cis preferential replication of *Lettuce infectious yellows virus* (LIYV) RNA 1: The initial step in the asynchronous replication of the LIYV genomic RNAs

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ABSTRACT

A series of *Lettuce infectious yellows virus* (LIYV) RNA 1 mutants was created to evaluate their ability to replicate in tobacco protoplasts. Mutants ΔEcoRI, ΔE-LINK, and Δ1B, having deletions in open reading frames (ORFs) 1A and 1B, did not replicate when individually inoculated to protoplasts or when co-inoculated with wild-type RNA1 as a helper virus. A fragment of the green fluorescent protein (GFP) gene was inserted into the RNA 1 ORF 2 (P34) in order to provide a unique sequence tag. This mutant, P34-GFP TAG, was capable of independent replication in protoplasts. Mutants derived from P34-GFP TAG having frameshift mutations in the ORF 1A or 1B were unable to replicate in protoplasts alone or *in trans* when co-inoculated with wild-type RNA1 as a helper virus. Taken together, these data strongly suggest that LIYV RNA 1 replication is *cis*-preferential.

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An important feature found for some positive-sense RNA viruses is *cis*-preferential RNA replication. The coupling of translation and replication has been proposed as a primary characteristic of *cis*-preferential replication in cases where the virus genomic RNA is translated to yield the virus-encoded component(s) of the replicase, and then, on the same RNA molecule, the replicase directs the transcription of a complementary copy of the template RNA, as the first step in replication (Bol, 2005; Mohan et al., 1995; Neeleman and Bol, 1999; Novak and Kirkegaard, 1994; Okamoto et al., 2008; Van Bokhoven et al., 1993; Weiland and Dreher, 1993; Yi and Kao, 2008; Zhou and Jackson, 1996). Furthermore, when virus RNAs exhibit *cis*-preferential replication, if virus-encoded replication-associated proteins are supplied in *trans*, they are unable to rescue the replication-deficient mutant virus RNAs, or in some cases, these mutants replicate only to low levels (Bol, 2005; Mohan et al., 1995; Neeleman and Bol, 1999; Novak and Kirkegaard, 1994; Okamoto et al., 2008; Van Bokhoven et al., 1993; Weiland and Dreher, 1993; Yi and Kao, 2008; Zhou and Jackson, 1996).

The bipartite *Lettuce infectious yellows virus* (LIYV) is the type member of the genus *Crinivirus* in the family *Closteroviridae* (Martelli et al., 2002). LIYV RNA 1 encodes replication-associated proteins, whereas RNA 2 encodes proteins involved in virion structure, translocation in the host, and whitefly transmission specificity (Klaassen et al., 1995; Ng and Falk, 2006a, 2006b; Tian et al., 1999). Using an LIYV protoplast replication system, it has been shown that RNA 1 is capable of independent replication, whereas RNA 2 can only replicate when co-

inoculated with RNA 1 (Klaassen et al., 1996). Each of the LIYV RNA 2-encoded proteins is dispensable for LIYV RNA replication and accumulation in protoplasts (Yeh et al., 2000). Furthermore, a number of LIYV RNA 2 defective RNAs (D RNAs), which contain deletions of RNA 2 coding sequences, have been identified (Rubio et al., 2000), but so far no LIYV RNA 1 D RNAs have been identified. Previous temporal studies also have demonstrated that LIYV RNAs 1 and 2 show asynchronous accumulation (Yeh et al., 2000). LIYV RNA 1 accumulates very soon after infection, with progeny RNAs detected as early as 12 h post-inoculation (hpi). However, the accumulation of LIYV RNA 2 or RNA 2 D RNAs is delayed ca. 24 h relative to that of RNA 1 (Yeh et al., 2000). This delay is also seen for recombinant LIYV RNA 2 D RNAs engineered to express the green fluorescent protein (GFP) where the timing of GFP expression correlated with the accumulation of the RNA 2 D RNAs (Yeh et al., 2001).

The determinants of LIYV RNA replication which may affect asynchronous accumulation of RNAs 1 and 2 have not been identified but some interesting characteristics are known. The two LIYV genomic RNAs show an unusual nucleotide sequence composition relative to most other positive-sense, multipartite ssRNA plant viruses. Both of the LIYV genomic RNAs have five identical nucleotides at their 5′-termini, and a stretch of 23 identical nucleotides within the first 150 nucleotides (Fig. 1). However, unlike the great majority of multipartite RNA plant viruses, the 3′ termini of the two LIYV genomic RNAs do not show sequence or structural homology/similarity (Klaassen et al., 1995). Whether these unusual sequence characteristics are involved in the asynchronous accumulation of LIYV RNAs is not yet known. Mutational analysis of LIYV RNA 1-encoded proteins has also revealed another unusual feature of the replication of the two LIYV genomic RNAs. RNA 1 encodes ORF 1A and 1B proteins which resemble the

