulation of the heat shock transcription factors HSFA2 and HSFB1 and of the genes they control. The proposed mechanism is reduced nuclear translocation of HSFA2 in infected cells, with viral proteins including the coat protein binding HSFA2 directly. If something similar operates in the ToLCNDV pathosystem, virus and heat are not two stresses a plant experiences additively; the infection erodes the plant's capacity to tolerate the temperature. Table 3 summarises the direction and threshold of each channel.

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Table 3. Thermal channels acting on the ToLCNDV–tomato–Cas9 system. Directions are given from the plant's perspective. Thresholds are approximate. Most of these studies used Arabidopsis, cassava or Nicotiana benthamiana rather than tomato with ToLCNDV, and extrapolation across pathosystems is a stated limitation of this synthesis.

Channel	Threshold	Direction	Principal evidence
Salicylic-acid-dependent basal immunity	~28 °C	Against the plant	GBPL3 condensate formation reduced; CBP60g and SARD1 transcription suppressed; ICS1-mediated SA biosynthesis falls (Kim et al., 2022)
Antiviral RNA silencing	25 → 30 °C	For the plant	Geminivirus-induced silencing increases; non-recovery-type viruses behave as recovery-type (Chellappan et al., 2005)
Introgressed Ty-1 resistance	High-temperature regime	Against the plant	Symptom severity in Ty-1 cultivars approaches that of susceptible cultivars with a virulent strain (Koeda and Kitawaki, 2024)
Host heat-stress response	Heat-stress regime	Against the plant	HSFA2 and HSFB1 down-regulated in begomovirus-infected tomato; HSFA2 nuclear translocation impaired
SpCas9 catalytic activity	22 → 37 °C	For the plant	About 5-fold higher somatic and up to 100-fold higher germline mutagenesis under 37 °C treatment; SpCas9 more active in vitro at 37 °C (LeBlanc et al., 2018)
Vector population growth	Warming season	Against the plant	Whitefly development and population turnover accelerate with temperature; epidemic range expanding with climate

A thermal window model

SpCas9 is a mesophilic enzyme in a cool plant

Streptococcus pyogenes grows optimally at 37 °C and its nuclease inherits that preference. Tomato grows well between 20 °C and 27 °C, so the enzyme routinely works below its optimum. LeBlanc and colleagues measured the cost: Arabidopsis heat-treated at 37 °C showed about five-fold higher CRISPR-induced mutation frequencies in somatic tissue and up to one hundred-fold higher frequencies in the germline compared with plants held at 22 °C. The effect reproduced in Citrus, and in vitro assays confirmed that SpCas9 generates double-stranded breaks more efficiently

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at 37 °C. Both enhanced Cas9 activity and increased guide RNA levels contributed. Kurokawa and colleagues later showed that a single 24-hour treatment at 37 °C is enough to raise mutation efficiency in Arabidopsis, and similar heat-driven gains have been reported in cotton, poplar and wheat.

Those experiments were designed to solve a laboratory problem, namely how to recover more edits when making transgenic lines. Their consequence for antiviral use has not been drawn out. If Cas9 cuts several times more efficiently at 35 °C than at 25 °C, a CRISPR-based antiviral defence is intrinsically heat-activated. It gets stronger exactly as the plant's salicylic-acid-dependent immunity gives way. Whether that compensation is sufficient, partial or overwhelmed has not been measured.

Three regimes with different logic

Figure 6 assembles these responses against canopy temperature. SpCas9 activity rises steeply through the moderate warming range and plateaus near 37 °C. Salicylic-acid immunity falls sharply above 28 °C. Virus-derived siRNA accumulation rises from 25 °C to about 30 °C, then attenuates. Vector pressure and viral replication rate climb until the vector's own thermal limits bite. These are normalised conceptual curves built from published directions and thresholds, not measured ToLCNDV data, and their quantitative shape should not be relied upon.

Below 27 °C, host immunity is intact and inoculum pressure is low while Cas9 works well below optimum. Resistance measured here will look good for reasons that have little to do with the construct, which bears on how the existing literature should be read. Between 28 °C and 33 °C both the nuclease gain and the immune loss are large, and their balance sets the phenotype; this is where evaluation should concentrate. Above 34 °C, cutting is maximal, the plant has almost no defensive backup, escape variants face the strongest selection, and off-target load peaks.

