Role of the beet western yellows virus readthrough protein in virus movement in *Nicotiana clevelandii*

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**Introduction**

Beet western yellows virus (BWYV) is a member of the family *Luteoviridae* (genus *Poleroviridae*), which comprises small icosahedral viruses with a single-stranded plus-sense RNA genome of about 5.7 kb (Mayo & Ziegler-Graff, 1996). Luteoviruses are obligately transmitted by aphids in a circulative non-propagative fashion and are typically limited to the phloem tissues of their plant hosts. The luteovirus capsid consists of two proteins: a major species of ~22 kDa, termed P3, and a minor species, termed the readthrough (RT) protein (Bahner et al., 1990; Martin et al., 1990; Filichkin et al., 1994; Brault et al., 1995; Wang et al., 1995). RT protein is a C-terminally extended form of P3 produced by suppression of P3 translation–termination (Veidt et al., 1988; Bahner et al., 1990; Tacke et al., 1990; Dinesh-Kumar et al., 1992), so that translation continues into the adjacent ~54 kDa RT domain (RTD) encoded by ORF 5 (Fig. 1).

The RT protein in virions is thought to be anchored into the capsid via its P3 moiety with the RTD projecting toward the exterior (Cheng et al., 1994; Brault et al., 1995). Sequences within the RTD are implicated in transmission of luteoviruses by their aphid vectors (Jolly & Mayo, 1994; Brault et al., 1995; Chay et al., 1996), but the RTD is also important for efficient accumulation of the virus in whole plants (Brault et al., 1995; Chay et al., 1996; Bruyère et al., 1997). Thus, *Nicotiana clevelandii* plants agro-infected with a BWYV mutant (BW6.4) in which the RTD had been deleted accumulated tenfold less virus than did plants agro-infected with the wild-type construct (Brault et al., 1995).

The fact that BW6.4 efficiently replicates and assembles into virions in *Chenopodium quinoa* mesophyll protoplasts (Reutenauer et al., 1993; Brault et al., 1995) suggested that the diminished accumulation of BW6.4 in whole plants reflects a requirement for the RTD for vascular transport of the virus (Brault et al., 1995). It is difficult, however, to eliminate the possibility that the low virus titre in whole plants is instead due to less efficient replication of the RTD-null mutant in the specialized nucleate cells of the phloem compartment where virus replication normally occurs. In this paper, we have addressed this question by comparing the distribution and level of multiplication of BW6.4 and wild-type BWYV in phloem cells of systematically infected leaves of agro-infected plants. Our findings indicate that deletion of the RTD greatly diminishes both the number and the size of infection foci in the phloem tissue but does not markedly reduce the virus levels attained within those cells which become infected.

**Methods**

- **Agro-infection and virus detection.** Binary vectors for agro-infection with infectious cDNA clones of wild-type BWYV (BW63) and RTD mutants (BW6.4, BW6.40 and BW6.106) have been described (Brault et al., 1995; Bruyère et al., 1997). Agro-inoculation of 6–8-week-old...
old *N. clevelandii* plants followed published procedures (Leiser et al., 1992). Virus titres in agro-infected plants were measured by DAS-ELISA (Brault et al., 1995) or by dot-blot hybridization of total leaf RNA with a 32P-labelled riboprobe complementary to residues 5445–5641 of the BWYV genome (Veidt et al., 1988). Radioactivity in the spots was quantified using a Fujix BAS1000 BioAnalyser.

**Tissue printing.** Petioles of systemically infected leaves of *N. clevelandii* were hand-cut with a razor blade and the freshly cut surface was gently pressed to a nitrocellulose membrane for several seconds. Serial prints were obtained by repeated application of this procedure at 1–2 mm intervals along the petiole. The membrane was incubated overnight at 4 °C in PBS (8 mM Na2HPO4, 1.5 mM KH2PO4, 150 mM NaCl, 2.5 mM KCl, pH 7.4) containing 0.5% Tween 20 and 5% dried milk (blocking solution). The membrane was next washed three times with PBS plus 0.5% Tween 20 and then incubated for 4 h at room temperature in blocking solution containing a 1:5000 dilution of a rabbit anti-rabbit secondary antibody (Bio-Rad). After three washes in PBS, bound antibody was rendered visible by incubating with the AP substrate Fast Red TR/Naphthol AS-MX (Sigma).

**Electron microscopy.** Transmission electron microscopy was carried out essentially as described (Ritzenthaler et al., 1995) on thin sections of glutaraldehyde-fixed leaf tissue from healthy or systemically infected leaves of *N. clevelandii*.

