Production and Pathogenicity of Isolates of Beet Necrotic Yellow Vein Virus with Different Numbers of RNA Components

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SUMMARY

Ten Japanese field isolates of beet necrotic yellow vein virus (BNYVV) were transmitted to Tetragonia expansa by inoculation with sap from rootlets of sugar-beet seedlings, to which the virus had been transmitted by the fungus Polymyxa betae. RNA extracted from BNYVV particles obtained from the T. expansa leaves was analysed by agarose gel electrophoresis. Some isolates contained RNA-1 (7.1 kb), RNA-2 (4.8 kb), RNA-3 (1.85 kb) and RNA-4 (1.5 kb) and the others contained, in addition, RNA-5 (1.4 kb). Further isolates, derived from single lesions produced by these isolates, had a variety of RNA compositions. Some contained only RNA-1 and RNA-2. Others contained, in addition, RNA-3, RNA-4, RNA-5 or RNA-6 (1.0 kb), or combinations of two or three of these components. Such isolates generally maintained their RNA composition on further subculture, and their particles had length distributions corresponding to their RNA components. Isolates containing RNA-1 + 2 + 3 caused yellow or strongly chlorotic local lesions in T. expansa, Beta vulgaris, B. macrocarpa and Chenopodium quinoa, and caused systemic stunting and yellow mosaic in B. macrocarpa and, occasionally, in B. vulgaris. In contrast, isolates containing RNA-1 + 2 + 4 or 1 + 2 + 5 induced chlorotic lesions, those containing RNA-1 + 2 + 6 or 1 + 2 induced faint chlorotic lesions, and none of these isolates easily infected B. macrocarpa systemically. Isolates containing different combinations of RNA-3, -4 and -5 induced more severe symptoms than those containing a single RNA. Such synergistic effects occurred between RNA-3 and RNA-4 or RNA-5, or between RNA-4 and RNA-5 or RNA-6, but not between RNA-3 and RNA-6, or between RNA-3 and RNA-6. These small RNA species therefore contain the genetic determinant(s) for lesion type and for ability to infect B. vulgaris and B. macrocarpa systemically. RNA-1 and RNA-2 are viral genome components. The other RNA components have some characteristics of viral satellite nucleic acids but they may not all be dispensable if the BNYVV isolates are to survive in nature.

INTRODUCTION

Beet necrotic yellow vein virus (BNYVV), which causes rhizomania disease of sugar-beet (Tamada & Baba, 1973; Tamada, 1975), has straight tubular particles, is transmitted by the soil-inhabiting plasmodiophoromycte fungus Polymyxa betae, and probably belongs to the furovirus group (Shirako & Brakke, 1984a). The virus particles consist of single-stranded plus-sense RNA associated with coat protein of about 22K (Putz, 1977). It has been shown that a French isolate of BNYVV contains four RNA species with lengths of 7100 (RNA-1), 4800 (RNA-2), 1850 (RNA-3) and 1500 (RNA-4) nucleotides (nt) (Richards et al., 1985). The sequences of all four BNYVV RNA species have been established (Bouzoubaa et al., 1985, 1986, 1987).

The four RNA species of BNYVV are always present in naturally infected sugar-beet roots (Koenig et al., 1986), but the two smaller RNA species, RNA-3 and RNA-4, may undergo

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deletion or disappear when the isolates are passed serially by mechanical inoculation of Chenopodium quinoa leaves (Bouzoubaa et al., 1985; Burgermeister et al., 1986; Kuszala et al., 1986). Although recent observations suggest that the full-length RNA-3 influences the production of strongly chlorotic lesions in C. quinoa (Kuszala et al., 1986) and that one or both of the small RNA species may play a role in vector transmission (Lemaire et al., 1988), more detailed experiments are needed to establish the functions of these small RNA species.

In earlier work (Tamada, 1975), Japanese isolates of BNYVV were found to have particles of two or three lengths, usually about 390 nm (long particles), about 270 nm (intermediate) and 65 to 105 nm (short). The length of the short particles differed between isolates. These isolates also differed in the type of lesions produced in inoculated leaves of Tetragonia expansa: yellow spots (YS), chlorotic spots (CS), necrotic spots (NS) or concentric rings (CR). The YS-inducing isolates were those which had particles 105 nm long and which caused systemic infection in sugar-beet and Beta macrocarpa (Tamada & Baba, 1973; Tamada, 1975). These findings suggested that virus particles of different lengths contain different genetic information, and that the short particles contain information that affects symptom expression. In this paper, we describe the RNA compositions of several Japanese field isolates of BNYVV, the production of laboratory isolates with different combinations of RNA species, and the symptoms induced by these laboratory isolates.

