Use of *Tomato yellow leaf curl virus* (TYLCV) *Rep* Gene Sequences to Engineer TYLCV Resistance in Tomato

Y. Yang, T. A. Sherwood, C. P. Patte, E. Hiebert, and J. E. Polston

University of Florida, Department of Plant Pathology, Gainesville 32611.
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ABSTRACT

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Tomato yellow leaf curl virus (TYLCV), a member of the genus *Begomovirus* (family *Geminiviridae*), causes severe losses in tomato production in the tropics and subtropics. In order to generate engineered resistance, eight different constructs of the TYLCV replication-associated protein (*Rep*) and *C4* gene sequences were tested in transformed tomato inbred lines. Transgenic plants were screened for resistance to TYLCV using viruliferous whiteflies. No symptoms were observed and no TYLCV genomic DNA was detected by both hybridization and polymer-

ase chain reaction in progenies of plants transformed with three constructs. This resistance was observed in plants that contained one of the following transgenes: 2/5Rep (81 nucleotides [nt] of the intergenic region [IR] plus 426 nt of the 5' end of the TYLCV *Rep* gene), Δ 2/5Rep (85 nt of the IR plus 595 nt of the 5' end of the TYLCV *Rep* gene in the antisense orientation), and Rep Δ 2/5Rep (81 nt of the IR, the entire *Rep* gene, and 41 nt 3' to the end of the *Rep* gene fused to Δ 2/5Rep). Our study differs from other transgenic *Geminivirus* resistance reports involving the *Rep* gene in that viruliferous whiteflies were used for challenge inoculation instead of agroinoculation or biolistic inoculation, and TYLCV resistance was evaluated under field conditions.

Tomato yellow leaf curl virus (TYLCV) belongs to the genus *Begomovirus* (family *Geminiviridae*). Begomoviruses are small, circular, single-stranded DNA plant viruses that are transmitted by the whitefly *Bemisia tabaci* (Genn.). TYLCV first was described in what is now Israel, and it is one of the most devastating viruses that can infect tomato (7,8). It now occurs in several locations around the world and has infected tomato in Florida since 1997 (24). Management of TYLCV in tomato production is very difficult, expensive, and has limited options. Host resistance to TYLCV has been difficult to find and develop (16). TYLCV-resistant tomato lines have been developed in Israel and in the United States over the last 20 and 12 years, respectively, using genes derived from wild species of *Lycopersicon* (11,17,22, 28,33). However, resistance often is unsatisfactory due to a linkage with poor fruit quality. Tolerant commercial cultivars collapse under early or severe infection pressure and require protection during early growth stages. These cultivars produce a yield in the presence of TYLCV, but still support the replication of TYLCV and can act as sources of TYLCV for susceptible crops (12,18).

Most of the strategies for genetically engineered resistance to begomoviruses have involved the replication-associated protein (*Rep*) sequences. Several studies have focused on pathogen-derived resistance using partial, entire, or mutated *Begomovirus Rep* genes. A *Tomato golden mosaic virus* (TGMV) *Rep* construct that contained 15 nucleotides (nt) upstream of the translation start codon, plus 185 nt downstream of the *Rep* 3' end (including part of the *REN* and *TrAP* genes) was transformed into tobacco (*Nicotiana tabacum*) (9). Transformed plants containing the construct in the sense orientation could not be obtained. Symptom expression was reduced and viral DNA replication was blocked by the antisense of this construct. Furthermore, levels of antisense RNA were correlated with symptom development (resistance). Resis-

tance to the monopartite *Tomato yellow leaf curl Sardinia virus* (TYLCSV) was produced in *N. benthamiana* plants using the TYLCSV *Rep* gene with a deletion of 420 nt (140 amino acid residues) from the 3' end (21). However, resistance was transitory. Tomato plants were transformed with the same construct and it was found that accumulation of high levels of the truncated *Rep* protein was required for resistance, that this accumulation resulted in a curled phenotype, and that the resistance did not extend to an unrelated begomovirus. Bedahmane and Gronenborn (3) tested transgenic tobacco expressing an antisense, truncated TYLCSV *Rep* (63-nt leader and 288-nt [5'] *Rep* sequences) transcript for virus resistance by agroinoculation. They found some resistant, symptomless lines with suppressed TYLCSV replication as well as some tolerant lines with reduced TYLCSV replication. Transcript analysis indicated that plants with elevated transgene transcript levels consistently exhibited a higher degree of virus resistance. Polston and Hiebert (23) transformed tomato with the *Tomato mottle virus* (ToMoV) *Rep* gene (sense orientation), including the upstream 113 nt of intergenic region (IR), and demonstrated that a single copy of the transgene in tomato provided stable and high levels of resistance to ToMoV under field conditions (evaluated in the R₁ through R₅ generations). Both tolerance and immunity to ToMoV were seen in transformed plants. Yields of transformed plants from the R₄ generation were found to be equal to or higher than those of the untransformed parent in the absence of virus, and more than twice as great in the presence of ToMoV.

