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ORIGINAL ARTICLE

Analysis of the Mild strain of tomato yellow leaf curl virus, which overcomes *Ty-2* gene–mediated resistance in tomato line H24

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Abstract In tomato line H24, an isolate of the Mild (Mld) strain of tomato yellow leaf curl virus (TYLCV-Mld [JR:Kis]) overcomes *Ty-2* gene–mediated resistance and causes typical symptoms of tomato yellow leaf curl disease (TYLCD). No systemic infection with visible symptoms or accumulation of viral DNA in the upper leaves was observed in H24 challenged with another isolate, TYLCV-IL (TYLCV-IL [JR:Osaka]), confirming that H24 is resistant to the IL strain. To elucidate the genomic regions that cause the breakdown of the *Ty-2* gene–mediated resistance, we constructed a series of chimeras by swapping genes between the two strains. A chimeric virus that had the overlapping C4/Rep region of the Mld strain in the context of the IL strain genome, caused severe TYLCD in H24 plants, suggesting that the overlapping C4/Rep region of the Mld strain is associated with the ability of this strain to overcome *Ty-2* gene–mediated resistance.

Introduction

Tomato yellow leaf curl virus (TYLCV) and related viruses cause tomato yellow leaf curl disease (TYLCD), which seriously impacts production of tomato (*Solanum lycopersicon* L.) worldwide [11, 22, 34]. TYLCV belongs to the genus *Begomovirus* in the family *Geminiviridae* [4, 5] and is transmitted by the whitefly *Bemisia tabaci* (Gennadius) [8, 10]. The monopartite, circular single-stranded DNA genome of this virus has two open reading frames (ORFs) on the virus-sense strand (V1 and V2) and four ORFs on the complementary strand (C1–C4). In monopartite begomoviruses, the replication-associated protein C1 (Rep) binds to the intergenic region (IR), which can form a stem-loop structure and is known to be the site of the origin of DNA replication, to initiate DNA replication and is indispensable for viral DNA replication [13]. The transcription activator protein C2 binds to ss- and dsDNA [25]. The replication enhancer protein C3 binds to C1 and is required for efficient DNA replication [35]. C4 is embedded in Rep in a different reading frame. In TYLCV, C4 has been implicated in viral movement in plant tissues and affects symptom severity in host plants [18, 32]. In tomato leaf curl virus (ToLCV), C4 is also involved in viral movement and symptom induction [21, 30, 31]. The coat protein V1 acts as a nuclear shuttle for transporting viral DNA [32] and is involved in virus transmission by the whitefly vector [7, 26]. V2 functions as a suppressor of gene silencing; C2 and C4 also have silencing-suppressor activity, which depends on the host plant [1, 3, 9, 14, 23, 45, 46].

In Japan, TYLCD was first found in tomato fields in 1996 in the central and south-western regions [20], and by 2014, it had spread and become endemic to the major tomato-cultivation areas in 38 prefectures. Several isolates of TYLCV belonging to either the Mild (Mld) or Israel (IL) strains have been reported from areas in Japan where tomato is cultivated year-round [39]. Resistance to TYLCV has been reported in wild tomato species. Introgression of resistance genes into cultivated tomato from wild tomato species is one of the best ways to manage TYLCD, and considerable efforts have been made in this respect in breeding programs. Genetic studies revealed that multiple genes of different origin control resistance of wild tomato species. For instance, the *Ty-1* and *Ty-3* alleles originated

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from *Solanum chilense*: *Ty-1* from accession LA1969 and *Ty-3* from LA1932 [17, 44]. *Ty-2* originated from *Solanum habrochaites* accession B6013 and has been introgressed into cultivated tomato [16, 19]. *Ty-2*, a dominant resistance gene, was mapped to the long arm of chromosome 11 in line H24 derived from *S. habrochaites*. This gene confers resistance to some monopartite begomoviruses (ToLCV-Tiawan, TYLCV-IL [IT:Sic:04], and TYLCV-IL [JR:Toc:07]) but not to other monopartite begomoviruses (tomato yellow leaf curl Sardinia virus; TYLCSV) or bipartite begomoviruses (tomato yellow leaf curl Thailand virus and tomato leaf curl New Delhi virus) [2, 16, 28, 29, 36, 38]. Commercial tomato cultivars that are resistant or tolerant to TYLCD have been released and are currently available in Japan. Analysis of these cultivars by using PCR-based markers suggested the involvement of *Ty-1*, *Ty-2*, or *Ty-3*, some of which are heterozygous [33]. Analysis of hybrid heterozygous cultivars (*Ty-2*/*ty-2*) with a PCR-based marker, TG0302 [12], has revealed that these cultivars became infected with the Mld strain of TYLCV in Japan [33]. Tomato plants harboring the *Ty-2* allele respond differently to different begomoviruses; however, little is known about whether the Mld strain overcomes resistance in tomato plants that are homozygous for *Ty-2* or which viral factors (genes) are involved in overcoming *Ty-2*-mediated resistance.

In the current study, we found that an isolate of the Mld strain of TYLCV infected and caused typical symptoms of TYLCD in the tomato line H24, which is homozygous for the *Ty-2* resistance gene. To investigate the ability of the virus to overcome *Ty-2*-mediated resistance, we constructed and tested chimeric clones in which the overlapping C4/Rep region of the IL strain was replaced with that of the Mld strain. This replacement caused systemic infection of the H24 plants that was indistinguishable from that caused by the Mld strain. We analyzed the leaf tissues of H24 plants agroinfiltrated with Mld and the chimeric strain and showed that viral DNA of both strains accumulated in the infected plant cells.

