RNA4-encoded p31 of beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots

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RNA3 and RNA4 of beet necrotic yellow vein virus (BNYVV) are not essential for virus multiplication, but are associated with vector-mediated infection and disease development in sugar beet roots. Here, a unique role for RNA4 in virus transmission, virulence and RNA silencing suppression was demonstrated. Mutagenic analysis revealed that the RNA4-encoded p31 open reading frame (ORF) was involved in efficient vector transmission and slight enhancement of symptom expression in some Beta species. No effects of RNA4 on virus accumulation in infected tissue were observed. Furthermore, the p31 ORF was involved in the induction of severe symptoms by BNYVV in Nicotiana benthamiana plants without affecting viral RNA accumulation. In contrast, RNA3-encoded p25, previously identified as a major contributor to symptom induction in sugar beet, had no such effect on N. benthamiana. In two different silencing suppression assays, neither p31 nor p25 was able to suppress RNA silencing in leaves, but the presence of p31 enhanced a silencing suppressor activity in roots without alteration in viral RNA accumulation. Thus, BNYVV p31 plays a multifunctional role in efficient vector transmission, enhanced symptom expression and root-specific silencing suppression.

INTRODUCTION

Beet necrotic yellow vein virus (BNYVV, genus Benyvirus) causes rhizomania disease of sugar beet and is transmitted by the soil-inhabiting plasmodiophorid Polymyxa betae. BNYVV has a multipartite, positive-sense, single-stranded RNA genome, which consists generally of four, or in some isolates five, distinct RNA species (Tamada, 1999). RNA1 and RNA2 encode housekeeping genes involved in viral RNA replication, assembly and cell-to-cell movement. All field isolates of BNYVV contain two additional RNAs, RNA3 and RNA4. RNA3 (1775 nt) encodes p25, which is involved in the induction of rhizomania symptoms in sugar beet (Koenig et al., 1991; Tamada et al., 1999) and a severe local lesion phenotype on leaves (Tamada et al., 1989; Jupin et al., 1992). The p25 protein is a nucleocytoplasmic shuttling protein (Vetter et al., 2004). RNA4 (1467 nt) is important for efficient transmission of the virus by the plasmodiophorid vector (Tamada & Abe, 1989), although it is unknown whether its protein product, p31, or replication of the RNA4 sequence is required for enhanced transmission efficiency. We observed previously that, in the absence of RNA3, virus isolates containing RNA4 produced strong chlorotic lesions in Tetragonia expansa leaves, whilst those lacking RNA4 produced only faint chlorotic lesions (Tamada et al., 1989). In addition, glasshouse inoculation tests using viruliferous P. betae showed that sugar beet plants infected with RNA4-containing virus had a greater yield loss in tap roots than those infected with virus lacking RNA4 (Tamada et al., 1990; Jupin et al., 1991). These results suggest that, although RNA3 has a major effect on symptom expression, RNA4 also has minor effects on symptom expression.

It is known that viral proteins associated with symptom severity often function as suppressors of RNA silencing (Brigneti et al., 1998; Voinnet et al., 1999; Silhavy & Burgván, 2004; Li & Ding, 2006). In the case of BNYVV, the RNA2-encoded p14 protein (cysteine-rich protein) has a transgene silencing suppressor activity in leaf tissue (Dunoyer et al., 2002; Kondo et al., 2005; Zhang et al., 2005). Because of the above-mentioned involvement of BNYVV RNA3 and RNA4 in symptom expression, it is of interest to know whether these RNA-encoding genes of p25 and p31 contribute to suppression of RNA silencing in shoots and roots. In fact, there is no information as to how the activities of RNA silencing suppressors differ in different organs such as shoots and roots.
In this study, we present evidence that the RNA4-encoded p31 open reading frame (ORF) is required for efficient vector transmission but is also involved in enhanced symptom expression in a host-specific manner. In addition, we show that neither p25 nor p31 is able to suppress RNA silencing in leaves, but that p31 enhances the ability of BNYVV to suppress silencing in roots.