Noncoding begomovirus sequences (IR) also have been tested for the generation engineered virus resistance. Pooggin et al. (25) demonstrated that transient expression of both sense and antisense *Vigna mungo yellow mosaic virus* (VMYMV) promoter sequences in the IR resulted in complete recovery in infected VMYMV plants. The recovery of the whole plant from VMYMV indicated that the interfering signal spread throughout the plant. They proposed that RNA interference (posttranscriptional gene silencing [PTGS]) as has been described for RNA viruses also was possible for a DNA virus.

Corresponding author: E. Hiebert; E-mail address: ehi@mail.ifas.ufl.edu

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The objective of this work was to explore the use of the TYLCV *Rep* and *C4* gene sequences to identify transgenes capable of generating tomato plants with strong resistance to TYLCV. We evaluated eight constructs containing the TYLCV *Rep* gene or modifications of the *Rep* gene or the *C4* gene. The transgenic tomato plants were tested for TYLCV resistance by exposing them at a young age to high populations of whiteflies reared on TYLCV-infected tomato plants. Here we report that tomato plants having one of three transgene constructs showed no symptoms of TYLCV infection, nor could TYLCV be detected by hybridization or polymerase chain reaction (PCR) in the transformed plants (phenotypically immune).

MATERIALS AND METHODS

Construction of transgene expression vectors. TYLCV *Rep* gene (C1) DNAs corresponding to full-length *Rep* or truncated *Rep* gene fragments were PCR amplified from the extracts of TYLCV-infected tomato plants from Florida with various primer pairs containing restriction endonuclease sites for subsequent cloning (Table 1). PCR was conducted under the following conditions: 93.5°C for 45 s, then 35 cycles of 54°C for 45 s, 54 to 72°C ramping for 1 min, and 72°C for 1 min. The reaction mixture was 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, and 1% Triton X-100), 12.5 mM dNTPs (Promega Corp., Madison, WI), 1 mM spermidine trihydrochloride (USB Corp., Cleveland, OH), 2.5 mM MgCl₂, and 1 unit of *Taq* DNA polymerase (Promega Corp.). PCR products first were cloned into the pGEM-T vector (Promega Corp.) for propagation of the target sequence. The amplified and cloned TYLCV *Rep* sequences were cleaved from the vector and directly ligated into binary vector pKYLX71:35S² (1,20) (Fig. 1A) or a modified vector with the nonenhanced 35S promoter (pKPN) in either sense or antisense (Δ) orientations (Fig. 1B). In this study, eight expression vectors were constructed, designated pKY2/5Rep, pKY Δ 2/5Rep, pKYC4, pKY Δ C4, pKYRep, pKYRep Δ 2/5Rep, pKPNRep, and pKPN Δ Rep. To construct pKPNRep and pKPN Δ Rep, the enhanced 35S promoter of pKYLX was removed by *Eco*RI and *Cla*I digestions and replaced by the pBI121 expression cassette (Clontech, Palo Alto, CA) containing regular 35S promoter and NOS terminator (Fig. 1B).