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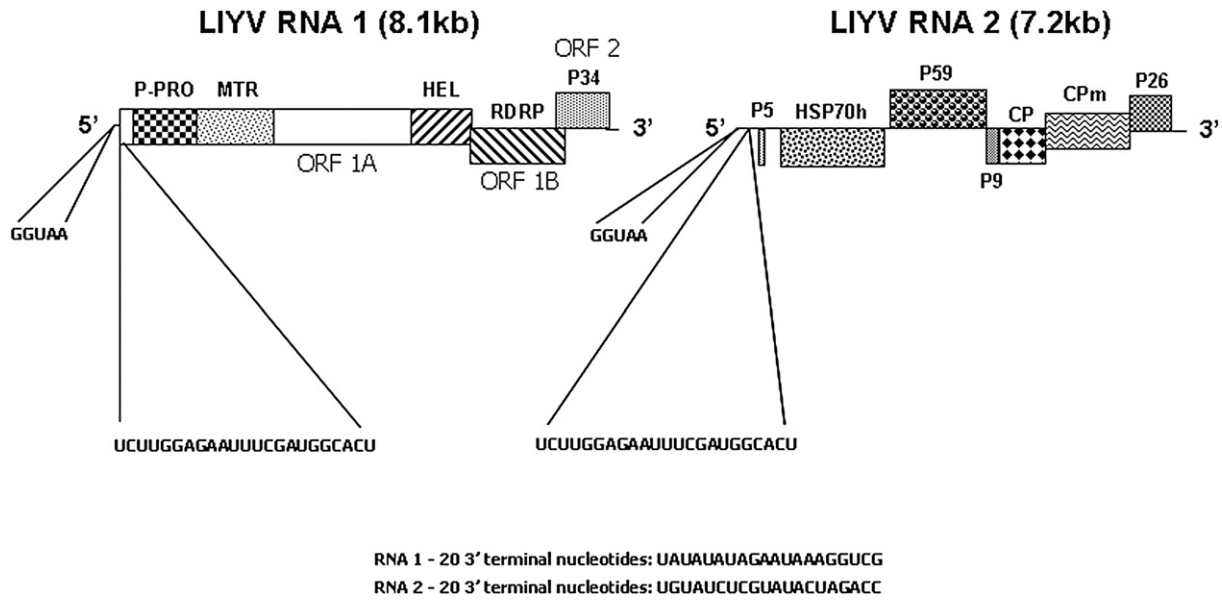*E-mail address:* bwfalk@ucdavis.edu (B.W. Falk).

Fig. 1. Schematic representation of *Lettuce infectious yellows virus* (LIYV) genomic RNAs. Rectangles represent ORFs encoded by LIYV genomic RNAs 1 and 2. LIYV RNA 1 ORFs 1A, 1B and 2 (encoding P34) are shown. P-PRO = papain-like protease; MTR = methyltransferase; HEL = RNA helicase; RDRP = RNA dependent RNA polymerase; HSP70h = homolog of HSP70 proteins; CP = major capsid protein; CPm = minor capsid protein. The 5′ terminal identical nucleotides (nts) located in RNA 1 (1–5, 83–105) and RNA 2 (1–5, 121–143) are indicated. The 3′ terminal 20 nucleotides of LIYV RNA 1 and RNA 2 are also shown.

replication-associated proteins of other positive-sense ssRNA plant viruses (Klaassen et al., 1995). However, RNA 1 also encodes a 34,000 MW protein (P34) in ORF 2 (Klaassen et al., 1995; Yeh et al., 2000), and while mutations that truncate this ORF have no effect on RNA 1 replication, they abolish or severely reduce the ability of RNA 2 to be replicated *in trans* (Yeh et al., 2000).
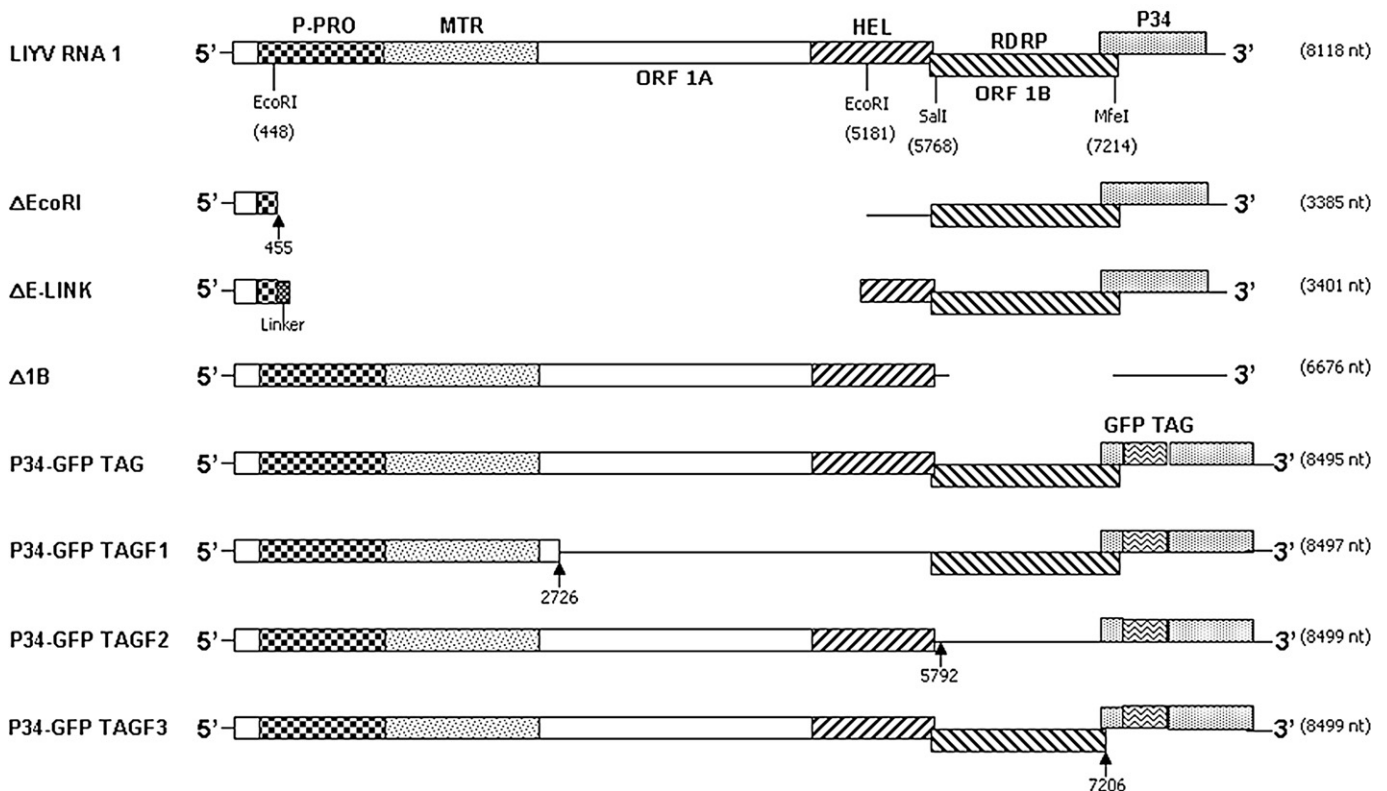

Fig. 2. Schematic representation of *Lettuce infectious yellows virus* (LIYV) RNA 1 and RNA 1 D RNA mutants. The LIYV RNA 1 genomic wild-type RNA is indicated at the top. The size (in nucleotides) of RNA 1 and each RNA 1 D RNA is indicated in parentheses. Blank areas indicate deletions in ORFs. Solid lines indicate mutations to abolish the ORFs while retaining nucleotide sequences.