**METHODS**

**Preparation of infectious transcripts of wild-type and mutant RNA4.** DNA manipulation and cloning were performed as described by Sambrook *et al.* (1989). A full-length RNA4 cdNA clone was amplified by RT-PCR from purified RNA of BNYVV field isolate O11. The forward primer 5′-CCAAGCTTATATAGACTCCTAGT-3′ (HindIII site in italics and T7 promoter sequence underlined) and reverse primer 5′-CTCTAGATCGTAAATTACTAGACGAACTGATAATATA-3′ (XbaI site in italics and oligo(dT) tract shown by T(26) site in italics) were used. The PCR products were cloned into the pGEM-T vector and subcloned into the HindIII and XbaI restriction sites of pUC19 to obtain a full-length cdNA clone, pUOF1-6. Five deletion mutants, ΔMd1, ΔMd2, ΔMd3, ΔCter and ΔORF (see Fig. 1 for detailed information on mutations of the constructs) were produced by PCR-based, oligonucleotide-directed mutagenesis within the pUOF1-6 clone. Mutant CCG (Fig. 1) was also obtained by substitution of a CCG triplet for the p31 AUG initiation codon was introduced in mutant CCG. The PCR-amplified fragment was cloned into the pUC19 vector to generate the p31 ORF sequence. All constructs were examined for sequence integrity.

**Fig. 1.** Constructs of BNYVV RNA4 mutants and their effects on symptom expression in *T. expansa* and *N. benthamiana*. The ORF of p31 on wild-type (wt) RNA4 is shown by the filled box that starts at position 380 and extends to position 1228. A CCG substitution for the p31 initiation codon was introduced in mutant CCG. Deletions in the RNA4 sequence are represented by the dashed lines and by map positions. Symptoms on *T. expansa* (*T.e*) and *N. benthamiana* (*N.b*) plants are indicated when inoculated with a mixture of O11-0 (RNA1+2) RNAs and each RNA4 mutant transcript indicated on the left, cs, Chlorotic spots; fcs, faint chlorotic spots; S, severe systemic symptoms; M, mild systemic symptoms.

<table>
<thead>
<tr>
<th>Constructs of RNA4 mutants</th>
<th>Symptoms</th>
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<tbody>
<tr>
<td>P31-ORF</td>
<td>cs S</td>
</tr>
<tr>
<td>ΔCter</td>
<td>Δ1109–1225 fcs M</td>
</tr>
<tr>
<td>ΔMd1</td>
<td>Δ788–1108 fcs M</td>
</tr>
<tr>
<td>ΔMd2</td>
<td>Δ464–802 fcs M</td>
</tr>
<tr>
<td>ΔMd3</td>
<td>Δ939–992 fcs M</td>
</tr>
<tr>
<td>ΔORF</td>
<td>Δ380–1225 fcs M</td>
</tr>
</tbody>
</table>

Plants, viruses and virus inoculations. *T. expansa*, sugar beet (*Beta vulgaris* subsp. *vulgaris*), *Beta macrocarpa*, *Beta vulgaris* subsp. *maritima* M8 and *Nicotiana benthamiana* were used for virus propagation or observation of symptom phenotypes (Tamada *et al.*, 1989; Tamada, 2007). The BNYVV field isolate O11 (RNA1 + 2 + 3 + 4) and the laboratory isolates O11-0 (RNA1 + 2), O11-3 (RNA1 + 2 + 3), O11-4 (RNA1 + 2 + 4) and O11-3d4 (RNA1 + 2 + 3d + 4) were used (Tamada, 2007). The mutant O11-3d4 contains a deletion from nt 726 to 1088 (363 nt) in a 3′-proximal region of the RNA4-encoded p25 ORF. These isolates were obtained from the original isolate O11 by single-lesion transfer in *T. expansa* leaves (Tamada *et al.*, 1989). Unless otherwise stated, virus isolates were propagated in inoculated leaves of *T. expansa*.

P. *betae* inoculation was conducted as described previously (Tamada & Abe, 1989; Tamada & Kusume, 1991). Sugar beet and *B. macrocarpa* were used as test plants for vector transmission tests. They were all grown in special test tubes (24 × 120 mm with a drainage hole) filled with quartz sand. The plants (one plant per tube) were maintained in a growth cabinet at 24 °C with a 16 h light/8 h dark cycle and watered every day with nutrient solution (Tamada *et al.*, 1989). To prepare virus-carrying *P. betae* cultures, RNA3-containing BNYVV isolates were rub-inoculated onto the leaves of *B. macrocarpa* plants. For RNA3-lacking or defective isolates, partially purified virus was carefully rub-inoculated onto the roots of *B. macrocarpa* plants (as these viruses are not systemically infected by *B. macrocarpa*). The ORF of the binary vector pBI121. This construct was transformed into *Agrobacterium tumefaciens* LBA4404 and subsequently into *N. benthamiana* plants.