Description of the constructs. Following is a description of the constructs used in this study. Diagrams of the constructs showing the details of their composition are shown in Figure 1A and B.

pKY2/5Rep: 81 nt of the IR (nucleotides 2,696 to 2,616 of GenBank accession no. AY530931) plus 426 nt of the 5' terminus of the TYLCV *Rep* gene; pKY Δ 2/5Rep: 85 nt of the IR (nucleo-

tides 2,701 to 2,616), 595 nt of the 5' terminus of the TYLCV *Rep* gene (encompasses the entire *C4* gene) in the reverse orientation; pKYRep Δ 2/5Rep: 81 nt of the IR, the entire *Rep* gene, followed by 41 nt downstream of the 3' end of the *Rep* gene, fused to the Δ 2/5Rep as above; pKYRep: 81 nt of the IR, the whole *Rep* gene, followed by 41 nt downstream of the 3' end of the *Rep* gene (includes the entire *C4* gene); pKNRep: (non-enhanced 35S promoter = N) 86 nt of the IR, the entire *Rep* gene, followed by 36 nt 3' to the *Rep* gene; pKN Δ Rep: (reverse orientation of NRep) 36 nt downstream of the 3' end of the *Rep* gene, the entire *Rep* gene, 85 nt of the IR; pKYC4: 81 nt of the IR, 151 nt of the *Rep* gene, then the entire *C4* gene, followed by 151 nt 3' to the *C4* gene; and pKY Δ C4: (reverse orientation) 151 nt 3' to the *C4* gene, the entire *C4* gene, followed by 104 nt 5' to the *C4* gene. The Δ C4 construct does not include any nucleotides of the IR.

Tomato transformation and screening. The binary vector containing the constructs was transformed into *Agrobacterium tumefaciens* LBA 4404 by a tri-parental mating approach (1). Cotyledonary tissues of tomato seedlings from Florida breeding lines Fla. 7613 and Fla. 7324 (provided by J. W. Scott) were transformed by *A. tumefaciens* LBA 4404 harboring the binary vector. Approximately 500 to 600 explants were used for each construct during transformation experiments. Putative transformants were screened on Murashige-Shoog media containing kanamycin (100 μ g/ml) (15). Regenerated (transgenic) plants (R₀) were grown in the greenhouse for transgene confirmation and seed production.

Molecular analysis of transgenes via PCR screening of transformants. DNA was extracted from R₀ regenerated plants (26) and was used for PCR amplification of the transgene. PCR was conducted under the same conditions as described above and with the same primer pair as used for transgene construction.

A different set of primer pairs was used to identify and distinguish the different constructs in R₁- and R₂-generation transformed plants. These would confirm whether the transgenes were present and allow the detection of different constructs in the same plant should any cross-pollination have occurred. The primer pairs and the expected size of the amplicon obtained with each construct and primer pair are shown in Table 2. Five pairs of primers were developed in which one primer would bind to the non-TYLCV *Rep* regions of the construct, and the other would bind in the *Rep* gene sequence. The primers used were JAP94 (5'-TCTCCATCCATTTCATTTCAC-3'), which binds in the Rubisco terminator; JAP28 (5'-GATAGTGGAAAAGGAAGG-3'), which binds in the CaMV 35S promoter; JAP62 (5'-GGATAAGCATGGAGATGTGG-3') and EH313 (5'-GGATTACTGCCT-

TABLE 1. Expression vectors and polymerase chain reaction primers used to construct the transformation cassettes

Construct	Primer sequence ^a	Primer no.	Restriction sites	Length of insert (bp)
pKY2/5Rep	5'-CC <u>AAAGCTT</u> AGCCATTAGGTGTCCAAG-3'	EH 312	<i>Hind</i> III	507
	5'-CTCTCGAGGGATTACTGCCTGAATTG-3'	EH 313	<i>Xho</i> I	
pKY Δ 2/5Rep	5'-CTCTCGAGCGACCCACTCTTCAAGTTC-3'	EH 315	<i>Xho</i> I	681
	5'-GCTCTAGAAAATAGCCATTAGCTGTCC-3'	EH 316	<i>Xho</i> I	
pKYC4	5'-CTCTCGAGCGACCCACTCTTCAAGTTC-3'	EH 315	<i>Xho</i> I	676
	5'-CC <u>AAAGCTT</u> AGCCATTAGGTGTCCAAG-3'	EH 312	<i>Hind</i> III	
pKY Δ C4	5'-CTCTCGAGCGACCCACTCTTCAAGTTC-3'	EH 315	<i>Xho</i> I	547
	5'-CCTCTAGACCCAATTGTTCTCTCTCTA-3'	EH 337	<i>Xba</i> I	
pKYRep	5'-CC <u>AAAGCTT</u> AGCCATTAGGTGTCCAAG-3'	EH 312	<i>Hind</i> III	1,192
	5'-CTCTCGAGGTAGTATGAGCAGCCACAG-3'	EH 330	<i>Xho</i> I	
pKYRep Δ 2/5Rep	5'-CC <u>AAAGCTT</u> AGCCATTAGGTGTCCAAG-3'	EH 312	<i>Hind</i> III	1,878
	5'-CTCTCGAGGTAGTATGAGCAGCCACAG-3'	EH 330	<i>Xho</i> I	
	5'-CTCTCGAGCGACCCACTCTTCAAGTTC-3'	EH 315	<i>Xho</i> I	
	5'-GCTCTAGAAAATAGCCATTAGCTGTCC-3'	EH 316	<i>Xba</i> I	
pKPNRep	5'-GTGAGCTCTAGTATGAGCAGCCACAG-3'	EH 317	<i>Sst</i> I	1,193
	5'-GCTCTAGAAAATAGCCATTAGGTGTCC-3'	EH 316	<i>Xba</i> I	
pKPN Δ Rep	5'-GCTCTAGAAAATAGCCATTAGGTGTCC-3'	EH 316	<i>Xba</i> I	1,193
	5'-GTGAGCTCTAGTATGAGCAGCCACAG-3'	EH 317	<i>Sst</i> I	

