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Plant proteins that bind to the 3′-terminal sequences of the negative-strand RNA of three diverse positive-strand RNA plant viruses

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Abstract The replication of positive-strand RNA plant viruses, which involves both virus-encoded and plant-encoded proteins, takes place in two stages: synthesis of a negative-strand RNA using the genomic positive-strand RNA as a template and synthesis of progeny positive-strand RNA using the negative-strand RNA as a template. Using gel mobility shift and photochemical crosslinking assays, we have identified three proteins of M_r 32K, 50K and 100K in extracts of tobacco and spinach leaves that bind to the 3′-terminal sequences of the negative-strand RNA of three diverse positive-strand RNA plant viruses. The 32K protein was purified to near homogeneity by chromatography on columns of Macro-prep high Q, heparin-sepharose, single-stranded DNA cellulose and poly(U)-sepharose. No binding of any of the three proteins to the 3′-termini of the positive-strand RNA or the 5′-termini of the positive-strand RNA or negative-strand RNA of any of the three viruses, or to the 3′-termini of the mRNAs of two chloroplast genes, *psb*A or *pet*D, could be detected. We propose that 3′-terminal negative-strand RNA binding proteins, which may be widespread in the plant kingdom, could be utilised by at least three different positive-strand RNA plant viruses for the initiation of positive-strand RNA synthesis.

Key words: Positive-strand RNA virus replication; 3′-Terminal negative-strand RNA binding protein; Initiation of positive-strand RNA synthesis

1. Introduction

The majority of plant viruses, many important animal viruses and some bacteriophages have genomes of positive-strand (messenger-sense) RNA [1]. The replication of positive-strand RNA viruses takes place in two stages: (i) the synthesis of a negative-strand RNA using the genomic positive-strand RNA as a template, and (ii) the synthesis of progeny positive-strand RNA using the negative-strand RNA as a template. The replication is catalysed by an RNA-dependent RNA polymerase (RdRp) encoded by the virus genome [2]. The replication of bacteriophage Qβ RNA requires four bacterial proteins in addition to the RdRp [3] and it is likely that eukaryotic cell proteins also play essential roles in eukaryotic virus RNA replication. Indeed, purified RdRp preparations from plants infected by cucumber mosaic virus or brome mosaic virus were found to contain several plant proteins in addition to virus-encoded proteins [4–7].

Initiation of the two stages of replication requires binding of the RdRp enzyme complex to promoters at the 3′-termini of the positive-strand and negative-strand RNAs respectively. However, for eukaryotic positive-strand RNA viruses it is not known whether virus-encoded or host-encoded proteins are involved in these processes. Recently proteins that bind to the 3′-terminus of the negative-strand RNAs of a number of animal positive-strand RNA viruses have been detected in animal cell extracts and postulated to play a role in the initiation of positive-strand RNA synthesis [8–10]. Whether analogous proteins exist in plants has not been investigated previously. Here we report the detection of three plant proteins that bind to the 3′-terminus of the cucumber mosaic virus negative-strand RNA and the purification of one of them to near homogeneity. We also show that the proteins bind to the 3′-termini of the negative-strand RNAs of two other plant viruses in different families and discuss this finding in relation to the replication of positive-strand RNA plant viruses.

