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Full Length Article

Trans-Dominant Interference by Synthetic Coat Protein of Tomato Yellow Leaf Curl Virus Expressed in Transgenic Tomato

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Abstract

Begomoviruses are the main biotic threat of tomatoes, resulting in substantial losses worldwide. The coat protein is the most conserved protein in begomoviruses and is essentially required by monopartite begomoviruses for infection in susceptible plants. Its expression in transgenic plants may interfere with the uncoating of the viral DNA upon infection. A study was conducted to investigate the effect of expressing viral coat protein in transgenic plants on the induction of resistance to *Tomato yellow leaf curl virus Oman* (TYLCV-OM). The synthetic codon-optimized coat protein (CP_{syn}) of TYLCV-OM was transformed into Tomato var. Pusa Ruby using *Agrobacterium.* CP_{syn} was expressed in transgenic tomato plants to avoid gene silencing of the transgene upon virus infection. T_1 transgenic lines were challenged with TYLCV-OM for resistance evaluation. Plants of three transgenic lines out of seven showed resistance response and most plants did not develop disease symptoms. Real-time quantitative PCR showed that the CP_{syn} helped reduce virus particles by 150-fold. Transforming tomato plants with CP_{syn} resulted in the induction of resistance to TYLCV-OM. The transgenic plants are valuable resource to understand the function of the coat protein and to provide resistance against the main begomoviruses infecting tomatoes. © 2020 Friends Science Publishers

Keywords: Coat Protein; TYLCV; CP_{syn}; leaf curl; Geminiviruses

Introduction

Geminiviruses have small, circular, single-stranded (ss) DNA genome encapsulated in twin icosahedral particles (Inoue-Nagata *et al.* 2016; Loriato *et al.* 2020). Over the past twenty years the disease incidence and severity caused by these little pathogens have enormously increased posing a potential threat to agriculture (Khalid *et al.* 2017; Ouattara *et al.* 2020; Zaidi *et al.* 2020; Zhang *et al.* 2020). The estimated yield losses of the infected plants are enormous (Osei *et al.* 2017; Tsai and Huang 2017). Pepper fields in Indonesia were affected by whitefly transmitted Geminiviruses, resulting in 20–80% yield losses (Hidayat *et al.* 2011; Kenyon *et al.* 2014). In Africa, cassava crops were heavily affected by *Cassava mosaic virus* (CMV) in 12 countries since the 1980s (Legg *et al.* 2011; Bruyn *et al.* 2016; Osei *et al.* 2017). Similarly, Tomato leaf curl disease (TLCD), caused by *Tomato leaf curl virus* (ToLCV), is the most prevailing disease of tomato and capable of 100% field destruction in many tropical and subtropical countries (Segbefia *et al.* 2018; Desbiez *et al.* 2019; Ouattara *et al.* 2020). The emergence of new recombinants associated with tomato has been recently found in Oman during field surveys (Al-Shihi *et al.* 2014; Al-Shihi *et al.* 2018a). More than one virus was found to be associated with tomato crops, resulting in 100% yield losses (Al-Shihi *et al.* 2016; Ammara *et al.* 2017).

Traditionally, whitefly transmitted begomoviruses are controlled by the excessive use of insecticides/pesticides that cause more damage to the ecosystem (Tsai and Huang 2017). The durable resistance in plants against these ever evolving geminiviruses is difficult to achieve by traditional plant breeding approaches. Thus, engineering resistance using molecular tools seems a more convenient option against these groups of viruses.

Geminiviruses have small genome contain either one or two genomic units (DNA A and B) and heavily rely on a host for replication of both viral and plant chromosomal DNA (More *et al.* 2019; Ouattara *et al.* 2020). CP gene is not only involved in viral genome packaging, but also found associated with a few other functions. The major functions include vector specificity (Khalid *et al.* 2017), protection of viral DNA in the vector (Azzam *et al.* 1994), virus spread (Felker *et al.* 2019) and transfer of viral DNA in/out of nucleus (Liu *et al.* 1999).

