A mutation in the *Lettuce infectious yellows virus* minor coat protein disrupts whitefly transmission but not *in planta* systemic movement.

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Running title: Whitefly transmission and *in planta* movement of LIYV CPₘ mutants.

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Abstract

The Lettuce infectious yellows virus (LIYV) RNA 2 mutant p1-5b was previously isolated from *Bemisia tabaci*-transmitted virus maintained in *Chenopodium murale* plants. The p1-5b RNA 2 contains a single nucleotide deletion in the minor coat protein (CPm) ORF that is predicted to result in a frameshift and premature termination of the protein. Using the recently developed agroinoculation system for LIYV, we tested RNA 2 containing the p1-5b CPm mutant genotype (agro-pR6-5b) in *Nicotiana benthamiana* plants. We showed that plant infection triggered by agro-pR6-5b spread systemically, and resulted in the formation of virions similar to those produced in p1-5b inoculated protoplasts. However, virions derived from these mutant CPm genotypes were not transmitted by whiteflies, even though virion concentrations were above the typical transmission thresholds. In contrast, and as demonstrated for the first time, an engineered restoration mutant (agro-pR6-5bM1) was capable of both systemic movement in plants and whitefly transmission. These results provide strong molecular evidence that the full-length LIYV-encoded CPm is dispensable for systemic plant movement, but is required for whitefly transmission.
Introduction

Members of the genus *Crinivirus* are emerging plant viruses in many parts of the world. An important factor contributing to the increase in these viruses is their association with and transmission by whitefly vectors that have increased in distribution in the last several decades. *Lettuce infectious yellows virus* (LIYV), the type member of the genus *Crinivirus* (family *Closteroviridae*), is specifically transmitted by the sweet potato whitefly, *Bemisia tabaci* biotype A, in a semi-persistent, non-circulative manner (6). The virus is confined to phloem cells within infected plants, and is not transmissible to plants by leaf-rub inoculation. The bipartite single-stranded positive sense LIYV genome components, consisting of RNA 1 (approx. 8.1 kb) and RNA 2 (approx. 7.2 kb) are separately encapsidated in flexuous filamentous particles that are characteristic of the family *Closteroviridae* (8, 11). These virions are comprised of four protein components: the major coat protein (CP), the minor coat protein (CPm), an Hsp70 homolog (Hsp70h), and a 59-kDa protein (P59). Like other viruses in the family *Closteroviridae*, LIYV has bipolar virions with a “body” composed mainly of the CP and a “head” that is formed by the assembly of CPm subunits (2, 4, 7, 22, 28). Hsp70h and P59 are detected in LIYV virions (22), but their locations have not been identified, as they are not readily detected by immunogold labeling and transmission electron microscopy (IGL-TEM). In two members of the family *Closteroviridae*, *Citrus tristeza virus* (CTV) and *Beet yellows virus* (BYV), the combination of Hsp70h, P61 (the homolog of LIYV P59 in CTV) or P64 (the homolog of LIYV P59 in BYV), and CPm encapsidate the 5’ end (~630-650 nt) of the RNA genome, demonstrating the complex interactions that exist among the capsid proteins and the genomic RNA (15, 21).
In our previous studies, we demonstrated the transmission of LIYV using an *in vitro* acquisition and whitefly transmission system (13, 22). Results from previous work implicated a role for LIYV CPm in whitefly transmission. Antibodies to CPm blocked *in vitro* acquisition/transmission of LIYV virion preparations by *B. tabaci* biotype A, while antibodies to the CP, Hsp70h, and P59 did not (22). The *in vitro* whitefly membrane-feeding system had also been used to demonstrate *B. tabaci* biotype A transmission of virions that were derived from cloned infectious cDNAs of LIYV RNA1 and RNA 2 of several genotypes, including pR6 [the first cloned wild type (WT) infectious cDNA of LIYV RNA 2 (10)], establishing for the first time that these cloned constructs contained all of the information necessary for protoplast infection, virion formation, whitefly transmission and infection in plants (12). In that study, the mutant p1-5b was among the cloned LIYV RNA 2 cDNAs derived from *B. tabaci* biotype A-transmitted virus maintained in *C. murale* plants.

