The Coat Protein of Beet Western Yellows Luteovirus is Essential for Systemic Infection but the Viral Gene Products P29 and P19 are Dispensable for Systemic Infection and Aphid Transmission

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The beet western yellows luteovirus genes encoding the major coat protein (open reading frame [ORF] 3), the putative protein P29 (ORF 0), and the candidate movement protein P19 (ORF 4) have been mutated to study the role of the corresponding gene products in transcript-infected *Chenopodium quinoa* protoplasts and agro-infected *Nicotiana clevelandii* plants. Mutations blocking synthesis of the major coat protein inhibited systemic infection of agro-inoculated plants, indicating that coat protein is required for systemic movement. Mutations in ORF 0 partially inhibited viral RNA amplification in both protoplasts and agro-infected plants but the mutant virus could spread systemically in *N. clevelandii* and be aphid-transmitted to other hosts. Thus, at present, no function can be assigned to the ORF 0 putative gene product. Viruses carrying mutations in ORF 4 accumulated to high levels in both protoplasts and agro-infected plants although symptom onset was delayed in plants and initial virus titers were somewhat lower. The ORF 4-mutant virus could be aphid-transmitted to other hosts. These findings rule out the possibility that P19 is an essential viral movement protein. A model in which beet western yellows virus can move in plants by two pathways is discussed.

Additional keywords: cell-to-cell movement, long distance movement, *Myzus persicae*.
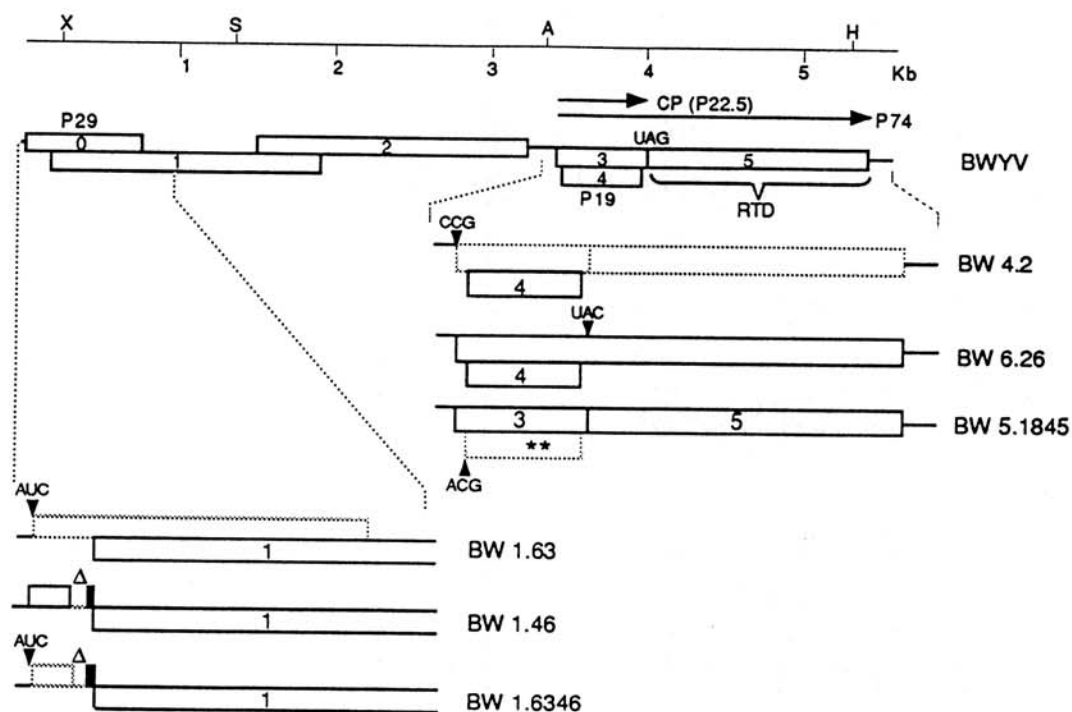
Beet western yellows luteovirus (BWYV) is a member of subgroup 2 of the luteoviruses, a group of obligately aphid-transmitted, phloem-limited viruses with small, isometric virions containing a plus-sense genome RNA of about 5.7 kb (Miller et al. 1995; Mayo and Ziegler-Graff 1996). Subgroup 2 members, including BWYV (genetic map shown in Figure 1), differ from subgroup 1 luteoviruses in the taxonomic affinities of the viral replicase and certain features of genetic organization (Habili and Symons 1989), the most important

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for this paper being the presence of an "extra" 5'-proximal open reading frame (ORF; note that the ORF numbering in this paper has been modified from our previous usage to conform to the system of Mayo and Ziegler-Graff [1996]), ORF 0, encoding an ≈29 kDa protein, P29. Important features common to all luteoviruses are the expression of the putative viral replicase by a −1 shift in reading frame in the region of overlap between ORFs 1 and 2 to produce an ORF 1–ORF 2 fusion protein (Brault and Miller 1992; Prüfer et al. 1992; Garcia et al. 1993; Kujawa et al. 1993), and the presence of a 3' gene cassette expressed from a subgenomic RNA (Tacke et al. 1990; Miller and Mayo 1991; Dinesh-Kumar et al. 1992) that encodes (i) the 22.5-kDa major viral coat protein (P22.5; ORF 3), (ii) a 19-kDa protein (P19) whose ORF (ORF 4) is embedded in ORF 3 in another reading frame and (iii) the "readthrough domain" (RTD; ORF 5), adjacent to and in the same frame as ORF 3. During infection, the RTD is expressed as a 74-kDa fusion protein (P74) by occasional readthrough of the major coat protein termination codon (Veidt et al. 1988; Tacke et al. 1990; Bahner et al. 1990; Dinesh-Kumar et al. 1992; Reutenauer et al. 1993). A protein derived from P74 is a minor component of virions (Bahner et al. 1990; Martin et al. 1990; Filichkin et al. 1994; Brault et al. 1995; Wang et al. 1995).

Full-length BWYV RNA transcripts readily infect protoplasts. With the protoplast infection system, knockout mutagenesis of each ORF has established that only ORF 1 and ORF 2 are required for viral RNA replication in single cells (Veidt et al. 1992; Reutenauer et al. 1993). Of the remaining genes, only that for P22.5 is required for virion formation in protoplasts (Reutenauer et al. 1993). Similar findings have been reported for the subgroup 1 luteovirus barley yellow dwarf virus–PAV (BYDV-PAV) (Mohan et al. 1995).

