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In vitro template-dependent synthesis of *Pepino mosaic virus* positive- and negative-strand RNA by its RNA-dependent RNA polymerase

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ABSTRACT

Pepino mosaic virus (PepMV)-infected tomato plants were used to develop an *in vitro* template-dependent system for the study of viral RNA synthesis. Differential sedimentation and sucrose-gradient purification of PepMV-infected tomato extracts resulted in fractions containing a transcriptionally active membrane-bound RNA-dependent RNA polymerase (RdRp). In the presence of Mg^{2+} ions, ^{32}P-labelled UTP and unlabelled ATP, CTP, GTP, the PepMV RdRp catalysed the conversion of endogenous RNA templates into single- and double-stranded (ds) genomic RNAs and three 3′-co-terminal subgenomic dsRNAs. Hybridisation experiments showed that the genomic ssRNA was labelled only in the plus strand, the genomic dsRNA mainly in the plus strand and the three subgenomic dsRNAs equally in both strands. Following removal of the endogenous templates from the membrane-bound complex, the purified template-dependent RdRp could specifically catalyse transcription of PepMV virion RNA, *in vitro*-synthesized full-length plus-strand RNA and the 3′-termini of both the plus- and minus-strand RNAs. Rabbit polyclonal antibodies against an immunogenic epitope of the PepMV RdRp (anti-RdRp) detected a protein of approximately 164 kDa in the membrane-bound and template-dependent RdRp preparations and exclusively inhibited PepMV RNA synthesis when added to the template-dependent *in vitro* transcription system. The 300 nucleotides long 3′-terminal region of the PepMV genome, containing a stretch of at least 20 adenosine (A) residues, was an adequate exogenous RNA template for RdRp initiation of the minus-strand synthesis but higher transcription efficiency was observed as the number of A residues increased. This observation might indicate a role for the poly(A)-tail in the formation and stabilisation of secondary structure(s) essential for initiation of transcription. The template-dependent specific RdRp system described in this article will facilitate identification of RNA elements and host components required for PepMV RNA synthesis.

1. Introduction

The replication of positive-strand RNA viruses takes place in membrane-associated multi-component replication complexes (RC) in a process that involves the virus genome, virus proteins and host factors (Buck, 1996; Lai, 1998). A central role is played by the viral RNA-dependent RNA polymerase (RdRp), which catalyses the synthesis of a complementary minus-strand RNA using the virus genomic, plus-strand RNA as a template and the synthesis of progeny virus genomic RNA using the minus-strand RNA as a template. *In vitro* RdRp systems utilize heterologous expression systems (Hong and Hunt, 1996; Li et al., 1998; Rajendran et al., 2002; Panaviene et al., 2004) or purified RdRp preparations from infected plants to identify replication-essential *cis*-acting RNA elements and host proteins. The purification of transcription-active and template-specific RCs from infected plants has been reported for plus-stranded RNA viruses, such as *Alfalfa mosaic virus* (Quadt et al., 1991), *Bamboo mosaic virus* (BaMV) (Cheng et al., 2001), *Brome mosaic virus* (Quadt and Jaspars, 1990; Kao and Sun, 1996), *Cereal yellow dwarf virus* (Osman et al., 2006), *Cucumber mosaic virus* (Hayes and Buck, 1990), *Potato virus* X (PVX) (Plante et al., 2000), *Tobacco mosaic virus* (Osman and Buck, 1996), *Tomato bushy stunt virus* (Nagy and Pogany, 2000), *Turnip crinkle virus* (Song and Simon, 1994) and *Turnip yellow mosaic virus* (Deiman et al., 1997; Singh and Dreher (1997)). For the prototype potexvirus PVX, *cis*-acting regulatory elements in the 3′-untranslated region (UTR) differentially affect minus-strand and plus-strand RNA accumulation (Pillai-Nair et al., 2003). It has also been reported that an 8-nucleotide U-rich motif within the PVX 3′-UTR is bound by two host proteins (of 28 and 32 kDa in size) to achieve efficient viral multiplication (Sriskanda et al., 1996). In another potexvirus, BaMV, both heterologous and infected plant-derived RdRp systems have identified 3′-terminal *cis*-acting elements and host factors essential

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for the initiation of complementary RNA synthesis (Huang et al., 2001; Cheng et al., 2002; Prasanth et al., 2011). Specifically, BaMV minus-strand RNA synthesis is initiated at multiple sites within the poly(A) tail, which participates in the formation of an RNA pseudoknot (Cheng et al., 2002). The glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is also part of the BaMV RC and inhibits RNA synthesis possibly by competing with the viral RdRp for binding the 3′-terminal pseudoknot (Prasanth et al., 2011).

