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Aphid transmission of beet western yellows luteovirus requires the minor capsid read-through protein P74

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Beet western yellows luteovirus is obligately transmitted by the aphid *Myzus persicae* in a circulative, non-propagative fashion. Virus movement across the epithelial cells of the digestive tube into the hemocoel and from the hemocoel into the accessory salivary glands is believed to occur by receptor-mediated endocytosis and exocytosis. Virions contain two types of protein; the major 22 kDa capsid protein and the minor read-through protein, P74, which is composed of the major capsid protein fused by translational read-through to a long C-terminal extension called the read-through domain. Beet western yellows virus carrying various mutations in the read-through domain was tested for its ability to be transmitted to test plants by aphids fed on agro-infected plants and semi-purified or purified virus preparations. The results establish that the read-through domain carries determinants that are essential for aphid transmission. The findings also reveal that the read-through domain is important for accumulation of the virus in agro-infected plants.

Key words: aphid transmission/beet western yellows virus/luteovirus/read-through protein

Introduction

Beet western yellows virus (BWYV) is a member of the luteovirus group, a group of plant viruses with monopartite plus-sense RNA genomes of ~5.6 kb encapsidated in small isometric particles (for reviews see Martin *et al.*, 1990; Mayo and Ziegler-Graff, 1995; Miller *et al.*, 1995). Luteoviruses are limited to phloem tissues of their hosts. Based on genetic organization (Habili and Symons, 1989; Martin *et al.*, 1990), the luteoviruses have been divided into two subgroups: subgroup 1, including the PAV and MAV isolates of barley yellow dwarf virus (BYDV), and subgroup 2, which includes BWYV, potato leafroll virus (PLRV), the RPV isolate of BYDV and others. The principal features distinguishing the two subgroups are associated with the 5′-proximal half of the genome. These include an extra 5′-proximal open reading frame (ORF) present only in the subgroup 2 viruses and different evolutionary affinities for the 'core' polymerase gene. On the other hand, a cluster of three genes near the 3′-terminus of the genome RNA are similar in all members of both subgroups. These genes, which are expressed from a subgenomic RNA (Smith and Harris, 1990; Tacke *et al.*, 1990; Miller and Mayo, 1991; Dinesh-Kumar *et al.*, 1992; Veidt *et al.*, 1992), include that encoding the ~22 kDa major capsid protein (ORF 4), an ORF for a 17–19 kDa protein which is embedded in the coat protein ORF in another reading frame and a long ORF (ORF 6) immediately downstream of and in the same reading frame as the coat protein cistron (Figure 1).

A distinctive feature of luteoviruses is their mode of transmission. Luteoviruses are obligately transmitted in a specific manner by one or a few species of aphid, *Myzus persicae* being the most efficient vector in the case of BWYV (Duffus, 1977). Transmission is non-propagative (no virus multiplication in the vector) and circulative (Rochow and Duffus, 1981; Gildow, 1987, 1991). Virus particles ingested during feeding pass through the epithelial cell lining of the digestive tube into the hemocoel ('acquisition') and diffuse through the hemolymph until they encounter the accessory salivary glands (ASG). There, the virions are transported into cells of the ASG, from which they are excreted into hosts during subsequent feeds (Gildow, 1991). In the case of BYDV, the hemocoel–ASG barrier has been shown to be the major locus of transmission selectivity (Gildow and Rochow, 1980; Gildow and Gray, 1993), although some discrimination, including that between luteoviruses and non-luteoviruses, is also believed to occur at the hindgut–hemocoel interface (Gildow, 1993).

The vector specificity of luteovirus transmission suggests that specific cellular receptors in the aphid interact with determinants on the capsid. The major component of the luteovirus capsid is the ~22 kDa polypeptide encoded by ORF 4. Some early studies (Rochow and Duffus, 1981; Murant *et al.*, 1985) reported the presence of a minor, higher M_r capsid component, but its origin and significance were uncertain. However, more recent work with both subgroup 1 and subgroup 2 luteoviruses has revealed that ORF 6 (Figure 1) is expressed as an ORF 4–ORF 6 fusion protein by occasional events of translational suppression of the coat protein amber termination codon (Veidt *et al.*, 1988; Bahner *et al.*, 1990; Dinesh-Kumar *et al.*, 1992; Reutenauer *et al.*, 1993). The resulting ~74 kDa 'read-through' protein (P74) has the capsid protein sequence at its N-terminus and the sequence of the ~52 kDa read-through domain (RTD), derived from ORF 6, at its C-terminus. A truncated form of the equivalent read-through fusion protein (apparently produced by loss of C-terminal sequences from the full-

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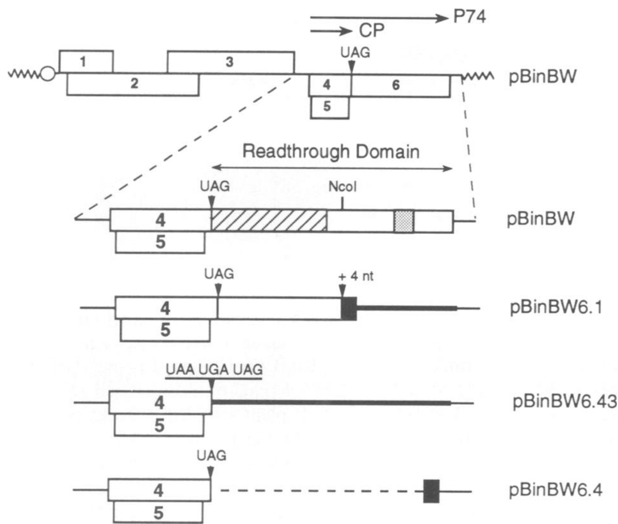

Fig. 1. Genome organization of BWYV and structure of the 3′-proximal gene cluster in the wild-type (pBinBW) and read-through domain (RTD) mutant (pBinBW6.1, pBinBW6.43 and pBinBW6.4) agro-infection vectors. The 22 kDa major capsid protein (CP) encoded by ORF 4 and P74, the ORF 4–ORF 6 fusion protein, are represented by arrows. The portion of the RTD with strong sequence similarities among all luteoviruses is hatched and the region in the RTD in which luteoviruses transmitted by *M.persicae* display similarity is shaded. Black rectangles represent mutation-induced missense amino acid sequences and the dashed line indicates the deletion in pBinBW6.4. Wavy lines flanking the BWYV insert in the upper portion of the figure represent vector sequences and the 35S promoter is represented by a circle.

length species during virus purification) has been detected in semi-purified preparations of PLRV and BYDV (Bahner *et al.*, 1990; Martin *et al.*, 1990). Sequence comparisons between highly aphid transmissible and poorly aphid transmissible isolates of PLRV have identified several amino acid substitutions in the RTD which might be associated with the changes in transmissibility (Jolly and Mayo, 1994).

