**Ty-1, a universal resistance gene against geminiviruses that is compromised by co-replication of a betasatellite**

**CRIEN M. VOORBURG**¹,¹, ZHE YAN²,¹, MARIA BERGUA-VIDAL³, ANNE-MARIE A. WOLTERS², YULING BAI² AND RICHARD KORMELINK †,¹,*

¹Laboratory of Virology, Wageningen University & Research, Droevendaalsesteeg 1, Wageningen, 6708PB, Netherlands
²Plant Breeding, Wageningen University & Research, Droevendaalsesteeg 1, Wageningen, 6708PB, Netherlands

**SUMMARY**

Tomato yellow leaf curl virus (TYLCV), a begomovirus, causes large yield losses and breeding for resistance is an effective way to combat this viral disease. The resistance gene Ty-1 codes for an RNA-dependent RNA polymerase and has recently been shown to enhance transcripational gene silencing of TYLCV. Whereas Ty-1 was earlier shown to also confer resistance to a bipartite begomovirus, here it is shown that Ty-1 is probably generic to all geminiviruses. A tomato Ty-1 introgression line, but also stable transformants of susceptible tomato cv. Moneymaker and *Nicotiana benthamiana* (*N. benthamiana*) expressing the Ty-1 gene, exhibited resistance to begomoviruses as well as to the distinct, leafhopper-transmitted beet curly top virus, a curtovirus. Stable Ty-1 transformants of *N. benthamiana* and tomato showed fewer symptoms and reduced viral titres on infection compared to wild-type plants. TYLCV infections in wild-type *N. benthamiana* plants in the additional presence of a betasatellite led to increased symptom severity and a consistent, slightly lowered virus titre relative to the high averaged levels seen in the absence of the betasatellite. On the contrary, in Ty-1 transformed *N. benthamiana* viral titres increased in the presence of the betasatellite. The same was observed when these Ty-1-encoding plants were challenged with TYLCV and a potato virus X construct expressing the RNA interference suppressor protein bC1 encoded by the betasatellite. The resistance spectrum of Ty-1 and the durability of the resistance are discussed in light of antiviral RNA interference and viral counter defence strategies.

**Keywords:** beet curly top virus, betasatellite, geminivirus, resistance, RNA interference, tomato yellow leaf curl virus, Ty-1.

**INTRODUCTION**

Tomato yellow leaf curl virus (TYLCV), a begomovirus, is the representative of the Old World monopartite begomoviruses within the family Geminiviridae. The virus belongs to the most devastating plant viruses worldwide and causes major losses in economically important crops like tomato. The virus mainly occurs in the (sub)tropical and Mediterranean regions worldwide due to the distribution of its vector, the whitefly *Bemisia tabaci* (*B. tabaci*) (Moriones and Navas-Castillo, 2000). TYLCV, and all monopartite begomoviruses, have a single, circular, single-stranded DNA genome of c. 2.7 kb in size. In the field, monopartite begomoviruses are frequently observed with co-replicating alpha- or betasatellites (Nawaz-ul-Rehman and Fauquet, 2009). Both satellites are approximately half the size of begomovirus genomes. While less is known about alphasatellites and their role in the pathogenesis of begomoviruses, co-replication of betasatellites often leads to more severe disease symptoms (Zhou, 2013); therefore, betasatellites are regarded as pathogenicity determinants. Betasatellites code for one single protein, bC1, that is known to suppress the antiviral defence mechanism transcriptional gene silencing (TGS) (Yang et al., 2011; Zhou, 2013).

Because the vector *B. tabaci* is difficult to control, the most effective way to combat TYLCV infections is to breed for resistance. Six resistance genes are known, namely Ty-1 to Ty-6, and some of these are widely used for introgression breeding. Five of these genes are derived from wild tomato species: Ty-1, Ty-3, Ty-4 and Ty-6 are from *Solanum chilense* (*S. chilense*), Ty-2 is from *S. habrochaites*, while ty-5 was identified in an old commercial tomato cultivar Tyking (Hanson et al., 2006; Hutton and Scott, 2014; Hutton et al., 2012; Ji et al., 2007, 2009; Lapidot et al., 2015; Zamir et al., 1994). On viral challenge, plants containing any of these genes do not seem to completely abolish infection as reduced virus titres are still observed in comparison to susceptible cultivars, nor is a hypersensitive response seen (Castro et al., 2005; Maruthi et al., 2003; Picó et al., 1999, 2000). In recent years several of these genes have been cloned and characterized; only Ty-4 and Ty-6 are not. Ty-2 encodes a nucleotide-binding leucine-rich repeat (NB-LRR) protein, while the ty-5 gene encodes an mRNA surveillance factor and contains a mutation that hampers viral protein translation (Lapidot et al., 2015; Yamaguchi et al., 2018). Ty-1 and Ty-3 are...
allelic and code for an RNA-dependent RNA polymerase (RDR) from the \( \gamma \)-class and distinct from the well-established RDRs from the \( \alpha \)-class with recognized functions in the amplification of the antiviral RNA interference (RNAi) response (Verlaan et al., 2012). Until recently, no function was assigned to any of the RDRs from the \( \gamma \)-class, but with the identification of Ty-1 a new class of resistance genes was unveiled.

Besides a major role in gene regulation and chromosome dynamics, RNAi (also named RNA silencing) presents a major antiviral defence mechanism in plants (Shou-Wei and Voinnet, 2007). The mechanism is induced by double-stranded (ds) RNA molecules, which arise from viral replicative intermediates or secondary RNA folding structures in genomic or messenger RNA molecules. After their processing by Dicer-like proteins (DCL) into small interfering (si) RNA molecules of 21–24 nucleotides (nt), one strand of the siRNA is uploaded into an RNA-induced silencing complex (RISC), leading to its activation (Hammond, 2005). When this complex is loaded with a 21 nt siRNA strand and contains an Argonaute 1 (AGO1) core component, it is able to target RNA molecules with sequence complementarity to the siRNA strand, leading to their degradation or translational arrest (Mallory and Vaucheret, 2010). This process is generally referred to as post-transcriptional gene silencing (PTGS). In contrast, AGO4 containing RISC is loaded with a 24 nt siRNA strand. This complex targets cytosine methyltransferases to complementary DNA molecules, causing cytosine methylation within the target sequence. This methylation leads to TGS of the DNA sequence (Mallory and Vaucheret, 2010).

