Effects of Mutations in the Beet Western Yellows Virus Readthrough Protein on Its Expression and Packaging and on Virus Accumulation, Symptoms, and Aphid Transmission


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Received December 3, 1996; returned to author for revisions December 23, 1996; accepted January 20, 1997

Virions of beet western yellows luteovirus contain a major capsid protein (P22.5) and a minor readthrough protein (P74), produced by translational readthrough of the major capsid protein sequence into the neighboring open reading frame, which encodes the readthrough domain (RTD). The RTD contains determinants required for efficient virus accumulation in agroinfected plants and for aphid transmission. The C-terminal halves of the RTD are not well conserved among luteoviruses but the N-terminal halves contain many conserved sequence motifs, including a proline-rich sequence separating the rest of the RTD from the sequence corresponding to the major coat protein. To map different biological functions to these regions, short in-frame deletions were introduced at different sites in the RTD and the mutant genomes were transmitted to protoplasts as transcripts and to Nicotiana clevelandii by agroinfection. Deletions in the nonconserved portion of the RTD did not block aphid transmission but had a moderate inhibitory effect on virus accumulation in plants and abolished symptoms. Deletion of the proline tract and the junction between the conserved and nonconserved regions inhibited readthrough protein accumulation in protoplasts by at least 10-fold. The mutants accumulated small amounts of virus in plants, did not induce symptoms, and were nontransmissible by aphids using agroinfected plants, extracts of infected protoplasts, or purified virus as a source of inoculum. Other deletions in the conserved portion of the RTD did not markedly diminish readthrough protein accumulation but abolished its incorporation into virions. These mutants accumulated to low levels in agroinfected plants and elicited symptoms, but could not be aphid-transmitted. A preliminary map has been produced mapping these functions to different parts of the RTD.

INTRODUCTION

Beet western yellows virus (BWYV), a member of luteovirus subgroup 2 (Habili and Symons, 1989), is, like other luteoviruses, phloem-limited and obligately transmitted by aphids in a circulative, nonpropagative manner (Miller et al., 1995; Mayo and Ziegler-Graff, 1996). The reactions between a luteovirus and an aphid vector during acquisition-transmission are complex. The virus, after ingestion by an aphid while feeding, must cross an epithelial cell barrier to enter the hemocoel from the digestive tract and then be taken up by epithelial cells of the accessory salivary glands (ASG) for secretion in saliva during subsequent feeding. Movement of virus into and out of the epithelial cells of the gut and the ASG occurs by receptor-mediated endocytosis and exocytosis (Gildow and Rochow, 1980; Gildow, 1982, 1987; Gildow and Gray, 1993). Interactions between the viral capsid and receptors displayed on the gut and ASG epithelial cell membranes of vector aphids are important in determining vector specificity, although the ability of the virus to penetrate the basal lamina surrounding the ASG is thought to be important as well (Gildow and Gray, 1993). Finally, interactions of the virus in the hemocoel with symbionin, a chaperonin secreted into the hemocoel by endosymbiotic bacteria of the aphid, may stabilize the virus in the hemolymph (Van den Heuvel et al., 1994).

Luteovirus particles are isometric and are composed of single-stranded viral genomic RNA plus two capsid proteins. The major capsid protein of BWYV is the 22.5-kilodalton (kDa) species (P22.5) encoded by ORF 3 (see Fig. 1 for a genetic map). The minor capsid protein is a readthrough (RT) protein (Bahner et al., 1990; Martin et al., 1990; Filichkin et al., 1994; Brault et al., 1995; Wang et al., 1995), derived from ORF 3 and the adjacent ORF 5 (Fig. 1) by translational readthrough of the ORF 3 stop codon (Veidt et al., 1988; Tacke et al., 1990; Bahner et al., 1990; Dinesh-Kumar et al., 1992). The part of the RT protein encoded by ORF 5 will be referred to as the readthrough domain (RTD). Full-length RT protein (P74) is readily detectable in protein extracts of BWV-infected tissue but, in preparations of purified virus, the RT protein exists as a C-terminally truncated form of about 54 kDa known as P74* (Brault et al., 1995). P74* analogs have been observed in purified virus and in crude extracts of plants and protoplasts infected with other luteoviruses.

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(Bahner et al., 1990; Martin et al., 1990; Filichkin et al., 1994; Wang et al., 1995), suggesting that cleavage of the RT protein is not due to accidental degradation but reflects a conserved, presumably biologically significant processing event.

We have shown previously that a BWYV mutant (BW6.4; see Fig. 1), in which the entire RTD had been eliminated by deletion and frameshift, could form virions but was incapable of being transmitted by Myzus persicae (Braault et al., 1995), indicating that the RTD harbors determinants which mediate circulation of the virus within the aphid. Elimination of the RTD also impaired accumulation of BWYV in Nicotiana clevelandii after agroinfection, possibly by interfering with long-distance movement of the virus (Braault et al., 1995; Ziegler-Graff et al., 1996). In this paper we have produced a set of BWYV mutants, mostly with short in-frame deletions at different locations in the RTD. The mutants have been used in protoplast and plant infection experiments to produce a preliminary map relating different regions of the RTD to different functions.

