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Citrus tristeza virus-based RNAi in citrus plants induces gene silencing in *Diaphorina citri*, a phloem-sap sucking insect vector of citrus greening disease (Huanglongbing)

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ABSTRACT

A transient expression vector based on *Citrus tristeza virus* (CTV) is unusually stable. Because of its stability it is being considered for use in the field to control Huanglongbing (HLB), which is caused by *Candidatus* Liberibacter asiaticus (CLas) and vectored by Asian citrus psyllid, *Diaphorina citri*. In the absence of effective control strategies for CLas, emphasis has been on control of *D. citri*. Coincident cohabitation in phloem tissue by CLas, *D. citri* and CTV was exploited to develop a novel method to mitigate HLB through RNA interference (RNAi). Since CTV has three RNA silencing suppressors, it was not known if CTV-based vector could induce RNAi in citrus. Yet, expression of sequences targeting citrus phytoene desaturase gene by CTV-RNAi resulted in photo-bleaching phenotype. CTV-RNAi vector, engineered with truncated abnormal wing disc (*Awd*) gene of *D. citri*, induced altered *Awd* expression when silencing triggers ingested by feeding *D. citri* nymphs. Decreased *Awd* in nymphs resulted in malformed-wing phenotype in adults and increased adult mortality. This impaired ability of *D. citri* to fly would potentially limit the successful vectoring of CLas bacteria between citrus trees in the grove. CTV-RNAi vector would be relevant for fast-track screening of candidate sequences for RNAi-mediated pest control.

1. Introduction

Virus-based vectors for expressing foreign genes in plants are routine laboratory tools (Porta and Lomonossoff, 2002; Gleba et al., 2007), generally developed for short term laboratory experiments in herbaceous plants or for making specialty products in these plants. However, the development of vectors that stably express foreign genes for years has opened up new opportunities in perennial plants (Folimonov et al., 2007; Kurth et al., 2012; Dawson and Folimanova, 2013; Dolja and Koonin, 2013). Virus-based vectors can be used to modify the existing generation of trees. One such opportunity is the use for field application to protect against disease or to treat infected plants. For example, the rapid spread of devastating disease of citrus, citrus greening also known as huanglongbing (HLB), which is threatening the survival of the citrus industry has changed the *Citrus tristeza virus* (CTV) vector from a laboratory tool to a potential management strategy for citrus groves (Hodges and Spreen, 2012; National Research Council, 2010). At this time, the one of the hopes for survival of the Florida citrus industry is the production of resistant or tolerant trees *via* transgene (http://www.nytimes.com/2013/07/28/science/a-race-to-save-the-orange-by-altering-its-dna.html?pagewanted=all&_r=0). But the time to make, evaluate, and amplify transgenic citrus trees is too long to save the industry. The viral vector can be deployed more quickly and is being considered as an interim approach (National Research Council, 2010).

The HLB disease manifestation requires both the phloem-limited pathogenic bacterium, *Candidatus* Liberibacter asiaticus (CLas), and phloem feeding Asian citrus psyllid insect vector, *Diaphorina citri* (Halbert and Manjunath, 2004). The disease can be controlled by suppressing either. Initial efforts have been to control the bacterium, but recent progresses in RNA interference (RNAi) in psyllids provide another possible approach (El-Shesheny et al.,

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2013, Wuriyanghan and Falk, 2013; Khan et al., 2013). It is now well-established that double-stranded RNA (dsRNA)-mediated gene silencing mechanism is conserved in many eukaryotes (Geley and Müller, 2004; Gordon and Waterhouse, 2007; Fire, 2007; Price and Gatehouse, 2008). Plant viral vectors have been utilized in virus-induced gene silencing (VIGS) by exploiting antiviral defense mechanism of the host plants (Ratcliff et al., 1997; Waterhouse et al., 2001; Lu et al., 2003). The dsRNAs generated by viral RNA polymerases as intermediates during replication specifically are targeted by host defense machinery (Tenllado and Díaz-Ruíz, 2001; Weber et al., 2006) thus, RNA viruses are inducers as-well-as targets of inherent RNA silencing machinery (Waterhouse et al., 2001). With VIGS vector carrying sequences of host gene, the defense machinery is targeted against the corresponding host mRNAs.

CTV is a member of the genus *Closterovirus* of the family *Closteroviridae*, the largest and the most complex plant viral family. Single-stranded RNA genome of ~19.3 kb is encapsidated by two coat proteins (CP) making a long flexuous virions (2000 nm by 10–12 nm) (Bar-Joseph et al., 1979; Karasev et al., 1995). CTV vector has been shown to be an efficient expression vector capable of expressing more than one foreign gene engineered at different positions in its genome either as extra gene or substitution of some non-essential genes using homologous and heterologous sub-genomic RNA (sgRNA) controller elements (Dawson and Folimanova, 2013; El-Mohtar and Dawson, 2014). However, plant virus-based vectors are notoriously unstable and tend to revert to wild type, with notable exception of CTV vector which has stably retained a foreign gene for more than a decade in citrus plants (Dawson and Folimanova, 2013). Many of the plant and animal viruses encode one silencing suppressor whereas CTV has been shown to encode three distinct suppressors of RNA silencing (Lu et al., 2004), which potentially protect CTV with such a large RNA genome from antiviral silencing machinery of the perennial woody citrus host. CTV open reading frames (ORFs) p23 and coat protein (CP) suppress the silencing pathway at intra- and inter-cellular level, respectively, while ORF p20, exhibits both at intra- and inter-cellular level silencing (Lu et al., 2004). There were serious concerns whether the CTV-based vector could effectively induce gene silencing. Yet, expression of sequences targeting citrus endogenous phytoene desaturase (*PDS*) gene by CTV-based vector resulted in photo-bleaching phenotype in citrus, thus demonstrating CTV as a gene silencing vector.