These characteristics suggest that RNA 1 replication may be *cis*-preferential. Here, we introduced a series of mutations into specific regions of LIYV RNA 1 to assess if the resulting RNA 1 mutants could replicate *in cis* when they are inoculated alone, or *in trans* when they are co-inoculated with wild-type LIYV RNA 1 as a helper virus. All LIYV RNA 1 ORF 1 mutants did not replicate in either case, and our data are consistent with *cis*-preferential replication of LIYV RNA 1.

Results

Replication of LIYV RNA 1 artificial D RNAs—deletion mutants

In order to assess effects of specific RNA 1 mutations, we inoculated protoplasts with wild-type RNA 1 and each of mutants (Fig. 2) ΔEcoRI, ΔE-LINK and Δ1B, respectively, having deletions in open reading frames (ORFs) 1A or 1B of LIYV RNA 1. These mutants were designed to test specific replication characteristics of RNA 1 artificial D RNAs. The ΔEcoRI construction served to truncate ORF 1A

after only 360 nucleotides, but retained the overlapping regions of ORFs 1A/1B, the complete ORF 1B and ORF 2. The ΔE-LINK mutant contained a 16 nucleotide linker sequence to restore the reading frame ORF 1A as well as the ORF 1A/1B overlap and ORF 2. Finally mutant Δ1B contained an intact ORF 1A and most of ORF2, but had most of ORF 1B deleted. After protoplast inoculation, northern hybridization analysis for positive-sense LIYV RNA 1 showed that only wild-type RNA 1 accumulated rapidly and to high levels (Fig. 3A, lanes 3 and 4) as expected because the mutants lacked essential replication genes. In all cases, the input inocula was detected at 0 hpi, but by 72 hpi only the wild-type RNA 1 and P34 subgenomic RNA were abundant. This indicated that none of the deletion mutants was capable of independent replication (Fig. 3A, lanes 5–10). Northern hybridization for negative sense RNA 1 confirmed replication was seen only for wild-type RNA 1 (Fig. 3B, lanes 3–10).

Because none of the above mutants was replication competent alone, we next tested if they could be replicated *in trans*, by complementation from wild-type RNA 1. The mutants were co-inoculated to protoplasts
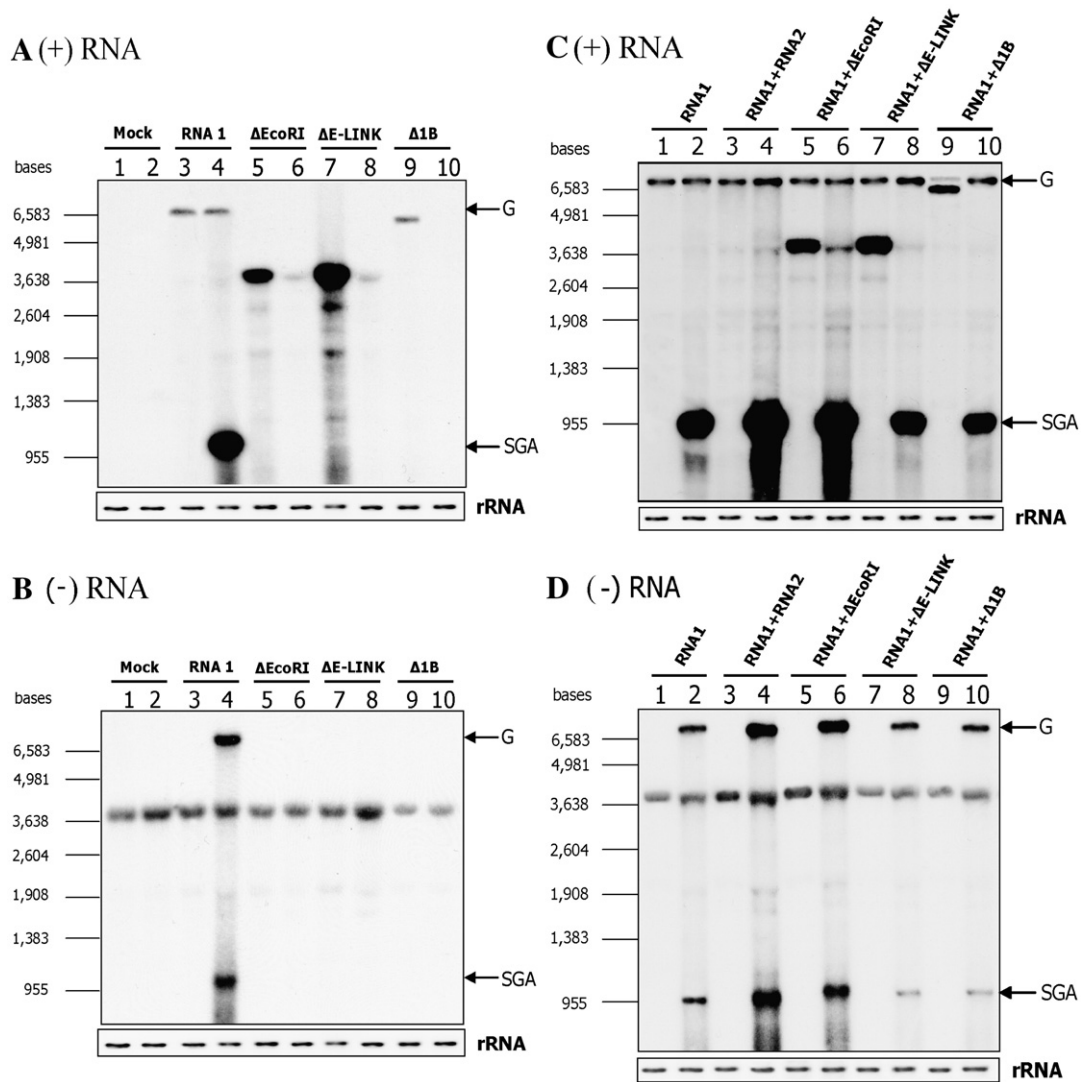

