

Chloroplast phosphoglycerate kinase, a gluconeogenic enzyme, is required for efficient accumulation of *Bamboo mosaic virus*

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ABSTRACT

The tertiary structure in the 3'-untranslated region (3'-UTR) of *Bamboo mosaic virus* (BaMV) RNA is known to be involved in minus-strand RNA synthesis. Proteins found in the RNA-dependent RNA polymerase (RdRp) fraction of BaMV-infected leaves interact with the radio labeled 3'-UTR probe in electrophoretic mobility shift assays (EMSA). Results derived from the ultraviolet (UV) cross-linking competition assays suggested that two cellular factors, p43 and p51, interact specifically with the 3'-UTR of BaMV RNA. p43 and p51 associate with the poly(A) tail and the pseudoknot of the BaMV 3'-UTR, respectively. p51-containing extracts specifically down-regulated minus-strand RNA synthesis when added to *in vitro* RdRp assays. LC/MS/MS sequencing indicates that p43 is a chloroplast phosphoglycerate kinase (PGK). When the chloroplast PKG levels were knocked down in plants, using virus-induced gene silencing system, the accumulation level of BaMV coat protein was also reduced.

INTRODUCTION

Small genome sizes restricts coding capacity in all RNA viruses, therefore, most processes of the infection cycle rely on the factors present/available in their hosts (1,2). In addition to the RNA-dependent RNA polymerase (RdRp), encoded by the RNA viruses, host factors are required for the formation of the replicase complex. In bacteriophage Q β -infected cells, the best-studied case, the replication complex needed to synthesize the plus-strand RNA consists of not only the viral RdRp but also the host translation elongation factors EF-Ts and -Tu and the ribosomal protein S1 (3). Moreover, a ribosome-associated protein, HF1, binding to the 3' end of the Q β genomic RNA, is required for the synthesis of the minus-strand RNA (4). Translation factors

also participate in viruses replicating in eukaryotic cells; e.g. translation elongation factor-EF1a was claimed to be involved in some viruses including *Tobacco mosaic virus* (TMV), *West Nile virus* (WNV) and *Turnip yellow mosaic virus* [TYMV; (5–7)].

Many host factors have been reported to be involved in virus translation and RNA replication processes (5,8–18). In addition, many host factors were demonstrated to physically interact with the *cis*-acting elements of the viral RNA in viruses including poliovirus, WNV and *Brome mosaic virus* [BMV; (5,19–21)]. In positive-sense RNA viruses, translation and replication of the same RNA templates must be regulated since the 5' to 3' movement of ribosomes on the RNA conflicts with the 3' to 5' movement of the RdRp on the template RNA. This could be achieved by the interaction of host and viral factors at both ends of and sometimes along the viral RNA (7,22,23).

Bamboo mosaic virus (BaMV), a member of *Potexvirus*, is a single-stranded positive-sense RNA virus with flexuous rod morphology (24). The RNA genome of BaMV consisting of 6366 nt [excluding the poly(A) tail], encoding five open reading frames (ORFs), is 5'-capped and 3'-polyadenylated (25). ORF1 encodes a 155 kDa replication-related protein that contains capping enzyme domain (26,27), helicase-like domain (28) and RdRp core domain (29). This protein may assemble on membrane along with some host factors into a replication complex (30). ORFs 2–4 are arranged within the triple gene block, which encodes proteins involved in cell to cell virus movement. ORF5 encodes the 25 kDa viral coat protein. Two major subgenomic RNAs, 2 and 1 kb in length, are produced to express proteins for the movement and encapsidation of viral RNA (25,31,32).

The 3'-UTR of the positive-strand RNA viruses contains essential *cis*-acting elements for the initiation of the minus-strand RNA synthesis. The 3'-UTR of BaMV RNA was determined to fold into a cloverleaf-like ABC domain, a major stem-loop (D domain), and a pseudoknot (E domain) containing part of the poly(A) tail [Figure 1; (33)]. The importance of these structures in the BaMV replication has been demonstrated *in vivo* and *in vitro* (30,33–35). The

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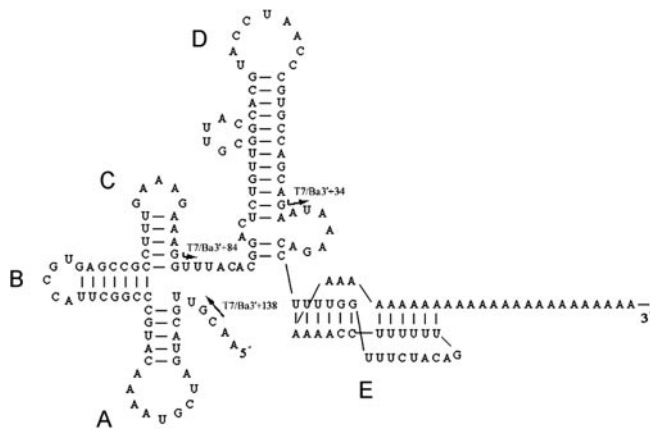


Figure 1. The sequence and the tertiary structure of the 3'-UTR of BaMV RNA. The tertiary structure of the 3'-UTR depicted includes the cloverleaf-like ABC domain, a stem-loop designated D domain, and a pseudoknot designated E domain. Arrows indicate the 5' ends of the 3'-UTR RNAs used in this study.

D domain and the pseudoknot were shown to strongly interact with a recombinant viral RdRp (36). In addition, it has been reported that synthesis of the minus-strand RNA initiates most frequently at the pseudoknot of the 3'-UTR, more specifically at the poly(A) residues of the pseudoknot (37).