p1-5b contains a single adenine residue deletion in the CPm ORF at nucleotide 592; a deletion that is predicted to result in a frameshift, 14 new amino acids, and premature termination of the protein (12). The predicted p1-5b CPm has 211 amino acids, compared to 453 amino acids in the wild type (pR6 genotype) protein. The p1-5b genotype also contains 3 other nucleotide changes in the CPm ORF relative to the pR6 infectious clone sequence (27), all of which result in amino acid changes. In contrast, the p1-5b CP, Hsp70h, and P59 sequences are identical to that of pR6 (12). Possible polymorphisms throughout the rest of the p1-5b clone were not characterized. In the prior study, *B. tabaci* biotype A transmission of p1-5b virions was not observed even though the mutation did not affect its infectivity in protoplasts (as determined by virion yields) and apparent particle morphology (12). However, these studies were disadvantaged by the necessity of
propagation in protoplasts to obtain specific genotypes from infectious cloned cDNAs. Protoplasts yield low quantities of virion relative to plants, and virion concentration is a critical parameter in whitefly transmission (13). Although virion concentrations in those experiments were above typical thresholds for whitefly transmission (12, 13), low concentrations may still be limiting for transmission, making negative transmission results difficult to interpret. Obtaining adequate virion concentrations of specific genotypes for whitefly transmission to plants has therefore been a significant hurdle to LIYV transmission studies.

The recently developed agroinoculation method for LIYV (24) permits study of systemic plant infection by distinct LIYV genotypes, including those that are whitefly transmission-deficient, and recovery of higher virion yields than were possible using protoplasts. The objective of this study was to further examine the function of the LIVY CPm by extending our observations of p1-5b. We constructed mutants with the CPm frameshift restored to determine if engineered mutations that either restored or disrupted the formation of an intact CPm also affected systemic plant infection, virion formation and B. tabaci biotype A transmission. Our study revealed that a mutant engineered with the restored CPm ORF produced a WT infection profile characterized by systemic virus movement within agroinoculated plants, and the generation of CPm-containing virions that were whitefly transmissible. Intriguingly, systemic virus movement was also observed for a mutant engineered to express the 1-5b CPm, but the virions lacked an identifiable CPm and were defective in whitefly transmission. These results represent a significant advance in addressing challenging questions and hypotheses about Crinivirus whitefly transmission properties not testable using earlier systems.


Materials & Methods

Engineering CPm mutants

To restore the CPm ORF in p1-5b (Fig. 1C), an adenine residue was engineered at p1-5b nucleotide position 5555 by polymerase chain reaction (PCR)-mediated mutagenesis, and the resulting construct was p1-5bM1 (Fig. 1C). Specifically, p1-5bM1 was derived using the complementary oligonucleotide primers LIYV 18: 5'-GTTTACACAAAAATGGGTA-3' and LIYV 17: 5'-GTTATAAGATAGGATACCATTATTGTGTAAC-3'; the adenine and its complementary thymine are indicated in bold. pR6-5b was constructed by replacing an 876 base pair NheI cut fragment in the cloned WT pR6 (between nucleotide positions 4884 and 5759, which includes the adenine nucleotide at position 5555 and encompasses the 3' end of the CP ORF as well as the 5' end of the CPm ORF) with the corresponding fragment of p1-5b that is missing the adenine nucleotide at position 5555 (Fig. 1C). Subsequent procedures employed in the transformation, colony analysis and plasmid purification were according to Sambrook and Russell (20). All engineered constructs were sequenced to confirm the introduced nucleotide change and to ensure that no spurious substitutions or deletions were present.

Construction of LIYV agroinoculation plasmids and agroinoculation

RNA 2 CPm mutant binary vector constructs were based on agro-pR6 (pR6 genotype RNA 2 agroinoculation construct, also named JW168) (24) (Fig. 1C). NheI fragments from p1-5b and p1-5bM1 were swapped into agro-pR6 and screened for directionality (Fig. 1A).
Resultant clones were named agro-pR6-5b (where *Nhe*I fragment source was p1-5b) and agro-pR6-5bM1 (where *Nhe*I fragment source was p1-5bM1) (Fig. 1C). Insert regions were sequenced to confirm genotype.

Transformation and preparations of *Agrobacterium tumefaciens* strain C58C1 were as previously described (24). Constructs were coinfiltreated with the wild-type RNA 1 agroinoculation construct JW100 (24) and either a 35S *Tomato bushy stunt virus* (TBSV) P19 silencing suppressor construct or a 35S *Tobacco etch virus* P1/HC-Pro silencing suppressor construct (5, 23) to *Nicotiana benthamiana* plants at about the 5-6 leaf stage as previously described (23). Plants were assayed for infection by symptom scoring and RT-PCR using primers P26-F and P26-r2 (13) at 2-4 weeks post-infiltration. Symptomatic plants were assayed for retention of engineered mutations by sequencing RT-PCR products obtained from infected plants at various time points between 2-12 weeks post-infiltration (see Results for further details on individual experiments).