Pepino mosaic virus (PepMV) is a mechanically transmitted potexvirus, which has become a significant threat for tomato crops (Hanssen and Thomma, 2010) after being first reported in pepino (*Solanum muricatum*) in Peru (Jones et al., 1980). PepMV has flexuous, rod shaped non-enveloped virions and possesses a 6.4 kb single-stranded (ss) RNA genome of positive polarity with a 5′-methylguanosine cap and a 3′ poly-A tail (Aguilar et al., 2002). The PepMV genome contains five open reading frames encoding a 164-kDa RdRp, three triple gene block (TGB) proteins of 26, 14 and 9 kDa, and the 25-kDa coat protein (CP). The viral replicase is expressed from the genomic RNA (gRNA), whereas the TGB proteins are expressed from subgenomic RNAs (sgRNAs) 1 and 2, and the CP from sgRNA3 (Aguilar et al., 2002; Sempere et al., 2011). Currently, four distinct PepMV genotypes (the original Peruvian, European, American and Chilean isolates) with nucleotide identity between 78% and 95% have been characterized (Hanssen and Thomma, 2010). Recent studies indicate that the European PepMV genotype is gradually being overtaken by the Chilean type in Europe (Gómez et al., 2009). PepMV infectious clones from both the European and Chilean genotypes have been produced to facilitate protoplast- and agrobacterium-based inoculation (Hasiów-Jaroszewska et al., 2009). A Polish isolate uniquely causes necrotic lesions in tomatoes due to a single amino acid mutation in TGB3 (Hasiów-Jaroszewska et al., 2011). Sempere et al. (2011) have constructed a PepMV-based vector to express recombinant proteins in plants and to facilitate functional analysis of virus and plant genes.

Here, we have used PepMV-infected extracts of tomato to establish an *in vitro* system that possesses RdRp activity. The system is able to specifically synthesize *de novo* RNA from the 3′-termini of both positive- and negative-strand PepMV RNA templates. This system and a fragment of its 3′-terminus comprising a minimal polyA-tail of 20 A residues will be used to further analyse *cis*-acting RNA elements and host proteins required for PepMV minus-strand RNA synthesis.

2. Materials and methods

2.1. Plant material and virus isolate

PepMV inoculum (isolate SP13) (Aguilar et al., 2002) and cDNA infectious clone pTOPO-T7 PepXL6 (Sempere et al., 2011) were kindly provided by Dr. M. Aranda, CEBAS/CSIC, Murcia, Spain. PepMV was mechanically inoculated to *Solanum lycopersicum* cv. *Boludo* seedlings (Seminis Vegetable Seeds Europe, Enkhuizen, The Netherlands) grown under standard greenhouse conditions. Following mechanical inoculation, the tomato plants were maintained at 25 °C with a 16 h photoperiod. Plants were harvested at various times over a ten-day period post-inoculation and fresh or frozen at (−70 °C) leaf tissue was used to prepare crude RdRp preparations. *Tomato mosaic virus* (ToMV)-infected tissue was kindly provided by Professor N. Katis, Aristotle University of Thessaloniki, Greece.