The foregoing observations, although not conclusive, are consistent with a role for the luteovirus RTD, when displayed on virus particles, in virus–vector interactions. We have shown previously that BWYV transcripts carrying mutations truncating or eliminating the RTD are infectious in protoplasts and produce virions (Reutenauer *et al.*, 1993). In this paper we have tested the ability of BWYV with such mutations to multiply in whole plants and to be transmitted by *M.persicae*. Our findings demonstrate conclusively that the RTD is required for aphid transmission of the virus, but also indicate that it has at least one other role as well; in particular, the RTD is required for the sustained accumulation of virus during infection of *Nicotiana clevelandii*.

Results

Mutations in the read-through domain

Three RTD mutants were used in this study (Figure 1). In mutant pBW6.1 (Veidt *et al.*, 1992), a frame-shift was introduced near the mid-point of the RTD by filling in an *Nco*I site at nucleotide 4822 of ORF 6. In mutant pBW6.4, the entire RTD was deleted (Reutenauer *et al.*, 1993). It has been shown previously that full-length transcripts produced by *in vitro* transcription of linearized pBW6.1 and pBW6.4 are replication-competent in *Chenopodium quinoa* protoplasts and produce viral proteins and progeny RNA in amounts comparable to those found in protoplasts infected with wild-type transcript (Veidt *et al.*, 1992; Reutenauer *et al.*, 1993; unpublished observations). The mutant progeny RNA is encapsidated in virions which appear similar to wild-type virions by immunosorbent electron microscopy (ISEM) (Reutenauer *et al.*, 1993). In the third mutant used in this study (pBW6.43), the leaky UAG codon separating the coat protein cistron from the RTD was converted to UAA and reinforced by creation of two additional stop codons immediately downstream (Figure 1). A +1 frame-shift was introduced during creation of the two extra stop codons. Transcripts of pBW6.43 were highly infectious in protoplasts (data not shown).

Effect of mutations in the read-through domain on infection of plants

Luteovirus particles and RNA cannot be transmitted by mechanical inoculation to whole plants. This obstacle has recently been overcome by use of agro-infection (also referred to as agro-inoculation), a procedure which takes advantage of the ability of *Agrobacterium tumefaciens* harboring a Ti plasmid to efficiently transfer to plant cells a T-DNA segment containing the viral genome in a form permitting it to escape and autoreplicate (Grimsley *et al.*, 1986; Leiser *et al.*, 1992). Full-length BWYV cDNA was inserted into a T-DNA binary cassette (pBin19) between a 35S transcription promoter and a NOS transcription termination signal so as to produce a viral RNA transcript which can be translated to produce viral RNA-dependent RNA replicase and then be replicated (Leiser *et al.*, 1992). In our original work there was concern that the 3′-non-viral extension of the primary transcript, derived from the NOS termination sequence, might interfere with the ability of the transcript to be recognized by the viral replication machinery. Therefore, a construct was produced with a ribozyme sequence immediately after the 3′-terminus of the cDNA in order to generate a molecule resembling authentic viral RNA as closely as possible (one extra nucleotide at the 3′-end). The efficiency of the ribozyme-containing construct in agro-infection experiments proved to be similar to that of a construct from which the ribozyme was omitted and, in both cases, the non-viral downstream sequences on the primary transcripts were eliminated during replication *in planta* (Leiser *et al.*, 1992). We have subsequently found that a third construct (pBinBW), which has neither the NOS terminator nor the ribozyme sequence at the 3′-terminus of the cDNA, is fully as infectious as either of the above (data not shown). pBinBW was used as starting material for the binary plasmid constructs (designated pBinBW6.1, pBinBW6.4 and pBinBW6.43) containing the RTD mutations described above.

The BWYV RTD mutants were agro-inoculated into *N.clevelandii* and the course of infection was followed over a period of 11 weeks by measuring the level of viral coat protein in randomly selected leaves of the plants by ELISA. The success rate of agro-infection with the mutants was generally in the range 30–50% of the inoculated plants, a rate which is somewhat lower than that obtained with the wild-type pBinBW, in which ~75% of the agro-

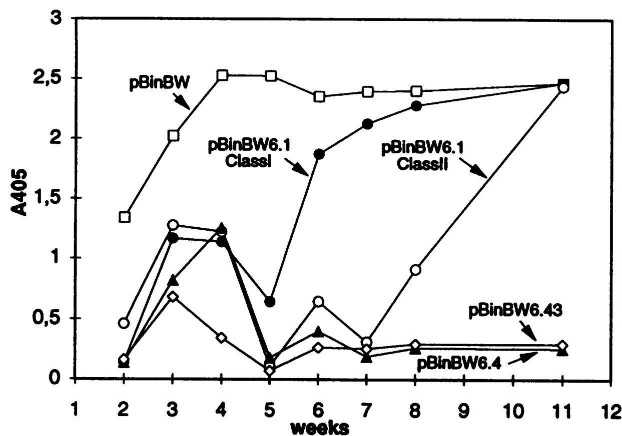

Fig. 2. Time course of appearance of BWYV coat protein as measured by ELISA of agro-infected *N.clevelandii*. For each time point, three randomly selected leaf samples from each plant were pooled before extraction and ELISA measurement and the values were averaged for the wild-type (pBinBW) and for each mutant. Only plants which gave an ELISA value above the threshold for infection (Leiser *et al.*, 1992) at 3 weeks post-agro-inoculation were considered to be successfully agro-infected and were included in the calculations (five plants for pBinBW, ten plants for pBinBW6.1, three plants for pBinBW6.43 and seven plants for pBinBW6.4). The plants agro-infected with pBinBW6.1 were further divided into those for which the ELISA measurement dipped for 1 week after week 4 before rising (Class I) and those for which the dip lasted ≥2 weeks (Class II). For each time point, the average ELISA background (0.12–0.14) obtained for four healthy plants tested in parallel has been subtracted.

