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Sequence variation in two genes determines the efficacy of transmission of citrus tristeza virus by the brown citrus aphid

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BRIEF REPORT

Sequence variation in two genes determines the efficacy of transmission of citrus tristeza virus by the brown citrus aphid

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Abstract Vector transmission is an important part of the viral infection cycle, yet for many viruses little is known about this process, or how viral sequence variation affects transmission efficacy. Here we examined the effect of substituting genes from the highly transmissible FS577 isolate of citrus tristeza virus (CTV) in to the poorly transmissible T36-based infectious clone. We found that introducing p65 or p61 sequences from FS577 significantly increased transmission efficacy. Interestingly, replacement of both genes produced a greater increase than either gene alone, suggesting that CTV transmission requires the concerted action of co-evolved p65 and p61 proteins.

Over past 30 years, significant progress has been made in the identification and characterization of virus-vector interactions [1, 2]. Viruses may be circulative, and move through the gut lining of the insect, replicating and persisting for the life of the vector, or more commonly, non-circulative, binding to structures within the insect vector's mouthparts or foregut. In some non-circulative virus-vector systems, the viral coat protein interacts with the cuticular intima of the hemipteran vector, whilst others use one or more non-virion helper proteins to bridge virus and vector [3, 4]. For most plant viruses, the mechanism by which virus and vector interact is unknown, or is described by analogy from better studied virus-vector systems.

One area that requires greater attention is the effect of mutation or variation in viral genes that control transmission. One of the best characterized systems is cucumber mosaic virus (CMV), in which it has been shown that transmission efficacy is determined by polymorphism within certain codons of the coat protein, and that these polymorphisms are aphid species-specific [5]. The effect of mutation in potyvirus transmission has also been described, where, unlike CMV, motifs in two proteins are crucial to transmission: the DAG motif in the coat protein [6], and the PTK and KITC motifs in HcPro [7].

The effect of polymorphism in other viruses is less well understood. One example is citrus tristeza virus (CTV) an aphid transmitted member of the family *Closteroviridae* with four major vectors: species *Aphis gossypii*, *Aphis spiraecola*, *Toxoptera aurantii,* and *Toxoptera citricida* [8]. CTV is a genetically diverse virus, with seven characterized strains that differ from one another by 10-20 % at the nucleotide level [9]. It is not presently known how this diversity, particularly in the p27, p65 and p61 genes, affects transmission efficacy of individual isolates. It has recently been demonstrated [10] that this virus uses three proteins to interact with the lining of the cibarium of the aphid: (1) p27, the minor coat protein, which has been reported to be involved in vector interaction in other characterized closteroviruses [11, 12], (2) p65, a HSP70-like molecular chaperone, and (3) p61, a HSP90-like molecular chaperone, both of which are required for virion assembly [13].

Here we examined the effect of substitution of sequences into an infectious clone developed from the poorly transmissible CTV T36 isolate, from a related yet genetically distinct and highly transmissible isolate, FS577. We

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found that substitution of either p61 or p65 (p27 is identical) from FS577 into T36 gave a minor increase in transmission, yet when both sequences were introduced simultaneously, transmission efficacy increased to near FS577 wild type levels, suggesting that aphid transmission of CTV requires not only the minor coat protein but the concerted action of these two proteins.

We began by examining differences in transmission efficacy between CTV isolates T36 and FS577, both of which were from the collection held at the University of Florida Citrus Research and Education Center. These two isolates were selected as they are members of the same strain lineage [9] and were known to be pure, single strains rather than mixtures of multiple strains [14]. The full length infectious clone of T36, 947R [15] was also tested to provide a baseline for subsequent hybrid generation. Six-month-old *Citrus macrophylla* were graft-inoculated with each of the source isolates, and virus presence confirmed by ELISA 6 weeks post-inoculation [14]. Aphid transmissions were conducted from these plants using *T. citricida*, with a 24 hour acquisition period, followed by transfer to six-week-old *C. macrophylla* in batches of 10 aphids per plant, and left to transmit for a further 24 hours. Seedlings were tested for the presence of CTV by ELISA at eight weeks post-transmission.

