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Viral and Host Factors Involved in Host Gain and Host Loss by Tomato Leaf Curl Begomoviruses in Tomato and Cucumbers

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ABSTRACT

Begomoviruses transmitted by whiteflies cause severe crop losses worldwide. Individual strains or isolates have a narrower host range, but collectively begomoviruses infect a wide range of plants. Begomovirus genomes undergo frequent recombination and mutations that confer a selective advantage in interactions with specific host factors facilitating host range adaptation, resulting in the rapid emergence of new strains with adapted host range. In this study, we examined the processes by which the begomoviruses can acquire and lose hosts by exchanging fragments of the viral genomes between a variant of tomato leaf curl New Delhi virus only infecting cucumber (ToLCNDV-C), tomato leaf curl Karnataka virus only infecting tomato (ToLCKV-T), and a ToLCNDV strain infecting both tomato and cucumber (ToLCNDV-T&C). We mapped the region responsible for tomato host loss to a 63 nucleotide (nt) region in the C-terminal of the transcriptional activator/replication enhancer protein (TrAP/REN) regions of ToLCNDV. We tested known host proteins reported to interact with this region using the yeast two-hybrid approach and found divergence in interactions with host proteins PCNA and AGO1. Finally, we found that the TrAP/REN region of DNA-A in conjunction with DNA-B can confer ToLCKV-T the ability to weakly infect its non-host, cucumber, and ToLCNDV-C to infect its non-host, tomato. Our studies reveal that multiple complex intra-virus interactions between viral proteins and virus–host interactions govern infectivity, virus accumulation and symptom severity.

1 | Introduction

Tomatoes (*Solanum lycopersicum*) and cucumbers (*Cucumis sativus*) combined make up about 20% of all vegetables produced in the world (Khoury et al. 2023). They are beset by several infectious diseases, notably by various begomoviruses. The tomato yellow leaf curl disease caused an 80% loss of tomato yield in the 1970s in the Middle East (Makkouk et al. 1979). In cucumbers, incidence of mosaic and leaf curl diseases caused by begomoviruses can reach up to 100% with severe yield loss (Charoenvilaisiri et al. 2020). In Spain, yield loss of up to 22% was reported on zucchini recently (Crespo et al. 2020), and they

have been a severe threat for vegetable production around the world for over 50 years.

Begomoviruses are single-stranded DNA viruses with a circular DNA-A genome of about 2700 nucleotides (nt) in size, while some of them are bipartite in nature with an additional DNA-B segment of equal size. DNA-A encodes about six proteins: replication protein (Rep), AC4 protein, which is a small multifunction protein countering plant defences; the transcriptional activator protein (TrAP), activating expression of viral sense strands by binding to host transcription factors; replication enhancer protein (REN), which aids Rep in replication; coat protein (CP); and

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precoat protein. DNA-B produces two proteins: the movement protein (MP) and nuclear shuttle protein (NSP) (Fondong 2013). Begomovirus proteins are multifunctional with additional roles in, for example, suppression of gene silencing and hijacking host metabolism to facilitate virus replication (Devendran et al. 2022).

Based on their geographic origin, begomoviruses are divided into New World and Old World groups, which are distinct. The New World begomoviruses are all bipartite and do not have a precoat protein. Both groups can be further grouped by their region and/or predominant host (Briddon et al. 2010). Mutations to virus genomes conferring improved interactions between viral and host proteins facilitate infection, or conversely, evade interactions with host proteins, which prevents infection, leads to the acquisition or loss of hosts (Nigam 2021). Begomoviruses' reprogramming of their host cells to favour homologous recombination during their replication leads them frequently to undergo intra- and interspecies recombination (Shakir et al. 2023). This propensity for recombination, along with individual mutations, which can be as high as those found in RNA viruses (Duffy and Holmes 2009), is hypothesised to allow them to readily jump to new hosts, increasing their host range (Fiallo-Olivé and Navas-Castillo 2023). Identifying viral determinants in the expansion of host range is key to finding unique factors involved in virus resistance or susceptibility.

Previous studies have swapped domains between begomovirus variants to identify the mutation or the domain responsible for differences in properties. Abutilon mosaic virus (AbMV) preference for *Phaseolus vulgaris* (common bean) was increased when it received the TrAP/REN domain along with a fragment of the DNA-B NSP promoter from the bean-preferring bean dwarf mosaic virus (BDMV) (Levy and Czosnek 2006). Tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) IR could confer tomato pathogenicity to pepper yellow leaf curl Thailand virus (An et al. 2021). Exchanges of CP between different begomovirus strains revealed epitopes responsible for specific preference of the viruses by different whitefly genotypes (Pan et al. 2020). Another study revealed a point mutation in the movement protein could determine mechanical transmissibility in cucurbits between two related begomoviruses (Lee et al. 2020). In this study, we used two strains of tomato leaf curl New Delhi virus (ToLCNDV) and a strain of tomato leaf curl Karnataka virus (ToLCKV) with differing host specificities. ToLCNDV was initially found infecting tomato in India in 1995; it was later found also infecting cucurbit crops (Cai et al. 2023). ToLCNDV is found within a clade of cucurbit- and tomato-infecting viruses, within a larger, primarily monopartite Asia/India grouping. The other well-studied viruses in ToLCNDV's clade, squash leaf curl China virus and tomato leaf curl Palampur virus, have isolates found infecting both tomatoes and cucurbits (Heydarnejad et al. 2009; Qiu et al. 2022). Variants of ToLCNDV have also been found infecting a wide range of plants including potato (Usharani et al. 2004), chilli (Hussain et al. 2004), cotton (Zaidi et al. 2016) and cucurbits (Janssen et al. 2022). Mediterranean isolates, referred to as ToLCNDV-ES, have adapted to cucurbits and are restricted in their ability to infect tomatoes (Janssen et al. 2022). It was recently found that a single mutation in their CP is responsible for ToLCNDV-ES's inability to infect tomato. An assay revealed that ToLCNDV-ES CP had an additional

interaction with a host factor, the ring-finger protein 44-like, which could be the reason for its inability to infect tomato (Vo et al. 2023).

In this work we selected viruses, defined their cucumber/tomato host preference, and exchanged domains to find and quantify virus factors in host range preference. The viruses are referred to as ToLCNDV-C (cucurbit strain) as it infects only cucurbits but not tomato; this strain is part of the ToLCNDV-BG that has recently emerged in India infecting bitter melon. It represents a distinct strain that has a, for ToLCNDV, unique Rep protein (Renukadevi et al. 2024). ToLCNDV-T&C (tomato and cucurbit strain) was previously described as 'Jessore strain' (Maruthi et al. 2005), known to primarily infect tomatoes, but this strain also infects both cucurbits and tomato. The strain ToLCKV-T (tomato strain) is another begomovirus from India infecting tomatoes (Otti et al. 2016); phylogenetically, it is found in a clade adjacent to the ToLCNDV clade. Unlike ToLCNDV, it is monopartite, meaning DNA-A alone can cause infection without the need for DNA-B, and the virus is not phylogenetically related to cucurbit-infecting viruses.

We prepared *Agrobacterium* infectious clones of the viruses and exchanged multiple regions between these begomoviruses, revealing that a few mutations within a 63-nt region in the C-terminal of TrAP and REN determine the tomato host loss or host gain. We then tested host factors known to interact with this region using yeast-2-hybrid assay and discovered a change in the strength of interactions between TrAP/REN C-terminal and host factors known to bind to with TrAP and REN. Additionally, we found that the TrAP/REN region and DNA-B can allow ToLCKV-T to infect cucumber, although only weakly, indicating that multiple adaptations are involved.

2 | Results

2.1 | Virus Diversity, Symptom Phenotype and Host-Specificity

Full sequencing of virus clones revealed that ToLCNDV-T&C (tomato and cucurbit strain, GenBank accession: PQ468836 and PQ468837) DNA-A and -B sequences were 99.7% and 99.9% identical to GenBank accession ToLCNDV-severe[Jessore], respectively (DNA-A: AJ875157 & DNA-B: AJ875158). ToLCNDV-C (cucurbit strain, GenBank accession: PQ468834 and PQ468835) DNA-A has 97% and 98% identity to ToLCNDV-[Er-BG2] (DNA-A: MW620975) and (DNA-B: MW620976), respectively. This makes both of them isolates of ToLCNDV according to the ICTV classification (Brown et al. 2015). Despite this, ToLCNDV-C and ToLCNDV-T&C DNA-A only have 86% pairwise identity with each other; most of the differences arise from the Rep protein and intergenic region (IR), which have approximately 60% nucleotide identity. ToLCKV-T (tomato strain, GenBank accession: PQ468833) has 98% identity to ToLCKV-[pBamA4] (MH577030), establishing it as an isolate of ToLCKV. This in turn only has 72% and 69% pairwise identity to ToLCNDV-T&C and ToLCNDV-C, respectively.