**METHODS**

**Virus sources.** Ten samples (listed in Fig. 1 legend, a and b), derived from rootlets of sugar-beet or soils collected from various areas throughout Hokkaido, were used as field isolates. Three of these cultures, from Shari (S), Tsubetsu (T) and Date (D) respectively (Abe & Ui, 1986), were selected for more detailed study. Inocula of all isolates consisted of dried rootlets containing resting spores of *P. betae*. These rootlets, which were air-dried and stored after careful washing to remove soil debris, were obtained from sugar-beet plants naturally infected with rhizomania or from seedlings grown in soils collected from different sites.

**Inoculation and isolation.** Sugar-beet seedlings (cv. Monohill) were used as test plants for *P. betae* inoculation. They were grown in special test tubes (24 mm wide, 120 mm long with a drainage hole), filled with quartz sand and were supplied with modified one-fifth strength Hoagland and Arnon solution (102 mg KNO₃, 136 mg KH₂PO₄, 236 mg Ca(NO₃)₂·4H₂O, 48 mg MgSO₄·7H₂O, 0.001 mg Fe-EDTA, 0.6 mg H₃BO₃ and 0.4 mg MnSO₄·4H₂O per litre, pH 7.0). Air-dried rootlets were added to rootlets of healthy young sugar-beet seedlings grown in test tubes. The inoculated seedlings were grown in a growth cabinet (kept at 25 °C in light of about 10000 lux for 16 h per day) for 1 to 2 months. Virus-carrying *P. betae* isolates from the original parent cultures were propagated in this system.

For the S, T and D field isolates, several pieces (about 5 mm long) of rootlets containing a large number of resting spores of *P. betae* were selected about 2 months after inoculation with *P. betae* and ground using a pestle with a small volume of distilled water in a mortar. About 100 resting-spore clusters in each suspension were collected by sucking into a glass capillary and then were inoculated to rootlets of healthy sugar-beet seedlings. In this way, other micro-organisms from the rootlets were excluded as far as possible.

To obtain mechanically transmitted virus isolates, rootlets (approx. 0·1 to 0·2 g) of sugar-beet seedlings inoculated with *P. betae* were ground individually with a pestle and mortar in 1 to 2 ml of 0·1 M-phosphate buffer pH 7·4, containing 0·5% 2-mercaptoethanol. The extract was inoculated into leaves of *T. expansa* plants. The inoculated leaves were rinsed immediately with water and the plants were kept in an insect-free glasshouse.

To obtain single-lesion isolates, the virus extract was inoculated at a series of dilutions to *T. expansa*. A single well separated lesion in an inoculated leaf was used as the virus source for the next inoculation. Each single-lesion isolate was passed at least five times consecutively through single lesions in *T. expansa*. The resulting single-lesion isolates produced uniform symptoms when propagated in inoculated *T. expansa* leaves, and these were frozen at −80 °C until they were used for analysis and for inoculum sources.

**Virus purification and RNA extraction.** Batches (5 g) of inoculated *T. expansa* leaves or sugar-beet rootlets were homogenized in 25 ml of 0·5 M-sodium borate buffer pH 9·0, containing 1 mM-Na₂EDTA. The extract was filtered through two layers of cheesecloth and centrifuged in a Hitachi RPR-20 rotor at 10000 r.p.m. for 10 min. After addition of Triton X-100 to 2%, the supernatant fluid was layered onto a pad of 6 ml of 20% sucrose in grinding buffer and centrifuged in a Centrikon TFF 70.38 rotor at 35000 r.p.m. for 1 h at 4 °C. The resulting pellet was resuspended in 0·5 ml distilled water and the suspension of virus particles was frozen at −20 °C until it was needed.