The RNAi response in plants, in contrast to insects and animals, is amplified by RDRs (Donaire et al., 2008; Wang et al., 2010). During this process aberrant RNA molecules, resulting from the first cleavage by the slicer activity of AGO1, are recognized by RDRs and converted into more dsRNA molecules of the target sequence. Their processing by DCLs produces a second generation of siRNAs. Due to this amplification a strong (antiviral) RNAi response is mounted. In the Arabidopsis thaliana genome, six RDR genes have been annotated, encoding RDR1 to RDR6. From those, RDR1, RDR2 and RDR6 represent the \( \alpha \)-class, whereas RDR3, RDR4 and RDR5 fall into the \( \gamma \)-class (Willmann et al., 2011). Silencing of RDR1 and RDR6 from the \( \alpha \)-class reduces the antiviral PTGS response and makes plants highly susceptible to RNA viruses, but the RNAi response against geminiviruses does not seem to be affected much by silencing of these RDRs (Aregger et al., 2012; Raja et al., 2008; Yu et al., 2003). This finding not only points to the involvement of other RDRs in the RNAi response against geminiviruses, but also supports the idea that the TGS pathway is the most important RNAi pathway against geminiviruses. In agreement with this hypothesis are findings showing that plants knocked out for proteins from the TGS pathway are hypersusceptible to geminivirus infection (Jackel et al., 2016; Raja et al., 2008).

After the identification of Ty-1 as an RDR \( \gamma \)-class member, its involvement in the RNAi amplification was demonstrated by an increase of siRNA production and an elevated rate of cytosine methylation in viral DNA collected from TYLCV-challenged Ty-1 tomato plants (Butterbach et al., 2014).

While the Ty-1 resistance gene is generally deployed to combat TYLCV, it has been shown to confer resistance to begomoviruses other than TYLCV (Barbieri et al., 2010; Butterbach et al., 2014; Pietersen and Smith, 2002; Prasanna et al., 2015; Shahid et al., 2013). However, whether the resistance is also effective against members of other genera, e.g. the Curtovirus genus, is still unknown. Furthermore, all previous studies on Ty-1 so far have been conducted using introgression lines and therefore it still remains to be questioned whether the resistance is solely due to the expression of Ty-1 or could involve other chromosomal introgression(s). Lastly, the earlier study by Butterbach et al. (2014) pointed towards an Achilles’ heel of Ty-1 resistance, namely suppression of TGS by viral RNAi suppressor proteins. In that study the Ty-1 resistance against TYLCV was compromised by a co-infection with cucumber mosaic virus (CMV) (Butterbach et al., 2014). This effect was attributed to the CMV-encoded RNAi suppressor 2b that was shown to inhibit AGO4 activity during TGS (González et al., 2010; Hamera et al., 2012). Since betasatellites encode suppressors of TGS, the compromising nature of betasatellites, by their encoded P1 protein, on Ty-1 resistance could be anticipated.

Here it is shown that Ty-1 from a tomato introgression line, but also after stable transformation into susceptible tomato Moneymaker (MM) and Nicotiana benthamiana (N. benthamiana), confers resistance to a completely distinct leafhopper transmitted curtovirus, beet curly top virus (BCTV). Furthermore, TYLCV resistance in transgenic Ty-1 plants is compromised not only by a co-replicating betasatellite, but also by transient co-expression of its encoded TGS suppressor protein only.

RESULTS

Broad-spectrum resistance against geminiviruses in Ty-1 introgression line

Ty-1 has been shown to confer resistance not only to the monopartite TYLCV, but also to the bipartite begomovirus tomato severe rugose virus (ToSRV) (Butterbach et al., 2014). In light of its role in the amplification of RNAi, a mechanism that is antiviral and generic to all viruses, it was anticipated that Ty-1 resistance would not be restricted to species from the TYLCV cluster. To test this hypothesis and determine the resistance spectrum of Ty-1, a Ty-1 breeding line was challenged with various TYLCV-like viral species, i.e. TYLCV isolates from Spain and China (both belonging to the TYLCV Israel species), tomato yellow leaf curl Sardinia virus (TYLCSV), as well as a
C. M. VOORBURG et al.

completely distinct geminivirus, namely the leafhopper-transmitted BCTV, which is a representative of the Curtovirus genus. Plants were monitored for 7 weeks by scoring disease symptoms and viral titres were determined. Whereas the susceptible control MM showed severe disease symptoms after a challenge with all (curto- and begomo-) viruses, the Ty-1 breeding line remained (almost completely) symptomless on a challenge with the TYLCV Almeria isolate, TYLCV-[CN:SH2], TYLCSV and BCTV (Fig. 1A). Subsequently, the virus titres were determined via quantitative PCR (qPCR) and the fold difference in titre

(A)

![Symptoms upon infection with mock-inoculation, TYLCV Almeria isolate (Alm), TYLCV-[CN:SH2], TYLCSV and BCTV in susceptible control (Solanum lycopersicum 'Moneymaker', MM) and Ty-1 introgression plants at 50 days post-inoculation (dpi). In the left upper corners the symptom scores are depicted as mean of five biological replicates ± the standard deviation.](image)

(B)

![Virus titre quantification of TYLCV-Alm, TYLCV-[CN:SH2], TYLCSV and BCTV in MM and the Ty-1 introgression line. Values were normalized relative to tomato EF1α and calibrated to the levels in MM plants (set to 1). Dots represent the relative virus titre of individual plants. Lines represent means and standard error of the mean of biological replicates. Asterisks indicate significant differences between Ty-1 introgression line and MM according to one-way analysis of variance (**P < 0.01, ***P < 0.001).](image)

Fig. 1 The Ty-1 introgression line is resistant against a broad range of geminiviruses. (A) Symptoms upon infection with mock-inoculation, TYLCV Almeria isolate (Alm), TYLCV-[CN:SH2], TYLCSV and BCTV in susceptible control (Solanum lycopersicum 'Moneymaker', MM) and Ty-1 introgression plants at 50 days post-inoculation (dpi). In the left upper corners the symptom scores are depicted as mean of five biological replicates ± the standard deviation. (B) Virus titre quantification of TYLCV-Alm, TYLCV-[CN:SH2], TYLCSV and BCTV in MM and the Ty-1 introgression line. Values were normalized relative to tomato EF1α and calibrated to the levels in MM plants (set to 1). Dots represent the relative virus titre of individual plants. Lines represent means and standard error of the mean of biological replicates. Asterisks indicate significant differences between Ty-1 introgression line and MM according to one-way analysis of variance (**P < 0.01, ***P < 0.001).
between the susceptible MM plants and the Ty-1 introgression line calculated using the ΔΔ Ct method (Livak and Schmittgen, 2001). While in MM plants titres were high for all four viruses (Ct values between 14 and 16), their amounts were significantly reduced in Ty-1 introgression lines (Fig. 1B). Ty-1 resistance seemed most effective to TYLCV and TYLCV-[CN:SH2], for which (hardly) no virus was detected in Ty-1 plants (Ct values between 32 and 34). TYLCV and BCTV were still clearly detected in Ty-1 plants (Ct values between 18 and 20), but TYLCV titres dropped c. 18-fold and BCTV titres c. 3-fold in Ty-1 plants compared to MM plants (Fig. 1B).

