Vascular movement of beet necrotic yellow vein virus in *Beta macrocarpa* is probably dependent on an RNA 3 sequence domain rather than a gene product

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RNAs 1 and 2 of beet necrotic yellow vein virus (BNYVV) carry the functions enabling viral RNA replication, cell-to-cell movement, virus assembly and vascular movement of the virus in the systemic host *Spinacea oleracea*. In *Beta macrocarpa*, the other hand, BNYVV RNA 3 is required for vascular movement. Replication-competent RNA 3 transcripts carrying various point mutations and deletions were co-inoculated with RNAs 1 and 2 to young leaves of *B. macrocarpa* and the ability of the virus to multiply on the inoculated leaves and to invade the plant systemically was examined. None of the RNA 3 mutants tested interfered with virus multiplication in the inoculated leaves. Point mutations designed to specifically block or truncate translation of the ORFs of the two known RNA 3 gene products, P25 and N, did not interfere with vascular movement. Vascular movement was not inhibited by deletions eliminating the short 5’-proximal ORF on RNA 3 (ORF A) or by point mutations blocking putative translation of the short 5’-proximal ORF (ORF S) on RNA 3sub, a subgenomic RNA derived from RNA 3. On the other hand, deletions in a ‘core region’ encompassing nucleotides 1033–1257 of RNA 3 completely blocked vascular movement of the virus while removal of sequences flanking the core region lowered its efficiency. The observations suggest that some feature of the RNA 3 sequence rather than an RNA-3 coded protein is important for vascular movement of BNYVV in *B. macrocarpa*.

Introduction

Phytoviruses can provoke severe diseases in plants, particularly when they are able to invade the entire host plant after infection. Spread of a virus from the primary site of infection to distant parts of a plant involves two steps. The infectious entity (virus or a viral RNA–protein complex) first moves symplastically through plasmodesmata into neighbouring cells until the phloem compartment is reached. This process is generally mediated by one or more viral non-structural movement proteins (MPs) of which the tobacco mosaic virus (TMV) 30 kDa protein is a well-studied prototype (Deom *et al*., 1992, 1994; Lucas & Gilbertson, 1994; Carrington *et al*., 1996). Once in the phloem compartment, rapid long-distance transport to other leaves occurs through the sieve elements (reviewed by: Gilbertson & Lucas, 1996; Séron & Haenni, 1996). For most but not all viruses that have been examined [exceptions include some geminiviruses, tombusviruses, tobacco stunt virus (TRV) and barley stripe mosaic virus (BSMV)] vascular movement requires viral coat protein. The coat protein may, however, do more than simply protect the viral genome by encapsidation. For example, specific interactions between coat protein and host proteins might govern plasmodesmatal gating for movement into, within, or out of the phloem compartment. Finally, there have been reports implicating viral nonstructural proteins other than the ‘conventional’ cell-to-cell movement proteins in vascular movement (e.g. S.-W. Ding *et al*., 1995; X. S. Ding *et al*., 1995).

The genome of beet necrotic yellow vein virus (BNYVV) consists of five plus-strand RNA components (Richards & Tamada, 1992). BNYVV RNAs 1 and 2 carry ‘house-keeping’ genes involved in replication, cell-to-cell movement and viral RNA encapsidation. RNAs 3, 4 and 5 are not required for infection of protoplasts or local lesion formation on leaves of *Chenopodium quinoa* and *Tetragonia expansa*. RNA 3 and RNA 4 are invariably present in field isolates of BNYVV. RNA 3 has been implicated in virus proliferation in roots of *Beta vulgaris*.
and symptom expression (rhizomania) (Tamada et al., 1989; Koenig et al., 1991) whereas RNA 4 is important for *Polymyxa betae*-mediated transmission of the virus (Tamada & Abe, 1989). RNA 5 is not present in all virus sources but it has been reported in some Japanese and European BNYVV isolates (Tamada et al., 1989; Kiguchi et al., 1996; Koenig et al., 1997). It can partially substitute for RNA 3 or interact synergistically with it when both are present (Tamada et al., 1989).