To obtain viral RNA, partially purified virus was disrupted by incubation at 60 °C for 5 min in 2%, SDS, 1%, 2-mercaptoethanol in E buffer (10 mM-Na₂HPO₄, 20 mM-Na₂HPO₄ and 1 mM-Na₂EDTA at pH 7·2). Protein was removed by emulsification with an equal volume of water-saturated phenol containing 0·1% 8-hydroxyquinoline.
Pathogenicity of BNYVV isolates

The RNA in the aqueous phase was denatured by incubation at 65 °C for 10 min in 2-2 M-formaldehyde at pH 7.0 (Boedtker, 1971; Lehrach et al., 1977).

RNA gel electrophoresis. A 50 μl RNA sample was loaded into each well of 1-3% agarose (Takara, Type H14) tube gels (7 mm × 110 mm) and electrophoresed at a constant current of 6 mA per tube for 2 to 2.5 h. Buffer was used in the gel and buffer reservoir. To get a flat surface the gels were placed upside down until set. After electrophoresis, the gels were stained with 0-005% Stains-all (Eastman Kodak) in 50% formamide and destained in distilled water under a dim light (Dahlberg et al., 1969). For Mr determination, tobacco mosaic virus RNA (Mr 2.21 × 106, 6.4 kb, for the Na salt; Goelet et al., 1982) and Escherichia coli ribosomal RNA species (Mr 1.01 × 106, 2.9 kb, and Mr 0.53 × 106, 1.5 kb for the Na salt; Brosius et al., 1978, 1980) were used as size standards.

Electron microscopy. Small pieces of inoculated leaf of T. expansa were cut on a glass slide in a drop of 2% phosphotungstic acid solution at pH 7.0. The droplet was transferred with a capillary to a carbon-Formvar coated grid, and the excess fluid was removed with a piece of filter paper. Air-dried preparations were examined immediately in the electron microscope (JEM-100CXII).

Symptoms in test plants. The symptoms produced by virus isolates were compared using manually inoculated test plants kept in a glasshouse at a mean temperature of about 23 °C. Inoculation tests were conducted in all seasons of the year. Infection of test plants was confirmed by ELISA (Tamada & Hagita, 1982) if the symptoms were doubtful.

RESULTS

Comparisons of RNA compositions of different field isolates of BNYVV

The 10 BNYVV-carrying P. betae samples used as inocula were originally collected from single fields in different areas in Hokkaido. Virus isolates were obtained from each sample by inoculating T. expansa leaves with sap from rootlets of sugar-beet seedlings, which had been previously inoculated with P. betae. Most of these root extracts produced only a few lesions per T. expansa leaf, because the virus content of rootlets was relatively small. Sap from a group of lesions produced by the first inoculation was therefore inoculated to further T. expansa, and the RNA was extracted from virus particles in inoculated leaves for analysis by agarose gel electrophoresis. The result (Fig. 1a) showed that four or more bands in agarose gels were detected in all the samples tested. The larger two and smaller two bands were considered to correspond to four RNA species: RNA-1, RNA-2, RNA-3 and RNA-4, described by Richards et al. (1985). In our tests, however, eight isolates contained a large amount of an RNA species a little smaller than RNA-4 (Fig. 1a, lanes 1, 3, 4, 5, 7, 8 and 9, and b, lane 1), whereas two isolates did not contain this additional component (Fig. 1a, lanes 2 and 6).

To ascertain whether the RNA patterns described above were obtained consistently, an attempt was made to detect RNA in a root extract from seedlings inoculated by P. betae. The result (Fig. 1b) showed that the RNA composition from the root extract was the same as that from T. expansa leaves inoculated with the same parent culture, although the relative amount of RNA-3 to other RNA species apparently increased. In many instances, however, it was difficult to obtain consistently such obvious bands in gels from root extracts, because their virus content was small. In further tests in which the T and I (Ikeda) field isolates were used, such RNA patterns were maintained after further two to four successive passages in T. expansa leaves (Fig. 1c). In addition, to ascertain whether there are differences between the RNA patterns obtained from different plants inoculated with the same field isolates, RNA preparations from 12 sugar-beet seedlings inoculated with the S field isolate by P. betae were analysed. No differences were found in the RNA patterns from different plants, although the relative amount of each RNA species varied (data not shown).