Twenty days after agroinoculation of *Nicotiana benthamiana* with 2-mer infectious clones of ToLCNDV-C, ToLCNDV-T&C



FIGURE 1 | Disease incidence for *Nicotiana benthamiana*, tomato cv. Moneymaker and cucumber cv. Marketmore following agroinfiltration with wild type or recombinant clones. Disease incidence is given as number of plants that developed infections out of the total number of plant inoculation. Infection was determined 20 days post-*Agrobacterium* inoculation using qPCR. The infectious virus clones are coloured according to the sequence origin: Red: ToLCNDV-T&C, green: ToLCNDV-C, blue: ToLCKV-T. Recombinant clones are fractionally coloured corresponding to the origin of each subsegment. Clones were inoculated together with or without DNA-B segments from ToLCNDV-C or ToLCNDV-T&C. The DNA-B used is indicated with the small green or red circles, for ToLCNDV-C or ToLCNDV-T&C, respectively.

and ToLCKV-T, 10 out of 10 plants had developed symptoms and subsequent quantitative PCR (qPCR) confirmed all to be infected. In the experiments on other host plants, ToLCNDV-T&C infected a total of 15/16 tomato and 16/16 cucumber, ToLCKV-T 5/9 tomato and 0/9 cucumber and ToLCNDV-C 0/19 tomato and 8/9 cucumber (Figure 1), with infection status being determined by qPCR. In *N. benthamiana* initial symptoms resulted in characteristic curled leaves, while ToLCNDV-T&C also produced vein clearing on the new leaves whereas ToLCNDV-C did not, as the disease progressed leaf size was greatly reduced and growth stunted (Figure 2). ToLCKV-T was less symptomatic with both curling and dwarfing being less pronounced than ToLCNDV strains. On tomato, ToLCNDV-T&C produced vein clearing, which was most pronounced on the first three true leaves; all later emerging leaves were dwarfed and curled. ToLCKV-T did not show any notable visible symptoms on tomato after 20 days. Relative virus accumulation in tomato was significantly lower for ToLCKV-T than ToLCNDV-T&C ($p=0.012$, Student's *t*-test) (Figure 3). On cucumber, both ToLCNDV-C and ToLCNDV-T&C produced similar symptoms 10–14 days after inoculation; emerging leaves showed mosaic, severe dwarfing and plant

stunting. In qPCR tests, ToLCNDV-T&C had higher virus accumulation than ToLCNDV-C ($p=0.009$, Student's *t*-test).

2.2 | Phylogeographical and Host Affinities of Global Begomoviruses

To investigate the possible host-related evolution of begomoviruses, we carried out phylogeographical analyses of global begomovirus sequences. The DNA-A sequences showed several clear branches/clades (Figure 4). There is a clade of primarily bipartite viruses infecting members of the Fabaceae family and three major clades encompassing viruses of African, Asian and Eurasian origins. The large Eurasian clade encompasses a subclade that mainly has monopartite begomoviruses infecting the Malvaceae family, two further subclades of primarily monopartite viruses predominantly infecting the Solanaceae family, and a clade of primarily bipartite viruses that are rich in viruses infecting Cucurbitaceae and/or Solanaceae. Both ToLCNDV-T&C and ToLCNDV-C are found within the Eurasian Cucurbitaceae clade and ToLCKV-T within the adjacent Solanaceae clade. By

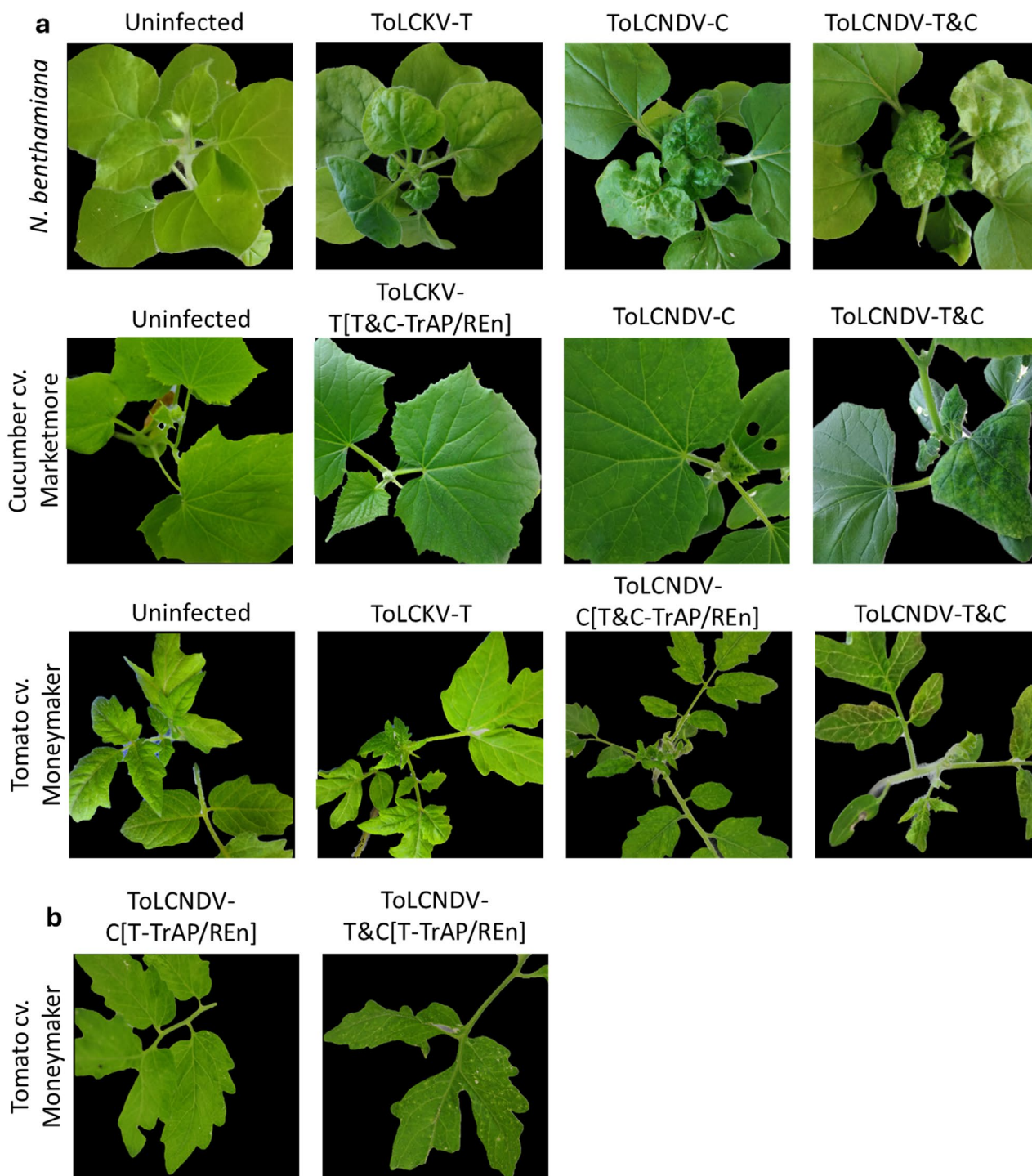


FIGURE 2 | (a) Characteristic symptoms produced by tomato leaf curl virus isolates and recombinant clones. ToLCNDV-C did not infect tomato so ToLCNDV-C[T&C-TrAP/REn] is shown instead and ToLCKV-T did not infect cucumber so ToLCKV-T[T&C-TrAP/REn] is shown instead. Symptoms on tomato plants (cv. Moneymaker) infected with either of the ToLCNDV isolates include yellowing, stunting, curling of developed leaves. Vein clearing was seen on plants infected with ToLCNDV-T&C regardless of TrAP/REn or CP swaps. Symptoms on cucumbers infected with either of the ToLCNDV isolates were mosaic spots, emerging leaves being severely deformed and cessation of plant growth. ToLCKV-T did not give any clear symptoms on tomato. Symptoms generally followed the main virus with the only exceptions being ToLCNDV-C[T-TrAP/REn] and ToLCNDV-T&C[T-TrAP/REn]. (b) ToLCNDV-C[T-TrAP/REn] infections were symptomless while ToLCNDV-T&C[T-TrAP/REn] only showed slight vein clearing symptoms and mosaic spots on lower leaves.