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The central part of coat protein sequence is very crucial for transmission (Höhnle *et al.* 2001; Malik *et al.* 2005), while half coat protein from N-terminal is the DNA binding domain (Unseld *et al.* 2004; Malik *et al.* 2005). The central part as well as C and N-terminal and sequences appear to be involved in CP multimerization (Liu *et al.* 2001; Unseld *et al.* 2001), which is essential for virus capsid assemblage and insect transmission (Zhang *et al.* 2001b; Hipp *et al.* 2016).

Coat protein (CP)-based resistance is widely used to confer resistance in plants against geminiviruses. Numerous crops have been reported and released for commercial cultivation by using viral CP (Dasgupta *et al.* 2003). However, it is important to note that in most of these examples the resistance was reported to be RNA based, rather than protein mediated. It was demonstrated that CP based resistance is somewhat specific as there is relationship between resistance sequence similarity between the CP of transgenic plants and the CP of challenging virus (Saxena *et al.* 2011).

Begomoviruses are often found associated with betasatellites, which are circular ssDNA satellite molecules, having half the size of their helper begomovirus (Xu *et al.* 2019). Betasatellites are dependent on their helper begomovirus for vector transmission, encapsidation and systemic movement in plants (Malathi *et al.* 2017). The major functions of betasatellites are symptom induction, host range determination and interaction with various host factors (Malathi *et al.* 2017).

In this study CP_{syn} was used to develop resistance against TYLCV-OM. The role of CP_{syn} was investigated in transgenic tomato plants against TYLCV-OM and TYLCV-OMB isolated from tomato fields in Oman.

Materials and Methods

Construction of CP_{syn}

A highly conserved 777 bp region of CP (V1 of TYLCV-OM) ORF, representing the whole coding sequence, was selected to design synthetic CP sequence. Codon optimization was acrried out by codon usage table for *Solanum lycopersicum* [gbpln]: 1452 CDS's (634390 codons) from NCBI-GenBank to increase the overall transaltional efficiency of codons without changing the amino acids sequence (Fig. 1–2). The synthetic CP gene was commercially synthesized by GenScript (GenScript Inc., New Jersey, USA) and was provided in pUC57 cloning vector. Synthesized CP gene was cloned in pGreen 0029 plant expression vector under pFMV promoter and G7 terminator at HindIII/ XbaI site to avoid promoter silencing. The synthetic CP possesses no sequence identity to the TYLCV-OM-CP; the low identity was done to avoid gene silencing.

Tomato transformation

The CP_{syn} construct was mobilized in *Agrobacterium tumefaciens* strain AGL1 by electroporation. Tomato var. Pusa Ruby was transformed with CP_{syn} by *Agrobacterium*-mediated tomato tissue culture according to a protocol described by Ammara *et al.* (2014). CP_{syn} gene-based primer pair was used to screen stably transformed T_0 tomato plants. PCR-positive plants were further analyzed for the presence of transgene by Southern blotting. All transgenic lines showed normal phenotype and produced viable seeds by self-pollination. T_1 transgenic lines were challenged with TYLCV-OM for resistance evaluation.