Materials and methods

Tomato yellow leaf curl virus and infectious clones

The Kisozaki isolate of the Mild strain of TYLCV (TYLCV-Mld [JR:Kis], GenBank accession no. AB116634) and the Osaka isolate of the Israel strain of TYLCV (TYLCV-IL [JR:Osaka], LC099965) were used [39]. TYLCV-IL [JR:Osaka] was originally provided by Dr. S. Ueda (National Agricultural Research Organization, Hokkaido Agricultural Research Center). In this manuscript, TYLCV-Mld [JR:Kis] is abbreviated as Mld-Ks and TYLCV-IL [JR:Osaka] as IL-Os. The viruses were maintained on a susceptible tomato cultivar (*S. lycopersicon* Mill 'House Momotaro', Takii & Co., Ltd, Kyoto, Japan) by transmitting the viruses by using viruliferous *B. tabaci* B biotype.

To produce infectious clones of both viruses, a head-to-tail partial dimer of each viral genome was constructed by cloning it into a binary plasmid vector using rolling-circle amplification and PCR. Total DNA from tomato plants infected with the virus was extracted using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Viral DNA was amplified by rolling-circle amplification using an Illustra TempliPhi Amplification Kit (GE Healthcare Sciences, Uppsala, Sweden) according to the manufacturer's instructions. Amplicons were digested with *Bam*HI, and the DNA fragments (~2.8 kb) were verified on and purified from agarose gels and ligated into the *Bam*HI site of pBI121. Cloned viral genomes in the resulting plasmids (pBI-Ks1.0 for Mld-Kis and pBI-Os1.0 for IL-Os) were sequenced. To obtain plasmids carrying the IR only, other regions of the viral genome were removed by digesting the recombinant plasmids with *Eco*RI. The *Eco*RI fragments containing the IR (and the 3′ region of the Rep ORF) were gel-purified and self-ligated to produce pIR Ks for Mld-Ks and pIR Os for IL-Os. *Bam*HI fragments from pBI-Ks1.0 or pBI-Os1.0 were cloned into the *Bam*HI sites of pIR Ks and pIR Os to obtain 1.3-mers (partial dimers), pBI-Mld-Ks1.3 and pBI-IL-Os1.3, respectively.

Construction of TYLCV chimeric constructs

Four chimeric constructs were created (Fig. 1). The first one, KsRep/Os, had the Rep ORF of Mld in the context of IL. Three overlapping DNA fragments were amplified using KOD-plus-Neo DNA polymerase (Toyobo, Tokyo, Japan). The first fragment (1414 bp) contained a sequence from the *Bam*HI site at the terminal end of the viral genome to the Rep start codon and was amplified with the F BamHI primer (corresponding to nucleotides 143–175 conserved in both strains) and the reverse primer R Os1. The middle fragment contained the Mld-Ks Rep gene region amplified with the primer set F KsRep/R KsRep. The last fragment, which contained the region from the Rep stop codon to the other *Bam*HI site at the other terminal end of the viral genome, was amplified with the primer set F Os2/R BamHI. The infectious clone pBI-IL-Os1.3 was used as a template to generate the first and last fragments, and pBI-Mld-Ks1.3 was used to generate the middle fragment. PCR-amplified fragments were gel-purified, and the middle fragment was mixed with either the first or the last fragment as a template for the second PCR with the primer set F BamHI/R KsRep or F KsRep/R BamHI, respectively. To create the full-length chimeric