MATERIALS AND METHODS

Construction of mutants

The transcription vector BW0 and agroinfection vector pBnBW0 containing the wild-type BWYV full-length cDNA insert have already been described (Veidt et al., 1992; Brault et al., 1995). Mutant BW6.51 contains a novel NheI site at nucleotides 4095–4100 followed by a deletion which eliminates nucleotides 4101–4157 (Fig. 1). An intermediate in the production of BW6.51 was BW6.4852, into which an Nhel site was introduced at residues 4095–4500 and an Xbal site at residues 4299–4304 by oligodeoxyribonucleotide-directed mutagenesis (Kunkel et al., 1987). The Xbal site was created by a single base substitution (C4302 replaced by A) which did not modify the amino acid sequence but creation of the Nhel site altered the amino acids at positions +2 and +3 with respect to the P22.5 termination codon (Fig. 1). BW6.4852 DNA was used as a template in the polymerase chain reaction (PCR) with primer 51 (nucleotides 4158–4179 plus a nonviral Nhel site at its 5’ end) and primer 48 (complementary to nucleotides 4290–4313) to produce a DNA fragment spanning nucleotides 4158–4313. The PCR product was cleaved with Nhel and Xbal and the resulting fragment was substituted for the Nhel–Xbal fragment of BW6.4852 to yield BW6.51.

Mutants BW6.106 and BW6.104 were constructed by PCR mutagenesis (Higuchi et al., 1988; Ho et al., 1989) using BW0 as template. The external primers for PCR were 83 (nucleotides 3914–3933) and 21 (complementary to nucleotides 4828–4846). For BW6.106, the internal mutagenic primers (106 and 107) were designed to delete nucleotides 4252 to 4269 and change A4270 to T to create a novel XhoI site spanning the deletion. The deletion in BW6.104 was created using primers 104 and 105 and eliminated nucleotides 4423 to 4467. For each mutant, the PCR fragment bearing the deletion was cut with BamHI (cuts at nucleotide 4006) and MscI (nucleotide 4544) and substituted for the wild-type BamHI–MscI fragment in BW0.

Mutant BW6.ΔMT (nucleotides 4547–4591 deleted) was made by cutting BW0 with MscI (nucleotide 4544) and Thnl111 (nucleotide 4588) and recircularizing the DNA after filling in recessed extremities by using the Klenow fragment of DNA polymerase I. Mutant BW6.ΔTB (nucleotides 4593–4708 deleted) was constructed on a BW0 BamHI–NcoI subclone (nucleotides 4006–4822) which was digested with Thnl111 and BsiWI (nucleotide 4708). Recessed extremities were filled in using the Klenow fragment and the DNA was circularized in the presence of an octameric SmaI linker. Insertion of the linker results in the introduction of the amino acid sequence ARA at the site of the deletion. The full-length clone was reconstructed by substituting the BamHI–NcoI fragment bearing the mutation for the wild-type sequence in BW0.

For mutant BW6.50 (nucleotides 4723–4821 deleted), a novel Xbal site was first created at residues 4595–4600 of BW0 by oligodeoxyribonucleotide-directed mutagenesis to produce BW6.49. The single base change required to create the Xbal site (G4598 replaced by A) does not modify the amino acid sequence of the RTD. A PCR fragment spanning nucleotides 4703–4497 was produced with primer 50 (complementary to nucleotides 4703–4722 and containing a nonviral NcoI site at its 5’ end) and primer 54 (nucleotides 4480–4497), with BW6.49 DNA as template. The PCR product was cut with Xbal and NcoI and the resulting fragment was used to replace the Xbal–NcoI fragment of BW6.49 to produce BW6.50.

Mutant BW6.ΔE1 was constructed by PCR mutagenesis using as external primers oligonucleotides 95 (nucleotides 4818–4834) and 94 (complementary to nucleotides 5360–5376). The mutagenic primers 100 and 101 were designed to replace the sequence between nucleotides 4996 and 5005 with the sequence 5’-GGCGTCTACTAGGCCC-3’, which creates novel EagI, Xbal, and NcoI restriction sites while retaining the reading frame. The final large PCR fragment was cut with EagI and NcoI to produce a DNA fragment extending from the viral NcoI site (nucleotide 4822) to the novel EagI site. After purification, this fragment was used to replace the original NcoI–EagI fragment (nucleotides 4823–5253) in BW0 to produce mutant BW6.ΔE1 (deletion of nucleotides 4996–5250). Mutant BW6.ΔMHD was created by PCR mutagenesis using the external primers 95 and 94. The pair of mutagenic primers 92 and 93 introduced seven point mutations between residues 5187–5200 (5’-CCCGGATATTCCAA converted to GCCGGCGTGCGCG). The full-length mutant was reconstructed by cutting the final PCR product bearing the mutations with NcoI and HindIII and
using this fragment to replace the wild-type NcoI–HindIII fragment in BW0.

