MOLECULAR CLONING OF dsRNAs ASSOCIATED WITH STRAWBERRY MOTTLE VIRUS

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Abstract

Strawberry mottle virus was transmitted from *Fragaria vesca* to *Chenopodium quinoa* and *Nicotiana occidentalis* 37B. In all plants, SMoV infection-associated dsRNA was detected but dsRNA patterns showed some differences between the herbaceous hosts. The differences were more evident when the dsRNA preparations were electrophoresed employing a SDS-PAGE method followed by silver staining of polyacrylamide gels than when employing traditional agarose gel electrophoresis and EtBr staining. Double-stranded RNA, purified from *N. occidentalis* 37B, was used to synthesize cDNA and for the development a cDNA library in *Escherichia coli*. Recombinant plasmids were analyzed by restriction enzyme digestion, and specific cDNA clones were selected by means of a ^{32}P-labelled probe prepared from SMoV dsRNA. After a miniprep step, the plasmids were digested and the length of the inserts was determined to be between 200 and 2000 bp. The largest inserts were selected and used for further research. Hybridization experiments confirmed the specificity of the cloned material for SMoV dsRNA.

1. Introduction

Within the major aphid-borne viruses, strawberry crinkle rhabdovirus and strawberry mottle virus (SMoV) still remain unidentified and largely uncharacterized. Recent progresses obtained by Leone, *et al.*, 1995 showed that, after transmission of SMoV from *Fragaria vesca* to *Nicotiana occidentalis* 37B plants, while virus particles in plant extracts or after purification trials were seldom present, two infection-associated dsRNA bands of 7.8 and 6.3 kbp could be readily detected. These results, together with the finding that infectivity was phenol extractable and RNase sensitive, showed that SMoV has properties similar to those of defective viruses (Falk, *et al.*, 1979). Preliminary attempts at molecular cloning of dsRNA of SMoV were reported by Schoen and Leone (1995).

Here we report the detection of dsRNAs in different host plants infected by SMoV and the successful cDNA cloning of dsRNA extracted from *N. occidentalis* 37B. The infection-associated dsRNA patterns showed some host dependence. Hybridization experiments confirmed that cloned material is specific for SMoV dsRNA.

2. Materials and methods

2.1. Virus maintainence and plant material

Several isolates of SMoV were maintained in *F. vesca* UC-5 or Witte vesca plants. Some isolates were mechanically transmitted to *Chenopodium quinoa* and, subsequently, to *N. occidentalis* 37B plants and propagated in these hosts as described by Leone, *et al.*, 1995.

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2.2. Isolation of dsRNA

Double-stranded RNA was extracted from *F. vesca*, *C. quinoa* and *N. occidentalis* 37B SMoV-infected or uninfected plants and analyzed by agarose gel electrophoresis as reported by Schoen and Leone (1995).

SDS-PAGE of dsRNA was performed in 5% polyacrylamide gels (T=5%; C=2.6%) as described by Tas (1990), running for 20 min at 70 V and 2 h at 120 V constant or at 50 V constant for 16 h. Gels were silver-stained following the procedure described by Bassam, *et al.*, (1991), taking care that formaldehyde concentration was $1.5x10^{-4}$% during the development step.

2.3. Production and analysis of cDNA clones

Synthesis of cDNA from dsRNA template of SMoV purified from *N. occidentalis* 37B was performed as described by Jelkmann, *et al.*, (1989). Blunt end cDNA fragments were cloned into the EcoR V site of pBluescript KS (Stratagene) and transformed into *Escherichia coli* DH5α . Molecular biology techniques were performed as described in Sambrook, *et al.*, (1989).