Luteovirus particles and RNA are not normally infectious to whole plants when mechanically inoculated, but *Agrobacterium tumefaciens*–mediated infection (agro-infection) of plants with cloned BWYV cDNA has been achieved (Leiser et al. 1992). Agro-infection experiments with BWYV cDNA encoding a truncated RTD have established that the RTD is required for virus to accumulate to normal levels in agro-

Fig. 1. Genome structure of beet western yellows luteovirus (BWYV) mutants. The genetic organization of BWYV is shown above with major open reading frames (ORFs) (hollow rectangles) identified by number and by calculated size of corresponding gene product (in kDa preceded by "P") for genes discussed in text. ORF numbering has been modified to conform to system described by Mayo and Ziegler-Graff (1996). Thus, what we have previously referred to as ORF 1 is now ORF 0, the previous ORF 2 is now ORF 1, etc. Arrows above the map represent major viral coat protein (CP) and readthrough protein (P74). RTD: readthrough domain. At top, some important restriction sites: X = *Xho*I; S = *Spe*I; A = *Afl*II; H = *Hin*dIII. Lower part of figure shows more detail in vicinity of the mutations. For point mutations, the mutated sequence is shown except for the two in-frame termination codons in ORF 4 of BW5.1845, which are indicated by asterisks. The position of the 29-nucleotide deletion in mutants BW1.46 and BW1.6346 is indicated by "Δ". ORFs eliminated by mutation of their initiation codons are represented by rectangles composed of dotted lines; the missense extensions of ORF 0 caused by the deletion in BW1.46 and BW1.6346 are represented by black rectangles.
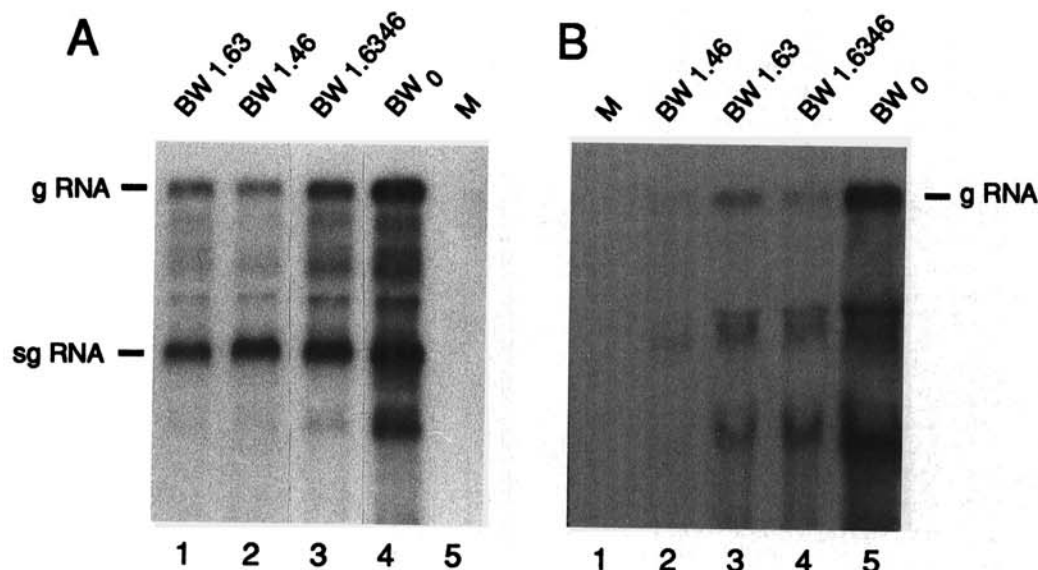
Fig. 2. Amplification of viral RNA in protoplasts (**A**) and whole plants (**B**) infected with beet western yellows luteovirus (BWYV) genome constructs bearing mutations in open reading frame (ORF) 0. **A,** Northern (RNA) blot analysis of appearance of progeny viral RNA 72 h after electroporation of *Chenopodim quinoa* Willd. protoplasts with plasmid DNA of the ORF 1–mutant constructs pBSBW1.63 (lane 1), pBSBW1.46 (lane 2), pBSBW1.6346 (lane 3), the wild-type construct pBSBW$_0$ (lane 4), or mock inoculation (lane 5). **B,** Northern blot analysis of appearance of progeny viral RNA 4 weeks after mock inoculation (lane 1) or agro-infection of *Nicotiana clevelandii* Gray. with the ORF 0–mutant constructs pBinBW1.46 (lane 2), pBinBW1.63 (lane 3), pBinBW1.6346 (lane 4), and the wild-type construct pBinBW$_0$ (lane 5). Viral RNA on the blots was detected with a ^{32}P-labeled antisense viral RNA probe (Reutenauer et al. 1993). gRNA: genomic RNA; sgRNA: subgenomic RNA. On the Northern blot of RNA from plants (**B**), the sgRNA is not easily detectable because of the presence of ribosomal RNA "shadow" bands (Palukaitis et al. 1983; Leiser et al. 1992). The band migrating more rapidly than sgRNA in lanes 1 to 4 (**A**) and lanes 2 to 5 (**B**) was not detected in all experiments and its origin has not yet been investigated.

infected plants and for aphid acquisition/transmission of the virus (Brault et al. 1995). In this paper we have examined the effect of knockout mutations in the other replication-dispensable BWYV genes (ORFs 0, 3, and 4) on virus function in agro-infected plants and on aphid transmission.

RESULTS

BWYV constructs carrying mutations in ORFs 0, 3, and 4 of BWYV.

Figure 1 shows the structures of the ORF 0, 3, and 4 mutants used in this paper. In mutant BW1.63, the ORF 0 initiation codon (nucleotides 32 to 34) was converted to AUC (the next in-frame AUG is 175 codons downstream). In mutant BW1.46, a 29-residue deletion (nucleotides 123 to 151 deleted) was introduced in ORF 0 upstream of the ORF 1 initiation codon (nucleotides 174 to 176) and in mutant BW1.6346 the two mutations were combined in the same construct. In mutant BW4.2, the ORF 3 initiation codon was changed to CCG (the next in-frame AUG is 107 codons downstream). In mutant BW6.26 the ORF 3 termination codon UAG was converted to UAC to produce the ORF 3–ORF 5 fusion protein P74 but no P22.5 (Reutenauer et al. 1993). Finally, in mutant BW5.1845, the ORF 4 initiation codon was modified to ACG and two in-frame stop codons were introduced near the middle of the ORF (the next in-frame AUG is 15 residues from the 3′ end of the ORF). None of the ORF 4 modifications alters the amino acid sequence of the ORF 3 gene product.