2. Materials and methods

2.1. Isolation and purification of the 32K 3′-terminal negative-strand RNA binding protein

Young leaves of tobacco (*Nicotiana tabacum*) or spinach (*Spinacea oleracea*) were homogenised in a Waring blender in two volumes of H buffer (20 mM HEPES pH 7.9, 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT and 5% glycerol) at 4°C. After filtering through two layers of muslin, the extract was centrifuged for 15 min at 12,000 rpm in a Beckman J2-21 rotor, and the supernatant re-centrifuged for 2 h at 50,000 rpm in a Beckman Ti70 rotor. The supernatant (50 ml) was then applied to a 5 ml Econo-Pac high Q column, containing the quaternary ammonium Macro-Prep high Q support (Bio-Rad). The column was developed with a gradient of 60–500 mM KCl in H buffer at a flow rate of 2 ml/min. Fractions were dialysed against H buffer and the proteins concentrated by centrifugation in Centricons (Amicon). Fractions were assayed for their ability to bind to the 3′-terminal 250 nucleotides of the negative strand of RNA 1 of cucumber mosaic virus by RNA mobility shift and photochemical cross-linking assays. Fractions containing this RNA-binding activity were applied to a 1 ml Hi-Trap Heparin-Sepharose column (Pharmacia). Proteins were eluted using a linear gradient of 60 mM–1 M KCl in H buffer at 1 ml/min. Fractions were dialysed against H buffer and the proteins concentrated by centrifugation in Centricons (Amicon). Fractions from the heparin column containing negative-strand RNA-binding proteins were applied to a 1 ml single-stranded DNA cellulose column (Pharmacia). Bound proteins were eluted using H buffer containing 1 M KCl, dialysed against H buffer and then applied to a 1 ml poly(U)-sepharose column (Pharmacia). The 32K 3′-terminal negative-strand binding protein binds weakly to poly(U)-sepharose and therefore the flow through was re-applied at least three times. Bound proteins were then eluted with H buffer containing 1 M KCl and dialysed against H buffer.

2.2. RNA synthesis

Regions corresponding to the 3′- and 5′-terminal 250 nucleotides of the positive and negative strands of virus RNAs were amplified using

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the polymerase chain reaction [11,12] using pairs of appropriate oligonucleotide primers, one of which contained the T7 RNA polymerase ϕ10 promoter [12] at its 5′ end, and cDNA clones of each virus RNA as templates: cucumber mosaic virus RNA 1, pCMV-A [12], red clover necrotic mosaic virus RNA 2, pTM34–210 [13], tobacco mosaic virus, pLFW3 [14]. The amplified DNA was then purified and used as a template for in vitro transcription by T7 RNA polymerase [5]. Transcripts from the 3′-terminal regions of the mRNAs of the chloroplast genes *psb*A and *pet*D were synthesised as described [15,16]. The transcripts were labelled using [^{32}P]UTP and purified as described previously [5].

2.3. RNA mobility shift assay

A solution of the protein (0.1–1 μg) in H buffer (10 μl), ^{32}P-labelled RNA transcript (3 μl, 25,000–100,000 cpm), prepared as in section 2.2, and wheatgerm tRNA (2 μl of a 10 mg/ml solution) were mixed and incubated at room temperature for 15 min. The products were then analysed by electrophoresis through a native 4% polyacrylamide gel to separate RNA–protein complexes from free RNA transcripts [5], followed by autoradiography.

2.4. Photochemical cross-linking assay

Protein and RNA solutions were mixed and incubated as in section 2.3. The samples were then irradiated in open tubes with 2 J of UV light in a Stratalinker 1800 (Stratagene). Uncrosslinked RNA was then removed by addition of 1 μl of a 0.5 mg/ml solution of ribonuclease A and incubation at 37°C for 20 min. Crosslinked protein–RNA complexes were then subjected to electrophoresis through a 10% SDS-polyacrylamide gel [5] and detected by autoradiography.

3. Results

To determine if tobacco cells contained a protein(s) that could bind to the 3′-termini of cucumber mosaic virus RNA, an extract of leaves of healthy tobacco plants was incubated with ^{32}P-labelled RNAs corresponding to the 3′-terminal 250 nucleotides of the negative-strand RNA 1 or the positive-strand RNA 1 of cucumber mosaic virus and the mixtures were analysed by electrophoresis through a native polyacrylamide gel. The detection of a band with retarded mobility (Fig. 1, lane 1, single-headed arrow), compared to that of the free RNA (double-headed arrow), indicated the formation of a specific complex between the 3′-terminal sequence of the negative-strand RNA and one or more plant proteins. No complex was detected between the 3′-terminal sequence of the positive-strand RNA and proteins in extracts of healthy tobacco (Fig. 1, lane 2); only the band of free RNA was detected. Furthermore the binding

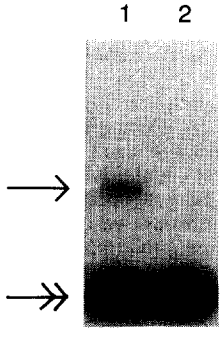

Fig. 1. Gel mobility shift analysis of the interaction of tobacco protein(s) with 3′-terminal sequences of the cucumber mosaic virus negative and positive RNA strands. The ^{32}P-labelled 3′-terminal 250 nucleotides (25,000 cpm) of the negative strand (lane 1) or positive strand (lane 2) of cucumber mosaic virus RNA 1 were incubated with a protein extract of healthy tobacco leaves (1 μg protein) and the mixtures were analysed for formation of protein–RNA complexes by native polyacrylamide gel electrophoresis and autoradiography.