^a Underlined sequences represent restriction sites.

GAATTG-3'), which bind in the middle of the antisense sequence of the TYLCV *Rep* gene; JAP83 (5'-TCCCACTATCCTTCG-CAAGACC-3'), which binds in the CaMV 35S promoter; JAP84 (5'-ATCATCGCAAGACCGGCAAC-3'), which binds in the NOS terminator; and EH316 (5'-AAATAGCCATTAGGTGTCC-3') and EH337 (5'-CCCAATTGTTCTCTCTA-3') (Table 1), which bind in the TYLCV *Rep* gene and were used to obtain some of the inserts.

All primer sets described above used the same PCR program to amplify products. The reaction conditions were as described above for the construction of the transgenes. The PCR program had an initial denaturing step at 92°C for 2 min, followed by 35 cycles of 1 min at 94°C for denaturing, 20 s at 60°C for annealing, and 30 s

at 72°C for elongation, ending with an extended elongation at 72°C for 7 min followed by an indefinite hold at 4°C (GeneAmp PCR System 9700; PE Applied Biosystems, Foster City, CA).

Plant inoculation and virus detection. TYLCV and whitefly cultures. The TYLCV isolate used in this study was obtained originally from an infected tomato plant growing in a commercial planthouse in Homestead, FL in 1997 (24). This virus has been fully sequenced (GenBank accession no. AY530931) and has been identified as an isolate of TYLCV first recognized in Israel (GenBank accession no. X15656) (7). Adult populations of *B. tabaci* biotype B, which were identified by isozyme analysis, were used in this study. Whiteflies were reared on TYLCV-infected tomato ('Florida Lanai') in a growth chamber (23 to 25°C, 40% relative humidity, 13 h of light). Whiteflies of mixed age and gender were obtained from infected plants by gently tapping the plants to dislodge whiteflies. Those that fell or flew onto a yellow plastic card were collected with an aspirator.

Inoculation of TYLCV to R_1 -generation plants. Approximately 15 R_1 progeny of every R_0 plant were evaluated for resistance to TYLCV in the greenhouse. Tomato seed were germinated in Todd planter flats (128 cells/flat) and were inoculated at the four- to six-true-leaf stage in a greenhouse at the Gulf Coast Research and Education Center in Bradenton, FL. Todd planter flats containing the tomato seedlings were placed on shallow trays for watering and enclosed in white nylon organdy bags with an internal support made of PVC plastic pipe. Whiteflies were collected from TYLCV-infected plants. An average of 10 adult whiteflies per test plant were added two times, 1 week apart. Tomato seedlings were watered daily by filling the tray with water poured through the sides of the bags so that whitefly feeding was not disturbed, and so that whitefly escape during the inoculation period was minimized. Whiteflies were allowed to remain on test plants for 14 days and then whiteflies were killed by applying a drench of imidacloprid (Admire 2F at 4 ml per liter of water) to the test plants. The tomato seedlings then were moved to a large enclosed greenhouse with natural light.

Both transformed and nontransformed plants were whitefly inoculated with TYLCV. Each tray contained six lines (five lines of interest plus the nontransformed control) with 15 plants each. Plants from the different lines were evenly distributed throughout the tray in order to allow a more effective inoculation.