building the phylogenetic tree based on Rep protein sequences, it became clear that ToLCNDV-C Rep belongs to a distinct clade that is different from the clade containing ToLCNDV-T&C and ToLCKV-T. The Rep from this clade is predominantly present in

the other Eurasian Solanaceae clade, and it has seemingly, occasionally, recombined into almost all other clades (Figure S1). In our RefSeq DNA-A tree, ToLCNDV-C type Rep is found in one other virus, luffa yellow mosaic virus (GenBank accession

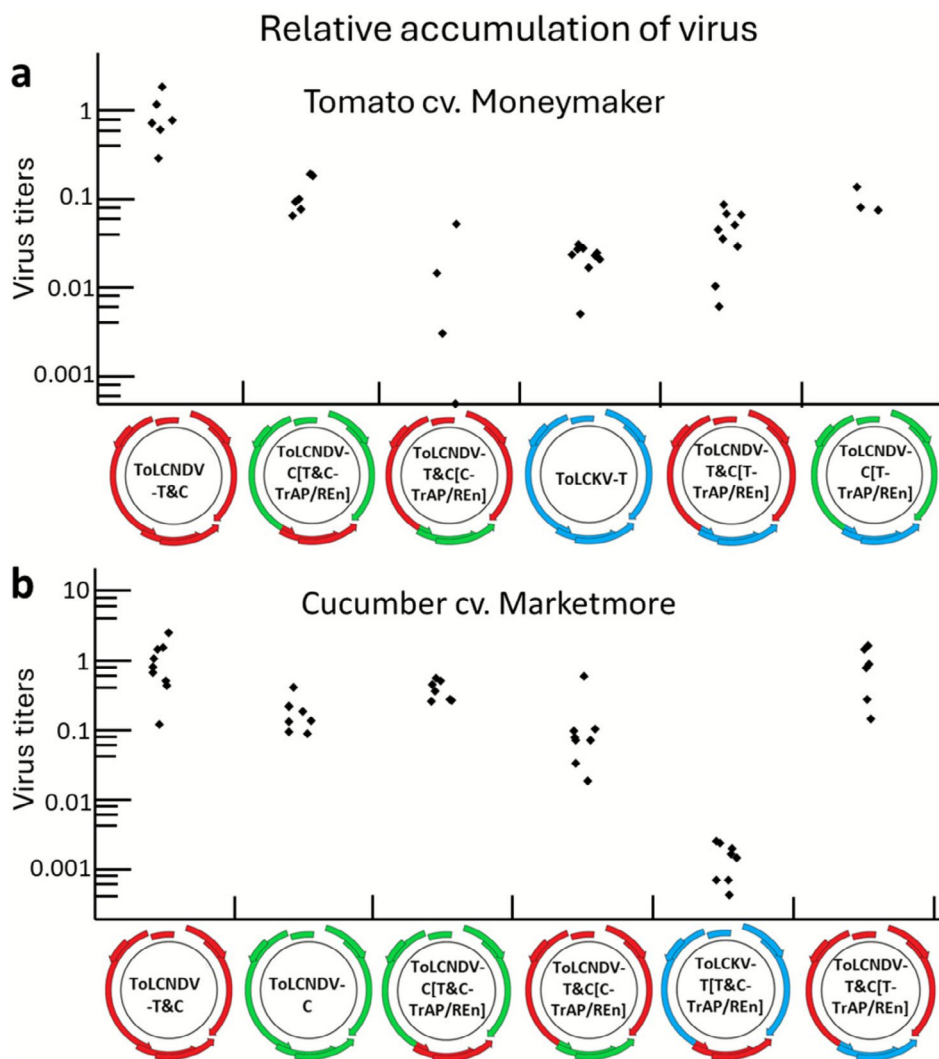


FIGURE 3 | Relative quantification of virus DNA-A in (a) tomato cv. Moneymaker and (b) cucumber cv. Marketmore. Samples were taken from systemic leaves 20 days post-infiltration and the amount of virus and plant DNA in each sample was measured by quantitative PCR. Virus levels are copies of virus divided by copies of a conserved region of plant chloroplast and mitochondrial DNA (Demesure et al. 1995). The units are arbitrary with the average virus level of ToLCNDV-T&C set as 1. The y-axis is logarithmic, and each dot represents one measurement from one plant.

NC_004824) to which it has 86% identity, within the DNA-A ToLCNDV-T&C and -C clades.

2.3 | Wild-Type DNA-A and DNA-B Swaps for Trans-Complementation

We initially exchanged DNA-A and DNA-B segments between ToLCNDV-T&C and ToLCNDV-C as well as with ToLCKV-T in co-inoculation experiments. Both strains of ToLCNDV DNA-A weakly infected *N. benthamiana* in the absence of DNA-B. However, none of these combinations infected cucumber or tomato, and only ToLCKV-T continued to infect its original host tomato plants as expected (Figure 1). The titres of ToLCNDV-T&C DNA-B in systemic leaves of *N. benthamiana* were one hundredth of ToLCKV-T DNA-A, while the ToLCNDV-C DNA-B was entirely lost in 4/4 *N. benthamiana* plants, demonstrating that their original DNA-Bs are essential for both ToLCNDV-T&C and ToLCNDV-C infections in crops. We also measured the ratio of DNA-A to DNA-B by qPCR following transient

co-infiltration in *N. benthamiana* (Figure 5). For ToLCNDV's, the non-cognate DNA-B showed a ratio around 100:1 DNA-A:DNA-B, whereas cognate DNA-Bs retained a ratio closer to 1:1. In the case of ToLCKV-T, the DNA-A:DNA-B ratio was closer to 1:1 for DNA-B of ToLCNDV-T&C while the DNA-B of ToLCNDV-C was poorly replicated, with a ratio close to 100:1, demonstrating that ToLCNDV-T&C and ToLCNDV-C DNA-As were unable to replicate each other's DNA-B, while ToLCKV-T DNA-A was unable to replicate ToLCNDV-C DNA-B well.

2.4 | Rep/AC4 Protein Exchanges

Recombinant infectious clones were created where the Rep region (which also contains the N terminal part of AC4), encompassing the start of the Rep protein coding sequence until the beginning of the TrAP coding sequence, was exchanged between ToLCNDV-C and ToLCNDV-T&C, producing ToLCNDV-C[T&C-Rep] and ToLCNDV-T&C[C-Rep]. These were not infectious in *N. benthamiana*. In the transient co-infiltration