2.2. Preparation of extracts containing RdRp activity

PepMV-infected tomato plants (100 g) were homogenized in a blender at 4 °C in 200 ml of buffer A (50 mM Tris–HCl [pH 7.4], 15 mM $MgCl_2$, 120 mM KCl, 1 μM pepstatin, 1 μM leupeptine, 20% [vol/vol] glycerol) and the homogenate was passed through muslin. The filtrate was centrifuged at 500 × *g* for 15 min at 4 °C and the supernatant was pelleted by centrifugation in a Sorval SA-600 rotor at 30,000 × *g* for 30 min at 4 °C. The resulting pellet was resuspended in 20 ml of buffer B (50 mM Tris–HCl [pH 8.2], 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μM leupeptin, 1 μM pepstatin) and layered on top of four 36 ml linear (20–60% [wt/vol]) sucrose density gradients in TED buffer (50 mM Tris–HCl [pH 8.0], 10 mM NaCl, 1 mM EDTA, 5% [vol/vol] glycerol) and centrifuged in a Sorval AH-629 rotor at 112,000 × *g* for 2 h at 10 °C. Fractions (5 ml) were collected and tested for RNA polymerase activity and stored at −70 °C without sucrose removal. Fractions with significant RNA polymerase activity were dialysed against buffer B; calcium acetate and micrococcal nuclease were added to a final concentration of 2 mM and 1 u/μl, respectively for 30 min at 30 °C. EGTA was added to a final concentration of 5 mM for 10 min at 4 °C.

The RNA polymerase preparation was added to buffer B containing 1 mM ATP, 1 mM CTP, 1 mM GTP, 10 mM UTP, 5 μCi of [α-^{32}P] UTP, bentonite (4.8 mg/ml) and if required, an RNA template (25 μg/ml) in a total volume of 200 μl. Reaction mixtures were incubated at 30 °C for 1 h. When required, the RNA polymerase transcription products were treated with S1 nuclease (1 u/μl) in buffer C (200 mM NaCl, 50 mM sodium acetate [pH 4.5], 1 mM $ZnSO_4$, 0.5% glycerol) at 37 °C for 20 min as described before (Bates et al., 1995). S1-treated and untreated transcription products were extracted by phenol/chloroform and analyzed on 5% polyacrylamide gel electrophoresis (PAGE) containing 8 M urea as described before (Osman and Buck, 1996). Gels were dried and exposed to film; band intensities on autoradiograms were measured using a Bio-Rad Gel Documentation XR System and Quantity-One software (version 4.6.5, Bio-Rad Laboratories).

PepMV genomic RNA was extracted from purified virions (Jones et al., 1980) and served as a template. Alternatively, a full length PepMV cDNA clone (Sempere et al., 2011) was utilized to generate specific sub-fragments of the PepMV genome by PCR. For these experiments, amplification was carried out using specific oligonucleotide primer pairs that incorporated the T7 promoter, allowing the generation of RNA transcripts of plus or minus polarity from each product. Using this approach, four approximately 300 nucleotide-long fragments derived from the 5′ and 3′-terminal sequences of the PepMV plus- and minus-strand RNA were amplified from a set of eight PepMV and one ToMV oligonucleotide primers (Table 1). The complete ToMV RNA genome was RT-PCR amplified from infected plants using Primescript reverse transcriptase and Pyrobest DNA polymerase (TAKARA) and primer pair ToMV-T23 and ToMV-T18 (Table 1) and also transcribed as above.

2.3. RdRp antiserum production and immunobloting

A rabbit polyclonal antiserum was raised against the PepMV RdRp epitope (amino acids 1176-1189) and the total IgGs were isolated using a protein-A column (Biogenes, Germany). Template-bound and template-dependent extracts from PepMV-infected plants, plus equivalent extracts from mock-infected controls were subjected to electrophoresis in a 12% SDS–PAGE system. Proteins were transferred to Immobilon membrane (GE Healthcare) and briefly stained with Ponceau S to mark the molecular weight standards. The membranes were incubated with the anti-RdRp epitope antiserum (1:1000), followed by alkaline phosphatase-conjugated goat anti-rabbit antibodies (1:5000). The proteins bound by the IgG were detected with an ECL chemiluminescence kit (Sigma-NEN).

Table 1
List of oligonucleotide primers used in this study for RNA synthesis.