Fig. 3. Replication in protoplasts of *Lettuce infectious yellows virus* (LIYV) RNA 1 deletion mutants alone (A and B) or co-inoculated (C and D) with LIYV RNA 1 as a helper virus. For A and B, lanes 1 and 2 are mock-inoculated control protoplasts, and transcripts of LIYV wild-type RNA 1 (lanes 3 and 4), ΔEcoRI (lanes 5 and 6), ΔE-LINK (lanes 7 and 8), Δ1B (lanes 9 and 10) were inoculated into protoplasts, respectively. For C and D, lanes 1 and 2 are protoplasts inoculated with LIYV RNA 1 wild-type transcripts. Lanes 3–10 are protoplasts inoculated with LIYV RNA 1 wild-type transcripts plus transcripts of LIYV wild-type RNA 2 (lanes 3 and 4), ΔEcoRI (lanes 5 and 6), ΔE-LINK (lanes 7 and 8), Δ1B (lanes 9 and 10), respectively. Lanes with odd numbers indicate samples taken at 0 h post-inoculation (hpi). Lanes with even numbers indicate samples collected at 72 hpi. Total RNAs were extracted and analyzed by northern hybridization using a LIYV RNA 1 3′ terminal minus-sense probe pSKL1 (A and C); and a positive-sense probe pSKL1 (B and D) (Yeh et al., 2000). The positions of migration of the wild-type RNA 1 genomic (G) and wild-type RNA 1 P34 subgenomic (SGA) RNAs are indicated. Numbers at left indicate positions of RNA markers. rRNA stained by ethidium bromide is the indication of total RNA loading.

with wild-type LIYV RNA 1 as a helper virus and northern hybridization was performed as before. Because the mutants all had specific deletions it was easy to differentiate them from wild-type RNA 1 by their smaller sizes. However, none of the mutants showed evidence of replication *in trans* (Figs. 3C and D, lanes 5–10). In contrast, wild-type RNA 2 was replicated efficiently in protoplasts demonstrating that while none of the RNA 1 D RNAs was replicated *in trans*, *trans* replication of LIYV RNA 2 occurred in these protoplasts (Fig. 5A, lanes 3 and 4).

Replication of LIYV RNA 1 artificial D RNAs—frameshift mutants

The above LIYV RNA 1 mutants all contained significant deletions of the LIYV RNA 1 nucleotide sequence. It is possible that some of the

deleted regions may have eliminated RNA 1 structural elements needed for replication. However, if we had generated mutants by only introducing frameshift mutations to abolish the RNA 1 ORFs but to retain nucleotide sequences, we would not have been able to differentiate mutant RNA 1 constructs from the wild-type RNA 1 helper virus in a standard northern hybridization analysis as all RNAs would be of similar size. Therefore, we inserted a marker nucleotide sequence to allow differentiation based on sequence composition, and the marker was inserted in a region not needed for RNA 1 replication. We inserted a fragment of the GFP sequence (as a tag) into ORF 2 (P34) of the P34-mutant (Yeh et al., 2000), thereby producing mutant P34-GFP TAG (Fig. 2). Upon protoplast inoculation, the mutant P34-GFP TAG was capable of independent replication (Figs. 4A and B, lanes 5
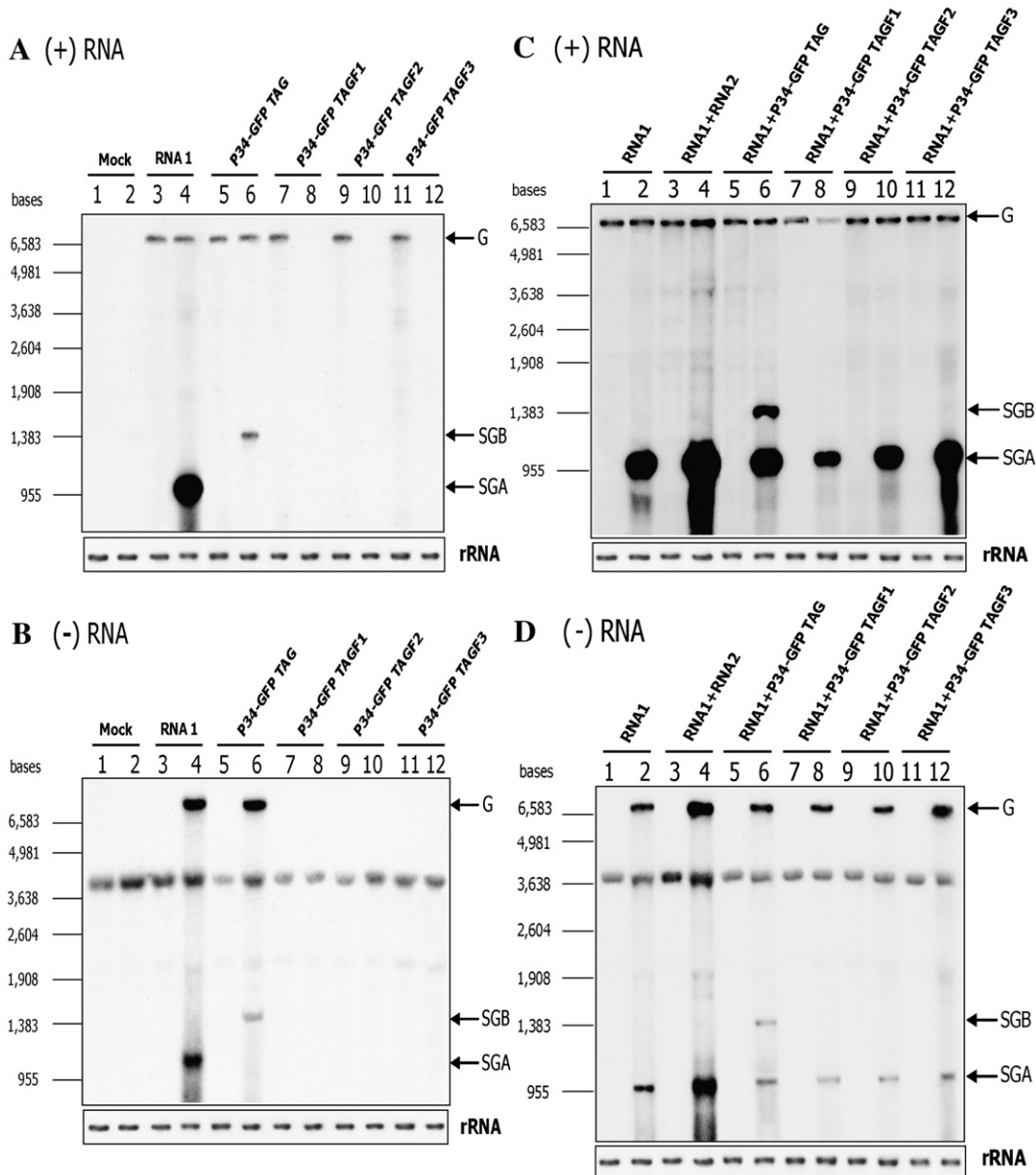