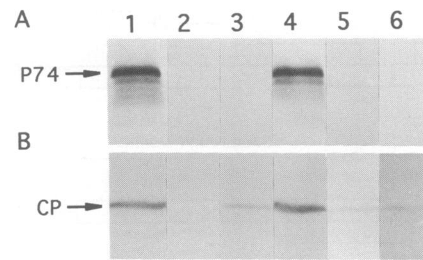

Fig. 3. Western blot analysis of BWYV capsid proteins in agro-infected plants. Protein samples were taken 4 weeks after agro-infection with pBinBW (lane 1), pBinBW6.1 (Class I plant) (lane 3), pBinBW6.43 (lane 5) and pBinBW6.4 (lane 6). Also shown are samples from a pBinBW6.1 (Class I) plant sampled 8 weeks post-agro-inoculation (lane 4) and a healthy plant (lane 2). After electrophoresis in a 10% polyacrylamide gel and electrotransfer to nitrocellulose, P74 was detected by developing the upper portion of the blot (**A**) with an anti-RTD serum (Reutenauer *et al.*, 1993) and the 22 kDa major capsid protein (CP) was detected by developing the lower portion of the blot (**B**) with anti-BWYV IgGs.

inoculated plants routinely became infected. In the positive plants inoculated with the RTD mutants, coat protein accumulated during the first 3–4 weeks, although to a lower level than in plants agro-infected with pBinBW (Figure 2). Thereafter, the coat protein titer in the plants agro-infected with mutants pBinBW6.4 and pBinBW6.43 progressively declined to near background levels (defined by ELISA measurements on healthy plants), while the levels attained in plants agro-infected with pBinBW remained high. Possible explanations for the fall in coat protein levels in plants agro-infected with the mutant will be discussed below.

The behavior of plants agro-inoculated with pBinBW6.1 was more complex. In some plants (Class I in Figure 2), there was a 1 week dip in coat protein titer after week 4, followed by a sustained climb up to high levels. Class II plants displayed similar bimodal behavior, but the dip in coat protein titer following week 4 was more pronounced and of longer duration (Figure 2).

Symptoms (interveinal leaf yellowing) on the agro-infected plants began to appear 3–4 weeks after inoculation with pBinBW and intensified thereafter. Plants agro-infected with pBinBW6.1 also developed symptoms, but with a time delay correlating with the second stage increase of coat protein titer in the plants. None of the plants agro-infected with pBinBW6.4 or pBinBW6.43 developed symptoms during the period of the experiment. *In situ* hybridization experiments with ^{33}P-labeled antisense viral RNA probes revealed that the location of virus in sections across the midribs and petioles of infected leaves was similar in plants agro-infected with pBinBW, pBinBW6.1 and pBinBW6.4, with the label associated exclusively with the phloem vasculature (data not shown).

Analysis of the progeny

The appearance of progeny viral RNA during infection of plants with the RTD mutants was investigated by Northern blot using a BWYV-specific ^{32}P-labeled riboprobe. Analysis of total plant RNA extracted 4 weeks post-agro-infection with pBinBW, pBinBW6.4, pBinBW6.43 or pBinBW6.1 showed the presence of genomic RNA of the expected size (data not shown). The 22 kDa major capsid protein (24 kDa apparent M_r) was also detected by Western blot analysis of total protein extracts from the infected plants (Figure 3B), although the level of coat protein in the plants infected with the mutants was significantly lower than in the wild-type control. No product in the crude extracts from the mutant agro-inoculated plants sampled 4 weeks post-agro-inoculation reacted positively with an antiserum directed against the RTD (Figure 3A, lanes 3, 5 and 6) and, in particular, the frame-shift-truncated P74, which was detected in protoplasts infected with pBW6.1 transcripts (see below), could not be detected in plants agro-inoculated with this mutant (Figure 3A, lane 3). This failure is probably due to the low level of coat protein produced in such plants, as this would be expected to result in synthesis of correspondingly lower amounts of the truncated P74.

Western blot analysis was also performed on protein extracted from a Class I plant agro-infected with pBinBW6.1 but sampled 8 weeks post-agro-inoculation, when the coat protein titer had risen to near wild-type levels. The blot revealed abundant amounts of coat protein (Figure 3B, lane 4), but no truncated P74. However, a prominent immunolabeled band was present which co-migrated with full-length P74 (86 kDa apparent M_r) found in plants agro-infected with pBinBW (Figure 3A, lane 4). Similar results were obtained for a pBinBW6.1-infected Class II plant sampled 11 weeks post-agro-inoculation (data not shown). As will be shown below, the appearance of full-length P74 in such plants is associated with pseudo-reversion. Western blot analysis of pBinBW6.4- and pBinBW6.43-agro-infected plants 8 weeks post-infection revealed very low amounts of capsid protein, but no detectable P74 (data not shown).

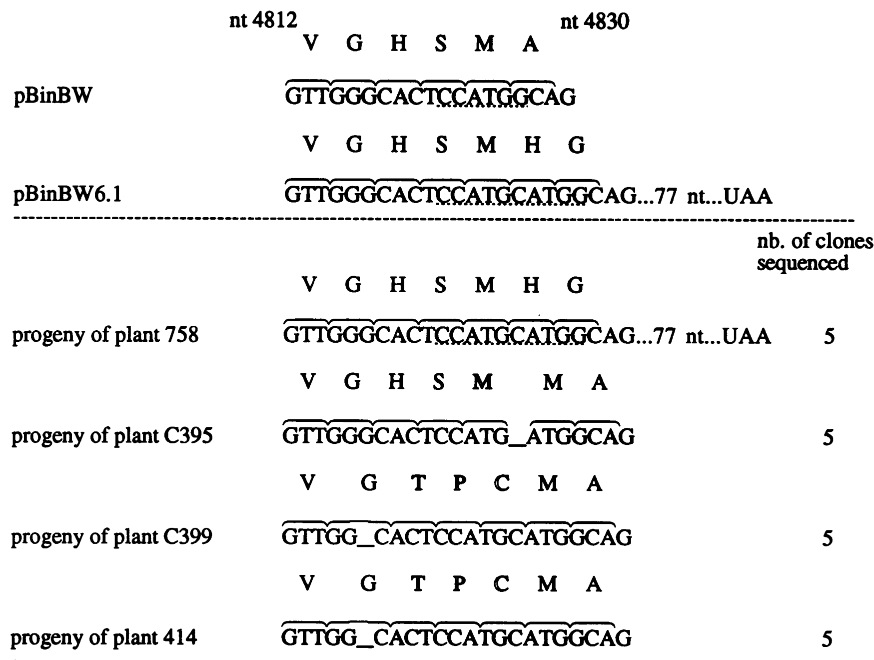

Fig. 4. Sequence analysis of viral progeny from plants agro-infected with pBinBW6.1. The nucleotide sequence and the corresponding amino acid sequence in the vicinity of the RTD *Nco*I site of the wild-type and pBinBW6.1 (*Nco*I site filled-in) are shown above. The lower portion of the figure shows the sequence of the corresponding region from cloned RT-PCR products obtained from the progeny of infection with pBinBW6.1 extracted 4 weeks (plant 758), 11 weeks (plants C395 and C399) and 8 weeks (plant 414) post-agro-infection. Five separate clones from a single RT-PCR amplification were sequenced for each plant. Amino acids which differ from the wild-type sequence because of the second-site mutation are in outlined text. Underlined spaces indicate deleted residues.