Transgenic expression of Ty-1 in tomato confers broad-spectrum resistance to geminiviruses

To rule out any other chromosomal introgression(s) being involved, and to ensure that the broad-spectrum resistance was solely due to the expression of the Ty-1 gene, susceptible tomato MM was transformed with a copy of the Ty-1 gene for a functional complementation study. Agrobacterium-mediated transformation of MM plants with a 35S promoter-driven Ty-1 construct resulted in nine primary transformants (T1). After selfing of the T1 plants, a T2 progeny was obtained that was challenged with TYLCV and monitored for phenotypic responses. In all the subsequent experiments, the Almeria isolate of TYLCV was used as representative of the Begomovirus genus, as was also done in previous Ty-1 research (Butterbach et al., 2014; Verlaan & Sons LTD, 2001). While in MM plants titres were high for all four viruses, when virus titres were determined a c. 100-fold result in both T4 lines was less profound than that of TYLCV (Fig. 2D). Plants from the two T4 lines that exhibited mild BCTV symptoms showed relatively higher BCTV titres compared to plants that remained symptomless.

Transgenic expression of Ty-1 in N. benthamiana confers resistance to TYLCV and BCTV

To further substantiate the findings on broad-spectrum resistance conferred by Ty-1, and to test whether the Ty-1 resistance gene also functions in other Solanaceae species, stable transformants of N. benthamiana plants were generated expressing Ty-1 driven by a 35S promoter. In analogy to the transformation of tomato MM (previous section), stable transformants were selected based on a TYLCV resistant phenotype and selfed until T4. Expression levels of Ty-1 were determined by RT-qPCR in four transgenic lines selected from two independent transformations (Fig. 3A). The four transgenic lines showed a comparable high level of expression of Ty-1. Whereas values for Ct were in the range of c. 25 for all four transgenic lines, no signal was obtained from wild-type (WT) N. benthamiana samples due to the absence of a ty-1 homologue in WT N. benthamiana that could be amplified by the primers used. The Ct values were normalized to the expression of the housekeeping gene Elongation Factor 1α (EF1α), and the ΔΔ Ct values were calculated to compare Ty-1 transgenic plants with WT N. benthamiana, showing clear and high expression levels in Ty-1 plants. On infection with TYLCV no clear symptoms were observed in all four lines (Fig. 3B), and when virus titres were determined a c. 100-fold reduction was observed in the transgenic lines compared to WT N. benthamiana plants (Fig. 3C).

When the stable Ty-1 N. benthamiana transformants were challenged with BCTV, only a slight curling and chlorosis (average symptom score 1.5) was observed at 18 dpi, whereas WT N. benthamiana revealed a strong curling and major chlorosis of systemic leaves (average symptom score 3.8) (Fig. 3B). BCTV titres showed a c. 15-fold reduction relative to the titres

© 2019 THE AUTHORS. MOLECULAR PLANT PATHOLOGY PUBLISHED BY BRITISH SOCIETY FOR PLANT PATHOLOGY AND JOHN WILEY & SONS LTD. MOLECULAR PLANT PATHOLOGY (2020) 21(2), 160–172
obtained from susceptible *N. benthamiana*, which was smaller than the difference in TYLCV titres (between 70- and 180-fold) (Fig. 3C). This smaller effect on BCTV compared to TYLCV was consistently observed during repeated experiments and resembled the observations in the *Ty-1* tomato introgression line and transgenic tomato.
Compromising the universal resistance of Ty-1

Earlier, a co-infection of CMV was shown to compromise Ty-1 resistance (Butterbach et al., 2014), likely due to the abrogation of AGO4 activity by the CMV 2b RNAi suppressor protein. Under natural field conditions TYLCV is often observed with co-replicating betasatellites, elements that encode suppressors of TGS (Yang et al., 2011). To test whether betasatellites also compromise Ty-1 resistance, susceptible MM and Ty-1 introgression tomato plants were co-infected with TYLCV and an ageratum yellow vein virus associated betasatellite (AYVB) via agroinfiltration. In parallel, WT N. benthamiana plants were infected with either TYLCV alone or in combination with the betasatellite. At 21 dpi, no differences in viral symptoms were observed between TYLCV singly infected and TYLCV plus betasatellite co-infected tomato plants (results not shown).
shown). However, PCR analysis of systemically infected tomato leaves for the presence of the betasatellite turned out negative (data not shown), indicating that the betasatellite did not co-replicate with TYLCV in tomato. In contrast to tomato, at 19 dpi curling and yellowing of systemically infected leaves was more severe in N. benthamiana plants co-infected with TYLCV and the betasatellite compared to plants only infected with TYLCV (Fig. 4A). The presence of the betasatellite in systemically infected leaves was confirmed by PCR (data not shown). For this reason, the compromising nature of betasatellites, and their encoded TGS suppressor protein, on Ty-1 resistance was further analysed using stably transformed N. benthamiana Ty-1 plants. To this end, Ty-1 stable transformants, next to WT N. benthamiana plants, were challenged with TYLCV singly or in a mixed setting with the betasatellite. At 19 dpi WT plants again showed yellowing and curling of the top leaves in the presence of TYLCV, whereas the transgenic Ty-1 N. benthamiana plants only exhibited very mild symptoms. In the additional presence of the betasatellite both WT and transgenic plants exhibited more severe yellowing and curling, although the symptoms in the transgenic plants infected with TYLCV and the betasatellite were less pronounced than in WT plants infected with TYLCV only (average symptom score WT + TYLCV of 2.7, Ty-1 + TYLCV + betasatellite of 2, see Fig. 4A). When virus titres were determined by qPCR, a co-infection with the betasatellite led to a c. 2-fold increase of TYLCV titres in systemically infected leaves from Ty-1 transgenic plants, while a reduction in virus titres was observed in WT (susceptible) plants (Fig. 4B). This difference was observed in all four transgenic lines and during two independent repetitions.