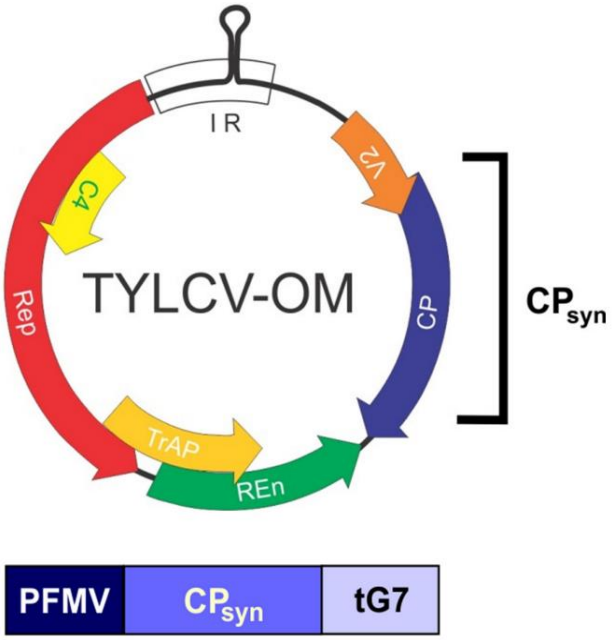

Fig. 1: The genome organization of TYLCV -OM. The CP gene of TYLCV-OM was used to prepare synthetic CP_{syn} to offer protein mediated resistance. The overall arrangement of CP_{syn} under its independent promoter and terminator is shown below the circular organization of TYLCV-OM.

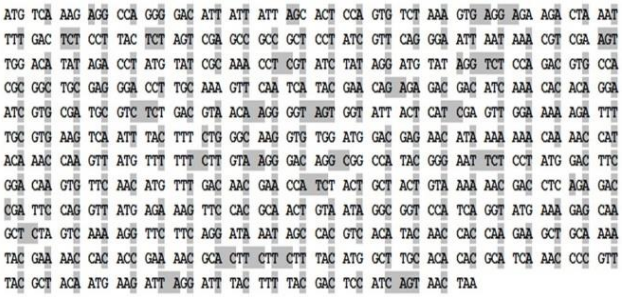

Fig. 2: A synthetic CP (CPsyn) sequence designed and synthesized by codon optimization of wild type viral sequence

Inoculation of transgenic lines with TYLCV-OM

Positive putative T_0 plants were selected for further screening and resistance evaluation, from which seeds were collected by self-pollination. Seeds from seven selected

lines were germinated on kanamycin media to get T_1 generation. Germinated seedlings on selection media were transferred to pots for T_1 resistance evaluation. Transgene was confirmed in these seedlings by PCR. Each independent line with ten replicates was germinated and maintained in a glass house under 28–29°C temperature with 80–90% relative humidity. These lines were infiltrated with Agro-infectious construct of TYLCV-OM (Acc. No. DQ644565.1) and TYLCV-OM/TYLCV-OMB (Acc. No. HE800544.1) as described by Llave *et al.* (2000).

Ten non-transgenic tomato plants of the same age were infiltrated with Agro-infectious construct of TYLCV-OM and TYLCV-OM/TYLCV-OMB as positive control. All agro-inoculated plants were kept in a glasshouse and were monitored for symptoms development and severity until harvesting stage. Leaf samples were collected at 30 days post inoculation (dpi) when all control plants developed full symptoms. DNA was extracted from leaf tissues by CTAB method (Doyle and Doyle 1990). The presence of TYLCV-OM was checked by PCR using FD-CP-382 /RD-CP-1038 primers while the universal primers Sat01 and Sat02 were used for TYLCV-OMB detection.

Southern hybridization

For the confirmation of transgene, 777bp fragment of CP gene was digested by BamHI and SalI, followed by gel purification and then labelling with digoxigenin using a DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche GmbH, Germany). For the detection of virus in transgenic lines, 650-bp fragment of CP gene of TYLCV-OM was used. Amplification was done using FDCP/RDCP primers, followed by labelling with digoxigenin. To detect betasatellite, 1084-bp fragment restricted byBamHI and XbaI of a betasatellite clone (Tb-1) was gel purified and labeled with digoxigenin.

Quantification of viral molecules in resistant transgenic plants by qPCR

Screening of transgenic lines harboring CP_{syn} was carried out by conventional PCR and southern hybridization. Three resistant lines, which were negative by southern hybridization, were further analyzed by qPCR to quantify the virus titer in these lines. SYBER green dye (IQ SYBER Green supermix by BIO-RAD U.S.A.) was used for this experiment. Primer pair were designed on CP gene of TYLCV-OM QF (5'TAAAAGGCGCACTAATGGGTAGACCGTAGA3') and QR (5'GGCGATAACCACCTTCCCG3') to amplify 150 bp product specific to TYLCV-OM.