Name[a]	Primer sequence (5′-3′)[b]	PepMV nucleotide position[c]
T7-PepMV-t1	*cgaaat***taatacgactcactatag**cttcttcgatggagtcacca	6121-6140
PepMV-t2	ttttttttttatttcaaagaaataa	*c*6396-6410-poly(A)$_{10}$
PepMV-t3	gaaaacaaaataaataaata	1-20
T7-PepMV-t4	*cgaaat***taatacgactcactatag**tggctgcggcatgtgtgtgt	*c*281-300
T7-PepMV-t5	*cgaaat***taatacgactcactatag**aaaacaaaataaataaata	1-20
PepMV-t6	tggctgcggcatgtgtgtgt	*c*281-300
T7-PepMV-t7	*cgaaat***taatacgactcactatag**ttttttttttttttta	*c*6410-poly(A)$_{15}$
PepMV-t8	cttcttcgatggagtcacca	6121-6140
PepMV-t31	ttttttttttttttttttttttttttttttttttttttttttatttcaaaga	*c*6401-6410-poly(A)$_{40}$
PepMV-t32	ttttttttttttttttttttttttttttttttatttcaaaga	*c*6401-6410-poly(A)$_{30}$
PepMV-t33	ttttttttttttttttttttatttcaaaga	*c*6401-6410-poly(A)$_{20}$
PepMV-t34	ttttttttttttttatttcaaaga	*c*6401-6410-poly(A)$_{15}$
T7-ToMV-t23	*cgaaat***taatacgactcactatag**gtatttttacaacaattacc	1-20
T7-ToMV-t17	*cgaaat***taatacgactcactatag**gtactggactgtacaatcag	6110-6128
ToMV-t18	tgggccccaaccgggggttc	*c*6366-6385
T7-ToMV-t21	*cgaaat***taatacgactcactatag**ggtaggctttggttgcaataa	*c*249-271
ToMV-t22	gtatttttacaacaattacc	1-20

[a] Names of oligonucleotide primers designed in fusion with the T7 promoter are written in bold.
[b] Stretches of 6 nucleotides upstream the T7 promoter are written in italics and bold, respectively.
[c] Numbers refer to the PepMV SP13 sequence (accession number 484251) and sequences complementary to the PepMV sequence are prefixed with a "*c*"

3. Results

3.1. Membrane-bound RdRp primarily synthesizes double-stranded RNA products

Homogenized extracts from PepMV-infected leaves were subjected to differential centrifugation and separation on 20–60% sucrose gradients and the resultant fractions, containing membrane-bound RdRp, were tested for RNA transcriptional activity using endogenous templates, namely incomplete, partially synthesized RNAs present in the RCs of infected plants. The ^{32}P-labelled products transcribed by the resuspended P30 pellet produced were four RNA species approximately 6.4, 2.0, 1.2, and 0.8 kbp in size (Fig. 1; lane 1) corresponding to products of the genomic and sgRNAs (TGB1, TGB2-3, CP) (Sempere et al., 2011). Following S1 nuclease treatment, all four RNAs were shown to be double-stranded (ds) in nature (Fig. 1, lane 2) similar to previous observations with two other potexvirus replicases including PVX (Plante et al., 2000) and BaMV (Lin et al., 2005). In all PepMV RdRp preparations, the peak of the RdRp activity was routinely found in the same position in sucrose gradients (fraction 4, data not shown). As compared to crude P30 RdRp preparations, the sucrose fractionated preparation not only catalysed the synthesis of the three sgRNAs but also an S1-sensitive ssRNA product (*cf.* Fig. 1, lanes 3 and 4) identical in size to PepMV RNA transcribed from a full-length infectious clone of the virus (Fig. 1, lane 5) and assumed to be virion genomic RNA. These results were consistent in five separate experiments. Testing RdRp activity obtained from tomato plant extracts every 48 h post inoculation with PepMV over a 10 day period revealed a peak of activity after 4 days similar to results obtained with BaMV RdRp from infected plants (Lin et al., 2005). No RNA synthesis was supported by any extracts prepared from mock-inoculated plants (Fig. 1, lane 6).