Fig. 4. Replication in protoplasts of *Lettuce infectious yellows virus* (LIYV) RNA 1 frameshift mutants alone (A and B), or co-inoculated (C and D) with LIYV RNA 1 as a helper virus. For A and B, lanes 1 and 2 are mock-inoculated control protoplasts, and transcripts of LIYV RNA 1 (lanes 3 and 4), P34-GFP TAG (lanes 5 and 6), P34-GFP TAGF1 (lanes 7 and 8), P34-GFP TAGF2 (lanes 9 and 10), P34-GFP TAGF3 (lanes 11 and 12) were inoculated into protoplasts, respectively. For C and D, lanes 1 and 2 are protoplasts inoculated with LIYV RNA 1 wild-type transcripts. Lanes 3–10 are protoplasts inoculated with LIYV RNA 1 wild-type transcripts plus transcripts of LIYV RNA 2 (lanes 3 and 4), P34-GFP TAG (lanes 5 and 6), P34-GFP TAGF1 (lanes 7 and 8), P34-GFP TAGF2 (lanes 9 and 10), P34-GFP TAGF3 (lanes 11 and 12), respectively. Lanes with odd numbers indicate samples taken at 0 hpi. Lanes with even numbers indicate samples collected at 72 hpi. Total RNAs were extracted and analyzed by northern hybridization using a LIYV RNA 1 3′ terminal minus-sense probe pSKL1 (A and C) and a positive-sense probe pSKL1 (B and D) (Yeh et al., 2000). The positions of migration of the wild-type RNA 1 genomic (G), wild-type RNA 1 P34 subgenomic (SGA), mutant RNA 1 subgenomic (SGB) RNAs are indicated. Numbers at left indicate positions of RNA markers. rRNA stained by ethidium bromide is the indication of total RNA loading.

and 6). Both positive-sense and negative-sense genomic RNAs were detected at 72 hpi. Also, northern hybridization analysis showed that both the positive-sense and negative-sense probes detected a prominent subgenomic RNA. This subgenomic RNA, P34-GFP, had a decreased electrophoretic mobility relative to the wild-type LIYV RNA 1 P34 subgenomic RNA. Wild-type P34 subgenomic RNA is ca. 1000 nucleotides (compare lanes 4 and 6 in Figs. 4A and B), while the estimated size for the P34-GFP subgenomic RNA is ca. 1400 nucleotides corresponding to the additional GFP TAG 377 nucleotide sequence inserted into the P34 ORF. Both subgenomic RNA P34-GFP and P34 were detected by northern hybridization when the mutant P34-GFP TAG was co-inoculated with wild-type LIYV RNA 1 (Figs. 4C and D, lane 6). The insertion of the GFP TAG into the P34-mutant without affecting RNA 1 replication is consistent with earlier work showing that the P34 ORF (or its encoded protein) is not required for LIYV RNA 1 replication.

We next generated individual frameshift mutations in the ORF 1A or 1B coding regions to create a new series of mutants, P34-GFP TAGF1, P34-GFP TAGF2 and P34-GFP TAGF3 (Fig. 2). The resulting mutants were then analyzed for replication alone, and by co-inoculation to protoplasts with wild-type LIYV RNA 1. Northern hybridization was performed first by using probes specific for LIYV sequences. We would not be able to differentiate RNA 1 mutant genomic RNAs from the wild-type RNA 1 helper virus genomic RNAs because both RNAs would be the similar size and would not be easily differentiated using specific probes for LIYV. However, presence of LIYV RNA 1 subgenomic RNA P34-GFP in northern hybridization would indicate that the respective mutant replicated. Thus, these data showed that the mutants, P34-GFP TAGF1, P34-GFP TAGF2, P34-GFP TAGF3 exhibited no evidence for replication, either alone or in trans with the helper virus, wild-type LIYV RNA 1 (Figs. 4A and B, lanes 7–12; Figs. 4C and D, lanes 7–12). In contrast, wild-type RNAs 1 and 2 replicated efficiently in protoplasts demonstrating efficient trans replication of RNA 2 (Fig. 5A, lanes 3 and 4). Furthermore, both the wild-type RNA 1 and P34-GFP TAG mutant replicated efficiently in co-inoculated protoplasts demonstrating that efficient replication of P34-GFP TAG mutant was not affected by the replication of wild-type RNA 1 (Figs. 4C and D, lanes 5 and 6).