Pseudo-revertants of pBinBW6.1

The nature of the progeny of agro-infection with pBinBW6.1 was further analyzed by cDNA synthesis followed by PCR amplification of genomic segments containing the mutation (RT-PCR). It will be recalled that the RTD of pBinBW6.1 was disrupted by filling in the *Nco*I site at position 4822 of the wild-type sequence (Figure 1). RT-PCR products encompassing this region (nt 4480–4982) were prepared from the RNA of five different pBinBW6.1-infected Class I plants sampled 8 weeks post-agro-inoculation and tested for the presence of this site by digestion with *Nco*I. As expected, the PCR fragments were not cleaved by *Nco*I, although the product of RT-PCR of RNA from a pBinBW-agro-inoculated plant was readily digested (data not shown). This finding rules out contamination with wild-type virus as a possible source of the P74.

To detect possible second-site mutations in the RTD, the RT-PCR products from several of the pBinBW6.1-agro-infected plants were cloned and sequenced (Figure 4). In five clones obtained from RNA extracted 4 weeks post-agro-inoculation from a Class I plant (plant 758, no P74 detectable), the filled-in *Nco*I site characteristic of the mutation was still present and no other sequence modifications from the wild-type were noted. On the other hand, the cloned RT-PCR products from plants 414 (Class I, sampled 8 weeks post-agro-inoculation), C395 and C399 (Class II, sampled 11 weeks post-agro-inoculation) contained single base deletions near the filled-in *Nco*I site (Figure 4). The deletions restore the reading frame and can account for the appearance of a species migrating like full-length P74 in these plants. It should be noted, however, that the second-site mutations modify several amino acids in the vicinity of the *Nco*I site (Figure 4), so that the resulting products are not strictly identical to wild-type P74.

Transmissibility of the P74 mutants from agro-infected N.clevelandii

Infected leaves of *N.clevelandii* agro-inoculated with the RTD mutants pBinBW6.1, pBinBW6.4 or pBinBW6.43 were used as virus source in aphid transmission tests. Non-viruliferous *M.persicae* nymphs (first instar for experiments 1, 5 and 6, fourth instar for the others) were allowed to feed on leaves of the agro-infected plants (3–4 weeks post-inoculation) for 1 day and eight nymphs were then transferred to each healthy test plant (*Montia perfoliata* or *Physalis floridana*) for 4 days. Virus infection of the test plants was assayed by ELISA 3 weeks later. Under these experimental conditions, 13 of 22 test plants were infected when the source plants had been agro-infected with pBinBW, but no transmission was observed from the source plants agro-infected with pBinBW6.1, pBinBW6.4 or pBinBW6.43 (Table I, experiments 1 and 2).

In an effort to increase the chances of transmitting the mutant viruses, the number of aphids per test plant was increased from 8 to 16–100. Under these conditions transmission from the plants agro-infected with pBinBW6.43 or pBinBW6.4 remained unsuccessful (Table I, experiments 3–6), but one transmission event was observed with pBinBW6.1 when the test plant was loaded with 100 aphids (Table I, experiment 6). Symptom development on this test plant was delayed by ~2 weeks compared with plants infected with the wild-type virus. Western blot analysis of protein extracted from the plant using the antiserum specific for the RTD revealed the

Table I. Aphid transmission of BWYV mutants from agro-infected plants

Experiment	1	2	3	4	5	6	7
pBinBW6.1	0/37				0/3 (50)	1/2 (100)	13/13
pBinBW6.43		0/6	0/9 (17–27)	0/2 (16–20)			
pBinBW6.4	0/43		0/4 (20–27)		0/3 (50)	0/2 (100)	
pBinBW	12/21	1/1	1/1 (31)	2/2 (37–47)	3/3 (50)	2/2 (100)	6/6

Experiments 1–6 were conducted 3–4 weeks after agro-inoculation and experiment 7, 11 weeks post-inoculation. After an acquisition access period of 1 day, eight nymphs of *M.persicae* (experiments 1, 2 and 7) or 16 to 100 nymphs (experiments 3–6) were transferred on *M.perfoliata* or *P.floridana* for an inoculation access period of 4 days. The numbers in brackets (experiments 3–6) indicate the range of the number of nymphs used in these experiments. Virus detection by ELISA was performed 3–4 weeks later. Results of transmission are presented as number of infected plants/ total number of plants tested.

presence of a species with the same mobility as wild-type P74. To eliminate the possibility that this transmission event was due to the presence in the source plant or in the aphid population of contaminating wild-type virus, RNA was isolated from the test plant and the viral sequence surrounding the *Nco*I site was amplified by the RT-PCR procedure as described above. The resulting fragment could not be cleaved by *Nco*I, indicating that the virus does not possess the wild-type sequence (*Nco*I site present) in this region. Thus we cannot exclude the possibility that the transmission event was due to the presence in the source plant of small amounts of pseudo-revertant which had regained the ability to synthesize full-length P74.

Aphid transmission experiments were also conducted 11 weeks after agro-inoculation using the pBinBW6.1-infected plants C395 and C399, in which pseudo-revertants were abundant (Figure 4). The transmission efficiency from these source plants was very high (Table I, experiment 7). Analysis by RT-PCR of the sequence of the viral progeny of four test *M.perfoliata* (22 clones sequenced) in the vicinity of the altered *Nco*I site revealed the presence of revertants which carried the same mutations (single base deletions) found in the source plants (see Figure 4). This result indicates that the amino acid sequence of P74 in the vicinity of the *Nco*I site can be altered, at least to some extent, without affecting transmission.

Transmission from infected protoplasts

An evident drawback of using agro-infected plants as virus source in the above transmission experiments is that the amount of coat protein and presumably virus in the source plants infected with the RTD mutants is three to four times lower than in plants agro-infected with wild-type virus. It is unlikely that the lower virus concentration is sufficient by itself to account for the inability of the RTD mutants to be transmitted, particularly at high inoculum pressures. However, concentration effects could mask weak transmission by the RTD mutants. To circumvent this problem, transcript-infected protoplasts were used as the virus source in transmission experiments.