CTV is limited to phloem and phloem-associated cells in citrus trees like *C*Las bacterium. Since *D. citri* are phloem feeders, they probe and suck phloem sap and existent alongside including *C*Las (when feeding on a diseased plant) and there by succor *C*Las transmission. This coincident cohabitation in the phloem tissue could be exploited to develop a method to combat HLB disease. In our previous study, *in vitro* topical application of dsRNAs of truncated abnormal wing disc (tAwd) gene to nymphs of *D. citri* induced wing deformation and reduced survivability in adults, both positively correlated with *Awd* gene down regulation (El-Shesheny et al., 2013). We hypothesized that; if *D. citri* could acquire the *C*Las bacteria from citrus phloem during feeding, it would acquire other components as well present in the phloem sap, such as virions (like virions of phloem limited CTV), virion RNAs, dsRNAs, small RNAs, etc. The objective of this study was to develop a novel method to mitigate HLB disease by controlling its insect vector, *D. citri*, through CTV-based plant-mediated RNA interference (RNAi). In the present study, gene silencing capabilities of CTV was exploited to express silencing triggers such as dsRNAs (replicative intermediates of both genomic and subgenomic RNAs) and small-interfering RNAs (siRNAs) specific to *D. citri* endogenous *Awd* gene in citrus phloem and associated cells. Silencing the *Awd* gene increased adult mortality and induced malformed wing phenotype which potentially would affect ability of psyllids to vector *C*Las. CTV-RNAi vector would therefore be relevant for fast-track screening of candidate sequences for RNAi-mediated pest control. By virtue of time, labor and cost, CTV-RNAi could be answer to the slow and difficult citrus transgenic approach in mitigating HLB. Besides it could be a valuable tool in functional genomics studies on citrus.

2. Materials and methods

2.1. Plant material

Nicotiana benthamiana plants were grown under controlled growth-room with temperature of 22–24 °C, 16/8 h daylight cycle and 60% humidity. One year old seedlings (approximately two feet tall & stem of a pencil thickness) of Alemow (*Citrus macrophylla*), Duncan grapefruit (*C. paradisi*) and Sour orange (*C. aurantium*) were maintained under a controlled greenhouse conditions at Citrus Research and Education Centre, Lake Alfred, FL.

2.2. Citrus tristeza virus (CTV)-based vectors

The infectious cDNA clone of *Citrus tristeza virus* (CTV isolate T36; GenBank accession no. AY170468) in the binary vector pCAMBIA-1380 was used as base plasmid for engineering all the constructs used in this study (Satyanarayana et al., 1999; Satyanayanana et al., 2001; Gowda et al., 2005; El-Mohtar and Dawson, 2014). This plasmid referred to as wild type, CTV-wt, contained CTV genomic RNA between the duplicated 35S promoter of *Cauliflower mosaic virus* in the 5′ end, a ribozyme sequence of *Subterranean clover mottle virus* satellite RNA at the 3′ end. Unique restriction sites, *Pac*I and *Stu*I were engineered at 5′ and 3′ end, respectively, to ligate the inserts under coat protein (CP) sub-genomic RNA controller element (CE) between ORF-p23 and 3′-untranslated region.

To clone truncated fragment of green fluorescent protein (*GFP*) and generate CTV-tGFP, *GFP* gene coding fragment corresponding to the nts 4–443 of the 30B-GFP-Cycle 3 (Shivprasad et al., 1999) was amplified by SpeedSTAR HS DNA polymerase (Takara Bio. Inc.) using primers GFP-*Pac*I (5′-CGAGTTAATTAAGCTAGCAAAGGAGAAGAACTTTTCACTG-3′) and GFP-*Stu*I (5′-GACAAGGCCTGAGTTATAGTTGTACTCGAGTTTGTGTC-3′) & CTV-GFP (Satyanayanana et al., 2001) as a template. The PCR product was digested with *Pac*I and *Stu*I restriction enzymes and cloned into similarly digested CTV-wt engineered with CTV CP CE and unique *Pac*I and *Stu*I sites to enable ligation of similarly digested tGFP product.

To clone truncated *PDS* gene (tPDS) and generate CTV-tPDS vector, primers were designed based on *C. sinensis PDS* gene (Genbank accession no. DQ235261.1). The truncated fragment corresponding to the nucleotides 4–395 of the *PDS* gene was amplified using total RNA from *C. macrophylla* as a template by SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Life Technologies Corp.) and primers PDS-*Pac*I (5′-CGAGTTAATTAAAGCCTTTGCTTCAGCGTTTCTGAAAGTGCTTTC-3′) and PDS-*Stu*I (5′-GACAAGGCCTGTCTCATACCAGTTCCCGTCCCCATCTTTCC-3′). The PCR product was digested with *Pac*I and *Stu*I restriction enzymes and cloned into similarly digested CTV-tGFP by replacing tGFP with tPDS fragment.

The truncated fragment corresponding to the nucleotides 4–462 of putative abnormal wing disc-like protein (*Awd*) gene (Genbank accession no. DQ673407.1) of *D. citri* was amplified from the total RNA isolated from the *D. citri* by SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Life Technologies Corp.) using the primers Awd-*Pac*I (5′-CGAGTTAATTAAGCCGAACCCAAGGAAAGAACTTTTCTCATG-3′) and Awd-*Stu*I (5′-GACAAGGCCTTTATTCATAGATCCAGGATTCACTGGCATTTG-3′). The PCR product was digested with *Pac*I and *Stu*I

restriction enzymes and cloned into similarly digested CTV-tPDS vector plasmid by replacing tPDS with tAwd fragment.