BNYVV forms local lesions on inoculated leaves of *C. quinoa* and *T. expansa*, but does not move systematically in these hosts. The virus can, however, readily move from inoculated to upper, noninoculated leaves of young *Beta macrocarpa* and *Spinacea oleracea* plants (Kuszala & Putz, 1977; Tamada et al., 1989). Although RNA 3 is not required for infection of the inoculated leaves of *B. macrocarpa*, it is essential for vascular movement in this host (Tamada et al., 1989). In this paper, we have used site-directed mutagenesis of cloned RNA 3 cDNA to produce a set of transcripts with deletions or point mutations throughout the central, replication-dispensable portion of the RNA 3 sequence. The various mutant RNA 3 transcripts were tested for their ability to assist vascular movement by inoculating them to *B. macrocarpa* along with wild-type BNYVV RNAs 1, 2 and 4. The experiments reveal that movement-essential sequences are situated in a domain between nucleotides 1033 and 1266 of RNA 3. Surprisingly, mutations designed to block expression of the two known RNA 3 gene products, P25 and N, and of the hypothetical translation products of the two small ORFs A and S (Fig. 1) did not interfere with vascular movement.

**Methods**

**Mutant RNA 3 transcription vectors.** Plasmids pB35AAS, pB35AEB, pB35AES, pB35CCG, pB35AEl, pB35APS, pB35ABA, pB35Aλexo3, pABC5 and pABC12 have already been described (Jupin et al., 1990, 1992; Gilmer et al., 1992; Balmori et al., 1993). Additional RNA 3 mutants were obtained using the wild-type plasmid pB35 (Ziegler-Graff et al., 1988), pB35A (Jupin et al., 1990) or a derivative as starting material. pB35A is identical to pB35 except for an insertion of 10 residues including a Smal site at position 1470. Some mutants were produced by digesting pB35 with BamHI (pB35BK), EcoRI plus StuI (pB35AES), EcoRI plus BamHI (pB35AEB), StuI plus BamHI (pB35ASB), Acl plus PmoI (pB35AAP) or EcoRI plus PmoI (pB35AEIP), filling-in recessed extremities with the Klengen fragment of DNA polymerase I and religation. Another series of mutants was produced by digesting pB35CCC with EcoRI plus Smal (pB35AES), Smal plus StuI (pB35SSS), Smal plus BamHI (pB35ASB), or Smal plus Acl (pB35ASA), filling-in recessed extremities with Klengen fragment and religation. In mutant pB35Xb2, ATG (1274–1276) at the beginning of ORF S (Fig. 1) was replaced with AGA by PCR mutagenesis (Higuchi et al., 1988; Ho et al., 1989). The sequence alteration created a novel XhoI site. PCR mutagenesis was also used to introduce a stop codon into ORF N (pB35N-Stop) by replacing AATT (nucleotides 1114–1118) with CTAG and into ORF S by replacing T129 with A (pB35S-Stop). In all cases, the mutant constructs were characterized by restriction enzyme digestion and sequence analysis.

The cDNA plasmids were linearized downstream of the insert and the 3′poly(A) by cleavage with HindIII and capped run-off RNA 3 transcripts were produced with bacteriophage T7 RNA polymerase (Ziegler-Graff et al., 1988). Transcripts will be referred to by the name of the parent plasmid prefixed by the letter t (e.g. t35, t35BK, t35CCG etc.). For infection experiments, 5 μg freshly prepared transcript was mixed with RNA from a transcript-derived BNYVV isolate containing RNAs 1, 2 and 4 (Stras124; Quillet et al., 1989) in a total volume of 10 μl and 8 μl was inoculated to each of the first true leaves of approximately 15-day-old *Beta macrocarpa* or *Spinacea oleracea*. The concentration of Stras124 RNA in the inoculum was adjusted in preliminary experiments so as to produce 50–150 local lesions per leaf on *C. quinoa* (Jupin et al., 1990).