Production of single-lesion isolates having different RNA components

When rootlets of sugar-beet inoculated by P. betae or systemically infected sugar-beet leaves were used as sources of sap to inoculate T. expansa leaves, various types of lesions appeared in inoculated leaves: NS, YS, CS and CR (Tamada, 1975). Therefore T. expansa was used as the test plant for better single-lesion isolation. Most leaves had a mixture of these lesion types (Fig. 2a), although the YS type predominated. When sap from a single yellow lesion of the S field isolate was inoculated to T. expansa plants, the leaves developed a mixture of YS and CS,
Fig. 1. Agarose gel electrophoresis of BNYVV RNAs from different field isolates and different preparations. (a) RNA preparations from leaves of *T. expansa* inoculated with different field isolates: lane 1, S (Shari); lane 2, T (Tsubetsu); lane 3, To (Toya); lane 4, M (Makkari); lane 5, So (Sobetsu); lane 6, B (Bihoro); lane 7, H (Hombetsu); lane 8, Sa (Saroma) and lane 9, I (Ikeda). (b) RNA preparations from D (Date) field isolate: lane 1, from inoculated leaves of *T. expansa*; lane 2, from root extract of sugar-beet seedlings inoculated by *P. betae*. (c) RNA preparations from different serial passages in *T. expansa*: lane 1 to 3, 2nd, 3rd and 4th passages of T field isolate; lane 4 to 6, 2nd, 3rd and 4th passages of I field isolate. Pointers 1 to 4 indicate RNA-1, RNA-2, RNA-3 and RNA-4, respectively.

whereas sap from chlorotic lesions induced chlorotic lesions without YS. During serial passages of isolates from yellow lesions, however, the lesions became uniformly bright yellow, although a few faint CS always developed at later time after inoculation. Variants cultured from such faint chlorotic lesions also induced only faint chlorotic ones.

In further experiments, RNA preparations from the single-lesion isolates were analysed by agarose gel electrophoresis. It was found that isolates causing the YS type of lesion contained RNA-1, RNA-2, RNA-3 and RNA-4 or RNA-1, RNA-2 and RNA-3, whereas those from plants showing the CS type of lesion contained RNA-1, RNA-2 and RNA-4 or RNA-1 and RNA-2. As noted above, in general, isolates with RNA-3 (without RNA-4) caused yellow lesions, those with RNA-4 caused chlorotic lesions, and those without either RNA-3 or RNA-4 induced faint chlorotic lesions. Thus single-lesion isolates obtained from the S field isolate were
Fig. 2. Symptoms induced by the S field isolate and by single-lesion isolates in inoculated leaves of T. expansa. Various types of lesions in leaf inoculated with root extract containing (a) S field isolate, (b) isolate S-3, (c) isolate S-4 and (d) isolate S-0.

referred to as S-34 (YS type with RNA-1+2+3+4), S-3 (YS, RNA-1+2+3), S-4 (CS, RNA-1+2+4) and S-0 (CS, RNA-1+2) (Fig. 2b, c and d). Such isolates maintained their different combinations of RNA components after several serial passages of bulk cultures in T. expansa. However, isolates having only RNA-1 and RNA-2 could always be obtained from some of the leaves produced by the other isolates (S-34, S-3 or S-4).

In a second set of experiments, similar single-lesion isolates were selected from the T field isolates, in which only four RNA species were detected originally. The resulting single-lesion isolates were referred to as T-3 (YS, RNA-1+2+3), T-4 (CS, RNA-1+2+4) and T-0 (CS, RNA-1+2) as shown in Fig. 3(b).

In the third set of experiments, single-lesion isolates were similarly obtained from the D field isolate: D-3 (YS, RNA-1+2+3), D-4 (CS, RNA-1+2+4) and D-0 (CS, RNA-1+2) (Fig. 3c, lanes 6, 7 and 10). However, from the D culture, in which RNA species smaller than RNA-4 were detected (Fig. 3c, lane 1), other isolates were obtained which had fifth and sixth RNA species (designated RNA-5 and RNA-6, respectively). Our hybridization tests show that cDNA specific to RNA-5 hybridized strongly with RNA-6 but did not hybridize with either RNA-1, RNA-2, RNA-3 or RNA-4 (Saito et al., 1988; Kiguchi et al., 1988, unpublished data). These new isolates were referred to as D-5 (CS, RNA-1+2+5) and D-6 (CS, RNA-1+2+6). During serial passages and agarose gel analysis, further isolates with other mixtures of RNA species were obtained: D-34 (YS, RNA-1+2+3+4), D-45 (YS, RNA-1+2+4+5), D-46 (YS, RNA-1+2+4+6), D-56 (CS, RNA-1+2+5+6) (Fig. 3c, lanes 2, 3, 4 and 5).