For mutant BW6.40 (nucleotides 4983–5366 deleted), a DNA fragment extending from nucleotide 4772 to nucleotide 4982 was produced by PCR using BW0 DNA as template and oligodeoxynucleotides 24 (nucleotides 4772–4789) and 40 (complementary to nucleotides 4963–4982) as primers. Primer 40 had a nonviral HindIII site built in at its 5’-terminus. The PCR fragment was cut with NcoI (nucleotide 4822) and HindIII and inserted into BW0 in place of the wild-type NcoI–HindIII fragment. Mutant BW6.41 (nucleotides 5130–5366 deleted) was created in a similar fashion except that the PCR fragment was produced using primers 24 and 41 (complementary to nucleotides 5110–5129). Escherichia coli DH5α was used in all transformations and manipulation of recombinant DNA employed conventional procedures (Sambrook et al., 1989). All stretches of DNA in the final constructs that were produced by PCR amplifications were completely sequenced.

Mutant constructs for agroinfection (pBinBW6.51, pBinBW6.106, pBinBW6.104, etc.) were made by replacing the SpeI–SalI fragment (extends from nucleotides 1350 to a point 32 nucleotides downstream of the insert 3’-terminus) of the wild-type construct pBinBW0 by the SpeI–SalI fragment from the corresponding mutant transcription vector. The resulting plasmids were introduced into Agrobacterium tumefaciens strain LBA4404 for agroinfection (Brault et al., 1995).

Infection of protoplasts and plants

Mesophyll protoplasts of Chenopodium quinoa were inoculated with viral transcript RNA as described (Reutenauer et al., 1993) except that a pulse of 180 V was used during electroporation. Agroinoculation of N. clevelandii was as described (Brault et al., 1995). Virus antigen in agroinfected plants was measured by DAS–ELISA using a rabbit polyclonal antiseraum raised against virus with absorbance measured at 405 nm (Brault et al., 1995). A0.5 readings were made at 30 min, 1 hr, and 2 hr after starting the colorimetric reaction and a plant was judged to be infected when the value measured at 2 hr was greater than the threshold value, defined as the mean absorbance observed for healthy plant extracts plus three times the standard deviation of the healthy plant values (Leiser et al., 1992). Virus purified (Van den Heuvel et al., 1991) from agroinfected plants was stored at −80°C in C buffer (0.1 M sodium citrate, pH 6.0) containing 25% (w/v) sucrose.

Detection of viral RNA and capsid proteins

BWYV RNA in RNA extracts of infected plants and protoplasts was detected by Northern blot using a 32P-labeled RNA probe complementary to the 3’-terminal 196 nucleotides of the viral RNA (Reutenauer et al., 1993).

Proteins extracted from plants and protoplasts were separated by electrophoresis in SDS–polyacrylamide gels (SDS–PAGE; Laemmli, 1970) as described (Brault et al., 1995) except that urea was omitted from the gel-loading buffer. Following electrottransfer to nitrocellulose, viral capsid proteins were immunodetected using an enhanced chemiluminescence kit (Amersham) and following the supplier's instructions. P22.5 was immunodetected using an antiseraum raised against virions but this antiseraum produced unacceptably high background reactions with host proteins in the region of the blot where wild-type RT protein and its deleted variants were located. Consequently, RT protein was immunodetected by cutting the nitrocellulose membrane in two following electrottransfer and reacting the upper part of the membrane with an antiseraum specific for the RTD (Reutenauer et al., 1993). Because of this procedure, the relative intensities of the signals for RT protein and P22.5 in a given lane do not reflect their relative abundance in the sample, although "horizontal" comparisons between the intensities of the P22.5 bands and the RT protein bands in different lanes from the same experiment are possible.

Aphid transmission assays

Nonviruliferous M. persicae nymphs reared on healthy pepper (Capsicum annuum) were used in all transmission experiments. The aphids were given a 24-hr acquisition access period (AAP) on one of three virus sources: (i) detached leaves from agroinfected N. clevelandii, (ii) crude extracts of infected C. quinoa protoplasts, or (iii) purified virus. The crude protoplast extract was obtained by harvesting 2 × 10^5 protoplasts 65 hr postinoculation (p.i.) by centrifugation at 1000 g for 3 min. The pellet was resuspended in 50 μl C buffer and sonicated for 20 sec using a Vibra-Cell (Bioblock Scientific) equipped with a 3-mm probe at power setting 10. The suspension was then supplemented with 150 μl artificial diet MP148 (Harrewijn, 1983). Aphids were allowed to feed on the protoplast extract or on solutions of purified virus (at dilutions of 5–50 μg/ml in MP148) across a Parafilm membrane (Van den Heuvel et al., 1991) for 24 hr. Eight to 30 aphids were then transferred per healthy Monia perfoliata test plant for a 4-day inoculation access period (IAP). Finally, the aphids were killed with an insecticide spray (4 ml/liter mevinphos; Agrishell) and the test plants were assayed for virus infection by ELISA 3–4 weeks later. The absence of contaminating wild-type virus in the test plants following successful aphid transmission of the RTD domain mutants was confirmed by agarose gel electrophoresis of DNA fragments produced by PCR following reverse transcription of the viral RNA (RT-PCR) with primer pairs flanking the deleted region.