**Co-expression of βC1 is sufficient to compromise Ty-1 resistance**

To test whether the compromising effect of the betasatellite on Ty-1 resistance against TYLCV was due to co-replication of the betasatellite with TYLCV or to suppression of TGS by the betasatellite encoded protein βC1, experiments were repeated, but this time Ty-1 stable transformants were challenged with TYLCV in the presence of a potato virus X (PVX) vector expressing the βC1 protein. Considering that so far βC1 protein TGS suppression activity has been reported only for tomato yellow leaf curl China virus associated betasatellite (TYLCCNB) and in the experiments here AYVB was used, TYLCCNB-βC1 and a functionally deficient mutant were included as positive and negative control, respectively (Cui et al., 2005; Yang et al., 2011). Twenty-five-day-old WT and Ty-1 transgenic N. benthamiana plants were infected with TYLCV via agroinfiltration and at 10 dpi were challenged with PVX from which no protein, the βC1 protein of AYVB, the βC1 protein of TYLCCNB or the mutated TYLCCNB-βC1 protein was expressed. The challenge with PVX was optimized at 10 days post-TYLCV agroinfection due to PVX causing systemic infections much faster than TYLCV. Eight days post-PVX infection plants were analysed for development of disease symptoms caused by TYLCV and/or PVX. Since PVX (empty vector) infections also caused chlorosis of...
the top leaves, these plants were more difficult to score for TYLCV symptoms. Whereas WT *N. benthamiana* plants infected with TYLCV showed symptoms as earlier observed (Fig. 5A), a mixed infection with PVX-TYLCCNB-βC1 or PVX-AYVB-βC1 intensified the curling and chlorosis (Fig. 5A) similarly to that during a co-infection with the betasatellite (Fig. 4). During a co-infection of TYLCV and the PVX-TYLCCNB-βC1 mutant (encoding a nonfunctional mutant βC1 TGS suppressor) only some necrosis and increased

![Fig. 5](https://example.com/fig5.png)

**Fig. 5** βC1 protein expressed via a PVX vector compromises resistance against TYLCV in Ty-1 transgenic *Nicotiana benthamiana*. (A) Wild-type (WT) and Ty-1 transgenic *N. benthamiana* plants were infected with TYLCV and at 10 days post-inoculation (dpi) were challenged with PVX expressing TYLCCNB-βC1, TYLCCNB-βC1 mutant, AYVB-βC1 or empty PVX vector. Eight days post-PVX infection, pictures were taken and plants were scored for disease symptoms, indicated in the bottom right corner. (B) From the plants described under 5a, samples of systemically infected leaves were collected, DNA was isolated and the titre of TYLCV was determined via quantitative PCR. The viral titre was normalized to the presence of genomic DNA (25S rRNA) and the fold difference was calculated according to the $2^{-\Delta\Delta C_t}$ method relative to WT plant infected with TYLCV only (set to 1). Every point represents one plant. Lines represent means and standard error of the mean of biological replicates. Note that the y-axis is split to show the low titres. Asterisks indicate significant differences according to one-way analysis of variance. In the analysis WT TYLCV-only infected plants were compared with the different WT co-infected groups and the same was done for the Ty-1 transformants (**P < 0.01; ***P < 0.001).
chlorosis was observed compared to TYLCV only. In the stably transformed Ty-1 N. benthamiana plants, TYLCV only caused some slight chlorosis, but the additional presence of PVX-AYVB-βC1 or PVX-TYLCCNB-βC1, and not of PVX-TYLCCNB-βC1 mutant, caused severe curling and chlorosis. To determine whether the increase in symptoms in the susceptible WT and Ty-1 transgenic N. benthamiana plants also correlated with a higher TYLCV titre, samples were collected from systemically infected leaves and DNA purified for qPCR analysis. In WT N. benthamiana no significant change in virus titres was observed with TYLCV only versus a mixed infection with TYLCV and one of the PVX constructs (Fig. 5B). In the case of a mixed infection with PVX-AYVB-βC1 a trend of increased TYLCV titres was observed, which contrasted with the reduced titres seen in the presence of AYVB. In the Ty-1 transgenic plants challenged with TYLCV, virus titres were again drastically reduced compared to WT plants, and these titres did not change with the addition of PVX-empty or PVX-TYLCCNB-βC1 mutant. However, in the additional presence of PVX-AYVB-βC1 or PVX-TYLCCNB-βC1 TYLCV titres increased 2.7- and 2-fold, respectively (Fig. 5B). These data indicate that the presence of a betasatellite compromises Ty-1 resistance by means of its encoded TGS suppressor, the βC1 protein.

**DISCUSSION**

Earlier we showed that Ty-1 confers resistance against the monopartite TYLCV (Israel strain, Almeria isolate) and the bipartite begomovirus ToSRV. We also showed that Ty-1 resistance involves an amplification of the antiviral RNAi response, leading to increased levels of viral siRNA production and concomitant cytosine methylation within the viral DNA genome (Butterbach et al., 2014). Here it is shown that, besides TYLCV, comitant cytosine methylation within the viral DNA genome leading to increased levels of viral siRNA production and conance involves an amplification of the antiviral RNAi response, symptoms in the susceptible WT and Ty-1 transgenic N. benthamiana βPVX-TYLCCNB-βC1, and not of PVX-TYLCCNB-βC1 mutant, caused severe curling and chlorosis. To determine whether the increase in symptoms in the susceptible WT and Ty-1 transgenic N. benthamiana plants also correlated with a higher TYLCV titre, samples were collected from systemically infected leaves and DNA purified for qPCR analysis. In WT N. benthamiana no significant change in virus titres was observed with TYLCV only versus a mixed infection with TYLCV and one of the PVX constructs (Fig. 5B). In the case of a mixed infection with PVX-AYVB-βC1 a trend of increased TYLCV titres was observed, which contrasted with the reduced titres seen in the presence of AYVB. In the Ty-1 transgenic plants challenged with TYLCV, virus titres were again drastically reduced compared to WT plants, and these titres did not change with the addition of PVX-empty or PVX-TYLCCNB-βC1 mutant. However, in the additional presence of PVX-AYVB-βC1 or PVX-TYLCCNB-βC1 TYLCV titres increased 2.7- and 2-fold, respectively (Fig. 5B). These data indicate that the presence of a betasatellite compromises Ty-1 resistance by means of its encoded TGS suppressor, the βC1 protein.

Ty-1 does not confer resistance to RNA viruses, as recently shown for tomato spotted wilt virus (TSWV) and CMV (Butterbach et al., 2014). Also in the study presented here, the symptoms caused by a PVX infection were not different between WT and stably transformed Ty-1 N. benthamiana. However, RNA viruses may compromise Ty-1 resistance against geminiviruses, as shown earlier during a co-infection of TYLCV and CMV. The observed increase in TYLCV titres in the latter case was explained to be due to inhibition of the TGS response by the CMV 2b RNAi suppressor protein (Butterbach et al., 2014; González et al., 2010; Hamera et al., 2012). This idea is strengthened by the current study, showing a compromising effect on Ty-1 resistance by AYVB, and also solely by the AYVB βC1 and TYLCCNB βC1 proteins expressed from a PVX virus vector. TYLCNV βC1 protein has been demonstrated to suppress TGS via inhibition of the enzyme S-adenosylhomocysteine hydrolase (SAHH), an enzyme that is required for production of the methyl donor used in the TGS pathway (Yang et al., 2011). CMV 2b in contrast has been shown to hamper TGS via inhibition of AGO4 (González et al., 2010; Hamera et al., 2012). Despite the fact that these viral suppressors act differently, the ability to inhibit TGS at (any) different step(s) of the pathway apparently is sufficient to compromise Ty-1-mediated resistance.