Here, host factors interacting with the BaMV 3'-UTR were detected using the electrophoretic mobility shift assay (EMSA) and the ultraviolet (UV) cross-linking technique. The results indicated that two host factors, p51 and p43, interact specifically with the 3'-UTR of the BaMV RNA. The LC/MS/MS analysis identified the p43 as the chloroplast phosphoglycerate kinase (PGK). The role of p43 and p51 in BaMV replication were investigated using *in vitro* RdRp assay and virus-induced gene silencing (VIGS).

MATERIALS AND METHODS

Templates for *in vitro* transcription and plasmid construction for virus-induced gene silencing

The DNA templates for transcript r138/40A, r84/40A and r34/40A were PCR amplified with 5' primers BaMV/T7+6228 (5'-TAATACGACTCACTATAGGGCGTTGCATGATCG-3'; T7 promoter was underlined), BaMV/T7 + 6282 (5'-TAATACGACTCACTATAGTTTACACGGACT-3') and BaMV/T7 + 6333 (5'-TAATACGACTCACTATAGGAATAAAGACCTTTT-3'), respectively, and a common 3' primer T40GG (5'-T₄₀GG-3') from a BaMV 3'-UTR cDNA clone, pBa6228/Bam (30). The templates for transcribing r34/10A and r34/noA were also amplified from pBa6228/Bam with a common 5' primer BaMV/T7 + 6333 and specific 3' primer BaMV 3' 10A (5'-TTTTTTTTTTGGAAAAACTGTAGAAA-3') and BaMV 3' noA (5'-GGAAAAACTGTAGAAA-3'), respectively. Template for transcript Ba-77 of BaMV minus-strand 3'-terminal 77 nt was amplified from the pBaMV-O (33) with the 5' primer BaMV/T7(-)77 (5'-TAATACGACTCACTATAGGGCGATTGTAG-3') and the 3' primer BaMV 5' + 1 (5'-GAAAACCACTCCAAACGAAA-3'). Finally, pT7CMV/tRNA (30) was linearized with BamHI and used for transcribing the 3' tRNA-like structure of *Cucumber mosaic virus*, CMV/3'TLS.

Tobacco rattle virus (TRV) based VIGS system was used to knock down the expression of host genes. Plasmids pTRV1, pTRV2 and pTRV2 with PDS (phytoene desaturase) gene were kindly provided by Dr Baulcombe (Sainsbury Laboratory, John Innes Centre, UK). The cDNA fragment corresponding to *Nicotiana benthamiana* chloroplast PGK was PCR amplified using primer pairs designed according to the sequences acquired from expressed sequence tag library of *N.benthamiana* deposited in the GeneBank and cloned into pTRV2. Two plasmids pTRV2/PGK-5 and pTRV2/PGK-3 containing 482 and 471 bp insert, respectively, were constructed to knock down chloroplast PGK. The 482 and 471 bp fragments corresponding to 54–535 nt and 1039–1509 nt of tobacco chloroplast PGK were RT-PCR amplified and cloned into pTRV2 using primer set PGK-5, with forward primer (5'-GGCATCAGCTACAGCTTCTC-3') and reverse primer (5'-GCAGTTCGGATAGCCTGG-3'), and PGK-3, with forward primer (5'-GAGAAGGCCAAGGCGA-3') and reverse primer (5'-GTACAAATGTTTTACACAGCAAC-3'), respectively. For VIGS assay, pTRV1 and pTRV2 or its derivatives were introduced into *Agrobacterium tumefaciens* strain C58C1 by electroporation.

In vitro transcription

To uniformly label the RNA transcripts, 10 μ l of *in vitro* transcription reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 125 mM NaCl, 2 mM spermidine, 10 mM DTT, 3 mM each of ATP, CTP and GTP, 30 μ M UTP, 40 μ Ci of [α -³²P]UTP (3000 Ci/mmol; Amersham), 10 U of RNasin (Boehringer Mannheim Biochemicals), 0.5 μ l of T7 polymerase (New England Biolabs) and 1 μ g of DNA template were incubated at 37°C for 2 h. The labeled-RNAs were electrophoresed and purified from polyacrylamide gel. To synthesize unlabeled transcripts, 200 μ l of reaction mixture was set at the same condition described above with normal 3 mM UTP, 20 U of RNasin, 10 μ l of T7 polymerase and 20 μ g of DNA template. RNA transcripts were treated with RNase-free DNase I (Roche) and gel purified.

RdRp extract preparation and activity assay

The purification of BaMV RdRp extract was described previously (30). In brief, the leaves of infected *N.benthamiana* plants were collected 5 days post-inoculation and stored at the -80°C. The frozen leaves were homogenized in buffer A (50 mM Tris-HCl (pH 7.6), 15 mM MgCl₂, 120 mM KCl, 1 μ M pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% β -mercaptoethanol and 20% glycerol), filtered, and ultracentrifuge at 30 k g (Ti70 rotor, BECKMAN) for 35 min to obtain the supernatant (S30) and the pellet (P30). To further purify the viral RdRp extract, P30 was fractionated through a 20–60% sucrose-gradient ultracentrifugation. Ten fractions were collected and numbered from top-to-bottom.

RdRp activity assays, using endogenous RNA templates bound to the enzyme complex when isolated or with the exogenous RNA templates (templates added externally after the removal of endogenous RNAs), were performed as described previously (30). For the exogenous template activity assay, the detergent-solubilized RdRp preparation was treated with micrococcal nuclease and subsequently used in the reaction containing [α -³²P]UTP and RNA templates.

The RNA products were electrophoresed on a 5% native polyacrylamide gel, and the radioactive signals were analyzed with the PhosphorImager (Fujifilm BAS-1500).

