Biologically active transcripts from cloned cDNA of genomic grapevine fanleaf nepovirus RNAs

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Transcripts were produced in vitro by run-off transcription from full-length cDNA of RNA1 and RNA2 of grapevine fanleaf nepovirus (GFLV; isolate F13) cloned downstream from a bacteriophage RNA polymerase promoter. These transcripts, which possess a 5' terminal cap structure and a non-viral G residue instead of the naturally occurring genome-linked viral protein (VPg), are infectious to Chenopodium quinoa protoplasts when inoculated by electroporation. Synthetic RNA1 alone replicated in protoplasts. Inoculation of C. quinoa plants with synthetic RNA1 plus RNA2 produced symptoms similar to, but weaker, than those observed in plants infected with natural GFLV 6 to 8 days post-inoculation. Co-inoculated RNA1 and RNA2 were able to replicate and spread systemically in plants but RNA1 alone produced no symptoms and was not detected in non-inoculated leaves, suggesting that virus spread requires RNA2. Analysis of the genomic RNAs in plants infected with transcripts showed that the non-viral G at their 5' ends was not retained in the progeny.

Introduction

Grapevine fanleaf virus (GFLV) is a nepovirus with a genome made up of two single-stranded positive-sense RNAs (RNA1 and -2) carrying a 5' genome-linked viral protein (VPg) and a 3' poly(A) tail (Pinck et al., 1988). RNA1 consists of 7342 nucleotides (nt) (Ritzenthaler et al., 1991) with a large open reading frame (ORF) encoding a 253K polyprotein (P1) which is processed by an RNA1-encoded proteinase (Margis et al., 1991). The putative genetic organization of RNA1 includes a 92K RNA-dependent RNA polymerase in the C-terminal part of P1 and a 24K cysteine protease (Ritzenthaler et al., 1991). Microsequencing of the purified VPg has allowed alignment of its sequence with residues 1218 to 1241 of P1 (Pinck et al., 1991). The 133K N-terminal part of P1 comprises at least two subdomains, a putative NTP-binding protein and a possible protease cofactor, by analogy with cowpea mosaic virus (CPMV). In vitro activity and characteristics of the proteinase have been reported by Margis et al. (1991).

RNA2 consists of 3774 nt with one ORF encoding a 122K polyprotein (P2). The 56K coat protein (CP), located in the C-terminal part of P2, is released from the 122K polyprotein at a dipeptide Arg/Gly (residues 680/681) (Serghini et al., 1990). Infectious RNA transcripts from full-length cloned genomic cDNAs of several picorna-like plant viruses have been reported: CPMV (Vos et al., 1988), tobacco vein mottling virus (TVMV, Domier et al., 1989) and plum pox potyvirus (PPV, Riechmann et al., 1990; Maiss et al., 1992), but no infectious transcripts corresponding to genomic RNA of nepoviruses have so far been described. Here, we report the construction of clones containing the full-length cDNA of GFLV (isolate F13) RNA1 and -2 under the control of bacteriophage T3 or T7 RNA polymerase promoters. The procedure used to produce full-length cDNA of RNA1 from two clones obtained from polymerase chain reaction (PCR) amplification will be described. We will show that the corresponding transcripts can be replicated in protoplasts and in Chenopodium quinoa plants.

Methods

Virus, bacteria and plasmids. GFLV (isolate F13) was routinely multiplied in C. quinoa. Virus and RNA extraction were as described previously (Pinck et al., 1988; Serghini et al., 1990). Escherichia coli strains NM522, JM101, JM103 and C6005K were transformed by the CaCl2 method (Sambrook et al., 1989) or by electroporation (Pfu & Youderian, 1990). All constructs were cloned in BlueScribe m13 plus (BS+, Stratagene), pUC9 and pUC18.

cDNA1: first strand synthesis and cloning. First strand cDNAs were obtained by primer extension on total virion RNA primed with primer P4180 (5' GGAATTCGCTACCCGGCGCCAGATCT[T]3o) complementary to part of the poly(A) tail and including KpnI, NotI and BglII restriction sites (italics) and primer P4207 (5' AATTCTCTGG TACCTTCATGA) (Fig. 1a) complementary to nt 4733 to 4748 of RNA1. Total purified GFLV-F13 RNA (5 μg) was used as the template
and reverse-transcribed with 500 units of Moloney murine leukaemia virus H-RT reverse transcriptase (Superscript, BRL) as described by the manufacturer.