Recently, Conflon et al. (2018) reported on the compromising effect of a co-replicating betasatellite on Ty-1 resistance. Ty-1 tomato lines co-infected with different TYLCV strains and a cotton leaf curl Gezira betasatellite revealed an increase in disease symptoms, but interestingly titres only increased in the mixed infection case with TYLCV-II, while those of TYLCV-Mld were decreased on addition of the betasatellite. How to explain these effects caused by one and the same betasatellite remains to be further investigated, although it is likely that this has to do with the virus strains and not the betasatellite. Considering that the efficacy of Ty-1 is compromised by trans-complementing TGS suppressors, the suppressors encoded by the geminiviral DNA genome of TYLCV-II and TYLCV-Mld will also affect Ty-1 resistance. If their suppressors of RNAi differ in strength, Ty-1-mediated TGS will be suppressed to varying degrees, leading to different levels of reduction in virus titres. Although speculative, whether this also explains the observed difference in efficacy of Ty-1 resistance towards TYLCV and BCTV remains to be analysed. To this end, initial attempts to detect TGS suppression by BCTV and TYLCV in 16C-TGS transgenic plants (as shown by Buchmann et al., 2009) failed and remain to be repeated.
Recently several articles have been published on Ty-1 resistance-breaking strains of TYLCV in cultivations of Ty-1-bearing tomato. Samples of Ty-1 plants showing TYLCV-like symptoms collected in Morocco, Italy and Spain revealed the presence of viruses derived from a recombination event between TYLCV and TYLCSV in which a noncoding region between the origin of replication and the start of the coding region of V2 were exchanged (Belabess et al., 2015; Granier et al., 2019; Panno et al., 2018; Torre et al., 2019). In Morocco, this recombinant replaced both parental strains, but also under laboratory conditions this recombinant was positively selected in Ty-1-bearing plants (Belabess et al., 2015, 2016). So far, the mechanism behind the improved fitness of this resistance-breaking strain is unknown, but the region of recombination covers the promoter region of the coat protein. The resistance breaking, therefore, might be due to (a combination of) changes in replication efficiency or viral gene expression, or the region of recombination might be less prone to TGS.

In conclusion, Ty-1 is a unique resistance gene that confers resistance to a broad spectrum of geminiviruses, as demonstrated for begomoviruses and a curtovirus. Although Ty-1 is likely to confer resistance to all geminiviruses, suppression of RNAi by co-replicating betasatellites, or other RNA viruses, but also by suppressors encoded from the geminiviral DNA genome itself, seems to present the Achilles’ heel of the resistance mechanism. Whether Ty-1 resistance can also be compromised by an infection involving a bacterial or fungal/oomycete pathogen, some of which are shown to either hypomethylate or suppress (the amplification of) RNAi (Hou et al., 2017; Navarro et al., 2018; Pave et al., 2006), remains an interesting question. The effect of co-infections on Ty-1 resistance is important in light of disease management strategies, and simultaneously underlines the importance for monitoring the presence of other pathogens that encode suppressors of RNAi and interfere at the level of TGS during cultivation of Ty-1-bearing tomato plants.

EXPERIMENTAL PROCEDURES

Plant material and virus sources

Throughout this study S. lycopersicum and N. benthamiana plants were maintained under greenhouse conditions at 23 °C during the day and at 21 °C at night (16 h light/8 h dark regime) and at a relative humidity of 60%. Tomato cv. Moneymaker (MM) was used as susceptible control and a Ty-1 introgression line was derived from S. chilense LA1969 (Verlaan et al., 2013). Plants were infected with geminiviruses by means of agroinoculation of infectious clones. Agroinfectious clones used in this study were from two isolates of TYLCV, namely the Israel strain isolated from Almeria, Spain, as described by Morilla et al. (2005) (GenBank AJ489258.1), and the Israel strain isolated from Shanghai, China, named TYLCV-[CN-SH2] [GenBank AM282874, (Zhang et al., 2009)], an isolate of TYLCSV [GenBank X61153.1 (Noris et al., 1998)] and an isolate of BCTV California Logan [GenBank M24597.2 (Stanley et al., 1986)]. Co-infections were performed with an agroinfectious clone of AYVB [GenBank AJ252072.1 (Saunders et al., 2000)].

Transformation of tomato and N. benthamiana plants with Ty-1

To generate a construct for transformation purposes, the Ty-1 full-length coding sequence was amplified from complementary DNA (cDNA) of Ty-1 introgression lines with forward primer Ty-1-CDS-F and reverse primer Ty-1-CDS-R (for primer sequences see Table S1) (Verlaan et al., 2013). PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA) and verified by sequence analysis prior to recombination into pK7WG2 via an LR reaction (Invitrogen). The resulting plasmid was transferred to Agrobacterium tumefaciens strain AGL1 by electroporation. Transformation of cotyledons from MM and N. benthamiana was carried out according to the method described by Appiano et al. (2015). Plants regenerated from kanamycin-resistant primary transformants (T₀) were selected and self-pollinated to produce T₁ families. From each segregating T₂ family, transgenic lines were selected by PCR amplification of the NPTII gene cassette (using primers NPT3 and NPT4 (Heilersig et al., 2006), amplifying a 722-bp fragment) and a partial Ty-1 gene sequence (using primers CaMV 35S promoter primer 35S-F and gene insert-specific primer Ty-1-R, amplifying a 822-bp fragment).

PVX constructs

PVX infectious clones encoding the betasatellite C1 protein gene were constructed as follows. The open reading frame of AYVB C1 was amplified from the infectious clone (GenBank AJ252072.1) using primers AYVB-C1-F and AYVB-C1-R (Table S1). Sequences for C1 and a C1 mutant (Cui et al., 2005) of the TYLCCNB were ordered as a gene block from Integrated DNA Technologies (IDT, Leuven, Belgium) and were based on GenBank accession AJ421621.1. The gene blocks were cloned into pJet1.2 according to the manufacturer’s protocol (Thermo Fisher, Waltham, MA, USA), and their sequences verified. Next, the TYLCCNB C1 gene was amplified with primers TYLCCNB-C1-F and TYLCCNB-C1-R containing a 5’ Clal or SalI restriction site. Amplicons were purified and subsequently digested with SalI and Clal prior to cloning into the SalI- and Clal-digested PVX vector pGR107 (Jones et al., 1999). Positive clones were selected and verified by sequence analysis. The Agrobacterium strain GV3101, containing the helper plasmid pSoup, was transformed with the constructs PVX-AYVB-C1, PVX-TYLCCNB-C1, PVX-TYLCCNB-C1mut or with the empty pGR107 plasmid for subsequent agroinfiltration of plants.