For aphid microinjection (Sylveste, 1988), calibrated 12 to 15-μm (outer diameter) glass capillaries were used to introduce purified virus (25 μg/ml in C buffer) into the
hemocoel of aphids (10 or 20 nl per aphid) using an Inject+ Ma.ic microinjector (Gabay Instruments, Geneva, Switzerland) and a pantograph M K1 micromanipulator (Singer Instruments, Somerset, England). Five microinjected aphids were transferred per healthy test plant and, after a 4-day IAP and elimination of the aphids as described above, the plants were tested for virus infection by ELISA 3–4 weeks later.

RESULTS
Mutations in the RTD

Sequence comparisons among different luteoviruses have revealed extensive sequence homology within the part of the RTD proximal to the P22.5 sequence (Guilley et al., 1995; Mayo and Ziegler-Graff, 1996). A few nucleotides downstream of the suppressible termination codon of P22.5, the RTD of BWYV, and other luteoviruses contain a C-rich sequence which encodes a succession of alternating proline residues with the general formula (XP)\textsubscript{15–16}, where X is often P, S, or T and the number of repeats is 8 for BWYV (Fig. 1B). This alternating proline tract, which has also been referred to as the proline hinge (Guilley et al., 1994), is followed by a region of about 210 amino acids which displays a high degree of homology among all luteoviruses (Fig. 1A). The C-terminal half of the RTD is not well conserved, although it contains a short region of homology, the "M. persicae homology domain," encoded by nucleotides 5139–5273 in BWYV (Fig. 1A)], unique to viruses which are efficiently transmitted by this vector (Guilley et al., 1994). The site of cleavage to produce the C-terminally truncated form of the RT protein (P74*) is not known but, based on its apparent Mr, is predicted to lie near the boundary between the conserved and nonconserved portions of the RTD.

The structures of the RTD deletion mutants studied in this paper are shown in Fig. 1A. In BW6.51, the 57-nucleotide deletion eliminated the proline tract plus the 3 amino acids immediately downstream (Fig. 1B). Note that the strategy used to produce the construct has also altered the sequence at positions +5 to +9 with respect to the P22.5 amber termination codon (Fig. 1B). In BW6.106 the sequence RFRYIED (residues conserved in all sequenced luteoviruses underlined) was replaced by a single leucine residue. The deletion in BW6.104 eliminated 15 residues including the conserved sequence GLIAY. The deletions in BW6.ΔMT and BW6.ΔTB eliminated, respectively, 15 amino acids (including 3 absolutely conserved residues) and 33 amino acids (including 9 absolutely conserved residues). The deletion in mutant BW6.50 eliminated 33 residues (including 6 conserved residues) spanning the junction between the conserved and nonconserved regions. Mutants BW6.ΔE1, BW6.Δ40, and BW6.41 contained overlapping deletions of 85, 128, and 79 amino acid residues, respectively, in the nonconserved region. Note that all three of these deletions eliminated all or part of the M. persicae homology domain (Fig. 1A). Finally, in mutant BW6.MHD, 4 amino acid residues in a stretch of 5 conserved residues in the M. persicae homology domain were replaced by alanines (PGYSK to AGAAA; Fig. 1A).

Infection of protoplasts with the RTD mutants

We have shown previously that deletion of the entire RTD did not inhibit viral RNA replication in transcript-inoculated C. quinoa protoplasts and that the progeny viral RNA could be assembled into virions (Reutenauer et al., 1993). C. quinoa protoplasts electroporated with transcripts containing the aforementioned RTD mutations also accumulated progeny viral RNA to levels similar to that observed in protoplasts inoculated with wild-type transcript (data not shown). Proteins were extracted from the infected protoplasts and tested for the presence of P22.5 by Western blot using a virus-specific antisera. Protoplasts infected with the RTD mutants were found to accumulate P22.5 to levels approximately similar to those observed in protoplasts infected with wild-type transcript (Fig. 2).

Western blot analysis revealed that certain RTD mutations had significant effects on accumulation of RT protein. Thus, RT protein in protoplasts infected with mutants BW6.51 (Fig. 2A, lane 2) and BW6.50 (Fig. 2A, lane 5) was below the level of detection unless long exposure times were used in conjunction with detection by enhanced chemiluminescence (ECL; Fig. 2B, lanes 2 and 3). Protoplasts infected with BW6.ΔMT (Fig. 2A, lane 3) and BW6.ΔTB (Fig. 2A, lane 4) reproducibly accumulated about half as much RT protein as observed in parallel infections with wild-type transcript (Fig. 2A, lane 8). No significant and reproducible inhibition of RT protein accumulation relative to wild-type levels was observed for the other mutants (Fig. 2).

The apparent Mr of wild-type RT protein (86 kDa), based on mobility during SDS–PAGE, is higher than predicted on the basis of the sequence (Reutenauer et al., 1993). The mutant RT proteins also had anomalous electrophoretic mobilities. For example, the BW6.ΔE1 and BW6.Δ40 RT proteins had apparent Ms of 81 and 71 kDa (64 and 60 kDa predicted) and the RT proteins of BW6.51, BW6.106, BW6.104, BW6.ΔMT and BW6.ΔTB, and BW6.50 had mobilities close to that of wild-type RT protein even though they were 1–9 kDa shorter (Fig. 2). Note that the deletions in BW6.ΔE1 and BW6.41 are of about the same extent (Fig. 1) but the resulting RT proteins have significantly different mobilities (Fig. 3, lanes 1 and 3), suggesting that the features presumably responsible for the anomalous electrophoretic behavior of the RT protein are not distributed uniformly along the polypeptide chain.

