Shortened forms of beet necrotic yellow vein virus RNA-3 and -4: internal deletions and a subgenomic RNA

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Beet necrotic yellow vein virus RNA-3 and RNA-4, produced as full-length biologically active transcripts in vitro, can undergo spontaneous internal deletions when inoculated onto Chenopodium quinoa leaves along with RNA-1 and -2. The deletion process is specific, giving rise to only a few major species, and can be rapid; deleted forms appear after only one or two passages in leaves. In one of the shortened forms of RNA-4, the deletion precisely eliminated one copy of a 15 nucleotide (nt) direct sequence repeat from the full-length prototype sequence, suggesting that 'copy-choice' switching of the replicase-template complex from one repeat to the other during RNA replication was responsible for the generation of this deletion. The deletion found in a major shortened form of RNA-3, on the other hand, did not occur near sequence repeats but began with GU and ended with AG like a nuclear intron sequence. Thus it is possible that the deleted sequence has been removed by splicing. However, two other deletions that were characterized were not associated with either of these types of sequence feature. An approximately 600 nt 5'-terminally truncated non-encapsidated form of RNA-3 was also detected in infected plant tissue. The evidence suggests that it is a subgenomic RNA derived from RNA-3.

Introduction

Beet necrotic yellow vein virus (BNYVV), a possible member of the furovirus group (Shirako & Brakke, 1984), has a quadripartite genome consisting of 5' capped, 3'-polyadenylated plus-sense RNA (Bouzoubaa et al., 1987). BNYVV is transmitted in the field by the soil-borne fungus Polymyxa betae and is found associated with roots of sugar beet displaying symptoms of rhizomania disease (Tamada & Baba, 1973). Under these conditions all four genome components are invariably present (Koenig et al., 1986; Bouzoubaa et al., 1988; Tamada et al., 1989). Isolates of BNYVV maintained in the laboratory, on the other hand, are customarily propagated by mechanical inoculation onto leaves of Chenopodium quinoa or Tetragonia expansa and in these circumstances neither of the two smallest genomic RNAs, RNA-3 [approximately 1850 nucleotides (nt) including the 3' poly(A) tail] and RNA-4 (approximately 1550 nt), is required for infection (Koenig et al., 1986; Kuszala et al., 1986; Burgermeister et al., 1986; Quillet et al., 1989). These observations are consistent with the idea that the two longest RNAs, RNA-1 and -2, code for basic functions required in all hosts while RNA-3 and -4 are functional in the natural infection process, that is, fungus-mediated infection of roots and proliferation within root tissue (Koenig et al., 1986; Lemaire et al., 1988; Tamada & Abe, 1989). Recent findings indicate that RNA-4 is essential for efficient fungal transmission (Tamada & Abe, 1989) while RNA-3 may facilitate viral multiplication and/or movement in roots (Koenig & Burgermeister, 1989; Tamada & Abe, 1989).

During serial propagation in leaves, BNYVV isolates sometimes lose one or both small RNAs. In other isolates, the small RNAs are present but appear to have undergone deletion. This deletion process is of interest because it results in the appearance of a limited number of discrete species rather than a heterogeneous population of shortened forms (Bouzoubaa et al., 1985; Kuszala et al., 1986; Burgermeister et al., 1986; Tamada et al., 1989). This means that there are 'hot spots' or preferred sites for deletion within RNA-3 and RNA-4 which can manifest themselves in the absence of selective pressure for maintenance of the full-length molecules. In previous studies we have characterized the deleted forms of
RNA-3 and RNA-4 found in several BNYVV isolates (Bouzoubaa et al., 1985, 1988). This work has shown that the deletions arise from a loss of a continuous stretch of sequence rather than several non-contiguous segments, and that the deletions lie within the RNA-3 and -4 molecules, leaving the extremities intact. The latter observation is consistent with the finding, based on deletion mutagenesis of a biologically active RNA-3 transcript, that the approximately 300 5'-terminal residues of RNA-3 are indispensable in cis for successful amplification of RNA-3 in infected leaves (Jupin et al., 1990).