**Analysis of viral RNA and proteins.** RNA was extracted (Jupin et al., 1990) from leaves 15–40 days post-infection (p.i.) using the Pol extraction procedure to obtain both encapsidated and nonencapsidated viral RNA or the TM extraction procedure to obtain only encapsidated viral RNA. RNA was extracted from roots as described (Lemaire et al., 1988). Viral RNAs were detected by Northern hybridization or dot-blot with a mixture of 32P-labelled probes specific for RNA 1, 2, 3 and 4 (Lemaire et al., 1988). For the RNA 3 mutants bearing point mutations, the persistence of the mutations following passage in plants was tested by analysis of DNA fragments produced by RT–PCR using RNA 3-specific primer pairs flanking the site of each mutation. In the case of t35CCG and t35BK, the presence of the mutations in the progeny was tested by digestion of the RT–PCR product with Smal and Clal, respectively. For the other mutants, the RT–PCR products were cloned and sequenced or directly sequenced with an Applied Biosystems 373 DNA sequencer.

For immunodetection of viral proteins, ten 10 mm2 disks were punched from leaves and pooled. Each disk was centred on a local lesion in the case of *C. quinoa* but samples were taken at random from the B. macrocarpa and *S. oleracea* leaves. The samples were homogenized in 100 μl of 2× concentrated gel loading buffer (Laemmli, 1970) and centrifuged briefly to remove insoluble material. The soluble proteins were heated to 95 °C for 5 min and loaded (1 μl for detection of coat protein, 10 μl for detection of P25) on a 12% polyacrylamide gel (Laemmli 1970). After PAGE and electrotransfer to Immobilon-P transfer membranes (Millipore), coat protein and P25 were immunodetected by enhanced chemiluminescence (Amersham) using specific rabbit polyclonal antisera (Niesbach-Kløsgen et al., 1990).

**Results**

**RNA 3 is required for long-distance movement of BNYVV in *B. macrocarpa* but not in *S. oleracea***

*B. macrocarpa* and *S. oleracea* (spinach) are systemic hosts of BNYVV. Using natural BNYVV isolates with different RNA
compositions, Tamada et al. (1989) have shown that, in addition to RNAs 1 and 2, RNA 3 is required to produce a systemic infection of *Beta macrocarpa*. We have confirmed this observation using transcript-derived BNYVV isolates (Quillet et al., 1989) containing RNAs 1 and 2 plus RNA 3 and/or RNA 4. When all four RNAs were present in the inoculum, yellow lesions appeared on the inoculated leaves of both young spinach and *Beta macrocarpa* within 5–7 days and yellow mosaic symptoms began to appear on systemically infected leaves 5–7 days later. At later times, leaf curling and severe dwarfing symptoms were also noted. Dot-blot hybridization on RNA extracted from noninoculated leaves and from the roots confirmed that vascular movement of the virus had taken place (Fig. 2). Typically, more than 90% of the plants responded systemically (see Table 1).

When RNA 3 was omitted from the inoculum (isolates Stras12 and Stras124), no symptoms were observed on the noninoculated leaves of *Beta macrocarpa* and viral RNA was never detected in such leaves or in roots, even 40 days p.i. (Fig. 2). RNA 4, on the other hand, was not required for vascular movement of the virus in *Beta macrocarpa* and neither small RNA was necessary for vascular movement in spinach (Fig. 2). These findings confirm that RNA 3 does indeed mediate long-distance movement of BNYVV and, furthermore, show that it operates in a host-specific manner.

BNYVV does not require virion assembly for cell-to-cell movement on *C. quinoa* leaves although coat protein and, presumably, virion formation are necessary for systemic infection of *S. oleracea* (Quillet et al., 1989). To determine if functional coat protein is required for vascular movement of BNYVV in *B. macrocarpa*, transcript of a BNYVV RNA 2 mutant, pB218A, which encodes 21 kDa viral coat protein rendered assembly-defective by a deletion (Schmitt et al., 1992), was inoculated along with wild-type transcripts of RNAs 1, 3 and 4 to leaves of *B. macrocarpa*. Infection of the inoculated leaves but no systemic infection was observed on any of 12 plants tested, indicating that a viral RNA–coat protein complex (presumably virions) is also the active entity for vascular movement of BNYVV in *B. macrocarpa*.