These results showed that all the field isolates of BNYVV, which were inoculated by the fungus vector P. betae, can give rise to isolates with and without each of RNA-3, RNA-4, RNA-
Fig. 3. Agarose gel electrophoresis of BNYVV RNA from leaves of *T. expansa* inoculated from different original field isolates and their single-lesion isolates. (a) Lane 1, S field isolate; lane 2, S-34; lane 3, S-3; lane 4, S-4; lane 5, S-0. (b) Lane 1, T field isolate; lane 2, T-3; lane 3, T-4; lane 4, T-0. (c) Lane 1, D field isolate; lane 2, D-34; lane 3, D-45; lane 4, D-45; lane 5, D-56; lane 6, D-3; lane 7, D-4; lane 8, D-5; lane 9, D-6; lane 10, D-0. Pointers 1 to 6 indicate RNA-1 (7.1 kb), RNA-2 (4.8 kb), RNA-3 (1.85 kb), RNA-4 (1.5 kb), RNA-5 (1.4 kb) and RNA-6 (1.0 kb), respectively.

5 and RNA-6, depending on the parent isolate. They also indicated that isolates having RNA-3 induce bright yellow lesions on leaves of *T. expansa* and those with RNA-4 or RNA-5 cause chlorotic lesions, whereas isolates with RNA-6 or without small RNA species cause faint chlorotic spots or rings. These results also support the suggestion (Bouzoubaa et al., 1985) that the RNA-1 and RNA-2 may be needed for virus infection, whereas RNA-3, RNA-4, RNA-5 and RNA-6 behave like satellite RNA.

Sizes of the virus particles and RNA species of single-lesion isolates

The $M_r$ values of BNYVV RNAs were determined after formaldehyde denaturation. A preliminary experiment revealed that a plot of $\log_{10}$ of $M_r$ against distance of migration after formaldehyde denaturation of TMV RNA and *E. coli* ribosomal RNA was linear when 1.3% agarose was used. The $M_r$ value and number of nucleotides of each RNA species of BNYVV
Pathogenicity of BNYVV isolates

Fig. 4. Particle-length distribution of leaf-dip preparations (stained with 2% phosphotungstic acid pH 7.0) of the following isolates of BNYVV: (a) S-3 (546 particles), (b) S-4 (584 particles), (c) D-5 (631 particles), (d) D-6 (588 particles) and (e) S-0 (573 particles). Tobacco mosaic virus particles (300 nm) were used as the standard. Particle lengths are divided into increments of 9 nm. Figures in parentheses are modal length (nm).

detected in this study was estimated as follows: RNA-1 Mr 2.3 × 10^6, 6746 nt excluding the poly(A) tail; RNA-2 Mr 1.6 × 10^6, 4612 nt; RNA-3 Mr 0.65 × 10^6, 1774 nt; RNA-4 Mr 0.54 × 10^6, 1467 nt; RNA-5 Mr 0.51 × 10^6, 1400 nt; RNA-6 Mr 0.36 × 10^6, 1000 nt. The numbers of nucleotides correspond to those in the four RNA species of a French isolate (Richards et al., 1985; Bouzoubaa et al., 1985, 1986, 1987). There was no apparent difference between the S, T and D field isolates, in the size of RNA-1, RNA-2, RNA-3 or RNA-4.

It was reported that Japanese isolates of BNYVV have particles of two or three predominant lengths, and that the lengths of the short particles differ with the isolate (Tamada, 1975). Further experiments were therefore made to compare the lengths of particles of single-lesion isolates containing different RNA species. Isolate S-0 (RNA-1 and 2) produced particles predominantly with lengths of 384 nm (long) and 264 nm (intermediate) (Fig. 4). Isolates with smaller RNA species contained many shorter particles, in addition to long and intermediate-sized particles of about the same lengths as in isolate S-0. The modal lengths of the short particles of isolate S-3 (containing RNA-3), S-4 (RNA-4), D-5 (RNA-5) and D-6 (RNA-6) were 105 nm, 91 nm, 82 nm and 64 nm, respectively (Fig. 4). Each of these isolates contained many more short particles than long or intermediate-sized ones. Thus it was confirmed that the presence of each viral RNA species corresponds with the presence of virus particles of the appropriate length.