3. Results

3.1. Analysis of dsRNA in plants infected with different isolates of SMoV

Double-stranded RNA was extracted from infected or uninfected *F. vesca* UC-5 or Witte vesca plants, *C. quinoa* and *N. occidentalis* 37B and analyzed by SDS-PAGE electrophoresis on 5% polyacrylamide gel. The equivalent of about 4 g of leaf material was analyzed. SDS-PAGE electrophoresis and silver staining of polyacrylamide gels were used to improve separation and detection of dsRNA bands compared to agarose gels. Similar results were obtained by agarose gel electrophoresis, but sensitivity of detection was not satisfactory because dsRNA concentration in strawberry plants was very low (results not shown). Both in uninfected and SMoV-infected strawberry plants, evident dsRNA bands ranging from 5.2 to 4.7 kbp were detected (Fig. 1). However, only in plants infected by four different SMoV isolates, four additional faint bands of infection-associated dsRNA were found. These infection-associated dsRNA bands were identical for the different isolates and were estimated to be 8.3, 7.8, 7.1 and 6.8 kbp. In six-day-old leaves of *C. quinoa* infected by SMoV, two bands of dsRNA were detected. In uninfected plants no such bands were detected (results not shown). The two infection-associated bands were estimated to be 7.1 and 6.8 kbp and were identical for two different SMoV isolates. Two additional, very large, infection-associated bands of about 35.5 and 15 kbp were detected in *C. quinoa,* and bands of about 37 and 16 kbp were also found in *N. occidentalis* 37B. All the infection-associated bands were characterized as dsRNA by DNase/RNase digestion in the presence of NaCl above 0.5 M.

3.2. Cloning of SMoV dsRNA

Attempts at cDNA synthesis from purified SMoV dsRNA after heat treatments and/or in the presence of DMSO or formaldehyde failed. Studies on the melting properties of dsRNA of SMoV showed a very high melting temperature (Tm). The Tm was of 77°C at 1.25 mM NaCl and 81°C at 5 mM NaCl (Fig. 2). This may partly explain unavailability of open template for cDNA synthesis under the conditions mentioned above.

Only at 20 mM methylmercuric hydroxide concentration was it possible to obtain sufficient cDNA synthesis for the generation of cDNA clones. Molecular cloning of dsRNA resulted in the analysis of 120 clones by plasmid isolation and restriction enzyme digestion. Insert sizes of these clones ranged between 200 and 2000 bp. The specificity of the generated clones for SMoV dsRNA was analyzed by spotblotting on nitrocellulose (Fig. 3). Clone SMoV12, containing a 2.6 kbp fragment, was oligo-labelled with [α-^{32}P]dATP and used to

probe other cDNA clones, dsRNA of SMoV and dsRNA of cucumber mosaic virus (CMV). The probe detected specifically dsRNA of SMoV and other cDNA clones but no hybridization occurred to dsRNA control of CMV. Clones pSMoV3 and pSMoV8 were also not detected, indicating that these two clones cover different regions of the SMoV genome. Detection of dsRNA of SMoV was possible only after treatment with methylmercuric hydroxide.

4. Discussion

Viral, infection-associated dsRNAs were found in SMoV-infected *F. vesca* plants, *C. quinoa* and *N. occidentalis* 37B. The dsRNA band patterns were consistent for the same host but showed some differences between the host plants. The origin of these differences is at the moment unknown as well as the origin of the very large dsRNA bands detected in SMoV-infected experimental hosts *C. quinoa* and *N. occidentalis* 37B. The lack of these bands in *F. vesca* might be due to an overall, much lower concentration of SMoV dsRNA in this plant. All the dsRNAs were characterized as such by DNase/RNase digestion at high salt concentration (Schoen and Leone, 1995). Differences in dsRNA patterns between hosts has been reported to occur for citrus tristeza virus by Dodds, *et al.*, (1986), but no explanation was given to this phenomenon.

Large-scale isolations of dsRNA in SMoV-infected *N. occidentalis* 37B yielded pure preparations of the 7.8 and 6.3 kbp bands (Schoen and Leone, 1995). Starting from these dsRNAs it was possible to generate cDNA clones only when the preparations were treated with methylmercuric hydroxide (Jelkmann, 1989). Previous attempts at cDNA synthesis without the use of this chemical were all unsuccessful (Schoen and Leone, 1995). The very high Tm of SMoV dsRNA can in part explain its stability to denaturation. The future availability of sequence information will help to understand this phenomenon.
Hybridization studies by spotblotting on nitrocellulose and detection with a ^{32}P-dATP oligo-labelled clone confirmed the specificity of the cDNA clones for SMoV dsRNA. Sequence analysis and genome organization studies are in progress and will be reported elsewhere.