EMSA

About 20 fmol of α -³²P-labeled RNA probe along with binding buffer [20 mM Tris-HCl (pH 7.9), 3 mM MgCl₂, 25 mM KCl, 20 U of RNasin, 0.1 mM EDTA, 2 mM DTT, 1 μ g of yeast total RNA and 4% glycerol] was added to the extracts derived from the first fraction of RdRp preparation (P30) of the sucrose-gradient, and incubated at 25°C for 10 min.

After incubation, the reaction mixtures were electrophoresed on a 5% native polyacrylamide gel, dried and autoradiographed. The radioactivity emitted by the probes was scanned and quantified using a PhosphorImager (Fujifilm BAS-1500).

UV cross-linking and competition assay

The reactions were performed as in EMSA before irradiated with a 254 nm-wavelength UV lamp (STRATAGENE, UV stratalinker TM 1800) on ice for 20 min. After irradiation, the samples were treated with 40 μ g of boiled RNase A for 20 min at 37°C, boiled in Laemmli buffer, and electrophoresed on a 12% SDS-polyacrylamide gel. In the competition reactions, the various amounts of unlabeled competitor RNAs were pre-incubated with the proteins for 10 min prior to the addition of ³²P-labeled RNA probe.

Purification and identification of host factors

The soluble fraction S30 from healthy plants was prepared as for RdRp extraction except replacing the buffer A to Heparin-buffer [50 mM Tris-HCl (pH 7.9), 20 mM KCl, 2.7 mM MgCl₂ and 10% glycerol], filtered, and passed through Heparin-Sepharose column (column volume, 5 ml; Amersham Pharmacia Biotech) with the flow rate of 2 ml/min. After washing with 50 mM NaCl, the bound proteins p43 and p51 were eluted with 50 ml of 50–350 mM NaCl gradient. Eluted p43 and p51 were dialyzed in binding buffer [20 mM Tris-HCl (pH 8.6) and 15 mM KCl] and subsequently passed through Q-column (column volume, 1 ml; Amersham Pharmacia Biotech) with the flow rate of 1 ml/min. The flow-through fraction contains p51 and was used in the exogenous RdRp assay. The bound p43 was eluted with 12 ml of 0–400 mM NaCl gradient. For all chromatography, 1 ml fractions were collected and the RNA-binding proteins were traced by UV cross-linking assay. The band corresponding to p43 (the fifth fraction) was used in the RdRp assay and also subjected to LC/MS/MS (Mission Biotech, Taiwan).

VIGS and virus challenging

N.benthamiana plants were grown in pots at 28°C in a growth chamber under 16 h light/8 h dark cycle. *A.tumefaciens* cultures at OD₆₀₀ = 1 containing pTRV1 or pTRV2 derivative plasmids were mixed in 1:1 ratio and infiltrated with a syringe onto three leaves of each plant. Ten days after infiltration, 10 μ l of 1 μ g BaMV or cytomegalovirus (CMV) virion RNA was mechanically inoculated on to the fourth leaf above the infiltrated leaves. Two days later, total protein was extracted from the virus-inoculated leaves of three independent plants. BaMV and CMV coat protein

accumulation was determined by western blot assay individually.

RNA isolation and real-time PCR

Total RNA was extracted from *N.benthamiana* plants using hot phenol extraction and LiCl precipitation (38). First strand cDNA was synthesized using 1 μ g of total RNA, 39 d(T) oligo primer and reverse transcriptase. In the real-time PCR experiment, the chloroplast-specific PGK is amplified and detected by a forward primer (5'-GGCATCAGCTACAGCT-TCTC-3') located in the transit peptide region and a reverse primer (5'-GCAGTTCGGATAGCCTGG-3'). PCR without prior reverse transcription was performed as a negative control. The levels of actin mRNA were also determined by real-time PCR using a forward primer (5'-GATGAAGATAC-TCACAGAAAGA-3') and a reverse primer (5'-GTGGTTTC-ATGAATGCCAGCA-3') for the normalization of specifically silenced gene.

RESULTS

Two cellular proteins interact with the 3'-UTR of BaMV RNA

To identify and characterize the BaMV 3'-UTR RNA-binding proteins, the potential minus-strand RNA synthesis regulators, protein extracts derived from BaMV-infected *N.benthamiana* leaves were prepared for the protein-RNA interaction studies. The 10 top-to-bottom fractions (F1–F10) collected from a 20–60% sucrose-gradient of P30 were tested for the endogenous RdRp activity and electrophoretic mobility shift (EMS) activity. Interestingly, the EMS activity colocalized with the RdRp activity in some fractions, with the endogenous RdRp activity found mainly in F5–F7 (Figure 2A) and the EMS activity mainly in F1 (F1) as well as in F5 to F7 but less prominent (Figure 2B). F1 which contains the most prominent EMS activity was further characterized. EMSA-based competition experiment was conducted using F1 as the protein source to analyze the specificity of RNA-protein interaction. Results showed that the EMS signals of the RNA-protein complexes can be competed out efficiently by r138/40A RNA, compared to the negative control, CMV 3' tRNA-like RNA structure (CMV/3'TLS; Figure 3A). r34/40A, which contains a portion of stem-loop D and pseudoknot structure (Figure 1), performed as efficient as r138/40A. However, the EMSA competition ability of r34/40A was diminished when the poly(A) tail was removed (r34/noA). Furthermore, the EMS signals of the complexes were also competed out efficiently by poly(A) RNA but not by double-strand poly(I/C) RNA (Figure 3B). These results suggest that the 3'-poly(A) tail is essential for the formation of the RNA-protein complex.