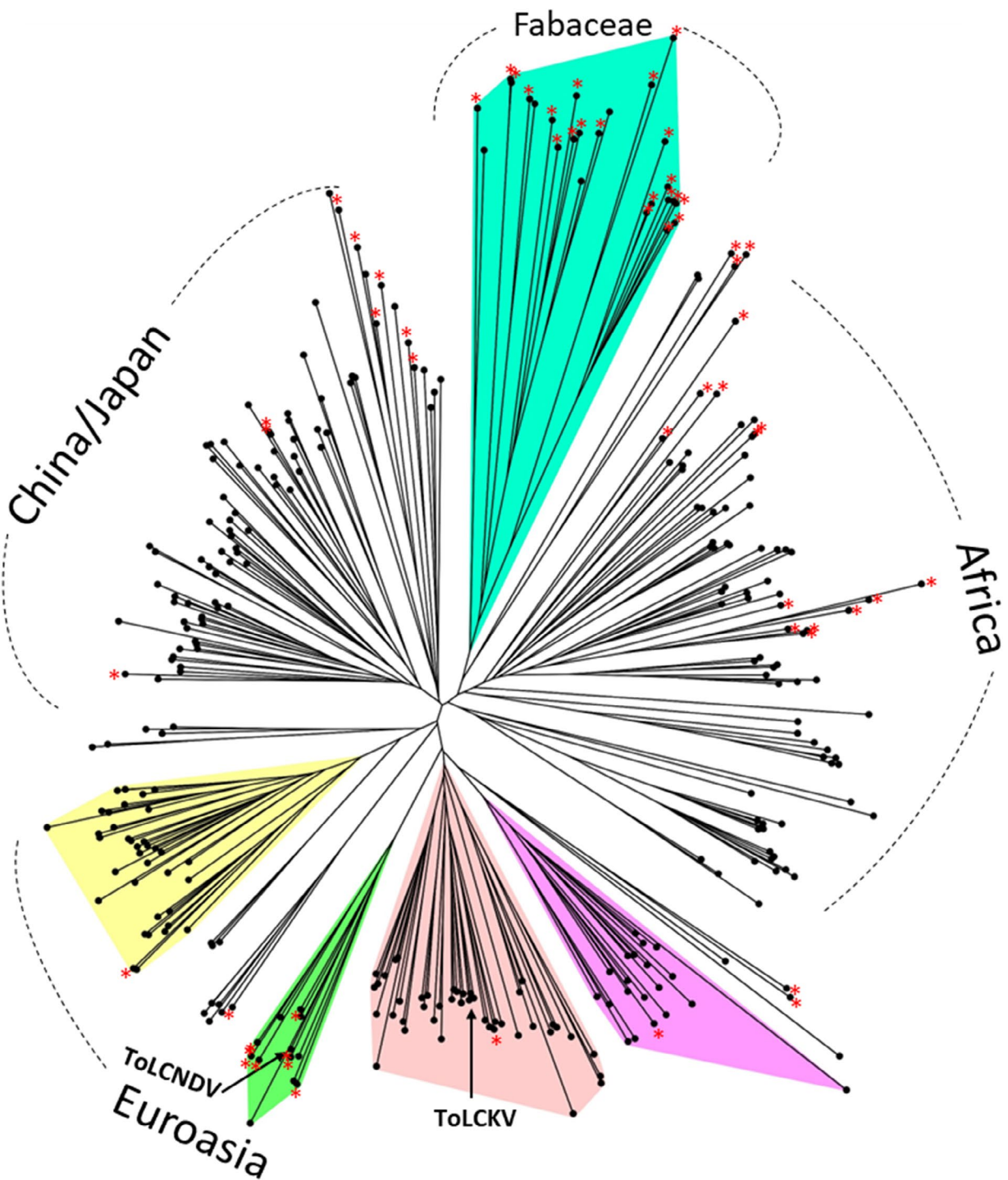


FIGURE 4 | Phylogenetic relationships between DNA-A of old world begomoviruses (RefSeq) inferred by neighbour-joining with bootstraps of 100 replications showing nucleotide distance by Jukes-Cantor. The model was made using CLC-Workbench. Select clades have been coloured. Green, ToLCNDV-clade representing the predominantly cucurbit infecting viruses; lilac, ToLCKV clade with predominantly monopartite viruses; purple, C-Rep clade over-representation of ToLCNDV-C type Rep; yellow, clade with over-representation of Malvaceae-infecting viruses; turquoise, clade with predominantly Fabaceae-infecting viruses. Prevalent geographic origin is also labelled. Viruses that have a known DNA-B component are marked as bipartite.

assay we found that ToLCNDV-C[T&C-Rep] DNA-A co-inoculated with ToLCNDV-T&C DNA-B shifts the DNA-A/DNA-B ratio heavily towards the DNA-B (Figure 5). This also held true for ToLCNDV-C DNA-B and Rep. These results clearly show that the Rep region determines specificity for the iteron/IR and these ToLCNDV strains have unique IR preference.

2.5 | Coat and Pre-Coat Protein Exchanges

We then swapped the entire coat and pre-coat protein coding sequence between ToLCNDV-C and ToLCNDV-T&C, producing ToLCNDV-C[T&C-CP] and ToLCNDV-T&C[C-CP]. Both these clones infected all *N. benthamiana* with symptoms identical

a

ToLCNDV-C	GGAGCTCTGGGTGC
ToLCNDV-T&C	GGTGT-CTGGAGTC
ToLCKV-T	GGTGTACTGGAGTC

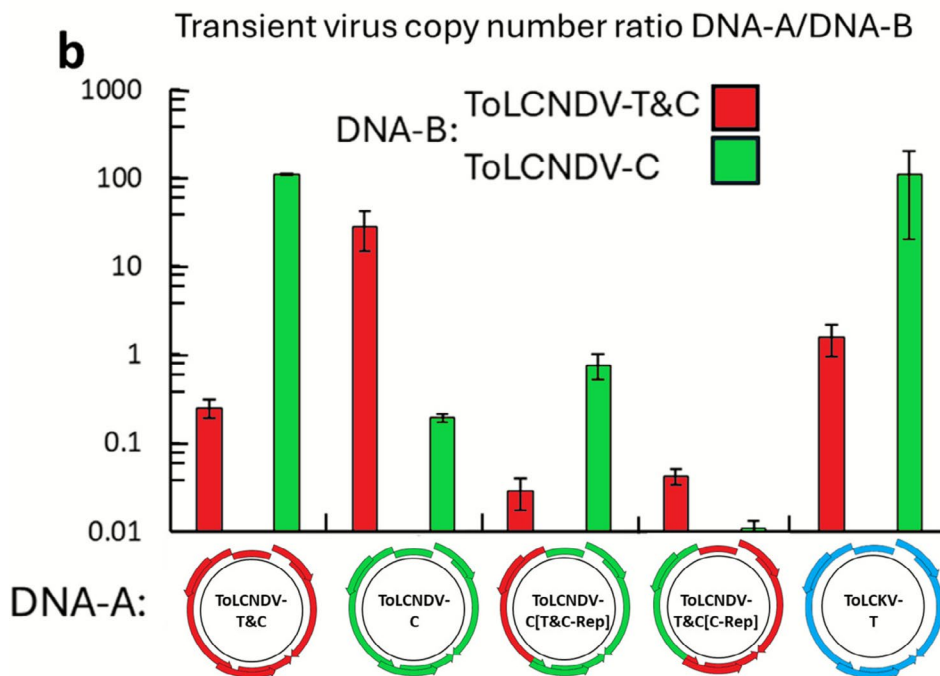


FIGURE 5 | (a) A comparison of the DNA-A iteron sequence region between ToLCNDV-C, ToLCNDV-T&C and ToLCKV-T with known iterons underlined (Chatterji et al. 1999). ToLCNDV-C's sequence is assumed from alignment of common region. (b) Transient trans-replication in *Nicotiana benthamiana*. *N. benthamiana* leaves were agroinfiltrated with different combinations of DNA-A and DNA-B. Six days post-inoculation, three samples were taken from the inoculated leaves and copies of DNA-A and DNA-B were determined by quantitative PCR (qPCR). Bars show DNA-A:DNA-B segment ratio as determined by qPCR amplification following transient co-inoculation. The difference between cognate and non-cognate DNA-B, or in the case of ToLCKV-T between ToLCNDV-C and ToLCNDV-T&C, is significant ($p < 0.05$, Student's *t*-test). Error bar signifies standard deviation and the y-axis is logarithmic. DNA-B segments are shown by bars coloured red: ToLCNDV-T&C or green: ToLCNDV-C.

to the wild-type viruses and both continued to be infectious in cucumber (Figure 1). However, ToLCNDV-C[T&C-CP] did not infect tomato, similar to ToLCNDV-C, while ToLCNDV-T&C[C-CP] infected as well as wild-type ToLCNDV-T&C. We also made ToLCKV-T[T&C-CP], which infected tomato but did not change severity, nor was it better able to maintain the DNA-B segment.

2.6 | ToLCNDV TrAP/REN Protein Exchanges

Recombinant infectious clones were created where the entire TrAP/REN protein coding sequence was exchanged between ToLCNDV-C and ToLCNDV-T&C. All constructs infected all *N. benthamiana*. For ToLCNDVs, both domain-swapped constructs were infective in cucumber, and both were also infective in tomato but with reduced disease incidence, infecting only 7/20 for ToLCNDV-T&C[C-TrAP/REN] and 6/17 for ToLCNDV-C[T&C-TrAP/REN] (Figure 1). ToLCNDV-C[T&C TrAP/REN] carrying ToLCNDV-T&C TrAP/REN region infected tomato for the first time, and thus we concluded that TrAP/REN was responsible for host jump from cucumber to tomato by the

ToLCNDV-C. The virus titres for ToLCNDV-T&C[C-TrAP/REN] were also lower than ToLCNDV-T&C in tomato and cucumber ($p = 0.018$ and $p = 0.032$, respectively, Student's *t*-test) (Figure 3). It would thus seem the TrAP/REN region is key to changes in host range. As *Agrobacterium* inoculation is not a natural method of infection, we also used whiteflies to transmit ToLCNDV-T&C, ToLCNDV-C, ToLCNDV-T&C[C-TrAP/REN] and ToLCNDV-C[T&C-TrAP/REN] from infected cucumber to tomato to confirm their infectivity (Figure 6). As expected, both ToLCNDV-T&C and ToLCNDV-C[T&C-TrAP/REN] transmitted to tomato plants by whiteflies, but none with ToLCNDV-T&C[C-TrAP/REN], further indicating the importance of the TrAP/REN region in host specificity.