The ability of the mutant constructs to be amplified in *Chenopodium quinoa* Willd. protoplasts was tested. For protoplast infection experiments with mutants pBW4.2, pBW6.26, and pBW5.1845, bacteriophage T7 RNA polymerase run-off transcripts were used as inocula. For the ORF 0 mutants, the inocula consisted of plasmid constructs containing full-length cDNA placed downstream of a cauliflower mosaic virus 35S promoter. As will be shown below, a plasmid (pBSBW$_0$) bearing the corresponding wild-type

cDNA is infectious when electroporated into protoplasts (see Figure 2). For agro-infection experiments, the mutant cDNA constructs were inserted into the T-DNA of the binary vector Bin 19 (Bevan 1984; Frisch et al. 1995) under transcriptional control of the 35S promoter. *A. tumefaciens* harboring the recombinant binary vector (referred to as pBinBW$_0$ for the wild-type, pBinBW1.63 etc. for the mutants) was then inoculated to midveins of *Nicotiana clevelandii* Gray. leaves (Leiser et al. 1992).

Infectivity tests with ORF 0 mutants.

Total RNA was extracted from *C. quinoa* protoplasts 70 h postinoculation (pi) with the ORF 0 mutant plasmids pBSBW1.63, pBSBW1.46, and pBSBW1.6346 and tested for the appearance of progeny viral RNA. For all three mutants, both full-length viral RNA (gRNA) and the major subgenomic RNA encoding ORFs 3, 4, and 5 (sgRNA) were readily detected on Northern (RNA) blots (Fig. 2A), confirming our earlier finding (Veidt et al. 1992) that the ORF 0 putative gene product is dispensable for virus replication in protoplasts. Viral capsid proteins could also be readily detected on Western blots (immunoblots) of proteins extracted from the infected protoplasts (data not shown). Although there was some variability in different experiments in the amounts of progeny RNA and coat protein synthesized, protoplasts inoculated with the ORF 0 mutants generally accumulated two to five times less progeny viral RNA and capsid proteins (P22.5 and P74) than did protoplasts inoculated with the wild-type construct.

The three ORF 0 mutant cDNAs were moved into Bin 19 and agro-inoculated to leaves of *N. clevelandii*. The course of infection was followed by enzyme-linked immunosorbent assay (ELISA) on randomly selected leaves over a period of 11 weeks. Viral coat protein was readily detected in plants inoculated with both the wild-type and the mutant constructs by 3 to 4 weeks pi and for the remainder of the measuring period. However, the titer of coat protein antigen in the plants agro-infected with the mutant constructs remained fivefold to
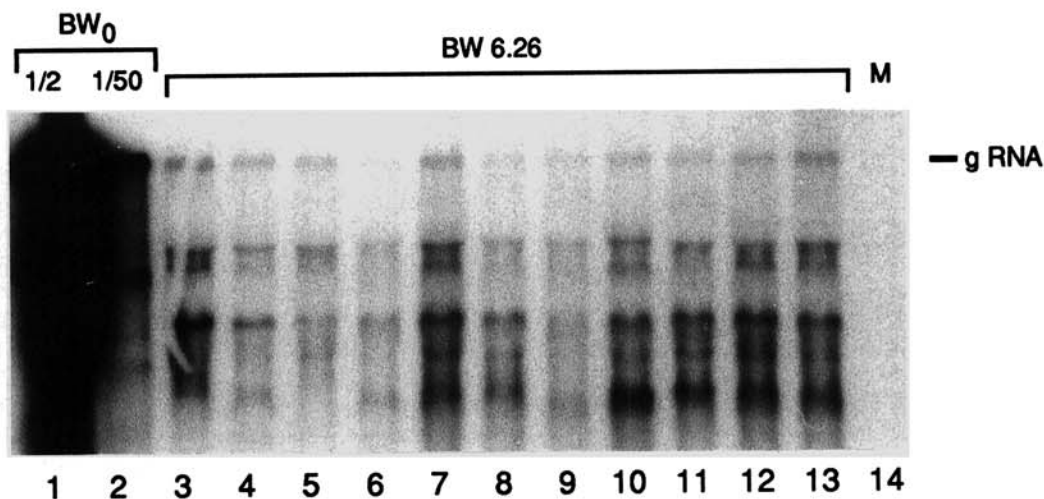

Fig. 3. Detection by Northern (RNA) hybridization of beet western yellows luteovirus (BWYV) RNA in midveins of *Nicotiana clevelandii* agro-inoculated with the wild-type construct pBinBW$_0$ (lanes 1 and 2) or the ORF 3–ORF 5 fusion construct pBinBW6.26. RNA samples were extracted 4 weeks postinoculation from 11 plants agro-inoculated with pBinBW6.26 (lanes 3 to 13) and from a mock-inoculated plant (lane 14). For the plant agro-infected with pBinBW$_0$, the amount of total RNA loaded was one half (lane 1) or one fiftieth (lane 2) of the amount loaded in lanes 3 to 14. Other details are as described in the Figure 2 legend.

sevenfold lower than in control plants (estimates based on ELISA measurements with diluted plant extracts). Plants agro-infected with the ORF 0 mutants developed symptoms (interveinal leaf yellowing) qualitatively similar to those observed on plants agro-infected with the wild-type construct pBinBW₀ but the symptoms were initially milder and their appearance was generally delayed 1 to 2 weeks.

The appearance of viral RNA in leaves of plants agro-infected with the three ORF 0 mutants was investigated by Northern blot analysis 10 weeks pi. Progeny viral RNA was readily detected in the leaves of the mutant-infected plants but accumulated to levels significantly lower than observed in the control (Fig. 2B), reflecting the lower titer of virus antigen detected in these leaves by ELISA.

The stability of the ORF 0 mutations during the virus infection cycle was studied by cloning and sequencing DNA fragments that had been obtained by reverse transcription–polymerase chain reaction (RT-PCR) amplification of the progeny viral RNA. The analysis was carried out on RNA extracted 7 weeks pi from one plant agro-infected with pBinBW1.63 (four clones sequenced), one plant agro-infected with pBin1.6346 (three clones sequenced), and two plants agro-infected with pBinBW1.46 (four clones from each plant sequenced). In every case the mutation was conserved and no second-site mutations were observed in the ≈250 residues flanking the mutations that were sequenced.

Mutation of the major capsid protein (P22.5).