### Results and Discussion

**Deletion of the BWYV RTD lowers virus accumulation levels in both agro-inoculated and systemically infected leaves of *N. clevelandii***

We have previously shown that *N. clevelandii* agro-infected with the BWYV RTD-null mutant BW6.4 (Fig. 1) accumulated about tenfold less viral antigen than did plants agro-infected with wild-type BWYV (BW0) (Brault et al., 1995). In these experiments, samples were collected from random leaves of each plant and pooled prior to ELISA. To determine if elimination of the RTD differentially affects virus accumulation in the inoculated and systemically infected leaves, duplicate samples were punched from the inoculated leaf of each BW0- and BW6.4-agro-infected plant 4 weeks p.i. and from two younger non-inoculated leaves at the same time or 1 or 2
Table 1. Virus titre in inoculated and systemically infected leaves after agro-inoculation of N. clevelandii with wild-type BWYV (BW₀) or the RTD-null mutant BW6.4

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Time p.i. (weeks)</th>
<th>Leaf sampled*</th>
<th>n†</th>
<th>Virus titre (arbitrary units ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW₀</td>
<td>4</td>
<td>Inoc.</td>
<td>3</td>
<td>159 ± 41</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Syst.</td>
<td>6</td>
<td>355 ± 272</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Syst.</td>
<td>6</td>
<td>314 ± 164</td>
</tr>
<tr>
<td>BW6.4</td>
<td>4</td>
<td>Inoc.</td>
<td>6</td>
<td>22 ± 16</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Syst.</td>
<td>12</td>
<td>22 ± 7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Syst.</td>
<td>12</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Expt 1</td>
<td>Syst.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW₀</td>
<td>4</td>
<td>Inoc.</td>
<td>8</td>
<td>274 ± 22</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Syst.</td>
<td>16</td>
<td>404 ± 129</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Syst.</td>
<td>16</td>
<td>348 ± 51</td>
</tr>
<tr>
<td>BW6.4</td>
<td>4</td>
<td>Inoc.</td>
<td>8</td>
<td>29 ± 22</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Syst.</td>
<td>16</td>
<td>67 ± 42</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Syst.</td>
<td>16</td>
<td>58 ± 28</td>
</tr>
<tr>
<td>Expt 2</td>
<td>Syst.</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

* Inoc., samples punched from agro-inoculated leaves acropetal of the inoculation site; syst., samples punched from systemically infected leaves.
† n, no. of samples tested in each experiment.

weeks later. The samples were processed separately and the viral RNA contents were determined by dot-blot hybridization using a 32P-labelled antisense RNA probe directed against a sequence common to both the wild-type and the mutant viral genome. Virus titre was measured by dot-blot hybridization rather than ELISA to avoid systematic errors due to possible differences in the antigenicity of virions with or without the RTD. Measurements from two independent experiments (Table 1) revealed considerable variability in virus levels in both the inoculated and systemically infected leaves, probably due mainly to differences in the amount of inoculum successfully delivered to phloem cells by agro-inoculation. Nevertheless, the observations are consistent with our earlier finding that virus levels are about an order of magnitude lower in BW6.4-infected plants than in BW₀-infected plants, for both the inoculated and systemically infected leaves (Table 1).

Detection of BW₀ and BW6.4 in immuno-imprints of petioles

The distribution of virus within petioles of systemically BW6.4- and BWₐ-infected N. clevelandii leaves at 4 weeks p.i. was studied by tissue print analysis (Holt, 1992). The loci of virus accumulation on the tissue prints stained reddish-brown and were readily visible against the faint brownish-green outline which defines the basic morphology of the section (Fig. 2a-f). Virus-specific staining was apparent in the phloem bundles of the petiole and in some lateral bundles within the leaf traces. Of the 120 BWₐ-agro-infected sections examined, 95% contained at least one stained bundle and the majority of these contained numerous stained bundles (Fig. 2a, b). About 60% of the 200 petiole sections examined from the BW6.4-agro-infected plants displayed immuno-staining but no more than one or a few bundles were infected (Fig. 2d, e, f). No staining was observed in the petioles of non-agro-infected plants (Fig. 2c). The intensity of staining at infected sites, estimated by visual examination of prints stained in parallel, was comparable for BWₐ- and BW6.4-infected petioles. We conclude that elimination of the RTD dramatically reduces the number of infection sites in the petioles but does not markedly reduce virus accumulation at the loci where the BW6.4 infection has taken hold.

Detection of BW₀ and BW6.4 in leaf veins

The immuno-print method was not suitable for detection of BWYV in cross-sections of leaf blades or whole leaf press-bLOTS. Therefore, virus distribution in systemic leaves of BW6.4- and BWₐ-infected N. clevelandii was studied in thin sections. Upper, non-inoculated leaves were detached from the agro-inoculated plants 1, 2 and 4 weeks p.i. Small leaf fragments that did not contain primary or secondary veins were excised, embedded in paraffin and cut into serial thin sections. The sections were then immuno-stained for virus and observed with a light microscope.