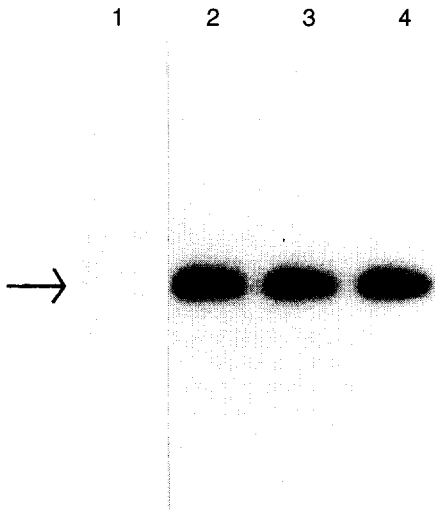

Fig. 2. Binding competition. The ^{32}P-labelled 3′-terminal 250 nucleotides (100,000 cpm) of the negative strand of cucumber mosaic virus RNA 1 were incubated with a protein extract of healthy tobacco leaves (1 μg protein) together with the unlabelled 3′-terminal 250 nucleotides of the negative strand (lanes 1 and 2) or positive strand (lanes 3 and 4) of cucumber mosaic virus RNA 1 at molar ratios (labelled/unlabelled) of 1 : 100 (lanes 1 and 3) or 1 : 1 (lanes 2 and 4) and the protein–RNA complexes formed were analysed by native polyacrylamide gel electrophoresis and autoradiography. Only the protein–RNA complex (single-headed arrow) is shown; the free RNA has run off the end of the gel.

of one or more proteins from healthy plants to the ^{32}P-labelled 3′-terminal sequence of the negative strand was competed out by an excess of the unlabelled 3′-terminal sequence of the negative-strand, but not by a similar excess of the unlabelled 3′-terminal sequence of the positive-strand RNA (Fig. 2; the free RNA band has run off the end of this gel). Hence the binding is specific for the 3′-terminal negative-strand RNA.

To characterize further the plant protein(s) with the ability to bind to the 3′-terminal sequence of the cucumber mosaic virus negative-strand RNA, protein extracts of healthy tobacco leaves were fractionated by chromatography on a variety of media. Fractions from each column were assayed for the ability to bind to the 3′-terminal 250 nucleotides of the negative strand, but not to the 3′-terminal 250 nucleotides of the positive strand, of cucumber mosaic virus RNA 1. The final procedure adopted, which resulted in the purification to almost homogeneity of a protein of M_r 32K, consisted of successive cycles of chromatograhy on columns of a strong quaternary ammonium anion exchanger (Macro-prep high Q), another anion exchanger (heparin-Sepharose), followed by single-stranded DNA cellulose and finally poly(U)-sepharose. The yield of the purified protein was ca. 1 μg/kg of leaf material.

Analysis of the purified protein by SDS-polyacrylamide gel electrophoresis (Fig. 3) indicated a major protein with M_r 32K together with a small amount of a protein of M_r 67K. The ability of the protein to bind to the ^{32}P-labelled 3′-terminal sequence of cucumber mosaic virus negative-strand RNA was assayed by photochemical cross-linking (Fig. 4). The 32K protein was clearly the major component with 3′-terminal negative-strand RNA binding activity, although two other fainter bands corresponding to proteins of M_r 50K and 100K were also detected. No binding to the 3′-terminal sequence of the positive strand or the 5′-terminal sequences of the positive or negative

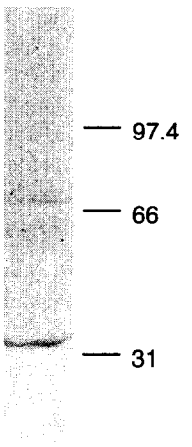

Fig. 3. Analysis of the purified 3′-terminal negative-strand binding protein from tobacco. The purified 3′-terminal negative-strand binding protein was subjected to electrophoresis through an 8% SDS-polyacrylamide gel and the gel was stained with Coomassie brilliant blue R250. The M_r values (K) of marker proteins are shown on the side of the gel.

strands of cucumber mosaic virus RNA was detected (Fig. 4). Furthermore the binding to the ^{32}P-labelled 3′-terminus of the cucumber mosaic virus negative-strand RNA was not competed out by an excess of unlabelled 3′-terminus of the cucumber mosaic virus positive-strand RNA or the 5′-terminus of the cucumber mosaic virus positive-strand or negative-strand RNAs (not shown), confirming that the cross-linking was not due to non-sequence-specific RNA binding.