2.7 | Identification of the Domain Responsible for Tomato Host Loss

To identify the specific subdomain responsible for tomato host loss, we created chimeric infectious clones with subdomains from N-terminal, C-terminal and middle domains: ToLCNDV-C[T&C-TrAP/REN-Nterm], ToLCNDV-C[T&C-TrAP/REN-Cterm] and

ToLCNDV-C[T&C-TrAP/REn-Mid], respectively. These were all infectious in cucumber, but only ToLCNDV-C[T&C-TrAP/REn-C-term] infected tomato, similar to ToLCNDV-C[T&C-TrAP/REn], demonstrating that the C-terminal region of TrAP/REn was responsible for host gain. We then proceeded to create new point mutations and minor domain swaps of ToLCNDV-C and -T&C, creating clones ToLCNDV-C[M1] to ToLCNDV-C[M9] (see Figure 7 for exact mutation). All except ToLCNDV-C[M7] were infectious in cucumber as a control; ToLCNDV-C[M7] was also unable to infect *N. benthamiana*, indicating it was completely faulty. None of the individual point mutations were able to infect tomato, whereas [M9] representing a 63-nt fragment was able to infect tomato, similar to ToLCNDV-C[T&C-TrAP/REn-Cterm].

2.8 | Identification of Tomato Host Factors

We chose known host factors that could potentially interact with the C-terminal of TrAP or REn and tested their interactions in a yeast two-hybrid experiment. HGold yeast was transformed

with the pGADT7 constructs and Y187 yeast with the pGBKT7 constructs and then mated to produce diploids with both constructs. None of the constructs tested produced colonies on selective media when mated with the empty vector. pGADT7-PolA mated with pGBKT7-REn grew well, confirming interactions, while pGADT7-RBR did not grow with any pGBKT7-REn. For pGADT7-PCNA we found high growth when mated to pGBKT7-REn[T], none when mated with pGBKT7-REn[T&C] or pGBKT7-REn[M9] and some when mated to pGBKT7-REn[C], regardless of whether it was PCNA from tomato or cucumber (Figure 8). This establishes that REn from ToLCNDV-T&C does not appear to interact with PCNA and the 63-nt fragment is responsible for this differentiation from ToLCNDV-C, which does appear to interact with PCNA.

pGBKT7-ATG7 or pGBKT7-rgsCaM grew when mated with any pGADT7-TrAP from any of the viruses tested, indicating some interactions. Meanwhile, pGBKT7-SIAGO1 had some growth only when mated with pGADT7-TrAP[C], while pGBKT7-CsAGO1 did not, which indicated some differential interactions and that the 63-nt fragment is responsible for the change.

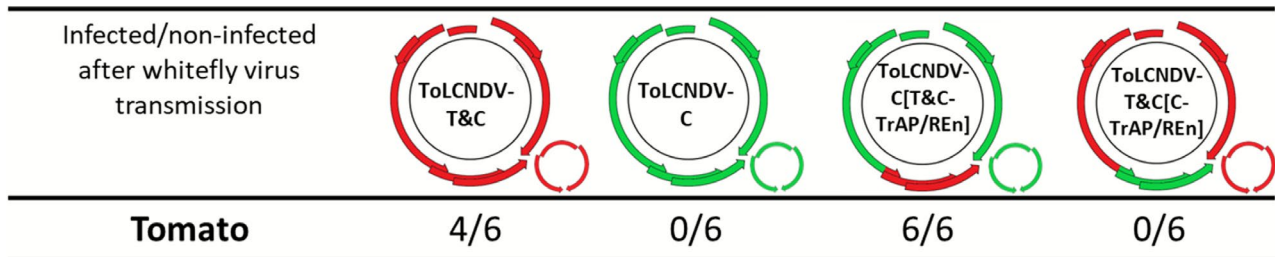


FIGURE 6 | Disease incidence for tomato cv. Moneymaker following whitefly transmission of ToLCNDV-T&C, ToLCNDV-C, ToLCNDV-C[T&C-TrAP/REn] and ToLCNDV-T&C[C-TrAP/REn]. Infection was determined 20 days post-inoculation using quantitative PCR. The infectious virus clones are coloured according to the sequence origin: Red: ToLCNDV-T&C, green: ToLCNDV-C, blue: ToLCKV-T. Recombinant clones are fractionally coloured corresponding to the origin of each subsegment. Clones were inoculated together with or without DNA-B segments from ToLCNDV-C or ToLCNDV-T&C. The DNA-B used is indicated with the small green or red circles, for ToLCNDV-C or ToLCNDV-T&C, respectively.

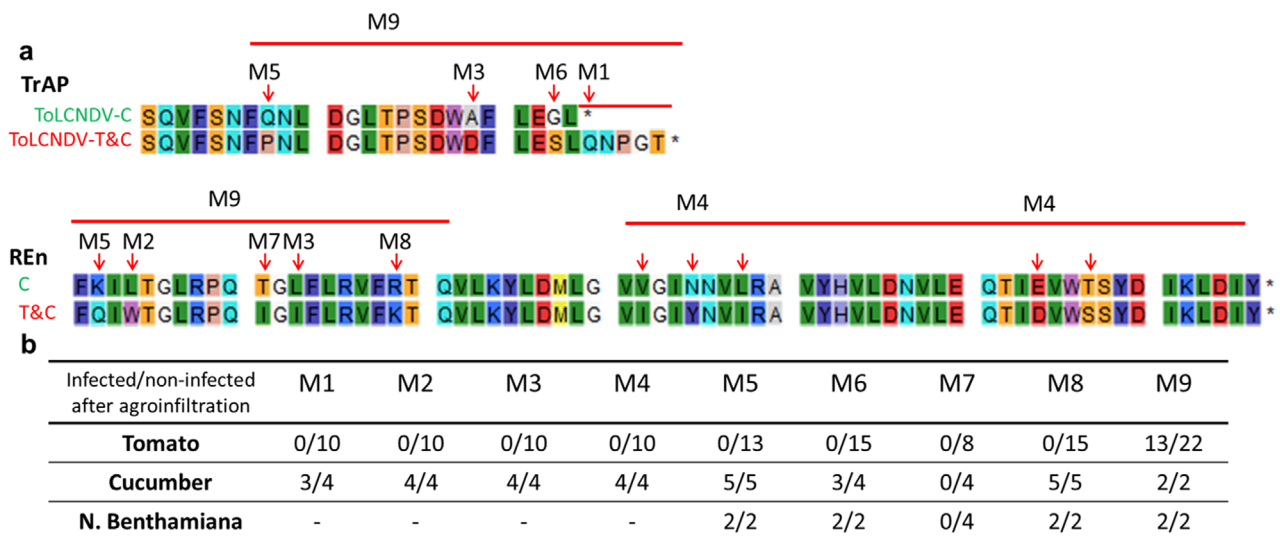


FIGURE 7 | (a) Amino acid sequence alignment for TrAP and REn of ToLCNDV-C and ToLCNDV-T&C. Mutations in which the amino acid of ToLCNDV-C was changed to ToLCNDV-T&C's are marked. For M9 and M4 the entire sequence, marked by red lines, of ToLCNDV-C was replaced with ToLCNDV-T&C's. (b) Disease incidence for *Nicotiana benthamiana*, tomato cv. Moneymaker and cucumber cv. Marketmore following agroinfiltration with the various ToLCNDV-C clones. Infection was determined 20 days post-inoculation using quantitative PCR.