**Replication-competent mutants of RNA 3 are capable of cell-to-cell movement in *B. macrocarpa***

Mutagenesis experiments were undertaken to locate the region on RNA 3 which mediates BNYVV vascular transport. Numerous RNA 3 mutants (Fig. 3) were available from previous work (Jupin et al., 1990; Gilmer et al., 1992; Balmori et al., 1993) or were purpose-built for this study. Before using these RNA 3 mutants to study vascular movement, their ability to replicate and move from cell to cell on the inoculated leaves of *B. macrocarpa* was evaluated. The mutant RNA 3 transcripts were inoculated along with BNYVV RNAs 1, 2 and 4 to the first two true leaves of young *B. macrocarpa* and the plants were observed for symptoms. All of the inoculations provoked the appearance of yellow local lesions on the inoculated leaves by 8 days p.i. and progeny viral RNAs, including RNA 3, were readily detected by Northern blot of total RNA extracted from such leaves (Fig. 4). The amounts of progeny viral RNA 3 (relative to RNAs 1 and 2) observed for the various RNA 3 mutants was approximately comparable to that observed in parallel experiments with wild-type RNA 3 transcript.

Sequences essential for assembly of RNA 3 into virus particles are situated near nucleotide 200 (Fig. 1) of RNA 3 (Gilmer et al., 1992); previous experiments have shown that the entire central portion of RNA 3 (nucleotides 381–1644) can be deleted without interfering with RNA 3 encapsidation (Jupin et al., 1990). Confirmation that the RNA 3 mutants used in this paper were assembly-competent was obtained by testing their sensitivity to RNase degradation in crude extracts of infected *B. macrocarpa* leaves (Jupin et al., 1990). All of the mutant RNA 3 progeny in such experiments proved to be RNase-resistant and are hence presumed to be stably packaged into virus particles (data not shown).

**P25 and the ORF N gene product are not required for vascular movement***

Two gene products are known to be encoded by RNA 3. The central portion of RNA 3 contains the open reading frame (ORF) of a 25 kDa protein (P25; Fig. 1) which has been serologically detected in the cytosolic fraction of infected *C. quinoa* leaves (Niesbach-Klösgen et al., 1990). Expression
Table 1. Effect of RNA 3 mutations on the efficiency of vascular movement in *B. macrocarpa* and the symptoms produced

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Position of mutation*</th>
<th>Symptoms produced in systemic leaves†</th>
<th>Systemically infected plants/inoculated plants</th>
</tr>
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<tbody>
<tr>
<td>t35</td>
<td>–</td>
<td>YM-ST-LC</td>
<td>53/58</td>
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<tr>
<td>t35CCG</td>
<td>445</td>
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<td>15/17</td>
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<td>t35BK</td>
<td>751</td>
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<td>8/10</td>
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<td>1115</td>
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<td>19/27</td>
</tr>
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<td>15/19</td>
</tr>
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<td>1328</td>
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<td>13/19</td>
</tr>
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<td>11/12</td>
</tr>
<tr>
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<td>A381–658</td>
<td>M</td>
<td>10/12</td>
</tr>
<tr>
<td>t35AB</td>
<td>A381–755</td>
<td>M</td>
<td>10/12</td>
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<td>A381–1033</td>
<td>S</td>
<td>2/19</td>
</tr>
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<td>A445–658</td>
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<tr>
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<td>A445–755</td>
<td>M</td>
<td>10/12</td>
</tr>
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<td>A445–1033</td>
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<td>3/12</td>
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<tr>
<td>t35ASt</td>
<td>A658–755</td>
<td>M</td>
<td>9/12</td>
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<tr>
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<td>A755–1033</td>
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<td>20/32</td>
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<td>t35AΔexo3</td>
<td>A1438–1492</td>
<td>S</td>
<td>3/23</td>
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</table>

* Single numbers refer to the RNA 3 nucleotide position at which a point mutation or frameshift was introduced. Pairs of numbers preceded by Δ refer to the coordinates of the nucleotides bordering a deletion.
† Symptoms on systemically infected leaves. YM, yellow mosaic; ST, stunting; LC, leaf curling; M, mild mosaic; S, symptomless; –, no systemic infection. Symptoms in parentheses were only observed occasionally.