cDNA1: second strand synthesis and amplification by PCR. Phenol-extracted first-strand cDNAs were used as a template for synthesis of the double-stranded cDNAs which were then amplified by PCR (Satki et al., 1988). The following pairs of primers were used: P4180 (see above) and P4203 (5' TCACTGAAGTACAGATGAAAT, identical to nt 4733 to 4748), or P4207 (see above) and P3725 (5' GCAAGATGGCATGACTGATAGCTCATAGATAAGAATTTCCTAACAGTTC, which corresponds to the first 13 nt, corresponding to PvuII and SalI restriction sites (italics), are followed by the T7 transcription promoter (underlined) (18 nt, underlined) and by 21 nt identical to the first nucleotides of RNA1. PCR was performed in a final volume of 100 μl containing the cDNA of a first-strand preparation, 1 μM-P4180 and -P4203, or 1 μM-P4207 and-P3725, and 0.2 mM of each dNTP, 10 μl 10 × PCR buffer (100 mM-Tris-Cl pH 8.3, 1.5 mM MgCl₂, 500 mM-KCl) and 5 units Taq DNA polymerase (Beckman). PCR was carried out in a programmable thermal cycler (Perkin Elmer). As P4180 and P3725 were not complementary over their entire length with the initial templates (see above), four cycles of denaturation (94 °C) and hybridization (60 s at 94 °C), hybridization (60 s at 35 °C for P4180 and 45 °C for P3725, calculated as indicated in Sambrook et al. (1989)], and elongation (180 s at 72 °C) were first carried out. This was followed by 10 cycles of PCR with the same denaturation and elongation temperature but with the hybridization at 48 °C for 60 s.

cDNA1: cloning of full-length cDNA. Full-length recombinant clones were constructed by combining the two cDNA fragments obtained by PCR through the Kpnl restriction site present in both fragments, as shown in Fig. 1 (a). The 5' end PCR fragment obtained with P3725 and P4207 was digested with SalI and Kpnl and cloned in pUC18 to obtain plasmid p5SK. The 3' end fragment obtained with P4180 and P4203 was digested with Kpnl and inserted in KpnI-linearized p5SK, the correct orientation of the inserted fragment being established by restriction analysis. The full-length recombinant clones were named pMV₁₀, where n corresponds to the specific number of each clone.

cDNA2 construction. A clone containing full-length cDNA of RNA2 was obtained by joining partial RNA2 cDNA clones in BS + as shown in Fig. 1 (b). Inserts of clone pG38 (nt 1123 to 3774 with a 3'-terminal sequence of 34 consecutive G residues) and pS38 including nt 78 to 1303 described previously (Serghini et al., 1990) were joined to obtain clone pN1.

The cDNA extending to the 5' end of RNA2 was synthesized using primer P638 (nt 1303 to 1286) and directly PCR-amplified with primers P638 and P1283 [P1283 is identical to the reported sequence of nt 1 to 20 of RNA2 but with an additional U at its 5' extremity to fit with the 5' consensus sequence of most nepovirus RNAs (Fuchs et al., 1989)]. Double-stranded cDNA was cloned into pUC9 to produce pAS76. The HindIII–HindIII fragment from pAS76 (Fig.1b) was subcloned into HindIII-linearized BS + to produce pAS3BS. This clone was mutagenized, using a kit (Amersham) based on the method of Nakamaye & Eckstein (1986), with oligodeoxynucleotide P1766 (5'-GAAAAACGTAACATTTCTACATTTAGTGGG) to eliminate a plasmid sequence of 35 nt between the T3 transcription promoter (underlined) and the beginning of the RNA2 cDNA insert in order to produce transcripts starting with GUAUGA (G being a non-viral 5' end nucleotide).

It had been shown that the RNA2 sequence of GFLV, unlike most nepovirus RNAs, starts with an A residue linked to VPg instead of UA as presumed from the consensus sequence (Fuchs et al., 1989)), GFLV RNA1 (a) and RNA2 (b) for in vitro transcription. (a) Construction of clone pMV₁₀ of RNA1. The empty box represents RNA, the thick lines correspond to PCR fragments. The hatched and dotted boxes are cDNA fragments. The thin lines represent parts of the pUC18 vector sequence. Restriction sites are indicated by arrows (B, BglII; K, Kpnl; S, SalI) and the T7 promoter by a black oval. (b) Construction of clone pACN RNA2. The thin lines represent parts of the pUC 18 vector sequence. Restriction sites are indicated by arrows

and reverse-transcribed with 500 units of Moloney murine leukaemia virus H-RT reverse transcriptase (Superscript, BRL) as described by the manufacturer.
NaCl, 10 mM-spermidine, 200 mM-Tris-HCl pH 8), 60 units RNase inhibitor (Promega) and 50 units T7 or T3 RNA polymerase (BRL) were mixed in a final volume of 100 μL. After 30 min incubation at 37 °C, 4 μL 12.5 mM-GTP and 50 units polymerase were added and incubation was continued for 90 min. After transcription DNA was removed by the addition of 20 units DNase I (Boehringer), 20 μL 10 x DNase buffer (100 mM-NaCl, 60 mM-MgCl₂ and 400 mM-Tris-HCl pH 7.5) in 200 μL. Following incubation for 20 min at 37 °C, the transcripts were extracted with phenol-chloroform (1:1) and ethanol-precipitated. Their size and integrity were analysed by agarose-formaldehyde gel electrophoresis (Gustafson et al., 1982).