Protoplast inoculation, virion purification, IGL-TEM, and whitefly transmission

Procedures for the isolation of protoplasts derived from cultured suspension cells of *N. tabacum* var. Xanthi were as previously described (14). The extraction of virion RNA and synthesis of capped transcripts of cloned constructs were as in prior work (9, 10, 26). Protoplasts were inoculated with capped transcripts of the cloned cDNA constructs of RNAs 1 and 2 as described before (10). Specifically, protoplasts were inoculated with capped transcripts of p9/55, the full-length cDNA clone of RNA1 (10), and one of the following full-length cDNA clones of RNA 2: pR6 (10), p1-5b(12), p1-5bM1, and pR6-5b, using ~2 µg (RNA 1 transcript) and ~ 6 µg (RNA 2 transcript) per half-million protoplasts.
Procedures for the purification of virions from infected protoplasts and plants, estimation of virion concentrations, western blot analysis, IGL-TEM and whitefly transmission were according to previously described methods (13, 22). The nomenclature of virions obtained from the in vitro transcript-inoculated protoplasts is according to previous convention (12). For example, p1-5b virions are those obtained from p9/55- (RNA 1) and p1-5b -(RNA 2) inoculated protoplasts. Virion concentrations were estimated by densitometry from SDS-PAGE or western immunoblots, as indicated in Table 2, using ImageJ 1.42q software (NIH, USA). Diluted proteins of known concentration were used to generate a linear formula for concentration, which was used to calculate virion (CP) concentrations.

Reverse transcription (RT)-PCR, immunocapture RT-PCR, cloning and sequencing

For the analysis of CPm nucleotides following protoplast inoculation, total RNAs were extracted from protoplasts using TRI Reagent (MRO) according to the manufacturer’s recommendations. The first strand cDNA synthesis by Superscript™ II reverse transcriptase (Invitrogen), followed by PCR amplification with Pfu DNA polymerase (Stratagene) and the direct sequencing of PCR-amplified products were performed using the primers LIYV 10, 11, 12, and 13 (12). Immuno-capture RT-PCR was according to the method previously described (18) with modifications. Following the pre-coating of PCR tubes with LIYV virion specific IgG at ~2.3 µg/mL for 30 minutes at 37°C, and rinsing with phosphate-buffered saline + 0.05% Tween 20 (PBST), tubes were incubated with a blocking buffer (PBST with 2% polyvinyl pyrrolidone and 2% bovine serum albumin) for 30 minutes at 37°C. After the second wash, tubes were incubated in blocking buffer with samples: greenhouse maintained wild type virions (100 ng), pR6 virions (10 ng), p1-5b
virions (5 ng), wild type virion RNAs (100 ng) and a 1/20 dilution of a purified preparation of protoplast-derived virions inoculated with the p9/55 (RNA 1) in vitro synthesized transcript. Following the final wash, RT-PCR was performed using the primers LIYV 38: 5’-TCACAATTACCATTGGGCGAAG-3’ and LIYV 39: 5’-TCCGCTCTTTAGTTGCAGCAG-3’, which amplify 273 nucleotides (from nucleotide position 6 to 278) of the LIYV RNA 2 5’ region; P26-F and P26-r2 (13), which amplify a 303 bp fragment of the LIYV RNA2 3’ region; LIYV 40: 5’-CCGTTGGACAAGGTAAAGATT-3’ and LIYV 41: 5’-GTCACACTCCAACCATTACC-3’, which amplify 486 nucleotides (from nucleotide position 19 to 504) of the LIYV RNA 2 5’ region; LIYV 42: 5’-GTCGCGCTGATTAGCATTG-3’ and LIYV 43: 5’-CGTTGCCAGCTAAGACGA-3’, which amplify 255 nucleotides (from nucleotide position 7085 to 7339) of the LIYV RNA 1 3’ region.

For the analysis of CPm nucleotides of LIYV with the 1-5bM1 genotype (agro-pR6-5bM1) after whitefly transmission, total RNAs were extracted from systemically infected lettuce leaves using the RNeasy Plant Mini Kit (Qiagen) and subjected to RT-PCR by MMLV reverse transcriptase (Promega) and Herculase II Fusion DNA polymerase (Agilent Technologies). RT-PCR was performed using the primers LIYV 19: 5’-GAAATATCAGCAATCGGGCATTGTCT-3’ and LIYV 20: 5’-CACCACTGCTCAGCCTCAACTCCTCG-3’, which amplify 1063 nucleotides (from nucleotide position 4847 to 5909) spanning the CPm ORF in which the mutation in 1-5bM1 was engineered. The resulting purified RT-PCR product was A-tailed by incubation with Taq polymerase for 30 min at 72°C with deoxyadenosine triphosphate (dATP) to facilitate subsequent cloning into the pGEM-T easy vector (Promega). cDNA clones from four randomly selected infected plant samples were sequenced in both directions.
Results

Western blot and IGL TEM analyses of virions recovered from inoculated protoplasts

Due to the presence of a premature stop codon in the CPm of the p1-5b mutant, only 211 encoded amino acids (<50% of the intact wild type CPm), corresponding to a peptide of 21 kDa, were expected to be expressed from the p1-5b genome (Fig. 1A) (12). This mutant was competent for replication in protoplasts, and yielded virions as determined by TEM analyses (12). However, western blot analyses of purified p1-5b virions did not show the complete CPm, nor a fragment corresponding to the expected 21 kDa CPm truncation, suggesting that they were absent on mutant virions (12).