The model makes a prediction that separates it from the null. If temperature affected only the plant, resistance would decay monotonically with warming; if it affected only the nuclease, resistance would improve monotonically. The model predicts neither. A well-designed multiplexed construct should hold roughly flat across the range because the two effects partly cancel, while a single-guide construct against a coding target should decay sharply, gaining the escape-selection penalty of heat without a durability reserve to absorb it. That difference is testable.

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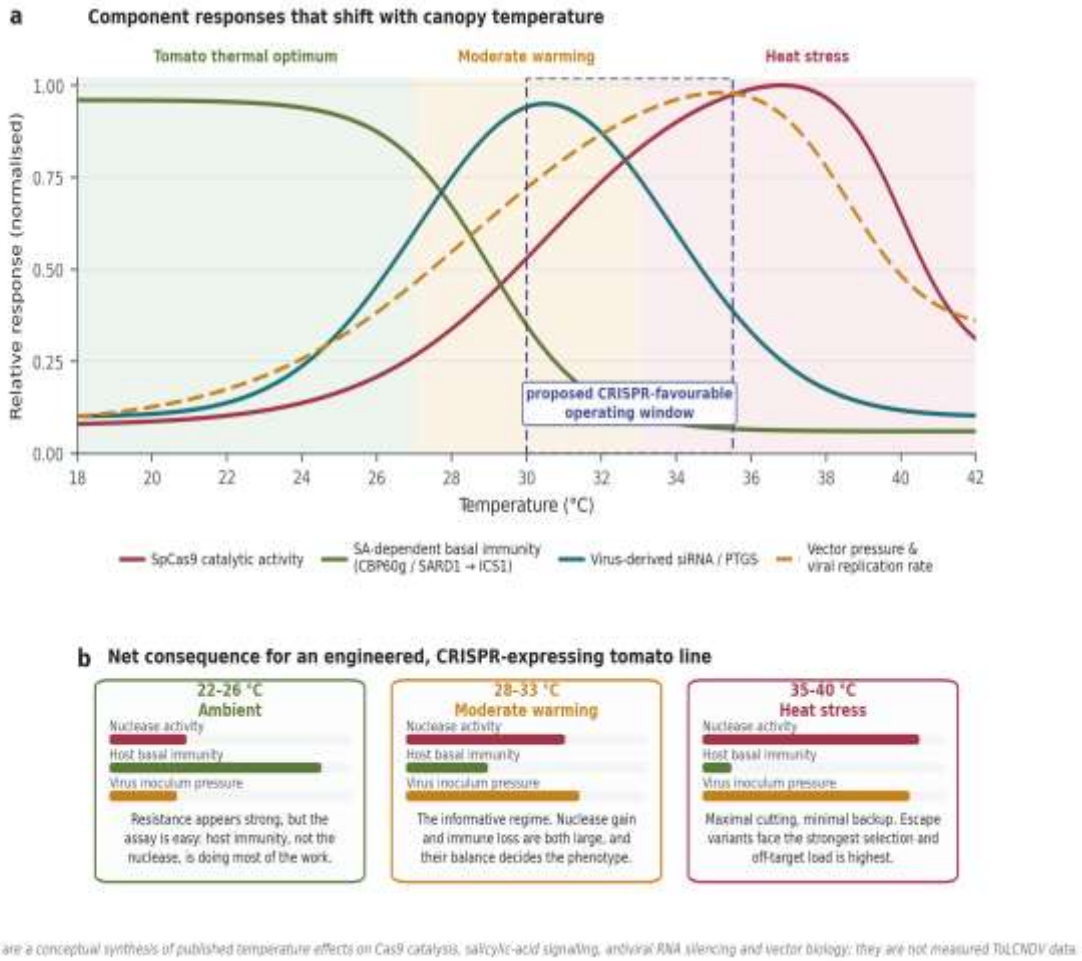


Figure 6. A thermal window model of CRISPR-mediated resistance to ToLCNDV. (a) Component responses against canopy temperature, showing that warming does not move the nuclease, host immunity, antiviral silencing and inoculum pressure in the same direction. (b) The consequence for an engineered line across three regimes. Curves and bars are a conceptual synthesis of published temperature effects rather than measured ToLCNDV data.