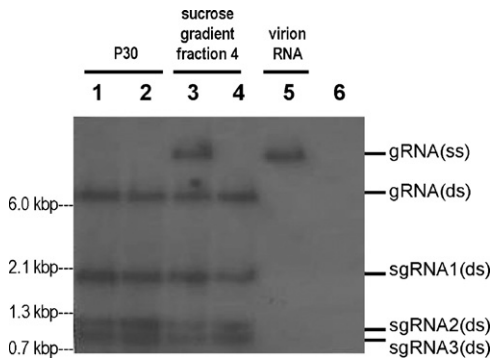

Fig. 1. 8% urea polyacrylamide gel analysis of PepMV ^{32}P-labelled RNA products catalysed by membrane-bound RdRp. Transcriptions were carried out using P30 (lanes 1–2) or sucrose gradient fraction 4 (lanes 3 and 4). RNA products were S1 nuclease treated (lanes 2 and 4) or untreated (lane 1 and 3) to reveal the single – (ss) or double-stranded (ds) nature of the genomic (g) and subgenomic (sg) RNAs. Lane 5: T7-transcribed full-length PepMV RNA from an infectious cDNA clone (courtesy of Dr. M. Aranda). Lane 6: Transcription products using P30 from health tomato plants. *Penicillium stoloniferum virus* and *Aspergillus foetidus virus* dsRNAs were used as molecular weight standard markers.

To characterize the genomic and sgRNAs produced by membrane-bound PepMV RdRp, the relative amounts of the positive- and negative-sense newly synthesized RNA molecules were examined. Each individual radioactive RNA species was excised from gels, purified and used as a probe in dot blot hybridization experiments for unlabelled, *in vitro* transcribed plus- and minus-sense PepMV RNAs as target RNAs (Fig. 2). In these experiments, genomic ^{32}P-labelled dsRNA transcribed from membrane-bound RdRp (*i.e.* P30 and fraction 4) predominantly hybridized to negative-sense PepMV 3′-terminal RNA transcripts (Fig. 2A), suggesting that the full length genomic, endogenous, newly synthesized dsRNA species mainly consist of positive-sense RNAs. Conversely the ^{32}P-labelled genomic ssRNA from fraction 4 exclusively hybridized to minus-sense PepMV RNA confirming its positive-sense polarity.

Using ^{32}P-labelled probes transcribed from P30 (Fig. 2B) or sucrose gradient fraction 4 (data not shown), all three subgenomic dsRNAs hybridized with similar intensities to identical amounts 5 μg of the negative-sense 5′-terminal and positive-sense strand 3′-terminal RNAs. No hybridisation occurred with the negative-sense 3′-terminal or positive-sense 5′-terminal RNAs (Fig. 2B). These results confirm that endogenous, newly synthesized 3′-co-terminal subgenomic dsRNAs are comprised of similar amounts of both RNA strands.

3.2. Specific plus- and minus-strand PepMV RNA synthesis by template-bound RdRp

Endogenous PepMV RNA present in the RdRp-enriched fraction 4 of the sucrose gradient was digested with the Ca^{2+}-dependent micrococcal nuclease, and subsequently inactivated by EGTA treatment. The resultant RdRp was incubated with a mixture of ^{32}P-UTP, unlabelled ATP, CTP, GTP, Mg^{2+} plus one of three different RNA

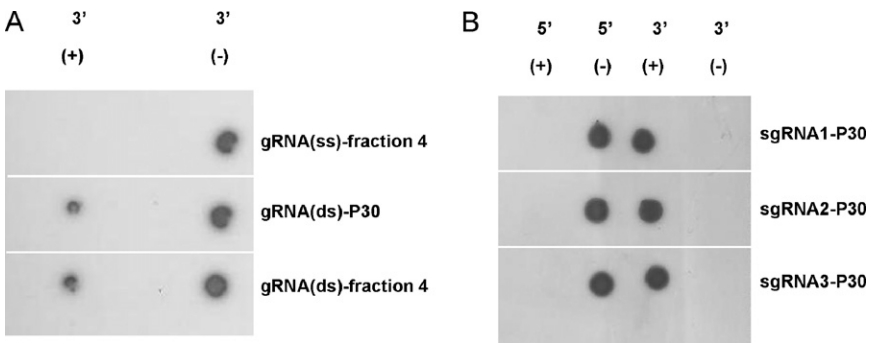

Fig. 2. Determination of membrane-bound RdRp-generated PepMV RNAs. Unlabelled single stranded RNAs representing positive- or negative-sense 3′-terminal (A) or 5′- and 3′-terminal RNA fragments (B) were dot blotted and hybridized to various membrane-bound RdRp catalysed ^{32}P-labelled RNA transcription products following analysis in an 8% polyacrylamide gel similar to that presented in Fig. 1.