Study of the deleted RNA-3 and RNA-4 forms in natural BNYVV isolates has two drawbacks. First, the RNA-3 and RNA-4 in such isolates almost certainly consist of a population of closely related sequences (Holland et al., 1982; Domingo & Holland, 1988) causing consequent uncertainty as to the exact nature of the form that has undergone deletion (sequence analysis of deleted molecules, of course, provides no information about the sequence of the segment deleted from the full-length form). Second, it is difficult to assess when, and how frequently, deletions occur in natural isolates, many of which have been propagated in leaves for long periods of time. It is evident that use of a BNYVV isolate produced by transcription of cloned viral cDNA circumvents such problems by providing a homogeneous starting material and a means of ‘setting the clock to zero’ with respect to the analysis of deletion. In this paper we describe experiments in which such a transcript-based isolate, derived from cloned cDNA of RNA of BNYVV isolate F3 (Ziegler-Graff et al., 1988; Quillet et al., 1989), was used to examine the deletion process. We have also characterized a 5'-terminally truncated RNA-3 species found in plants infected with either natural or transcript-derived BNYVV isolates. This species, termed RNA-3sub, is not encapsidated and corresponds to the approximately 600 3'-terminal residues of RNA-3. It is probably a subgenomic RNA.

**Methods**

**Virus.** BNYVV isolate F3 and infectious run-off transcripts of BNYVV RNA-1 to -4 have been described elsewhere (Ziegler et al., 1985; Ziegler-Graff et al., 1988; Quillet et al., 1989). Virus isolates were propagated by mechanical inoculation of local lesions homogenized in 50 mM-potassium phosphate pH 7-2, or mixtures of transcripts (Quillet et al., 1989) onto leaves of C. quinoa (Fig. 1 a) and RNA-4 (Fig. 1 b) in infected leaf tissue after each passage. In addition to full-length RNA-3 and -4, which were present in all samples, discrete shortened RNA-3- and RNA-4-related forms could be detected after the first or second passage. The deleted RNA-3 species, termed RNA-3a, was about 1500 nt in length, and the deleted RNA-4 species were about 1250 nt (RNA-4a) and 1100 nt (RNA-4b) in length. Fig. 1 also shows the RNA-3 and RNA-4 content of C. quinoa leaves

Internal deletions in transcript-derived RNA-3 and RNA-4

BNYVV isolates maintained by mechanical inoculation onto leaves often accumulate specific deleted forms of RNA-3 and RNA-4. We have shown previously that bacteriophage T7 RNA polymerase run-off transcripts produced from cloned cDNA and corresponding to RNA-1 to -4 of BNYVV isolate F3 are infectious and induce symptoms in leaves indistinguishable from those induced by natural BNYVV isolates (Quillet et al., 1989). In a first experiment, a mixture of RNA-1, -2, -3 and -4 transcripts was used to infect C. quinoa leaves. These leaves in turn served as inocula to initiate four additional serial passages of the virus; the inoculum for each passage was an extract of combined local lesions from the preceding passage. Fig. 1 shows the amounts of RNA-3 (Fig. 1 a) and RNA-4 (Fig. 1 b) in infected leaf tissue after each passage. In addition to full-length RNA-3 and -4, which were present in all samples, discrete shortened RNA-3- and RNA-4-related forms could be detected after the first or second passage. The deleted RNA-3 species, termed RNA-3a, was about 1500 nt in length, and the deleted RNA-4 species were about 1250 nt (RNA-4a) and 1100 nt (RNA-4b) in length. Fig. 1 also shows the RNA-3 and RNA-4 content of C. quinoa leaves
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Fig. 1. Appearance of deleted forms of RNA-3 (a) and RNA-4 (b) during serial passage of a BNYVV isolate produced by inoculating BNYVV RNA-1 to -4 transcripts to C. quinoa leaves. Formaldehyde-denatured RNA from leaves infected with natural isolate F3 (lane F3) and from passages 1 to 5 (lanes 1 to 5, respectively) of the transcript-derived isolate were subjected to agarose gel electrophoresis. Viral RNA was detected with the RNA-3-specific antisense RNA-3 probe 3A (a) or the RNA-4-specific probe 4A (b). The positions of full-length RNA-3 and -4 are indicated by asterisks. The deleted forms of RNA-3 and -4 referred to in the text are indicated by 3a, 3b, 4a and 4b.