2.3. Agroinfiltration of CTV constructs into N. benthamiana

Procedures for agroinfiltration of CTV constructs into *N. benthamiana* was followed as described previously (Gowda et al., 2005; Ambrós et al., 2011; El-Mohtar and Dawson, 2014). ORF p22 silencing suppressor from *Tomato chlorosis Crinivirus* (ToCV) ligated in place of hygromycin gene was used in the binary vector pCAMBIA1380 to help establish the CTV infection in the infiltrated leaves (El-Mohtar and Dawson, 2014).

2.4. CTV virion isolation and inoculation to citrus

Systemic leaves from *N. benthamiana* that tested positive for CTV by ELISA, were harvested after 4–6 weeks post infiltration and used to isolate CTV virions for bark-flap inoculation of *C. macrophylla* as described previously (Gowda et al., 2005; Robertson et al., 2005). An additional ultracentrifugation step at 50,000 rpm for 60 min at 4 °C was carried out in Beckman Optima™ TL 100 to further concentrate the virions.

2.5. Large RNA Northern blot hybridization

Total RNA was extracted from 100 mg of *C. macrophylla* tissues using RNeasy Mini Kit (Qiagen) and used in large RNAs Northern hybridizations as described previously (Satyanarayana et al., 1999). The negative-stranded riboprobe with digoxigenin-labeled UTP specific to 3′-untranslated region of CTV genomic RNA (273 nucleotide long) was used for hybridization.

2.6. Small RNA isolation

Total RNAs were extracted from 1 g of *C. macrophylla* tissue using TRIzol® Reagent (Life Technologies Corp.) and was further purified by extracting 1–2 times with Phenol:Chloroform:IsoAmylAlcohol (25:24:1) (Chomczynski and Sacchi, 1987) and separated into large and small RNA fractions by following mirVana™ miRNA Isolation Kit (Life Technologies Corp.). To enrich small RNAs, the RNA sample was brought to 25% ethanol concentration. The lysate/ethanol mixture was passed through a glass-fiber filter to immobilize large RNAs and the ethanol concentration of the filtrate was increased to 55%, and passed through a second glass-fiber filter to immobilize small RNAs. Both glass-fiber filters were washed to elute small and large RNAs separately.

2.7. Small RNA Northern blot hybridization

Detection of small interfering RNAs (siRNAs) by Northern blot was followed as described in the manual of mirVana™ miRNA Isolation Kit (Life Technologies Corp.) with few modifications. One μg small RNA enriched sample was run on a 15% denaturing polyacrylamide gel (urea/TBE) at 150 volts for 90–120 min or until the dye front reaches bottom of the gel. Semi-dry method was employed to transfer small RNAs to positively charged nylon membrane at 100 mA for 60 min and the RNA was immobilized on membrane by UV crosslinking. Full-length cDNA sequence of *GFP* (720 bp), *PDS* (1662 bp) and *Awd* (462 bp) genes were cloned into pGEM®-T Easy Vector (Promega) and negative-stranded DIG-labeled riboprobes were generated using DIG RNA labeling mix (Roche Applied Science) and T7 RNA polymerase. These probes were further hydrolyzed into 50–100 nt long RNA pieces by treating with sodium carbonate buffer as described (Dalmay et al., 2000) and used for hybridization. Prehybridization and hybridization were done at 41 °C using ULTRAhyb™ solution (Life Technologies Corp.) of 10 mL per 100 cm^2 of membrane. The rest of the Northern protocol was followed as described previously (Satyanarayana et al., 1999) except the high stringency wash at 41 °C. Synthetic 5′-DIG-labled oligonucleotide of 18 and 21 mer, which ran as 20 and 22 nucleotides respectively, were used as siRNA size markers in small RNA Northern blot hybridizations.

2.8. Reverse transcription quantitative PCR (RT-qPCR) for plant tissue

The large RNA isolated from mirVana™ miRNA Isolation Kit was used in SYBR Green-I based RT-qPCR to measure the level of down-regulation of *PDS* mRNAs due to gene silencing by CTV-based silencing vector in comparison to CTV-wt control plants. Citrus actin (*ACT*) gene expression was used as an internal control to normalize gene expressions among treatments for RT-qPCR reactions. The level of *PDS* mRNA from control plants infected with CTV-wt was arbitrarily set to a value of one (1) and the level of the *PDS* mRNA from plants infected with CTV-tPDS was estimated as a relative number to this reference value (Hajeri et al., 2011). Similar procedures were followed to measure the level of down-regulation of *GFP* mRNAs from *N. benthamiana* line 16c due to gene silencing by CTV-tGFP vector.

2.9. Insect bioassay

Asian citrus psyllid, *D. citri* used in this study were collected from citrus groves, Polk Co., FL and maintained on Valencia sweet orange, *C. sinensis* (L.) (Osbeck), at 28 ± 1 °C, 60 ± 2% RH and 16/8 h photoperiod. One year old *C. macrophylla* seedlings (approximately two feet tall & stem of a pencil thickness) were used for insect bioassay. In feeding experiments, each of the *C. macrophylla* seedling infected with either CTV-tAwd or CTV-wt control was exposed to 100 *D. citri* adults caged in insect rearing cages (30 in. × 15.5 in. × 15 in.) and kept in growth rooms in conditons as described above. One-month post exposure, all adults and nymphs were removed and egg masses were left. Two weeks later, newly emerged adults were counted, collected and examined for wing malformation and photographed using a Canon Power Shot S3IS digital camera, Leica M3Z stereomicroscope. Five replicative treatments for each experiment were used and compared statistically by the use of *t* test the number of adults with malformed wings to total adults.

2.10. Gene expression analysis in D. citri

Total RNA was isolated using TRIzol® Reagent (Life Technologies Corp.) from total of 10 *D. citri* for each treatment. Single-stranded RNA was purified from the total RNA by ssDNA/RNA Clean & Concentrator™ (Zymo Research) and expression levels of *Awd* was determined using SYBR Green-I based RT-qPCR in triplicate for each biological replicate. Alpha-tubulin (*TubA*) was used as a non-target gene control and we normalized gene expression of actin (*Act*) to compare the relative gene expression levels among treatments. The level of *Awd* transcripts in *D. citri* adults exposed to CTV-wt plants was arbitrarily set to the value one and the level of *Awd* transcripts in CTV-tAwd were presented as relative value to this reference value (Hajeri et al., 2011). Means and standard deviation of experiments in triplicate are presented.

