Subgenomic RNAs of bamboo mosaic potexvirus-V isolate are packaged into virions

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Purified virions of bamboo mosaic potexvirus-V isolate (BaMV-V) were found to contain three major RNA species, the 6-4 kb genomic RNA and two RNAs of 2-0 and 1-0 kb, in addition to associated satellite RNA (0-85 kb). Results of Northern blot hybridization, primer extension analysis and cDNA sequencing showed that the packaged 2-0 and 1-0 kb RNAs of BaMV-V were subgenomic RNAs. In contrast, in the BaMV-O isolate, only genomic RNA was packaged and encapsidated subgenomic RNAs were not detectable. The transcription initiation sites for the 2-0 and 1-0 kb subgenomic RNAs of BaMV-V were located 11 and 16 nt upstream of the initiation codon of open reading frames (ORFs) 2 and 5, respectively. The 2-0 and 1-0 kb subgenomic RNAs functioned as messengers for the ORF2 protein and capsid protein, respectively. Packaging of the 1-0 kb subgenomic RNAs resulted in the formation of rod-shaped particles about 70 nm in length. Our results indicate that BaMV isolates have evolved distinctly for packaging of subgenomic RNAs.

Introduction

Bamboo mosaic potexvirus (BaMV) has a single-stranded, positive-sense RNA genome of 6-4 kb which contains five conserved open reading frames (ORFs) and belongs to the alphavirus superfamily (Lin et al., 1994; Yang et al., 1997). ORF1 encodes a putative RNA-dependent RNA polymerase (RdRp) of 156 kDa (Lin et al., 1992, 1994). The triple gene block, ORFs 2-4, encodes proteins of 28, 13 and 6 kDa, respectively (Lin et al., 1994; Yang et al., 1997), which are required for virus cell-to-cell movement (Beck et al., 1991). The product of ORF5 is the capsid protein of 25 kDa. Genomic RNA and the two major subgenomic RNAs, which are about 2-0 and 1-0 kb in size, could be detected in inoculated protoplasts and plants (Lin et al., 1992, 1996). Like subgenomic RNAs associated with other potexviruses, the 2-0 and 1-0 kb subgenomic RNAs may be responsible for expression of the ORF2 protein and capsid protein, respectively (White & Mackie, 1990). The mechanism of expression of ORFs 3 and 4 is not yet clear.

The complete cDNA sequences have been determined for two BaMV isolates: BaMV-O, originally isolated from infected green bamboo (Bambusa oldhamii Munro) (Lin et al., 1994), and BaMV-V, isolated from common bamboo (Bambusa vulgaris McClure) (Yang et al., 1997). BaMV-V contains an additional satellite RNA (satBaMV), the only satellite RNA known to be associated with potexviruses (Lin & Hsu, 1994). Comparisons between BaMV-O and BaMV-V show a difference of 10-0% at the nucleotide level and of 3-2% at the amino acid level (Yang et al., 1997).

It is believed that subgenomic RNAs are transcribed from an internal site of the minus strand template by the viral RdRp (Miller et al., 1985; Gargouri et al., 1989; Kao & Sun, 1996). The subgenomic RNAs of the alphavirus superfamily are often found to be encapsidated into virions although in various amounts (Maia et al., 1996). For example, the subgenomic RNAs of brome mosaic virus (Ahlquist et al., 1981), alfalfa mosaic virus (Brederode et al., 1980), cucumber mosaic virus (Gould & Symons, 1982) and turnip yellow mosaic virus (Guilley & Briand, 1978) are encapsidated as efficiently as genomic RNA. However, for virions with rod-shaped morphology, subgenomic RNAs of alphaviruses are generally found to be inefficiently encapsidated (Maia et al., 1996). In the case of tobacco mosaic virus, the subgenomic RNAs are encapsidated in certain strains depending on the position of the packaging signal (Dawson & Lehto, 1990). Few subgenomic RNAs of potexviruses are encapsidated (Maia et al., 1996) since the packaging signal of potexviruses is located at the 5’ end of...
the genomic RNA, a region that is not transcribed into subgenomic RNAs (Sit et al., 1994). However, recent studies have indicated that the subgenomic RNA of Aura alphavirus is encapsidated into virus particles (Rumenapf et al., 1994), even though the packaging signals of several alphaviruses are located at the 5' termini of the genomic RNA (Frolova et al., 1997). In this paper, we report that the 2.0 and 1.0 kb subgenomic RNAs of BaMV-V, but not of BaMV-O, are efficiently encapsidated into virions. The characteristics of these encapsidated subgenomic RNAs are also described.