**Symptom expression by single and mixed infections of single-lesion isolates**

Because the single-lesion isolates containing different RNA species induced different types of lesion in *T. expansa* (Fig. 2, Table 1), the symptom differences between isolates S-3, S-4, D-5, D-6 and S-0 were determined in other manually inoculated plant species in glasshouse conditions. In *C. quinoa*, isolate S-3 induced severe chlorotic (later yellowish) lesions, whereas the other isolates (S-4, D-5, D-6 and S-0) caused chlorotic lesions. In *C. amaranticolor*, all the
Table 1. Symptoms produced in two host species by single and mixed infections of single-lesion isolates of BNYVV containing different RNA species

<table>
<thead>
<tr>
<th>Inoculum source</th>
<th>RNA components</th>
<th>T. expansa</th>
<th>B. macrocarpa</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-3</td>
<td>1 + 2 + 3</td>
<td>YS 0</td>
<td>YM ST</td>
</tr>
<tr>
<td>S-4</td>
<td>1 + 2 + 4</td>
<td>CS 0</td>
<td>fCS 0</td>
</tr>
<tr>
<td>D-5</td>
<td>1 + 2 + 5</td>
<td>CS 0</td>
<td>CS (YM)</td>
</tr>
<tr>
<td>D-6</td>
<td>1 + 2 + 6</td>
<td>fCS 0</td>
<td>fCS 0</td>
</tr>
<tr>
<td>S-0</td>
<td>1 + 2</td>
<td>fCS 0</td>
<td>fCS 0</td>
</tr>
<tr>
<td>S-3 + S-4 + D-5</td>
<td>1 + 2 + 3 + 4 + 5</td>
<td>YS (NS) 0</td>
<td>YM ST</td>
</tr>
<tr>
<td>S-3 + S-4</td>
<td>1 + 2 + 3 + 4</td>
<td>YS (NS) 0</td>
<td>YM ST</td>
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<tr>
<td>S-3 + D-5</td>
<td>1 + 2 + 3 + 6</td>
<td>YS 0</td>
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<td>YM ST</td>
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<td>CS (YM)</td>
</tr>
<tr>
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<td>1 + 2 + 4 + 6</td>
<td>YS 0</td>
<td>CS 0</td>
</tr>
<tr>
<td>D-5 + D-6</td>
<td>1 + 2 + 5 + 6</td>
<td>CS 0</td>
<td>fCS 0</td>
</tr>
</tbody>
</table>

* I, inoculated leaves; S, systemically infected leaves; CS, chlorotic spots; fCS, faint chlorotic spots or symptomless; YS, yellow spots; NS, chlorotic or yellow spots which later become whitish or necrotic; YM, yellow mosaic or mottle; ST, stunting; 0, not infected; parentheses indicate symptoms produced occasionally.

isolates caused chlorotic and necrotic lesions, although the lesions of S-4, D-5, D-6 and S-0 tended to be more necrotic than those of S-3. In sugar-beet (B. vulgaris), isolate S-3 induced chlorotic and yellowish lesions on inoculated leaves. The yellow lesions enlarged and tended to coalesce, spreading along the veins. A few plants that were inoculated as young seedlings became systemically infected, and developed typical yellow vein symptoms (Tamada & Baba, 1973). In contrast, isolates S-4, D-5, D-6 and S-0 caused faint chlorotic lesions or no symptoms in inoculated leaves, and did not invade the plants systemically. In B. macrocarpa, isolate S-3 caused yellow lesions in inoculated leaves, followed in many plants by systemic infection and by severe stunting and systemic mosaic and mottle (Table 1). In contrast, isolates S-4, D-5, D-6 and S-0 caused faint chlorotic lesions or no symptoms in inoculated leaves, and S-4, D-6 and S-0 did not become systemic, but D-5 later produced systemic mild mottle in some plants (Table 1). Systemic infection by these isolates was also confirmed by ELISA. The other isolates (T-3, T-4, T-0, D-3, D-4 and D-0) induced almost the same reaction as the isolates from the S culture containing RNA species of the same size. In general, the symptoms were more intense and appeared more rapidly in the summer than in the winter. These results indicate that the presence of RNA-3 resulted in yellow symptoms in several species of host plant, and also enabled systemic infection to occur in sugar-beet and B. macrocarpa, whereas the other smaller RNA species had little or no effect on symptom expression.