Photochemical cross-linking also showed that the 32K protein, and the two minor proteins, bound to the 3′-terminal sequences of the negative-strand RNAs of two other viruses in different taxonomic groups [1,17], tobacco mosaic virus and red clover necrotic mosaic virus (Fig. 4). Again no binding to the 3′-terminal sequences of the positive-strands, or the 5′-terminal sequences of the positive or negative strands, of either of these two viruses (Fig. 4) could be detected. As further controls, the ability of the purified 32K protein to bind to the 3′-terminal sequences of the mRNAs of two chloroplast genes, *psb*A (which encodes the thylakoid D1 protein of photosystem II) [15,16] and *pet*D (which encodes subunit IV of the cytochrome b_6f complex) [15,16], was tested. No binding could be detected (Fig. 4).

When extracts of healthy spinach leaves were fractionated as for the tobacco leaves, a protein of M_r 32K was isolated. This protein also had the ability to bind to the 3′-termini of the negative-strand RNAs of cucumber mosaic virus, tobacco mosaic virus and red clover necrotic mosaic virus, but not to the 3′-termini of the positive-strand RNA or the 5′-termini of the positive-strand RNA or negative-strand RNA of any of these three viruses, or to the 3′-termini of the mRNAs of the *psb*A or *pet*D genes, two plastid mRNAs known to bind ribonucleoproteins [15,16].

4. Discussion

We have shown that 32K proteins from tobacco and spinach bind to the 3′-terminal sequence of the negative strand of cucumber mosaic virus RNA, but not to the 3′-terminal sequences of the positive strand or the 5′-terminal sequences of the positive or negative strands. This is the first report of the detection of RNA binding proteins with these properties from plants and the first report of the purification of such proteins from eukaryotic organisms. The purification of 32K proteins with similar binding properties from plants in two diverse families (tobacco, Solanaceae; spinach, Chenopodiaceae) suggests that such proteins may be widespread in the plant kingdom.

Although the 32K 3′-terminal negative-strand binding protein has been purified to near homogeneity, small amounts of other proteins were present in the purified protein preparation. One of these with M_r 67K, which could be detected after SDS-polyacrylamide gel electrophoresis by staining the gel with Coomassie brilliant blue (Fig. 3), did not have 3′-terminal negative-strand RNA binding activity, because no band corresponding to this M_r could be detected in the photochemical cross-linking assay (Fig. 4). Two other proteins, which could not be detected by staining the gel with Coomassie brilliant blue, had 3′-terminal negative-strand RNA binding activity as shown by the photochemical cross-linking assay (Fig. 4). These proteins, cross-linked to radiolabelled nucleotides, migrated in SDS-polyacrylamide gel electrophoresis with apparent M_r values of 50K and 100K; the M_r values of the uncrosslinked pro-