Chenopodium quinoa protoplasts were inoculated by electroporation with bacteriophage T7 RNA polymerase transcripts derived from pBW, pBW6.1 and pBW6.4 (Reutenauer, *et al.*, 1993). Three days post-inoculation, the protoplasts were disrupted by sonication and the viral capsid protein contents of a fraction of each extract were analyzed by Western blot. As shown in Figure 5B, lanes 2–4, similar amounts of 22 kDa coat protein were present

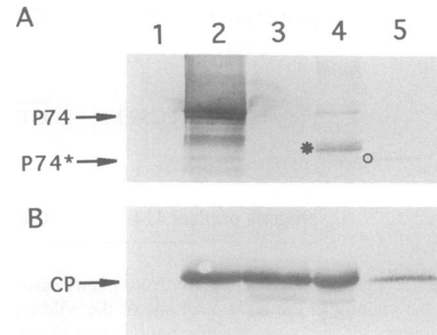

Fig. 5. Western blot analysis of BWYV capsid proteins in transcript-infected *C.quinoa* protoplasts. Protein was extracted from 50 000 protoplasts 72 h after mock-inoculation (lane 1) or inoculation with transcripts from pBW (lane 2), pBW6.4 (lane 3) or pBW6.1 (lane 4). Lane 5 contains 50 ng of purified BWYV. P74 and related products were detected in the upper portion of the blot (**A**) with the anti-RTD serum and the 22 kDa coat protein (CP) was detected in the lower portion of the blot (**B**) with anti-BWYV IgGs. The positions of full-length P74 and the C-terminally truncated species P74* found in purified virus (lane 5A, circle) are indicated on the left. The asterisk in lane 4A shows the ~60 kDa truncated read-through protein produced by a frame-shift in pBW6.1. Other details as in Figure 3.

in all samples, in agreement with our previous finding (Reutenauer *et al.*, 1993) that mutations in the RTD do not affect virus replication in protoplasts. In the protoplasts inoculated with pBW6.1 transcript, a product with an apparent M_r of 60 kDa was detected by the antiserum directed against the RTD (Figure 5A, lane 4). This product is presumably the truncated P74 species of pBW6.1 (predicted M_r 53 kDa). Note also that in addition to the 60 kDa species, a small amount of P74 was also detected in this experiment, presumably as a consequence of a low level of pseudo-reversion in the protoplasts.

We have shown previously (Reutenauer *et al.*, 1993) that virions from *C.quinoa* protoplasts infected with mutants pBW6.1 or pBW6.4 appeared as 'normal' isometric particles by ISEM. In an effort to visualize the presumed association between P74 and virions, virions from protoplasts infected with pBW, pBW6.1 and pBW6.4 transcripts were decorated with RTD-specific IgGs which had been conjugated to colloidal gold beads of 12 nm average diameter. About 20% of the virions from protoplasts infected with wild-type virus were tagged with the RTD-specific antiserum (Figure 6A), indicating that the RTD is accessible on the surface of at least a portion of the virions. Virions from the protoplasts infected with pBW6.1 displayed very low levels of labelling (~0.1% of the

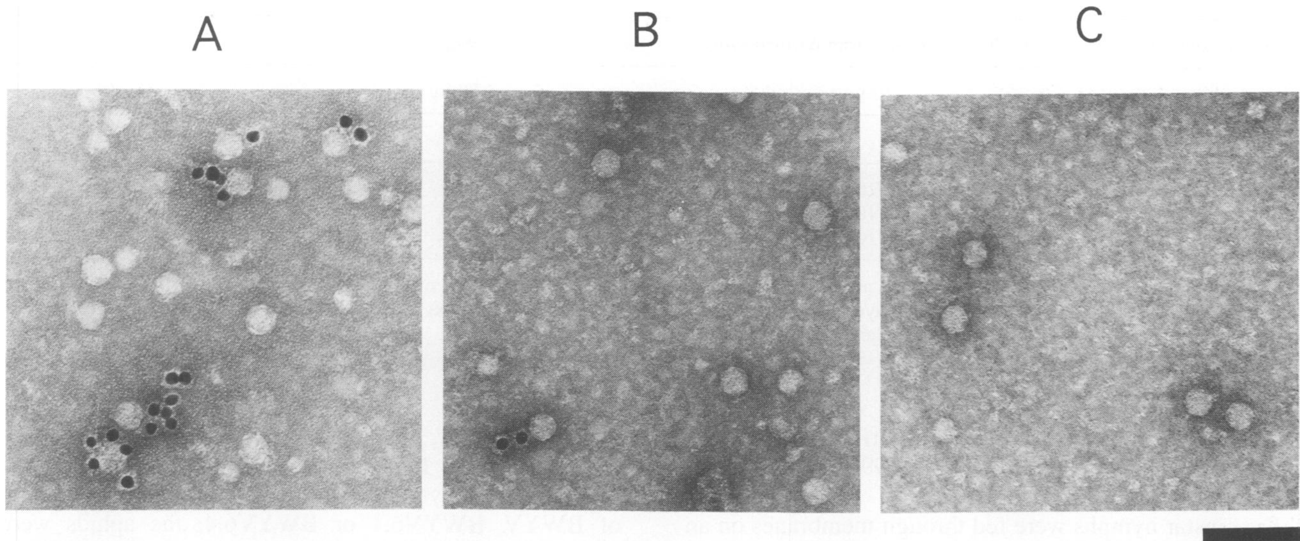

Fig. 6. Detection of the RTD in virions by immunogold labeling. Virions from protoplasts infected with transcripts of pBW (**A**), pBW6.1 (**B**) or pBW6.4 (**C**) decorated with anti-RTD IgGs conjugated to colloidal gold beads of 12 nm average diameter. The fields shown in (A) and (C) are typical. The field in (B) was selected to show one of the rare immunogold-labeled particles detected in the pBW6.1 preparation. The bar corresponds to 100 nm.

Table II. Aphid transmission of BWYV mutants from infected protoplasts

Transcripts used for electroporation of protoplasts	Aphids per plant	Infected plants/plants tested
pBW	10–12	5/6
pBW6.1	10–14	0/10
pBW6.4	10–15	0/5

Semi-purified virions were prepared from protoplasts as described in Materials and methods and fed to aphids through membranes for 30 h. 10 to 15 aphids were then transferred for 4 days on *P.floridana*. The presence of the virus was assessed 3 weeks later by ELISA.

particles; Figure 6B), while no labeling whatsoever was observed with the pBW6.4 virions (Figure 6C). The rare gold-labeled particles observed in the pBW6.1 virion preparation may be pseudo-revertants. Whatever the nature of the rare decorated particles, however, the low overall level of labeling obtained for BWYV6.1 may also indicate that the C-terminally truncated form of the RTD is less efficiently incorporated into virions than full-length P74 (see below) or that the truncated form is present in virions but is less rich in epitopes recognized by the anti-RTD IgGs than is the full-length form.

The virus in extracts of 200 000 infected protoplasts was concentrated by polyethylene glycol precipitation and fed to *M.persicae* nymphs (first instar) through parafilm membranes for 30 h. The aphids were then transferred to healthy *P.floridana* test plants for 4 days and the presence of virus in the test plants was assessed by ELISA 3 weeks later. No transmission was observed with the virus from the protoplasts infected with pBW6.4 or pBW6.1 (Table II), although the efficiency of transmission of virus from protoplasts infected with pBW was high (5/6). We conclude that the failure of the RTD mutants to be transmitted from agro-infected plants is not related to their low concentration in such plants, but is a consequence of a basic inability of the mutants to be acquired and/or delivered by the aphid vector.