We have shown previously that full-length BWYV RNA transcripts carrying the coat protein knockout mutation BW4.2 and the ORF 3–ORF 5 fusion construct BW6.26 are amplified in *C. quinoa* protoplasts, albeit to significantly lower levels (≈25%) than the wild-type control (Reutenauer et al. 1993). The appearance of virus in randomly selected leaves of *N.*
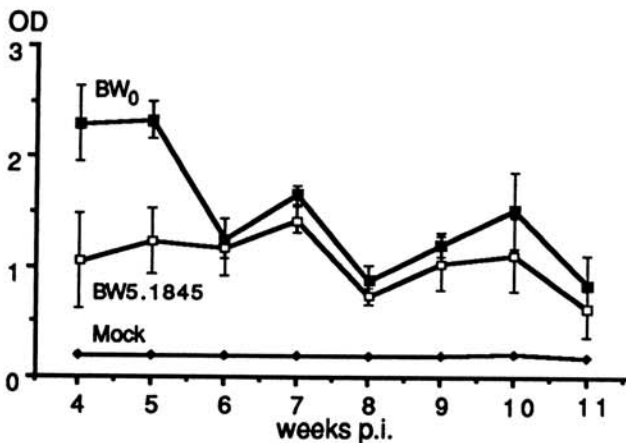

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Fig. 4. Time course of beet western yellows luteovirus (BWYV) appearance as measured by enzyme-linked immunosorbent assay (ELISA) of agro-infected *Nicotiana clevelandii* Gray. For each time point, four randomly selected leaf samples from each plant were pooled for extraction; ELISA values were averaged for the plants agro-infected with the wild-type construct pBinBW₀ (filled squares; values are average of four plants) and the open reading frame (ORF) 4 mutant pBinBW5.1845 (empty squares; values are average of six plants). ELISA values obtained for healthy plant extracts indicated by filled circles. Procedures as described (Brault et al. 1995) except that extracts from infected tissue were diluted a further 10-fold prior to ELISA measurements.

clevelandii agro-inoculated with the major coat protein knockout mutant pBinBW4.2 and the ORF 3–ORF 5 fusion mutant pBinBW6.26 was monitored by ELISA 3 to 7 weeks pi. No virus antigen could be detected in 16 plants agro-inoculated with pBinBW4.2 or in 28 plants agro-inoculated with pBinBW6.26 whereas 15 of 23 control plants agro-inoculated with pBinBW₀ contained coat protein. Total RNA samples were extracted from randomly selected leaves of eight of the plants agro-inoculated with pBinBW4.2 (samples taken 4, 6, and 9 weeks pi) and from six plants agro-inoculated with pBinBW6.26 (samples taken 8 weeks pi). No viral RNA could be detected by Northern blot or dot blot in any of these samples whereas viral RNA was readily detected in samples taken in parallel from plants agro-infected with the wild-type construct (data not shown).

To determine whether low levels of infection with the capsid protein mutant constructs could be detected near the sites of agro-inoculation, RNA was extracted from the midveins of agro-inoculated *N. clevelandii* leaves from 11 plants agro-inoculated with pBinBW6.26 (samples taken 4 weeks pi). Viral RNA could be detected in all samples by Northern blot analysis but in several-hundred-fold lower amounts than in midveins from a plant infected with the wild type (Fig. 3). Similar results were obtained with the mutant pBinBW4.2 (data not shown). No viral RNA signal for the coat protein mutants could be detected in RNA extracted from midveins of noninoculated leaves or midvein sections from the apices of leaves that had been inoculated near their bases (data not shown).

Mutation of ORF 4.

A full-length transcript of construct BW5.1845, containing multiple mutations in ORF 4 (elimination of the initiation codon and introduction of two in-frame termination codons; Fig. 1), replicated in *C. quinoa* protoplasts with approximately the same efficiency as wild-type transcript (data not shown), confirming our earlier finding made with mutant BW5.18 (initiation codon mutated) that the ORF 4 gene product is replication-dispensable (Reutenauer et al. 1993). *N. clevelandii* plants agro-infected with pBinBW5.1845 developed typical symptoms although their appearance was sometimes delayed 1 to 2 weeks compared with the wild type. The time course of accumulation of virus following agro-inoculation was monitored by ELISA on randomly selected leaves over a period from 4 to 11 weeks pi. Virus antigen was found to accumulate to high levels in both the plants agro-infected with pBinBW5.1845 and those agro-infected with pBinBW₀ (Fig. 4). pBinBW5.1845-infected plants contained about 50% of the virus levels in wild-type controls at early times (4 to 5 weeks pi) but similar levels were present at later times. Northern blot (Fig. 5A) and Western blot (Fig. 5B) analysis likewise revealed relatively little difference between RNA and capsid protein levels in the two types of infected plant, particularly at later times. Sequence analysis of cloned cDNA (13 clones sequenced) following PCR amplification of the viral RNA sequence in the vicinity of the mutations showed that the mutations were still present in the progeny RNA (RNA samples were taken from three plants 10 weeks pi). Finally, no P19 could be detected in protein extracts from pBinBW5.1845-infected plants with the use of a P19-specific antiserum (Fig. 5C), providing additional evidence that the mutations introduced into ORF 4 had not reverted.

Aphid transmission studies.

Newly formed infected leaves from *N. clevelandii* that had been agro-infected with the ORF 0 mutants pBinBW1.63, pBinBW1.46, and pBinBW1.6346 or with the ORF 4 mutant pBinBW5.1845 were used as source of virus in aphid transmission experiments. Nonviruliferous nymphs of *Myzus persicae* Sulz. were allowed to feed on the leaves for 1 day before being transferred to healthy *Montia perfoliata* (eight nymphs per plant). The test plants were assayed for virus infection 3 to 4 weeks later by ELISA. The experiments revealed that the ORF 0 mutants could be aphid-transmitted to *M. perfoliata* with efficiencies ranging from 60 to 100% (Table 1). The infected plants developed typical symptoms. Analysis of cloned RT-PCR products from RNA from four plants infected with BW1.46 revealed that the original mutation was maintained in 14/14 clones sequenced although four of the cloned PCR fragments contained secondary point mutations in the vicinity of the original deletion. The ORF 4 mutant BW5.1845 was also efficiently transmitted by the vector (Table 1). The stability of the ORF 4 mutations in the progeny viral RNA after aphid transmission was analyzed by sequencing cloned RT-PCR products from RNA from three plants (eight clones sequenced) and the original mutations were found to be conserved in all cases.

Aphids that had acquired wild-type, ORF 0-mutant, or ORF 4-mutant virus by feeding on agro-infected *N. clevelandii* were used as inoculum source in infection tests with different viral host plants. Virus titer was measured by ELISA 3 to 4 weeks later. For the ORF 0 mutants, the host plants tested were *Capsella bursa-pastoris* (L.) Medik., *Physalis floridana* Rybd., spinach (*Spinacia oleracea* L.), and lettuce (*Lactuca sativa* L.), the host from which our isolate of BWYV was

originally obtained (Veidt et al. 1988). Aphid transmission occurred in every case (Table 2). The ORF 4 mutant BW5.1845 was likewise aphid-transmitted to spinach, *C. bursa-pastoris*, and *P. floridana* (Table 2; transmission to lettuce was not tested).