Production of p31 ORF-transgenic *N. benthamiana* plants. Transgenic *N. benthamiana* plants carrying the p31 ORF sequence were produced as described previously (Andika *et al.*, 2005). The p31 ORF sequence was amplified by PCR using forward primer 5′-TCTAGAATCTAAATACGCTAGCTATAAATATAAATATA-3′ (XbaI site in italics) and reverse primer 5′-AGTCCTAATCTGCGAGAGCACTTTATAA-3′ (SacI site in italics). The PCR-amplified fragment was cloned into the pZERO-2 cloning vector and inserted between the 35S cauliflower mosaic virus promoter and nopaline synthase terminator sequence of the binary vector pBI121. This construct was transformed into *Agrobacterium tumefaciens* LBA4404 and subsequently into *N. benthamiana* plants.

Silencing suppression assay. For the patch co-infiltration assay, each of the p31 and p25 ORFs was amplified by PCR on the BNYVV cdNA clones and inserted between the XbaI and BamHI restriction sites of the pBin61 binary Ti vector (Voinnet *et al.*, 2000) to obtain pBin-p31 and pBin-p25. pBin-HC-Pro, which carries the coding domain of HC-Pro (a known silencing suppressor of potato virus Y), was used as a positive control. The *Agrobacterium* (strain C58C1) culture containing pBin-p31, pBin-p25 or pBin-HC-Pro was mixed with bacteria carrying pBin-GFP and the mixtures were infiltrated into the leaves of *N. benthamiana* plant line 16c, as described by Voinnet *et al.* (1998). For another silencing suppression assay, leaves of *N. benthamiana* plant line 16c were infiltrated with the bacteria containing pBin-GFP. After 20–25 days, when silencing was achieved against GFP transcripts in the whole plant, systemic leaves were inoculated with BNYVV isolates. At 14–17 days post-inoculation
(p.i.), when systemic symptoms began to appear, roots of the infected plants were partially removed and the plants were transplanted into new pots to enhance root growth. GFP fluorescence in leaves and new roots was examined under a UV lamp and with a fluorescence microscope (Zeiss Axioskop), respectively.

**Northern and Western blot analyses.** Northern blot analysis of total RNA from leaf or root tissue was performed as described previously (Andika *et al.*, 2005). The blot was hybridized with digoxigenin-labelled DNA probes specific for the BNYVV RNA1 (nt 3815–6531), RNA2 (nt 144–711), RNA3 (nt 444–1104) and RNA4 (nt 377–1225) sequences. GFP mRNA was detected by hybridization with a digoxigenin-labelled DNA probe. Equal loading was verified by visual estimation of ethidium bromide-stained 28S rRNA. Western blot analysis was the same as for a tissue imprinting assay described previously (Andika *et al.*, 2005). Rabbit anti-BNYVV polyclonal IgG and rabbit anti-p14 protein serum were used for detection of the coat protein and p14 protein, respectively.

**ELISA and RT-PCR.** ELISA and RT-PCR were conducted for quantitative and qualitative detection of virus content and viral RNA, as described previously (Andika *et al.*, 2005).

### RESULTS

**BNYVV p31 ORF is needed for efficient transmission by P. betae**

Our previous study showed that wild-type (wt) RNA4 is required for efficient transmission of BNYVV by the plasmodiophorid *P. betae* (Tamada & Abe, 1989). In an attempt to map its activity domain, six RNA4 mutants, ΔMd1, ΔMd2, ΔMd3, ΔCter, ΔORF and ΔCG, and wt RNA4 (Fig. 1) were used for transmission tests. To allow virus-free *P. betae* to acquire the virus isolates containing RNA4 mutants, transcripts of these RNA4 mutants were mixed with O11-3 RNAs (RNA1, RNA2 and RNA3) and the mixtures were inoculated into *B. macrocarpa* plants, which is a systemic host of the virus after foliar rub-inoculation (Tamada & Kusume, 1991). One week after virus inoculation, virus-free *P. betae* was infested into the roots of these plants. At 30 days after *P. betae* inoculation, virus and viral RNA accumulation in roots was assessed by ELISA and Northern blots. No dramatic differences in virus accumulation in roots were found between wt and mutant viruses (Table 1). Northern blot analysis also showed no apparent differences in accumulation levels of RNA1, RNA2 and RNA3 among these virus isolates (data not shown). Although the reasons remain unknown, the progeny of the ΔORF mutant underwent an internal deletion in the p31-coding region, whilst the ΔORF mutant did not contain the expected short sequence; therefore, these two mutant viruses were excluded from transmission experiments. As controls, the original field isolate O11 and laboratory isolates O11-4 (RNA1 + 2 + 4) and O11-3d4 (RNA1 + 2 + 3d + 4) were also used for transmission in parallel (Table 1). For O11-4 and O11-3d4 isolates, virus-carrying cultures were obtained by rub-inoculation onto the roots of *B. macrocarpa* plants. The virus content of these two isolates in the roots amounted to levels almost similar to those of the wt and mutant viruses (Table 1).