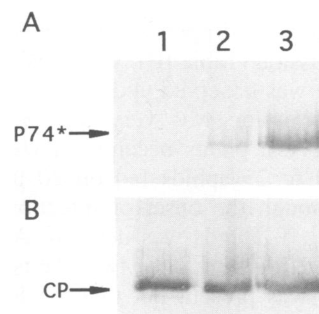

Fig. 7. Western blot analysis of proteins associated with virus purified from plants agro-infected with pBinBW6.4 (lane 1), pBinBW6.1 (lane 2) or pBinBW (lane 3). The truncated P74* species was detected in the upper portion of the blot (**A**) with the anti-RTD serum and the 22 kDa coat protein (CP) was detected in the lower portion of the blot (**B**) with anti-BWYV IgGs.

Transmission of purified virus

Additional evidence for intervention of the RTD in aphid transmission has come from membrane feeding experiments with purified virus. Virus was purified 3 weeks after agro-inoculation from plants infected with pBinBW, pBinBW6.1 or pBinBW6.4. The purified viruses will be referred to as BWYV, BWYV6.1 and BWYV6.4 respectively. Western blot analysis using the anti-RTD antiserum detected a truncated form of P74 (P74*) of ~54 kDa apparent M_r in purified BWYV and in smaller amounts in BWYV6.1 (Figure 7, lanes 3 and 2). P74* was immunolabeled by an antiserum raised against a portion of the coat protein sequence expressed as a fusion protein in bacteria (data not shown), indicating that the sequence loss has occured mostly, if not exclusively, from the C-terminus of the RTD. As noted in the Introduction, truncated CP–RTD fusion proteins of about this size have

Table III. Aphid transmission of BWYV mutants from purified virus

	10 μg virus/ml			20 μg virus/ml			
Nb of aphids per test plant	1	5	10	1	5	10	25
BWYV	1/10	3/5	4/4	3/10	5/5	5/5	ND
BWYV6.1	0/10	0/5	0/5	0/10	0/5	1[a]/5	1/1
BWYV6.4	0/10	0/5	0/5	0/10	0/4	0/3	ND

Aphids were fed through membranes for 30 h on artificial diet (MP148) containing 10 or 20 μg/ml of purified BWYV, BWYV6.1 or BWYV6.4. 1 to 25 aphids were transferred to *P.floridana* for 4 days. The presence of the virus was assessed 2 weeks later by ELISA. The results indicate the number of plants positive in ELISA/number of plants tested. ND, not determined.
[a]Plant positive in ELISA after 5 weeks.

also been detected in purified or semi-purified preparations of PLRV and BYDV (Bahner *et al.*, 1990; Martin *et al.*, 1990).

First instar nymphs were fed through membranes on an artificial diet containing purified virus at concentrations of 10 and 20 μg/ml. After a 30 h access period, different numbers of aphids were transferred to healthy *P.floridana* test plants for 4 days and transmission was assessed by ELISA on the test plants 3 weeks later. Starting with purified virus, even a single aphid was capable of transmitting wild-type BWYV to 4/20 test plants and using larger numbers of aphids (5–10) per test plant resulted in highly efficient transmission (Table III). With BWYV6.4 virions, no transmission was observed under any conditions. Efficiency of transmission was very low with BWYV6.1 virions, but two test plants became infected when challenged with 10 or 25 aphids fed on 20 μg/ml of virus (Table III), although the onset of infection of the plant challenged with 10 aphids was delayed. Analysis of the viral protein and RNA in these two plants revealed that full-length P74 was present but that the RNA sequence was not wild-type (*Nco*I site not present on PCR-amplified cDNA), indicating that transmission to these two plants was associated with the generation of pseudo-revertants.

We have shown previously that, in protoplasts, the packaged genome RNAs of the RTD mutants pBW6.1 and pBW6.4 are protected from the action of endogenous RNases released during extraction (Reutenauer *et al.*, 1993). To assess whether the mutant virions are stable under conditions prevailing in the aphid digestive tract, aphids were fed on purified virus solutions as described above and honeydew was collected for a period of 8 h. For each sample, virus from equivalent volumes of honeydew was immunotrapped on electron microscope grids and particles were counted in five randomly chosen fields. The concentration of particles in the honeydew of the aphids fed on the wild-type and mutant virions was the same (19 ± 2 virions/field for BWYV and BWYV6.4; 18 ± 2 virions/field for BWYV6.1), indicating that the non-transmissibility of the RTD mutants cannot be attributed to virus degradation in the digestive tube of the aphid.

Microinjection of virus into aphids

The foregoing experiments demonstrate that the RTD mutations interfere with the ability of the virus to complete the acquisition/transmission circuit within the aphid, but they do not identify the step or steps for which the mutants are defective. To test the importance of the RTD for steps subsequent to movement across the digestive tube–hemocoel barrier, we have microinjected purified virions directly into the hemolymph of non-viruliferous 6-day-old *M.persicae* nymphs. After microinjection with ~0.5 ng of BWYV, BWYV6.1 or BWYV6.4, the aphids were permitted to feed on *P.floridana* for 4 days (1 aphid/plant) and virus transmission was monitored by ELISA 3 weeks later. Wild-type BWYV was transmitted efficiently by this procedure (9/16 test plants infected), while BWYV6.4 was not transmitted (0/16). For mutant BWYV6.1, one of 20 test plants was infected. The infected plant showed very mild symptoms and was only weakly positive in ELISA. The progeny virus in this plant was not characterized but, in view of the fact that the virus preparation used in this experiment (the same as was used in the experiment shown in Table III) is known to harbor pseudo-revertants, it is possible that the virus detected in the infected test plant was a pseudo-revertant. We conclude that the RTD is important for virus stability in the hemolymph and/or for efficient movement of the virus from the hemocoel into the ASG.

Discussion

In this paper we have demonstrated that the RTD of BWYV contains determinants important for BWYV transmission by *M.persicae*. Much of our knowledge of luteovirus–vector interactions is based on work with BYDV and cereal aphids (for reviews see Gildow, 1987, 1991), but what evidence there is indicates that the main findings apply to other luteovirus–vector pairs as well. As noted in the Introduction, there are thought to be two critical steps in the transmission process; movement of virus from the digestive tube into the hemocoel and passage into and out of the ASG. Electron microscope observations indicate that BYDV uptake across the apical plasmalemma into the epithelial cell cytoplasm at the hindgut occurs at coated pits, suggestive of a receptor-mediated process, and that the virions are transported through the cytoplasm in coated vesicles and tubular structures (Gildow, 1993). Virions move into the hemocoel compartment by fusion of these transport vesicles with the basal plasmalemma. For PLRV, acquisition occurs by a similar process, but has been reported to take place at the intestine, rather than the hindgut, of *M.persicae* (Garret *et al.*, 1993). The site of acquisition of BWYV is not known.