infected with BNYVV isolate F3, the isolate initially used to prepare the cDNA clones employed to synthesize the RNA-3 and RNA-4 transcripts (Ziegler-Graff et al., 1988). Since then, isolate F3 has been carried through 30 successive bulk passages in leaves over a 3 year period. It can be seen that, in addition to full-length RNA-3 and RNA-4, the isolate has accumulated two deleted forms of RNA-3 and a deleted RNA-4. One of the RNA-3-related forms comigrated with RNA-3a from the transcript-derived isolate; the deleted version of RNA-4 had a mobility similar to that of the shortest transcript-derived RNA-4 form.

In order to gain further insight into the frequency with which deletions occur, individual local lesions from leaves inoculated with the RNA-1 to -4 transcript mixture were extracted separately and their RNA-3 and RNA-4 content was evaluated by Northern hybridization (Fig. 2). In addition to full-length RNA-3, which was abundant in all local lesions, a species with a mobility like that of RNA-3a was present in small amounts in lesions 3 to 5 and a 1200 nt species (RNA-3b) was detected in local lesion 2 (Fig. 2a). When the same RNA samples were probed with an RNA-4-specific probe, full-length RNA-4 was detected in all cases except for local lesions 8 and 10 (Fig. 2b). The former contained no RNA-4-related species but the latter contained the approximately 1100 nt RNA-4b.

Mapping the internal deletions

The above results illustrate that discrete shortened forms of BNYVV RNA-3 and RNA-4 can appear spontaneously in a transcript-derived BNYVV isolate even after only one or two passages in leaves. The deleted forms of RNA-3 and RNA-4 observed previously in several natural BNYVV isolates have been shown to arise by elimination of internal sequences. Northern blot hybridization with probes corresponding to different portions of the RNA-3 and RNA-4 sequence has revealed this also to be the case for the various shortened transcript-derived species observed in Fig. 1 and 2. Examples of such analysis are shown in Fig. 3. All of the RNA-3-specific probes except probe 3B hybridized with RNA-3a (Fig. 3a), indicating that the RNA-3 sequence eliminated by the deletion process encompasses nt 754 to 1035. Similarly, only probes 4A and 4C hybridize with the approximately 1100 nt RNA-4b species in BNYVV
isolate F3 and in local lesion 10 of Fig. 2(b), indicating that the sequence between nt 858 and 1186 was lost in the deletion process. The RNA preparation shown in Fig. 3(a) also contained an approximately 600 nt RNA-3-related species (indicated by S in Fig. 3a) which hybridized only with the two probes representing the 3'-terminal portion of the RNA-3 sequence. This species is discussed more fully below.

The boundaries of the deletions in several of the shortened RNA-3 and -4 variants were located more precisely by sequence analysis of cloned cDNA. In one experiment, virions were purified from passage 4 of the experiment shown in Fig. 1 and RNA extracted from the virions was used to generate cDNA clones by the method of Heidecker & Messing (1983). Clones containing long RNA-3 cDNA inserts were selected by colony hybridization with probe 3A. The 73 RNA-3-positive clones detected in this way were then screened with probe 3B to counterselect for RNA-3 inserts that fail to hybridize with this segment. In spite of the fact that RNA-3a was almost as abundant as full-length RNA-3 in the RNA preparation only two of the recombinant cDNA plasmids did not hybridize with probe 3B and were therefore apparently derived from the deleted form. Sequence analysis of the cDNA inserts of these two plasmids revealed that both were identical to full-length RNA-3 except for the loss of a segment between nt 713 and 1051 (Fig. 4a). In the same fashion, virus was prepared from leaves inoculated with RNA from either local lesion 2 or local lesion 10 in Fig. 2 and cDNA plasmids generated from the viral RNA were probed for RNA-3 or RNA-4-specific sequences, respectively. A single clone containing an internal deletion (nt 438 to 1090) was obtained in the former case and in the latter case six clones containing internally deleted RNA-4 sequences were observed. The deleted RNA-4 sequences fell into two classes. Four plasmids lacked the sequence between approximately nt 724 and 1164 [the exact limits of the deletion cannot be specified because of sequence redundancy at the deletion termini (Fig. 4a)] and two lacked the sequence between nt 697 and 1194.