In addition, northern hybridization was performed by using probes specific for the GFP sequence. No hybridization signal was obtained using the GFP sequence specific probe for the mutants, P34-GFP TAGF1, P34-GFP TAGF2 and P34-GFP TAGF3 (Fig. 5B, lanes 3–5 and 8–10). In contrast, the P34-GFP TAG mutant showed accumulation of the genomic RNA and predicted subgenomic RNA P34-GFP (Fig. 5B, lanes 2 and 7).

Discussion

Here we present data suggesting that replication of LIYV RNA 1 is cis-preferential. In contrast, as has been shown previously (Rubio et al., 2000; Yeh et al., 2000, 2001), replication of LIYV RNA 2 and RNA 2 D RNAs occurs in trans. Previous evidence suggesting that LIYV RNA 1 replication may be cis-preferential included: LIYV progeny RNA 1 accumulated rapidly after inoculation, whereas accumulation of progeny RNA 2 was considerably delayed (Yeh et al., 2000, 2001); and numerous LIYV RNA 2 D RNAs can be found in natural LIYV infections (Rubio et al., 2000), whereas natural LIYV RNA 1 D RNAs have yet to be identified. Herein we show that all artificial LIYV RNA 1 D RNA ORF 1A and 1B mutants generated by us failed to be replicated in trans by wild-type LIYV RNA 1. These data suggest that LIYV RNA 1 replication depends on translation of both the ORF 1A and 1B polypeptides, essential for RNA 1 replication, from the same RNA 1 molecule, and thus RNA 1 replication is cis preferential. In cis preferential RNA 1 replication, the same LIYV RNA 1 molecule switches from translation to replication, not allowing the translation products to direct RNA synthesis from another template.

Several reports have suggested that the coding/translation capacity of virus RNAs is important for their replication in trans (de Groot et al., 1992; Mawassi et al., 2000a; Tzeng et al., 2001; van der Most et al., 1995; White et al., 1992; Yi and Kao, 2008). In these examples, the size of the first ORF on the RNA, and likelihood of its ability to be translated appear to be correlated with the efficient replication in trans. It is unlikely that this is the case for LIYV RNA 2 as the first ORF encodes only a 5 kD protein (Klaassen et al., 1995; Yeh et al., 2000), and some LIYV RNA 2 D RNAs that lack this ORF are efficiently replicated in trans
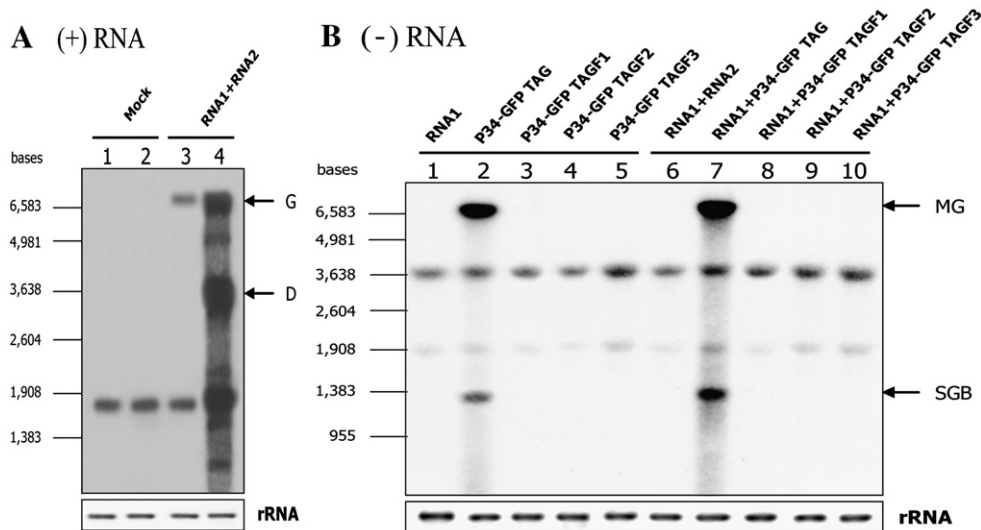

Fig. 5. Replication of *Lettuce infectious yellows virus* (LIYV) wild-type and mutant RNAs in protoplasts. (A) Northern hybridization results from protoplasts inoculated with transcripts of LIYV RNAs 1 and 2 and collected at 0 and 72 hpi (lanes 3 and 4). Lanes 1 and 2 are mock-inoculated control protoplasts. Lanes with odd numbers indicate samples taken at 0 hpi. Lanes with even numbers indicate samples collected at 72 hpi. Total RNAs were extracted and analyzed by northern hybridization using a LIYV RNA 2 3′ terminal minus-sense probe pSKL16 (Yeh et al., 2000). The positions of migration of the wild-type RNA 2 genomic (G) and RNA 2 defective (D) RNAs are indicated. (B) Northern hybridization results from protoplasts inoculated with LIYV RNA 1 (lane 1), P34-GFP TAG (lane 2), P34-GFP TAGF1 (lane 3), P34-GFP TAGF2 (lane 4), P34-GFP TAGF3 (lane 5), LIYV RNA 1 and 2 (lane 6), LIYV RNA 1 and P34-GFP TAG (lane 7), LIYV RNA 1 and P34-GFP TAGF1 (lane 8), LIYV RNA 1 and P34-GFP TAGF2 (lane 9), LIYV RNA 1 and P34-GFP TAGF3 (lane 10). Samples were all collected at 72 hpi and hybridization was performed using a ^{32}P-labeled GFP probe in order to detect minus-sense RNAs. The positions of migration of mutant RNA 1 genomic (MG), mutant RNA 1 subgenomic (SGB) RNAs are indicated. Numbers at left indicate positions of RNA markers. rRNA stained by ethidium bromide is the indication of total RNA loading.