3. Results

3.1. CTV-induced gene silencing in N. benthamiana line 16c

N. benthamiana is a non-natural host of CTV. To demonstrate the gene silencing capabilities of CTV, transgene green fluorescent

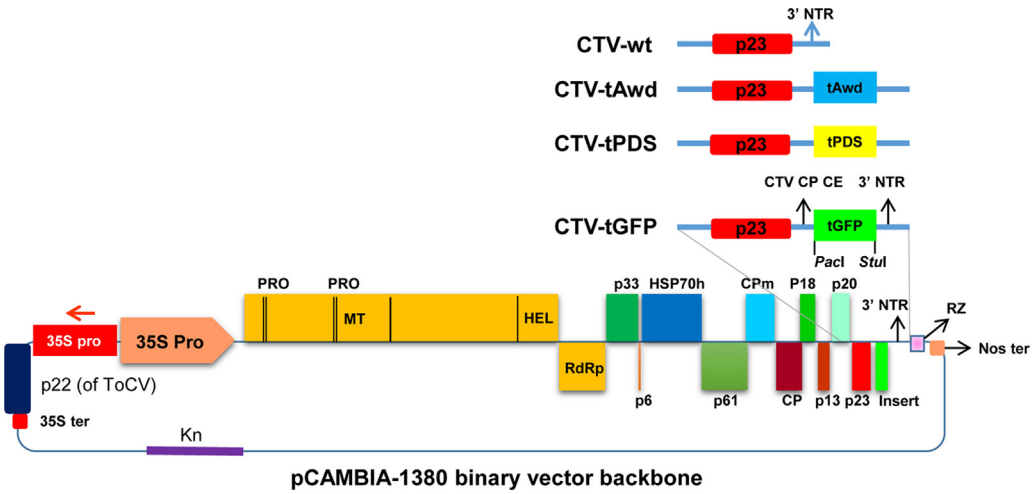

Fig. 1. Schematic representation of *Citrus tristeza virus* (CTV) genome in a binary vector. Schematic representation of full-length infectious cDNA clones of *Citrus tristeza virus* (CTV) with its open reading frames (ORF) placed between enhanced 35S promoter of *Cauliflower mosaic virus* at the 5′ end, ribozyme (RZ) of *Subterranean clover mottle virus* satellite RNA and nopaline synthase terminator (Nos ter) at the 3′ end in the binary vector pCAMBIA-1380. The vector plasmid referred to as wild type CTV (CTV-wt) is based on CTV isolate T36. Unique restriction sites, *Pac*I and *Stu*I at 5′ and 3′ end, respectively, to ligate the inserts under coat protein (CP) sub-genomic RNA controller element (CE) between ORF-p23 and 3′-nontranslated region (NTR). Truncated green fluorescent protein (tGFP) was cloned using unique restriction sites *Pac*I and *Stu*I to generate CTV-tGFP, similarly, truncated phytoene desaturase (tPDS) and truncated abnormal wing disc (tAwd) were cloned to generate CTV-tPDS and CTV-tAwd respectively. ORF p22 silencing suppressor from *Tomato chlorosis Crinivirus* (ToCV) driven by 35S promoter & 35s terminator (35S ter). PRO, papain-like proteases; MT, methyltransferase-like domain; HEL, helicase-like domain; RdRp, RNA-dependent RNA polymerase domain; and the ten 3′-end ORFs p33, p6, HSP70h, p61, CPm, CP, p18, p13, p20, and p23.