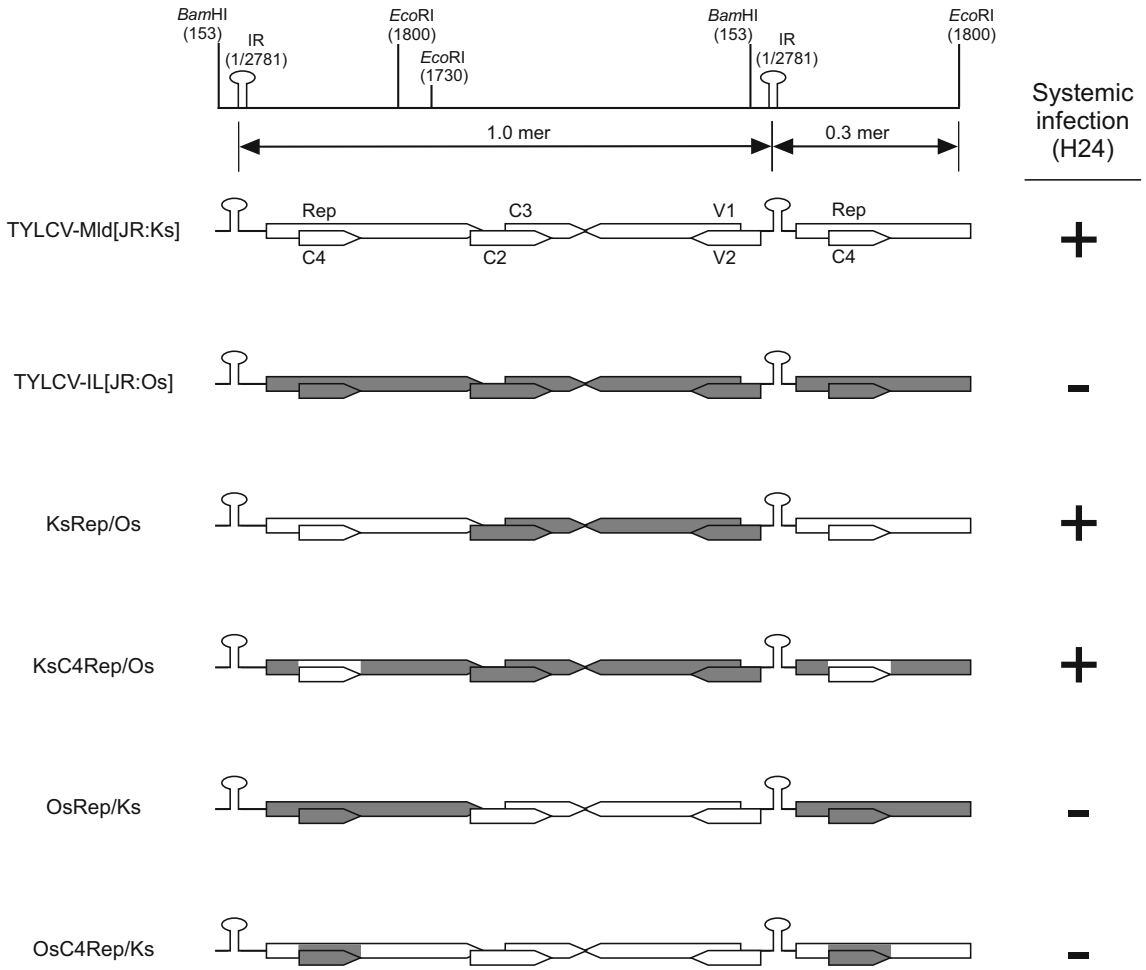

Fig. 1 Schematic representation of infectious constructs of the Mld and IL strains of tomato yellow leaf curl virus (TYLCV) and their chimeras, and analysis of systemic infection in tomato line H24. A partial dimeric (1.3-mer) TYLCV genome was cloned into the *Bam*HI/*Eco*RI sites of the binary plasmid pBI121. Arrows represent TYLCV open reading frames and their orientation. The genome of the Mld strain is shown as gray arrows, and that of the IL strain as white arrows. The response of the tomato line H24 following agroinoculation with each infectious clone is indicated on the right. +, systemic symptoms of tomato yellow leaf curl disease; −, no symptoms. Virus infection was evaluated by PCR diagnosis for the virus in upper leaves and by visual observation of the disease symptoms. Nucleotide numbering is for TYLCV-IL [JR:Osaka] (GenBank accession no. LC099965)

genome, the two overlapping PCR products were purified, mixed, and used as a template for the third PCR with the primer set F BamHI/R BamHI. The PCR product was cloned into the pCR4-BluntII TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced. The *Bam*HI fragment containing the 1.0-mer of the chimeric viral genome was cloned into the *Bam*HI site of pIR Os to produce the 1.3-mer of pBI KsRep/Os 1.3.

The chimeric construct OsRep/Ks was created by using the same strategy. The first fragment was amplified with the primer set F BamHI/R Ks1 and the last fragment with F Ks2/R BamHI; for both fragments, pBI-Mld-Ks1.3 was used as a template. The middle fragment was amplified with F OsRep/R OsRep and pBI-IL-Os1.3 as a template. The full-length chimeric construct was generated by consecutive overlapping PCR, cloned, and sequenced. The final construct was obtained by cloning the *Bam*HI fragment into pIR Ks to generate pBI OsRep/Ks 1.3.

The third chimeric construct, KsC4Rep/Os, had the overlapping C4/Rep region of Mld in the context of IL. Full-length genomes of the both strains (1.0-mer) were amplified using KOD-plus-Neo polymerase and pBI-Ks1.0 or pBI-Os1.0 as a template. The amplicons were cloned into pCR4-BluntII TOPO to create pCR Ks1.0 or pCR Os1.0, respectively. The viral genomic sequences in both plasmids were sequenced. These plasmids were used as templates in the following process. Three genomic DNA fragments were amplified by PCR as above with some modifications. The first fragment included the region from the *Not*I site (in the multiple cloning site of the plasmid) to

13 nucleotides downstream of the C4 start codon; it was amplified with the primer set 1F04 OsKs NotI/2R01 Os. The middle fragment included the overlapping C4/Rep sequence of Mld-Ks; it was amplified with the primer set 2F02 KsC4/2R02 KsC4. The last fragment included the region from the C4 stop codon to the internal *Xba*I site of the viral genome; it was amplified with the primer set 2F03 Os/1R03 OsKs XbaI. The infectious clone pCR Os1.0, was used as a template to generate the first and last fragments, and pCR Ks1.0 was used to generate the middle fragment. Using an In-Fusion HD Cloning Kit (TaKaRa BIO Inc., Shiga, Japan), we inserted the three fragments into the *Not*I/*Xho*I sites of the plasmid pCR Os1.0 to generate pCR KsC4Rep/Os1.0 and sequenced. The infectious clone (1.3-mer) was generated as described above, and the resultant plasmid was named pBI KsC4Rep/Os1.3.

The chimeric construct OsC4Rep/Ks was created by using the same PCR strategy. The first and last fragments were amplified with the primer sets 1F04 OsKs NotI/1R01 Ks and 1F02 OsC4/1R02 OsC4, respectively; pCR Ks1.0 was used as a template. The middle fragment was amplified with the primer set 1F03 Ks/1R03 OsKs XbaI and pCR Os1.0 as a template. Using an In-Fusion HD Cloning Kit, we inserted the three fragments into the *Not*I/*Xho*I sites of the plasmid pCR Ks1.0 to obtain pCR OsC4Rep/Ks1.0, which was sequenced. The infectious clone (1.3-mer) was generated as above and named pBI OsC4Rep/Ks1.3. The primer sequences are listed in Table 1.