DISCUSSION

Analysis of gene function of viruses with an RNA genome has been greatly facilitated by the availability of biologically active transcripts (Boyer and Haenni 1994), which provide a substrate for mutagenesis studies. Agro-infection represents a means of extending such analysis to the study of gene function for viruses such as the luteoviruses, which cannot be transmitted by mechanical inoculation to whole plants (Leiser et al. 1992). With this approach, we have previously shown that truncation or elimination of the BWYV RTD not only blocks aphid transmission of the virus but also impedes virus accumulation in agro-infected *N. clevelandii*. (Brault et al. 1995).

The major coat protein.

In this paper we have investigated the function of the other BWYV replication-dispensable genes in whole plant infections. Mutant BW4.2, which does not produce the major viral coat protein P22.5 due to mutation of the ORF 3 initiation codon, and mutant BW6.26, which expresses P22.5 but only as part of P74, both multiplied in protoplasts (Reutenauer et al. 1993), although progeny viral RNA accumulated to about one fourth the level observed for wild-type control. Similar observations have been reported for barley protoplast infections with major coat protein mutants of BYDV-PAV (Mohan
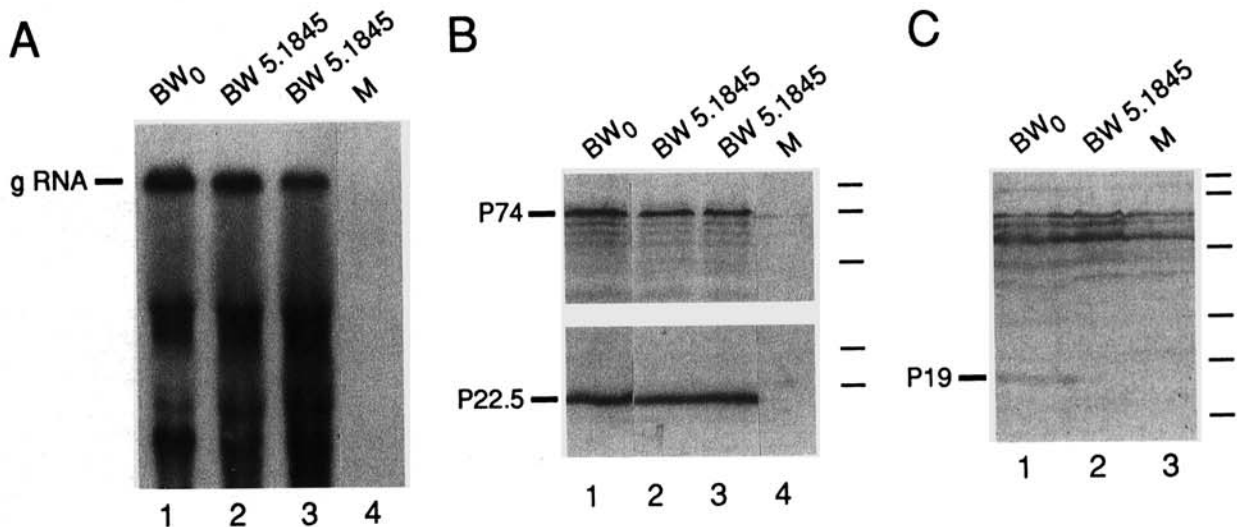
Fig. 5. Analysis of plants agro-infected with open reading frame (ORF) 4 mutant pBinBW5.1845 for appearance of progeny viral RNA (**A**), viral capsid proteins (**B**), and P19 (**C**). **A,** Northern (RNA) blot analysis of total RNA extracted 10 weeks postinoculation from randomly selected tissue samples from a plant agro-inoculated with pBinBW$_0$ (lane 1), two plants agro-inoculated with pBinBW5.1845 (lanes 2 and 3), and a mock-inoculated plant (lane 4). Viral RNA on blots was detected with a ^{32}P-labeled antisense viral RNA probe. gRNA: genomic RNA. **B,** Western blot (immunoblot) analysis of appearance of viral structural proteins P22.5 and P74 in the agro-infected plants. Total protein was extracted from a plant agro-infected with pBinBW$_0$ (lane 1), two plants agro-infected with pBinBW5.1845 (lanes 2 and 3), and a mock-inoculated plant (lane 4). An antiserum specific for the readthrough domain (Reutenauer et al. 1993) was used to identify readthrough protein (P74) in upper part of blot and a virus-specific antiserum (Brault et al. 1995) was used to identify P22.5 in lower part of blot. A weak cross-reaction between the readthrough domain–specific antiserum and a plant protein that migrates slightly more rapidly than P74 is visible in lane 4 in upper part of blot. **C,** Total protein extracts from a plant agro-infected with pBinBW$_0$ (lane 1), pBinBW5.1845 (lane 2), and a mock-inoculated plant (lane 3) were immuno-probed for the ORF 4 gene product P19 with a P19-specific antiserum (Reutenauer et al. 1993). Mobility markers whose positions are shown to the right in **B** and **C** have molecular masses of 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa, from top to bottom.

et al. 1995). The less efficient accumulation of progeny RNA for the coat protein mutants could be due to instability of the nonpackaged RNA in the cytosol of the protoplasts (De Varennes and Maule 1985; Chapman et al. 1992; Van Bokhoven et al. 1993; Boccard and Baulcombe 1993) or to operation of a hypothetical feedback inhibition cycle in which the rate of viral RNA synthesis is regulated by the concentration of nonpackaged viral RNA.

Plants agro-infected with pBinBW4.2 and pBinBW6.26 accumulated very small amounts of progeny viral RNA in tissue extracts enriched for agro-inoculated midveins but no viral RNA could be detected in total extracts of noninoculated leaves or in midvein-enriched fractions from such leaves. These observations suggest that the coat protein mutants can replicate in those cells that have initially taken up the binary vector ("subliminal infection") and, perhaps, in close neighbors, but that the infection does not move much beyond the site of inoculation.

We have shown earlier that deletion or truncation of the RTD of P74 does not diminish replication in protoplasts but impedes accumulation of virus in agro-infected plants (Brault et al. 1995). Deletion of the RTD of the subgroup 1 luteovirus BYDV-PAV has recently been shown to have a similar effect (Chay et al. 1996). These observations suggest that the RTD is required for efficient long-distance movement of the virus and/or enhances long-term stability of the virions, although a number of alternative explanations (e.g., an effect on replication in cells of the phloem compartment) cannot yet be strictly ruled out. In any event, this finding raises the possibility that the inhibition of accumulation of mutant pBinBW4.2 in plants could be related to the absence of P74 rather than the major coat protein since the start codon mutation blocking P22.5 translation initiation will also block P74 synthesis. This possibility is ruled out, however, by the failure of pBinBW6.26, which synthesizes P74 but no P22.5 (Reutenauer et al. 1993), to invade the plant. Thus it appears that synthesis of both viral structural proteins (and presumably virion formation) is required for efficient spread of BWYV in planta. Note that, in this respect, luteoviruses differ from the phloem-limited bipartite geminiviruses, for which the coat protein is dispensable for systemic movement (Gardiner et al. 1988; Sanderfoot and Lazarowitz 1995).