To test the hypothesis that a complete CPm is required for positive detection on virions, we analyzed p1-5b, and the engineered mutants, p1-5bM1 (p1-5b engineered to express a complete CPm) and pR6-5b (an engineered mutant in which the p1-5b CPm gene had been incorporated within the cloned WT [pR6] background [Figs. 1A and 1C]). As a comparison, LIYV virions purified from pR6-infected protoplasts and/or lettuce plants, and greenhouse maintained WT-infected lettuce plants were included in the study. Western blot analyses of pR6 virions identified proteins with sizes corresponding to those of the intact CP and CPm (Fig. 1B, lanes 1 and 3), consistent with our published results (12). A complete CP and CPm were also identified for virions of p1-5bM1 in the same western analyses (Fig. 1B, lane 4). Thus, the mutation engineered in the CPm gene of p1-5b removed the premature stop codon and resulted in the restoration of an intact CPm while retaining the other nucleotide polymorphisms of the p1-5b clone relative to the wild type pR6 clone. However, western blot analyses of purified pR6-5b virions positively identified...
only the CP but not the CPm (Fig. 1B, lane 2), indicating that loss of detection of the CPm for the 1-5b CPm genotype was irrespective of the genetic background of the LIYV isolate. We also used antibodies specific to the CP and CPm in IGL-TEM to obtain physical evidence for the presence of the CP and CPm on virions purified from protoplasts. These virions were derived from protoplasts inoculated with the in vitro synthesized transcripts of LIYV RNA1 (p9/55) and one of the following LIYV RNA 2 constructs: pR6, p1-5b, p1-5bM1, or pR6-5b. IGL-TEM observation of virions prepared from the p1-5b, p1-5bM1 and pR6-5b inoculated protoplasts revealed the presence of virions that were similar in length and morphology to greenhouse maintained WT and pR6 virions, consistent with results from our previous observations (Fig. 2A – J)(12). Positively labeled particles were counted and the percentage of labeling was expressed as the number of labeled particles over the total number of particles observed (Table 1 and Fig. 2). When antibodies to the CP were used, specific labeling was readily and abundantly (100%) observed along the entire extent of the “body” of the particles for greenhouse maintained WT and pR6 virions regardless whether the latter virions were purified from protoplasts or plants (Table 1 and Figs. 2A and 2C). Specific labeling (at one end of the particle) was also seen for greenhouse maintained WT and pR6 virions when antibodies to the CPm were used, although the percentage of labeling was lower (44-88%) relative to that of particles tested using antibodies to the CP (Table 1 and Fig. 2A-D). Specific labeling (92%) was seen when p1-5b virions were tested using antibodies to the CP (Table 1 and Fig. 2E). In contrast, none of the 50 virions examined were specifically labeled when tested using antibodies to the CPm (Table 1 and Fig. 2F). Similarly, IGL-TEM analyses positively identified the CP (100%) but not the CPm (0%) from purified pR6-5b virions (Table 1 and Fig. 2I, J). This was consistent
with the western blot results for pR6-5b virions and was expected since p1-5b lacked a detectable full-length CPm. Therefore, we used the virions of p1-5bM1 to determine whether the restoration of an intact CPm resulted in restoration of detectable specific labeling on p1-5b virions. Abundant (100%) specific labeling was observed when p1-5bM1 virions were tested using antibodies to the CP (Table 1 and Fig. 2G). With CPm antibodies, up to 82% of the virions examined were labeled (Table 1 and Fig. 2H).

**Immunocapture RT-PCR assay for mutant p1-5b virions purified from protoplasts**

Closterovirus encapsidation studies have revealed that the polar end of these virions in which the CPm is located correspond to the 5’ end of the encapsidated genomic RNAs (15, 21). Thus, it was of interest to determine if the 5’ ends of genomic RNAs 1 and 2 were present in the p1-5b virions since an intact p1-5b CPm was not detectable based on the above analyses. Immunocapture RT-PCR of p1-5b virions was performed using antibodies raised against whole LIYV virions and primers specific to the 5’ and 3’ ends of both RNAs 1 and 2 (Fig. 3; see also Materials and Methods). Greenhouse maintained WT and pR6 virions were included as positive controls, while virion RNAs and the *in vitro* transcript of LIYV RNA 1 (p9/55), were used as negative controls in the assay (Fig. 3). Our results indicated that 486 and 273 nucleotides at the 5’ end of RNAs 1 and 2, respectively, as well as 255 and 303 nucleotides at the 3’ end of RNAs 1 and 2, respectively, of wild type controls and p1-5b (and the pR6 positive controls) were detected (Fig. 3), suggesting that these regions in the genomic RNAs were present in the virions.