Agrobacterium transient transformation assay

Agrobacterium transient transformation assays were performed following a slightly modified protocol of Bucher et al. (2003). In brief, A. tumefaciens was grown overnight at 28 °C in 3 mL LB3 (10 g/L
tryptone, 5 g/L yeast, 4 g/L NaCl, 1 g/L KCl, 3 g/L MgSO₄·2H₂O medium containing proper antibiotic selection pressure. From this culture, 600 μL was transferred and incubated overnight in 3 mL induction medium [10.5 g/L K₂HPO₄, 4.5 g/L KH₂PO₄, 1 g/L(NH₄)₂SO₄, 0.5 g/L sodium citrate, 2H₂O, 1 mM MgSO₄·7H₂O, 0.2% (w/v) glucose, 0.5% (v/v) glycerol, 50 μM acetylthiocholine, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.6]. The next day bacteria were pelleted by centrifugation (15 min, 2670 g) and resuspended in M5 MES buffer [Murashige and Skoog medium (Duchefa Biochemie, Haarlem, Netherlands) supplemented with 150 μM acetylthiocholine, 10 mM MES and 87 mM sucrose] at an OD₆₀₀ of 0.5 per construct. One hour after plants were watered in excess, the first two true leaves were fully infiltrated with this mixture by pressure inoculation with a needleless syringe on the abaxial side of the leaf.

Disease assessment

Plant responses were scored several times during the whole disease assay on viral symptom development. The time span of the disease assay varied per experiment and lasted up to 50 days. Depending on the lines and experiments, five to ten plants were challenged. Each plant was rated using a 0 to 4 disease severity index (DSI) described by Friedmann et al. (1998), where 0 indicates no viral disease symptoms and 4 means severe symptoms. Intermediate scores (0.5, 1.5, 2.5 and 3.5) were incorporated for more precise scoring.

Nucleic acid purification

Systemically infected leaves were harvested and snap frozen in liquid nitrogen. The samples were ground either by mortar and pestle or by using precellys (Bertin Instruments, Montigny-le-Bretonneux, France) for 10 s at 5000 rpm. To determine geminivirus titres, DNA was isolated following the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with slight modifications as described by Fulton et al. (1995). In brief, ground plant material was incubated for 1 h at 65 °C with CTAB buffer (0.1 M Tris, 0.7 M NaCl, 0.01 M EDTA, 2% CTAB). After chloroform/isooamyl alcohol extraction and spinning of the samples, the upper aqueous phase was transferred to a new tube and the DNA precipitated following the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with slight modifications as described by Fulton et al. (1995). In brief, ground plant material was incubated for 1 h at 65 °C with CTAB buffer (0.1 M Tris, 0.7 M NaCl, 0.01 M EDTA, 2% CTAB). After chloroform/isooamyl alcohol extraction and spinning of the samples, the upper aqueous phase was transferred to a new tube and the DNA precipitated following the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with slight modifications as described by Fulton et al. (1995). In brief, ground plant material was incubated for 1 h at 65 °C with CTAB buffer (0.1 M Tris, 0.7 M NaCl, 0.01 M EDTA, 2% CTAB). After chloroform/isooamyl alcohol extraction and spinning of the samples, the upper aqueous phase was transferred to a new tube and the DNA precipitated following the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with slight modifications as described by Fulton et al. (1995). In brief, ground plant material was incubated for 1 h at 65 °C with CTAB buffer (0.1 M Tris, 0.7 M NaCl, 0.01 M EDTA, 2% CTAB). After chloroform/isooamyl alcohol extraction and spinning of the samples, the upper aqueous phase was transferred to a new tube and the DNA precipitated following the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with slight modifications as described by Fulton et al. (1995). In brief, ground plant material was incubated for 1 h at 65 °C with CTAB buffer (0.1 M Tris, 0.7 M NaCl, 0.01 M EDTA, 2% CTAB). After chloroform/isooamyl alcohol extraction and spinning of the samples, the upper aqueous phase was transferred to a new tube and the DNA precipitated following the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with slight modifications as described by Fulton et al. (1995). In brief, ground plant material was incubated for 1 h at 65 °C with CTAB buffer (0.1 M Tris, 0.7 M NaCl, 0.01 M EDTA, 2% CTAB). After chloroform/isooamyl alcohol extraction and spinning of the samples, the upper aqueous phase was transferred to a new tube and the DNA precipitated following the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with slight modifications as described by Fulton et al. (1995). In brief, ground plant material was incubated for 1 h at 65 °C with CTAB buffer (0.1 M Tris, 0.7 M NaCl, 0.01 M EDTA, 2% CTAB). After chloroform/isooamyl alcohol extraction and spinning of the samples, the upper aqueous phase was transferred to a new tube and the DNA precipitated following the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with slight modifications as described by Fulton et al. (1995). In brief, ground plant material was incubated for 1 h at 65 °C with CTAB buffer (0.1 M Tris, 0.7 M NaCl, 0.01 M EDTA, 2% CTAB). After chloroform/isooamyl alcohol extraction and spinning of the samples, the upper aqueous phase was transferred to a new tube and the DNA precipitated following the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987) with slight modifications as described by Fulton et al. (1995).

Detection of the betasatellite

DNA of AYVB was amplified using AYVB-specific primers AYVB-F1, AYVB-F2 and AYVB-R (Table S1). The combination of AYVB-F1 and AYVB-R resulted in a product of 823 bp, whereas AYVB-F2 and AYVB-R resulted in a product of 457 bp. The PCR was performed with GoTaq polymerase according to the manufacturers’ protocol (Promega, Madison, WI, USA), using the following cycling conditions: 2 min at 95 °C; 40 cycles of 30 s at 95 °C, 30 s at 55 °C, 60 s at 72 °C; 7 min at 72 °C. Amplified PCR products were visualized in a 1% agarose gel using ethidium bromide.