ORF 0.

Mutations disabling ORF 0 (mutants BW1.43 and BW1.63 and the double mutant BW1.6346) multiplied and moved systemically within agro-infected *N. clevelandii*, although viral RNA accumulation levels were significantly lower (five- to sevenfold) than in controls infected with the wild type. Lower accumulation levels were also observed in protoplast infection experiments, suggesting that the mutations are primarily affecting viral RNA replication rather than virus movement. These findings may indicate that the putative ORF 0 gene product P29 (the ORF 0 gene product has not been detected in infected plants or protoplasts for any of the subgroup 2 luteoviruses) enhances viral RNA replication rates but is not an essential component of the replication complex. Another possibility is that the observed inhibitory effect of the ORF 0 mutations is a consequence of modification of *cis*-acting replication signals since the mutations in BW1.43, BW1.63, and BW1.6346 all fall within the 5′-proximal 151 residues of the viral RNA, a region where such signals might be located. Finally, it is possible that the mutations affect replication by interfering with translation initiation of the downstream ORF 1, which is known to be replication-essential (Reutenauer et al. 1993; Mohan et al. 1995).

The ORF 0s of the subgroup 2 luteoviruses are the viral genes that display the least sequence homology (Miller et al. 1995; Mayo and Ziegler-Graff 1996). This has led us to suggest that the putative gene product of ORF 0 might play a role in determining host range (Veidt et al. 1992). Although not conclusive, the findings described in this paper do not support such a hypothesis since the various P29 knockout mutants not only replicated and spread in *N. clevelandii* but in four other hosts as well. Furthermore, the mutations did not interfere with virus transmission by *M. persicae*. Thus, although P29 may intervene in a nonobligatory fashion in viral replication, its exact function still remains a mystery.

ORF 4 and virus movement.

Spread of a virus from its initial site of infection to neighboring cells through plasmodesmata requires the intervention of a virus-coded movement protein (MP) (Deom et al. 1992; Citovsky and Zambryski 1993; Lucas and Gilbertson 1994).

Table 1. Transmission by *Myzus persicae* of beet western yellows luteovirus (BWYV) carrying mutations in open reading frame (ORF) 0 and ORF 4 from agro-infected *Nicotiana clevelandii* to *Montia perfoliata*

Inocula[a]	Experiment						
	1	2	3	4	5	6	7
BW1.46	7/7[b]	4/4	11/11	4/4			
BW1.63		3/4		3/4			
BW1.6346		3/4		1/3			
BW5.1845					7/8	13/13	11/17
BW0	6/6	4/4	6/6	4/4	7/8	9/9	6/6

[a] Inocula were supplied to aphids as leaves of *N. clevelandii* agro-infected with the wild-type construct (pBinBW0), ORF 0–mutant constructs (pBinBW1.46, pBinBW1.63, pBinBW1.6346), and the ORF 4–mutant construct (pBinBW5.1845).
[b] All values refer to the number of *M. perfoliata* plants infected (numerator), as measured by enzyme-linked immunosorbent assay, and the number of aphid-inoculated plants (denominator) for each test.

Table 2. Transmission by *Myzus persicae* of beet western yellows luteovirus (BWYV) carrying mutations in open reading frame (ORF) 0 and ORF 4 from agro-infected *Nicotiana clevelandii* to different hosts

Inocula[b]	Host[a]			
	L. sativa	*C. bursa-pastoris*	*P. floridana*	*S. oleracea*
BW1.46	5/8[c]	8/8	6/8	5/6
BW1.63				5/6
BW1.6346				8/8
BW5.1845		2/8	7/8	6/8
BW0	8/8	8/8	8/8	8/8

[a] Tested were *Lactuca sativa* L. (lettuce, cv. Apollo), *Capsella bursa-pastoris* (L.) Medik., *Physalis floridana* Rybd., and *Spinacia oleracea* L. (spinach, cv. Géant d'Hiver).
[b] Inocula were supplied to aphids as leaves of *N. clevelandii* agro-infected with wild-type BWYV (pBinBW0), ORF 0–mutant virus (pBinBW1.46, pBinBW1.63, pBinBW1.6346), or ORF 4–mutant virus (pBinBW5.1845).
[c] All values refer to the number of plants infected (numerator), as measured by enzyme-linked immunosorbent assay, and the number of aphid-inoculated plants (denominator) for each test.

Currently, two major types of viral cell-to-cell movement have been recognized, differentiated by whether or not coat protein is required. In capsid protein–independent movement, as typified by tobacco mosaic virus, the viral MP increases the size exclusion limit of plasmodesmata and can form a complex with viral RNA that can pass through the modified plasmodesmata to neighboring cells. Other viruses, such as cowpea mosaic virus, require the capsid protein and virion formation for movement. Host cells infected with these viruses develop tubules, composed partly or wholly of MP, that provide a conduit for transit of virions through the plasmodesmata (Van Lent et al. 1990, 1991). Other viruses require coat protein for cell-to-cell movement but apparently do not form tubular structures (Chapman et al. 1992; Boccard and Baulcombe 1993; Dolja et al. 1995) while some viruses, notably cauliflower mosaic virus, may be able to move by both coat protein–dependent and coat protein–independent mechanisms (Citovsky et al. 1991; Perbal et al. 1993; Thomas and Maule 1995).

Long distance movement of a virus through the vascular system is less well understood. For most but not all viruses, the coat protein is required (Hamilton and Baulcombe 1989; Quillet et al. 1989; Allison et al. 1990; Saito et al. 1990; Suzuki et al. 1991; Heaton et al. 1991; Hilf and Dawson 1993; Dolja et al. 1995; Flasinski et al. 1995; Vaewhongs and Lommel 1995) although it often remains an open question whether the coat protein serves simply to protect the viral RNA by encapsidation or if it (also) specifically interacts with and modifies the properties of the specialized plasmodesmata characteristic of vascular tissue (Hull 1991; Maule 1991; Leisner and Turgeon 1993; Lucas and Gilbertson 1994). Finally, examples are known in which a viral nonstructural protein distinct from the cell-to-cell movement protein plays a role in long distance movement (e.g., Ding et al. 1995; Cronin et al. 1995).