No immuno-labelled veins were detected in sections of either the BW6.4- or BWₐ-infected tissue sampled 1 week p.i. (Table 2). At 2 and 4 weeks p.i., immuno-labelled phloem cells associated with minor veins were observed for both the BW6.4-infected and the BWₐ-infected samples. The intensity of staining of the cells in the BW6.4-infected (Fig. 2g–i) and the BWₐ-infected (Fig. 2k–m, o–q) samples was comparable but the number of veins labelled was markedly different. Thus at 2 weeks p.i., only 5% of the veins observed in section in the BW6.4-infected tissue were immuno-labelled whereas 80% of the veins in the BWₐ-infected tissue contained at least one immuno-labelled cell (Table 2). In the few BW6.4-infected veins that were observed, immuno-labelling was always confined to a single cell (Fig. 2g–i). In the BWₐ-infected veins, labelling was occasionally observed in single cells at 2 weeks p.i. (Fig. 2k, l)), but it was much more common to observe clusters of two or more adjacent immuno-labelled phloem cells when the vein was observed in transverse section (Fig. 2m), or when the same vein was traced through a series of sections (not shown).

The situation was similar at 4 weeks p.i. (Table 2). The fraction of BW6.4-infected veins increased to ~ 10%, but in every case only isolated cells were immuno-labelled (data not shown). In the BWₐ-infected tissue, on the other hand, more than 90% of the veins were immuno-labelled and in virtually
Fig. 2. For legend see facing page.
Table 2. Virus-specific immuno-labelling of minor vein phloem cells of BW<sub>0</sub>- and BW6.4-infected <i>N. clevelandii</i> leaves at different times after agro-inoculation

<table>
<thead>
<tr>
<th>Time post-agro-inoculation (weeks)</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW&lt;sub&gt;0&lt;/sub&gt;-infected</td>
<td>0 (80)*</td>
<td>95 (118)</td>
<td>173 (189)</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>80.5%†</td>
<td>92.1%</td>
</tr>
<tr>
<td>BW6.4-infected</td>
<td>0 (80)</td>
<td>7 (136)</td>
<td>53 (539)</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>5.1%</td>
<td>9.8%</td>
</tr>
<tr>
<td>Healthy</td>
<td>0 (30)</td>
<td>0 (34)</td>
<td>0 (94)</td>
</tr>
</tbody>
</table>

* Number of minor veins containing at least one infected cell; values in parentheses indicate the total number of veins examined in each experiment.
† Percentage of minor veins containing infected cell(s).

Immuno-labelling of veins after agro-infection with RTD mutants

<i>N. clevelandii</i> plants were also agro-infected with BW6.106 and BW6.40 (Fig. 1), mutants with an in-frame deletion in either the conserved N-terminal half (BW6.106) or the non-conserved C-terminal half (BW6.40) of the RTD. We have shown previously that neither of these mutations inhibited synthesis of RT protein but that, in the case of BW6.106, the mutated RT protein was not incorporated into virions and accumulated to near wild-type levels following agro-infection (Bruyère et al., 1997).

Examining leaf thin-sections taken 4 weeks p.i. revealed a pattern of immuno-labelled cells in the BW6.40-infected leaves similar to that observed in BW<sub>0</sub>-infected leaves, i.e. staining typically appeared in numerous contiguous cells (Fig. 2n). In the BW6.106-infected systemic leaves, on the other hand, the distribution of immuno-labelled phloem cells was similar to that observed in the BW6.4-infected leaves, i.e. stained cells were infrequent and isolated (Fig. 2i). Thus, even a relatively small deletion in the RTD can interfere with virus movement provided it falls within the conserved portion of the RTD. At present, we cannot conclude whether the inefficient spread of BW6.106 is due to the deletion of an important sequence motif or to the fact that the RT protein is not incorporated into virions.

Elimination of the RTD does not alter the infected cell types

The major cell types in veins of <i>N. clevelandii</i> are companion cells (C), phloem parenchyma cells (PP), sieve elements (SE), xylem parenchyma cells and xylem tracheary elements, all within a sheath of bundle cells (BS) (Ding et al., 1988). BWYV infection is generally confined to the C and PP cells. Immuno-labelling of small cells lining the sieve tubes was observed for both the BW6.4- and the BW<sub>0</sub>-infected samples (e.g. Fig. 2g, h, k, l, q). Their small size and their proximity to the SE suggest that these cells are C cells. We also observed, for both types of inoculum, immuno-labelling of large rounded cells which are probably PP cells (Fig. 2i, o). Finally, some of the smaller immuno-labelled cells which were not immediately adjacent to an SE in the section cannot be placed with confidence in either category.