Transmission efficiencies of all of these virus strains were compared using 10-fold dilutions of inoculum sources. Table 1 shows that O11, O11-4 and O11-3d4 isolates, harbouring wt RNA4, were transmitted frequently by *P. betae*, even when the inocula were diluted 1000-fold, whereas O11-3 viruses containing RNA4 deletion mutants or lacking the wt RNA4 sequence were transmitted by *P. betae* only when high levels of viral sources were used.

### Table 1. Transmission of BNYVV isolates containing RNA4 mutants by *P. betae*

<table>
<thead>
<tr>
<th>Virus inoculum*</th>
<th>RNA3</th>
<th>RNA4</th>
<th>Virus content of source roots†</th>
<th>Transmission efficiencies at inoculum dilutions‡</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>×1</td>
</tr>
<tr>
<td>O11</td>
<td>wt</td>
<td>wt</td>
<td>220 (180–300)</td>
<td>5/5</td>
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<tr>
<td>O11-4</td>
<td>–</td>
<td>wt</td>
<td>104 (55–160)</td>
<td>5/5</td>
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<tr>
<td>O11-3d4</td>
<td>del</td>
<td>wt</td>
<td>185 (100–220)</td>
<td>5/5</td>
</tr>
<tr>
<td>O11-3/wt</td>
<td>wt</td>
<td>wt</td>
<td>162 (90–210)</td>
<td>5/5</td>
</tr>
<tr>
<td>O11-3/ΔMd1</td>
<td>wt</td>
<td>ΔMd1</td>
<td>210 (180–270)</td>
<td>13/15</td>
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<tr>
<td>O11-3/ΔMd2</td>
<td>wt</td>
<td>ΔMd2</td>
<td>180 (120–210)</td>
<td>9/15</td>
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<tr>
<td>O11-3/ΔCter</td>
<td>wt</td>
<td>ΔCter</td>
<td>120 (90–180)</td>
<td>10/15</td>
</tr>
<tr>
<td>O11-3/ΔMd3</td>
<td>wt</td>
<td>ΔMd3</td>
<td>148 (80–200)</td>
<td>9/15</td>
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<tr>
<td>O11-3</td>
<td>wt</td>
<td>–</td>
<td>192 (100–280)</td>
<td>11/25</td>
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</table>

*Inocula were prepared as described in the text. The original crude homogenate [100 ml (g fresh root tissue)⁻¹] and a series of 10-fold dilutions were added to test tubes (1 ml per tube) in which healthy sugar beet seedlings were growing.

†Virus content is the mean value from five *B. macrocarpa* roots used as inocula, expressed as µg (g fresh tissue)⁻¹. Figures in parentheses give the range.

‡Virus infection was confirmed by ELISA 3–4 weeks after inoculation. Results are given as the number of sugar beet plants infected/number of sugar beet plants tested. NT, Not tested.
No significant differences in transmission efficiency were found either between the field isolate O11 and the ‘reconstituted’ isolates O11-3/wt (with in vitro-synthesized wt RNA4 transcript) or between viruses with RNA4 deletion mutants and those without RNA4. Thus, RNA4-encoded p31 is required for efficient transmission, whereas, in contrast, efficient transmission does not require the presence of RNA3.