Serial dilution of a plasmid which contains the TYLCV-OM genome was used to obtain standard. Thus, series of dilutions were prepared and 5 of them were used in order to get a standard curve. There were 10-fold decreases in each dilution (10 ng, 1 ng, 0.1 ng, 0.01 ng and 0.001 ng). ABI iCycler software (version 3.1) was used to handle data acquisition and analysis which automatically calculates the threshold cycle *C*t values and the parameters of the standard curves.

The reaction mixture (25 μL) contained 0.4 μL of each primer (QF and QR; 4 picomole), 1X iQ SYBR Green Supermix and 2 μL DNA sample (5 ng/μL for all known samples). Each DNA sample was carefully measured by nanodrop before setting up reactions. In order for the standard curves to fall within the range, DNA concentration was then adjusted up to 10 ng/μL for each sample. PCR was performed in the Real-Time PCR Detection System (ABI) according to Azhar *et al.* (2010).

Results

Construction of CP_{syn}

In this study the ability of CP_{syn} to develop resistance against a heterologous monopartite TYLCV-OM has been investigated. Synthesized CP gene was cloned in pUC57 vector containing pFMV promoter and G7 terminator at HindIII/ XbaI site. The whole cassette of 1596 bp (CP gene with promoter and terminator) was lifted by I-CeuI homing enzyme and cloned in modified pGreen plant expression vector at I-CeuI site.

Tomato transformation with CP_{syn} construct

All lines showed a single band representing a single integration site for each line (Fig. 3). All positive transgenic lines were then self-pollinated and grown on kanamycin selection medium to get T1 generation. Each line with ten replicates was generated to test transgene efficiency against TYLCV-OM.

Resistance evaluation of transgenic CP_{syn} tomato lines

The typical symptoms of TYLCV infection (downward/upward curling and yellowing) started to appear on non-transgenic Pusa Ruby plants after 28 days post inoculation (dpi) with TYLCV-OM and TYLCV-OM/TYLCV-OMB. The non-transgenic Pusa Ruby plants developed severe symptoms (yellowing, curling, crumpling) of TYLCV-OM disease in newly developed leaves by 28 dpi (Fig. 4). Transgenic lines expressing CP_{syn} inoculated with TYLCV-OM were resistant at 28 dpi except line # 41, 66 & 67 (Table 1). A small number of replicates started developing milder symptoms at 60 dpi in all transgenic lines showing resistance response. All lines were resistant at 60 dpi when inoculated with TYLCV-OM but started developing milder symptoms when co-inoculated with TYLCV-OM/TYLCVOMB. PCR analysis showed the presence of TYLCV-OM and TYLCVOMB in most replicates. All PCR positive replicates were then analyzed by Southern hybridization. Breakdown of resistance was

Table 1: Infectivity of TYLCV-OM/TYLCVOMB in transgenic tomato plants harbouring CP_{syn}

Treatments	Exp	CP_{syn} transgenic lines							Non transgenic control		
		41	43	44	55	66	67	101	N. C*	P. C**	P.C***
TYLCV-OM	I	1/10	2/10	6/10	5/10	0/10	1/10	1/10	0/10	10/10	10/10
	II	0/10	1/10	5/10	6/10	1/10	0/10	1/10	0/10	10/10	10/10
TYLCV-OM+ TYLCVOMB	I	3/10	4/10	8/10	5/10	2/10	3/10	3/10	0/10	10/10	10/10
	II	2/10	3/10	9/10	8/10	3/10	2/10	4/10	0/10	10/10	10/10
Southern$ for TYLCV-OM		-	+	+++	+++	-	-	+++	-	+++	+++
Southern$ for TYLCVOMB		-	-	+++	+++	+	-	-	-	+++	+++