Plant materials and virus inoculation

Tomato line H24 (*Ty-2*/*Ty-2*) [15, 16] was provided by Asian Vegetable Research and Development Center (AVRDC); the line was evaluated by PCR for the molecular marker TG0302 for *Ty-2* [12]. *S. lycopersicon*, 'House Momotaro', is susceptible to both strains (Mld and IL) and showed typical disease symptoms.

Recombinant plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 (TaKaRa BIO Inc., Shiga, Japan) by electroporation. Stems of tomato plants (three-leaf growth stage) were inoculated with *A. tumefaciens* cultures ($OD_{600} = 1.0$) by using agroinfiltration. For analysis of local viral DNA accumulation, *A. tumefaciens* cultures ($OD_{600} = 0.1$) were infiltrated into the leaves by using needleless syringes.

After acquisition-access feeding for 72 h on 'House Momotaro' plants infected with either IL or Mld, viruliferous adult whiteflies were used for inoculation of H24 plants (10 to 15 adults per plant). The whiteflies were allowed to feed on test plants for 72 h, and the plants were then sprayed with an insecticide to kill the whiteflies. All inoculated plants were kept in an environmental room at 25 °C with a 16-h photoperiod. Seven weeks after inoculation, stems of the plants were collected and tested for virus infection as below.

Virus detection

PCR diagnosis and quantitative PCR (qPCR) for detection of TYLCV in infected plants were carried out as described previously [27, 43]. For direct tissue print immunoassay (TPIA), the main stem was cut with a razor blade below the branch carrying the youngest expanding leaf at the apex of inoculated plants; a new blade was used for each plant. The cut surface was pressed onto nitrocellulose membranes for 10–15 s, and the membranes were air-dried at room temperature. The membranes were placed into a mixture of Tris-buffered saline and Tween-20 (TBST) (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.6, 0.05 % (v/v) Tween-20), washed three times (5 min each), and blocked with 0.1 × blocking reagent (Roche, Mannheim, Germany) in TBST for 2 h at room temperature or overnight at 4°C. The membranes were then incubated with the anti-TYLCV monoclonal antibody (TYLCV Reagent Set, Neogen Europe Ltd, Glasgow, Scotland) diluted 1:1000 in the blocking buffer for 2 h at 37 °C. The membranes were washed with the blocking buffer and incubated with goat anti-mouse IgG antibody conjugated with alkaline phosphatase (Sigma–Aldrich, St. Louis, MO, USA) diluted 1:1000 in blocking buffer for 1 h at 37 °C. Membranes were washed three times with TBST, rinsed twice in TBS (TBST without Tween-20), and immersed in the substrate (VECTOR Blue Alkaline Phosphatase Substrate Kit; Vector Laboratories, Burlingame, CA, USA). Incubation was stopped by washing the membranes in deionized water when color appeared in positive samples.

Southern blot analysis of total plant DNA was performed with a DIG-High Prime DNA Labeling and Detection Starter Kit II and a PCR DIG Probe Synthesis Kit (Roche) according to the manufacturer's instructions. Discs (8-mm diameter) were cut from infiltrated leaf areas with a disposable biopsy punch (Kai Corporation, Tokyo, Japan). Genomic DNA was extracted from the discs by using a DNeasy Plant Kit (QIAGEN) according to the manufacturer's instructions. The DIG-labelled V1 gene–specific probe was generated using the Outer F and R primers [27] and the plasmid pBI-Os1.0 as a template; this 561-bp probe recognized the V1 ORF region conserved between the two TYLCV strains. DNA (10 μg) was separated in a 1.0 % agarose gel and transferred to a positively charged nylon membrane (Roche). The blots were hybridized with the probe, and the signal was visualized with CSPD chemiluminescent substrate (Roche) according to the manufacturer's instructions.