Luteoviruses and geminiviruses represent a special situation with respect to long distance movement since they are delivered by their vector to the phloem compartment and are normally confined there. During infection, such viruses may move "horizontally" via plasmodesmata between neighboring phloem cells and "vertically" via the sieve elements, but they are not confronted with the problem of moving back and forth across the bundle sheath cell barrier as are viruses that can invade mesophyll tissues (Leisner and Turgeon 1993; Lucas and Gilbertson 1994). BWYV and related luteoviruses have been observed in companion cells, phloem parenchyma cells, and sieve elements of infected leaves (Esau and Hoefert 1972; D'Arcy and de Zoeten 1979; Shepardson et al. 1980), although virus replication is probably limited to nucleate cells. Virus particles have been observed in the plasmodesmata connecting the three cell types (Esau and Hoefert 1972; D'Arcy and de Zoeten 1979; Shepardson et al. 1980). The plasmodesmata that contained virions were devoid of their desmotubules but specialized tubular structures such as those associated with movement of cowpea mosaic virus were not present. Thus, the electron microscope observations indicate that luteovirus particles can enter and exit phloem cells via plasmodesmata and are consistent with our finding that capsid protein mutants are movement-defective. They do not, however, provide information concerning the possible role of viral nonstructural proteins in the process nor tell us whether a

viral RNA-MP complex can also participate in cell-to-cell movement, perhaps limited to certain cell or tissue types or certain developmental stages of the plant.

The ORF 4 gene product is an attractive candidate for a luteovirus MP. The ORF is present in all luteoviruses and displays sequence similarity ranging from 42 to 90% identity for the characterized subgroup 2 members (Mayo and Ziegler-Graff 1996). In the case of the potato leafroll luteovirus (PLRV), the 17-kDa ORF 4 gene product has been shown to bind single-stranded nucleic acids (Tacke et al. 1991), to form homodimers and higher polymers, to undergo phosphorylation in vivo, and to be primarily localized in a membrane-enriched fraction from transgenic potato plants expressing the protein (Tacke et al. 1993). These properties are similar to those of several known viral movement proteins (Deom et al. 1992; Fujiwara et al. 1993), leading Tacke et al. (1993) to suggest that the ORF 4 gene product is involved in cell-to-cell movement of the virus in the phloem compartment. Furthermore, recent work with an infectious transcript of the subgroup 1 luteovirus BYDV-PAV has provided direct evidence that the ORF 4 gene product of this virus is essential for a whole plant infection (Chay et al. 1996).

In view of the aforesaid observations, the failure of the P19 mutation in BW5.1845 to block virus accumulation in agro-infected N. clevelandii and in other hosts following aphid inoculation was quite unexpected. Our findings rule out an essential role for P19 as a component of the BWYV movement machinery. They do not, however, eliminate certain other possibilities. Evidently, P19 may have some function unrelated to movement which remains to be discovered. Another possibility is that BWYV and possibly other subgroup 2 luteoviruses employ two parallel movement pathways, one independent of P19 and the other requiring the protein (Fig. 6). We suggest that the hypothetical P19-independent pathway would involve movement of virions. One possibility is that the RT domain of P74 molecules incorporated into the virion acts as an MP to modify plasmodesmata and facilitate passage of the particle. Alternatively, movement of virions might require the major coat protein but no specialized viral MP. In this latter case, the RTD might simply serve to stabilize virions. Both of these variants can account for the observed inhibition by RT domain mutations of virus accumulation in plants (Brault et al. 1995; Chay et al. 1996) and also explain how virions, after being initially delivered into sieve elements by the aphid vector, can move through plasmodesmata into nucleate phloem cells for replication in the absence of a nonstructural viral movement protein.

The P19-dependent movement pathway, on the other hand, would presumably involve a viral RNA-P19 complex as an active agent. This type of movement might be limited to certain types of plasmodesmata; e.g., those connecting nucleate phloem cells (Fig. 6). The majority of companion and phloem parenchyma cells are believed to have at least a few plasmodesmatal connections to sieve elements (Behnke 1989). Hence, even if P19 is absolutely required for movement through the plasmodesmata connecting nucleate phloem cells, it should be possible for P19-defective mutant viruses produced in a given nucleate cell to eventually invade other cells by transiting via a sieve element with connections to both. The delay in symptom onset and the lower initial virus titers observed in plants agro-infected with mutant BW5.1845

(Fig. 4) could be a consequence of the indirect path from cell to cell that the virus must take in the absence of functional P19. Knockout mutations in the RTD, on the other hand, would allow nucleate phloem cells in the vicinity of an agro-infection site to become infected via P19-mediated movement of viral RNA but would impede "vertical" systemic infection by inhibiting entry and/or exit of virions into the sieve elements and/or diminishing their stability there. Ultrastructural comparisons of the pattern of appearance of wild-type and mutant virus in different cell types and in different types of phloem tissue during the course of infection should provide a means of testing this hypothesis.

MATERIALS AND METHODS

Plasmids.

Unless otherwise noted, the transcription vector pBW$_0$ containing wild-type BWYV cDNA (Veidt et al. 1992) was

Fig. 6. Schematic illustration of possible modes of beet western yellows luteovirus (BWYV) movement among different cell types of the phloem compartment. Shown are a portion of a sieve element (SE), a companion cell (CC), and a phloem parenchyma cell (PP). Surrounding bundle sheath cells, which are not efficiently infected by luteoviruses, are not included in illustration. Plasmodesmata joining companion cells and phloem parenchyma cells are symbolized by short parallel lines traversing cell wall, and "candelabra" plasmodesmata (Esau 1969) joining nucleate phloem cells and sieve element are symbolized by "V"s. Virions are symbolized by black circles with a protruding spike, which represents a molecule of P74 incorporated into the virion. P19/RNA complexes are represented as strings of beads. Possible association between P19 and plasmodesmata to alter the latters' size exclusion limit is not shown.
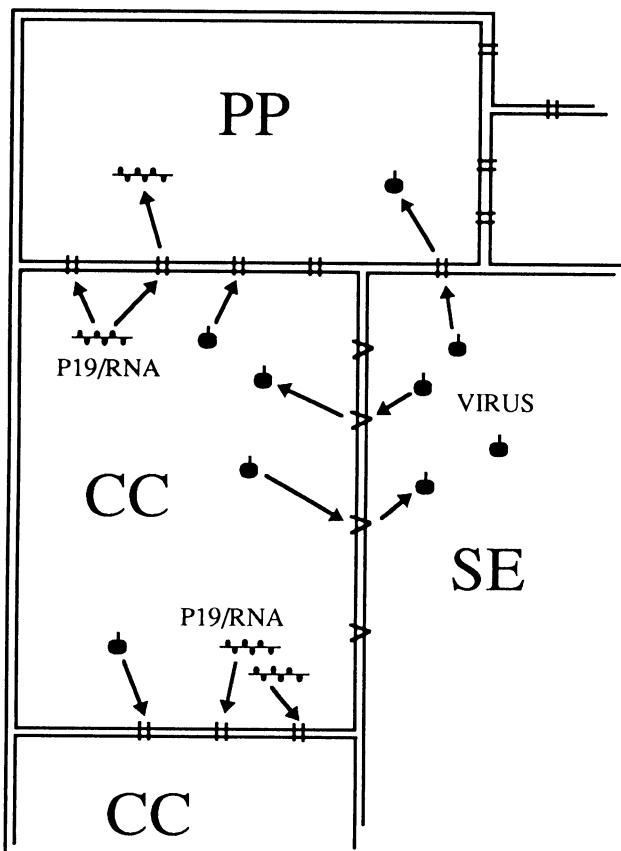
used as starting material for mutant constructs. Mutants pBW4.2 and pBW6.26 have already been described (Veidt et al. 1992; Reutenauer et al. 1993). Point mutations disrupting ORF 4 (conversion of the AUG initiation codon [nucleotides 3514 to 3516] to ACG; production of two in-frame UAA codons by conversion of C_{3812} to A and G_{3818} to A) were introduced by oligonucleotide site-directed mutagenesis of a DraI fragment (nucleotides 3313 to 5565) that had been cloned into the SmaI site of pBluescribe (Stratagene, La Jolla, CA) and expressed as single-stranded phagemid DNA. The mutated sequence was transferred as an AflII-HindIII fragment (nucleotides 3339 to 5367) back into pBW$_0$ to produce pBW5.1845.