To test for interactions between the small RNA species, further experiments were made in which plants were inoculated with mixtures of isolates containing RNA species of different size. Table 1 shows the effects of these inocula on the two plant species. When a mixture of two or three isolates such as S-3, S-4 and D-5, S-3 and S-4, or S-3 and D-5, was used as inoculum, the symptoms were usually more severe than those produced by isolate S-3 alone. Some chlorotic and yellow spots that developed in inoculated leaves of T. expansa later developed into whitish or necrotic lesions in which all the cells were killed. B. macrocarpa plants were also more severely stunted, and later leaves became necrotic and frequently the plants died. When isolate S-4 was mixed with isolate D-5 or D-6, yellow (strongly chlorotic) lesions were produced in inoculated leaves of T. expansa, but the mixed infection of D-5 and D-6 caused only chlorotic lesions. The yellow lesions produced by these mixed inoculations were somewhat paler than those produced by S-3. A similar effect to that of mixing isolate S-4 and D-5 was also observed by inoculating mixtures of D-5 and T-4 or D-4, which contained RNA species of the same size as S-4. Thus isolates containing combinations of two or three small RNA species have a stronger effect on symptom expression than each alone, and in the mixtures each RNA species remained after
serial passages in the same host species, but RNA-5 tended to disappear during serial passages in isolates which contained RNA-5 and RNA-6. This fact indicates that there are synergistic interactions between some of the smaller RNA species.

**DISCUSSION**

In this paper, we described the RNA compositions of 10 field isolates of BNYVV in Japan, production of single-lesion isolates using *T. expansa* as the isolation and propagation host and their pathogenicity on several host plants.

Because the virus content of sugar-beet roots is small, the virus was multiplied in *T. expansa* plants by inoculation with sap from rootletts of sugar-beet. RNA extracted from BNYVV particles obtained from inoculated leaves was analysed by agarose gel electrophoresis. One result indicated that the RNA composition of virus purified directly from infected sugar-beet roots was maintained well after two passages in *T. expansa* leaves (Fig. 1b). Another result showed that RNA patterns were stable even after several more passages of the isolates in this host plant (Fig. 1c). Thus RNA patterns obtained after two passages in *T. expansa* leaves are considered to represent those in sugar-beet roots affected by rhizomania in the field, although the possibility of minor change in relative amounts of RNA species is not excluded. From the RNA analysis of the 10 field isolates, we found that two isolates contained only RNA-1, RNA-2, RNA-3 and RNA-4, the sizes of which correspond to those reported for a French F2 isolate (Richards *et al.*, 1985) i.e. 7-1 kb, 4-8 kb, 1-85 kb and 1-5 kb, respectively, whereas eight isolates contained, in addition, RNA species smaller than RNA-4 (Fig. 1a).

In our work, an RNA smaller than RNA-4 and named RNA-5 (1-4 kb) was found in the D field isolate. Our hybridization experiments (Saito *et al.*, 1988; Kiguchi *et al.*, 1988, unpublished results) show that cDNA copies corresponding to RNA-5 do not hybridize to either RNA-3 or RNA-4, indicating that the RNA-5 is not formed by loss of materials from either of these two RNA species. This RNA-5 which is associated with a modal particle length of 82 nm has not been reported from BNYVV isolates from Europe or the U.S.A. Further hybridization tests (data not shown) have shown that RNA-5 can also be detected in all eight of the field isolates noted above, although single-lesion isolates containing RNA-5 were not obtained from the S isolate culture in the experiments illustrated in Fig. 3a. This suggests that RNA-5 seems to be prevalent in fungus-infested fields in Hokkaido.