* Non-transgenic Pusa ruby plants as healthy control.
** Pusa ruby plants inoculated with Agrobacterium culturesharbouringpGreen0029
# TYLCV-OM was detected in DNA extracted from plants by PCR using FD-CP/RD-CP primers (Table 1)
$ Southern hybridization results are given as strong hybridization (+++), weak hybridization (+), and no hybridization detected (−)

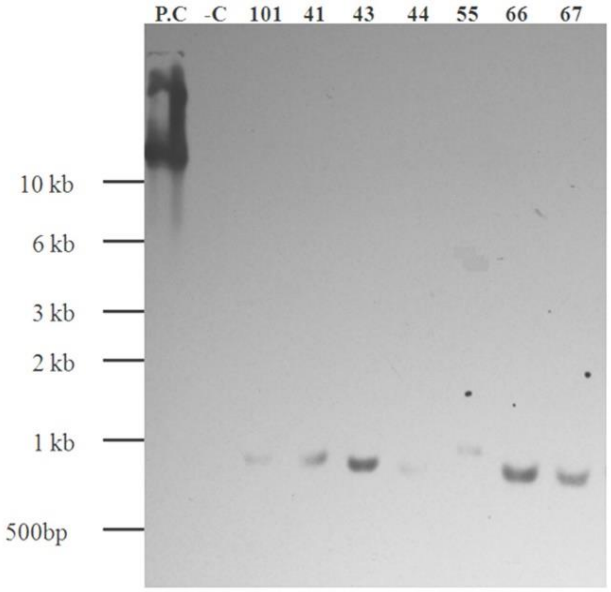

Fig. 3: Southern blot analysis for the confirmation of transgene in CP_{syn} transgenic lines. Genomic DNA ~10 μg digested with EcoRI for PCR positive lines probe with ~777 bp fragment of CP_{syn} clone. A DNA size marker was electrophoresed in lane 1 and superimposed on membrane. All seven transgenic lines showed single integration in genome by giving ~777 bp single band

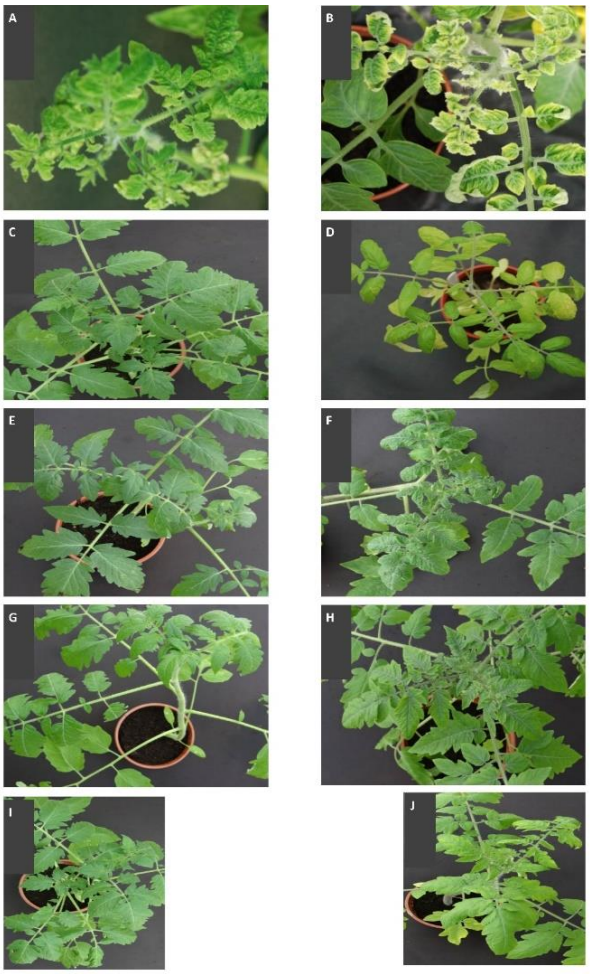

Fig. 4 A. Pusa ruby control plants showing TYLCV-OM **B.** Pusa ruby control plants showing TYLCV-OM Panel **C**, **E**, **G** and **I** are Line 41, 43 66 and 67 inoculated with TYLCV-OM. While panel **D**, **F**, **H** and J are line 41, 43, 66 and 67 inoculated with TYLCV-OM/TYLCV-OMB. Photographs were taken at 60 dpi