Table 1 Primers used for generating infectious clones and PCR analysis

Primer name	Sequence (5′ - 3′)*	Position, nt**			Purpose
F BamHI	GCAATATGTGGGATCCACTTCTTAATGAATTTCC	143	-	175	Cloning of 1.0-mer viral genome, chimera construction
R BamHI	GGAAATTCATTAAGAAGTGGATCCCACATATTGC	175	-	143	
F Ks2	AAATAAACGAGGCATATGGTCAATGAGTACCGATTGACTAATATTTTTAC	2601	-	2657	Chimera construction
R Ks1	ACCCATAAGGCGTAAGCGTGTAGACCTAGACTGTGGCTGCTCATAC	1556	-	1511	Chimera construction
F OsRep	TCTAGGTCTACACGCTTACGCCTTATGGGTTTCTTCTTGGCTATC	1527	-	1571	Amplification of the Rep ORF of IL-Os
R OsRep	GTACTCATTGACCATATGCCTCGTTTATTTAAAATATATGCC	2629	-	2589	
F KsRep	TCTAGGTCTACACGCTTACGCCTTATTGTTTTCTTCTTGGCTATC	1527	-	1571	Amplification of the Rep ORF of Mld-Ks
R KsRep	CCGATTCATTTCAACATGGCTCCCCCTAAGCGCTTCCAAATAAATTGC	2630	-	2589	
R Os1	AACAATAAGGCGTAAGCGTGTAGACCTAGACTGTGGCTGCTCATAC	1556	-	1511	Chimera construction
F Os2	CTTAGGGGGAGCCATGTTGAAATGAATCGGTGTCCCTCAAAGCTC	2602	-	2645	Chimera construction
1F04 OsKs NotI	TTGAATTTAGCGGCCGCGAA		***		Chimera construction
1R01 Ks	TTTGCCTTCGAATTGGATAAGCACATGGAG	2421	-	2450	Chimera construction
1F02 OsC4	TTATCCAATTCGAAGGCAAATACCAATGTA	2440	-	2411	Amplification of the C4 ORF of IL-Os
1R02 OsC4	AATCTTTTGGGGCCTTCTCTTTTAATATAT	2150	-	2179	
1F03 Ks	AGAGAAGGCCCCAAAAGATTATATTTTACA	2169	-	2140	Chimera construction
1R03 OsKs XbaI	TTTTTCTTCGTCTAGATATTCCCTATATGA	1665	-	1694	Chimera construction
2R01 Os	TTTCCCTTCAAATTGGATAAGCACATGGAG	2421	-	2450	Chimera construction
2F02 KsC4	TTATCCAATTTGAAGGGAAATTCAAGTGCC	2440	-	2411	Amplification of the C4 ORF of Mld-Ks
2R02 KsC4	AGTCCTTTGGCATTAATTCTTTAATGATTC	2150	-	2179	
2F03 Os	AGAATTAATGCCAAAGGACTACATTTTACA	2169	-	2140	Chimera construction
Outer F	GCCCGTGACTATGTCGAAGCGACCA	298	-	322	PCR diagnosis for detection of tomato yellow leaf curl virus
Outer R	ATTTCCTCATCACTTGAAACCTATCCCGC	857	-	829	
Qv1F447	CAGCCCAATGGATTTTGGAC	754	-	773	Quantitative PCR for detection of TYLCV
Qv1R662	TACTTGGCTGCCTCCTGATG	969	-	950	
TUBint_f	ATTAGATCGGATGGATGTCCATAGT		****		Quantitative PCR for detection of tomato tubulin gene
TUBint_r	TTCTGCGCCCATCTCTATGGT		****		