P25 is associated with the appearance of bright yellow local lesions (Tamada et al., 1989) on inoculated leaves of hosts such as *C. quinoa* and *T. expansa* (Tamada et al., 1989; Jupin et al., 1992) and the development of rhizomania symptoms in sugar beet roots (Tamada et al., 1990; Koenig et al., 1991). RNA 3 also contains a short ORF called N (nucleotides 1052–1231; Fig. 1), which overlaps the 3′ terminal portion of the P25 ORF and has the potential to encode a protein of 6·4 kDa. N is not detectably expressed from full-length RNA 3 but is translationally activated by spontaneous or experimental deletion of the upstream portion of the P25 ORF (Jupin et al., 1992). When so expressed, N provokes the appearance of necrotic local lesions on *T. expansa* and *C. quinoa* (Jupin et al., 1992).

To determine if P25 intervenes in vascular movement, RNA 3 mutants were produced in which the P25 ORF was disabled. In pB35CCG, the first initiation codon of the ORF, ATG_{147}, was changed to CCG by site-directed mutagenesis. The next in-frame initiation codon is ATG_{690}. In p35BK, a four residue insertion near the middle of the 25 kDa ORF was produced by filling-in a BamHI site at nucleotide 750. When inoculated along with RNAs 1, 2 and 4 to *B. macrocarpa*, the corresponding transcripts, t35CCG and t35BK, promoted virus movement to upper noninoculated leaves with efficiency comparable to that of t35 (Fig. 5 A, lanes 4–5; Table 1). The symptoms produced on the systemically infected leaves resembled those observed in an infection with BNYVV containing wild-type RNA 3. No P25 could be detected by immunoblot of protein extracted from such leaves (Fig. 5 B, lanes 4 and 5) and sequence analysis in the vicinity of each mutation following RT–PCR amplification of the progeny RNA 3 in systemically infected leaves confirmed that the mutations were conserved (data not shown).

Site-directed mutagenesis was also performed to determine if the ORF N gene product is required for vascular movement. There are five ATG codons within ORF N, all within the first 16 codons of the reading frame. A plasmid (pB35N-Stop) containing a point mutation was constructed with an in-frame TAG codon inserted six codons downstream of the last ATG of the ORF (Fig. 3). The resulting truncated N gene product, if produced, will be a maximum of only 21 amino acids in length.
BNYVV RNA 3-mediated vascular movement

Fig. 3. Positions of point mutations (A) and deletions (B) in the BNYVV RNA 3 mutants. The ORFs referred to in the text are indicated by hollow rectangles. At the top of (B), the positions of relevant restriction sites are indicated: E, EcoRI; St, StuI; B, BamHI; Ac, AccI; P, PmlI; Sm, SmaI. The SmaI sites (in parentheses) at the beginning of the P25 ORF and at nucleotide 1470 are not present in pB35 but were introduced by mutagenesis into the derivatives pH35 CCG and pH35A, respectively. Asterisks in (B) represent sites of mutagenesis-induced premature translation termination.

versus 59 residues for the full-length product. Nevertheless, t35 N-Stop supported efficient long-distance movement of BNYVV in B. macrocarpa (Fig. 5A, lane 6; Table 1), producing wild-type systemic symptoms. Analysis of RT–PCR products revealed that the mutation was conserved in progeny RNA 3 isolated from the systemically infected leaves (data not shown). The evidence thus indicates that neither of the known RNA 3 gene products, P25 and N, are essential for long-distance movement of BNYVV in B. macrocarpa.