Inoculation of TYLCV to R_2 -generation plants. R_2 -generation plants from 13, 5, and 2 R_1 -generation plants transformed with 2/5Rep, $\Delta 2/5$ Rep, and the Rep $\Delta 2/5$ Rep transgenes, respectively, were evaluated for resistance to TYLCV under field conditions. In the case of the 2/5Rep construct, we evaluated the progeny of 13 R_1 -generation plants selected from a total of 31 R_1 -generation parents. These 13 were selected to represent lines where there was low, intermediate, and high frequencies of resistance. Plants were seeded as for R_1 -generation plants. Plants were inoculated at the four- to six-true-leaf stage with nontransformed plants (20 per flat) located randomly in the flats in order to measure the inoculation rate. Plants were inoculated in small, enclosed greenhouses using whiteflies which were shaken from TYLCV-infected tomato plants each morning for a 3-week inoculation period. Additional infected plants containing whiteflies were added 1 week into the inoculation period to increase the supply of viruliferous whiteflies. Seedlings were lightly brushed and trays were rotated twice each day to decrease whitefly aggregation. The seedlings were hardened off during the last week of the inoculation period. The inoculation period was ended with an application of imidacloprid. Plants then were planted in the field.

Tomato seedlings were planted in the field at a plant spacing of 0.6 m and a row spacing of 1.5 m. Imidacloprid (0.03% Admire 2F) and fertilizer (4 g of 20-20-20 per liter) were added in the setting water. Standard production practices were followed for the remainder of the season. A total of 23 R_2 -generation lines plus two nontransformed lines (Fla. 7613 and Fla. 7324) were evaluated.

Constructs not to scale

Fig. 1. Diagram demonstrating the development of Tomato yellow leaf curl virus (TYLCV) gene expression constructs engineered into pKYLX71:35S² vector or nonenhanced promoter vector. **A**, Gene fragment containing the full-length sequence of the TYLCV *Rep* gene was cloned directly into pKYLX:35S². **B**, A modified (nonenhanced 35S promoter) binary vector pKYLX into which the full-length sequence of the TYLCV *Rep* gene was cloned. In the generic construct, LB = left border and RB = right border of the T-DNA; 35S² = modified CaMV 35S promoter with a duplicated "enhancer" region; TYLCV insert = insertion site of the various TYLCV constructs; RBCS Ter = rbcS-terminator; and NPT II = kanamycin resistance gene. In the construct names, T stands for "truncated"; Δ stands for antisense. TYLCV sequence GenBank accession no. AY530931. Capital letters are carryovers from restriction sites used in construction. Constructs are not to scale.

Approximately 60 plants per line were evaluated in a randomized complete block design, with approximately 20 plants per block.

Determining resistance to TYLCV in R₁- and R₂-generation transformed tomato plants. *R₁ generation.* Plants were screened for virus symptoms beginning 3 weeks after the end of the inoculation access period and continuing weekly until plants were discarded after seed collection. Tissue samples of young leaves were collected from all plants at 4, 8, and 12 weeks after the inoculation access period and were tested by spot hybridization (4 and 8 weeks) and by PCR (12 weeks). Plants were screened for the presence or absence of TYLCV symptoms. The probe used in the nucleic acid spot hybridization assay was a 351-nt portion of the TYLCV genome that did not overlap with the TYLCV sequences used in the transgenes. The probe DNA is composed of nucleotides 2,750 to 320, which represent a region 10 nt upstream of the stemloop to 18 nt into the coat protein gene. Standard hybridization conditions and stringent washing conditions consisted of 65°C hybridization overnight; two washes of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature (RT) for 5 min each; two washes of 0.2× SSC, 1.0% sodium dodecyl sulfate (SDS) at 65°C for 15 min each; followed by two washes of 0.1× SSC, 1.0% SDS at RT for 15 min each. PCR screening used primers PCRv181 and PAL1c496, which would produce an amplicon of approximately 500 nt (27). Plants infected with TYLCV were discarded. Resistant plants were tested for the presence of the transgene at 12 weeks after inoculation and once more just before seed was collected.

R₂ generation. Resistance was evaluated by symptom expression, dot spot hybridization, and PCR. Tissue samples of young leaves were collected from all plants at 4, 8, and 12 weeks after the inoculation and were tested by nucleic acid spot hybridization (4 and 8 weeks) and by PCR (12 weeks). Plants were tested for infection with TYLCV as described for the R₁ generation.