Virus titration

Relative virus titres of the TYLCV Almeria isolate, TYLCV- [CN:SH2] and BCTV were determined by qPCR using 25S rRNA (N. benthamiana) or EF1α (S. lycopersicum, Solyc06g005060) as internal control. To measure samples of N. benthamiana, the reaction mixture contained 1× Sybr Select (Applied Biosystems, Foster City, CA, USA), 375 nM forward primer, 375 nM reverse primer and 10 ng genomic DNA, while for S. lycopersicum samples the iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA) was used. For PCR amplification the following primers were used (Table S1): for TYLCV Almeria isolate primers TYLCV-Alm-qPCR-F and TYLCV-Alm-qPCR-R (Powell et al., 2012), for TYLCV primers TYLCV-qPCR-F and TYLCV-qPCR-R, for TYLCV-[CN:SH2] primers TYLCV-CN-qPCR-F and TYLCV-CN-qPCR-R, for BCTV either primers BCTV-qPCR-F1 and BCTV-qPCR-R1 or BCTV-qPCR-F2 and BCTV-qPCR-R2. During qPCR 25S rRNA was amplified using primers 25S-qPCR-F and 25S-qPCR-R, and EF1α using primers SIEF1a-qPCR-F and SIEF1a-qPCR-R.

The qPCR was performed in a Bio-Rad CFX384 using the following cycling conditions: 2 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by a melting curve with 0.5 °C steps from 60 to 95 °C to determine PCR specificity. Relative viral titres were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Values were normalized relative to the internal control 25S rRNA or EF1α, and calibrated to levels of the control plants, which were set as 1.

Ty-1 gene expression analysis

Prior to gene expression analysis 1 μg of purified total RNA of N. benthamiana samples was treated with TURBO DNase (Invitrogen) following the manufacturer’s instructions. First-strand cDNA was synthesized using random hexamers (Roche, Basel, Switzerland) and M-MLV reverse transcriptase according to the manufacturers’ protocol (Promega, Madison, WI, USA). The RT-qPCR was performed in a total volume of 10 μL, containing 1 μL of 5X diluted cDNA, 1X Sybr Select (Applied Biosystems), 375 nM forward primer and 375 nM reverse primer. Total RNA of S. lycopersicum was treated with DNase I, Amplification Grade (Invitrogen) following the manufacturer’s instructions. cDNA was synthesized using the iScript CDNA Synthesis Kit (Bio-Rad) and RT-qPCR performed with the iQ SYBR Green supermix (Bio-Rad). To determine Ty-1 expression levels, either Ty-1-qPCR-F1 and Ty-1-qPCR-R1 or Ty-1-qPCR-F2 and Ty-1-qPCR-R2.
and Ty-1-qPCR-R2 primers were used. The gene expression was measured relative to the housekeeping gene EF1α by using the primers nbEF1α-qPCR-F and nbEF1α-qPCR-R in the case of N. benthamiana samples or SlEF1α-qPCR-F and SlEF1α-qPCR-R in the case of S. lycopersicum samples (Table S1). RT-qPCR was performed in a Bio-Rad CFX384 using the same conditions as for the virus titration. Relative gene expression was calculated using the formula 2^−(ΔCt Ty-1 (Ct Ty-1 – Ct EF1α) − ΔCt MM (Ct Ty-1 – Ct EF1α)) (Livak and Schmittgen, 2001).

ACKNOWLEDGEMENTS

The authors would like to thank Prof. Eduardo Rodríguez Bejarano (Universidad de Málaga, Málaga, Spain) for providing the infectious TYLCV (Almería isolate) clone, Dr Emanuela Noris (Institute of Plant Virology, Torino, Italy) for providing the infectious clone of TYLCV and Dr Keith Saunders (John Innes Centre, Norwich, UK) for providing the infectious clone of BCTV and AYVB. The authors would like to thank Annelies E.H.M. Loonen for making the pri-
tative TYLCV (Almeria isolate) clone, Dr Emanuela Noris (Institute

REFERENCES

Appiano, M., Pavan, S., Catalano, D., Zheng, Z., Bracuto, V., Lotti, C., Visser, R.G.F., Ricciardi, L. and Bai, Y. (2015) Identification of candidate MLO powdery mildew susceptibility genes in cultivated Solanaceae and functional character-

ization of tobacco NIMLO1. Transgenic Res. 24, 847–858.

Aregger, M., Borah, B.K., Seguin, J., Rajeswaran, R., Gubaeva, E.G., Zvereva, A.S., Windels, D., Vazquez, F., Blevins, T., Farinelli, L. and Pooggin, M.M. (2012) Primary and secondary siRNAs in geminivirus-induced gene silencing. PLoS Pathog. 8, e1002941.

Barbieri, M., Acciarri, N., Sabatini, E., Sardo, L., Accotto, G.P. and Pecchioni, N. (2010) Intergression of resistance to two Mediterranean virus species causing tomato yellow leaf curl into a valuable traditional tomato variety. J. Plant Pathol. 92, 485–493.

Belabess, Z., Dallot, S., El-Montaser, S., Granier, M., Majde, M., Tahiri, A., Blenzar, A., Urbino, C. and Peterschmitt, M. (2015) Monitoring the dynamics of emergence of a non-canonical recombinant of tomato yellow leaf curl virus and displacement of its parental viruses in tomato. Virolgy, 486, 291–306.

Belabess, Z., Peterschmitt, M., Granier, M., Tahiri, A., Blenzar, A. and Urbino, C. (2016) The non-canonical tomato yellow leaf curl virus re-combinant that displaced its parental viruses in southern Morocco exhibits a high selective advantage in experimental conditions. J. Gen. Virol. 97, 3433–3445.

Bucher, E., Sijen, T., Haan, P.D., Goldbach, R. and Prins, M. (2003) Negative-

study toplospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. J. Virol. 77, 1329–1336.

Buchmann, R.C., Asad, S., Wolf, J.N., Mohannath, G. and Bisaro, D.M. (2009) Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation. J. Virol. 83, 5005–5013.

Butterbach, P., Verlaan, M.G., Dullemans, A., Lohuis, D., Visser, R.G.F., Bai, Y. and Kormelink, R. (2014) Tomato yellow leaf curl virus resistance by Ty-

1 involves increased cytosine methylation of viral genomes and is compromised by cucumber mosaic virus infection. Proc. Natl. Acad. Sci. USA. 111, 12942–12947.

Castro, A.P.D., Diez, M.J. and Nuez, F. (2005) Evaluation of breeding tomato lines partially resistant to tomato yellow leaf curl Sardinia virus and tomato yellow leaf curl virus derived from Lycopersicon chilense. Can. J. Plant Pathol. 27, 268–275.

Conflon, D., Granier, M., Tiendrébéogo, F., Gentil, P., Peterschmitt, M. and Urbino, C. (2018) Accumulation and transmission of alphastatellite, betasatel-

light and tomato yellow leaf curl virus in susceptible and Ty-1-resistant tomato plants. Virus Res. 253, 124–134.