Once within the hemocoel, the virus is thought to diffuse freely until it reaches the ASG, where a rather similar receptor-mediated process occurs, but in reverse

(Gildow 1982; Gildow and Gray, 1993). Virions first accumulate at the ASG basal lamina, which the virus must penetrate to reach the underlying basal plasmalemma. Both penetration of the ASG basal lamina and association with the basal plasmalemma appear to be vector-specific, at least in BYDV (Gildow and Gray, 1993). Cognate virions move into plasmalemmal invaginations, which form tubular structures that in turn bud off coated vesicles containing virions. These vesicles move within the cell until they fuse to the apical plasmalemma at sites where it composes the membrane of the microvilli of the salivary canal. The virions are liberated there and can enter the salivary duct for delivery to a new host during feeding.

Except for the fact that luteoviruses do not disassemble and replicate in their vectors, there are many points of similarity between the aforesaid process and the manner in which non-enveloped animal viruses enter and exit host cells. In particular, the association of luteoviruses with coated pit structures at the plasmalemma indicates that virus transport involves receptor-mediated endocytosis and exocytosis. Attachment of animal viruses to host cell membranes is mediated by the binding of a virus-encoded attachment protein (VAP) to specific cell surface receptor molecules, which may be either protein or carbohydrate (Lentz, 1990; Haywood, 1994). The specificity of the interaction between VAP and receptor is important in determining host range and tissue tropism. For a number of animal viruses it has been possible to identify a domain in the VAP with sequence similarity to the ligand which is the normal substrate for the target receptor (Lentz, 1990).

Our findings indicate that the RTD is the site of one or more 'VAP-like' domains governing luteovirus movement within its vector. The simplest model predicts the existence of two such domains, one governing movement from the digestive tract into the hemocoel and the other movement from the hemocoel into the ASG. Each VAP-like domain could map to a distinct portion of the RTD or could be composed of motifs scattered along the sequence and juxtaposed by three-dimensional folding. Sequence comparisons (Guilley *et al.*, 1994) between the RTDs of different luteoviruses provide some support for the former hypothesis. Motifs shared by all sequenced luteoviruses are located almost exclusively within the N-terminal half of the RTD (Figure 1). These sequences could participate in virus recognition at the hindgut or intestine epithelial cell basal plasmalemma. The C-terminal half of the RTD, on the other hand, is much less conserved, although a subdomain has been identified there (Figure 1) which is similar among luteoviruses transmitted by *M.persicae* (Guilley *et al.* 1994). Possibly, this domain may be important in conferring specificity to the transmission process. Finally, monoclonal antibodies specific for the major capsid protein of PLRV have been shown to block transmission when mixed with virions prior to their being fed to aphids (van den Heuvel *et al.*, 1993), suggesting that the major capsid protein also contains determinants involved in virus−vector interactions.

We have shown that preparations of purified wild-type BWYV contain a C-terminally truncated form of P74, referred to as P74*, apparently generated by proteolytic cleavage during the extraction procedure. As noted in the Introduction, a similar truncated read-through protein is also associated with purified or semi-purified PLRV and BYDV particles. In contrast to the situation with BWYV6.1, in which rare transmission events are apparently associated with pseudo-reversion, purified wild-type BWYV containing P74* can be readily transmitted when acquired orally or microinjected into the hemocoel. It remains to be determined if the transmission observed by us and others with purified luteovirus preparations results from the presence of trace amounts of non-truncated P74 or if P74* contains the necessary transmission signals. In the latter case, however, it is difficult to understand why BWYV6.1 is not likewise efficiently transmitted, unless its impaired transmissibility is due to a low level of incorporation of the truncated form of P74 into virions rather than lack of transmission signals *per se*. Transmission bioassays with additional RTD deletion mutants may help to resolve this question.

The inhibitory effect of the RTD mutations on the long-term accumulation of virus in agro-infected plants was unexpected in view of the fact that such mutations did not inhibit virus replication in protoplasts. However, the replication behavior of the mutants in mesophyll protoplasts of *C.quinoa* may not necessarily reflect their behavior in the cell types (sieve elements, vascular parenchyma and companion cells) in which they multiply in intact host plants (Esau and Hoefert, 1972; D'Arcy and de Zoeten, 1979). Indeed, it has been speculated that the impeded long distance movement of the masked strain of tobacco mosaic virus (TMV) might be caused by impaired replication of the virus in cells of the phloem compartment (Nelson *et al.*, 1993). A similar cautionary remark should be made concerning possible differences in virus stability in protoplasts and intact plants, although the RTD mutant and wild-type virions from agro-infected plants did not differ noticably in their behavior during virus purification.

Another possibility is that the RTD may play a role in virus movement in *N.clevelandii*. Invasion of a plant by a phloem-limited virus such as BWYV probably involves both cell−cell movement between cells of the phloem domain and long distance movement within the sieve elements. Little is known concerning the plasmodesmata between nucleate phloem cells, but the branched plasmodesmata joining phloem companion cells and sieve elements appear to have unusual properties (Kempers *et al.*, 1993; Lucas *et al.*, 1993). Thus, virus movement within the phloem compartment and, in particular, movement into and out of the sieve elements almost certainly involves different mechanisms than movement between mesophyll cells (Hull, 1989; Maule, 1991; Leisner and Turgeon, 1993).

Some viruses, notably TMV, require virion assembly for long distance movement, while others do not (Maule, 1991; Leisner and Turgeon, 1993). If BWYV belongs to the former category, it is easy to imagine that the RTD, present on the surface of virions, could intervene in long distance movement. In the study of the time course of appearance of BWYV after agro-infection shown in Figure 2, the ELISA measurement for each plant was made on a pooled extract of tissue collected from three randomly selected leaves of that plant. The samples taken at early times were largely drawn from leaves near the point of agro-inoculation (the petioles and midribs of leaves of young plants) but, at later times, senescence of these leaves and removal of tissue from them for the preceding

measurements often precluded their being further sampled. Consequently, later time samples were drawn in large part from newly formed, upper leaves of the plants. Evidently, failure of BWYV6.4 and BWYV6.43 to move efficiently into such leaves could produce curves for their accumulation such as those observed in Figure 2. Furthermore, long distance virus movement through phloem channels is believed to follow the flow of photoassimilates from source to sink leaves and sink–source relationships change during plant development (see Leisner et al., 1992, 1993). Relatively small changes in the rate of replication or movement in source leaves provoked by the RTD mutations could be amplified if they cause the rate of virus production in such leaves to lag behind the rate of plant development. Additional experiments will clearly be required to gain a better understanding of the behavior of the RTD mutants in plants following agro-infection.