Heat should accelerate escape

Ali and colleagues showed that guides against coding sequences of several geminiviruses produce viral variants that still replicate and move systemically. Non-homologous end joining repairs the cut, and a fraction of the resulting indels preserve the reading frame while destroying the protospacer or its adjacent motif, leaving a virus that works but can no longer be cut. Guides against the intergenic region behaved differently: interference occurred, but mutated variants were recovered inefficiently, which limited the supply of replication- and movement-competent escapees. Figure 7a sets out the two routes.

Mehta and colleagues then demonstrated the failure mode at scale. Working with African cassava mosaic virus in cassava, CRISPR-Cas9 transgenics showed no effective resistance under glasshouse inoculation, and between 33% and 48% of

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edited viral genomes carried a conserved single-nucleotide mutation conferring resistance to cleavage. In N. benthamiana, replication of the mutant depended on wild-type virus being present. Rybicki disputed the causal claim, arguing that the mutation could have arisen spontaneously rather than having been selected by Cas9 activity. The distinction matters for interpretation but not for design: whether the nuclease creates the variant or enriches it, a single guide against a coding target applies selection to a fast-replicating population and can be expected to lose.

Two mechanisms suggest heat makes this worse. More cutting means more repair events, and escape variants come from repair. Faster viral replication at higher temperature enlarges the population in which a variant can be sampled and fixed. Against those, stronger nuclease pressure leaves fewer surviving genomes, which shrinks the effective population available for adaptation. Which dominates cannot be determined from existing data, and the projection in Figure 7b is a hypothesis rather than a finding. What is known is that Mehta's 33–48% was obtained under standard glasshouse conditions with a single guide. If temperature accelerates the process at all, the durability margin for single-guide designs is thinner in the field than the literature suggests.