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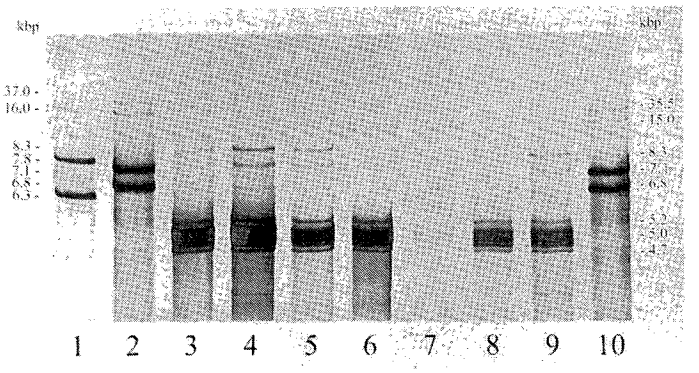

Figure 1. SDS-PAGE in a 5% polyacrylamide gel of dsRNA from SMoV-infected *Chenopodium quinoa*, *Fragaria vesca* and *Nicotiana occidentalis* 37B plants and from uninfected strawberry indicator plants. Lane 1, *N. occidentalis* 37B infected with isolate 1134; lane 2, *C. quinoa* infected with isolate 1134; lane 3, *F. vesca* UC-5 infected with isolate 1134; lane 4, *F. vesca* UC-5 infected with isolate 1265; lane 5, *F. vesca* UC-5 infected with isolate 1280; lane 6, *F. vesca* UC-5 uninfected; lane 7, *F. vesca* UC-4 uninfected; lane 8, *F. vesca* Witte vesca uninfected; lane 9, *F. vesca* Witte vesca infected with isolate 1279; lane 10, *C. quinoa* infected with isolate 1279.

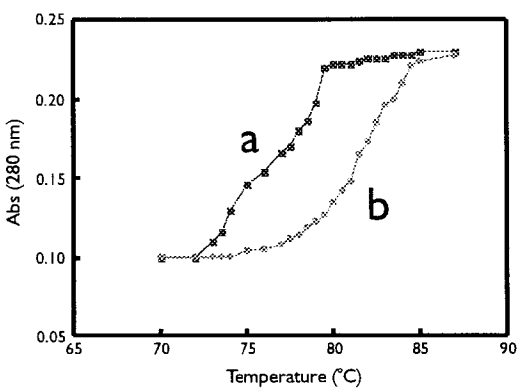

Figure 2. Melting temperature curves of dsRNA of SMoV. A: dsRNA in 1.25 mM NaCl; B: dsRNA in 5 mM NaCl.

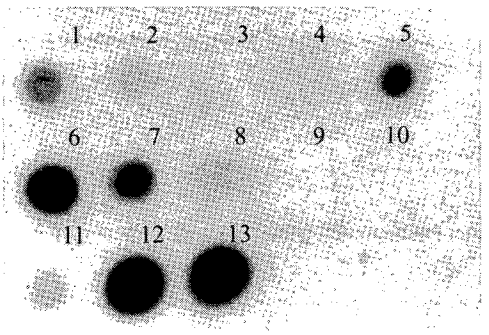

Figure 3. Autoradiogram of spotblotting hybridization with [α-^{32}P]dATP labelled cDNA clone pSMoV12. 1, pSMoV65; 2, SMoV dsRNA (denaturation step: 5 min at 100°C); 3, CMV dsRNA (denaturation step: 5 min at 100°C); 4, SMoV dsRNA (denaturation step: formamide/formaldehyde); 5, SMoV dsRNA (denaturation step: methylmercuric hydroxide); 6, pSMoV12; 7, pSMoV45; 8, pSMoV37; 9, CMV dsRNA (denaturation step: formamide/formaldehyde); 10, CMV dsRNA (denaturation step: methylmercuric hydroxide); 11, pSMoV24; 12, pSMoV 89; 13, pSMoV76.