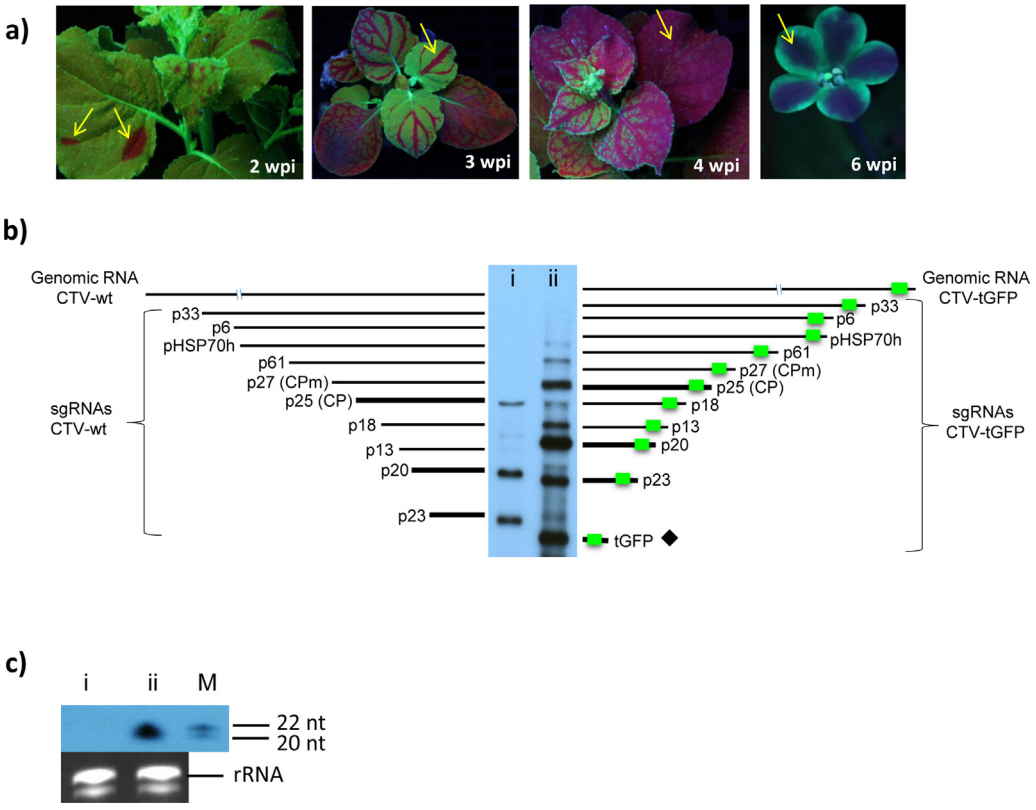

Fig. 2. *Citrus tristeza virus* (CTV)-induced gene silencing in *Nicotiana benthamiana* transgenic line 16c. Transgene green fluorescent protein (*GFP*) of *Nicotiana benthamiana* line 16c was silenced by *Citrus tristeza virus* (CTV)-based virus-induced gene silencing vector carrying truncated GFP (tGFP). (a) Progression of GFP silencing in the systemic leaves, stems and flowers at 2, 3, 4 and 6 weeks post infiltration (wpi) was photographed under handheld long wave fluorescent UV lamp. GFP Silenced areas appear as red, indicated by arrow mark, due to autofluorescence of chlorophyll. (b) Schematic representation of the subgenomic RNA (sgRNA) profile of CTV from plants infected with wild type CTV (CTV-wt) control (left), and CTV-tGFP (right). Abundantly accumulating sgRNAs for p23, p20 and CP are shown in thick lines. Northern blot shows the 3′ sgRNAs and the extra sgRNA for tGFP, indicated by a diamond symbol, accumulated in CTV-tGFP plants (ii; on right) compared to CTV-wt plants (i; on left). The blot was hybridized with digoxigenin labeled minus-sense ribo-probe specific to the 3′-nontranslated region of CTV. (c) Accumulation of GFP-specific small interfering RNAs (siRNAs) in CTV-tGFP plants (ii) compared to CTV-wt (i). Ethidium bromide stained rRNA in polyacrylamide gel electrophoresis as a loading control is shown at the bottom. Synthetic 5′-DIG-labled oligonucleotide of 18 and 21 mer, which ran as 20 and 22 nucleotides, respectively, were used as siRNA size markers (M). The blot was hybridized with digoxigenin labeled minus-sense ribo-probe specific to full-length sequence of *GFP* gene.

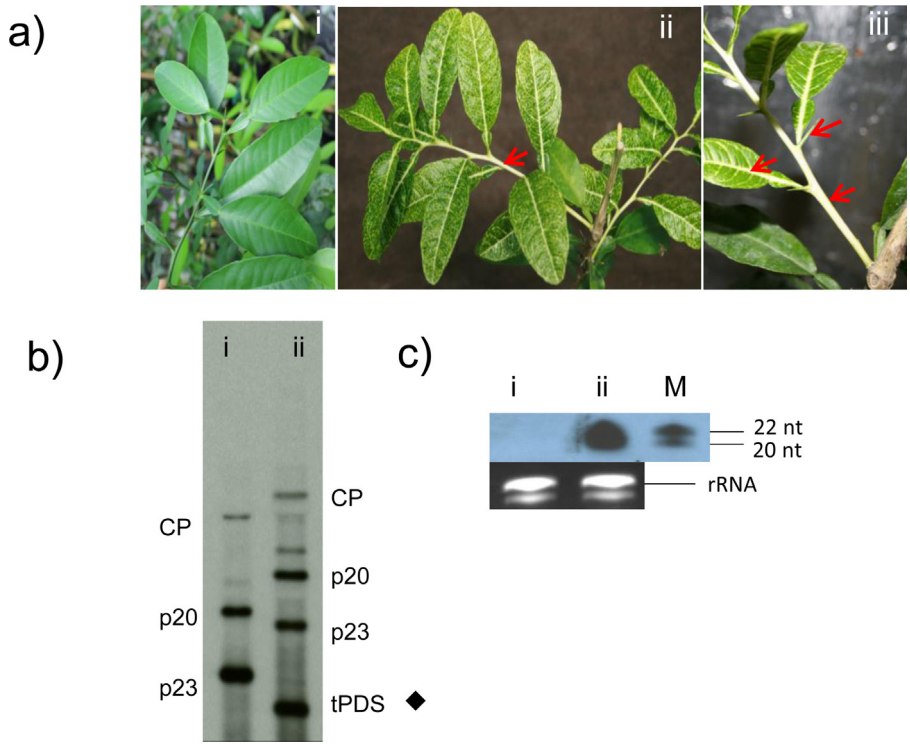

Fig. 3. *Citrus tristeza virus* (CTV)-induced gene silencing in citrus. *Citrus macrophylla* endogenous gene, phytoene desaturase (*PDS*) was silenced by CTV-based virus-induced gene silencing (VIGS) vector carrying truncated PDS (tPDS). (a) Photo-bleaching phenotype observed in the newly emerging leaves, stem and thorns, indicated by arrow marks (ii and iii), of *C. macrophylla* infected with CTV-tPDS compared to control wild type CTV (CTV-wt) (i). (b) Northern blot shows the 3′ subgenomic RNAs (sgRNAs) and the extra sgRNA for tPDS, indicated by a diamond symbol, accumulated in CTV-tPDS plants (ii; on right) compared to CTV-wt plants (i; on left). The blot was hybridized with digoxigenin labeled minus-sense ribo-probe specific to the 3′nontranslated region of CTV. (c) Accumulation of PDS-specific small interfering RNAs (siRNAs) in CTV-tPDS plants (ii) compared to CTV-wt (i). Ethidium bromide stained rRNA in polyacrylamide gel electrophoresis as a loading control is shown at the bottom. Synthetic 5′-DIG-labled oligonucleotide of 18 and 21 mer, which ran as 20 and 22 nucleotides respectively, were used as siRNA size markers (M). The blot was hybridized with digoxigenin labeled minus-sense ribo-probe specific to full-length sequence of *PDS* gene.