The fact that the top fraction F1 appears to contain the most EMS signal implies that the RNA-interacting proteins may exist largely in the soluble fraction. Subsequently, EMS activity comparable to that of F1 was detected in the soluble fractions (S30) prepared from the BaMV-infected as well as from the healthy plants, indicating that the RNA-binding proteins are not virally encoded. UV cross-linking experiments were then performed to detect the presence of

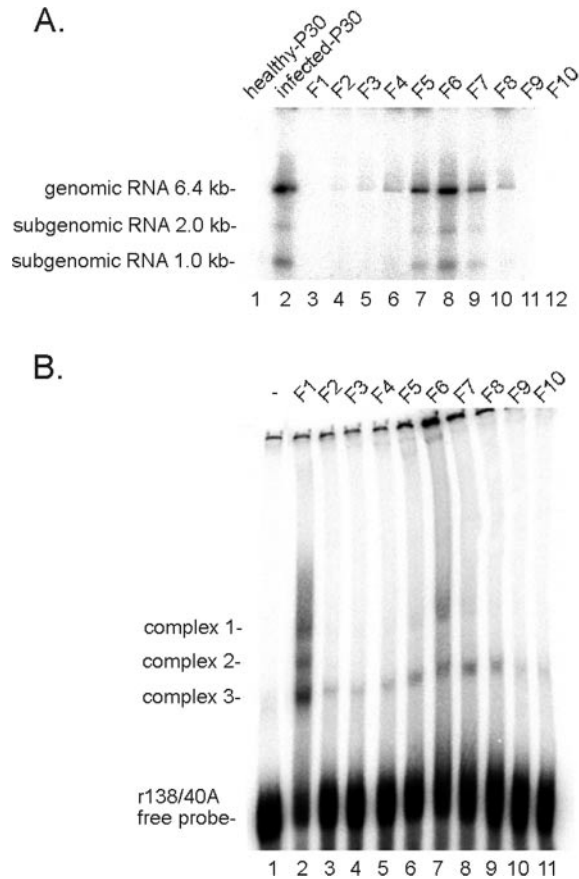


Figure 2. Co-fractionation of the RdRp and the RNA shifting activities. (A) The endogenous RdRp assay in detecting the activity of the polymerase in membrane fractions. [α - 32 P]UTP along with four ribonucleotides was incubated with the fractions to incorporate the radioactive uridines into the replicating RNAs. Lanes 1 and 2, membrane fractions (P30) derived from healthy and infected plants, respectively; lanes 3 to 12, the 10 top-to-bottom sucrose-gradient fractions described in Materials and Methods. The double-stranded products of genomic and two subgenomic RNAs are indicated. (B) Detection of the formation of BaMV 3'-UTR RNA-protein complexes by the EMSA. Probe r138/40A, the 3'-UTR of BaMV RNA with 40 As, alone was loaded as a control (lane 1). The same 10 sucrose-gradient fractions were incubated with the probe and analyzed as described in Materials and Methods. Three complexes are indicated.

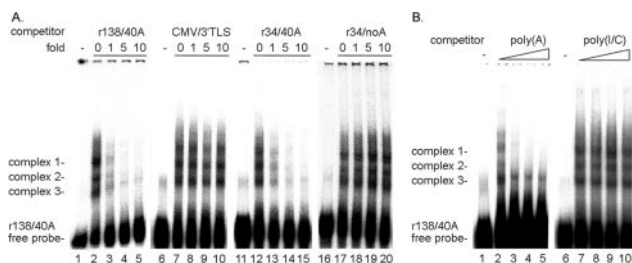


Figure 3. Characterization of the RNA elements responsible for complex formation using EMSA-based competition experiment. The BaMV 3'-UTR RNA, r138/40A, was used as a probe to characterize the RNA elements required for the formation of the gel-shifting complexes with fraction F1. In EMSA reactions, F1 was incubated with labeled r138/40A RNA in the presence of (A) 0x-, 1x-, 5x- and 10x-molar excess of the competitors r138/40A (lanes 2-5), CMV/3'TLS (lanes 7-10), r34/40A (lanes 12-15) or r34/noA (lanes 17-20) and (B) 0, 0.5, 2.5 and 5.0 ng of the competitors of poly(A) (lanes 2-5) and poly(I/C) (lanes 7-10). Probe alone was loaded in (A) lanes 1, 6, 11 and 16; and in (B) lanes 1 and 6.

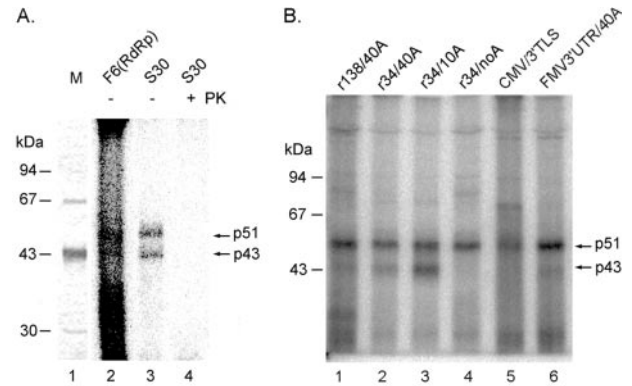


Figure 4. Characterization of the BaMV 3'-UTR-binding proteins by an UV cross-linking assay. (A) The r138/40A RNA probe was labeled with [α - 32 P]UTP and incubated with the RdRp containing membrane fraction F6 from BaMV-infected leaves (lane 2) and the soluble fraction S30 from healthy leaves (lanes 3 and 4) prior to UV cross-linking. Before 12% SDS-PAGE, one of the reactions was treated with proteinase K (lane 4). Protein size markers are on lane 1 as indicated. The arrows indicate the position of the two RNA-binding protein designated p51 and p43. (B) The heparin column-eluted protein extracts were incubated with equal amounts of radioactive probes: r138/40A (lane 1), r34/40A (lane 2), r34/10A (lane 3), r34/noA (lane 4), CMV/3'TLS (lane 5) and FMV3'-UTR/40A (lane 6).

any 3'-UTR-binding proteins in the S30 fraction from uninfected leaves and in the membrane-associated RdRp fraction (F6) from BaMV-infected leaves (Figure 4A). A 51 kDa and a 43 kDa protein, designated p51 and p43, respectively, were observed in both F6 and S30 fractions, indicating that they are present in cells as membrane-associated (F6) as well as soluble proteins (S30).