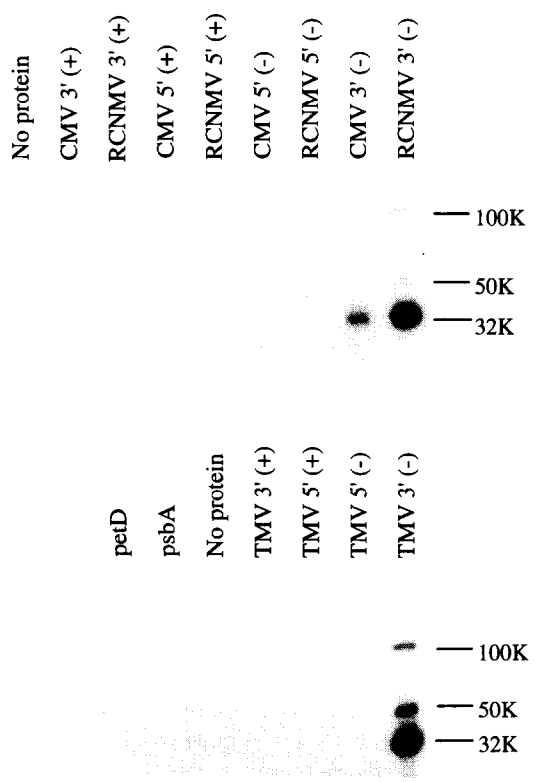

Fig. 4. Binding of the purified 3′-terminal negative-strand binding protein to terminal sequences of the negative- and positive-strand RNAs of different viruses. The purified 3′-terminal negative-strand binding protein from tobacco (0.1 μg) was incubated with the ^{32}P-labelled 3′-terminal or 5′-terminal 250 nucleotides (50,000 cpm) of the negative strand (−) or positive strand (+) of cucumber mosaic virus (CMV) RNA 1, red clover necrotic mosaic virus (RCNMV) RNA 2, tomato mosaic virus (TMV) RNA, *pet*D mRNA or *psb*A mRNA. No protein controls were carried out with the CMV (top) or TMV (bottom) 3′-terminal negative strands. After photochemical cross-linking, uncrosslinked RNA was removed with ribonuclease and protein-nucleotide complexes were analysed by SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

teins may be about 2K smaller [18]. Although the bands were fainter, because these two proteins were present in only trace amounts in the purfied 32K protein preparation, it is possible that they bound the 3′-terminal negative-strand RNA sequences as strongly as the 32K protein.

Animal cell proteins that bind to the 3′-terminal sequence of the negative-strand RNAs of animal viruses have also been reported [8–10]. The finding of 3′-terminal negative-strand RNA binding proteins in animals and plants suggests that cellular proteins may generally play a role in the initiation of positive-strand RNA synthesis in the replication of positive-strand RNA viruses. The animal cell 3′-terminal negative-strand RNA binding proteins had M_r values of 97K, 79K and 56K (Vero African green monkey cells) [8], 42K, 44K and 52K (chicken embryo fibroblasts) [9], and 50K and 52K (mosquito, *Aedes albopictus*, cells) [10]. None of these has yet been purified and further work will be needed to determine if any of them are related to any of the plant 3′-terminal negative-strand RNA binding proteins described here.

Our finding that the 32K plant protein can bind to the 3′-terminal sequences of the negative-strands of two additional positive-strand RNA viruses in taxonomic groups different from that of cucumber mosaic virus, i.e. tobacco mosaic virus and red clover necrotic mosaic virus, is noteworthy. Binding of animal cell proteins to the 3′-terminal sequences of the negative strands of different animal positive-strand RNA viruses has also been reported [10], although the viruses were all in the same family. Comparison of the three plant virus 3′-terminal negative-strand sequences using the GAP and BESTFIT programs [19] did not reveal any significant sequence identity between any of them. It is possible that different domains of the 32K protein could recognise different sequences in the three negative-strand RNAs or that the RNAs could fold into similar secondary or tertiary structures that are recognised by the same protein domain. Analysis with the FOLDRNA program [19] showed that all three 3′-terminal negative-strand RNA sequences could be folded into secondary structures containing stem-loops. However a stem–loop structure *per se* is clearly insufficient for binding, since no binding was obtained with the 3′-terminal regions of the *psb*A and *pet*D mRNAs or the 3′-terminal positive-strand RNAs of the three viruses, all of which contain sequences that can be folded into stem–loop structures [16, 20–22]. Similar considerations apply to the 50K and 100K proteins.

Binding of cellular proteins to the 3′-terminal negative-strand sequences of three different viruses could have important implications for the replication of positive-strand RNA viruses. It has generally been found that isolated RdRps are template-specific, i.e. they will catalyse the synthesise of negative-strands using the RNA templates of only the same or closely related viruses [1,2]. It is likely therefore that binding of the RdRp to the 3′-terminal sequences of the positive-strand RNA (to initiate negative-strand RNA synthesis) is specified by a virus-encoded protein. We propose that binding of the RdRp to the 3′-terminal sequence of the negative-strand RNA could be specified by one or more cellular proteins able to function in the initiation of positive-strand RNA synthesis with at least three, and possibly many, different viruses.

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