The ORF A and ORF S putative gene products are dispensable for vascular movement

In addition to the P25 ORF and ORF N, RNA 3 contains a number of other short ORFs. Although there is no evidence that any of these ORFs are expressed during infection, we have tested the potential role of two of them, ORF A and ORF S (Fig. 3), in vascular movement. There are five ATGs upstream of the P25 initiation codon at nucleotide 445. Of these, ATG54 and ATG230 are followed immediately by termination codons and the ORF headed by ATG234 is only seven codons in length. ATG234 initiates an ORF of 25 codons (ORF A; Fig. 3) and is thus the first ORF of significant length which would be encountered by a ribosome scanning RNA 3 from the 5' terminus. [The fifth initiation codon, ATG250, is codon number three in ORF A.] Two previously described mutants, t35ΔBC5 and t35ΔBC12 (Gilmer et al., 1992), contain short deletions which completely eliminate ORF A. Both mutants replicate when inoculated with BNYVV RNAs 1, 2 and 4 to B. macrocarpa (Fig. 4) and both are efficient in promoting vascular movement of the virus to noninoculated leaves (Table 1), producing wild-type symptoms there. We conclude that ORF A, if expressed, does not intervene in RNA 3-mediated vascular movement.

The other ORF tested for a possible role in vascular movement lies downstream of ORF N. RNA 3 produces a subgenomic RNA, RNA 3sub, during infection (Bouzoubaa et al., 1991). The 5' terminus of RNA 3sub maps to nucleotide 1230 (Bouzoubaa et al., 1991) (Fig. 1) and the 5'-proximal reading frame on RNA 3sub is ORF S (nucleotides 1274–1393), corresponding to a putative protein of 4.6 kDa (Fig. 3). While
there is no evidence that ORF S is expressed in planta, we have tested the ability of mutants in which ORF S has been eliminated or truncated to support BNYVV vascular movement. In pB35-XB2 (Fig. 3), the ORF S initiation codon was converted to AGA, creating an XbaI site in the process; in pB35 S-Stop (Fig. 3), a termination codon was introduced near
the middle of the ORF by site-directed mutagenesis. Neither
mutation interfered with systemic movement of the virus in B.
macrocarpa or symptom expression (Fig. 5 A, lanes 7 and 8; Table 1). Analysis of the progeny RNA 3 in the systemically
infected leaves following RT–PCR revealed that the mutation in t35 5-Stop was stable (data not shown). In the case of t35-
Xb2, on the other hand, analysis of 12 clones obtained from the
RT–PCR products revealed that only two retained the original
mutation, while in eight clones the AGA had been replaced by
CGA and in two clones by UGA. The significance of these
alterations in the progeny RNA 3 is not known but it should
be noted that in no case was a functional ORF regenerated. We
conclude that the ORF S gene product, if it is expressed, is not
implicated in RNA 3-mediated vascular movement.

Mapping vascular movement determinants on RNA 3 by deletion mutagenesis

Earlier experiments have shown that essential cis-acting
replication signals on RNA 3 are confined to the 5'-proximal
292 residues and the 3'-terminal 70 residues upstream of the
poly(A) tail (Jupin et al., 1990; Gilmer et al., 1992). To better
define the RNA 3 sequences involved in vascular movement we
have tested the ability of transcripts containing various
deletions within the replication-dispensable central region to
promote spread of BNYVV from inoculated to upper B.
macrocarpa leaves. First to be tested was a series of RNA 3
mutants with deletions extending from either the EcoRI site at
nucleotide 381 upstream of the 25 kDa ORF or from an
introduced Smal site at the beginning of the ORF (nucleotide
445) to various downstream restriction sites (see Fig. 3). Transcripts with deletions extending from either of these
positions to nucleotide 658 (pB35ΔESt and pB35ΔSSt) or
nucleotide 755 (pB35ΔEB and pB35ΔSB) permitted the virus to
systemically invade B. macrocarpa with efficiency comparable
to that observed with wild-type RNA 3 (Table 1). Since most of
the aforesaid deletion mutants remove large segments of the
P25 ORF, they provide independent confirmation of our
previous conclusion excluding P25 from a role in vascular
movement.