Accumulation of virus in agroinfected plants

For agroinfection experiments, the cDNAs containing the various RT domain mutations were placed under the
transcriptional control of the cauliflower mosaic virus 35S promoter in the binary vector Bin19 and *A. tumefaciens* harboring the agroinfection constructs were inoculated to midveins of *N. clevelandii* (Leiser et al., 1992). Plants agroinfected with the wild-type construct BW0 and BW6.MHD were slightly stunted and began to display typical interveinal yellowing symptoms 4–5 weeks p.i. Plants infected with the agroinfection constructs corresponding to mutants BW6.106, BW6.104, BW6.ΔMT, and BW6.ΔTB developed leaf symptoms 6–7 weeks p.i. but no stunting. The plants infected with the other mutants (BW6.51, BW6.50, and the mutants containing deletions...
FIG. 2. Detection of BWYV capsid proteins in extracts of C. quinoa protoplasts infected with BWYV transcripts. Total SDS-soluble protein was prepared from 10,000 protoplasts infected with the transcript indicated at the top of each lane. The proteins were fractionated by SDS-PAGE in a 10% polyacrylamide gel and transferred to nitrocellulose. The blot was cut in two perpendicular to the direction of migration and P74-related products (RT) in the upper section of the blot were immunodetected by ECL with an antiserum raised against a fusion protein containing the RTD sequence (Reutenauer et al., 1993). In the lower part of the blot, P22.5 (CP) was immunodetected by ECL with an antiserum raised against virions. Band intensities can be compared horizontally between lanes of a given blot but not vertically between the upper and lower sections of a blot. The sample in lane 1 of each panel is a mock-inoculated control. The images in B, lanes 1–3, are the same as in A, lanes 1, 2, and 5, but with a longer exposure time to permit detection of the RT protein produced by mutants BW6.50 and BW6.51. C comes from a separate experiment. The arrowhead to the left of A and the right of C indicates a background band due to cross-reaction of the secondary antibody in the immunodetection kit with a host protein. The positions of molecular weight markers (M, in kDa) are indicated between B and C.

in the nonconserved region) grew normally and did not develop leaf symptoms, even at later times p.i.

Virus antigen was measured by ELISA on leaf samples from each plant at various times p.i. Values measured at 5 weeks p.i. are shown in Table 1. Note that, for infections with both the wild-type and the mutant constructs, there was considerable variation in the A_{405} values from plant to plant as indicated by the large standard deviations associated with the measurements. This variability may reflect differences in the amount of inoculum originally delivered by agroinfection and/or differences in the rate of spread of the virus within the plant from the initial site(s) of infection.

We have reported previously that deletion of the entire RTD (mutant BW6.4; Brault et al., 1995) dramatically reduced but did not completely abolish virus accumulation

TABLE 1

<table>
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<tr>
<th>Construct</th>
<th>Mean absorbance</th>
<th>N</th>
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<tbody>
<tr>
<td>pBinBW6.4</td>
<td>0.259 ± 0.102</td>
<td>29</td>
</tr>
<tr>
<td>pBinBW6.51</td>
<td>0.232 ± 0.094</td>
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</tr>
<tr>
<td>pBinBW6.106</td>
<td>0.236 ± 0.049</td>
<td>19</td>
</tr>
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<td>pBinBW6.104</td>
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<tr>
<td>pBinBW6.ΔMT</td>
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</tr>
<tr>
<td>pBinBW6.ΔTB</td>
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</tr>
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<td>pBinBW6.50</td>
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</tr>
<tr>
<td>pBinBW6.ΔE1</td>
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<tr>
<td>pBinBW6.41</td>
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</tr>
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<td>pBinBW6.40</td>
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</tr>
<tr>
<td>pBinBW6.MHD</td>
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<td>126</td>
</tr>
<tr>
<td>Uninfected</td>
<td>0.120 ± 0.018</td>
<td>55</td>
</tr>
</tbody>
</table>

* Plants were tested about 5 weeks after inoculation with the indicated construct. Only values from plants judged to be infected as described in the text were included in the analysis.

# Mean absorbance ± standard error at 405 nm after 30 min of substrate incubation.

| Number of plants.