protein (*GFP*) of *N. benthamiana* line 16c was silenced by CTV-VIGS vector carrying truncated GFP (tGFP; Supplementary data 1a). We engineered tGFP into CTV to express 400 nucleotides of *GFP* under CTV CP sgRNA controller element (CE) using unique *Pac*I and *Stu*I restriction sites (Fig. 1). *N. benthamiana* plants were inoculated with a binary plasmid vector carrying CTV-tGFP through agro-infiltration of fully expanded true leaves. Wild type CTV (CTV-wt) was used as a control. Progression of GFP silencing was monitored in the leaves, stems and flowers by fluorescence observation under long wave UV (Fig. 2a). Northern blot analysis of total RNA from the systemic leaves showed accumulation of the extra sgRNA in CTV-tGFP plants compared to CTV-wt plants. The tGFP sgRNA was the most abundantly accumulated sgRNA and the tGFP sequence was present as a component of all sub-genomic and genomic RNAs (Fig. 2b). The GFP silencing was further confirmed by reverse transcription quantitative PCR (RT-qPCR) showing 4–5-fold down-regulation of GFP mRNA (data not shown), the extent of GFP-mRNA down regulation does not represent a true value because the total RNA isolated for RT-qPCR represents a mixture from silenced and non-silenced regions. Further, Northern blots hybridization showed accumulation of GFP-specific ~21 nucleotide small interfering RNAs (siRNAs) from plants infected with CTV-tGFP compared to CTV-wt control plants (Fig. 2c).

3.2. CTV-induced gene silencing in citrus

To test the silencing induced by CTV in citrus, its natural host, citrus endogenous gene, phytoene desaturase (*PDS*) was targeted by CTV-VIGS vector carrying truncated PDS (tPDS; Supplementary data 1b). We engineered tPDS into CTV to express 392 nucleotides of PDS under CTV CP sgRNA CE using unique *Pac*I and *Stu*I restriction sites (Fig. 1). *N. benthamiana* plants were inoculated with a binary plasmid vector carrying CTV-tPDS through agro-infiltration of fully expanded true leaves and wild type CTV (CTV-wt) was used as a control. CTV virions were isolated from symptomatic systemic leaves of *N. benthamiana* four weeks post infiltration. *C. macrophylla* plants inoculated with CTV-tPDS virions showed a photo-bleaching phenotype in the newly emerging leaves, stems and thorns (Fig. 3a) compared to control CTV-wt plants. Northern blot analysis of RNA showed accumulation of the extra sgRNA in CTV-tPDS plants compared to CTV-wt plants (Fig. 3b). Further, RT-qPCR showed a 2.5–3-fold down-regulation of *PDS* mRNA in infected leaves (data not shown). Additionally PDS-specific siRNAs were detected from plants infected with CTV-tPDS compared to CTV-wt (Fig. 3c).

Graft-transmissibility of CTV-VIGS vector and photo-bleaching phenotype to other citrus cultivars was tested. Source plant, *C. macrophylla*, harboring CTV-tPDS vector, used for side and leaf graft inoculations to Duncan grapefruit (*C. paradisi*) and Sour orange (*C. aurantium*), which induced photo-bleaching phenotype in the newly emerged systemic leaves (Fig. 4).

3.3. CTV-based citrus plant-mediated RNAi in phloem-sap sucking insect D. citri

The results presented above suggested that CTV vector could be successfully used as an efficient silencing vector. We designed CTV-RNAi vector, CTV-tAwd, to express 459 nucleotides sequence of *D. citri Awd* gene (tAwd; Supplementary data 1b) in citrus similar to CTV-tPDS (Fig. 1). CTV-tAwd virions were isolated from

Fig. 4. Graft-transmissibility of *Citrus tristeza virus* (CTV)-based virus-induced gene silencing (VIGS) vector and photo-bleaching phenotype to other citrus cultivars. Source plant, *Citrus macrophylla*, harboring CTV-VIGS vector expressing truncated phytoene desaturase gene of *C. macrophylla* and inducing photo-bleaching phenotype. *C. macrophylla* source plant used for side and leaf graft inoculations to Duncan grapefruit (*C. paradisi*) and Sour orange (*C. aurantium*), which induced typical photo-bleaching phenotype in the newly emerged systemic leaves.

symptomatic systemic leaves of *N. benthamiana* and inoculated to *C. macrophylla* plants similar to CTV-tPDS. Northern analysis of the total RNA isolated from newly emerged systemic leaves of *C. macrophylla* plants, which were inoculated with CTV-tAwd, showed the accumulation of an extra sgRNA for tAwd compared to CTV-wt (Fig. 5a). Awd-specific siRNAs were detected in CTV-tAwd plants compared to CTV-wt (Fig. 5b). One hundred adult *D. citri* (per plant) were allowed to feed on five individually caged *C. macrophylla* infected with CTV-tAwd. One-month post exposure, all *D. citri* adults and nymphs were removed and egg masses were left. Two weeks later, we calculated the total number of resulting *D. citri* adults in the new generation. Statistically significant differences (t test and evaluated at $P < 0.05$) were observed in number of newly emerged adults between CTV-tAwd and CTV-wt plants (Fig. 5c). Among the new generation of *D. citri* adults that emerged from nymphs, some displayed wing-malformed phenotype. Nearly 15% of the nymphs fed on CTV-tAwd plants developed into severe wing-malformed adults (Fig. 5d and f-ii) and another 30% of nymphs developed varying degrees of wing-malformation.