**Systemic plant infection, sequence retention and virion formation of CPm mutants**
LIYV agroinoculation constructs with the p1-5b and p1-5bM1 CPm genotypes (referred to here as agro-pR6-5b and agro-pR6-5bM1, respectively) were tested compared to a wild type (pR6 genotype) RNA 2 agroinoculation construct, agro-pR6, in all cases coinfiltrated with an RNA 1 construct (24). In these experiments, we repeatedly observed systemic infection of plants agroinfiltrated with agro-pR6-5b, agro-pR6-5bM1, or the positive control (agro-pR6). Infiltrated *N. benthamiana* plants showed typical LIYV symptoms, including interveinal chlorosis beginning at lower leaves and progressing upward, and a quality of leaf brittleness. Western blotting and/or RT-PCR confirmed LIYV infection of non-inoculated leaves (data not shown). Plants infected by each of the genotypes listed above showed indistinguishable symptoms and timing of symptom appearance, suggesting no noticeable delay or defect in infection for the CPm mutants.

To determine whether infected plants retained the expected CPm genotypes, including the CPm frameshift in agro-pR6-5b, we tested plants by RT-PCR and nucleotide sequence analysis. All plants tested within 6-8 weeks post-infiltration exhibited the expected genotypes for the entire CPm ORF (based on results from testing approx. 16 plants). When three symptomatic plants infiltrated with the agro-pR6-5b construct were tested by RT-PCR and sequencing at 3 months post-inoculation (mpi), all consensus sequences had the expected genotype. However, when a set of eight plants including the same three tested at 3 mpi were tested again a month later, 2/8 (including 1 of the 3 sequenced prior) exhibited a compensatory frameshift mutation in the CPm gene: an adenine residue insertion in a 4-adenine tract seven nucleotides upstream of the mutant adenine deletion. This insertion was not a bona fide "reversion", since the site of nucleotide insertion was not the same as the mutant deletion site. However, it was expected to restore
the CPm frame for translation of a full-length CPm with two additional amino acid changes: M to N at CPm amino acid 196 and N to Y at amino acid 198. Other polymorphisms of the CPm genotype in agro-pR6-5b relative to the wild type infectious clone agro-pR6 were retained in these compensatory mutants. These late-appearing compensatory mutants were not further tested, as mixed infections arising from viruses with the revertant and the 5b genotypes were likely present in these plants, thereby possibly confounding virion concentration-dependent transmission results.

We recovered agro-pR6 and agro-pR6-5b virions from the systemically infected leaves of agroinfiltrated plants with expected sequences and subjected them to IGL-TEM analysis using antibodies to the LIYV CP and CPm (Table 1 and Fig. 2). The results indicated that the CP was readily detected in 100% of these virions (Table 1 and Fig. 2K and 2M), consistent with results obtained using virions purified from protoplasts (Table 1 and Fig. 2C and 2I). In contrast, although the CPm was readily detected in 94% of the agro-pR6 virions, it was not observed in any of 50 particles of agro-pR6-5b (Table 1 and Fig. 2L and 2N). These results were consistent with those obtained using virions purified from protoplasts (Table 1 and Fig. 2D and 2J).

Whitefly transmission of CPm mutant virions

In our previous study, the p1-5b virions were defective in transmission by B. tabaci biotype A, while transmission of the greenhouse maintained WT and pR6 virions was observed (12). Here, we tested virions of the restoration mutant p1-5bM1, which expresses complete CPm, for transmission by B. tabaci biotype A. Tobacco protoplasts were inoculated with the in vitro synthesized transcripts of p9/55, the cloned cDNA construct of
LIYV RNA1, and that of p1-5bM1, and the resulting purified virions were subjected to in vitro acquisition and transmission by *B. tabaci* biotype A. In four transmission experiments in which the average concentration of p1-5bM1 virions was approx. 7.9 ng/μl and hundreds of whiteflies were used per transmission, no transmission was observed in a cumulative total of 30 test plants. However, it should be noted that although the average virion concentrations in these experiments were within the range that supports virion transmission (13), they were close to limiting concentrations for efficient transmission.