*T. expansa* plants were found to be most useful for separating single-lesion isolates of BNYVV, because they showed at least four types of distinguishable local lesions in inoculated leaves, i.e. necrotic YS, YS, CS and faint CS. Based on these different lesion types, virus isolates were established by several single-lesion transfers. All the single-lesion isolates contained RNA-1 and RNA-2 with or without the other smaller RNA species, indicating that the presence and the kind of smaller RNA species affect lesion types. In one particular case, one isolate obtained from some subcultures of the D field isolates contained an RNA of 1-0 kb which could not be detected in the original culture and was named RNA-6.

Both RNA-5 and RNA-6 detected in this study are different from small RNA species so far detected from extracts of sugar-beet roots and *C. quinoa* leaves by Northern blots with cDNA probes specific to RNA-3 and RNA-4 (Burgermeister *et al.*, 1986; Koenig *et al.*, 1986; Kuszala *et al.*, 1986). The small RNA species detected directly from extracts by Northern blots are most likely to be deleted forms or subgenomes of RNA-3 or RNA-4, because they cross-hybridize with cDNA probes specific to RNA-3 or RNA-4. Our preliminary data using cDNA probes specific to each RNA species indicate that RNA-5 is not a deleted form of either RNA-1, RNA-2, RNA-3 or RNA-4, and therefore it is a distinct RNA species. RNA-6 hybridized with cDNA probes specific to RNA-5, suggesting that this RNA-6 may be derived from RNA-5 (Kiguchi *et al.*, 1988, unpublished results), as with the deleted forms of RNA-3 and RNA-4 of German G1 isolate (Bouzoubaa *et al.*, 1985) and the deletion mutants of RNA-2 of soil-borne wheat mosaic virus (Shirako & Brakke, 1984b). They are packaged in virus particle protein, so that each RNA species gives rise to virus-like particles of characteristic length. RNA-5 seems to be a common component of BNYVV in fields in Hokkaido. RNA-6 might also be present at undetectable
levels in the fields or might have arisen during the passages in *T. expansa*. We are currently conducting further experiments using Northern blotting with specific cDNA probes to answer these questions.

In terms of effects of small RNA species on pathogenicity, RNA-3 was found to increase symptom severity in inoculated leaves of *T. expansa*, *B. vulgaris*, *B. macrocarpa* and *C. quinoa*, whereas RNA-4, RNA-5 and RNA-6 have little or no effects. Kuszala *et al.* (1986) likewise reported that isolates containing full-length RNA-3 caused strongly chlorotic spots on *C. quinoa* but isolates in which RNA-3 is deleted or lost caused milder symptoms. RNA-3 was also shown to confer the ability to infect *B. macrocarpa* and *B. vulgaris* systemically. In some cases, isolates containing RNA-5 also infected *B. macrocarpa* systemically, but not as efficiently as those containing RNA-3. In addition, our results indicate that there are some synergistic effects between smaller RNA species which affect symptom expression. For example, the presence of RNA-3 with RNA-4 and/or RNA-5 in the inoculum causes strongly yellow spots (together with necrotic spots occasionally) in *T. expansa*. The presence of RNA-4 with RNA-5 or RNA-6 causes strongly chlorotic spots. However, no synergistic effect was found between RNA-3 and RNA-6, corresponding to the findings that these two RNA species are related in their nucleotide sequences.

The fact that neither RNA-1 nor RNA-2 could be eliminated from cultures suggests that they are both needed for virus infection. In contrast, our results and those of Bouzoubaa *et al.* (1985) show that the small RNA species are not necessary for replication of RNA-1 and RNA-2, or for production of virus particles. In this respect and, for the French isolates, in having nucleotide sequences different from RNA-1 and RNA-2 (Bouzoubaa *et al.*, 1985, 1986, 1987), they resemble satellite RNA (Murant & Mayo, 1982). However, the fact that RNA-3 and RNA-4, and probably RNA-5, were detected in all the original field isolates suggests that these small RNA species may confer some properties required for virus survival, such as fungus transmissibility (Lemaire *et al.*, 1988; Tamada *et al.*, 1988) and multiplication of virus in roots of sugar-beet. In this respect, further investigations using the established single-lesion isolates are in progress.

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**REFERENCES**


Pathogenicity of BNYVV isolates


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