observed in Line #44 and 55. In line # 43, 44, 55 and 101 high titers of TYLCV-OM and TYLCV-OMB were observed by Southern blot hybridization (results not shown). However, in transgenic line # 41, 66 and 67 level of both TYLCV-OM and TYLCV-OMB was negligible in comparison to control and unable to detect by southern hybridization.

Quantitative PCR to determine virus titer in inoculated plants

The results of Q-PCR are summarized in Fig. 5. Although the virus was detected in all three lines but the level of virus was significantly lower than the control plants T1–T3 in Fig. 5. It is clear from qPCR results that in the presence of betasatellite, the number of virus particles has increased even in all the inoculated resistant transgenic lines.

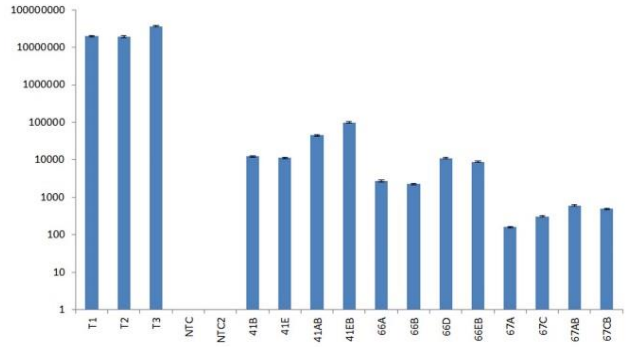

Fig. 5: Quantitative RT-PCR to quantitate viral DNA particles in transgenic and non-transgenic tomato plants inoculated with TYLCV-OM and TYLCV-OMB. Blue bar represents viral DNA concentration in inoculated plants. T1 and T2 are control non-transgenic plants inoculated with TYLCV-OM and T3 is infected non-transgenic plant inoculated with TYLCV-OM/TYLCV-OMB while NTC is non-transgenic healthy control plant. Transgenic lines 41, 66 and 67 were analyzed for the quantification of virus as these lines were negative by Southern hybridization. Each bar is the mean of three replicates and the error bars indicate standard deviation

There is almost 150-fold less virus present among transgenic lines but there is only 10-fold difference between plants inoculated with TYLCV-OM and with TYLCV-OMB. The non-transgenic negative control was also used to see the overall efficiency of reaction and no detectable virus particles were found in these controls. The overall reaction efficiency of qPCR was 98.4%. The Ct values with non-transgenic negative control and without template DNA were equal to the number of cycles used in the qPCR reaction. In contrast, TYLCV-infected control (non-transgenic) Pusa Ruby plants showed amplification in very low Ct values and contain relatively large amounts of viral DNA (Fig. 5). The melt curve analysis resulted in single peak and represents that single product was amplified and all PCR products melted at single temperature.

Discussion

The first successful demonstration of CP based resistance was achieved in *Nicotiana tabacum* against *Tobacco mosaic virus* (TMV) (Abel *et al.* 1986). The model to explain resistance is that transgenically expressed CP assembles to form virus-like particles (VLPs) to block the uncoating of virus. Alternatively, CP inhibits virus disassembly by shifting the disassembly-assembly reaction in favor of assembly, thereby preventing virus infection in the inoculated cells (Register III and Beachy 1988). Later on, it was suggested that the CP inhibits disassembly of challenged viruses in the initial infected cells (Bendahmane *et al.* 1997).