In order to confirm the above transmission data in a system where much higher virion yields could be obtained, *N. benthamiana* plants were systemically infected with LIYV agro-pR6-5b and agro-pR6-5bM1. It should be noted that *N. benthamiana* plants were used for agroinoculation because methods so far have not succeeded in delivering LIYV to lettuce or other preferred feeding hosts of the LIYV whitefly vector *B. tabaci* biotype A (24). Because these whiteflies feed very poorly on *N. benthamiana* plants and this negatively affects virus transmission, virions were purified from agroinfected plants and used for membrane feeding in transmission experiments. In this way, virion concentrations used for feeding could also be measured and compared between experiments. Since the 1-5b mutation was not 100% retained following agroinoculation, particularly at time points after 3 mpi, the expected genotype in all plants used for subsequent whitefly transmissions were confirmed by RT-PCR and sequencing within 1 week prior to transmission experiments, and plants were used for virion purification between 6-8 weeks post infiltration (at which time points no mutations were found). Virion concentrations were estimated by SDS-PAGE and western blotting or Coomassie staining and comparison with standards of known concentration (data not shown). In three experiments, with
concentrations ranging from 32 to 78 ng/μl, transmission of agro-pR6-5bM1 virions was observed (Table 2, Experiments 1-3) while transmission of agro-pR6-5b virions, with concentrations ranging from 15 to 21 ng/μl, was not (Table 2, Experiments 1 and 2). pR6-5bM1 CPm genotypes in four randomly-selected lettuce plants was verified after transmission by RNA isolation and sequencing (see Materials and Methods; data not shown). Transmission of agro-pR6-5b virions was not observed even when the concentration exceeded that of agro-pR6-5bM1 by more than 9 fold (Table 2, Experiment 3). Transmission of virions from greenhouse maintained WT or plants infiltrated with agro-pR6 RNA 2 construct was also observed (Table 2, Experiments 1 and 2). Because of the higher virion concentrations obtained in these experiments, the transmission of agro-pR6-5bM1 virions demonstrated here (Table 2) may be taken with greater confidence over that of protoplast-derived p1-5bM1 virions reported above.

Discussion

The current study extends our previous findings on the mutant p1-5b by comparing this LIYV genotype to an engineered restoration genotype, p1-5bM1, and assessing virion structure (encapsidation), systemic plant infection by agroinoculation, and transmissibility by *B. tabaci* biotype A. In immunoblot and IGL-TEM analyses of p1-5b and p1-5bM1 virions, CPm was not detected in the truncation mutant virions, consistent with the notion that a complete CPm is likely the preferred form to be incorporated into the assembled virion. In order to not be biased by our own observations, it was necessary for the IGL-TEM analyses to be both qualitative and quantitative. Therefore, we counted every particle, labeled or otherwise (depending on the virions and the antibody used), in our analysis and...
the data presented in Table 1 and Fig. 2 represent our observations of the similarity and
dissimilarity in the specific labeling characteristics between p1-5b and p1-5bM1 virions.
Studies on CTV and BYV have shown that genomic RNA encapsidation involves a
complex interaction between the Hsp70h, P61 (the homolog of P59 in CTV)/P64 (the
homolog of P59 in BYV) and CPm, and the interaction occurs near the 5’ end of the genomic
RNA (16, 21). How and/or whether similar interactions occur for LIYV is not known;
however, given the similarity in the structural compositions among these viruses, it may be
that LIYV behaves similarly. However, our immunocapture/RT-PCR on LIYV virions
indicates that 5’ and 3’ ends of LIYV RNAs 1 and 2 are present even in virions derived from
the 1-5b CPm truncation genotype. Similar results have been reported for BYV, for which
CPm mutants still had complete RNA protection, likely due to compensation by the major
CP (4). It is also possible that the CP or other capsid proteins (Hsp70h or P59) compensate
in encapsidating the CPm mutant, or that the truncated LIYV CPm is still incorporated into
virions but lacks immunogenicity thereby precluding detection by immunoblot or
immunogold labeling assays.
Based on transmission experiments from plant- and protoplast-derived virions, it is
clear that the p1-5b genotype, which lacks a complete CPm, is not transmissible to plants
by B. tabaci biotype A. Restoration of the CPm mutation in p1-5b was performed by
engineering p1-5bM1, so that it had an intact CPm but retained other sequence
polymorphisms of the 1-5b CPm relative to wild type pR6. The agroinoculated pR6
chimeras containing these CPm genotypes (agro-pR6-5b and agro-pR6-5bM1) both moved
systemically in plants when co-inoculated with an RNA 1 construct, but only agro-pR6-
5bM1 virions were whitefly transmissible (Table 2). These results provide strong evidence
that CPm is a whitefly transmission determinant for LIYV, and are in agreement with previous work implicating CPm in whitefly transmission (12, 22). We also have preliminary data indicating that one basis for the defect in whitefly transmission of virions derived from the mutant CPm genotype in p1-5b (agro-pR6-5b) is impaired virion retention in the whitefly's foregut (J. Ng, unpublished data). Continuing work will reveal the relationship of 1-5b LIYV virions with whiteflies, including retention of p1-5b versus wild type and p1-5bM1 virions. Further analyses will also be necessary to delimit the portions of the CPm, apparently in the C-terminal 31 kDa, that are required for whitefly transmission.