Materials and methods

Plasmids

The wild-type transcription vector pBW (previously referred to as pBW_0) and the read-through domain mutants pBW6.1 and pBW6.4 have already been described (Veidt et al., 1992; Reutenauer et al., 1993). In pBW6.43, the coat protein UAG stop codon was changed to UAA and two additional stop codons and a +1 frame-shift were introduced immediately downstream by site-directed mutagenesis (Kunkel et al., 1987). Mutagenesis was performed on single-stranded phagemid DNA of BS(−) (Stratagene) containing a *Dra*I fragment (nt 3312–5565) encompassing the target sequence for mutation. The mutated sequence was introduced as an *Afl*III–*Hin*dIII fragment (nt 3339–5367) into pBW. The ribozyme and NOS sequence of pBW.RN (Leiser et al., 1992) were deleted by digesting the plasmid with *Xba*I and *Sal*I. Recessed ends were filled in with *Escherichia coli* DNA polymerase Klenow fragment and the DNA was recircularized with bacteriophage T4 DNA ligase. The large *Kpn*I–*Sal*I fragment from this plasmid was introduced into the binary vector pBin19 (Bevan, 1984) to produce pBinBW. The read-through domain mutations in pBW6.1, pBW6.4 and pBW6.43 were introduced into pBinBW on a *Spe*I-*Sal*I fragment (nt 1350–3′-end of insert) to produce pBinBW6.1, pBinBW6.4 and pBinBW6.43.

Protoplast inoculation and agro-infection

Chenopodium quinoa protoplasts were inoculated with transcripts by electroporation as described previously (Veidt et al., 1992). For agro-infection, recombinant binary vectors were introduced into *A.tumefaciens* LBA4404 (Hoekema et al., 1983) by electroporation (Mozo and Hooykaas, 1991). The bacteria were then agro-inoculated by injection into leaf midribs and petioles of *N.clevelandii* (Leiser et al., 1992).

Virus from protoplasts and agro-infected leaves

Semi-purified virus for aphid transmission bioassays was extracted from infected protoplasts 72 h post-infection by resuspending 200 000 protoplasts in 400 µl 0.1 M sodium citrate, pH 6.0, followed by sonication for 3 min at 4°C. Debris was eliminated by low speed centifugation and virions were precipitated with 10% polyethylene glycol for 1 h on ice. The precipitate was collected by centrifugation and taken up in 100 µl artificial diet MP148 (Harrewijn, 1983). Virus was also purified from 150–200 g *N.clevelandii* leaves 3 weeks post-agro-inoculation. The purification procedure was essentially as described (Van den Heuvel and Peters, 1990), except that the final step was a 20–50% sucrose gradient.

Analysis of progeny viral RNA and proteins

Virus infection following agro-inoculation and aphid transmission experiments was assessed by double antibody sandwich ELISA (Herrbach et al., 1991) using a BWYV-specific polyclonal antiserum. The appearance of viral RNA in protoplasts and agro-infected plants was monitored by Northern hybridization with a ^{32}P-labeled antisense RNA probe specific for the 3′-terminal region of the viral RNA (Veidt et al., 1992). For Western blots, 500 mg leaf tissue from agro-infected plants was ground in a morter with 500 µl 2× concentrated gel loading buffer (Laemmli, 1970) supplemented with 6 M urea. Samples were boiled for 15 min and insoluble material was eliminated by centrifugation for 5 min at 5000 *g*. The proteins were separated by SDS–PAGE in 10% polyacrylamide gels and viral coat protein and P74 were immunodetected (Niesbach-Klösgen et al., 1990) using purified BWYV-specific IgGs. Proteins containing the RTD were specifically immunodetected with a polyclonal antiserum raised against a recombinant fusion protein containing the N-terminal portion of the bacteriophage λ cI protein fused to the entire RTD (Reutenauer et al., 1993).

RT-PCR (Veidt et al., 1992) on RNA from plants agro-infected with pBinBW6.1 was carried out using an oligonucleotide complementary to nt 5394–5412 to prime cDNA synthesis. PCR amplification was performed using an oligonucleotide complementary to nt 4963–4982 (with a *Hin*dIII extension at its 5′-terminus) and an oligonucleotide corresponding to nt 4480–4497. The amplified DNA fragment was purified on an agarose gel and, after digestion with *Nco*I, the fragments were analyzed by electrophoresis in a 10% polyacrylamide gel. For sequence analysis, the PCR-amplified fragment was digested with *Hin*dIII and cloned into *Hin*dIII–*Sma*I-cut BS(−) (Stratagene). Unless otherwise noted, conventional recombinant DNA procedures (Sambrook et al., 1989) were employed.

Virus transmission bioassays

For virus transmission tests, nymphs of *M.persicae* were allowed to feed on leaves from agro-infected plants for 1 day before transfer to healthy *P.floridana* or *M.perfoliata* (Leiser et al., 1992). Membrane feeding experiments were carried out as described by van den Heuvel et al. (1991) using the virions from protoplast extracts or purified virus (10–20 µg/ml) from agro-infected plants suspended in artificial diet MP148.

Characterization of virions in honeydew by ISEM

Honeydew excreted by 2 day-old nymphs feeding on artificial diet MP148 containing 10 µg/ml purified virus was collected on parafilm membranes. Samples consisting of 100 honeydew droplets were resuspended in 20 µl 0.1 M sodium citrate buffer. For ISEM, electron microscope grids were coated with anti-BWYV IgGs (at 2 µg/ml in phosphate-buffered saline, pH 7.4). The activated grids were then allowed to adsorb virions from the honeydew for 3 h at room temperature before staining with 2% uranyl acetate and examination with a Philips CM12 electron microscope.

Immunogold labeling of virions

Virions were extracted from 100 000 infected protoplasts (suspended in 100 µl 0.1 M sodium citrate, pH 6.0) by two 1 min bursts of sonication at 4°C. Virions in the extract were allowed to immunoadsorb to activated electron microscope grids as described above. The immobilized virions were then immunolabeled for 2 h with anti-RTD IgGs conjugated to colloidal gold beads (Garaud et al., 1982) of 12 nm average diameter. Virus on the grids was stained with 2% uranyl acetate and examined with the electron microscope.

In situ hybridization

Tissue from agro-infected plants was fixed overnight in 2% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2. Sections (25 µm) were cut with a Reichert-Jung 2800 N cyromicrotome and processed as described (Meyerowitz, 1987). Hybridization (Jackson, 1992) was performed at 50°C overnight using a ^{33}P-labeled riboprobe ($\sim 2\times10^5$ counts/min/slide) complementary to the 3′-terminal 196 residues of BWYV RNA. The slides were coated with Ilford K5 nuclear track emulsion. After development, the sections were stained with toluidine blue and examined with a light microscope.

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