RESULTS

R₀-generation plants. We were able to obtain plants transformed with all eight constructs (Fig. 1) but the number of viable (seed-bearing) transformants for each construct varied (Table 3). Plants transformed with the C4 construct had a highly altered phenotype with greatly reduced lateral growth and flowers growing from the main stem. No viable transformants were obtained with this construct. The R₁ generation progeny of all viable R₀ plants were evaluated with the exception of R₀ plants containing the 2/5Rep transgene. Because of the large number of R₀ 2/5Rep plants, only the progenies of 34 out of 46 lines were evaluated (Table 4).

Greenhouse evaluation of R₁-generation plants. An average of 15 plants in each R₁-generation line were evaluated for resistance to TYLCV (Table 4). We determined that the transgene was transferred to the next generation in a majority of the R₁-generation lines with the exception of the Rep and NΔRep, where only one line of each had transformed plants. The majority of the R₁-generation lines transgenic for either the 2/5Rep (21/31) or the

Δ2/5Rep (15/19) had plants with no symptoms of TYLCV (Fig. 2). For the RepΔ2/5Rep transgene, only 3/10 R₁ lines were symptomless. No TYLCV DNA could be detected by either spot hybridization or PCR in symptomless plants. These plants had normal phenotypes. No resistant plants were observed in R₁-generation lines transformed with ΔC4, Rep, NRep, or NΔRep.

The resistance obtained using the 2/5Rep, Δ2/5Rep, and the RepΔ2/5Rep constructs varied in frequency among the lines transformed with the same construct (Table 4). There was a significant amount of segregation for resistance in the R₁-generation lines, as expected. The mean frequency of resistant plants among all lines evaluated varied from 26.3% for Δ2/5Rep-transformed plants to 47.7% for RepΔ2/5Rep-transformed plants. In plants that contained the transgene, the frequency of resistance was much higher, with a low of 60.7% for Δ2/5Rep-transformed plants and a high of 79.7% for 2/5Rep-transformed plants. Not all transformed plants were resistant to TYLCV. Seed was collected from selected resistant plants for evaluation in subsequent generations.

Some R₁ plants were observed to have a mildly altered horticultural phenotype. Fruit were not correctly shaped, and leaves showed some mild epinasty. These altered phenotypes were not associated with any one transgene and appeared at a low incidence in some lines. These off types were not selected for advancement to the next generation.

Field evaluation of R₂-generation plants. R₂-generation plants from 13, 5, and 2 R₁-generation plants transformed with 2/5Rep, Δ2/5Rep, and the RepΔ2/5Rep transgenes, respectively, were evaluated for resistance to TYLCV under field conditions. Nontransformed plants were 100% infected with TYLCV by 4 weeks after the end of the inoculation access period. R₂-generation plants were considered resistant when they showed no symptoms of TYLCV infection, tested negative for TYLCV DNA by nucleic acid hybridization at 4, 8, and 12 weeks after the inoculation period, and tested negative for TYLCV DNA by PCR at 12 weeks after the inoculation period. Resistant plants (Fig. 3) were detected in all lines evaluated. As expected, plants in this generation were still segregating for resistance. The frequency of

TABLE 3. Number of R₀-generation tomato plants transformed with *Tomato yellow leaf curl virus Rep* gene constructs from which seed was obtained

Transgene ^a	R ₀ plants ^b
2/5Rep	46
Δ2/5Rep	24
RepΔ2/5Rep	17
ΔC4	18
C4	0
Rep	1
Nrep	10
NΔRep	7

^a Approximately 500 to 600 explants were used for each construct during transformation attempts.

^b Number of plants that produced seed.

TABLE 2. Primers used to detect transgenes in R₁- and R₂-generation transformed plants and expected sizes generated by each transgene^a

Construct	JAP94, EH337	JAP28, EH316	JAP94, EH313	JAP83, JAP84	JAP28, JAP62
pKY2/5Rep	592	...	...	...	539
pKYΔ2/5Rep	...	971	698	...	...
pKYC4	761	...	...	...	539
pKYΔC4	...	...	564	...	...
pKYRep	1,275	...	...	...	539
pKYRepΔ2/5Rep	...	...	698	...	539
pKPNRep	...	...	...	1,343	539
pKPNΔRep	...	...	...	1,343	...