Methods

Virus isolates. BaMV-O and BaMV-V were obtained from infected green bamboo (B. oldhamii Munro) (Lin & Chen, 1991) and common bamboo (B. vulgaris McClure) (Lin & Hsu, 1994), respectively. BaMV-L is an isolate derived from BaMV-V and is free from satellite RNA (Lin & Hsu, 1994; Lin et al., 1996). BaMV-O and BaMV-L were maintained and propagated in Chenopodium quinoa (Lin et al., 1994).

Virus purification and RNA extraction. Purification of BaMV virions from infected C. quinoa and extraction of viral RNA were described previously (Lin & Chen, 1991; Lin et al., 1992). RNAs extracted from purified virions were separated in 1% low-melting agarose gel in Tris–borate buffer by electrophoresis (Sambrook et al., 1989); the gel was stained with ethidium bromide. The subgenomic RNAs of 2.0 and 1.0 kb were isolated from gel slices by phenol extraction followed by ethanol precipitation. The purified RNAs were dissolved in sterile distilled water, quantified by UV absorption, and stored at −70°C until use.

Northern blot analysis. Glycylolation and Northern blot analysis were carried out as described (Lin et al., 1992), except that RNA probes were used. The RNA probes included an ORF-1 probe specific for ORF1 of genomic RNA or BS24 [5' CCCTTTCTGAG(T)13 3' for cloning 2.0 kb subgenomic RNA] or BS24 [5' CTCTCTGAG(T)13 3' for cloning 1.0 kb subgenomic RNA]. 1 µl reaction buffer and 100 U Moloney murine leukemia virus reverse transcriptase (supplied with the kit). The double-stranded cDNA synthesis and PCR reaction were carried out according to kit protocol. The PCR reaction products were directly ligated into the pCRII vector of the TA Cloning kit (Clontech). The first-strand cDNA synthesis reaction contained 0.1 µg gel- eluted subgenomic RNA, 1 µl CapSwitch oligonucleotide, 0.1 µg primer B53 (5' GAGCCCAAAGGCCAGTT 3' for cloning 2.0 kb subgenomic RNA) or BS24 [5' CTCTTTCTGAG(T)13 3' for cloning 1.0 kb subgenomic RNA], 1 µl reaction buffer and 100 U Moloney murine leukemia virus reverse transcriptase (Promega). The extension reaction was carried out at 42°C for 1 h in a mixture of 15 µl labelled products and 1 µl 10 mM dNTP. Extension products were analysed by electrophoresis in a 6% polyacrylamide/7 M urea gel containing 1× TBE (133 mM Tris–HCl, pH 8.3, 44 mM boric acid and 2.5 mM EDTA). The image was exposed and analysed by PhosphorImager using ImageQuant version 3.3 (Molecular Dynamics).

cDNA construction of 5' termini of subgenomic RNAs. cDNAs corresponding to 5' termini of BaMV subgenomic RNAs were constructed by utilizing the Capfinder PCR cDNA Library Construction kit (Clontech). The first-strand cDNA synthesis reaction contained 0.1 µg gel- eluted subgenomic RNA, 1 µl CapSwitch oligonucleotide, 0.1 µg primer B53 (5' GAGCCCAAAGGCCAGTT 3' for cloning 2.0 kb subgenomic RNA) or BS24 [5' CTCTTTCTGAG(T)13 3' for cloning 1.0 kb subgenomic RNA], 1 µl reaction buffer and 100 U Moloney murine leukemia virus reverse transcriptase (supplied with the kit). The double-stranded cDNA synthesis and PCR reaction were carried out according to kit protocol. The PCR reaction products were directly ligated into the pCRII vector of the TA Cloning kit (Invitrogen). The DNA sequences of 5' termini of subgenomic RNAs were determined by the chain-termination procedure using T7 Sequenase (Amersham).