Fig. 3. Mapping the deletions in RNA-3a (a) and RNA-4b (b) by Northern hybridization using different antisense RNA probes. The position of each probe on the RNA-3 and -4 sequence is indicated in the lower portion of the figure. (a) Samples of RNA from passage 5 in Fig. 1 were hybridized with probe 3A (lane 1), probe 3b (lane 2), probe 3C (lane 3) or probe 3D (lane 4). S indicates the position of RNA-3sub and the arrow RNA-3a. The band in lane 4 denoted by an open circle arises from cross-hybridization of RNA-2 with the 3'-terminal homologous domain (Bouzoubaa et al., 1987) present in probe 3D. (b) RNA from isolate F3 (lanes 1, 3 and 5) and from local lesion 10 from Fig. 2 (lanes 2, 4 and 6) were hybridized with probe 4A (lanes 1 and 2), probe 4B (lanes 3 and 4) or probe 4C (lanes 5 and 6). The arrowhead indicates RNA-4b. The positions of full-length RNA-3 and -4 are indicated by asterisks.
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Characterization of RNA-3sub

As shown in Fig. 3(a), total RNA extracted from leaves infected with a transcript-derived BNYVV isolate contains, in addition to full-length and internally deleted forms of RNA-3, a second RNA-3-related species of about 600 nt which does not hybridize with probes specific for the 5' terminal portion of the RNA-3 sequence. This species, which we refer to as RNA-3sub, is similar in size and is presumably identical to an RNA-3-related species previously observed but not characterized by Burgermeister et al. (1986). RNA-3sub appeared (Fig. 5c) in the first passage after inoculation of full-length RNA-3 transcript, along with RNA-1 and -2, and was present in leaves infected with all natural RNA-3-containing BNYVV isolates that we have tested (data not shown). It was also present in the roots of sugar beet infected with BNYVV using viruliferous P. betae (Fig. 5d). RNA-3sub could not be detected in RNA extracted from purified virions (Fig. 5a) and was sensitive to degradation by endogenous nucleases in crude homogenates of infected leaves (data not shown) indicating that RNA-3sub, unlike the various internally deleted RNA-3 and RNA-4 species, is not encapsidated.