^a One primer binds in the non-*Tomato yellow leaf curl virus* (TYLCV) Rep regions of the construct, the other binds in the Rep. JAP94 binds in the Rubisco terminator, JAP28 binds in the CaMV 35S promoter, JAP83 binds in the CaMV 35S promoter, JAP84 binds in the NOS terminator, EH313 and JAP62 bind in the antisense sequence of the Rep, and EH316 and EH337 bind in the TYLCV Rep gene; ... = no amplicon produced.

TABLE 4. Greenhouse evaluation of R₁-generation tomato plant lines transformed with *Tomato yellow leaf curl virus* (TYLCV) gene constructs for resistance to infection by TYLCV

Transgene	Number of R ₁ -generation				Resistance		
	Plants evaluated ^a	Lines tested	Lines with transgene	Lines with transgene and resistance	Frequency (%)	Mean frequency (%)	Mean frequency in plants with transgene (%)
2/5Rep	437	34	31	21	7–91	42.7	79.7
Δ2/5Rep	258	23	19	15	8–50	26.3	60.2
RepΔ2/5Rep	166	14	10	3	14–69	47.7	77.7
ΔC4	192	18	14	0	0	0	0
Rep	28	2	1	0	0	0	0
NRep	152	12	8	0	0	0	0
NΔRep	40	3	1	0	0	0	0

^a Excludes plants that died during the evaluation.

Fig. 2. Phenotype of the R₁-generation tomato plants being screened for resistance to *Tomato yellow leaf curl virus*. Plant on the left is a nontransformed parent (Fla. 7613), plant on the right is transformed with pKY2/5Rep.

resistant plants varied among R₂-generation lines transformed with the same construct (Table 5). The highest frequency of resistance, 80%, was observed in a line transformed with 2/5Rep. However, plants transformed with the RepΔ2/5Rep construct had the highest mean frequency of resistance (69.6%). Lines transformed with the Δ2/5Rep construct had the lowest mean frequency of resistance (22.3%) compared with the other two constructs. Resistant plants were tested for the presence of the transgene by PCR to confirm both its presence and identity. All resistant plants had a transgene and the identity was confirmed. No altered phenotypes (as observed in the R₁ generation in the greenhouse) were observed in the R₂-generation plants in the field.

DISCUSSION

The best resistance was obtained using constructs that contained IR and 2/5 *Rep* gene sequences of TYLCV in either the sense or antisense orientation (Table 1). Resistance was observed at high frequency in both the R₁ and R₂ generations. The absence of detectable viral DNA by either nucleic acid hybridization or PCR in TYLCV-inoculated plants suggested that the resistance may be immunity.

Several transgenes (ΔC4, Rep, NRep, and NΔRep) did not produce resistance. However, in some cases, the limited number of transformed plants tested does not preclude possible resistance if more transformants had been obtained. R₀ plants that were trans-

Fig. 3. Transgenic tomato showing resistance in the field. Tomato plants inoculated with *Tomato yellow leaf curl virus* and transplanted in the field: A, nontransformed plant of Fla. 7613 and B, R₂-generation plant transformed with pKY2/5Rep (Fla. 7613 genetic background). Picture was taken approximately 60 days after transplanting.

formed successfully with the C4 transgene failed to produce viable seed. The altered phenotype in these plants suggests that the C4 transgene product may have been toxic and caused the plants to be sterile. The presence of the C4 gene sequence in the Rep transgene also may have suppressed the transformation efficiency of this construct (Table 3). The difficulty in obtaining plants transformed with the Rep transgene may have other causes. Day et al. (9) were unable to obtain tobacco plants transformed with a full-length Rep of TGMV and Bendahmane and Gronenborn (3) were unable to regenerate plants that expressed a sense copy of the complete TYLCSV Rep. Bendahmane and Gronenborn (3) ascribed this failure to possible deleterious affects of the Rep protein on the cell.