A comparison of the CPm amino acid sequences among the criniviruses revealed a low to moderate level of identity among them (19), with more similarities being seen in the C termini (alignment not shown). Although we have not yet identified any distinct motifs that can be linked to the whitefly specific transmission of these viruses it is plausible to speculate that, if all Criniviruses require CPm for whitefly transmission, sequence variations in the CPm might play a role in differences in whitefly transmission specificity as has been suggested previously (25).

Using the agroinoculation method to bypass protoplast inoculation and whitefly transmission to deliver specific genotypes to plants, we have also been able to assess systemic plant movement for these mutants. Our results show that full-length CPm is dispensable for systemic LIYV movement in agroinoculated N. benthamiana plants, since systemic infection of the 1-5b genotype was repeatedly identified and retained in non-inoculated tissues. We have recently also found systemic infection in plants agroinoculated with another engineered CPm genotype, R6M6 (27) containing an 5' double stop codon insertion for an earlier truncation than in the 1-5b genotype, for a predicted CPm fragment...
of only 71 amino acids (data not shown). In contrast, in repeated experiments we have not
detected systemic infection or symptom development of Hsp70h, P59, or CP knockout
mutants by agroinfiltration. However, confirmation of local replication is necessary to
validate negative results for these mutants.
Systemic movement of the LIYV CPm mutants is intriguing and somewhat
surprising, since all four conserved capsid components (CP, CPm, Hsp70h, and the P59
homolog) have been shown to be required for cell-to-cell movement of another
closterovirus, BYV, in a local lesion host (1, 3). The requirements and nature of movement
components for LIYV have not been accessible prior to development of the agroinoculation
system, and long-distance movement mechanisms for the phloem-limited LIYV are likely to
have important distinctions versus the cell-to-cell movement mechanisms for the non-
phloem-limited BYV, including perhaps the role of CPm in movement. We must also
consider the possibility that systemic movement without an intact CPm is host-specific for
N. benthamiana, as plant delivery methods without the use of whiteflies are not yet
available for any other hosts. It is also noteworthy that a compensatory mutation predicted
to restore full-length CPm production appeared over time in some of the agro-pR6-5b-
infected plants after systemic infections had been established. This suggests there may be
selection for full-length CPm for some as yet undefined role in plants. For example, it is
tempting to speculate that the full-length CPm may be required to confer an increased,
albeit subtle competitive ability in some aspects of replication, movement, and/or tissue
tropism beyond what our estimation of purified virion concentrations could discern.
Similarly, predicted compensatory mutations without selection by aphid transmission has
been reported in viruses carrying mutations in a transmission-determining structural
protein (P3/P5) in the polerovirus *Potato leafroll virus* (PLRV) (17). The PLRV P5 read-through protein is also associated with other roles in host plants, including establishment of phloem limitation.

The results presented here clearly elucidate how the complex nature of the LIYV virions (and similarity for other viruses of the *Closteroviridae*) regulates their transmissibility by insect vectors. They also provide insight into potentially novel movement requirements for LIYV relative to other studied members of the family *Closteroviridae*.