(Rubio et al., 2000). However, we tested whether or not this might be important for artificial LIYV RNA 1 D RNAs but the LIYV RNA 1 ORF 1A deletion mutant ΔEcoRI, having an ORF of 358 nucleotides was not replication competent. Another LIYV RNA 1 ORF 1A deletion mutant ΔE-LINK, in which artificial D RNA had a relatively large ORF of 903 nucleotides, also failed to be replicated. Furthermore, LIYV RNA 1 ORF 1B deletion mutant Δ1B, which contained the complete wild-type ORF 1A of 5619 nucleotides but lacked ORF 1B, was not capable of *in trans* replication.

It is also possible that structural features of the LIYV RNA 1 sequence may be necessary for replication, or they may even inhibit RNA 1 replication *in trans* (Buck, 1996; Chen et al., 2001; Miller and White, 2006; Osman and Buck, 2003; Pogany et al., 2005; Ray and White, 2003; Sun and Simon, 2006; Yi and Kao, 2008; Zhang and Simon, 2005). We attempted to address both of these possibilities. The first series of mutants generated here (ΔEcoRI, ΔE-LINK, Δ1B) were not replication competent. However these mutants were generated by deletions of the RNA 1 sequence and, thus conceivably could be missing sequence signals required for *trans* replication. This seems somewhat unlikely as the additional frameshift mutants generated by us (mutants P34-GFP TAGF1, P34-GFP TAGF2, P34-GFP TAGF3), which retained the RNA 1 sequences and had frameshift mutations on ORF 1A or 1B, still were not replicated *in trans* when co-inoculated with wild-type RNA 1 as a helper virus. Although for some viruses including *Tobacco mosaic virus* and *Tomato bushy stunt virus*, internal sequences have been identified that are inhibitory to helper-mediated *trans* replication of artificial D RNAs (Lewandowski and Dawson, 1998; Pogany et al., 2003; Qiu et al., 2001), this may not be the case for LIYV RNA 1. The ΔEcoRI, ΔE-LINK, Δ1B mutants contained different, large sequence deletions at different positions, but still failed to replicate *in trans*.

cis-preferential replication has been reported for several monopartite and multipartite RNA viruses and has been proposed that it may serve as a means to maintain selection for viable progeny RNAs, and prevent accumulation of defective-interfering RNAs (DI RNA) and defective RNAs (D RNA) generated by recombination in the progeny (Neeleman and Bol, 1999; Novak and Kirkegaard, 1994; Okamoto et al., 2008; Van Bokhoven et al., 1993; van Rossum et al., 1996; Weiland and Dreher, 1993; Zhou and Jackson, 1996). The accumulation of D- or DI-RNAs might compete with the replication of RNA 2 (both are replicated *in trans*). If replication of LIYV RNA 1 is *cis*-preferential and RNA 2 is replicated *in trans*, this would be consistent with our observation that numerous LIYV RNA 2 D RNAs that are produced during infection, and our inability to find naturally-occurring RNA 1 D RNAs (Rubio et al., 2000). For the monopartite ssRNA virus, *Tobacco mosaic virus* (TMV), there were no reports of naturally occurring D RNAs (Lewandowski and Dawson, 1998). However, some artificial D RNAs (deletion mutants) could replicate *in trans* with TMV wild-type as a helper virus, whereas the full-length D RNAs (frameshift mutants) could not (Lewandowski and Dawson, 1998). In addition, the inability of *trans* replication for some TMV D RNAs, inability to move in plants, or lack of ability for mechanical transmission to other plants resulted in elimination of TMV D RNAs (Knapp et al., 2001; Lewandowski and Dawson, 1998). For the monopartite *Citrus tristeza virus* (CTV), which like LIYV is a member of the *Closteroviridae*, the situation appears more complicated. The ORFs 1A and 1B of CTV are organized similarly to those of LIYV. However, CTV infections contain abundant D RNAs ranging from <2 kb to almost full length (Mawassi et al., 2000b). Furthermore, studies on the fitness of CTV D RNAs showed that *trans*-replication competent D RNAs required not only at least the genomic RNA 5′ proximal ~1 kb and 3′ 270 nucleotides (Mawassi et al., 2000a), but also a continuous ORF, even of nonviral nucleotide sequence (Mawassi et al., 2000a). Although the accumulation of CTV D RNAs may compete with the replication of wild-type CTV, CTV infections do not appear to be compromised. In contrast to the situation for the monopartite CTV, LIYV RNA 2 must be replicated *in trans*, and

expression of RNA 2 genes are required to complete the LIYV infection cycle in plants.