Transformants that contained the ΔC4 transgene, which only differed from the Δ2/5Rep construct by the absence of IR sequences and a deletion of 48 nt from the 5' end of the Rep, did not produce resistance in the R₁ generation. This indicated that sequences of the IR and possibly the 5' end of the Rep may be correlated with TYLCV immunity. The presence of a nonenhanced promoter (constructs NRep and NΔRep) did not increase the fre-

TABLE 5. Field evaluation of R₂-generation tomato plants transformed with different *Tomato yellow leaf curl virus* (TYLCV) gene constructs for resistance to infection by TYLCV

Transgene	Number of R ₂ -generation			Resistance	
	Lines tested	Plants tested ^a	Lines with resistance	Frequency (%)	Mean frequency (%)
2/5Rep	13	347	13	26.6–80.0	53.8
Δ2/5Rep	4	135	5	12.5–50.0	27.8
RepΔ2/5Rep	2	50	2	66.9–72.2	69.6

^a Excludes plants that died during the evaluation.

quency of resistant plants. Because ΔRep with an enhanced promoter has been shown by others to generate resistance (9), the failure of NΔRep to be effective suggests that the resistance may be dose dependent (30).

Studies were focused on the most basic of the constructs, the 2/5Rep. We evaluated the progeny of 13 R₁-generation plants selected from a possible 31 R₁-generation parents in the field and all showed similar strong resistance or immunity to TYLCV. Under field conditions, the plants were exposed to possible natural variants of TYLCV and to possible resistance suppression by infection with other viruses. Plants tested during three growing seasons for TYLCV resistance all showed normal-looking phenotypes and fruit.

The resistance or immunity associated with the Δ2/5Rep transgene indicates that expression of the Rep protein is not necessary for resistance. This is in contrast to the studies of Brunetti et al. (4,5), which found a strong correlation between TYLCV resistance in transgenic tomato and high levels of Rep protein expression.

The three TYLCV *Rep* gene constructs that presented TYLCV resistance all contained sequences (IR) upstream of the expected start of the *Rep* gene transcript. The upstream sequence contains sequence specific elements (iterons) for Rep protein binding during the rolling circular replication of begomoviruses (13). Iterons consist of 8 to 12 nt-direct and -inverted repeats upstream from the transcription start of the *Rep* (2). TYLCV iterons are arranged such that there is one in the sense direction and three in the opposite direction. The inverted repeats are capable of forming hairpin structures when transcribed to RNA. In addition, the RepΔ2/5Rep, a construct which would be expected to form a large hairpin structure in the transcript (the antisense 2/5Rep segment binding to sense segment of the 5' end of the full-length *Rep*, leaving nucleotides 1,505 to 2,020 to form a large loop), also showed high frequencies of immunity in transgenic lines. The VMYMV interfering DNA constructs described by Pooggin et al. contain the iteron region of VMYMV (25). Constructs that express both sense and antisense RNA and thus form double-stranded RNA hairpin loops have been shown to be very effective in gene silencing, often generating immunity (29,31,32). Both the hairpin structures (14,32) and direct and indirect repeats (19) have been shown to be efficient triggers of PTGS. Han and Grierson (14) found that adding inverted repeats to the 5' end of the untranslated region of their transgene led to stronger silencing.

The virus resistance attributed to PTGS often is expressed as immunity, is associated with transgene transcript suppression, and is characterized as narrow spectrum (6). Characteristics of TYLCV resistance expressed by our constructs implicate gene silencing. The resistance associated with the 2/5Rep transgene showed immunity and transgene transcript suppression (10). Studies are in progress to determine the breadth of resistance and whether this resistance is due to PTGS.

The resistance to TYLCV demonstrated in this study is superior to that of commercially available resistances derived from wild *Lycopersicon* spp. due to the absence of virus in resistant plants, and the ability to resist infection in spite of early and intense inoculation pressure. Our study differs from other transgenic *Geminivirus* resistance reports involving the *Rep* gene in

that viruliferous whiteflies were used for challenge inoculation instead of agroinoculation or biolistic inoculation, and in the use of the IR sequences in our transgene constructs. Another distinctive feature of this study was the evaluation of the transgenic resistance for TYLCV under field conditions. The minimal-sized, 2/5 TYLCV *Rep* constructs generated resistance to TYLCV that is more robust than commercially available resistances. This construct also was tested in transgenic tobacco and found to confer similar immunity to TYLCV, indicating that this construct is a strong inducer of TYLCV resistance in independent transformation events and in a different genetic background (10). The presence of the transgenes was not associated with an altered horticultural phenotype. Further development of this immunity has great potential in the commercialization of cultivars with superior resistance to whitefly transmitted geminiviruses (begomoviruses). Patent protection of this construct has been initiated.

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