The pseudoknot domain in the 3'-UTR is responsible for p51 and p43 binding

To enrich the host factors, S30 was passed through a heparin column. The host proteins p51 and p43 were eluted in the same fractions from a salt gradient and used for later analysis. To localize the RNA region responsible for protein binding, several RNA probes, representing different regions of the 3'-UTR, were prepared for the UV cross-linking experiment (Figure 4B). r34/40A containing the pseudoknot domain retained the protein-binding activity. In addition, a stronger signal of p43 was observed using a shorter poly(A) tailed RNA probe, i.e. r34/10A, and this probe was used for all later UV cross-linking experiments. However, r34/noA with the poly(A) tail deletion failed to bind p43. Based on these observations, it is likely that p43 is a poly(A)-binding protein. In addition, the 3'-UTR (107 nt) of *Foxtail mosaic virus* (FMV), (which is genetically close to BaMV) RNA with 40As could also be cross-linked with p51 and p43; however, CMV/3'TLS [a genetically unrelated virus RNA that has no poly(A) tail] could not be cross-linked with p43, though weakly with p51 (Figure 4B).

p43 is a poly(A)-specific binding protein

Since the 3' poly(A) tail appeared to be the target binding site for p43, four homoribopolymers used as competitors in two concentrations (0.5 and 5.0 ng in a 10 μ l reaction; Figure 5) were examined by UV cross-linking competition

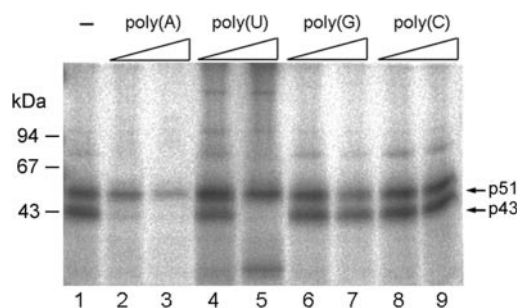


Figure 5. Characterization of the poly(A) RNA-binding activity of p43. The heparin column-eluted proteins were incubated in the UV cross-linking reactions with labeled r34/10A RNA probe (lane 1) or the addition of 0.5 ng and 5 ng of unlabeled poly(A) RNA (lanes 2 and 3), poly(U) RNA (lanes 4 and 5), poly(G) RNA (lanes 6 and 7) and poly(C) RNA (lanes 8 and 9). Protein size markers are indicated. The arrows indicate the position of the two RNA-binding protein p51 and p43.

assay. Only poly(A) RNA can compete out p43 from binding to the labeled RNA probe (r34/10A) at a lower concentration. However, the competing activity of poly(U) RNA at a higher concentration observed in the reaction could be due to the annealing of the competitor to the poly(A) tail of the probe. The results strongly support the conclusion that p43 is a novel poly(A)-specific binding protein. In addition, probe binding to p51 was also partially competed out at higher concentration of unlabeled poly(A) RNA, suggesting that the poly(A) tail may also be involved in the p51–RNA interaction.

p51 could specifically interact with the 3'-UTR of BaMV RNA

The interaction between p51 and the 3'-UTR of BaMV RNA was characterized by the UV cross-linking competition assay (Figure 6A). At 2-fold molar excess of the cold competitor r138/40A (about 40 fmol), 85% of the ³²P-labeled probe (r138/40A) was competed out, whereas only 10% competition was observed with the CMV/3'TLS or Ba-77 (the 3' end 77 nt of minus-strand BaMV RNA) as the competitor, suggesting specific interactions between p51 and BaMV 3'-UTR.

To localize the p51-binding region, 3'-UTR deletion RNAs, r84/40A and r34/40A were also used as competitors. Results indicated that the competing ability of r34/40A did not differ from that of r138/40A and r84/40A, but is much more efficient than that of r34/noA RNA (Figure 6B). Taken together, these results suggested that p51 mainly binds to the 3' pseudoknot region, although r34/40A contains about 11 nt derived from stem D, which might contribute some interaction with p51.

p51-containing fraction inhibited the minus-strand RNA synthesis *in vitro*

It has been reported that the poly(A) region within the pseudoknot structure in the 3' end of BaMV RNA (Figure 1) could specifically interact with the recombinant RdRp(Δ 893) protein *in vitro* (30). In addition, the minus-strand RNA initiation sites were located at the 3' poly(A) tail of the BaMV genomic RNA (37). As the results of our previous experiments indicate that host factors p51 and p43 interact with the pseudoknot and the poly(A) tail region, respectively, it raised the question