Alpha-tubulin (*TubA*) and actin (*Act*) were used as a non-target control gene and internal control gene, respectively to quantify *Awd* expression level between the treatments by t-test analysis. *TubA* expression did not change between treatments while *Awd* gene expression was down-regulated approximately 1.5–2-fold in wing-malformed adults of *D. citri* compared to control *D. citri* (Fig. 5e). Acquisition of CTV-specific dsRNAs by *D. citri* was confirmed by conventional two-step RT-PCR using sense or antisense primer generated cDNAs from RNAs isolated from *D. citri* fed on CTV-tPDS and CTV-tAwd plants (unpublished data).

4. Discussion

During replication, CTV accumulates abundant amounts of genomic and sub-genomic (sg) replicative intermediates as double-stranded RNAs (Dodds and Bar-Joseph, 1983; Hilf et al., 1995) and copious amounts of siRNAs (Scott and Dawson, unpublished), the latter possibly the consequence of antiviral silencing activity. The sgRNAs for ORFs closer to the 3′-ends accumulated in abundance compared to ORFs away from the 3′-end (Navas-Castillo et al., 1997). Additionally, the sgRNAs for p23, p20 and CP with dedicated sgRNA controller elements are produced in higher abundance compared to other sgRNAs (Hilf et al., 1995). It is thus possible to augment the abundance of silencing triggers, such as dsRNAs and siRNAs, by engineering sequence of interest at the 3′end and foster CTV as a gene silencing vector. We have demonstrated the gene silencing capabilities of CTV vectors by silencing transgene *GFP* in *N. benthamiana* line 16c and endogenous gene *PDS* in citrus. Thus, CTV-based VIGS vector could be a useful tool for reverse genetics to study the functions of citrus genes involved in basic cellular functions, metabolic pathways, developmental biology, and plant-microbe interactions.

The observations that the three RNA silencing suppressors do not prevent CTV-induced gene silencing, that CTV accumulates to high levels in phloem and phloem-associated cells, that CTV produces large amounts of dsRNAs, and that *D. citri* nymphs suck large amounts of fluid from the phloem of young shoots encouraged us to target psyllid genes using CTV-based RNAi vector.

Bt (*Bacillus thuringiensis*) toxin expressing transgenic plants have been effectively controlling chewing insects such as lepidopteran and coleopteran pests (Naranjo, 2011; Shelton et al., 2002). However, for phloem sap-sucking insects, such as psyllids, aphids, whiteflies, planthoppers and plant bugs, pesticides are still the major method to control (Walker and Allen, 2010; Gatehouse and Price, 2011). Therefore, in order to control phloem sap-sucking insects, novel methodologies such as RNAi-based technology must be considered in order to rein in economic and environmental damage (Zhang et al., 2013).

The two major challenges in deploying RNAi-based technology for pest control are effective target gene selection and reliable dsRNA delivery. We targeted *D. citri* endogenous *Awd* gene for silencing; because, inhibition of the *Awd* gene would induce altered wing development, a visible phenotype and down regulation of wing development of *D. citri* would impair its ability to fly and potentially limit the successful vectoring of the bacterial pathogen between citrus trees in the grove. Once the target gene is identified, the reliable and convenient dsRNA delivery system is prerequisite for pest control at field level. Delivery of dsRNA could be achieved by micro-injection, micro-application (topical application), soaking or by feeding as a dietary component (El-Shesheny et al., 2013; Zhang et al., 2013). However, these methods can only be used in laboratory experiments. Spraying dsRNA targeting specific insect pest could be a viable approach at the field level (Gan et al., 2010), if dsRNA can be cheaply mass produced. Expression of dsRNAs in transgenic plants has been shown to induce RNAi effects on target insects (Huang et al., 2006; Baum et al., 2007; Mao et al., 2007; Gottula and Fuchs, 2009). However, transgenic approach in citrus is slow and difficult. By virtue of its time, labor and cost efficiency, transient expression system of CTV-based plant-mediated RNAi provides major advantage over stable transformation in citrus since the CTV vector has been shown to be stable for several years in trees. This remarkable stability of CTV vector could be used in silencing insect genes or other pest genes directly in the field as an integrated pest management practice. Graft-transmissibility of CTV-tPDS vector and its silencing triggers to other citrus cultivars suggested that the silencing trait against insect pests induced by CTV-RNAi vector could also be transferable to other commercial cultivars of citrus through vegetative grafting which is not possible with transgenic lines with such traits.

Even in case of preference of transgenic approach over CTV-based RNAi, the CTV vector would act as a tool in fast-track screening of candidate genes/sequences related to insect's survivability, flight, or reproduction and ultimately affect the vectoring potential of insect vector in developing transgenic citrus. Thus CTV-based silencing vector would hasten the process of selecting right candidate sequences for stable transformation. On the other hand, CTV-based silencing vector could be used as an interim solution in mitigating the HLB disease manifestation at present in the field. The species specificity is the critical issue that needs to be addressed before using RNAi-based pest control measures in the