Acknowledgements

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References


FIG. 1 Predicted sizes of the minor coat protein (CPm) encoded by wild type (WT) and mutants of *Lettuce infectious yellows virus* (LiYV) and immunoblot analysis of virions. (A) Genome organization of *Lettuce infectious yellows virus* RNA 2 and amino acid positions of expected translation products encoded by the CPm gene. Expected translation product encoded by: (1) the full-length CPm gene of whitefly transmissible cloned WT, pR6, and the CPm restoration mutants p1-5bM1 and agro-pR6-5bM1; and (2) the CPm gene of the whitefly non-transmissible mutants, p1-5b, pR6-5b, and agro-pR6-5b, including 14 predicted novel amino acids (colored in green) at the C-terminus due to the frameshift preceding termination. The complete CPm corresponds to an intact protein with 453 amino acids whereas that from p1-5b, pR6-5b, and agro-pR65b correspond to truncated proteins with 211 amino acids. The relative positions of the LiYV RNA 2 ORFs encoding P5, HSP70h, P9, P9, CP, CPm and P26 are indicated. Arrows indicate *NheI* sites used for construction of agroinfiltration plasmids. (B) Western blot analysis of virions. pR6 virions (lanes 1 and 3) purified from lettuce plants inoculated by whitefly transmission of virions from pR6-infected protoplasts; p1-5bM1 and p1-5b virions (lanes 2 and 4) purified from inoculated protoplasts. Virion proteins were detected with antisera raised against the CPm (top panels) or whole virions (bottom panels). Positions of the CP (28 kDa) and CPm (52 kDa) are indicated. CPm antiserum frequently detected both the expected 52 kDa protein and a ca. 35 kDa protein, which may be a CPm degradation product (B, lanes 1, 3, and 4). Migration of molecular weight markers are indicated with colored text and arrows. Separate arrows are shown for 50 kDa and 25 kDa marker locations for each blot in top panel. (C) Summary of constructs used for experiments described herein.
FIG. 2. Immunogold labeling TEM analyses of LiYV virions. Virions were tested using antibodies against the LiYV major coat protein (CP) and minor coat protein (CPm) followed by goat-anti-rabbit IgG labeled with 5 nm gold particles. (A) and (B), greenhouse maintained wild type virions purified from infected lettuce plants; (C) and (D), pR6 virions purified from protoplasts of N. tabacum var. Xanthi; (E) and (F), p1-5b virions purified from N. tabacum var. Xanthi protoplasts; (G) and (H), p1-5bM1 virions purified from N. tabacum var. Xanthi protoplasts; (I) and (J), pR6-5b virions purified from N. tabacum var. Xanthi protoplasts; (K) and (L), agro-pR6 virions purified from agroinoculated N. benthamiana; (K) and (L), agro-pR6-5b virions purified from agroinoculated N. benthamiana. Bars each represent 200 nm. Arrows indicate gold labeling sites.
FIG. 3. Immuno-capture RT-PCR assay for virions. Virion preparations were obtained from protoplasts inoculated with the *in vitro* transcripts derived from the cDNA of LiYV RNA 1 (p9/55; lanes 4, 9, 14 and 19) alone, or along with p6 RNA 2 (lanes 3, 8, 13 and 18) or p1-5b RNA 2 (lanes 2, 7, 12 and 17). Greenhouse maintained wild type virions (lanes 6, 11, 16 and 21) and virion RNAs (lanes 5, 10, 15 and 20) purified from LiYV infected plants were included as positive and negative controls, respectively. Immuno-captured samples were subjected to RT-PCR using primers specific to the 5′ region: nucleotide position 6 to 278, and nucleotide position 19 to 504, of RNA 2 (lanes 2 to 6) and RNA 1 (lanes 12 to 16), respectively; and primers specific to the 3′ region: nucleotide position 6235 to 6537, and nucleotide position 7085 to 7339, of RNA 2 (lanes 7 to 11) and RNA 1 (lanes 17 to 21), respectively. Size in base pairs (bp) for DNA standards (lanes 1 and 22) is as indicated. Asterisk indicates the position of a non-specifically amplified product present in samples in lanes 17 to 21.
TABLE 1. Immunogold labeling TEM analysis of LiYV.

<table>
<thead>
<tr>
<th>LiYV genotype</th>
<th>Major coat protein (CP)</th>
<th>Minor coat protein (CPm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Labeled&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>pR6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>pR6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>p1-5b&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46</td>
<td>42</td>
</tr>
<tr>
<td>p1-5bM1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>pR6-5b&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>agro-pR6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>agro-pR6-5b&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>a</sup>Virions purified from plants (WT: greenhouse maintained wild type virus; pR6: cloned infectious wild type virus inoculated by whitefly transmission of virions purified from infected protoplasts).

<sup>b</sup>Virions purified from inoculated <i>N. tabacum</i> var. Xanthi protoplasts.

<sup>c</sup>Virions purified from agroinoculated <i>N. benthamiana</i> plants.

<sup>d</sup>Immunogold labeling of virions was as previously described (20), using primary antibodies specific to the major and minor coat proteins.
<table>
<thead>
<tr>
<th>RNA 2 genotype</th>
<th>Experiment 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Experiment 2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Experiment 3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>agro-pR6-5b</td>
<td>28 ng/μl</td>
<td>0/4</td>
<td>0/5</td>
<td>21 ng/μl</td>
</tr>
<tr>
<td>agro-pR6-5bM1</td>
<td>55 ng/μl</td>
<td>0/4</td>
<td>0/5</td>
<td>37 ng/μl</td>
</tr>
<tr>
<td>agro-pR6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 ng/μl</td>
<td>1/4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WT&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>68 ng/μl</td>
<td>0/5 lettuce</td>
</tr>
</tbody>
</table>

<sup>a</sup>Virion concentrations estimated by densitometry relative to protein standards from Coomassie-blue stained SDS-PAGE gels.

<sup>b</sup>Virion concentrations estimated by densitometry relative to virions of known concentration from western blots.

<sup>c</sup>Virions derived from clones with the CPm genotypes agro-pR6, agro-pR6-5b and agro-pR6-5bM1 were prepared from systemically infected agroinoculated *Nicotiana benthamia* plants after CPm genotypes in systemic tissue were confirmed by RT-PCR and sequencing.

<sup>d</sup>Wild type pR6 clone in binary vector.

<sup>e</sup>Greenhouse-maintained wild type LIYV.

<sup>f</sup>Greenhouse maintained WT virions were prepared from host plants *Chenopodium murale* or *Lactuca sativa* L. (lettuce) as indicated in experiment 2.