BNYVV p31 ORF enhances symptom expression in B. macrocarpa, B. vulgaris subsp. maritima M8 and T. expansa

BNYVV RNA3 is responsible for rhizomania symptoms (massive proliferation of rootlets and yellowing of shoots) in sugar beet (Tamada et al., 1999), but the role of RNA4 in symptoms remains unknown. First, we examined the effect of RNA4 on symptoms using viruliferous P. betae. P. betae cultures carrying O11, O11-3d4 or several RNA4 mutants as described above were inoculated into the roots of B. macrocarpa and B. vulgaris subsp. maritima M8, which are host plants for distinguishing the virulence of BNYVV isolates (Tamada, 2007). Two to three weeks after vector inoculation, BNYVV O11 produces yellowing symptoms on B. macrocarpa leaves, a characteristic leaf symptom of BNYVV infection. Compared with infection by O11-3/wt or O11, plants inoculated with O11-3/∆Md1, O11-3/∆Md2, O11-3/∆Md3 and O11-3/∆Cter had slightly milder yellowing symptoms (data not shown). In contrast, infection by O11-4 or O11-3d4 failed to induce yellowing symptoms and the infected plants were indistinguishable from the mock-inoculated plants. In parallel experiments carried out in B. vulgaris subsp. maritima M8, similar results were obtained (data not shown). Northern blot analysis of virus-infected roots of B. macrocarpa plants showed no dramatic differences in levels of viral RNA accumulation (RNA1, RNA2 and RNA3) between O11 and O11-3 with any of the RNA4 mutants (Fig. 2). In addition, the presence or absence of RNA3 had no influence on accumulation levels of RNA1, RNA2 and RNA4 (Fig. 2). These results indicated that RNA3-encoded p25 is essential for yellowing symptoms in B. macrocarpa, whilst RNA4-encoded p31 is involved in an increase in yellowing symptoms in the presence of p25 without alteration in accumulation of viral RNA.

Next, we examined the effect of RNA4 on symptom expression in T. expansa by foliar rub-inoculation. Each of the mutated RNA4 transcripts shown in Fig. 1 was mixed with O11-0 RNAs (RNA1 and RNA2) and inoculated onto the leaves of T. expansa plants. Infection by O11-0 with wt RNA4 induced chlorotic spots within 14 days p.i., whereas O11-0 plus each of the RNA4 mutants induced only faint chlorotic spots (Fig. 1), indistinguishable from those of O11-0 (Tamada et al., 1989). No differences in viral RNA1 and RNA2 accumulation were found between O11-0/wt and O11-0 with any RNA4 mutant (data not shown). Thus, the presence of p31 was associated with induction of stronger chlorotic symptoms. Taken together, we concluded that, although RNA3-encoded p25 protein has a major role in symptom expression (Tamada et al., 1989; Jupin et al., 1991), RNA4-encoded p31 also has an effect on the pathogenicity of BNYVV.

BNYVV RNA4 but not RNA3 is associated with severe symptoms in N. benthamiana

N. benthamiana is a systemic host of BNYVV (Andika et al., 2005), in which BNYVV induces a downward curling of the upper leaves by 10–12 days p.i. Infected plants are stunted and the first leaves to undergo curling gradually wilt. After 3 weeks or more, the symptoms change to a severe mosaic with leaf distortions (called severe symptom) (Fig. 3a, top left panel). This is characteristic of BNYVV infection of N. benthamiana. Nevertheless, much milder symptoms are observed occasionally, in which infected plants are not stunted and show only mild symptoms (called mild symptom) (Fig. 3a, top right panel).

Preliminary experiments suggested that the presence of RNA4 was associated with expression of the severe symptoms in N. benthamiana. One example is shown in Fig. 3(b). Of 18 plants inoculated with BNYVV O11, wt RNA4 was detected by RT-PCR in 14 plants with severe symptoms (Fig. 3b, lanes 1–10, 12–14 and 18), but not in the other four plants with mild symptoms (Fig. 3b, lanes 11 and 15–17), except for one (lane 16), which had a deleted form of RNA4. RNA3 was detected in only three plants.

![Fig. 2. Effects of BNYVV RNA4 mutation on foliar symptoms and viral RNA accumulation in B. macrocarpa roots inoculated with viruliferous P. betae. The P. betae cultures carried O11 (RNA1 + 2 + 3 + 4), O11-3 (RNA1 + 2 + 3), O11-3 plus each RNA4 mutant (∆Md1, ∆Md2 or ∆Cter), O11-4 (RNA1 + 2 + 4) or O11-3d4 (RNA1 + 2 + 3d + 4). 3d indicates an internal deletion of RNA3. A mock-inoculated plant (Mock) is shown. The results of Northern blot analysis of total RNA from the roots of plants are shown. Each lane shows an individual plant. The rows show detection of BNYVV RNA1 and RNA2, RNA3, RNA4 and a loading control of ethidium bromide-stained 28S rRNA. Symptoms in shoots were yellowing (Y), mild yellowing (y) and no symptoms (–) as indicated.](image-url)
(Fig. 3b, lanes 1, 16 and 18). When sap from leaves containing RNA3 in this experiment was reinoculated onto *N. benthamiana* plants, RNA3 was detected in only a few plants. These results suggest that RNA3 may be eliminated spontaneously during virus propagation in *N. benthamiana*.