RNA-3sub binds to an oligo(dT)-cellulose column (data not shown) indicating that, like RNA-3, it possesses a 3' poly(A) tail. In order to map the 5' terminus of the small RNA with precision, RNA-3sub and full-length RNA extracted from infected C. quinoa leaves were separated from one another by sedimentation through a sucrose gradient. Fractions highly enriched in either RNA-3sub or RNA-3 were pooled and used as templates for primer extension by reverse transcriptase. The synthetic 5'-32P-labelled oligodeoxyribonucleotide which served to prime cDNA synthesis was complementary to nt 1356 to 1376 of the RNA-3 sequence. Upon extension of the primer with reverse transcriptase two abundant cDNA run-off species (differing by about one nucleotide residue in mobility) were produced (Fig. 6, lane 1) which were absent when full-length RNA-3 was the template (Fig. 6, lane 2). The lengths of the run-off transcripts were estimated at 145 or 146 nt by reference to a DNA sequence ladder. This fixes the 5' terminus of RNA-3sub at about nt 1230 of RNA-3 provided that RNA-3sub and RNA-3 are collinear. Direct evidence for collinearity between RNA-3sub and RNA-3 was obtained by sequence analysis of the cDNA synthesized from the RNA-3sub template. When 5'-32P-labelled run-off cDNA such as that shown in Fig. 6 was sequenced by partial chemical degradation (Maxam & Gilbert, 1977) the resulting sequence was identical to the RNA-3 negative strand sequence up to nt 1234, the point at which the sequence ladder becomes unreadable because it merges with the band of non-degraded DNA at the top of the ladder (data not shown). Similar sequence analysis of cDNA primed further downstream on RNA-3sub extended the portion of the sequence which was shown
Fig. 6. Mapping the 5' extremity of RNA-3sub by primer extension. The 5'-32P-labelled primer was complementary to nt 1356 to 1376 of RNA-3. The products of reverse transcription were separated by electrophoresis through a 6% polyacrylamide sequencing gel alongside a DNA sequence ladder (not shown) to provide size markers. The templates for reverse transcription were sucrose gradient-enriched RNA-3sub (lane 1) or full-length RNA-3 (lane 2). Distances in nt from the primer binding site are indicated on the left. The arrowhead shows the position of the pair of closely spaced abundant run-off reverse transcripts of RNA-3sub.

by direct sequence analysis to be collinear with RNA-3 to within about 110 nt of the 3' poly(A) tail. Determination of the sequence closer to the 3' extremity in this way was not possible because the RNA-3sub preparation contained some RNA-4 and the extensive sequence homology between RNA-3 and -4 near the 3' terminus (Bouzoubaa et al., 1985) precludes design of a synthetic oligodeoxynucleotide primer which will bind specifically to RNA-3 in this region.

Discussion

In this paper we have shown that full-length BNYVV RNA-3 and RNA-4 produced by in vitro transcription of cloned cDNA can, when co-inoculated with RNA-1 and -2, undergo internal deletions after one or two passages in *C. quinoa* leaves. Analysis of the viral RNA content of infected leaves by gel electrophoresis reveals discrete bands of deleted RNA-3 and -4 rather than a continuum of shortened forms. In the case of RNA-3 the deleted species RNA-3a and -3b correspond in size to the abundant deleted forms present after extensive passage in leaves of BNYVV isolate F3, the isolate from which the cDNA clones used to generate the prototype full-length RNA-3 and -4 transcripts were derived. Thus our observations suggest that the rapid deletion events observed with transcript-derived RNA-3 can account for the appearance of the similar forms in the natural isolate. Comparable findings have been reported for D-satellite (D-sat) RNA of cucumber mosaic virus where a point sequence heterogeneity, appearing at the same site at which heterogeneity occurs in natural D-sat populations, can develop within two to four passages after inoculation with transcript RNA (Kurath & Palukaitis, 1990).

In the case of BNYVV RNA-4 the 1100 nt RNA-4b was the only RNA-4-related species detected in one local lesion from a leaf infected with the transcript-derived inoculum. Bulk serially passaged material on the other hand contained in addition to RNA-4b, a deleted RNA-4 species of about 1250 nt which clearly had no counterpart in the natural isolate. Perhaps this 1250 nt species is a quasi-stable intermediate which is subject to further deletion to produce the 1100 nt form.

In order to fix the boundaries of some of the deletions more precisely, two clones with deletion-bearing cDNA inserts were obtained from viral RNA containing RNA-3a, a single clone for RNA-3b and six clones for RNA-4b. The limits of the deletions, defined by sequence analysis of such clones (Fig. 4a), were consistent with the observed lengths of the deleted forms. It is evident, however, that not enough clones were analysed in the case of RNA-3 to allow conclusions to be drawn about possible fine-scale heterogeneity at the deletion boundaries. Indeed, such heterogeneity was detected for the RNA-4b where four of six clones examined were of one type (nt 724 to 728 to nt 1164 to 1168 deleted; the uncertainty concerning the exact site of the deletion in the prototype sequence is due to the presence of a 4 nt sequence redundancy at the deletion boundaries) whereas the sequence deleted in the other two spanned the same region but was slightly longer (nt 697 to 1194 deleted).