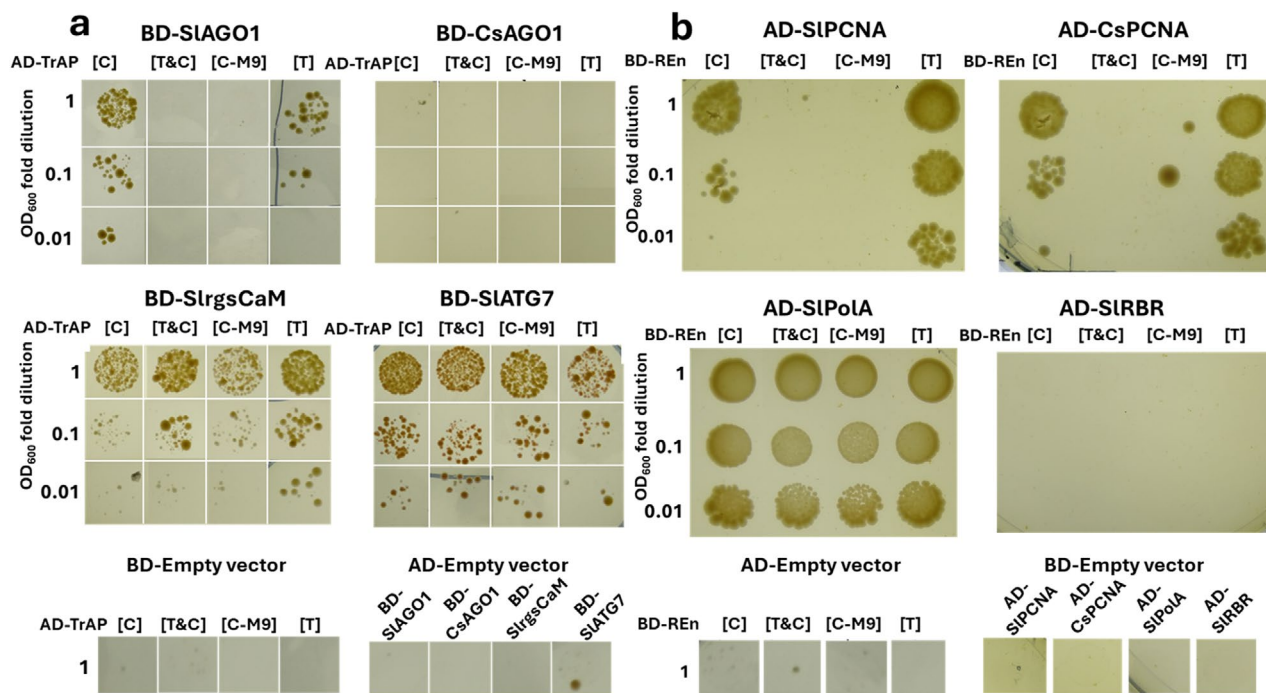


FIGURE 8 | Yeast 2-hybrid interaction assay of the interaction between (a) TrAP from ToLCNDV-C ([C]), ToLCNDV-T&C ([T&C]), ToLCKV-T ([T]) and ToLCNDV-C[M9] ([C-M9]) and SIAGO1, CsAGO1, Slrgs-CaM and SIATG7. (b) Ren from ToLCNDV-C, ToLCNDV-T&C, ToLCKV-T and ToLCNDV-C[M9] and SIPoLA, SIRBR, CsPCNA and SIPCNA. TrAP was expressed as a GAL4-activating domain fusion protein in HGold yeast and mated with Y187 yeast expressing the plant protein as a fusion protein to the GAL4-DNA binding domain. The figure shows the mated yeast plated on triple dropout (–Trp/–Leu/–His) plates as selection for interaction. Empty vector controls are HGold expressing the empty vector pGADT7-AD or Y187 expressing the empty pGBKT7-BD vector.

2.9 | Role of TrAP/REN and DNA-B in Host-Specificity

To investigate the role of TrAP/REN in host jump beyond the ToLCNDV species, additional recombinant infectious clones of ToLCKV-T with TrAP/REN domains from ToLCNDVs were made and they all infected *N. benthamiana* with symptoms characteristic of ToLCKV-T. ToLCKV-T[T&C-TrAP/REN] (ToLCKV-T with TrAP/REN from ToLCNDV-T&C), infected tomato similar to wild-type ToLCKV-T (lacking visible symptoms). ToLCKV-T[T&C-TrAP/REN] did not infect cucumber, despite carrying a cucumber-infecting TrAP/REN domain. However, co-inoculating cucumber with ToLCKV-T[T&C-TrAP/REN] with ToLCNDV-T&C DNA-B resulted in infections in 30/35 of the inoculated plants (Figure 1), as detected by qPCR. However, the cucumber plants did not display any visual symptoms. In addition to being symptomless, the ToLCKV-T[T&C-TrAP/REN] virus accumulation in cucumber was two orders of magnitude lower than ToLCNDV-T&C (Figure 3) and while DNA-A/DNA-B was always present in infected cucumbers, the DNA-A/DNA-B ratio was variable at $1:7.2 \pm 6.5$ compared to the ratio for wild-type ToLCNDV-T&C DNA-A/B, which was close to 1:1 (1.4 ± 0.5), with 10 and 9 samples, respectively. Additionally, the ratio was higher for ToLCKV-T[T&C-TrAP/REN], but with a *p*-value from Student's *t*-test at 0.04. ToLCKV-T[C-TrAP/REN], did not infect tomato and we only barely detected it in 3 out of 27 cucumber plants.

In the reverse experiments where ToLCNDVs received TrAP/REN from ToLCKV-T, both clones had unique behaviour.

ToLCNDV-T&C[T-TrAP/REN] stayed infectious in tomato infecting 10/10, but virus accumulation was on a par with wild-type ToLCKV-T and symptoms were distinct, with vein clearing present on the lower leaves while the remainder of the plant had mild to no visible symptoms. ToLCNDV-C[T-TrAP/REN] also infected its unnatural host, tomato, although only 3/10, and the symptoms and virus accumulation were similar to ToLCKV-T (Figures 1–3), which provided further evidence on the role of TrAP/REN in host switch.

Finally, in cucumber, ToLCNDV-T&C[T-TrAP/REN] behaved like wild-type ToLCNDV-T&C giving similar virus accumulation, disease incidence and symptoms, indicating further complex interactions between the viruses for host specificity. In cucumber, ToLCNDV-C[T-TrAP/REN] never gave symptoms and we only found 2 plants out of 18 with very low virus levels.

3 | Discussion

ToLCNDV is found within a clade of cucurbit- and tomato-infecting viruses, within a larger, primarily monopartite Asia/India grouping. Variants of ToLCNDV have also been found infecting a wide range of plants, and at the same time there are variants which struggle to infect tomatoes. Yet, the molecular factors involved in host range are rarely identified. Often, the host range information available is merely what is found by running PCR checks in fields, which has made it difficult to relate defined sequence information with infectivity in defined hosts. In this work, we selected viruses, defined their cucumber/tomato

host preference and exchanged domains to find and quantify virus factors in host range preference. Ultimately, we sought to identify the mutation causing the host range jumps of ToLCNDV as it is a rising threat to cucurbit production. However, in the end, we found that multiple mutations within the C-terminal domains of TrAP/REn region of DNA-A contributed to this host range jump.

ToLCNDV-C infects cucumber, but not tomato. However, the heterologous Rep region is also found in croton yellow vein mosaic virus and papaya leaf curl virus, where the region has approximately 90% identity and those two viruses can infect tomato (Pant et al. 2022; Pramesh et al. 2013), thus shedding doubts on the role of Rep in host specificity. Given the phylogenetic spread of the ToLCNDV-C Rep probably extends to Fabaceae and Malvaceae, its recombination into a new strain is unlikely to be a major factor of host change from tomato to cucurbits.

Previously, an arginine to tryptophan point mutation in the coat protein was identified as responsible for Mediterranean ToLCNDV-ES reduced infectivity in tomato (Vo et al. 2023). However, this exact mutation was not present in our virus clones and we saw no changes in infectivity in any of our swaps of coat and pre-coat. The TrAP/REn region was key for the host range of our clones. Both ToLCNDV-T&C and ToLCKV-T's TrAP/REn region enabled ToLCNDV-C to infect its unnatural host, tomato. We obtained a double confirmation of this when ToLCNDV-T&C's TrAP/REn region also allowed ToLCKV-T to infect cucumber (its unnatural host) together with the ToLCNDV-T&C's DNA-B. It is not entirely surprising that DNA-B is required for cucumber infectivity as, to our knowledge, no cucumber infectious clone of a monopartite begomovirus exists and there is only limited evidence of monopartite cucurbit infectious begomoviruses (Xie and Zhou 2003). Yet, it is clear there are more factors involved in host range determination, as ToLCNDV-C's TrAP/REn region does not fully prevent ToLCNDV-T&C from infecting tomato, although it stopped spread by whitefly and greatly reduced virus titres.