In vitro translation. The transcripts were translated in a wheat germ system as described by Godefroy-Colburn et al. (1985) and their translation products were analysed in 10% polyacrylamide gels as described by Laemmli (1970).

Protoplast infection and RNA extraction. Approximately 2 × 10⁶ C. quinoa protoplasts prepared according to Hans et al. (1992) were inoculated by electroporation in 0.5 ml 0.6 M-mannitol, 0.1 mM-CaCl₂, pH 5.6 with capped transcripts obtained from 10 μg cDNA1 and/or 7.5 μg cDNA2. After 30 min incubation on ice the protoplasts were washed and incubated at 22 °C in the presence of 0.3 mg/ml carbenicillin for 72 h. Total RNA from protoplasts was extracted as described by Hans et al. (1992).

Plant inoculation, RNA extraction and analysis. The two leaves of the third pair of leaves from young C. quinoa plants were dusted with celite and inoculated with capped transcripts transcribed from 5 μg cDNA1 and/or 3.5 μg cDNA2. The inocula in 25 mM-sodium phosphate buffer pH 7 were applied in 15 μl aliquots onto the leaves and gently rubbed with a sterile tip. Inoculated plants were left for 2 weeks in a glasshouse. Total RNAs were then extracted from 2 g of upper non-inoculated leaves by the method of Jackson & Larkins (1976) and further purified by precipitation in 2 M-LiCl (Pinck et al., 1991). The RNAs were separated by electrophoresis under denaturing conditions on agarose-formaldehyde gels and transferred to Hybond N membranes (Amersham). The viral RNAs were detected with labelled riboprobes as described by Hans et al. (1992).

Results and Discussion

Construction of full-length cDNA clones of GFLV genomic RNAs

Full-length cDNA1 was obtained exclusively from PCR products by joining two fragments containing, respectively, the 3' and 5' portions of the sequence. The PCR product containing the 3' extremity was produced using a primer corresponding to part of the poly(A) tail (P4180) and the other identical to nt 4733 to 4748 (P4203). The PCR product containing the 5' extremity was produced using a primer corresponding to the 5' extremity plus a T7 RNA polymerase promoter sequence (P3725) and a primer complementary to nt 4733 to 4748 (P4207) (Fig. 1a). The two PCR products were joined via a KpnI site in the region of sequence overlap. This strategy allows an orientated cloning and the synthesis of transcripts without additional nucleotides between the transcription initiation site and the RNA1 sequence. Inclusion of the transcription initiation sequence avoided time-consuming mutagenesis steps difficult to carry out on large cDNA clones. Primer P4180 contains a 30 dT stretch followed by two restriction sites which were used to linearize the recombined vector and a KpnI site for cloning (Fig. 1a). Six full-length cDNA clones of RNA1 (the pMVn clones) were obtained by this method. The resulting transcripts will be referred to as trMVn. The clone pMVn chosen for further experiments (see below), was characterized by sequence analysis of its extremities.

One full-length cDNA2 clone was obtained by joining partial cDNA clones in the BS+ transcription vector (Fig. 1b). Oligonucleotide-directed mutagenesis was carried out to delete 35 non-viral nucleotides between the T3 RNA polymerase transcription initiation site and the RNA2 sequence. A second mutagenesis was carried out in order to delete the additional 5' U introduced during the initial cloning step (see Methods) and to substitute the G in position +10 by a U in order to remove the AUG in positions 8 to 10 present in clone pAS3BS (R. Margis, unpublished results).

In vitro transcription and translation of viral RNA transcripts

We chose to cap the transcripts since it has been shown by several authors that capping generally increases the infectivity of transcripts. For instance, capping of CPMV transcripts doubled their infectivity (Eggen et al., 1989).

The pMVn clones contain the complete RNA1 sequence downstream from the T7 transcription site. Transcripts (trMV) obtained from these BglII-linearized clones possess a cap and have one extra G residue at the 5' terminus instead of a VPg. The 3' end comprises a stretch of 30 A residues followed by 4 nt generated by BglII linearization of pMV DNA.