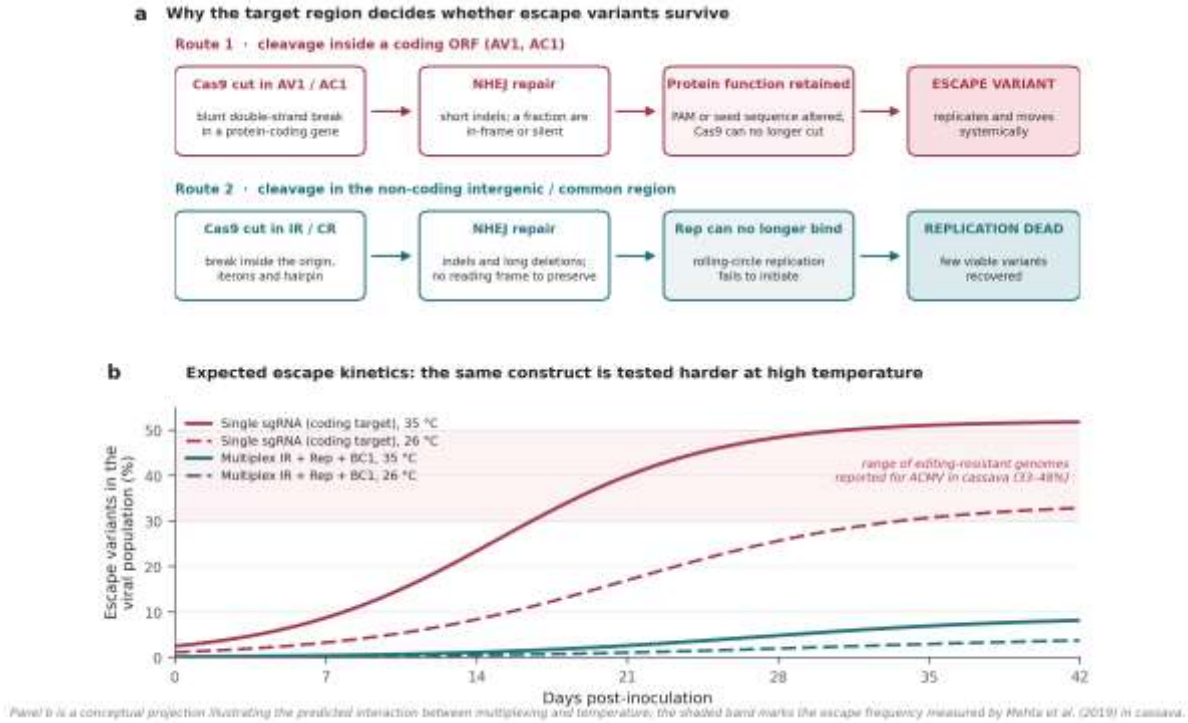


Figure 7. Target region, escape route, and the predicted interaction with temperature. (a) Cleavage inside a coding frame permits repair outcomes that preserve protein function while destroying the protospacer, producing a replication-competent escape variant. Cleavage in the non-coding region has no reading frame to preserve and disrupts elements required for replication initiation. (b) Projected escape kinetics; the curves are conceptual, and the shaded band marks the escape frequency measured by Mehta et al. (2019) in cassava.

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Off-target load and expression architecture

Heat-enhanced Cas9 activity carries a symmetrical cost. LeBlanc and colleagues reported that the regime raising on-target mutagenesis also raised off-target editing, though this was avoidable by ensuring the seed region differed from other genomic sites by at least two base pairs. Kurokawa and colleagues used a single shorter treatment partly to limit it. For a construct expressed constitutively through the life of a field crop the exposure is a whole season rather than a brief treatment, so off-target assessment carried out at 25 °C will understate the burden. Off-target sequencing under the hottest regime should be mandatory rather than optional.

One design consequence follows. If Cas9 activity, guide abundance and host immune competence all vary with temperature, constitutive expression from a 35S or ubiquitin promoter delivers a defence whose strength depends on the weather. Heat-inducible expression from a heat shock protein promoter would concentrate nuclease activity where host immunity is weakest and inoculum pressure highest, while limiting cumulative off-target exposure during cool periods. Phloem-specific expression would concentrate activity in the tissue the virus occupies. Neither has been tested for a geminivirus, and both are compatible with the multiplexed architecture proposed below.

What the two analyses imply for construct design

The sequence data and the thermal literature converge on the same conclusion from different directions, and they also create a tension that has to be resolved explicitly.

Multiplexing is required, not optional. Roy and colleagues tested this directly against chilli leaf curl virus, building nine duplex and two triplex constructs from six guide spacers. Simultaneous targeting at two or more sites eliminated the viral genome without recurrence of functional escape mutants, and duplex constructs consistently outperformed single-guide systems. The logic is combinatorial: the virus must repair every cut in a way that preserves function and destroys recognition at each site, and the probability of that falls steeply with the number of sites. Our conservation data add a second, independent reason. Since no single protospacer covers the whole panel, redundant guides are needed to maintain recognition across isolates even before escape is considered.

The choice of region has to be made against both criteria at once. The escape literature favours the intergenic region; our conservation data show it is the worst-covered region in both components. We would resolve this by pairing rather than choosing: anchor the construct on the two well-conserved DNA-A coding sites at positions 2306 and 1432, which reach 98.9% and 95.5% exact conservation, and add an intergenic guide designed against a regional isolate panel to supply the escape resistance that coding targets lack. That combination accepts a regionally restricted guide in exchange for a durability property no coding target provides.

DNA-B remains the weak point and should be acknowledged as such rather than designed around. With no site above 83% seed conservation, covering DNA-B across the species is not currently possible with one guide. Two options exist. Redundant guides against BV1 and BC1 raise combined coverage at the cost of construct size and off-target surface. Alternatively, guides can be selected against a defined regional panel, accepting that a construct built for Punjab will not work in Almería. Given the

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geographic structure in Figure 3, the second option is more honest about what these constructs can do.

Figure 8 shows why nuclease choice interacts with all of this. Cas12a processes its own crRNA array, so multiplexing needs a single transcript rather than independent promoters for every guide. Both LbCas12a and AsCas12a are temperature-sensitive in plants and perform poorly at the low end of the plant growth range, and Malzahn and colleagues showed that high-temperature regimes are needed for efficient Cas12a editing in rice, Arabidopsis and maize. That sensitivity is inconvenient for routine editing at ambient temperature and close to ideal for a hot-season antiviral application. A Cas12a array carrying the two conserved DNA-A sites plus redundant DNA-B guides would combine easier multiplexing with a thermal optimum overlapping epidemic conditions. We regard it as the most promising untested configuration for this pathosystem. Cas13 targets RNA, so for a DNA virus it addresses transcripts while the replicative form persists; it can suppress but is unlikely to clear.