Sedimentation of BaMV particles and electron microscopy. The partially purified virus preparations of BaMV-O and BaMV-L were centrifuged in a 10–40% linear sucrose density gradient in 0.05 M borate buffer (pH 8.0) at 25 000 r.p.m. for 3–5 h in a Hitachi SP-8200A rotor. The gradient was fractionated with an ISCO Model 185 density gradient fractionator, and absorption zones were collected with the aid of an ISCO UA-5 absorbance monitor. The sedimented particles were pelleted by centrifugation at 43 000 r.p.m. for 3 h in a Beckman TL-100 ultra-centrifuge and resuspended in borate buffer. RNAs were extracted from each fraction and dot–blot hybridization was performed as described (Lin & Hsu, 1994). Fractionated particles were stained with 0.2% phosphotungstic acid and examined with a Zeiss 109 electron microscope.

Results

Presence of 2.0 and 1.0 kb RNA in virus particles

RNAs extracted from purified virions of BaMV-O, BaMV-V, and BaMV-L were separated in a 1% agarose gel by electrophoresis and stained with ethidium bromide. As shown in Fig. 1(A), the BaMV-O isolate contains only a single species of genomic RNA of 6.4 kb in length (lane 1) whereas the BaMV-V contains an additional RNA of 0.85 kb in length, which has been identified as a satellite RNA (satBaMV RNA; lane 2) (Lin & Hsu, 1994). In the BaMV-L isolate, two RNAs (2.0 and 1.0 kb in length) were observed in addition to the 6.4 kb genomic RNA (Fig. 1A, lane 3). An in vitro transcript of satBaMV RNA is shown as a molecular mass marker (Fig. 1A, lane 4) (Lin et al., 1996).

To identify the two small RNAs (2.0 and 1.0 kb) detected in the BaMV-L isolate, Northern blot hybridizations of virion RNAs of various isolates were performed. The blot was probed either with the ORF-1 probe, specific for ORF1 of genomic RNA (Fig. 1B), the L-probe, specific for the 3’ end of genomic RNA (Fig. 1C) or with the S-probe, specific for satBaMV RNA (Fig. 1D). The ORF-1 probe detected the 0.4 kb genomic RNA in all isolates, but it did not hybridize with the small RNAs of the BaMV-L isolate (Fig. 1B, lane 3), the satBaMV RNA of reverse transcriptase (Promega). The extension reaction was carried out at 42°C for 1 h in a mixture of 15 µl labelled products and 1 µl 10 mM dNTP. Extension products were analysed by electrophoresis in a 6% polyacrylamide/7 M urea gel containing 1× TBE (133 mM Tris–HCl, pH 8.3, 44 mM boric acid and 2.5 mM EDTA). The image was exposed and analysed by PhosphorImager using ImageQuant version 3.3 (Molecular Dynamics).
Subgenomic RNAs of BaMV-V are encapsidated

Fig. 1. Gel electrophoresis (A) and Northern hybridization (B, C, D) of virion RNAs of BaMV. Virion RNAs were extracted from BaMV-O (lane 1), BaMV-V (lane 2) or BaMV-L (lane 3) isolates. Lane 4 displays the in vitro transcription products of pBSF4, which contain the satBaMV RNA sequence (Lin et al., 1996). (A) RNA (1 µg) was fractionated on a 1% agarose gel in Tris–borate buffer, and stained with ethidium bromide. (B, C, D) Northern hybridization of BaMV virion RNA. RNA (100 ng) was glyoxylated and fractionated on a 1% agarose in phosphate buffer, blotted onto a nylon membrane and hybridized to riboprobe ORF-1, specific for the ORF1 of genomic RNA (B), L-probe, specific for the genomic RNA 3' end (C), or S-probe, specific for the satBaMV RNA (D). Positions of the BaMV genomic RNA (G), 2.0 kb subgenomic RNA (2.0 kb), 1.0 kb subgenomic RNA (1.0 kb) and satellite RNA (satBaMV) are indicated on the right.

BaMV-V isolate (Fig. 1B, lane 2), or the satBaMV RNA transcripts (Fig. 1B, lane 4). Besides the genomic RNA, the L-probe detected the 2.0 and 1.0 kb RNAs in BaMV-V (Fig. 1C, lane 2) and BaMV-L isolates (Fig. 1C, lane 3), but did not hybridize to the satBaMV RNA (Fig. 1C, lanes 2 and 4). On the other hand, the S-probe detected only the satBaMV RNA of BaMV-V isolate (Fig. 1D, lane 2) and the satBaMV RNA transcripts (Fig. 1D, lane 4); it did not hybridize with the genomic RNAs or the two small RNAs of BaMV-V and BaMV-L isolates.