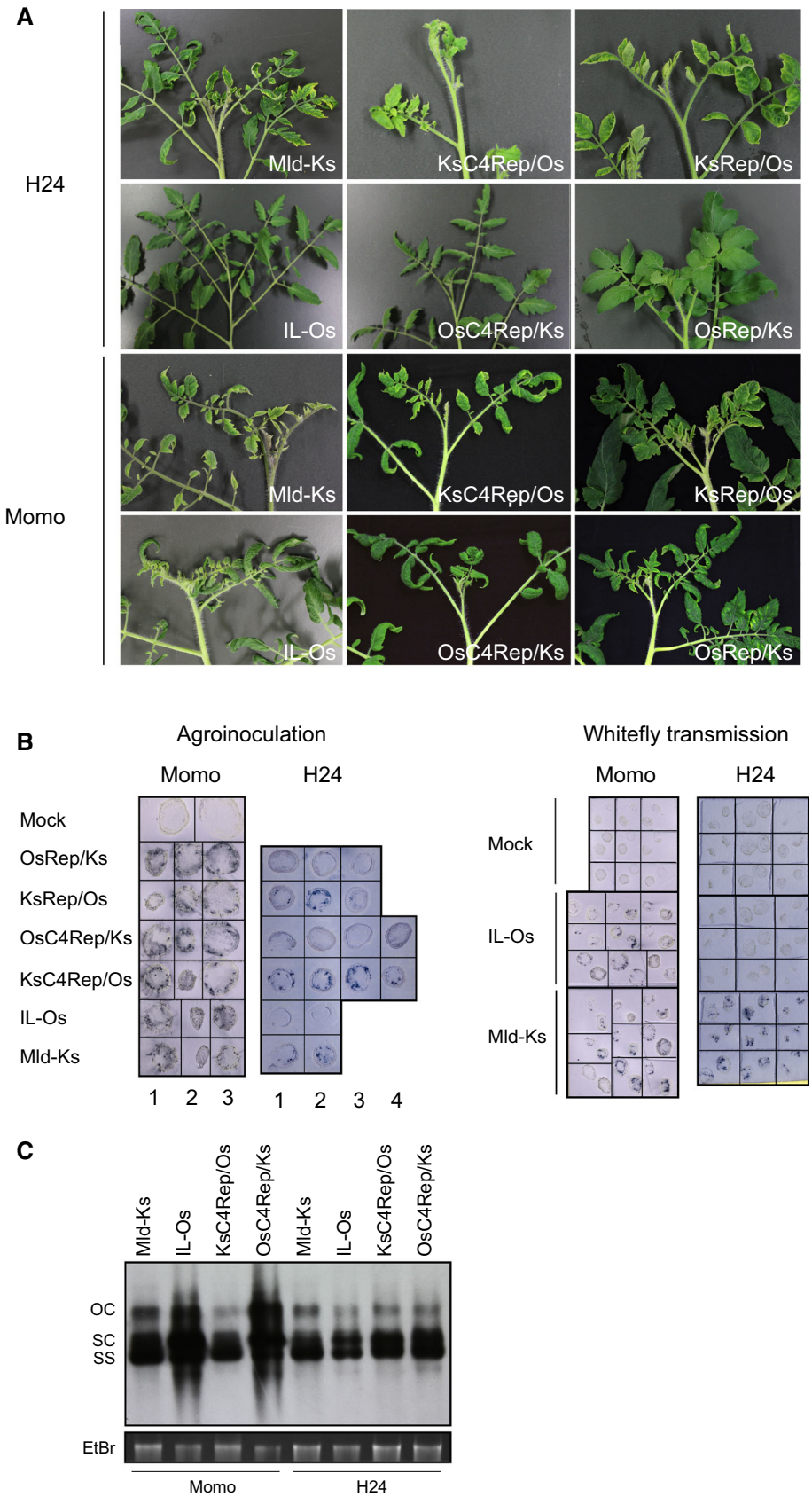

Fig. 2 Analysis of tomato line H24 inoculated with the Mld and IL strains of tomato yellow leaf curl virus (TYLCV) and their chimeras. (a) Responses of H24 plants agroinoculated with the indicated infectious clones 9 weeks after inoculation. Typical TYLCD symptoms were observed in plants infected with the Mld strain or chimeras containing Rep or the overlapping C4/Rep region of Mld in the IL backbone. (b) Detection of TYLCV in main stems of H24 plants by direct tissue print immunoassay. Main stems below the branch carrying the youngest expanding leaf at the plant apex were agroinoculated (two to four plants per experiment), or H24 plants were inoculated by viruliferous or non-viruliferous whiteflies (nine plants per experiment). Stems were collected 7 weeks after inoculation. Mouse monoclonal anti-TYLCV antibody (1:1000) and goat anti-mouse IgG secondary antibody (1:1000) conjugated with alkaline phosphatase were used. (c) Southern blot detection of viral DNAs in locally inoculated leaves of resistant (H24) and susceptible 'House Momotaro' (Momo) plants infected by leaf agroinfiltration. The blot was hybridized with a probe specific for a conserved region of the TYLCV V1 gene. The positions of open circular (OC) and supercoiled (SC) dsDNA forms and single-stranded (SS) DNA forms are indicated. Ethidium bromide staining is shown below the blot

Table 2 Response of tomato cultivar agroinoculated with infectious constructs of the Mld and IL strains of tomato yellow leaf curl virus (TYLCV) and their chimeras

Virus construct	House Momotaro		H24	
	Systemic infection***		Systemic infection	
IL-Os	27/27*	(6/6, 3/3, 6/6, 5/5, 4/4, 3/3)**	0/21	(0/8, 0/4, 0/4, 0/5)
Mld-Ks	22/22	(6/6, 3/3, 3/3, 5/5, 5/5)	17/17	(6/6, 3/3, 4/4, 4/4)
OsRep/Ks	11/11	(5/5, 3/3, 3/3)	0/11	(0/3, 0/3, 0/5)
OsC4Rep/Ks	19/19	(6/6, 3/3, 7/7, 3/3)	0/16	(0/3, 0/4, 0/5, 0/4)
KsRep/Os	17/17	(5/5, 3/3, 5/5, 2/2, 2/2)	16/16	(5/5, 3/3, 4/4, 4/4)
KsC4Rep/Os	15/15	(5/5, 3/3, 2/2, 2/2, 3/3)	13/13	(4/4, 4/4, 3/3, 2/2)

Response of tomato plants were based on the development of virus symptoms and systemic virus infection

All of the infected plants evaluated by PCR diagnosis developed systemic symptoms of tomato yellow leaf curl disease

* Results of all inoculation experiments are given as number of plants infected/number of plants inoculated

** Numbers in parentheses show the results of each independent inoculation experiment

*** Viral presence was tested by PCR diagnosis using DNA extracted from newly emerged upper leaves of inoculated plants

Table 3 Response of tomato cultivar inoculated by *Bemisia tabaci*-mediated transmission of the Mld and IL strains of tomato yellow leaf curl virus (TYLCV)

Virus construct	House Momotaro		H24	
	Systemic infection***		Systemic infection	
IL-Os	19/19*	(9/9, 5/5, 5/5)**	0/17	(0/9, 0/4, 0/4)
Mld-Ks	18/18	(9/9, 4/4, 5/5)	17/17	(9/9, 4/4, 4/4)

Adult whiteflies were given an acquisition-access feeding for 72 h on susceptible tomato plants infected with either IL or Mld strains, and viruliferous adults were then allowed to feed on test plants for 72 h (10 adults per plant). All test plants were sprayed with an insecticide after inoculation by viruliferous whiteflies

The response of tomato plants were based on the development of virus symptom and systemic virus infection

All of the infected plants evaluated by PCR diagnosis developed systemic symptoms of tomato yellow leaf curl disease

* Results of all inoculation experiments are given as number of plants infected/number of plants inoculated

** Numbers in parentheses show the results of each independent inoculation experiment

*** The presence of virus was tested by PCR using DNA extracted from newly emerged young leaves of inoculated plants at 7 weeks after inoculation

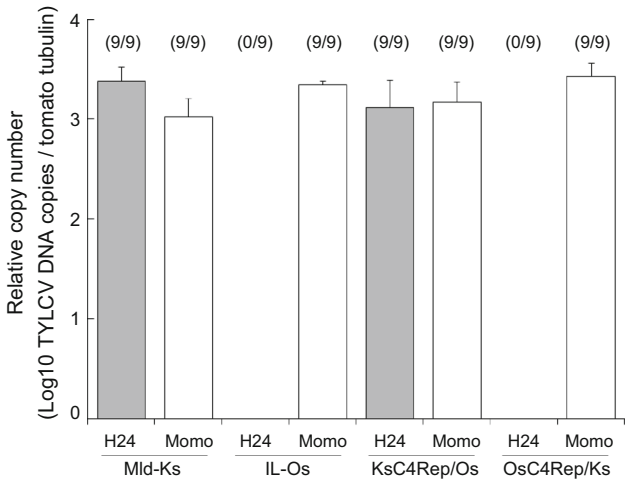

Fig. 3 Quantification of viral DNA of the two TYLCV strains and their chimeras accumulated in the leaves of tomato plants of line H24 (resistant) and cultivar 'House Momotaro' (Momo; susceptible). Plants were agroinoculated with infectious clones. Viral DNA in newly developed leaves 9 weeks after agroinoculation was quantified by real-time quantitative PCR. Results are expressed as copy numbers of viral DNA relative to those of the tomato tubulin gene. Data from three different experiments are shown. ND, not detected. Values are mean ± SD ($n = 9$). Numbers in parentheses represents total number of plants systemically infected / number of plants inoculated