In the present study, CP_{syn} was used to develop resistance against TYLCV-OM and TYLCV-OM/ToLCB-OM isolated from tomato fields in Oman. The results of the study showed that the transient expression of CP_{syn} with begomovirus-betasatellite complex showed 100% resistance phenotype while in transgenic plants challenged with TYLCV-OM and TYLCV-OM/ToLCB-OM symptom development and infectivity was slightly impaired. However, three transgenic lines remained symptomless at 90 dpi; qPCR detected low virus level in these specific lines. The possible reason for this differential behavior is the transgene copy number and expression. Further detailed studies are required to fully characterize this differential behavior of CP_{syn} transgenic tomato plants especially with relation to gene copy number and level of resistance.

TYLCV-OM is a monopartite begomovirus and for such viruses the CP is essential for infectivity (Shakir *et al.* 2018). CP is not essential for bipartite begomoviruses infectivity, although viruses lacking the CP have longer latent periods among inoculation and symptoms development, consistent with the CP having an important, if not essential, role in virus movement (Zhang *et al.* 2001a, b). For this reason, it is presumed that a CP-mediated resistance strategy against bipartite begomoviruses would not be successful. Consistent with this statement *N. benthamiana* plants expressing the ACMV CP did not show resistance (Frischmuth and Stanley 1998). Here in this study CP was used to develop resistance against monopartite virus *i.e.,* TYLC-OM and consistent with earlier studies resistance was achieved. Sinisterra *et al.* (1999) were unable to show expression of the CP. Resistance in this case correlated with CP gene transcript, suggestive of RNA mediated effect (RNA silencing) rather than a protein-mediated effect. However, in the present study a synthetic gene was used that has less identity to wild type CP and thus leads to *trans*-dominant negative interference rather than gene silencing (Lin *et al.* 2012; Fondong 2017). Thus, the reduced amount of CP and CP_{syn} would be competing for the uncoating of viral genome to initiate viral replication. The combined action of both CP possibly reduced the replication rate by coating the viral genome.

One possibility that has not yet been investigated is that transgenic expression of the CP could interfere with insect transmission. For geminiviruses the CP determines vector specificity and is presumed to interact with specific receptors in the digestive tract of vector (insects) to mediate acquisition of virions (Sattar *et al.* 2013). Thus, transgenic expression of CP could potentially be used to competitively block virus receptors in insects, thereby reducing the rate of transmission. Consistent with this hypothesis it has been shown that purified MSV virions treated with formaldehyde (making them non-infectious to plants) significantly reduced transmission by the vector *Cicadulina mbila* when mixed with infectious virions and fed to the insects through a membrane (Briddon *et al.* 1990). Presumably the non-infectious virions competed with the infectious virions for receptors which mediated acquisition, resulting in viruliferous vectors harboring a reduced virus inoculum for onward transmission. It would be interesting to study the

virus acquisition by whiteflies in CP_{syn} transgenic plants in future to understand the mode of action of this construct.

Conclusion

Our findings show that the expression of CP_{syn} in tomatoes resulted in the induction of resistance to TYLCV-OM. Further studies are warranted to understand the precise mechanism of resistance in transgenic tomato plants developed during this study. The expression of transgene with correlation to gene copy number and virus resistance level should be studied. Additionally, all transgenic plants should be assessed for their ability to provide protection against heterologous viruses reported from Oman including; ToLCOMV, ToLCSDV-OM, ToLCABV, ToLCBrV, OKLCuV, WmcSV, ChLCV-OM (Khan *et al.* 2013, 2014; Ammara *et al*. 2015, 2017; Al-Shihi *et al.* 2018b).

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Author Contributions

Um E Ammara: planned work; conducted experiments, analyzed data and wrote the manuscript; Shahid Mansoor: planned work; proof read the manuscript; Muhammad Saeed: planned work; proof read the manuscript; Abdullah M. Al-Sadi: planned work; supervised work, proof read the manuscript.

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