FIG. 3. Detection of BWYV capsid proteins in leaves of N. clevelandii agroinfected with BWYV RTD mutants. Leaf samples were taken 5 weeks after agroinfection with the construct indicated at the top of each lane and total SDS-soluble proteins corresponding to 10 mg of leaf were fractionated by SDS-PAGE in a 10% polyacrylamide gel. Immunodetection of RT protein (RT) in the upper part of the blot and P22.5 (CP) in the lower part of the blot were as described in the legend to Fig. 2. The arrowhead to the left indicates a background band due to cross-reaction of the secondary antibody in the immunodetection kit with a host protein. The positions of molecular weight markers (M, in kDa) are indicated to the right.
in agroinfected plants. The mutants containing short deletions in the conserved portion of the RTD (BW6.51, BW6.106, BW6.104, BW6.ΔMT, BW6.ΔTB, and BW6.50) behaved in a similar fashion, with small but measurable amounts of virus antigen present. ELISA values in such plants were at least 10-fold lower (net) than in plants agroinfected with the wild-type construct BW0 (Table 1; ELISA values for plants agroinfected with BW6.4 are also shown for comparison). Virus antigen titers remained low for the mutants when samples were taken at later times p.i. (data not shown).

Plants agroinfected with the mutants BW6.ΔE1, BW6.40, BW6.41, and BW6.MHD accumulated higher levels of virus antigen than did the mutants bearing modifications in the conserved portion of the RTD (Table 1). ELISA measurements made upon dilutions of the crude extracts in several experiments indicated that the virus antigen titer in plants agroinfected with BW6.ΔE1, BW6.40, and BW6.41 was two to three times lower than for plants agroinfected in parallel with BW0 (data not shown).

Both P22.5 and RT protein were readily detected by Western blot analysis of total proteins extracted from plants agroinfected with the variable domain mutants BW6.ΔE1, BW6.40, BW6.41, and BW6.MHD (Fig. 3). The low virus titers in plants agroinfected with the conserved region mutants hampered detection of RT protein by Western blot but a faint infection-specific band of the expected size could be detected in plants agroinfected with BW6.106, BW6.104, BW6.ΔMT, and BW6.ΔTB (data not shown). A RT protein band could not be reproducibly detected in plants agroinfected with BW6.50 and BW6.51.

Virus was also purified from plants agroinfected with the various RTD mutants and the protein contents were examined by Western blot. Mutants BW6.ΔE1 (Fig. 4A, lane 6), BW6.MHD (Fig. 4A, lane 7), BW6.40 (Fig. 4B, lane 2), and BW6.41 (Fig. 4B, lane 3) contained, in addition to P22.5, the C-terminally truncated RT protein P74*, which was also present in comparable levels in the wild-type virus (Fig. 4A, lane 1; Fig. 4B, lane 1).

Low levels of P74* were detectable in the BW6.50 virus (Fig. 4B, lane 4) but no full-length or truncated RT protein was visible in the virus of mutants BW6.106, BW6.104, BW6.ΔMT, or BW6.ΔTB (Fig. 4A, lanes 2 – 5) or BW6.51 (Fig. 4B, lane 5).

Aphid transmission tests using agroinfected plants as virus source

Young fully expanded leaves of agroinfected N. clevelandii plants (4–6 weeks p.i.) were used as a source of wild-type and mutant virus in aphid transmission tests. Nonviruliferous M. persicae nymphs were allowed a 24-hr AAP on the leaves before transfer to healthy M. persiculata, using either "standard" inoculum pressure (8 nymphs per test plant) or "high" inoculum pressure (30 nymphs per test plant) for a 4-day IAP. Three to 4 weeks later, viral infection of the test plants was assayed by ELISA. For the conserved subdomain mutants BW6.51, BW6.106, BW6.104, BW6.ΔMT, BW6.ΔTB, and BW6.50, no transmission was observed with either standard or high inoculum pressure (Table 2). For the variable subdomain mutants BW6.ΔE1, BW6.40, and BW6.41, no transmission was observed with standard inoculum pressure (Table 2, experiments 1 – 3), but some transmission events were observed at the high inoculum pressure (Table 2, experiments 4 – 7). Transmission of mutant BW6.MHD occurred with standard inoculum pressure (Table 2, experiment 3).

Transmission tests using protoplast extracts and purified virions

A drawback of the above transmission experiments is that the virus contents of plants agroinfected with many of the RTD mutants, particularly those with deletions in the conserved region, were much lower than in plants...
Aphid Transmission of BWYV Mutants from Agro-Infected Plants

<table>
<thead>
<tr>
<th>Construct</th>
<th>Experimenta</th>
<th>8 nymphs/test plant</th>
<th>30 nymphs/test plant</th>
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<td>pBinBW6.51</td>
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<td>pBinBW6.104</td>
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<tr>
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<td>0/6</td>
<td>0/5</td>
</tr>
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<td>0/6</td>
<td>0/5</td>
</tr>
<tr>
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<td>0/8</td>
<td>0/5</td>
</tr>
<tr>
<td>pBinBW6.MHD</td>
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<td>6/10</td>
<td>0/5</td>
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<td>pBinBW0</td>
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</tbody>
</table>

*a After a 24-hr acquisition access period (AAP), eight *M. persicae* nymphs (experiments 1–3) or 30 nymphs (experiments 4–8) were transferred to *M. perfoliata* for an inoculation access period (IAP) of 4 days. Virus detection by ELISA was performed 3–4 weeks later. Results of transmission are presented as number of infected plants/number of plants tested.

b The virus source was *N. clevelandii* leaves 4–6 weeks after agro-infection with the indicated construct.