To confirm the effects of RNA3 and RNA4 on symptoms and viral RNA accumulation, BNYVV RNAs extracted from leaves infected with O11-3, O11-4 or O11-0 were inoculated onto *N. benthamiana* leaves. Infection by O11-4 induced severe symptoms in all 20 plants tested, whereas infection by O11-3 and O11-0 produced mild symptoms in all plants (each of 20 plants tested) (Fig. 3a, and data not shown). Accumulation levels of RNA3 in O11-3-infected plants differed among individual plants ranging from high to below detectable levels (Fig. 3c, lanes 3–13). Even plants with relatively high levels of RNA3 accumulation showed mild symptoms (Fig. 3c, lanes 3 and 8). Accumulation levels of viral RNA1 and RNA2 fluctuated slightly, but no correlation was found between amount and symptom severity (Fig. 3c). Thus, BNYVV RNA4 is associated with symptom severity in *N. benthamiana*, whereas RNA3 is not implicated in these severe symptoms.

**BNYVV p31 ORF is required for expression of severe symptoms in *N. benthamiana***

To determine whether the p31-coding region is responsible for symptom severity, each of the mutated RNA4 transcripts was mixed with O11-0 RNAs and inoculated into *N. benthamiana* seedlings. Plants infected with RNA4 mutant ΔMd1, ΔMd2, ΔMd3, ΔCter, ΔORF or CCG had mild symptoms, indistinguishable from those induced by O11-0, whereas plants infected with O11-0/wt or O11-4 had severe symptoms (Table 1, Fig. 3a). Northern blot analysis showed accumulation of wt and mutant RNAs of the expected sizes (Fig. 3d). As observed in Fig. 3(c, d),...
accumulation levels of viral RNAs did not correlate with symptom severity on *N. benthamiana* plants. Thus, BNYVV p31 is required for expression of severe symptoms in *N. benthamiana*.

**Analysis using transgenic plants encoding the p31 ORF sequence**

To examine further the effect of the p31 ORF sequence on severe symptom expression in *N. benthamiana*, transgenic *N. benthamiana* plants expressing the p31 ORF sequence were produced by *Agrobacterium*-mediated transformation. A total of 24 transgenic lines (T1) were inoculated with O11-0 or O11-4 and subjected to Northern blot analysis. Levels of expression of transgene transcripts varied with transgenic lines (Fig. 4a, only nine lines are shown). Some lines showed morphological abnormalities, which were not associated with levels of transgene transcripts. Almost all of the transgenic plants infected with O11-0 had mild symptoms similar to O11-0-infected non-transgenic plants. When inoculated with O11-4, many transgenic lines had severe symptoms similar to O11-4-infected non-transgenic plants, irrespective of expression levels of the transgene transcripts. However, some lines such as R4-7 with an undetectable level of transcripts (Fig. 4a) showed mild symptoms, in which the plants did not contain wt RNA4, but some contained deleted RNA4 (Fig. 4b). The failure of both the p31 transgene and full-length inoculated RNA4 to accumulate suggests that both the p31 transgene and the region of RNA4 containing p31 in the O11-4 inoculum were targeted by silencing. Silencing of the transgene in this R4-7 line was also confirmed by the methylation status of the transgene (data not shown). These results support the proposal that the p31 ORF sequence is involved in expression of severe symptoms on *N. benthamiana*.

**Expression of the p31 protein does not suppress RNA silencing in leaves, but the presence of p31 increases a silencing suppressor activity in roots**

First, we employed a patch co-infiltration assay to determine whether the products of BNYVV p31 and p25 could suppress silencing in *N. benthamiana*. Co-infiltration of 16c plant leaves with two *Agrobacterium* cultures carrying pBin-GFP and pBin-HC-Pro resulted in bright green fluorescence at 5 days post-infiltration (Fig. 5a), whereas co-infiltration with two cultures carrying pBin-GFP and the empty vector pBin61 gave no visible fluorescence under the same conditions due to efficient silencing of the GFP gene (Fig. 5a). When a mixture of *Agrobacterium* cultures containing pBin-GFP and pBin-p31 or pBin-p25 was infiltrated, the infiltrated leaf tissues showed no visible fluorescence (Fig. 5a). Northern blot analysis also revealed that levels of GFP mRNA were greatly reduced in poorly fluorescent areas of leaves co-infiltrated with *Agrobacterium* cultures containing pBin-GFP and pBin-p31, pBin-p25 or the empty vector (Fig. 5b), whereas GFP mRNA was abundant in the green fluorescent tissue co-infiltrated with cultures containing the GFP and HC-Pro genes (Fig. 5b). Thus, expression of both p31 and p25 failed to suppress RNA silencing in the infiltrated leaves of *N. benthamiana*.