Detection of the 2.0 and 1.0 kb RNAs with the L-probe indicates that these RNAs are 3' co-terminal with genomic RNA. Since they did not react with the ORF-1 probe, this rules out the possibility that the 2.0 and 1.0 kb RNAs are encapsidated, defective RNAs and suggests rather that these RNAs are encapsidated subgenomic RNAs of BaMV. The
amount of subgenomic RNAs in BaMV-V particles was lower than that in BaMV-L (Fig. 1A and C). This may be due to interference of satBaMV RNA with genomic RNA replication (N. S. Lin, unpublished data) and the consequent decrease in transcription of 2±0 and 1±0 kb subgenomic RNAs in the virus isolate carrying a satellite. Thus, the BaMV-L isolate was chosen for further analysis of the two subgenomic RNAs.

The 2±0 and 1±0 kb RNA can translate ORF2 protein and capsid protein in vitro

After electrophoresis of virion RNAs from BaMV-L on a native agarose gel (Fig. 1A, lane 3), the 2±0 and 1±0 kb RNAs were purified from gel slices. The gel-eluted RNAs were translated in RRL and their translation products were separated by 15% SDS–PAGE. As shown in Fig. 2A, the translation products of the 2±0 kb RNA were a doublet with apparent molecular masses of 30 and 28 kDa (lane 2) whereas the translation product of the 1±0 kb RNA had an apparent molecular mass of about 28 kDa (lane 3).

To identify these translation products, immunoblot analyses were performed. Both of the translation products of the 2±0 kb RNA reacted positively with anti-ORF2 protein serum (Fig. 2B, lane 1), but not with anti-capsid protein serum (Fig. 2C, lane 1). The upper band of the doublet had an electrophoretic mobility similar to that of the ORF2 protein detected in proteins extracted from a BaMV-L-infected plant (Fig. 2B, lane 3), suggesting that the lower band is a degradation product of the ORF2 protein. The translation products of the 1±0 kb RNA reacted with anti-capsid protein serum (Fig. 2C, lane 2), but not with anti-ORF2 protein serum (Fig. 2B, lane 2), and co-migrated with the capsid protein from the BaMV-L-infected plant (Fig. 2C, lane 3). No serological reactions were detected with the total proteins extracted from healthy plants when they were reacted with the anti-ORF2 protein and anti-capsid protein sera (data not shown). These results suggest that the 2±0 and 1±0 kb RNAs purified from BaMV-L virions are mRNAs for the ORF2 protein and capsid protein, respectively.

Identification of the 5′ termini of the encapsidated subgenomic RNAs

Primer extension experiments were performed to determine the 5′ termini of the encapsidated subgenomic RNAs by using total RNAs from BaMV-L-infected C. quinoa plants or gel-eluted viral RNAs as templates. These RNAs were annealed with primer B18, which is complementary to a sequence in the ORF2 gene, or with primers B56 and B34, both complementary to a sequence in the capsid protein gene. With primer B18, two predominant extension products were obtained with the template RNAs from infected plants (Fig. 3A, lane 1) and from

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Fig. 3. Identification of the 5′ termini of BaMV-L subgenomic RNAs by primer extension. Total RNA (lane 1) and gel-eluted virion RNA (lane 2) were extracted from BaMV-L-infected C. quinoa as the template for primer extension. Primers B18 (complementary to a site starting 159 nt downstream of the ORF2 gene initiation codon) (A) and B56 (complementary to a site starting 132 nt downstream of the CP gene initiation codon) (B) were used for primer extension and the products were displayed on a 6% polyacrylamide/7 M urea sequencing gel. The gel was fixed and dried and the exposed image was analysed with a PhosphorImager (Molecular Dynamics). In (A), the maximum pixel intensity used in lane 2 was five times higher than that in lane 1. Lanes A, C, G and T contained the products of a dideoxynucleotide sequencing reaction performed on cDNA clone pBa19 (Yang et al., 1997) of BaMV-V genomic RNA with primer B18 (A) and B56 (B). The arrowheads indicate the position of subgenomic RNA 5′ termini. The sequences correspond to the region upstream of the initiation codon of ORF2 (A) or capsid protein gene (B), respectively. The initiation codons are underlined.