Electron microscopy can be used to better discriminate between C cells and PP cells because C cells typically have much smaller vacuoles than PP cells and contain a higher density of ribosomes. Thin sections of systemic leaves of <i>N. clevelandii</i> agro-infected with BW6.4 and BW<sub>0</sub> were examined and cells in small veins that contained pseudocrystalline arrays of virus-like particles (VLP) were located. In agreement with earlier observations (Esau & Hoefert, 1972; D’Arcy & de Zoeten, 1979; Shepardson et al., 1980), arrays of VLP were visualized in both C cells and PP cells in the BW<sub>0</sub>-infected tissue (data not shown). The same cell types also contained VLP arrays in the BW6.4-infected tissue (Fig. 3a, b), but no such arrays were observed in comparable sections from healthy tissue (data not shown). Although not enough infected cells were examined by electron microscopy to allow statistical...
Fig. 3. For legend see facing page.
analysis, we conclude that the RTD is not absolutely required for infection of either cell type. Furthermore, the presence of extensive arrays of VLPs in both the BW6.4- and the BWr-infected cells is consistent with the above observations of immuno-labelled cells by light microscopy, which indicate that, once a C or PP cell is infected, progeny virions accumulate abundantly even when the RTD is absent. In several sections from BW6.4-infected leaves, VLPs were observed in the plasmodesmata connecting a C cell and an SE (Fig. 3c, e) or a PP cell and an SE (Fig. 3d, f). Such images provide additional evidence that the RTD is not strictly essential for movement of virions through plasmodesmata.

While the immuno-labelling and electron microscope observations described above do not lend themselves to precise quantification, we feel that, taken together, they make a strong case that the low virus titres associated with deletion of the RTD principally reflect inefficient movement of the virus to new infection foci and their enlargement rather than low multiplication levels in individual infected cells. Luteovirus replication and assembly are believed to be limited to the nucleate cells of the vasculature, the C and PP cells. Long-distance movement of the virus to other parts of the plant would hence involve exit from such cells via plasmodesmata into the SE, ‘vertical’ movement through the SE along with the flow of photoassimilates and entry through plasmodesmata into nucleate phloem cells at another site. Both site-directed mutagenesis experiments (Ziegler-Graff et al., 1996) and electron microscopic visualization of VLPs in the specialized plasmodesmata connecting the SE to C and PP cells of BWVV-infected leaves (Esau & Hoefert, 1972; D’Arcy & de Zoeten, 1979; Shepardson et al., 1980) indicate that virions are the infectious entity engaged in vascular transport. We suggest that the RTD on the surface of a wild-type virus particle acts as a movement protein to facilitate virus transit in one or the other direction (or in both directions) through the plasmodesmata connecting nucleate phloem cells and the SE. The apparent ability of BW6.4 to invade, albeit inefficiently, non-inoculated leaves of N. clevelandii, however, argues that the RTD is not absolutely required for long-distance movement. Thus it is possible that RTD-dependent and -independent movement pathways operate in parallel.

We suggest that wild-type BWVV can move from an infected C or PP cell to a neighbouring cell either directly via connecting plasmodesmata, or indirectly, by transiting through an SE with connections to both cells. The abundance of isolated infected cells in the BW6.4-infected systemic leaves could reflect a strong requirement for the RTD for cell-to-cell movement of the former type. An alternative explanation would be that inefficient vascular movement of BW6.4 has sufficiently diminished the rate of virus movement into and out of the SE that transit via the vasculature to a neighbouring phloem cell is unlikely to occur during the experimental observation period. Evidently, it will be necessary to learn more about the relative importance in a wild-type infection of the cell-to-cell and vascular movement pathways in order to distinguish between these possibilities.

References


Fig. 3. Localization of virus-like particles (VLPs) in BW6.4-infected tissue by electron microscopy. (a) Pseudo-crystalline arrays of VLPs in a degenerating companion cell. (b) VLPs in arrays and associated with a chloroplast in a phloem parenchyma cell. (c) VLPs in a plasmodesma connecting a sieve element and a companion cell [(e) enlargement of the area indicated by the rectangle]. (d) VLPs in a plasmodesma connecting a sieve element and a phloem parenchyma cell [(f) enlargement of the area indicated by the rectangle]. C, companion cell; PP, phloem parenchyma cell; SE, sieve element; arrows, VLPs. Bars correspond to 250 nm in (a), (b), (e) and (f) and to 2.5 µm in (c) and (d).


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