Cui, X., Li, G., Wang, D., Hu, D. and Zhou, X. (2005) A begomovirus DNAJ- encoded protein binds DNA, functions as a suppressor of RNA silencing, and targets the cell nucleus. J. Virol. 79, 10764–10775.

Donaire, L., Barajas, D., Martínez-García, B., Martínez-Priego, L., Pagán, I. and Llave, C. (2008) Structural and genetic requirements for the biogenesis of tobacco rattle virus-derived small interfering RNAs. J. Virol. 82, 5167–5177.

Doyle, J.J. and Doyle, J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.

Friedmann, M., Lapidot, M., Cohen, S. and Pilowsky, M. (1998) A novel source of resistance to tomato yellow leaf curl virus exhibiting a symptomless reaction to viral infection. J. Am. Soc. Hortic. Sci. 123, 1004–1007.

Fulton, T.M., Chunwongse, J. and Tankelsky, S.D. (1995) Microprop protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol. Biol. Report. 13, 207–209.

González, I., Martínez, L., Rakitina, D.V., Lewsey, M.G., Atencio, F.A., Llave, C., Kalinina, N.O., Barr, J.P., Palukaitis, P. and Canto, T. (2010) Cucumber mosaic virus 2b protein subcellular targets and interactions: their significance to RNA silencing suppressor activity. Mol. Plant–Microbe Interact. 23, 294–303.

Granier, M., Tomassoli, L., Mangili, A., Nannini, M., Peterschmitt, M. and Urbino, C. (2019) First report of TYLCV-IS141, a tomato yellow leaf curl virus recombinant infecting tomato plants carrying the Ty-1 resistance gene in Sardinia (Italy). Plant Dis. 103, 1437.

Hamara, S., Song, X., Su, L., Chen, X. and Fang, R. (2012) Cucumber mosaic virus suppressor 2b binds to AGO4-related small RNAs and impairs AGO4 activities. Plant J. 69, 104–115.

Hammond, S.M. (2005) Dicing and slicing: the core machinery of the RNA inter-

ference pathway. FEBS Lett. 579, 5822–5829.

Hanson, P., Green, S.K. and Kuo, G. (2006) Ty-2, a gene on chromosome 11 conditioning geminivirus resistance in tomato. Tomato Genet. Coop. Rep. 56, 17–18.

Heiligers, H.J.B., Loonen, A., Bergervoet, M., Wolters, A.M.A. and Visser, R.G.F. (2006) Post-transcriptional gene silencing of GBSS1 in potato: effects of size and sequence of the inverted repeats. Plant Mol. Biol. 60, 647–662.

Hou, Y., Zhai, Y., Feng, L., Karimi, H.Z., Rutter, B.D., Zeng, L., Choi, D.S., Zhang, B., Gu, W., Chen, X., Ye, W., Innes, R.W., Zhao, J. and Ma, W. (2019) A Phytophthora effector suppresses trans-kingdom RNAi to promote disease sus-

ceptibility. Cell Host Microbe. 25, 153–165.

Hutton, S.F. and Scott, J.W. (2014) Ty-6, a major begomovirus resistance gene located on chromosome 10. Rep. Tomato Genet. Coop. 64, 14–18.
Hutton, S.F., Scott, J.W. and Schuster, D.J. (2012) Recessive resistance to tomato yellow leaf curl virus from the tomato cultivar Tyking is located in the same region as Ty-5 on chromosome 4. *HortScience*, 47, 324–327.

Jackel, J.N., Storer, J.M., Coursey, T. and Bisaro, D.M. (2016) Arabidopsis RNA polymerases IV and V are required to establish H3K9 methylation, but not cytosine methylation, on geminivirus chromatin. *J. Virol.*, 90, 7529–7540.

Ji, Y., Schuster, D.J. and Scott, J.W. (2007) Ty-3, a begomovirus resistance locus near the Tomato yellow leaf curl virus resistance locus Ty-1 on chromosome 6 of tomato. *Mol. Breed.*, 20, 271–284.

Ji, Y., Scott, J.W. and Schuster, D.J. (2009) Molecular mapping of Ty-4, a new tomato yellow leaf curl virus resistance locus on chromosome 3 of tomato. *J. Am. Soc. Hortic. Sci.*, 134, 281–288.

Jones, L., Hamilton, A.J., Voinnet, O., Thomas, C.L., Maule, A.J. and Baulcombe, D.C. (1999) RNA–DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell*, 11, 2291–2301.

Lapidot, M., Karniel, U., Gelbart, D., Fogel, D., Evenor, D., Kutsher, Y., Makkabash, Z., Nahon, S., Shlomo, H., Chen, L., Reuveni, M. and Levin, I. (2015) A novel route controlling begomovirus resistance by the messenger RNA surveillance factor Pelota. *PLoS Genet.*, 11, e1005538.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the −2 ΔΔCt method. *Methods*, 25, 402–408.

Mallory, A. and Vaucheret, H. (2010) Form, function, and regulation of ARGOUANTE proteins. *Plant Cell*, 22, 3879–3889.

Maruthi, M.N., Czosnek, H., Vidavski, F., Tarba, S.Y., Milo, J., Levitavos, S., Venkatesh, H.M., Padmaja, A.S., Kulkarni, R.S. and Muniyappa, V. (2003) Comparison of resistance to tomato leaf curl virus (India) and tomato yellow leaf curl virus (Israel) among *Lycopersicon* wild species, breeding lines and hybrids. *Eur. J. Plant Pathol.*, 109, 1–11.

Morilla, G., Janssen, D., García-Andrés, S., Moriones, E., Cuadrado, I.M. and Bejarano, E.R. (2005) Pepper (*Capsicum annuum*) is a dead-end host for tomato yellow leaf curl virus. *Phytopathology*, 95, 1089–1097.

Moriones, E. and Navas-Castillo, J. (2000) Tomato yellow leaf curl virus, an emerging virus complex causing epidemics worldwide. *Virus Res.*, 71, 123–134.

Navarro, L., Jay, F., Nomura, K., He, S.Y. and Voinnet, O. (2012) Suppression of defense. *Mol. Plant–Microbe Interact.*, 25, 344–350.

Noris, E., Vaira, A.M., Caciagli, P., Masenga, V., Gronenborn, B. and Accotto, G.P. (1999) RNA–DNA interactions and DNA methylation as an epigenetic defense against geminiviruses. *J. Virol.*, 73, 964–967.

Venkatesh, H.M., Padmaja, A.S., Kulkarni, R.S. and Muniyappa, V. (2013) Advances in understanding begomovirus satellites. *Annu. Rev. Phytopathol.*, 51, 357–381.

**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Table S1 List of sequences of primers used in this research.