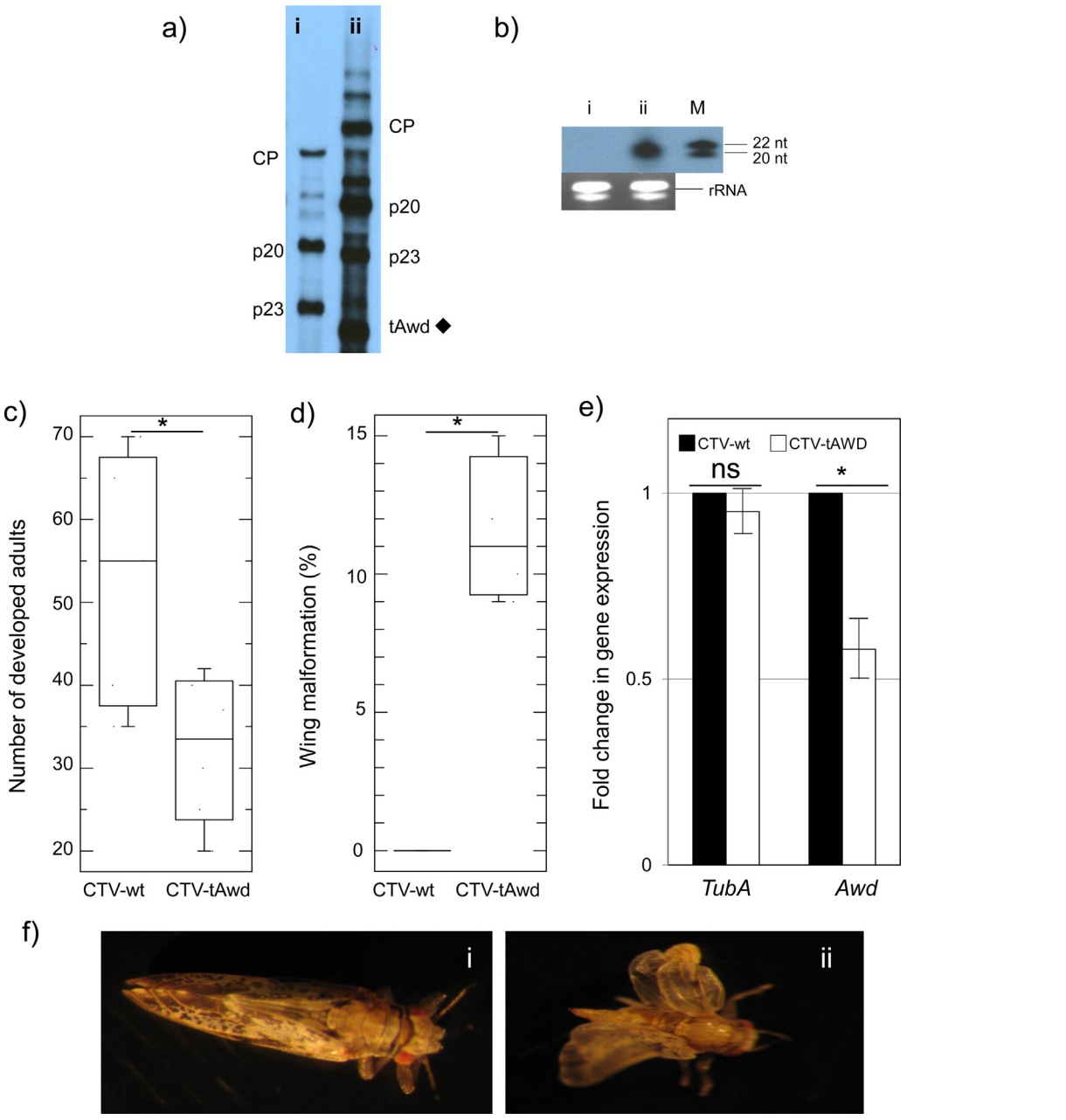

Fig. 5. *Citrus tristeza virus* (CTV)-based plant-mediated RNAi in phloem-sap sucking insect *Diaphorina citri.* (a) Northern blot analysis of total RNA from systemic leaves of *Citrus macrophylla* plants infected with wild type CTV (CTV-wt) control (i) and truncated abnormal wing disc gene (tAwd) expressing CTV vector (CTV-tAwd) (ii). Accumulation of an additional subgenomic RNA (sgRNA), tAwd, in plants infected with CTV-tAwd is indicated by a diamond symbol. The blot was hybridized with digoxigenin labeled minus-sense ribo-probe specific to the 3′ nontranslated region of CTV. (b) Accumulation of Awd-specific small interfering RNAs (siRNAs) in CTV-tAwd plants (ii) in comparison to CTV-wt (i). Ethidium bromide stained rRNA in polyacrylamide gel electrophoresis as loading control is shown at the bottom. Synthetic 5′-DIG-labeled oligonucleotide of 18 and 21 mer, which ran as 20 and 22 nucleotides respectively, were used as siRNA size markers (M). The blot was hybridized with digoxigenin labeled minus-sense ribo-probe specific to full-length sequence of abnormal wing disc (*Awd*) gene. (c) Box plot shows the number of *Diaphorina citri* adults developed from nymphs fed on CTV-wt and CTV-tAwd plants after one month exposure. (d) Percentage of wing-malformed adults on CTV-wt and CTV-tAwd plants, (e) expression of *Awd* in *D. citri* adults exposed to CTV-wt and CTV-tAwd plants. Alpha-tubulin (*TubA*) and actin (*Act*) were used as a non-target gene and an internal control gene, respectively. The level of *Awd* transcripts in *D. citri* adults exposed to CTV-wt plants was arbitrarily set to the value one and the level of *Awd* transcripts in CTV-tAwd were presented as relative value to this reference value. Means and standard deviation (as bars) of experiments in triplicate are presented. Asterisks indicate statistically significant difference ($p < 0.05$) and 'ns' as non-significant. (f) Images of *D. citri* adults developed from nymphs after exposure to CTV-wt (i) CTV-tAwd (ii) plants.

field. But RNAi technology has the potential to address this problem by producing sequence specific and species specific RNAi pesticide (Whyard et al., 2009).

5. Conclusions

Three RNA silencing suppressors of CTV do not prevent CTV from inducing gene silencing in *Citrus* and *N. benthamiana* transgenic line 16c. CTV-based plant-mediated RNAi induces gene silencing in phloem-sap sucking insect *D. citri*, which vectors bacterial disease HLB. Thus CTV-based RNAi vector could be a valuable tool for fast-track screening candidate sequences in developing transgenic citrus against citrus pest and diseases. Because of the slow and difficult transgenic methodology in citrus, CTV-RNAi vector could be an interim solution in mitigating the spread of HLB disease in the field.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.02.010.

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