Transcripts of mutant plasmids with deletions extending
from the EcoRI or Smal sites to the AccI site at nucleotide 1033
(mutants pB35ΔEA and pB35ΔSA) supported systemic move-
ment in only about 10% or 25%, respectively, of the plants
inoculated (Table 1). In the plants that became systemically
infected by these mutants, the onset of infection in the upper
leaves was generally delayed by 7–10 days and virus titres in
the systemically infected leaves were usually lower than in
leaves infected with the control isolate containing t35 (data not
shown). Mutant t35ABA (nucleotides 755–1033 deleted) promoted systemic movement of the virus with somewhat
higher efficiency (Table 1) although a 7–10 day delay in
appearance of the virus in the systemically infected leaves was
typically observed. No vascular movement of BNYVV
occurred when the viral RNA inoculum was supplemented

Discussion

In this paper we have shown that BNYVV RNA 3 functions
in a host-specific manner to enable vascular movement of
BNYVV. Like many plant viruses, BNYVV vascular movement
requires viral coat protein and we assume that virions rather
than viral RNA or a viral RNA–protein complex is responsible
for spread of the infection to non-inoculated leaves of the
plant. It has been shown for several plant viruses that a viral
protein other than the capsid protein and the conventional cell-
to-cell movement protein can also intervene in vascular
transport. For example, the attenuated systemic movement of
the masked strain of TMV has been attributed to inefficient
virus replication in cells of the phloem compartment (Nelson et
al., 1993; X. S. Ding et al., 1995). The helper component-
proteinase is required for systemic movement of tobacco etch
virus (TEV; Cronin et al., 1995), and both the polymerase
component encoded by RNA 1 (Gal-On et al., 1994) and the
protein encoded by ORF 2b (S.-W. Ding et al., 1995) regulate
the rate of systemic spread of cucumber mosaic virus (CMV),
at least in some hosts. The BSMV viral replicase component
encoded by the α gene also intervenes in systemic movement
(Weiland & Edwards, 1994, 1996) and a small ORF in the 3'-
leader sequence of RNA7 of the type-strain of BSMV has been
shown to prevent vascular movement in Nicotiana benthamiana
by down-regulating translation initiation of the replicase
component encoded by the downstream γa gene (Petty et al.,
1990).
BNYVV RNA 3 clearly contains a determinant which is essential for vascular movement in B. macrocarpa but our findings, unexpectedly, have failed to assign this activity to a specific RNA 3 gene product. Thus the P25 ORF can be disrupted by deletion, frameshift or point mutation without disabling long-distance movement. Deletions in the short ORF S which heads RNA 3sub (mutants t35A61 and t35APS) significantly lowered the efficiency of long-distance movement but point mutations which specifically knock-out or truncate ORF S (mutants t35-Xb2 and t35S-Stop) did not inhibit systemic movement, suggesting that the movement-inhibiting effect of the deletion is due to alterations in the RNA sequence or folding rather than loss of the ORF.

The region of RNA 3 between the Acl and Pml sites (nucleotides 1033–1257) shown by deletion analysis to harbour the vascular movement determinant encompasses ORF N. We have shown elsewhere, however, that expression of the N protein is activated only when deletion of the P25 ORF positions ORF N near the 5’ end of RNA 3 (Jupin et al., 1992). Internal deletions of RNA 3 which translationally activate ORF N can occur spontaneously during multiple steps of virus propagation in planta (Koenig et al., 1986; Bouzoubaa et al., 1991) but such deleted forms were not detected in B. macrocarpa (Fig. 4). Furthermore, introduction of a point mutation (mutant t35N-Stop) which prematurely terminates ORF N after translation of a maximum of 21 of the 59 residues in the ORF did not disable vascular movement. Although not excluded by our experiments, we regard it as unlikely that the N-terminal segment by itself would be sufficient to support systemic movement. Our results therefore suggest that a feature or features of the RNA 3 sequence between the Acl and Pml sites rather than an RNA 3 gene product per se is the critical factor.

At present we can only speculate about the mechanism by which the RNA 3 sequence intervenes in a host-specific manner in vascular movement. One possibility is that the region between nucleotides 1033 and 1257 interacts with cellular or viral factors which govern plasmodesmatal gating for entry into, movement within, or egress from the vascular compartment. Alternatively, the RNA 3 sequence could promote systemic movement indirectly by specifically activating viral RNA replication or viral protein expression in cells of the vascular compartment of B. macrocarpa. Evidently, more experiments will be needed to identify important features of the movement core domain and to determine how the domain functions.

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References


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