Secondly, we compared silencing suppressor activity of BNYVV isolates in leaves and roots using GFP-silenced *N. benthamiana* line 16c plants. Plants that had been completely silenced by agro-infiltration of pBin-GFP were inoculated with O11-4 and O11-0. No green fluorescence was observed in the leaves of plants infected with O11-0 or O11-4 (Fig. 5c, panels 3 and 4). However, it was interesting to note that obvious green fluorescence was observed in most of the new emerging roots infected with O11-4 (Fig. 5c, panel 8), whereas weak green fluorescence was seen in some parts of roots infected by O11-0 (Fig. 5c, panel 7). As controls, in mock-inoculated plants, faint fluorescence was seen only in root tips (Fig. 5c, panel 6), whilst no green fluorescence was observed in shoots (Fig. 5c, panel 2). In GFP-expressing 16c plants, strong green fluorescence was observed in both shoots and roots (Fig. 5c, panels 1 and 5). In further experiments, when inoculated with RNA4 deletion mutants ΔMd1 and ΔCter, no obvious green fluorescence such as seen in O11-4-infected roots was observed (data not shown). In Northern blot analysis, GFP mRNA was abundant in roots infected with O11-4, whereas a lower level of GFP was detected in roots infected with O11-0 (Fig. 5d, right panel). In contrast, GFP.

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**Fig. 4.** Northern blot analysis of BNYVV p31 ORF transgenic *N. benthamiana* plants. (a) Detection of the transgene p31 mRNA in total RNA from leaves of the nine independent transgenic plant lines and non-transgenic (NT) plants. (b) Detection of viral RNAs in total RNA from the leaves of transgenic (line R4-7) and NT plants inoculated with O11-4. Each lane shows an individual plant. Systemic symptoms of each plant are shown beneath the lane as severe (S) or mild (M). Loading controls of ethidium bromide-stained 28S rRNA are shown.
mRNA was not detected in leaves infected with either O11-4 or O11-0 (Fig. 5d, left panel). Thus, GFP mRNA accumulation levels in the leaves and roots were in accordance with the visual observations. In either leaves or roots of the silenced plants, RNA1 and RNA2 were readily detected, regardless of the presence or absence of RNA4 (Fig. 5d).

BNYVV RNA2-encoded p14 protein is known to be a silencing suppressor and therefore we tested whether p31 affected expression of the p14 gene. Western blot analysis showed no difference in expression of the p14 protein or the coat protein between roots infected with O11-4 and O11-0 (Fig. 5e). Taken together, our results indicate that BNYVV p31 does not suppress RNA silencing in the leaves of N. benthamiana, but that p31 enhances the ability of BNYVV to suppress RNA silencing of a transgene specifically in roots.

**DISCUSSION**

Here, we have demonstrated a multifunctional role of BNYVV RNA4-encoded p31 in efficient vector transmission, symptom induction and RNA silencing suppression in roots. The presence of full-length RNA4 in the inoculum sources resulted in approximately 100 times more efficient transmissibility by P. betae than when the inoculum sources contained mutated or no RNA4 (Table 1). The observation that mutation of any portion of RNA4 caused detrimental effects on vector transmission suggests that full-length wt p31 is required for the levels of transmission efficiency exhibited by BNYVV isolate O11 or O11-4. The dispensability of RNA3 in efficient vector transmission is consistent with our previous findings (Tamada & Abe, 1989).