We regard copy choice during replication, i.e. switching of the replicase-nascent strand complex from one point on the template strand to another point further downstream on the same or another RNA molecule, as the most plausible mechanism for generation of the internal deletions in RNA-3 and -4. Such a mechanism is the currently favoured mechanism for viral RNA
recombination (Kirkegaard & Baltimore, 1986) and the formation of defective interfering RNA (Holland, 1986).

In view of the apparent specificity of the deletion process in BNYVV we have examined the prototype RNA-3 and RNA-4 sequences for unusual features at the deletion boundaries. In the case of the more abundant of the two characterized RNA-4 deletion variants, a 15 nt direct sequence repeat (with two mismatches) is found at the boundaries, with the deletion having occurred in such a way that one of the two repeats is eliminated (Fig. 4a). Evidently, a sequence repetition of this sort would aid reassociation of a detached replicase-nascent strand complex to the new site on the template, although it does not explain why disassociation occurred at this point in the first place. It is also noteworthy that the copy of the sequence repeat conserved during the deletion process is the one nearer the 3' terminus, indicating that the putative template switch occurred during negative strand RNA synthesis.

No extensive sequence duplications were present at the limits of the other RNA-3 and -4 deletions characterized. We have noted, however, that the sequence deleted in RNA-3a resembles a nuclear pre-mRNA intron in that it begins with GU and ends with AG (Fig. 4a). Certain other features at the deletion boundaries of RNA-3a also resemble splice junction consensus sequences (Brown, 1986), most notably the U-rich tract preceding the 3' boundary. Thus a post-replication mechanism involving splicing cannot be ruled out for the generation of RNA-3a. There is no evidence, however, for a nuclear phase in the BNYVV replication cycle or for any particular association with subcellular organelles such as chloroplasts or mitochondria. Thus encounters between RNA-3 and the nuclear or organelle splicing machinery, if they occur, would presumably be infrequent. In any event, it will now be possible to test the role of the boundary sequences and other features of RNA-3 and -4 in the deletion process by altering the sequence by site-directed mutagenesis at the cDNA level and exploring the effects of such changes when RNA-3 and -4 molecules carrying such alterations are propagated on plants.

In this paper we have shown that, in addition to full-length and internally deleted forms of RNA-3 and RNA-4, BNYVV-infected tissue also contains another type of RNA-3-related species, termed RNA-3sub. RNA-3sub is collinear with most of (presumably all of) the 3'-terminal portion of RNA-3 with its 5' terminus mapping at about nt 1230 (Fig. 4b). RNA-3sub is distinct from the internally deleted forms of RNA-3 in that it always appeared in infected tissue whenever natural RNA-3 or RNA-3 transcript was present in the inoculum, it lacks the 5'-terminal portion of the RNA-3 sequence and it was not encapsidated. We have shown elsewhere (Jupin et al., 1990) that a 5'-terminal domain of about 300 nt is necessary for productive replication of RNA-3 in plants. Thus it appears likely that RNA-3sub cannot exist independently but is synthesized de novo from RNA-3 in the course of infection, i.e. that RNA-3sub is a subgenomic RNA. Site-directed mutagenesis of the RNA-3 prototype sequence should allow us to map sequences important in cis for its synthesis.

The failure of RNA-3sub to undergo encapsidation suggests that the 5'-terminal portion of RNA-3 which is absent from the subgenomic RNA must contain a sequence indispensable in cis for packaging. Recent analysis of RNA-3 variants produced by site-directed mutagenesis of the prototype cDNA clone has mapped such a signal to near nt 200 (unpublished observations). An open reading frame for an Mr 4600 protein begins near the RNA-3sub 5' terminus (Fig. 4b) but there is no evidence as yet either for or against its expression in vivo. Thus the role, if any, of RNA-3sub in the viral infection cycle remains to be established.

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References


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