Results

Mld-Ks, but not IL-OS, systemically infects tomato plants harboring the *Ty-2* gene

Mld-Ks agroinoculated into H24 plants (*Ty-2*/*Ty-2*) caused systemic infection with typical symptoms of TYLCD (Fig. 2A, Table 2); symptom severity was similar to that in susceptible 'House Momotaro' plants infected with the virus (Fig. 2A). Using TPIA, we detected that Mld-Ks accumulated in the upper stem phloem, similar to its accumulation in susceptible tomato plants (Fig. 2B, left part). Virus infection was evaluated by PCR diagnosis in the upper leaves of these test plants (Table 2). Similar results were obtained when viruliferous adult whiteflies harboring either Mld-Ks or IL-Os were used for inoculation (Fig. 2B, right part, Table 3). Using qPCR, we showed that Mld-Ks accumulated at similar levels in both H24 and susceptible ('House Momotaro') tomato plants (Fig. 3), whereas no visible symptoms of TYLCD were detected in

H24 plants inoculated with IL-Os (Fig. 2A). Accumulation of IL-Os DNA (Fig. 3) and V1 protein (Fig. 2B) was not detected in the H24 plants. Thus, H24 plants were resistant to IL-Os but not to Mld-Ks. This observation was consistent with previous reports that tomato plants harboring *Ty-2* respond differently to different species of monopartite bemogomoviruses [2, 16]. Inoculation by using viruliferous whiteflies also showed that resistance of H24 to IL-Os was not conferred by resistance to the whitefly *B. tabaci*, because the H24 plants became infected after inoculation by the viruliferous adults, which had been given an acquisition feeding on tomato plants infected with Mld-Ks (Fig. 2B, Table 3).

A chimeric viral clone carrying the overlapping C4/Rep region of Mld-Ks infects tomato plants harboring the *Ty-2* gene

A comparison of the genome sequences of the two strains used in this study showed multiple nucleotide (and occasional amino acid) differences. The amino acid sequence identity of the C1 protein between the two strains was 88.3 %, and that of the C4 proteins was 46 %. These values were much lower than those for the other proteins (99.2 % for V1, 99.1 % for V2, 98.5 % for C2, and 97 % for C3). The gene(s) with a high degree of sequence differences could be responsible for the phenotypic differences described above. To identify the region of the viral genome responsible for breaking *Ty-2*-mediated resistance, we generated chimeric constructs by swapping genome regions between Mld-Ks and IL-Os. The chimera KsC4Rep/Os, which had the overlapping C4/Rep region of Mld in the context of IL (Fig. 1), was used to inoculate H24 plants. The plants showed typical symptoms of TYLCD that were indistinguishable from those caused by Mld-Ks (Fig. 2A). In TPIA, this chimera was detectable in the phloem tissue of the main stem of H24 plants (Fig. 2B). KsC4Rep/Os DNA accumulated in the upper leaves of H24 plants at levels similar to those of Mld-Ks (Fig. 3). In contrast, no disease symptoms were observed when H24 plants were inoculated with another chimeric clone, OsC4Rep/Ks, which contained the overlapping C4/Rep region of IL in the Mld backbone (Fig. 1). OsC4Rep/Ks DNA was not detected by qPCR analysis (Fig. 3), and accumulation of V1 protein was not observed by TPIA (Fig. 2B). Similar observations were obtained when H24 plants were inoculated with the chimeric constructs KsRep/Os and OsRep/Ks, in which the Rep ORFs were swapped between Mld-Ks and IL-Os (Fig. 2A and B). Thus, we showed that the overlapping C4/Rep region of the Mld strain is responsible for breaking the *Ty-2*-mediated resistance of line H24, causing systemic infection and TYLCD development.

IL-Os and the chimeric construct OsC4Rep/Ks accumulate locally in tomato plants with the *Ty-2* gene

Local accumulation and replication of viruses in resistant (H24) and susceptible ('House Momotaro') plants were examined in leaves agroinfiltrated with KsC4Rep/Os, OsC4Rep/Ks, and their parental infectious clones. Southern blot analysis of total leaf DNA from agroinfiltrated areas showed the presence of viral DNA intermediates and genomes of all viruses, both in 'House Momotaro' and H24 plants (Fig. 2C). This indicates that IL-Os, Mld-Ks, and their chimeras were able to replicate and accumulate locally in H24 plants. These observations raise the possibility that *Ty-2*-mediated resistance to IL-Os restricts viral spread within the plant.