Fig. 4. The 5′ end sequences of cDNA clones of BaMV-L subgenomic RNAs. The cDNA clones containing the 5′ end sequences of gel-eluted 2±0 kb (A) and 1±0 kb (B) subgenomic RNAs were constructed using the Capfinder PCR cDNA Library Construction kit (Clontech). Lanes A, C, G and T contained the products of a dideoxynucleotide sequencing reaction performed with pUC/M13 forward primer (5′ GTTTTCCCCATCGAGAC 3′). The arrowheads indicate the position of subgenomic RNA 5′ termini. The bold lettering corresponds to the sequence of CapSwitch oligonucleotides (Clontech), and the italics to the sequence upstream of the initiation codon of ORF2 (A) or capsid protein gene (B), respectively. The initiation codons are underlined.
Subgenomic RNAs of BaMV-V are encapsidated.

Fig. 5. Sedimentation profiles, dot–blot hybridization and electron microscopy of sedimenting virus particles partially purified from BaMV-infected C. quinoa. (A, B) Sedimentation profiles of partially purified BaMV-O (A) and BaMV-L (B) particles in 10–40% linear sucrose density gradients. (C) Dot–blot hybridization of RNA purified from BaMV-O and BaMV-L sedimenting virus fractions with the L-probe. (D) Electron micrograph of sedimenting virus fraction S1 of BaMV-L, stained with 2% phosphotungstic acid. Bar, 100 nm.

purified virions (Fig. 3A, lane 2). They terminated at nt 4207 and 4208, respectively. The one nucleotide difference in length of the extension products may arise from insertion of a residue complementary to the cap structure as suggested by White & Mackie (1990). The signal produced for extension products on total RNAs (Fig. 3A, lane 1) was weaker than that for the gel-eluted 2±0 kb RNAs (lane 2). This may be due to a relatively low concentration of 2±0 kb subgenomic RNA in the infected plants. Similar analysis of the 5’ termini of 1±0 kb subgenomic RNA with primer B56 produced two extension products terminating at nt 5481 and 5482, respectively, when either total RNA (Fig. 3B, lane 1) or gel-eluted 1±0 kb viral RNA (lane 2) was used as a template. Similarly, primer B34 extended to the same points when either total RNA or gel-eluted 1±0 kb RNA was used as a template (data not shown). These results suggest that transcription initiation of the 2±0 and 1±0 kb subgenomic RNAs occurs at nt 4208, 11 bases upstream of the initiation codon of the ORF2 gene, and nt 5482, 16 bases upstream of the initiation codon of capsid protein gene, respectively. Our data also indicate that the 2±0 and 1±0 kb subgenomic RNAs are 5’ capped and encapsidated.

To further confirm the 5’-end sequence of the subgenomic RNAs, cDNA was constructed using the gel-eluted subgenomic RNAs as template by the Capfinder PCR cDNA Library Construction kit. The nucleotide sequences containing the 5’ termini of the 2±0 and 1±0 kb subgenomic RNAs are shown in Fig. 4. The sequences of the 2±0 and 1±0 kb subgenomic RNAs switch to that of the CapSwitch oligonucleotide at nt 4208 and 5482, respectively. This indicates that the subgenomic RNAs are 5’ capped and the transcription initiation site starts from nt 4208 and 5482, the same results as from primer extension experiments.