Clearly, the replication features of the LIYV genomic RNAs are different from those currently recognized for other multipartite plant viruses having positive-sense ssRNA genomes. LIYV RNA 1 accumulation proceeds quickly after infection, most likely as a result of *cis*-preferential replication, whereas RNA 2 replication is *in trans* and accumulation is delayed relative to RNA 1 (Yeh et al., 2000, 2001). Furthermore, the efficient *trans* replication of LIYV RNA 2 is dependent on the RNA 1-encoded P34, whereas P34 is not needed for replication of RNA 1 (Yeh et al., 2000). This strategy of LIYV RNA replication also could serve to temporally regulate LIYV gene expression. Obviously, the replication-associated proteins, encoded by RNA 1, are needed first in infection. Thus, ORFs 1A and 1B are translated first from the LIYV genomic RNA 1, and these are sufficient for RNA 1 replication (Yeh et al., 2000). The LIYV RNA 1 ORF 2-encoded P34, the *trans* enhancer of RNA 2 replication, is translated from the subgenomic RNA P34, but only after generation of minus-strand RNA 1, the likely template for subgenomic RNA synthesis. It is interesting to note also that the accumulation of the P34 subgenomic RNA is very high; it is the most abundant viral RNA found in LIYV-infected plants or protoplasts. The finding that P34 is a *trans* enhancer of RNA 2 replication (Yeh et al., 2000), could be partially explained by *in cis* replication of RNA 1, in which LIYV ORF 1A and 1B-encoded replication-related proteins could bind to the proper region of the same RNA 1 molecule directly after translation. It is possible that the binding of replication-related proteins to RNA 1 could result in a structural change of RNA 1. Similar models have been proposed for protein:RNA interactions and replication for some other ssRNA plant viruses (Daros and Carrington, 1997; Guogas et al., 2004; Okamoto et al., 2008; Olsthoorn et al., 1999; Petrillo et al., 2005; Pogany et al., 2005). However, the efficient RNA 2 replication requires the *trans* supply of P34. How P34 functions to assist RNA 2 replication is not yet known but recent studies demonstrate that it is a ssRNA binding protein (Wang et al., unpublished). LIYV genomic RNAs 1 and 2 do not show homology at their 3′ termini (Klaassen et al., 1995). Perhaps the LIYV RDRP needs other factors including P34 for template RNA selection and efficient RNA 2 replication. Thus, LIYV RNA 1 *in cis* replication is the initial step in the asynchronous replication of the LIYV genomic RNAs. Then RNA 2-encoded proteins (with the possible exception of P5 encoded by the 5′-most ORF) would be expressed later in the infection cycle after RNA 2 replication and generation of corresponding subgenomic RNAs (Rubio et al., 2000; Yeh et al., 2000).

Materials and methods

Construction of LIYV RNA 1 mutants

Seven LIYV RNA 1 mutants were constructed (Fig. 2). The ΔEcoRI mutant was created by deleting a 4732 nucleotide EcoRI fragment (nucleotides 448–5181) from ORF 1A in the full-length LIYV RNA 1 infectious clone p9/55 (Klaassen et al., 1996). The digested DNA was re-ligated using T4 DNA ligase. This resulted in truncation of ORF 1A at nucleotide 455. A second ORF 1A mutant, ΔE-LINK was created by digesting the ΔEcoRI mutant with EcoRI and inserting a 16 nucleotide EcoRI-digested linker (5′-CTC*GAATTC*AAGGATCCATG*AATTC*TTA-3′; EcoRI sites in italics) to restore the reading frame (Fig. 2), resulting in an ORF 1A of 903 nucleotides. An ORF 1B mutant, Δ1B, was created by digesting p9/55 with SalI and MfeI to remove a 1447 nucleotide fragment within ORF 1B (positions 5768–7214 of RNA 1). The digested DNA was treated with Klenow fragment DNA polymerase and re-ligated using T4 DNA ligase.

We next constructed an insertion mutation in the LIYV RNA 1 ORF 2. Primer Afl-F 5′-TTATCTTAAGGTTTCAGTAAAGGAGAAGAA-3′ and primer Afl-R 5′-TTATCTTAAGGTTTCAGTAAAGGAGAAGAA-3′ were used as primers in the polymerase chain reaction (PCR) with pAT1 as the template (Yeh et al., 2001). PCR was then performed to amplify the partial coding

region of GFP gene. The resulting fragment was digested using AflII and ligated into LIYV RNA 1 P34-mutant predigested using AflII at 7247 (Yeh et al., 2000). This gave clone P34-GFP TAG, which contained only 377 nucleotides of the GFP sequence inserted in the reverse orientation. Three frameshift mutants, P34-GFP TAGF1, P34-GFP TAGF2 and P34-GFP TAGF3, were generated by digesting P34-GFP TAG with ClaI at position 2716, SalI at position 5768, and AgeI at position 7134, respectively, then treating with Klenow fragment DNA polymerase and religating. This served to introduce two additional nucleotides in P34-GFP TAGF1, and four nucleotides in P34-GFP TAGF2 and P34-GFP TAGF3, resulting in frame-shifts and stop codons at positions 2726, 5792, 7206, respectively. All of the above mutations were confirmed by nucleotide sequence analysis.

LIYV inocula and protoplast manipulation

Capped transcripts corresponding to wild-type LIYV RNAs 1 and 2, and LIYV RNA 1 mutants were synthesized as described (Yeh et al., 2000). Protoplasts were prepared from cultured *Nicotiana tabacum* suspension cells and inoculated using transcripts essentially as previously described, except that 5×10^5 cells were used per inoculation (Yeh et al., 2000).

Analysis of LIYV genomic and defective RNA replication

Protoplasts were collected and analyzed as described (Yeh et al., 2000, 2001). Aliquots containing ca. 5×10^4 cells were collected at different times post-inoculation, and total RNA was extracted by using TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Generally, 2 μg of total RNA from each protoplast sample, respectively, was used for northern hybridization. Positive-sense and negative-sense ^{32}P-labeled probes of LIYV RNA 1 and 2 were generated from pSKL1 and pSKL16 respectively (Yeh et al., 2000). The ^{32}P-labeled GFP minus-sense probe was generated with T7 RNA polymerase using EcoRI-linearized pAT1 (Yeh et al., 2001). Since P34-GFP TAG contained the GFP sequence as a marker, the ^{32}P-labeled GFP probe was used to detect minus-sense RNAs of mutants, P34-GFP TAG, P34-GFP TAGF1, P34-GFP TAGF2 and P34-GFP TAGF3.

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