The TrAP/REn region was also previously indicated to play a role in the legume host range as AbMV's performance in beans was enhanced when it received the TrAP/REn region from BDMV (Levy and Czosnek 2006). In another study, the TrAP/REn region of tomato golden mosaic virus (TGMV) enhanced the disease severity and accumulation of bean golden mosaic virus (BGMV) in *N. benthamiana*, while TrAP/REn of BGMV did not allow TGMV to infect beans (Gillette et al. 1998), again showing that a unidirectional and complicated virus–host specific interactions can occur. In another study, tobacco host preference of BGMV also involves the BV1 promoter region from TGMV (Morra and Petty 2000), further indicating more complex interactions in host gain or loss. TrAP has an activator domain, but binds to BV1 through unknown host transcription factors, and this interaction is suggested to have a virus–host dependence (Sun et al. 2020). We found a strong connection between TrAP and DNA-B. Both ToLCKV-T[C-TrAP/REn] and ToLCNDV-C[T-TrAP/REn] have very limited infectivity, and they combine TrAP with its non-cognate DNA-B. While our swaps of ToLCNDV-T&C TrAP/REn region to ToLCNDV-C and ToLCKV-T resulted in simple host gain, swapping ToLCKV-T

TrAP/REn region to ToLCNDV-T&C gave more muddled results. ToLCNDV-T&C[T-TrAP/REn] did not lose cucurbit infectivity at all, while at the same time it took on ToLCKV-T's characteristics in tomato infections (mild symptoms). We hypothesise that potentially ToLCKV-T's low affinity for replicating ToLCNDV DNA-B limits infectivity, but the addition of ToLCNDV-T&C TrAP increases this affinity sufficiently for low-level infections in cucumber. We noted a possible reduction in disease incidence for ToLCNDV-C[T&C-TrAP/REn-Mid] and ToLCNDV-C[T&C-TrAP/REn-N-term], and we believe this combination of sequences is unfavourable for the complete structure of the protein.

We narrowed down the relevant region that enables ToLCNDV-C to infect tomato to a 63-nt fragment in the C-terminal of TrAP/REn, the very C-terminal of TrAP. We reason that the change in infectivity may be caused by a change in interaction between host factors and TrAP or REn. We did an initial screening of known interactors against TrAP/REn. We chose host factors that are known to interact with TrAP/REn in yeast 2-hybrid experiments and have straightforward homologues in tomato while omitting the host factor with interactions mapped outside the C-terminal region, such as Peapod2 (Cao et al. 2023; Guerrero et al. 2020; Settlage et al. 2005; Veluthambi and Sunitha 2021; Yong Chung et al. 2014) (see Table S4 for gene identifiers and sequences). TrAP functions as a transcriptional activator and when fused to the Gal4-binding domain in pGADT7, it will auto-activate the yeast 2-hybrid assay (Hartitz et al. 1999); for this reason, TrAP was fused to the Gal4 activator domain. For PCNA, we used a 55-amino acid N-terminally truncated PCNA similar to Settlage et al. (2005) because the full-length protein is toxic to the yeast (Castillo et al. 2003).

We expected to see ToLCNDV-C proteins fail to bind to a tomato host factor as it is not a host. On the contrary, we found that ToLCNDV-T&C failed to bind with both cucumber and tomato PCNA, and tomato AGO1. It appears that only ToLCNDV-C TrAP interacts weakly with tomato AGO1 and that this interaction is abolished by the 63-nt fragment. The AGO1 result could be an artefact of the activator domain and having swapped around the yeast-2-hybrid assay to have the prey on the DNA-binding domain. As we saw poor overall yeast growth, AGO1 is responsible for cleavage of miRNA targets, and it has a role in resistance to some viruses. However, suppression of AGO1 leads to upregulation of other Argonautes and loss of AGO1 leads to resistance against bamboo mosaic virus (BaMV) in *Arabidopsis* (Zhao et al. 2023). Solanaceous plants have additional Argonaute duplications and functionalisation with distinct roles in protection against other viruses (Liao et al. 2020). The astute reader will also note that as a DNA virus, AGO4 and not AGO1 plays a pivotal role in transcriptional gene silencing and AGO4 is inhibited by the AC4 protein (Kumar and Dasgupta 2023). It is possible that 'fine tuning' between the virus's suppressors of silencing and Argonautes leads to susceptibility. Our guess is thus that the AGO1 interaction results in reduced miRNA silencing of other Argonautes, which leads to an unfavourable disease outcome. The PCNA experiment showed ToLCNDV-T&C and ToLCKV-T REn binding strongly with PolA and PCNA, respectively, which adds credence to the result. PCNA should be crucial for begomovirus infections as it is a DNA clamp that recruits many other proteins involved in replication. Yet, it also interacts with Rep

(Castillo et al. 2003), and there was no difference in the interactions between cucumber and tomato PCNA. This leads us to believe that this difference for PCNA may not play a direct role in host range. Rather it is playing a role in the complete replication complex, where ToLCNDV-C and ToLCNDV-T&C Rep's are more divergent.

In conclusion, we have found changes in the TrAP/REN region partially facilitate a host jump. The clade with ToLCNDV is probably mainly adapted to cucurbits, and multiple mutations are probably involved in acquiring cucurbits as a host. Once a virus has adapted to live in cucurbits as a host, it can then acquire individual mutations that cause the loss of the previous host, tomato. Host jump or specificity of a virus is probably not dependent only on the acquisition of specific genes through recombination or mutations, but several gene-to-gene interactions, both within the virus and between virus and host, that will contribute to virus multiplication and eventual infection, and their spread in an ecosystem.

4 | Experimental Procedures

4.1 | Initial Virus Isolates and Detection

ToLCNDV-T&C (tomato and cucurbit strain), and its sequence information was previously obtained (Maruthi et al. 2005), while ToLCKV-T (tomato strain) was collected from the University of Agricultural Sciences, Bengaluru, India in 2022, and the ToLCNDV-C (cucurbit strain) from the village Midatharahally, Madhugiri Taluk, Tumkur District, India, all in 2022. Leaf samples were collected from infected tomato and cucumber plants from open fields. DNA was extracted using DNeasy plant mini-prep kit (Qiagen) in the UK and viruses detected subsequently using rolling circle amplification (RCA) (Wu et al. 2008).

RCA products, of ToLCNDV-C and ToLKV-T, digested with KpnI or BamHI produced a single approximately 3 kb band on a DNA agarose gel. The single bands were cloned into pJet using the CloneJet PCR cloning kit (Thermo Scientific) using the sticky end protocol. The resulting pJet plasmids were then Sanger sequenced using the primers provided (pJET1.2 Forward Sequencing Primer and pJET1.2 Reverse Sequencing Primer) with the kit.

4.2 | Construction of Infectious Viral DNAs

Obtained sequence information was used to design primers (Table S3) to clone 2-mer constructs of ToLCKV-T, ToLCNDV-T&C and ToLCNDV-C using NEBuilder. In the cloning of ToLCNDV-C DNA-A, genomic DNA fragments, F2 and F3, were obtained using CloneAmp HiFi PCR Premix (Takara) and the primer pairs p49/p55, and p56/p6 together with the vector backbone fragment, F1, obtained from PCR on pCambia backbone with primer pair p1/p2. A full list describing all cloning reactions, fragments obtained and primer pairs is provided in Table S1. In the case of ToLCKV-T DNA-A and ToLCNDV-C DNA-B, the vector backbone fragment, F6, was obtained from a restriction enzyme digest with SalI and SacI. Fragments were gel purified with NucleoSpin Gel and PCR Clean-up mini kit

(Macherey-Nagel) and then combined with NEBuilder (New England Biolabs) according to manufacturer's protocol.

4.3 | Construction of Chimeric Viral DNAs

From the existing infectious clones, new chimeric clones were prepared by swapping several domains between the viruses independently. We swapped AC4/Rep, pre-CP/CP and TrAP/REN domains between the three viruses. Similar to the creation of the original infectious clones, viral chimeric clones were made by combining PCR fragments obtained using CloneAmp HiFi PCR Premix. For example, in the case of creating ToLCNDV-T&C[C-Rep] (Rep region from ToLCNDV-T&C replaced the corresponding region of ToLCNDV-C strain), the infectious clone was assembled from four PCR products: two obtained from PCR on ToLCNDV-T&C with primer pairs p107/p13 and p111/p12; one PCR fragment from ToLCNDV-C obtained with primer pair p110/p46; and the backbone fragment obtained from PCR on pCambia backbone with primer pair p105/p106. Fragments were gel purified with NucleoSpin Gel and PCR Clean-up Mini kit (Macherey-Nagel) and then combined with NEBuilder (New England Biolabs) according to manufacturer's protocol. All other clones were prepared similarly using different primer combinations (Table S2). Full plasmid sequences were verified by Oxford Nanopore sequencing (Plasmid-Ez Genewiz).