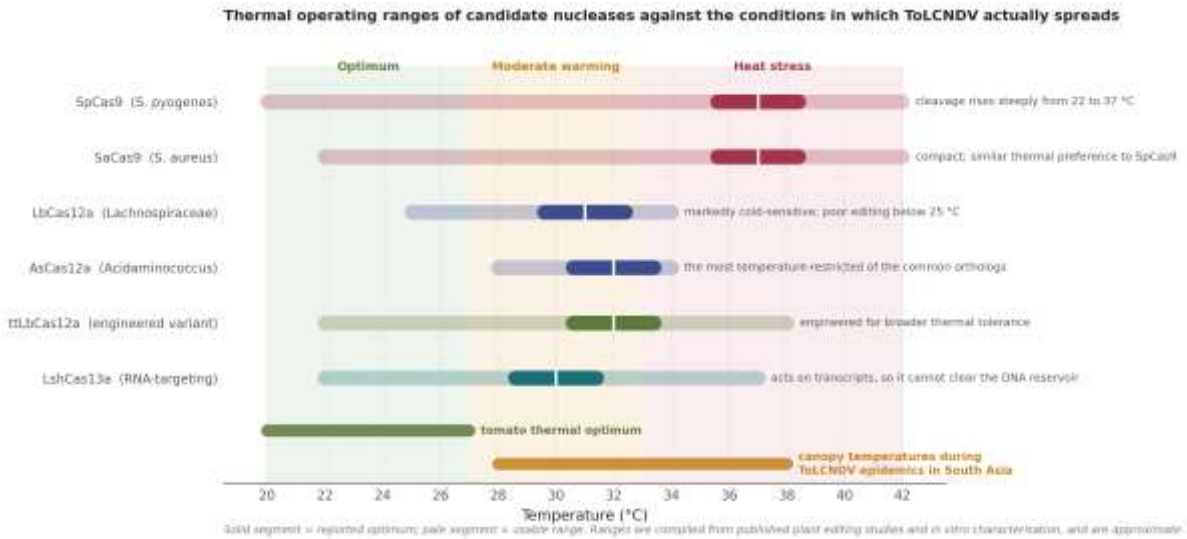


Figure 8. Thermal operating ranges of candidate nucleases set against tomato growth conditions and the canopy temperatures at which ToLCNDV epidemics occur. Solid segments mark reported optima, pale segments the usable range. Ranges are compiled from published plant editing studies and in vitro characterisation and are approximate. The gap between the conditions in which editing is usually validated and those in which the disease occurs is the practical point.

A different route is to edit the plant instead of the virus. Geminiviruses recruit host factors for replication, trafficking and defence suppression, and knocking out a susceptibility gene gives recessive resistance that the virus cannot escape by point mutation, since no target remains to mutate away from. The regulatory advantage is decisive: a susceptibility knockout can be segregated free of the editing machinery, leaving a plant carrying a small indel and no transgene, whereas a virus-targeting construct must express Cas9 and its guides continuously and stays transgenic. The cost is that susceptibility factors are host genes doing host work, and removing them often carries a yield penalty. No validated ToLCNDV susceptibility target exists in tomato yet, though transcriptomic comparison of resistant and susceptible melon

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genotypes and the mapping of the cucumber Cy-1 locus to a DFDGD-class RNA-dependent RNA polymerase both point in useful directions. Figure 9 sets out our appraisal of nine configurations against seven criteria.



Figure 9. Comparative appraisal of nine CRISPR-based configurations against seven criteria relevant to ToLCNDV. Scores are the authors' qualitative appraisal of the literature cited here, not measured values. The configurations expected to perform best carry the least published evidence, which is the gap the programme in Section 8 addresses.

A proposed experimental framework

The claims in Sections 6 and 7 are testable with a programme of ordinary size. Figure 10 sets it out in six phases and Table 4 specifies regimes and readouts. Four commitments carry the design.