The pACN clone contains the complete sequence of RNA2 under control of a bacteriophage T3 RNA polymerase promoter. Transcripts (trACN) derived from XbaI-linearized pACN possess the same 5' end structure as trMV and a stretch of 34 A residues plus 24 non-viral residues at the 3' end. These capped transcripts had the same electrophoretic mobility as purified GFLV F13 RNAs on a denaturing agarose-formaldehyde gel (data not shown). They were translated in cell-free wheat germ extracts to produce proteins which comigrated with those translated from authentic virion RNAs (Fig. 2). Their translation efficiency is about half that of the corresponding virion RNA as estimated from the translation patterns (data not shown).
2 x 10^5 C. quinoa protoplasts were inoculated by electro-peroration with capped RNA1 and RNA2 transcripts either together or separately. RNAs extracted from infected protoplasts were tested by Northern hybridization for the presence of newly synthesized viral RNAs.

In a first analysis, protoplasts were inoculated with each trMV transcript and RNAs were extracted 72 h post-inoculation (p.i.). The Northern blot revealed that all the trMV replicated but at variable levels (data not shown). The transcript trMV_{3a} displayed the highest level of replication; clone pMV_{13} was therefore selected for further experiments.

In order to determine the optimal replication time, the RNAs from protoplasts inoculated with trMV_{13} were analysed at various times p.i. Synthesis of RNA1 was detected 48 h.p.i. (data not shown) and the amount of RNA1 increased up to 72 h.p.i. (Fig. 3a, lane 2). Transcripts of clone pACN were also analysed for their ability to infect protoplasts. Unlike trMV_{13}, trACN was not replicated independently in protoplasts but only when co-inoculated with trMV_{13} (Fig. 3a, lanes 4 and 3, respectively), demonstrating that replicase functions are carried by RNA1 in GFLV.

To confirm the results obtained in protoplasts, C. quinoa plants were also used to test the infectivity of the transcripts. Two leaves per plant were inoculated with trMV_{3a} and trACN either together or separately. Following inoculation the course of infection was followed by visual identification of GFLV symptoms. A characteristic yellow mosaic appeared on the upper leaves about 6 to 8 days p.i. on plants inoculated with...
trMV\textsubscript{13} + trACN. Symptom appearance was delayed by almost 2 days compared with the GFLV F13 RNA control and the symptoms were weaker. In contrast, plants inoculated with either trMV\textsubscript{13} or trACN alone did not develop symptoms. Crude extracts from plants infected with trMV\textsubscript{13} + trACN were infectious when inoculated into C. quinoa plants and similar symptoms developed but remained attenuated and delayed in comparison with a GFLV F13 inoculum used as control. The presence of a satellite RNA in this control may be responsible in part for these differences. Additional experiments are needed to determine the role of the satellite RNA in the symptomatology. Molecular tests were used to confirm that these symptoms reflect GFLV replication. Two weeks after inoculation RNAs were extracted from 2 g of leaves and analysed for the presence of viral RNAs by Northern hybridization. In non-inoculated upper leaves with symptoms, the presence of viral RNAs was detected (Fig. 3b, lanes 1 and 2). In plants infected with either trMV\textsubscript{13} or trACN alone no RNA was detected (Fig. 3b, lanes 3 and 4 respectively) as expected from the absence of symptoms. The presence of RNA1 was also tested in leaves inoculated with trMV\textsubscript{13}, but no RNA1 was detected (data not shown). This result suggests that RNA1 may be able to replicate in inoculated cells only but cannot spread in the absence of RNA2, which is presumed to carry the diffusion functions. Analysis by primer extension of the 5' end of both RNA1 and RNA2 recovered from plants infected with trMV\textsubscript{13} and trACN showed that, in both RNAs, the non-viral G residue at the transcript's 5' extremity is removed during replication (data not shown). These experiments demonstrate that both synthetic RNAs of pMV\textsubscript{13} and pACN clones are infectious in protoplasts as well as in C. quinoa plants and that the VPg is not required for infectivity. This is in agreement with observations made for a number of other viruses possessing a 5' VPg (Van der Werf et al., 1986; Vos et al., 1988; Domier et al., 1989; Riechmann et al., 1990; Young et al., 1991). Moreover, the presence of a cap structure could increase infectivity, directly by increasing translation of viral proteins and indirectly by protecting RNAs from exonucleolytic degradation in cells. The presence of an extra G residue at the 5' end of trMV\textsubscript{13} and trACN as well as non-viral residues at the 3' end of the transcripts did not alter their infectivity. Once the transcripts are translated in the infected cell, VPg is produced and replication can then proceed as for natural RNA.

The availability of artificial infectious transcripts of the GFLV genomic RNAs has allowed us to obtain an artificial GFLV isolate free of satellite RNA which will be a useful helper strain to investigate the role of the satellite RNA in the F13 isolate of GFLV, for which an infectious transcript is available (Hans et al., 1992). These transcripts will also be useful in elucidating the role of various viral proteins in virus multiplication.

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References


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