4.4 | Construction of Clones for Yeast Two-Hybrid Assay

We use the Matchmaker Gold Yeast Two-Hybrid System (Takara) to screen interactions between begomovirus and plant proteins. Full-length open reading frames (ORFs) of TrAP were obtained from ToLCNDV-C, ToLCNDV-C[M9], ToLCNDV-T&C and ToLCKV-T plasmids and cloned into pGADT7-AD (Takara), fusing them with the Gal4 activator domain. Full-length ORFs of REN were obtained from ToLCNDV-C, ToLCNDV-C[M9], ToLCNDV-T&C and ToLCKV-T plasmids and cloned into pGBKT7-BD, fusing with the Gal4 DNA-binding domain (Takara: 630443). Full-length ORFs of Argonaute 1 (AGO1), regulator of gene silencing calmodulin (rgs-CaM) and autophagy-related gene (ATG7) were obtained from plant cDNA and cloned into pGBKT7-BD (Takara). Full-length ORFs of DNA polymerase α subunit 2 (PolA) and retinoblastoma-related protein (RBR), and ORF lacking the first 55 amino acids of proliferation cell nuclear antigen (PCNA) were obtained from plant cDNA and cloned into pGADT7-AD (Takara).

The plant RNA was obtained from healthy 40 days old tomato cv. Moneymaker leaves and cucumber cv. Marketmore using Qiagen RNeasy Plant Mini Kit (Qiagen: 74903). cDNA was prepared using LunaScript RT SuperMix Kit (New England Biolabs: E3010) according to manufacturer's protocol. pGADT7-AD was linearized in a PCR reaction with primer p330 and p378. pGBKT7-BD was linearized with EcoRI-HF and BamHI-HF (New England Biolabs: R3101S and R3136S). Full length cDNA fragments of plant genes were obtained using CloneAmp HiFi PCR Premix with primer pairs listed in (Table S2). Full length virus genes fragments were obtained from PCR on infectious clones using CloneAmp HiFi PCR Premix with primer pairs listed in

(Table S2). Fragments were gel purified with NucleoSpin Gel and PCR Clean-up Mini kit (Macherey-Nagel: 740609) and then combined with NEBuilder (New England Biolabs, UK: E5520S) according to manufacturer's protocol. All other clones were prepared similarly using different primer combinations (Table S2). Full plasmid sequences were verified by Oxford Nanopore sequencing (Plasmid-Ez Genewiz).

4.5 | Yeast Two-Hybrid Experiments

The yeast strain Y2H gold and Y187 (Takara: 630498) were transformed with 1 µg of pGADT7-AD and pGBKT7-BD carrying respective genes or empty vector using a modified lithium acetate method (Gietz et al. 1992). Transformants were selected on dropout (DO) media lacking tryptophan or leucine initially. Colonies were collected and resuspended to an OD₆₀₀ of 1; 20 µL of each clone suspension was added to 2 mL of YPDA and grown overnight at 28°C, 180 rpm shaking for mating. For estimating interactions, colonies were pooled from double DO (-Trp/-Leu) plates, resuspended in water and dilutions were plated on triple DO (-Trp/-Leu/-His) plates.

4.6 | Agroinfection

Agrobacterium tumefaciens LBA4404 (pAL4404) was transformed with infectious clone plasmids using a heat shock protocol (Jyothishwaran et al. 2007). *Agrobacterium* clones PCR positive for the construct were streaked out on Luria Bertani (LB) agar plates with kanamycin selection (50 µg/mL) and grown for 20 h at 28°C. *Agrobacterium* culture was collected from LB agar plates with a cell spreader and resuspended in infiltration buffer (10 mM MgCl₂, 100 µM acetosyringone, 10 mM 2-(*N*-morpholino) ethanesulphonic acid (MES), pH 5.5) to a final concentration of OD₆₀₀ of 0.3. When DNA-A and DNA-B were co-inoculated, their individual OD₆₀₀ was 0.15 each. *Agrobacterium* suspension was injected into the tender stem of seedlings of tomato cv. Moneymaker or cucumber cv. Marketmore at first or second true-leaf stage. For *N. benthamiana*, the *Agrobacterium* suspension was pressed into leaf lamina at the 4–8-leaf stage using a needleless syringe. The virus trans-replication assay was carried out similarly with a 1:1 mix of DNA-A and DNA-B segments from different viruses with a total OD₆₀₀ of 0.3. The plants were grown in (50:50 mix of John Innes and multipurpose mix) soil with a 16-h day 8-h night cycle at 23°C for symptom expression.

4.7 | Whitefly Transmission

We used a colony of Asia II-5 *Bemisia tabaci* population, originally collected from India, for virus transmission experiments (Rekha et al. 2005). Adult *B. tabaci* were collected using an aspirator from a colony established on eggplant in cages (30 × 30 × 30 cm) and placed on 30-day-old virus-infected cucumber plants of chosen strain for virus acquisition (24 h). Ten viruliferous whiteflies were then released onto 2-week-old tomato plants cv. Moneymaker enclosed in cylindrical plastic cage (3 × 8 cm) for virus inoculation (24 h). Leaf samples were collected 20 days after inoculation for testing for virus infection.

4.8 | Detection of Virus and Quantitative Analysis

Leaf samples were taken from the upper systemic leaf 20 days after inoculation, except for the *N. benthamiana* transreplication assay where samples were taken from infiltrated leaf area 6 days after inoculation. Genomic DNA was extracted from 2 to 5 cm² of leaf using a CTAB precipitation protocol essentially according to Otti et al. (2016). Infection status and virus titres were determined by qPCR using a C1000 Touch thermal cycler with CFX96 Optical Reaction Module. qPCR was set up in 10 µL reactions with 1 µL of diluted sample, 5 pmol of each primer and 5 µL PowerUp SYBR Green Master Mix. Estimation of virus titres was done by amplifying a conserved region in the CP of ToLCNDV, with primer pair p190/p191, or ToLCKV-T, with primer pair p262/p263. Estimation of DNA-B concentration was done using primer pair p260/p261. Estimation of plant DNA was done by amplifying a conserved region (Demesure et al. 1995) using primer pair p14/p15. All primers are listed in Table S3.

4.9 | Bioinformatics

Available Begomovirus refseq sequences were downloaded from the NCBI Virus database (<https://www.ncbi.nlm.nih.gov/labs/virus/vssi>), along with host information. Alignment of DNA-A and protein sequences was carried out using CLC Workbench v. 23.0.2 (Qiagen Aarhus). Sequence alignments and pairwise comparisons were subsequently carried out to create phylogenetic trees. The neighbour-joining method with bootstrapping for 100 replicates was used for tree construction. As Old and New World begomoviruses are divergent from each other, only viruses phylogenetically belonging to the Old World group, a total of 318 sequences, were used in the final trees.

Author Contributions

M. N. Maruthi: conceptualisation, funding acquisition, writing – original draft preparation, writing – review and editing; **Svenning Rune Möller:** investigation, writing – original draft preparation, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The sequence of constructs used in this study are openly available in GenBank (see [Supporting Information](#)). Other data that support the findings of this study are available from the corresponding author, M.N. Gowda, upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Phylogenetic relationships between Replication protein (Rep) (A) or Replication enhancer protein (REn) (B) of Old World begomoviruses (RefSeq). Clades are coloured to illustrate their approximate relation of most viruses to Figure 4. Location of ToLCNDV-T&C, ToLCNDV-C and ToLCKV-T is marked. (C). Phylogenetic relationships between DNA-A segment of Old World begomoviruses (RefSeq), identical to Figure 4 but viruses which are present in the purple clade with ToLCNDV-C in A labelled as C-Rep or Green in B as C-REn to indicate the spread of the ToLCNDV-C class Rep and REn. Trees were inferred by neighbour-joining with bootstraps of 100 replications showing nucleotide distance by Jukes-Cantor. The model was made using CLC Workbench. **Table S1:** List of vectors for initial infectious clones and the fragments used to generate them. The primer sets and templates used in the PCR to create those fragments are also listed. **Table S2:** List of vectors for recombinant infectious clones and the fragments used to generate them. The primer sets and templates used in the PCR to create those fragments are also listed. **Table S3:** List of primers used in this study. **Table S4:** List of vectors for yeast-2-hybrid and the fragments used to generate them. Tomato and cucumber gene ID are also provided. The primer sets and templates used in the PCR to create those fragments are also listed.