Sedimentation of BaMV-L isolate particles

Since the BaMV-O isolate does not contain any encapsidated subgenomic RNAs (Fig. 1C, lane 1; Lin et al., 1992), the virus particles of the BaMV-O isolate sedimented as a single UV-absorbing band in a sucrose density gradient after centrifugation (designated G) (Fig. 5A). In contrast, the virus preparations of the BaMV-L isolate sedimented as three UV-absorbing bands, slow-, middle- and fast-sedimenting fractions (designated S1, S2 and G, respectively) (Fig. 5B). The fast-sedimenting fraction of the BaMV-L isolate had the same sedimentation rate as that of the G band of BaMV-O isolate, suggesting that it contains the genomic RNA particles. This was confirmed by dot hybridization in which the L-probe (specific to the 3’ end of genomic RNA) hybridized to RNAs in fractions G from two isolates and to RNAs in fractions S2 and S1 from BaMV-L isolate (Fig. 5C), suggesting that these RNAs are 3’ co-terminal. The RNAs extracted from bands G, S2, and S1 had the same electrophoretic mobility in an agarose
gel as genomic RNA and the 2.0- and 1.0-kb subgenomic RNAs of the BaMV-L isolate, respectively (data not shown). We conclude that the particles in the fast-sedimenting band G contain the genomic RNA, and the particles in the middle- and slow-sedimenting bands S2 and S1, contain the 2.0- and 1.0-kb RNA, respectively.

The sedimented fractions were further examined by electron microscopy. Electron microscopy of the negatively stained G fraction revealed predominantly full-length flexuous particles 500 nm long (data not shown). In contrast, rod-shaped particles with an average length of 70 nm were observed in the S1 fraction (Fig. 5 D), which corresponds closely with the subgenomic size. Few virus particles were observed in the S2 preparation, probably due to the low quantity of material in the fraction.

Discussion

In this study, BaMV-L, which is derived from isolate BaMV-V and is free of satBaMV RNA, was used to prepare subgenomic RNAs and the virus particles encapsidating them. Since satBaMV RNA and the 1.0-kb subgenomic RNA are similar in size and satBaMV RNA usually replicates to a high level (Lin & Hsu, 1994), it is difficult to purify the subgenomic RNAs from BaMV-V isolate, although virions of BaMV-V contain subgenomic RNAs (Fig. 1C, lane 2). Evidence for the encapsidation of the subgenomic RNAs into virus particles in BaMV-L is as follows: the 2.0- and 1.0-kb subgenomic RNAs could be purified from virions and hence were not artefacts; the purified 2.0- and 1.0-kb subgenomic RNAs from encapsidated BaMV-L particles could direct the translation of ORF2 and the capsid protein gene, respectively, in vitro; the 5' termini of the 2.0- and 1.0-kb subgenomic RNAs, as determined by primer extension, were located 11 and 16 bases upstream from the initiation codon of ORF2 and the capsid protein gene, respectively; the cDNA cloning of the 5' termini of subgenomic RNAs by the Capfinder PCR cDNA Library Construction kit indicated that the 5' termini of subgenomic RNAs were capped; and electron microscopy revealed that the fractionated 1.0-kb subgenomic RNAs were encapsidated into particles of the predicted length of 70 nm.

Sit et al. (1994) reported that the in vitro initiation of papaya mosaic virus assembly requires a packaging signal located at the 5' end of the genomic RNA. The minimum initiation sequence of 47 nt is rich in A+C and very poor in U nucleotides. It also contains an AA dinucleotide at positions 3 and 4 of the 9 consecutive pentamers within first 45 nucleotides. Such an A+C-rich sequence and an AA dinucleotide at positions 3 and 4 are also seen within the first 9 pentamers of clover yellow mosaic virus (CyMV), potato virus X (PVX) (Sit et al., 1994) and BaMV-V (Yang et al., 1997). Furthermore, CyMV has been shown to be packaged in a polar fashion following the addition of capsid protein starting at the 5' terminus (Abou-Haidar, 1981). All of these results strongly suggest that there is indeed a packaging signal located at the 5' end of the genomic RNAs in the potexvirus group. Such packaging signals are not found in subgenomic RNAs among potexviruses. However, the subgenomic RNAs of narcissus mosaic virus (Short & Davies, 1983), CyMV (White & Mackie, 1990) and PVX (Price, 1993) have been reported to be encapsidated. Two possible explanations for the encapsidation of the subgenomic RNAs of BaMV-V come to mind. One possibility is that the subgenomic RNAs may contain packaging signals in addition to the putative signal at the 5' end of genomic RNA. The 5' untranslated regions of the 2.0- and 1.0-kb subgenomic RNAs of BaMV-V are in fact A+C-rich (9 A+C nt out of 11 nt for the 2.0 kb subgenomic RNA and 12 A+C nt out of 16 nt for the 1.0 kb subgenomic RNA; see Fig. 6). This is true even though the amounts of A+C residues in these regions of BaMV-O isolate do not differ greatly from those in the BaMV-V isolate (Lin et al., 1994; Yang et al., 1997). Possibly, the genomic RNA of the BaMV-V isolate contains a weak packaging signal at the 5' terminus, but one or several other unknown signals may also be present in the 3'-proximal half of genomic RNA.