Having determined that FS577 was transmitted at significantly greater frequency than T36, or its infectious clone, we constructed a series of hybrid infectious clones between the two isolates. As previous research had shown that the p65, p61, and p27 genes are involved in the interaction between virus and aphid [10], we focused on these genes. First, a single replacement from within p6 to within p18 (bases 11661 to 17300) was constructed through amplification (Table 1) of a fragment from FS577-1-8 cDNA, and substituted into the T36 infectious clone [15] using PmeI-PstI restriction sites. Three hybrids, replacing the p61, p65, and both p61 and p65 genes, were constructed through the amplification of three fragments: (a) from base 11647 to the 5' end of the gene being replaced, (b) the gene being replaced, and (c) from the 3' end of the gene of interest to base 17681 (Table 1). These amplicons were assembled into a contiguous fragment by overlap PCR, and substituted into the T36 vector via the PmeI-PstI restriction sites, as above. No hybrid was constructed for the replacement of p27 as the sequence of this gene in both FS577 and T36 is identical . All hybrids were inoculated into *C. macrophylla* seedlings as previously described [16], and successful inoculation confirmed by ELISA. To ensure sufficient source plants for transmission studies, hybrids were then sub-propagated into additional *C. macrophylla* and virus presence confirmed by ELISA. Plants were then cut back to force new flush growth suitable for aphid feeding. Aphid transmission assays were conducted as described above. Finally, to determine whether differences in transmission efficacy of the hybrids were attributable to differences in viral load in the source plants, we tested the CTV titer in the donor plants used in the aphid transmission assays using real time RT-qPCR, as previously described [14]. To support this we also used ELISA to approximate virion titer.

To examine the effect of sequence variation on transmissibility we compared the transmission efficacy of two CTV isolates, T36 and FS577. We found that T36, the eponymous type member of the strain transmitted at a frequency of 0.5 % (2 positive from 380 transmissions) using batches of 10 *T. citricida*, whereas FS577, a member of the same sequence group as T36 [9], was transmitted at a frequency of 24 % (95 of 394 transmissions). While these two isolates differ by only $\sim$2 % at the nucleotide level, these genetic differences translate into phenotypic differences. Most importantly, T36 contains nine amino acid substitutions in p61 and seven in p65, two genes previously shown to be involved in aphid transmission of CTV [10] that are not found in FS577 or other extant members of that strain.

We examined the effect of these mutations on aphid transmission efficacy through a construction of a series of T36-FS577 hybrid infectious clones (Figure 1). We first compared a T36-based hybrid with replacement of the

Table 1 Primers used to amplify products for the substitution of FS577 fragments into the full-length T36 infectious clone

Gene/region	Sense	Sequence (5′-3′)	Binding site
Outer primers	+	ACTAGTTAGTGCTGTCTCTCCGTA	11647-11670
	-	GTAGACTCTAGTTATCGCAAGGTAAG	17656-17681
Start of P65	+	GACTGTCTAAGCGGTATGGTGCTTTT	12020-12045
	-	CGAAGTCTAAACCCAAAAGCACCATA	12034-12059
End of p65 / Start of P61	+	TGGAAAGAATACCTCTCTGAATCAAC	13800-13825
	-	CATCGAAATTTCGAGTTGATTCAGAG	13814-13839
End of P61	+	CCTTATCATGGCAGGTTATACAGTAC	15318-15343
	-	CATCGGTTTTAGGAAGTACTGTATAA	15333-15358

Primer binding sites are given as per the sequence of isolate FS577 (NCBI GenBank Accession No. KC517488)