Three thermal regimes rather than two. A two-temperature design cannot distinguish a monotonic relationship from the non-monotonic one predicted here, and since the siRNA response appears to invert between 30 °C and 34 °C, sampling only the ends produces an uninterpretable result. We propose day/night regimes of 26/20 °C, 32/24 °C and 38/28 °C, with the middle regime placed on the inflection rather than to either side of it.

Stable homozygous lines rather than agroinfiltration. Transient assays measure interference over days, while durability emerges over weeks, and escape frequency means nothing without a complete infection cycle. Advancing to T1 or T2 homozygous lines before challenge costs months and is not optional.

Both inoculation routes. Agroinoculation with infectious DNA-A and DNA-B clones gives dose control and reproducibility. Whitefly-mediated inoculation gives the natural route and dose, together with vector behaviour that varies with temperature in its own right. Discrepancies between the two are themselves informative.

Amplicon deep sequencing of every target site at every temperature. Titre and symptom scores describe the outcome; only sequencing describes the mechanism. Without indel spectra and escape frequency there is no way to separate a construct

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that is working from one that has been evaded, and both can produce similar intermediate titres.

Table 4. Proposed thermal regimes, readouts and pass criteria. Regimes are day/night. The criteria are deliberately strict; a configuration failing any one of them should return to design rather than proceed to field evaluation.

Regime	Day / night	Rationale	Expectation under test
R1 Ambient	26 / 20 °C	Approximates the conditions under which most published geminivirus editing work was done; the comparability baseline.	Host immunity intact, Cas9 sub-optimal. Resistance should look strong across most configurations, weak ones included.
R2 Moderate warming	32 / 24 °C	Spans the inflection where salicylic-acid immunity falls and siRNA accumulation peaks.	Configurations should begin to separate. Single-guide coding-target constructs predicted to lose ground; multiplexed constructs to hold.
R3 Heat stress	38 / 28 °C	Peak-season canopy temperatures in South Asian tomato production, and the conditions of natural epidemics.	Maximal Cas9 activity, minimal host backup. Highest escape frequency and off-target load expected here.

Readout	Timing	Purpose
Viral titre by qPCR, DNA-A and DNA-B separately	7, 14, 21, 28 dpi	Quantifies interference and shows whether one component is cleared while the other persists.
Symptom severity index, biomass, fruit yield	28 dpi and at harvest	Separates resistance from tolerance. A construct that suppresses symptoms without suppressing titre creates a symptomless inoculum reservoir.
Cas9 protein and guide RNA abundance	Each regime	Establishes whether a thermal effect on performance comes from enzyme activity or from expression level.
Amplicon deep sequencing, all target sites	14 and 28 dpi	Indel spectra and escape frequency; the only readout separating a working construct from an evaded one.

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Readout	Timing	Purpose
Off-target sequencing, ten top-ranked sites	Under R3	Assesses off-target burden under the condition that maximises it.
Target-site sequencing of the challenge isolate	Before inoculation	Confirms the guide matches the isolate used, given the conservation limits reported in Section 4.