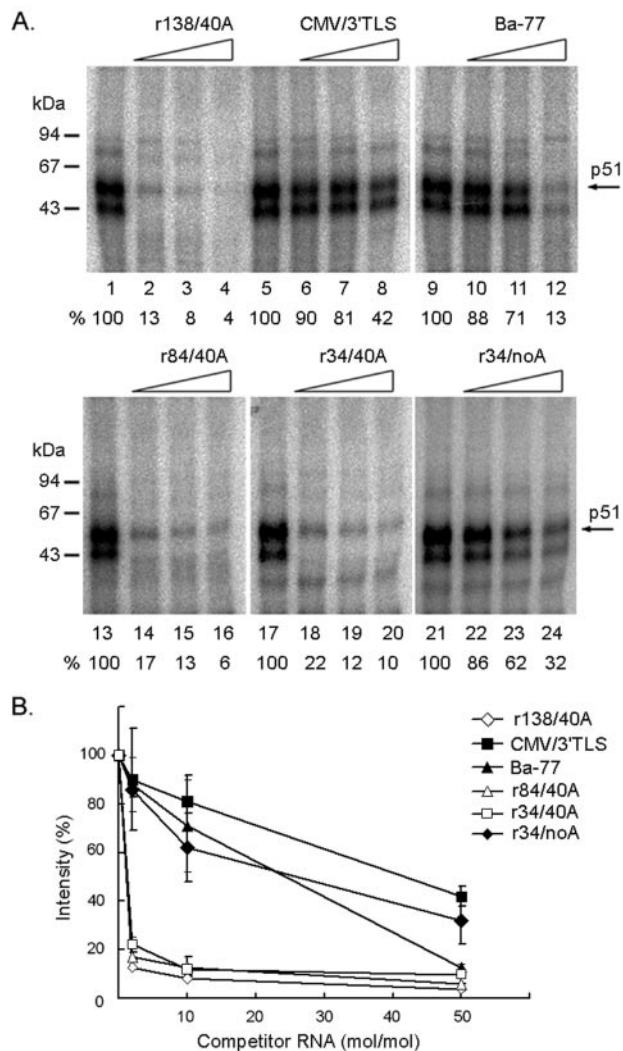


Figure 6. Characterization of the RNA-binding activity of p51. (A) A competition UV cross-linking assay. The heparin column-eluted proteins were incubated with the labeled r34/10A RNA (lanes 1, 5, 9, 13, 17 and 21) or in the presence of 2 \times -, 10 \times - and 50 \times -molar excess of the RNA competitors, r138/40A (lanes 2–4), CMV/3'TLS (lanes 6–8), Ba-77 (lanes 10–12), r84/40A (lanes 14–16), r34/40A (lanes 18–20) and r34/noA (lanes 22–24). Protein size markers are indicated. The arrow indicates the position of the RNA-binding protein p51. The percentage shown under each lane number is the intensity of p51 relative to that of the reaction without competitor in each set of experiment. (B) The intensities of the bands p51 in (A) were plotted. The intensity without a competitor RNA was defined as 100%. SDs shown were based on three independent experiments.

that whether p51 and p43 are involved in minus-strand RNA synthesis. To investigate this possibility, we used an *in vitro* RdRp assay to test if the addition of the fractions containing p51 and p43 has an effect on the RNA synthesis of minus-strand. Q-column chromatography was used to separate p51 and p43. The addition of the p51-fraction (Q-FT, unbound fraction) reduced the minus-strand RNA synthesis from the r138/40A template to 42% of the control reaction that did not receive any supplements (Figure 7, lanes 1 and 2). In addition, this inhibition was dose-dependent (data not shown). In contrast, the addition of the same amount of p51-fraction exerted only a minor effect on the plus-strand

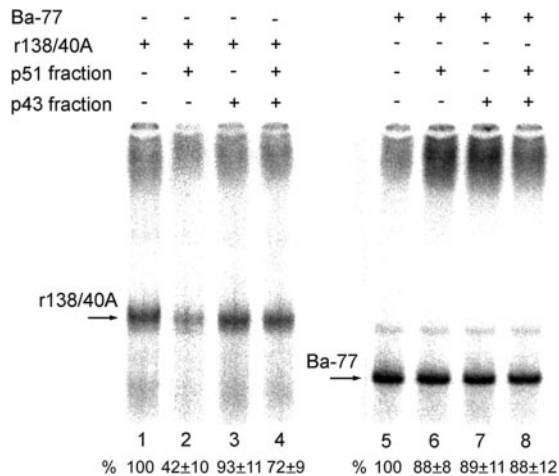


Figure 7. Functional studies of the host proteins in an *in vitro* RdRp assay. *In vitro* RdRp assay reactions described in Materials and Methods were performed RNA templates, r138/40A (lanes 1–4) and Ba-77 (lanes 5–8). The p51- (lanes 2 and 6) and p43-enriched fractions (lanes 3 and 7) were added in the reactions as indicated above each lane. In some of the reactions (lanes 4 and 8), the p43-enriched fraction was pre-incubated with the RNA for 10 min before the addition of the p51-enriched fraction into the reaction. The percentage of RNA synthesis was shown under each lane with the SD based on at least three independent experiments. The intensity of the RdRp products without the addition of protein fractions (lanes 1 and 5) was defined as 100%.

Table 1. Correlation of chloroplast PGK mRNA reduction and BaMV replication

pTRV2 constructs	Chloroplast PGK mRNA accumulations (%) ^a	BaMV CP accumulation (%) ^b	CMV CP accumulation (%) ^c
pTRV2	100 ± 13	100 ± 12	100 ± 24
pTRV2/PDS	85 ± 12	84 ± 20	73 ± 11
pTRV2/PGK-5	48 ± 6	93 ± 3	69 ± 21
pTRV2/PGK-3	13 ± 5	45 ± 16	85 ± 16

All data were averages (±SDs) of three independent experiments and normalized to that of pTRV2.