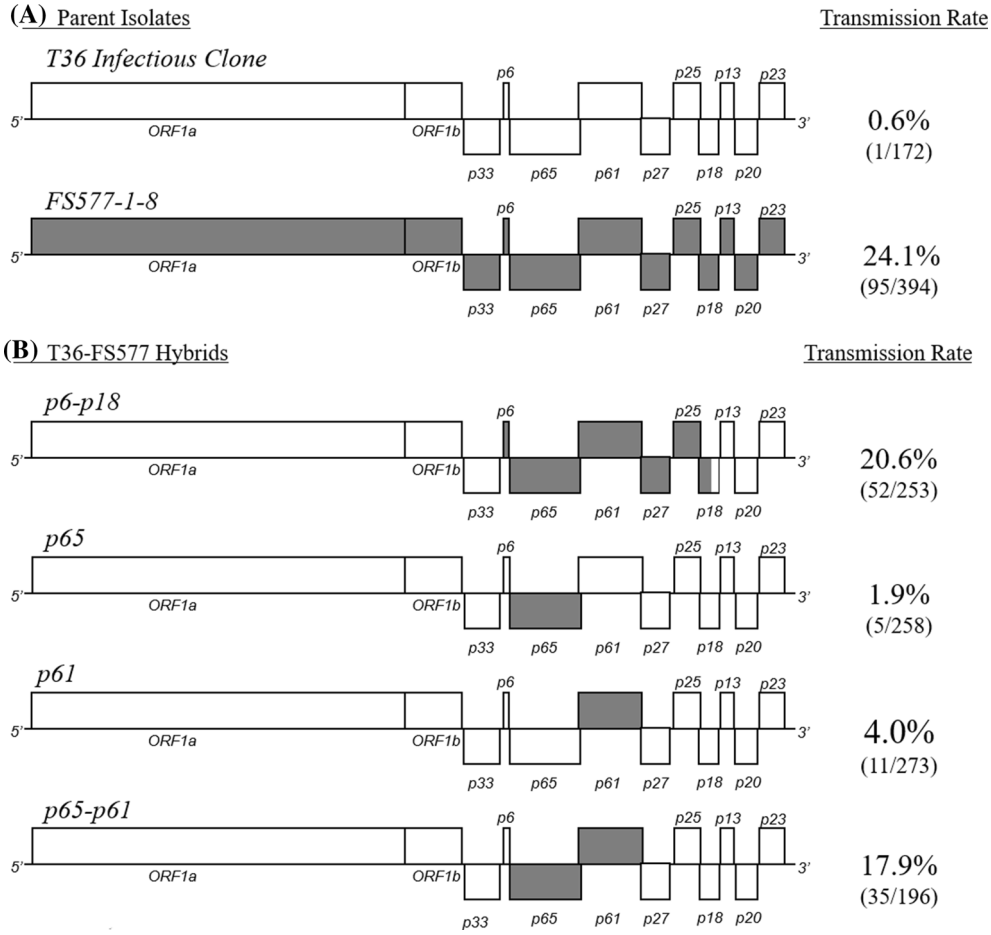

Fig. 1 Diagrammatic representation of genes substituted from isolate FS577-1-8 (grey) into the full-length T36-based infectious clone (white), and their effect on the rate of aphid transmission by *T. citricida*

partial p6 through partial p18 ORFs (bases 11661 to 17300) from FS577, to the unmodified T36 clone and found that substitution of genes within this region resulted in a marked increase in transmission efficacy from 0.6 (1 positive from 172 transmissions) to 21 % (52/253 transmissions). Of the genes previously identified as being involved in aphid transmission within this region, only p61 and p65 differ between T36 and FS577; the sequence of p27 is identical when comparing both isolates. Therefore, the p61 and p65 genes of FS577 were both separately and simultaneously inserted into the T36 infectious clone. Transmission with *T. citricida* revealed that substitution of p61 increased transmission efficacy from 0.6 to 4 % (11/273), while substitution of p65 gave an increase to 2 % (5/258), confirming that the sequence of these genes controls transmission efficacy. Interestingly, simultaneous substitution of both genes increased transmission efficacy to 18 % (35/196), which suggests that aphid transmission requires the concerted action of these two genes through an unknown mechanism, and further, that this requires compatible sequences in these two genes.

Given the role of these two proteins in virion assembly, we tested whether this increase was due to a difference in viral titer of the hybrids relative to each other or the controls by using both real time RT-qPCR and ELISA. Quantification of viral RNA by RT-qPCR (Figure 2a) showed that there was no significant difference (Tukey HSD $P > 0.05$) in virus replication and accumulation caused by the insertions. Examination by ELISA gave similar results (Figure 2b); while this method is non-linear, and at best can be an approximation of virion copy number, it does indicate that there is little difference in virus titer between the hybrids and their parental isolates. Cumulatively, this suggests the substitutions did not affect virus accumulation, therefore we propose that the increase in efficacy is due to the polymorphisms contained within the substituted p61 and p65 genes.

The interaction between viruses and their vectors is highly specific, involving the precise interaction of viral coat or helper proteins to receptors in the vector's mouthparts or foregut [4, 17]. Mutation of key motifs within the viral vector-binding proteins has been shown to reduce or