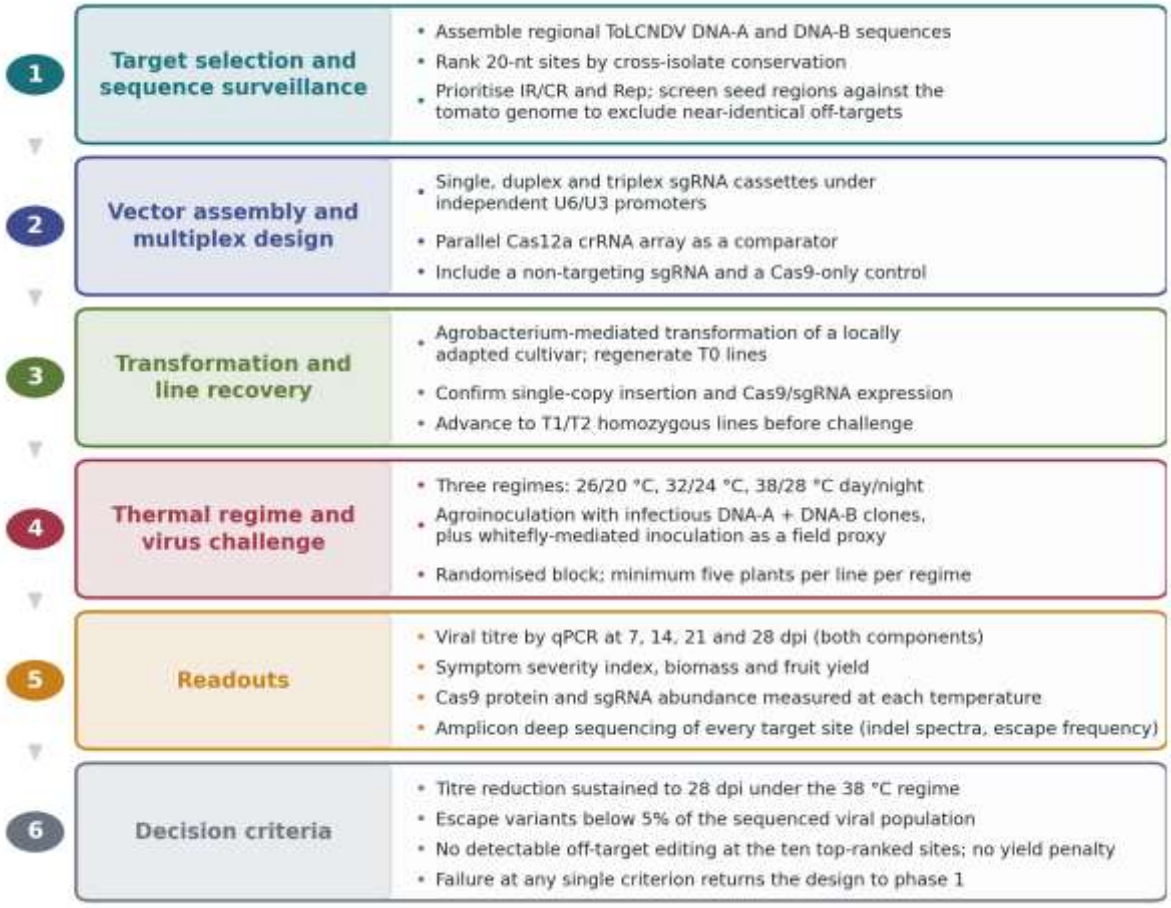


Figure 10. A six-phase framework for developing and evaluating CRISPR-based ToLCNDV resistance under defined thermal regimes. Phases are ordered by dependency, each supplying an input to the next, and the criteria in phase six return a failing design to phase one rather than to field evaluation. Phase four is what distinguishes this from published protocols: temperature is an experimental variable rather than a controlled background.

Biosafety, regulation and deployment

The two strategies discussed above sit in different regulatory positions. A virus-targeting construct must express Cas9 and its guides continuously to intercept incoming virus, so the machinery cannot be segregated away and the plant is transgenic under any framework. In much of South Asia that is a serious barrier to

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release. A susceptibility-gene knockout can be segregated free of the transgene, leaving a plant carrying only a small indel, which in jurisdictions regulating the product rather than the process may fall outside transgenic regulation entirely.

Escape variants also raise a biosafety question distinct from the agronomic one. Deploying a nuclease against a replicating viral population across a landscape applies selection at a scale no glasshouse reproduces. Mehta and colleagues framed their result as a risk attached to CRISPR-Cas9 virus immunity in eukaryotes and recommended that groups engineering virus-resistant plants sequence viral populations to detect comparable mutations. That should be treated as a minimum standard. A variant selected in a deployed crop does not stay in that crop; the vector moves it.

The most defensible deployment position is not to rely on the nuclease alone. Stacking a multiplexed construct onto Ty-based resistance and onto vector resistance, such as the acylsucrose-mediated whitefly resistance derived from Solanum pimpinellifolium, spreads the selective pressure across mechanisms with independent escape routes. That is standard resistance-gene pyramiding logic, and genome editing has no exemption from it.

Limitations

The sequence analysis inherits the biases of GenBank. Sampling is uneven, with China, India and France contributing 79 of 88 DNA-A sequences and Africa and the Middle East absent, so the conservation values are estimates for the sequenced population rather than for the species. Duplicate isolate designations were collapsed, but near-identical sequences from the same outbreak remain and will inflate apparent conservation. The protospacer scan enumerated sites present in the two reference genomes and asked whether other isolates carry them; sites present in most isolates but absent from the reference were not counted, which is a conservative bias.