In addition, we showed that BNYVV p31 is also involved in slight enhancement of symptom expression in Beta species.
and T. expansa (Table 1). Interestingly, the presence or absence of wt RNA4 did not affect apparent viral RNA accumulation in roots, suggesting that p31 may not be involved in an increase in virus replication and cell-to-cell movement within roots. We also found that BNYVV p31 is associated with the production of severe mosaic symptoms in N. benthamiana. However, RNA3 had no such effect on symptom severity. In N. benthamiana plants, RNA3 was either eliminated during the initial infection process or disappeared spontaneously during virus propagation (Fig. 3b, c). This is somewhat surprising given the fact that BNYVV p25 facilitates virus multiplication and is responsible for severe yellow local lesion symptoms in its natural host (Beta species) or in differential hosts such as T. expansa (Tamada et al., 1989, 1999; Jupin et al., 1992). Thus, both p31 and p25 are involved in symptom development, but their effects are different and host specific.

Many viral proteins involved in the enhancement of viral pathogenicity are known to function as silencing suppressors (Brigneti et al., 1998; Silhavy & Burgián, 2004; Li & Ding, 2006). In the case of BNYVV, RNA2-encoded p14 protein has been shown to have a transgene silencing suppressor activity in a patch co-infiltration assay on N. benthamiana (Dunoyer et al., 2002; Kondo et al., 2005). In this study, we showed that expression of either p31 or p25 failed to suppress RNA silencing in the co-infiltrated leaves (Fig. 5).

To test further whether BNYVV genes are involved in silencing suppression in roots, we employed a silencing suppression assay in which GFP-silenced N. benthamiana plants (16c) were inoculated with the virus (Voinnet et al., 1999). Generally, in this assay, variation in the tissue specificity of different suppressors has been observed previously, ranging from suppression in all tissues of all infected leaves to suppression only in the veins of new emerging leaves (Voinnet et al., 1999), but there has been no information as to how silencing suppressors are manifested in roots. Our results showed that, in BNYVV-infected plants, neither GFP fluorescence nor an increase in GFP mRNA level was observed in the shoots (or in the veins of new emerging leaves) (Fig. 5). However, it was of particular interest to note that, in the presence of wt RNA4, obvious green fluorescence and an increase in GFP mRNA levels were observed in the roots, whereas BNYVV lacking wt RNA4 or carrying mutated RNA4 gave much weaker green fluorescence and reduced GFP mRNA accumulation (Fig. 5, and data not shown). The observation that silencing suppression occurred in only new emerging roots when the virus arrived there suggests that p31 or the replication of RNA4 may prevent the establishment of silencing in the emerging roots. Taken together, our results indicate that wt RNA4 is able to contribute to silencing suppression of a transgene in the roots, but not in the leaves. This is the first evidence that a viral factor is involved in RNA silencing suppression in a root-specific fashion.

It has been shown that some plant viruses belonging to the family Clustroviridae encode more than one RNA silencing suppressor (Lu et al., 2004; Kreuze et al., 2005). In the case of sweet potato chlorotic stunt virus, one protein enhances silencing suppression activity of another protein (Kreuze et al., 2005). The BNYVV p14 protein is able to suppress RNA silencing in leaves (Kondo et al., 2005; H. Kondo, unpublished data) and, therefore, it could be hypothesized that RNA4 enhanced expression levels of the p14 protein in roots. However, this possibility can be ruled out because the presence of RNA4 did not influence either levels of p14 expression or viral RNA2 accumulation (Fig. 5d, e).

Another possibility is that p31 alone or in combination with other viral factor(s) enhances suppressor activities in roots. To test this idea, additional experiments will be required.

Another question arises as to how the silencing suppression activity of p31 is related to efficient transmissibility by P. betae. The RNA2-encoded p75 readthrough protein is known to be essential for vector transmission (Tamada et al., 1996), whilst p31 has an indirect effect on vector transmission. There is no clear indication that p31 enhances levels of virus accumulation in root tissues, suggesting that the transmission inefficiency of the p31-defective virus is not due to impaired virus spread in host plants. Therefore, it is anticipated that p31 itself or its interaction with other viral factor(s) positively impacts a step(s) of BNYVV transmission, e.g. acquisition, retention or the inoculation process. Alternatively, p31 may augment virus replication as an enhancer of RNA silencing suppression at a particular infection stage of root cells and tissues where the zoospores of P. betae acquire the virus and thereby increase the chance of virus transmission by the vector. One may argue that enhanced virus accumulation should be observed in root cells infected by RNA4-carrying BNYVV. However, the fact that there was no clear augmentation of virus replication in roots may be attributed to saturating levels of virus replication in root cells with lower RNA silencing activities, irrespective of whether p31 is expressed.

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REFERENCES


transgene silencing in *Nicotiana benthamiana*. EMBO J 17, 6739–6746.