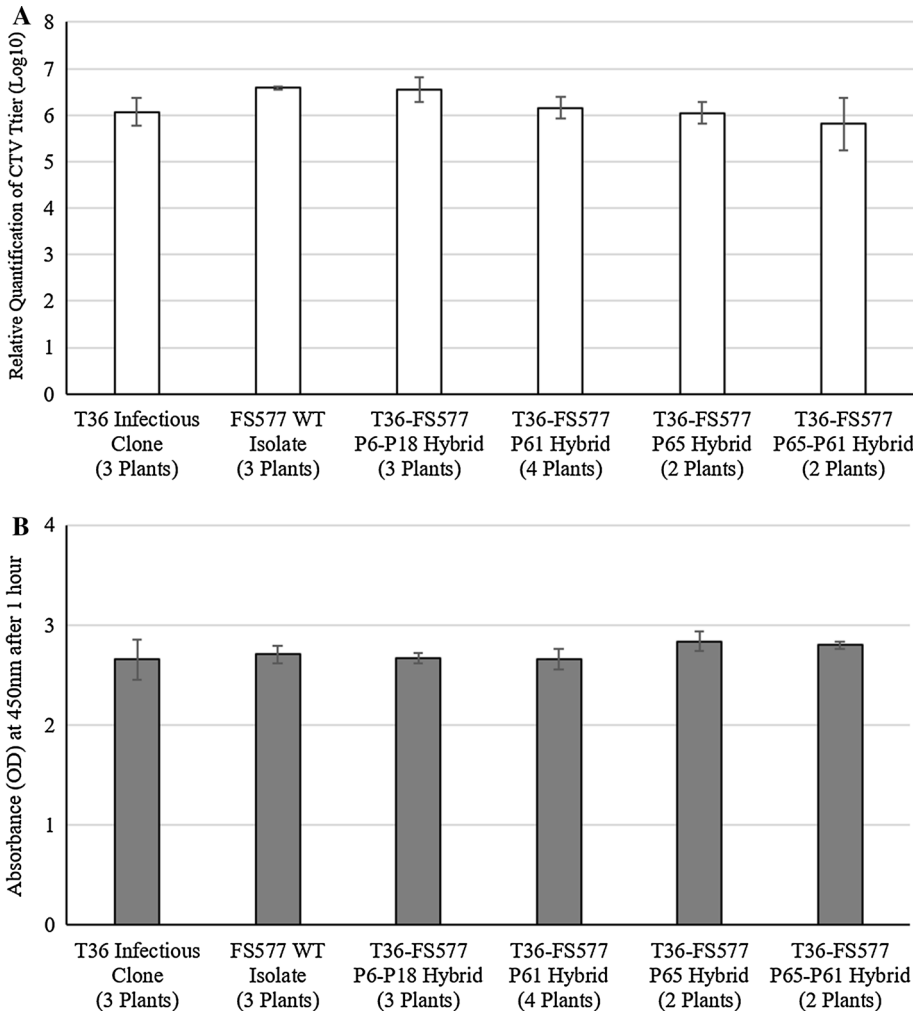

Fig. 2 The approximate titer of CTV T36-FS577 hybrid infectious clones, determined by **a** real time RT-qPCR, and **b** ELISA, in flush tissue of *C. macrophylla* seedlings used as aphid transmission sources, as compared to the unmodified T36 infectious clone, and FS577 parental isolate

abolish transmission [7, 18]. Unsurprisingly, viral proteins involved in transmission are under strong selective pressure [19], and although in the absence of transmission, such as repeated mechanical propagation, non-transmissible isolates have been noted to emerge [20].

Following the recent discovery that CTV uses three proteins, p27, p65, and p61, to interact with its aphid vector [10], we asked what effect polymorphism within these genes has on transmissibility. We focused on the poorly transmissible T36 isolate, which has been graft-propagated in greenhouse conditions for over 30 years (S. Garnsey, personal communication), and compared it to FS577, a highly transmissible member of the same genetic lineage. While the sequence of p27 is identical between the two isolates, there are seven and five nonsynonymous substitutions present in the p61 and p65 proteins of isolate T36, respectively, that are not found in either FS577 or other T36-like isolates obtained from field trees in Florida [9]. Interestingly, the sequence of these two proteins in field isolates are much more conserved, and differ from one another by between 2-6 and 0-2 nonsynonymous substitutions respectively. It is likely that the absence of selection for transmission has allowed T36 to accumulate mutations not found in other isolates. We found that replacement of the p65 or p61 sequences of the T36 infectious clone gave a minor increase in transmission, but when both were replaced simultaneously, transmission efficacy increased significantly. This suggests that aphid transmission requires the concerted activity of compatible p61 and p65 proteins.

The marked increase of CTV transmission by *T. citricida* observed after simultaneous substitution of p61 and p65 suggests co-evolution between these proteins, and potential interaction during transmission. It has previously been shown that these two proteins are essential for virion assembly, and will only restrict the minor coat protein to the 5' end of the virion when both are present [13]. It is plausible that they interact in a similar manner to effect aphid transmission by an unknown mechanism, and the requirement for co-evolved sequences is a requirement for compatible protein-protein binding sites. Interestingly, the polymorphisms in these two genes, between isolates T36

and FS577, do not appear to affect virion assembly; all hybrids infected *C. macrophylla* systemically and accumulated to similar titers.

How the p65 and p61 proteins interact with one another, and with the aphid vector requires further research, although it is interesting to note that homologues of these proteins are present in all extant members of the family *Closteroviridae* [21], and have reported to be components of virions of lettuce infectious yellow virus [11] and beet yellows virus [22]. Are p65 and p61 part of the virion? Do they, with p27, form a structure for binding to the aphid? Many questions remain to be answered about how members of the family *Closteroviridae* are transmitted by their hemipteran vectors.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. No human or animal subjects were used during this research.

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