The Bin 19–based binary vector pBinBW$_0$ containing full-length wild-type BWYV cDNA has already been described (Brault et al. 1995). The mutations in the transcription vectors pBW4.2, pBW6.26, and pBW5.1845 were introduced into pBinBW$_0$ on a SpeI-SalI fragment (nucleotides 1350 to the 3' end of the cDNA insert) to produce pBinBW4.2, pBin BW6.26, and pBinBW5.1845.

The Bluescribe-based construct pBSBW$_0$ carries full-length BWYV cDNA fused to the transcription initiation site of a 35S promoter (Brault et al. 1995). A DNA fragment extending from the polylinker KpnI site upstream of the 35S promoter of pBSBW$_0$ to the PstI site at nucleotide 2013 of the cDNA insert was cloned into Bluescribe to produce pBW.KP. An inconvenient XhoI site at position 1680 was then eliminated by deleting the sequence between the BglII sites at positions 1544 and 1711 to produce pBW.KPΔB. Plasmids bearing mutations in ORF 0 (pBSBW1.63, pBSBW1.46, and pBSBW1.6346) were constructed by PCR mutagenesis (Higuchi et al. 1988; Ho et al. 1989). The DNA template was pBW.KP for pBSBW1.63 and pBSBW1.46. The external primers for PCR were the universal primer (hybridizes upstream of the 35S promoter) and an oligonucleotide complementary to nucleotides 357 to 376 of BWYV. The internal mutagenic primers were designed to convert the ORF 0 initiation codon (nucleotides 32 to 34) to AUC in pBSBW1.63, or to introduce a 29-residue deletion (nucleotides 123 to 151 deleted) in pBSBW1.46. In the latter mutant, G_{153} was also converted to T to create a novel NheI site spanning the deletion site. For the double mutant pBSBW1.6346, the template DNA was pBSBW1.46 and the same mutagenic primers used to produce BSBW1.63 were employed.

The PCR fragments bearing the various mutations were treated with KpnI (cuts upstream of the 35S promoter) and XhoI (BWYV position 255) and substituted for the wild-type KpnI-XhoI fragment in the intermediate vector pBW.KPΔB. A KpnI-SpeI fragment (containing the 35S promoter and nucleotides 1 to 1350 of the BWYV sequence) from the intermediate vector bearing each of the ORF 0 mutations was then substituted for the corresponding wild-type fragment in pBSBW$_0$ to generate pBSBW1.63, pBSBW1.46, and pBSBW1.6346. The same set of KpnI-SpeI fragments was used to introduce the ORF 0 mutations into pBinBW$_0$ to produce pBinBW1.63, pBinBW1.46, and pBinBW1.6346.

Manipulation of DNA followed conventional techniques (Sambrook et al. 1989). All viral sequences generated by PCR were totally sequenced and the final structures of full-length mutant constructs were verified by restriction enzyme analysis.

Protoplast infection and agro-infection of plants.

Protoplasts of *C. quinoa* were prepared and inoculated with BWYV RNA transcripts as described (Veidt et al. 1992; Reutenauer et al. 1993) except that a pulse of 180 V rather than 300 V was used during electroporation. DNA inoculations were carried out in a similar manner except that the protoplasts were preincubated 30 min at 0° with plasmid DNA (30 μg per 200,000 protoplasts) followed by electroporation with a 300 V pulse. Procedures for agro-inoculation of *N. clevelandii* were as described (Leiser et al. 1992; Brault et al. 1995) except that *A. tumefaciens* strain C58C1 (Koncz and Schell 1986) instead of strain LBA4404 was used for experiments with the ORF 0 mutants. Extraction of protein and RNA from protoplasts and leaves and their analysis by Western blot or Northern blot followed published procedures (Veidt et al. 1992; Reutenauer et al. 1993; Brault et al. 1995). For Northern blots of RNA extracted from plants, 10 μg of RNA (as determined by spectrophotometry) was loaded per lane. Following electro-transfer, equal loading and transfer was confirmed by visualization of ethidium bromide–stained ribosomal RNAs on the nitrocellulose filter (Sambrook et al. 1989). The RNA probe used to detect viral RNA on Northern blots was complementary to the 3′-terminal 185 residues of the viral RNA (Reutenauer et al. 1993). ELISA measurements of virus content in leaves were carried out on 0.2-ml aliquots of tissue homogenate (0.2 g of tissue homogenized in 1 ml of buffer) as described (Brault et al. 1995) except that in some cases the homogenate was further diluted 10-fold prior to carrying out the analysis.

The stability of mutations in progeny viral RNA following agro-infection or aphid transmission experiments was examined by amplifying a DNA fragment spanning the mutation site by RT-PCR (Brault et al. 1995). The PCR fragment was cloned into pBluescript with restriction sites built into the PCR primers and the insert sequence was determined for randomly selected clones.

Virus transmission.

Aphid transmission experiments (Brault et al. 1995) were carried out by allowing nymphs of *M. persicae* to feed on leaves of agro-infected plants for 24 h before transfer (generally eight aphids per plant) to healthy *Montia perfoliata*. Aphids were eliminated after 72 h and the presence of virus 3 to 4 weeks pi was assayed by ELISA. A similar protocol was applied for tests of virus transmission to other hosts.

ACKNOWLEDGMENTS

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