It is also possible that the capsid proteins of BaMV-V and BaMV-O have different encapsidation activity. In Sindbis virus, the RNA binding domain (aa 76–132) of the capsid protein is involved in production of the encapsidated subgenomic RNA (Geigenmuller-Gnirke et al., 1993; Owen & Kuhn, 1996). When a capsid protein deletion mutant loses the selectivity for recognition of the packaging signal, the ratio of the encapsidated subgenomic RNA to genomic RNA increases. It has been shown that the capsid protein of BaMV-V has RNA binding activity and its binding is much stronger than that of BaMV-O (Y. H. Hsu & M. C. Lin, unpublished observations) although the two capsid proteins share 92.6% identity at the amino acid level (Lin et al., 1994; Yang et al., 1997). If the packaging signal recognized by the capsid protein is not present at the 5' terminus of genomic RNA nor in the subgenomic RNAs, it is possible that the capsid protein of...
BaMV is able to assemble any negatively charged RNA into virus-like particles. Thus, the capsid protein of BaMV-V may contain a stronger recognition activity for packaging than that of BaMV-O. It is of particular interest to note that satBaMV associated with BaMV-V is encapsidated with the capsid protein of BaMV-V (Lin & Hsu, 1994), although the packaging signal for satBaMV remains to be characterized.

The transcription initiation site of subgenomic RNAs of plant viruses is usually similar to that of the genomic RNA (Maia et al., 1996). It is noteworthy that both the 2-0 and 1-0 kb subgenomic RNAs of BaMV-V have adenosine at their 5′ termini (Figs 3 and 4), which is different from the genomic RNA, which begins with guanosine (Yang et al., 1997). For other potexviruses, such as CyMV (White & Mackie, 1990), PVX (Skryabin et al., 1988), Plantago asiatica mosaic virus (PIAMV) (Solovyev et al., 1994) and foxtail mosaic virus (FMV) (Bancroft et al., 1991), the 5′ termini of their 1-0 kb subgenomic RNAs are guanosine (Fig. 6). The 2-0 kb subgenomic RNAs of CyMV (White & Mackie, 1990) and PVX (Kim & Hemenway, 1996) also start with guanosine (Fig. 6). It has been shown that the level of the 2-0 kb, but not of the 1-0 kb, subgenomic RNA of PVX was significantly reduced in inoculated protoplasts when the putative transcription initiation sites were changed from G to an A and U, respectively (Kim & Hemenway, 1997). On the other hand, the 5′ termini of the 2-0 and 1-0 kb subgenomic RNAs of BaMV-V are capped like the genomic RNA. Moreover, the conserved hexamer motif (5′ ACUUAAG), which is common at the 3′ end region of potexvirus genomic RNA (White et al., 1992), is also found in the negative strand upstream of the subgenomic RNAs of the BaMV-V and BaMV-O isolates. The complementary conserved hexamer motif (5′ TTAAG) is located 14 nt upstream from the transcription initiation sites of both 2-0 and 1-0 kb subgenomic RNAs (Fig. 6). This suggests that genomic and subgenomic RNAs may share the same mechanism of transcription by RdRp and/or the same host factors, although the difference in the 5′-terminal nucleotide initiation and possible structural differences of the promoter-like regions between genomic RNA and subgenomic RNAs may differentially regulate the synthesis of RdRp, movement protein and capsid protein.

We have demonstrated here that the subgenomic RNAs of BaMV-V are efficiently packaged into virus particles. To our knowledge, this is the first report that packaging of subgenomic RNAs varies from isolate to isolate within the potexvirus group. These results lead to the notion that different BaMV isolates can evolve to display distinct selectivity for packaging of subgenomic RNAs and that there are specific signals within the subgenomic RNAs of BaMV-V to dictate packaging. The identification of these packaging signals, if any, would be of great interest although the importance of packaging of these RNAs into virus particles in virus evolution remains to be determined.

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