^aRelative accumulation of chloroplast PGK mRNA in various gene-knock down plants. The mRNA level of chloroplast PGK was normalized to that of actin.

^bBaMV coat protein accumulation at 48 h after BaMV RNA inoculation, detected by western blotting with anti-BaMV CP serum.

^cCMV coat protein accumulation at 48 h after CMV RNA inoculation, detected by western blotting with anti-CMV CP serum.

RNA synthesis from the Ba-77 template (Figure 7, lanes 5 and 6). On the other hand, the addition of the p43 fraction appeared to have no effect on the minus-strand RNA synthesis for all concentrations we tried. Interestingly, when the template was pre-incubated with p43 fraction for 10 min before the addition of p51-fraction, the minus-strand RNA synthesis was reduced to 72% to that of the control reaction (Figure 7, lane 4) thereby increasing the activity by 30% by countering the effect of p51 addition. These results suggested that p51 and p43 share the overlapped binding site, the poly(A) sequence, and that they might compete each other for the binding site and play opposing roles in regulating minus-strand RNA synthesis.

p43 was identified as the chloroplast phosphoglycerate kinase

After heparin column purification, p43 protein was further purified by an anionic exchange column (Q-column). UV cross-linked and silver-stained (Figure 8) protein bands were aligned. Although the products of fractions 5 and 6 eluted from a Q-column (Figure 8, lanes 1 and 2, respectively) contained a 100 kDa unknown protein, a clear protein band with 43 kDa in size was observed. Then the band corresponding to p43 in the fraction 5 was subjected to LC/MS/MS analysis. Sequence analysis with results obtained from p43 LC/MS/MS results show perfect similarity to the nucleus-encoded chloroplast PGK of *Nicotiana tabacum* (Supplementary Figure S1). Although the predicted size of the chloroplast PGK precursor of *N.tabacum* is 50.1 kDa, the mature PGK excluding the transit peptide is 42.6 kDa, in close agreement with the observed size of p43.

Chloroplast PGK is involved in BaMV accumulation in plants

The TRV-based VIGS system was used to generate specific gene-knockdown plants to investigate the importance of these host factors in BaMV RNA accumulation in plants. Two constructs were created for the chloroplast PGK (the 5' and 3' targeting regions, designated as PGK-5 and PGK-3) as described in Materials and Methods. The phytoene desaturase (PDS) gene construct was used as the knockdown control. Six days after the infiltration process, the PDS-knockdown plants started to display the photobleaching phenotype on the fourth leaf above the infiltrated leaves. All of the chloroplast PGK-knockdown plants grew normally and had only minor visible changes on their leaves (Supplementary Figure S1). Real-time PCR results showed that the levels of the chloroplast PGK mRNA in the PGK-5 and PGK-3 knockdown plants were reduced to about 50 and 10% to those of the control plants, respectively (Figure 9A and Table 1). To investigate the effects of the chloroplast PGK-knockdown plants on the BaMV replication, about 1 µg of the BaMV or CMV RNA purified from virions was inoculated onto the fourth leaf above the infiltrated leaves. Total proteins were extracted 48 h post-inoculation and analyzed on the western blots for the accumulation of viral coat protein. Comparative analysis of the protein extracts indicated a reduced level (45%) of BaMV coat protein in the PGK-3 knockdown plants, and no significant alteration in the control plants and the PDS-knockdown plants (Figure 9B). However, the amount of PGK required for an efficient accumulation of BaMV is relatively low since BaMV can accumulate to wild-type level in PGK-5 with 50% PGK-knockdown and accumulate to 45% in PGK-3 with almost 90% PGK-knockdown. On the other hand, a minor non-specific interference on the accumulation of CMV coat protein was observed in any host gene-knockdown plants (Figure 9C). Taken together, these results suggested that the chloroplast PGK is required for efficient BaMV accumulation, but not important for unrelated viruses, such as CMV.

DISCUSSION

Host-derived components were shown to be involved in the replication of RNA viruses (39,40). Translational factors EF-Tu, -Ts and the ribosomal protein S1 were shown to play a structural role in the formation of stable and active replicase complex of bacteriophage Q β (3,41). Translation factors were also shown to bind viral proteins or RNAs. The barley homolog of eIF-3 p41 subunit was shown to be co-purified with the BMV replication complex from infected plants and to enhance the RdRp activity *in vitro* (42). Translational elongation factor 1a, EF1a, was reported to bind the viral polymerase of *Vesicular stomatitis virus* (17) and the 3' end viral RNAs including TMV, TYMV and WNV (5,43–45).

For positive-sense RNA viruses, the translation process conflicts with the viral RNA replication. It is obvious that

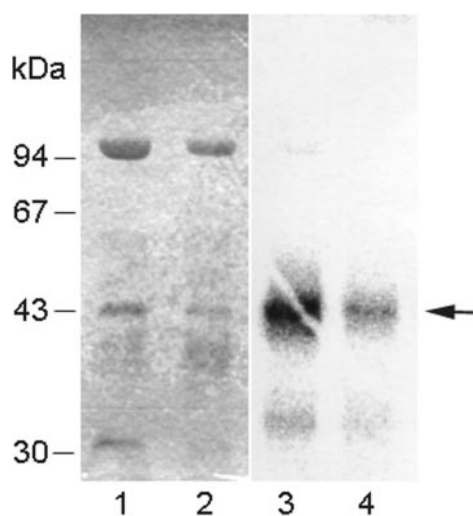


Figure 8. Purification of p43 by heparin and Q-chromatography columns. p43 was purified from S30 fraction by a heparin column, and then a Q-column. The Q-column-eluted fraction 5 and 6 (lane 1 and 2, respectively) were dialyzed and concentrated for electrophoresis and silver-staining. A UV cross-linking assay was performed using r34/10A probe and Q-column-eluted fraction 5 and 6 (lanes 3 and 4, respectively). The arrow indicates the protein band corresponding to the UV cross-linked p43 signal.

the translating ribosomes on the RNA template moving from the 5' toward to the 3' would somehow inhibit the viral RNA replication which moves in the opposite orientation to prevent collision (46–49). This conflict apparently can be solved through the coordination of the viral and host factors. In *Alfalfa mosaic virus*, the viral coat protein binding to the 3'-terminal region of the genomic RNA can inhibit the minus-strand RNA synthesis *in vitro* (50,51). The minus-strand RNA synthesis of TYMV was claimed to be repressed upon the binding of EF1a to the valylated viral RNA (7).