Discussion

We studied the response of tomato line H24 (*Ty-2*/*Ty*-2) to the IL and Mld strains of TYLCV, the virus that causes TYLCD in tomato. We showed that H24 has strain-specific resistance to TYLCV. We found that systemic infection with the IL strain was prevented in H24, which is consistent with the data on effective resistance to the IL strain of TYLCV in tomato hybrids heterozygous for the *Ty-2* gene [36]. Thus, either heterozygous or homozygous *Ty-2* confers resistance to the IL strain of TYLCV in tomato. However, H24 was susceptible to Mld-Ks, and systemic infection with typical symptoms of TYLCD developed after agroinoculation (Fig. 2A). Therefore, *Ty-2*, even when homozygous, does not confer resistance to the Mld strain, which caused typical TYLCD symptoms that are indistinguishable from those of diseased susceptible tomato plants. In cultivation areas where multiple virus strains are endemic, resistance with a narrow spectrum conferred by a single gene, such as *Ty-2*, is expected to be the least effective [28, 29]. Prasanna et al. reported that combining *Ty-2* and *Ty-3* introgressed from wild tomato species extended the resistance of tomato hybrids against some isolates of both monopartite and bipartite begomoviruses [28, 29]. Pyramiding multiple resistance genes introgressed from wild tomato species into related cultivars accomplished by marker-assisted selection is likely to be effective against some, but not all, isolates of monopartite and bipartite begomoviruses [28, 29, 41] .

The two closely related virus strains used in this study differed mainly in the region covering the Rep gene (in which the C4 gene is embedded) and the IR sequence [24]. The C4/Rep region is responsible for the induction of disease symptoms caused by ToLCV [21, 31] and TYLCSV [18] in tomato plants. This region is also the viral

determinant of systemic infection by Mld-Ks in resistant *S. habrochaites* accessions (EELM-388 and 889, see below), which have two loci that are different from the known *Ty* resistance loci [37]. We examined whether the overlapping C4/Rep region is responsible for overcoming the *Ty-2*-mediated resistance to Mld-Ks. The two chimeras, KsRep/Os and KsC4Rep/Os, which have the entire Rep region and the C4/Rep overlapping sequence of Mld-Ks in the IL-Os background, respectively, induced systemic infection and disease symptoms in line H24 that were indistinguishable from those induced by Mld-Ks (Fig. 2). This observation indicates that the overlapping C4/Rep region is responsible for the induction of TYLCD in line H24. A similar observation was made by Tomás et al., who found that two lines (EELM-388 and 889) derived from *S. habrochaites* were resistant to an isolate of the IL strain of TYLCV but were susceptible to an isolate of the Mld strain [37]. The authors reported that the overlapping C4/Rep gene region of the Mld strain was associated with the induction of systemic infection in the two lines. In these lines, the resistance was conferred by two independent loci, one dominant and one recessive, which were distinct from the previously identified *Ty-1* gene, but whether other *Ty* resistance genes were involved remained unknown [37]. It would be interesting to determine whether resistance in these lines was conferred by previously unknown gene(s).

In agroinfiltrated tomato leaves, DNA of the two strains and their chimeras accumulated similarly in H24 and susceptible plants (Fig. 2C). Thus, *Ty-2*-mediated resistance in H24 is unable to inhibit replication of the TYLCV genome in infected cells. Functional analyses of genes of monopartite begomoviruses (TYLCV, ToLCV, and TYLCSV) have suggested that C4 is associated with symptom induction and may be involved in cell-to-cell movement of the virus within the phloem [18, 21, 31, 32]. Disrupted expression of TYLCSV C4 abolishes systemic symptoms and virus movement [18], whereas disrupted expression of ToLCV C4 reduces symptom development in susceptible tomato plants [31]. Furthermore, constitutive expression of ToLCV C4 in transgenic tomato plants leads to systemic virus-like symptoms in the absence of virus [21]. Taken together, these data and our study suggest the presence of molecular mechanisms that recognize TYLCV C4 in *Ty-2*-mediated resistance. Although the fundamental function of the viral factor(s) interacting with *Ty-2*-mediated resistance were not ruled out in this study, our results suggest that the resistance to the IL strain in H24 suppresses viral infection after replication of the virus, resulting in suppression of systemic infection and transport of the virus.

In addition to its involvement in symptom induction and viral transport, C4 has been implicated in suppression of gene silencing. C4 and AC4 (its positional analog in bipartite begomoviruses) function as suppressors of RNA silencing, generally referred to as post-transcriptional gene silencing (PTGS) [23, 31, 40] or transcriptional gene silencing (TGS) [42]. Luna et al. showed that symptom induction and suppression activity of C4 varied among TYLCV strains and plant hosts [23]. The V2 protein from the Mld and IL strains of TYLCV strongly suppresses gene silencing in *Nicotiana benthamiana* and susceptible tomato. In contrast, C4 from IL functions as a suppressor in *N. benthamiana* but not in tomato, whereas C4 from Mld has no suppression activity in *N. benthamiana* [23]. Constitutive expression of C4 from different strains of TYLCV in transgenic *Arabidopsis thaliana* resulted in abnormal leaf size and shape, which are characteristic symptoms of TYLCD; the IL strain caused more severe defects than the Mld strain did [23]. Symptom severity may be linked to the efficiency of silencing suppression, which depends on the virus strain–host plant combination [23]. The difference in gene silencing suppression activity and symptom severity induced by the C4 from the two strains may be associated with breaking of *Ty-2*-mediated resistance in line H24.

Butterbach et al. showed that, in tomato plants with the *Ty-1*/*Ty-3* genes, resistance to the IL strain of TYLCV was conferred by enhanced TGS [6] and suggested that TGS is unlikely to be involved in resistance to the IL strain conferred by *Ty-2*, because the siRNA profiles specific to the IL strain were similar in *Ty-2* plants and susceptible plants, but not in *Ty-1*/*Ty-3* plants [6]. Elucidation of the molecular mechanisms for breaking resistance to TYLCV in tomato harboring *Ty-2* and analysis of viral factor(s) involved in breaking the resistance will be an important challenge to be addressed in future studies.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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