Seed-plus-PAM matching is a proxy for cleavage competence, not a measurement of it. Cas9 tolerance of mismatches depends on position, identity and chromatin context, and a site scoring 95% by our criterion may still fail against particular isolates. Nothing here substitutes for testing guides against the isolates they will meet. No off-target screening against the tomato genome was performed, and that must precede any use of the sites in Table 2.

On the thermal side, the four channels in Section 5 were characterised in different systems: salicylic-acid suppression in Arabidopsis with a bacterial pathogen, geminivirus siRNA responses in cassava and N. benthamiana, Cas9 thermal sensitivity in Arabidopsis and Citrus with reporter constructs, and Ty-1 breakdown in tomato with TYLCV rather than ToLCNDV. Assembling them into one model of the ToLCNDV–tomato interaction is a reasonable inference and remains an inference. Every quantitative element of Figures 6 and 7b is conceptual, marked as such in the figures so it is not mistaken for data.

We have also treated temperature as if mean canopy temperature were the relevant variable. In a field it is not. Diurnal amplitude, time above threshold and the timing of heat episodes relative to infection all plausibly matter, and Kurokawa's finding that a single 24-hour treatment suffices to raise Cas9 activity suggests transient episodes may count as much as sustained regimes. The three-regime design does not resolve

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this, and a follow-up on episode structure would be needed. Vector thermal biology is treated only in outline, though begomovirus acquisition is known to alter whitefly thermal tolerance, adding a feedback the model does not represent.

Conclusions

CRISPR/Cas editing is a credible route to ToLCNDV resistance and the proof of concept in tomato exists. Whether the nuclease can cut the viral genome is settled; it can. What remains open is whether a construct keeps working in a diverse viral population at field temperature, and both halves of that question have gone unmeasured.

The sequence analysis answers part of it. Across 88 DNA-A and 77 DNA-B genomes, no protospacer is universally conserved, DNA-B has no site above 83% seed conservation, and the non-coding regions that the escape literature recommends are the least conserved parts of both components. Guide design for this virus is therefore a trade between durability and coverage, and single-reference design will produce constructs that fail against isolates a few hundred kilometres away.

Temperature is the other part. It acts on the nuclease, on host immunity, on antiviral silencing, on the vector and on the virus, in directions that do not agree. A construct validated at 25 °C has been tested in the regime that flatters it most: immunity intact, inoculum pressure low, selection on the virus weak. The same construct in a 35 °C field meets a plant with little salicylic-acid defence, a larger and faster-replicating virus population, and stronger selection for escape, offset by a nuclease finally working near optimum. Whether that trade is favourable is unknown.

Three recommendations follow. Multiplex across both components, anchoring on the conserved DNA-A sites at positions 2306 and 1432 and accepting regionally restricted guides for DNA-B and for the intergenic region. Evaluate across three thermal regimes spanning the inflection where host immunity fails, with amplicon sequencing at every regime rather than titre alone. Stack the construct onto existing genetic and vector resistance instead of deploying it alone. None of this requires new technology; it requires that the experiments be run against the sequence diversity and the temperatures the crop actually meets.

Declarations

Author contributions. R.M. conceived the study, designed and performed the computational analysis, developed the thermal window model and drafted the manuscript. S.S. contributed the sections on host immunity and vector biology, assembled the comparative appraisal of editing configurations, and revised the manuscript. P.H. [contribution to be specified by the author]. M.B. [contribution to be specified by the author]. All authors read and approved the final version.

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Conflict of interest. The authors declare no competing financial or non-financial interests.

Data availability. All sequences analysed are publicly available from the NCBI Nucleotide database. The reference accessions are PX996129.2 (DNA-A) and OR678743.1 (DNA-B); the complete accession list for the 165 panel sequences,

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together with the Python analysis scripts, is available from the corresponding author on request. Figures 6 and 7b are conceptual models built by the authors from the published relationships cited in the text.

Ethics statement. Not applicable. This study involved no human participants, animals, or field release of engineered organisms.

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