## DISCUSSION

Earlier work has established that the luteovirus RTD is important for aphid transmission of the virus (Jolly and Mayo, 1994; Brault et al., 1995; Wang et al., 1995; Chay et al., 1996) and influences virus accumulation levels in plants (Brault et al., 1995; Chay et al., 1996). In this paper,
TABLE 3

Aphid Transmission of BWYV Mutants from Extracts of Infected Protoplasts

<table>
<thead>
<tr>
<th>Construct</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>8</th>
<th>Total</th>
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<tbody>
<tr>
<td>BW6.51</td>
<td>12</td>
<td>10-15</td>
<td>15-20</td>
<td>20</td>
<td>20</td>
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<td>30</td>
<td>30</td>
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<td>0/5</td>
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<td>0/18</td>
<td>0/9</td>
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<td>0/8</td>
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</tr>
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<td>0/4</td>
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<td>9/9</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BW6.MHD</td>
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<td>9/9</td>
<td>3/3</td>
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<td>11/16</td>
<td>9/9</td>
<td>3/3</td>
<td>2/5</td>
<td>3/5</td>
<td>10/13</td>
<td>2/6</td>
</tr>
</tbody>
</table>

* After a 24-hr AAP, nymphs were transferred to Montia perfoliata (experiments 1 – 3 and 6 – 9), to Nicotiana clevelandii (experiment 4), and to Physalis floridana (experiment 5) for an IAP of 4 days and the presence of virus was assayed by ELISA 3 – 4 weeks later. In experiments 3 – 8, the extracts from the protoplasts infected with the mutants were twice as concentrated as in the corresponding BW0 transcript-infected controls. The number of aphids per test plant is indicated. Results of transmission are presented as number of infected plants/number of plants tested.

b The virus source was an extract of transcript-infected protoplasts sandwiched between Parafilm membranes.

effects of mutations in different parts of the BWYV RTD on these properties as well as on the accumulation of RT protein, its incorporation into virions, and symptom expression in agroinfected plants. The results are summarized in Figs. 5 and 6.

An important finding reported here is that mutations in the conserved and nonconserved portions of the RTD differ dramatically in their effect on the protein content of virions. The virions produced by the mutants targeting the nonconserved region contained wild-type relative amounts of P22.5 and RT protein (P74*), but the mutants in which sequences from the conserved...

TABLE 4

Aphid Transmission of BWYV Mutants Supplied as Purified Virus

<table>
<thead>
<tr>
<th>Virus concentration (µg/ml)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aphids per plant</td>
<td>5</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>50</td>
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</tbody>
</table>

* After a 24-hr AAP, the indicated number of aphids was transferred to M. perfoliata (experiments 1 – 3 and 6 – 9) or to Physalis floridana (experiments 3 and 5) for a 4-day IAP and the presence of virus was tested by ELISA 3 – 4 weeks later. Results of transmission are presented as number of infected plants/number of plants tested.

b Virus was purified from plants agro-infected with the indicated construct. Purified virus was diluted to the indicated concentration in artificial diet MP148 and sandwiched between Parafilm membranes.

c Virus concentration of BW6.ΔMT was 170 µg/ml.
region (including the proline tract) had been eliminated produced virions containing little or no RT protein. The mutants which package little (in the case of BW6.50) or no detectable RT protein into virions fall into two categories: (i) those which accumulated only low amounts of RT protein during infection and (ii) those in which RT protein was produced but was not incor- porated into virions. Members of the first category are the proline tract deletion mutant (BW6.51) and mutant BW6.50. Brown et al. (1996) have recently shown that the C-rich sequence encoding the proline tract of the BYDV-PAV forms part of the signal governing the efficiency of suppression of the coat protein termination codon. Elimination of the corresponding sequence in BW6.51 may be at least in part responsible for the observed low levels of accumulation of RT protein with this mutant. Furthermore, the BW6.51 mutation has also altered the last two bases of the triplet at the +2 position following the suppressible stop codon (Fig. 1). This triplet forms part of the highly conserved context surrounding the stop codon (Miller et al., 1995) and its modification in BW6.51 may contribute to the decreased efficiency of readthrough, although modification of the corresponding bases in BYDV-PAV had no such effect (Brown et al., 1996).

Deletion of an ~60-nucleotide sequence about 700 residues downstream of the coat protein termination co-don also reduced readthrough at least 10-fold in BYDV-PAV (Brown et al., 1996). The deletion in BW6.50 removed the corresponding region of the BWYV RTD and a similar long-distance effect on readthrough may be responsible for the reduced accumulation of RT protein observed in BW6.50-infected protoplasts. Preliminary cell-free translation experiments with P22.5-RTD transcripts containing the BW6.50 and BW6.51 mutations have indicated that the inhibition of RT protein expression provoked by these deletions in vivo also occurs in vitro (J. Mutterer, personal communication). It is not yet known if the ~50% lower level of RT protein accumulation observed for BW6.50MT and BW6.50TB is due to somewhat less efficient termina-tion codon suppression or to diminished stability of the observed low levels of accumulation of RT protein with this mutant. Furthermore, the BW6.51 mutation has also altered the last two bases of the triplet at the +2 position following the suppressible stop codon (Fig. 1). This triplet forms part of the highly conserved context surrounding the stop codon (Miller et al., 1995) and its modification in BW6.51 may contribute to the decreased efficiency of readthrough, although modification of the corresponding bases in BYDV-PAV had no such effect (Brown et al., 1996).