Using EMSA and UV cross-linking competition technique, host proteins p51 and p43 were demonstrated to bind specifically to the 3'-UTR of BaMV RNA. Results of the RdRp assay indicated that p51-containing extract specifically inhibited the minus-strand RNA synthesis *in vitro*. Regrettably, we were unable to attribute this inhibitory effect to p51 since we couldn't purify p51 to homogeneity. However, the band corresponding to the size of p51 in silver-stained gel was also subjected to LC/MS/MS and matched to the EF1a of *Nepenthes paniculata* (Supplementary Figure S1). EF1a, a 50 kDa protein, has been claimed to function in the negative-regulation of TYMV minus-strand RNA synthesis (7) and also been reported to bind the tRNA-like structure of BMV (52). CMV/3'TLS has the similar structural properties as that of BMV, therefore, the interaction with p51 (Figure 4B) implies that the identity of p51 is EF1a. However more effort is needed for the purification and identification of p51 which consequently will reveal how the conflict between virus translation and virus replication is modulated.

Results of LC/MS/MS and BLAST indicate that the protein sequences of p43 matched to the host chloroplast PGK. Cytosolic PGK, a cytosolic isozyme of chloroplast PGK, is a ubiquitous monomeric protein that has been isolated from diverse species ranging from human, plants to bacteria (53). Some glycolytic enzymes appear to possess multiple functions in addition to glycolysis. For example, PGK and lactate dehydrogenase were found to reside in nuclei of mammalian cells and exert functions other than glycolysis (54,55). PGK was identified to be a subunit of primer recognition proteins, which are cofactors of DNA polymerase α and may have a role in lagging strand DNA synthesis in nuclei (55,56). In Sendai virus replication, PGK was reported to stimulate

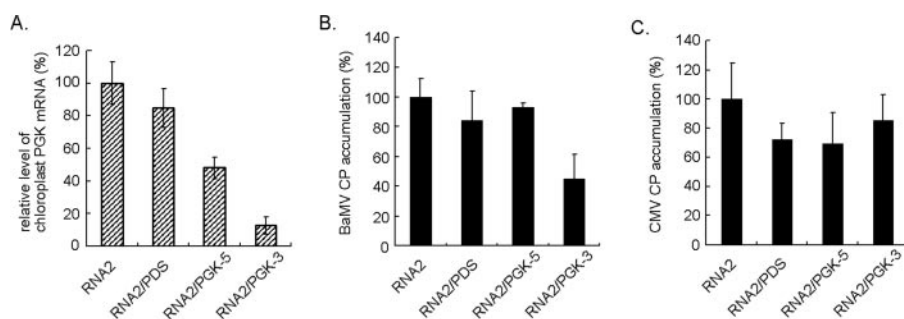


Figure 9. Chloroplast PGK gene expression knock down experiment by VIGS system. The control (RNA2) and knock down plants (RNA2/PDS, RNA2/PGK-5 and RNA2/PGK-3) were challenged with 1 μ g of the BaMV or CMV viral RNAs at the fourth leaf above the infiltrated leaves. Total RNAs and proteins were extracted from the virus-inoculated leaves after 48 h of post-inoculation. (A) The accumulation levels of chloroplast PGK mRNA were measured by real-time PCR. The primer set for the amplification is targeting to the transit peptide region of the gene to measure the chloroplast-form mRNA. The accumulation levels of BaMV (B) and CMV (C) coat proteins were measured by western blotting analysis with specific antisera. All data were the averages (\pm SDs) of three independent experiments and normalized to that of pTRV2.

mRNA transcription at the elongation step, probably through the interaction with tubulin that has been integrated into the replication complex (57). PGK was also found to bind the urokinase-type plasminogen activator receptor mRNA for a post-transcriptional regulation (58). These observations suggest that PGK, a glycolytic enzyme, may regulate cellular as well as viral processes in addition to glycolysis.

PGK is a well known ATP-generating enzyme that is part of the glycolytic, gluconeogenic and photosynthetic pathways (59,60). The chloroplast PGK gene, which is nucleus-encoded, is translated to produce a 50 kDa PGK precursor protein, and then processed into the mature 43 kDa PGK in the chloroplast. PGK precursor may have a half-life too short to detect. In VIGS experiment, PGK was shown to be required for efficient BaMV accumulation. Results derived from real-time PCR and western blot analyses indicated that the reduction of chloroplast PGK reduced the accumulation of BaMV coat protein.

It has been speculated that the replication complex of BaMV RNA is associated with chloroplast (N.-S. Lin). The interaction between the chloroplast PGK and the BaMV RNA seems to suggest that chloroplast PGK could assist the viral RNA in targeting it to the chloroplast membrane. Features of chloroplast PGK namely poly(A) binding and chloroplast localization will lead to further investigation into the possible functions of chloroplast PGK in viral protein translation and viral RNA localization.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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