The second class of mutants in the conserved portion of the RTD (BW6.106, BW6.104, BW6.1MT, and BW6.1TB) accumulated plentiful amounts of RT protein in infected protoplasts (Fig. 2) but did not detectably incorporate it into virions (Fig. 4). Packaging of the mutant RT protein could be inhibited by aberrant folding of the mutated RT protein or by its sequestration, perhaps into aggregates. As noted above, elimination of the entire RTD of BWYV (mutant BW6.4) has been shown to markedly diminish virus accumulation levels in planta (Brault et al., 1995), leading to the hypothesis that the RTD contains se-quences which promote transport and/or stability of the virus in the sieve elements (Brault et al., 1995; ZieglerGraff et al., 1996). The fact that BW6.106, BW6.104, BW6.1MT, and BW6.1TB accumulated virus poorly in planta even though RT protein synthesis is not greatly impaired represents circumstantial evidence that the RT protein must be incorporated into virions in order to exert an effect on virus accumulation levels. Evidently, the ab-
sence (or near absence in the case of mutant BW6.50) of RT protein from virions can also account for the failure of the conserved region mutants to be aphid-transmitted, although our findings do not rule out the possibility that some or all of the deleted sequences may be directly implicated in virus-vector interactions. It will be interesting to determine if RTD mutants can be discovered in which the effect of RT protein incorporation into virions is decoupled from effects on virus accumulation in planta and/or aphid transmission.

In contrast to the mutants in the conserved region, the mutants in the nonconserved region (BW6.ΔE1, BW6.40, BW6.41, and BW6.MHD) incorporated normal or near normal amounts of RT protein into virions. The RT protein detected in the virus preparations was exclusively the truncated form, P74*, indicating that extensive sequences in the nonconserved region are dispensable for the cleavage process. A similar finding has been reported for the RT protein of BYDV-PAV (Filichkin et al., 1994).

Although careful comparisons have not yet been carried out, BW6.MHD appears to accumulate virus in agroinfected plants and to be aphid transmitted in a manner similar to the wild-type virus. Thus, the significance, if any, of the conserved sequence motif targeted by this mutant remains to be discovered. The other mutants in the nonconserved region (BW6.ΔE1, BW6.40, and BW6.41), on the other hand, were poorly transmitted from agroinfected leaves, although they were readily transmissible when supplied to aphids as purified virus or as extracts of infected protoplasts. The poor transmission of these mutants from leaves may in part be related to the two- to threefold lower concentration of virus in the leaves compared to wild-type. We believe it unlikely, however, that this concentration difference is sufficient to account entirely for the difference in transmissibility. Another factor that could influence transmission is the distribution of virus in different compartments of the agroinfected plant. If, as suggested above, the RT protein mediates transport and/or stability of the virus in the sieve elements, alterations in the nonconserved portion of the RTD might possibly diminish the amount of virus in the sieve elements available for uptake by the aphid. Evidently, delivery of mutant virus to aphids in the form of an extract of infected protoplasts, as purified virus, or by microinjection would overcome such a compartmentalization effect.

The manner in which BWYV elicits symptoms in hosts is not understood, but the absence of symptoms following agroinfection of N. clevelandii with mutants BW6.ΔE1, BW6.40, and BW6.41 (large segments of the nonconserved region deleted) and mutants BW6.50 and 6.51 (in which accumulation of RT protein relative to P22.5 is inhibited) suggests that as yet unidentified sequences within the nonconserved portion of the RTD must be present in the plants for symptoms to appear. The M. persicae homology domain itself is apparently not involved in symptomatology because BW6.MHD, where 4 of 13 conserved residues in this region have been altered, produced symptoms like the wild-type. Finally, it should be noted that viral symptoms were observed on plants agroinfected with the conserved region mutants BW6.106, BW6.104, BW6.ΔMT, and BW6.ΔTB, even though virus titers in these plants were very low. This represents further evidence that it is the appearance of a particular viral polypeptide sequence in the infected plant rather than high concentrations of virus per se which elicits symptoms.

In conclusion, this paper confirms and extends our earlier finding (Brault et al., 1995) that the BWYV RTD is multifunctional (Fig. 6). Mutations in different parts of the RTD have been shown to have distinct effects on aphid transmission, accumulation of virus within plants, and symptomatology. Furthermore, the RTD has been shown to contain sequences which contribute in an indirect fashion to the formation of functional virions by affecting (i) the synthesis of RT protein and (ii) its incorporation into virions. Evidently, the association of multiple, potentially interacting functions with the RTD will make the high-resolution genetic analysis of this region a challenging endeavor.

ACKNOWLEDGMENTS

The authors thank O. Lemaire (INRA, Colmar) and H. Smith (IACR, Broom's Barn) for gifts of virus-specific antisera. This research was financed in part by NATO Collaborative Research Grant 950521.

REFERENCES