templates (Fig. 3A), namely a full-length PepMV RNA transcript (lane 3), PepMV virion RNA (lane 4), and a full length ToMV RNA (lane 5). In these experiments, both full-length *in vitro*-synthesized PepMV RNA and PepMV virion RNA were successfully used as templates for the synthesis of full-length genomic RNA products (Fig. 3A; lanes 3 and 4). No synthesis of any other labelled RNA transcripts was detected when water or ToMV RNA was added to the system confirming the absence of endogenous RNA template, and the template specificity of the system (Fig. 3A; lanes 2 and 5). The template-dependent PepMV RdRp was then programmed with several different PepMV RNA templates, including positive- and negative-sense RNA strands *ca*. 300 nucleotides in length derived from the 5′ or 3′-termini of PepMV RNA and the 3′-termini of ToMV RNA plus and minus polarity (Fig. 3B; Table 1). These experiments revealed specific recognition of the 3′-termini of both positive- and negative-sense PepMV RNA transcripts (Fig. 3B; lanes 3 and 4) but not of the 5′-termini of either polarity of PepMV RNA (Fig. 3B; lanes 2 and 5) or ToMV RNA 3′-termini of the plus or minus strands (lanes 6 and 7). Thus, the PepMV RdRp preparation described has been demonstrated to be both template-dependent and specific for the 3′-termini of PepMV RNA of either polarity.

3.3. Inhibition of the template-dependent transcription by antibodies against PepMV RdRp

A fourteen amino acid long peptide epitope (MARYLRKMRQRFQP) representing an immunogenic stretch (positions 1176-1189) of the PepMV RdRp was synthesized and used to raise a polyclonal antibody to PepMV RdRp. The purified total IgGs detected the PepMV RdRp in the membrane-bound and template-dependent fractions as a single band of molecular weight *ca*. 164 kDa (Fig. 4A–B; lanes 1 and 2, respectively). No reaction was observed in similarly prepared extracts from mock-infected plants (Fig. 4B; lane 3). In subsequent experiments, anti-RdRp IgGs (1 and 2 μg) were added in to RNA assays performed as described above, in both the presence and absence of the peptide antigen (Fig. 4C). Transcription by the membrane-bound RdRp was unaffected by the presence of 1 μg of RdRp-epitope IgGs (lane 1) but the activity of the template-dependent RdRp was significantly reduced by the presence of the anti-PepMV RdRp epitope IgG in a concentration-dependent manner (Fig. 4C; lanes 5 and 6). Template-dependent RdRp activity was unaffected by the addition of pre-immune serum, the peptide epitope, or 1 μg of IgG together with a molecular excess (1 μg) of the peptide epitope (Fig. 4C, lanes 2, 3 and 4, respectively).

3.4. A poly(A)-tail with twenty adenosine residues is sufficient for minus-strand RNA synthesis

To examine RNA synthesis from the 3′-terminus of the PepMV genome and to permit the study of replication-essential *cis*-acting RNA elements, the template-dependent transcription system was used to replicate otherwise identical templates with poly(A)-tails of different lengths. Identical amounts of T7-driven RNA transcripts representing the positive-stranded 3′-terminus of an RNA template together with either a 10, 15, 20, 30 or 40-mer poly(A) tail were introduced into the template-dependent RdRp replication system (Fig. 5). These experiments indicated that a poly(A) tail of more than 15 adenosine residues was necessary for minus-strand synthesis. The addition of 30 or 40 A's resulted in higher levels of progeny RNA, 1.3 and 2-fold, respectively compared to the template with 20 A's (Fig. 5; compare lane 4 to lanes 5 and 6). As was the case for BaMV, our data also suggest a role in increasing transcriptional

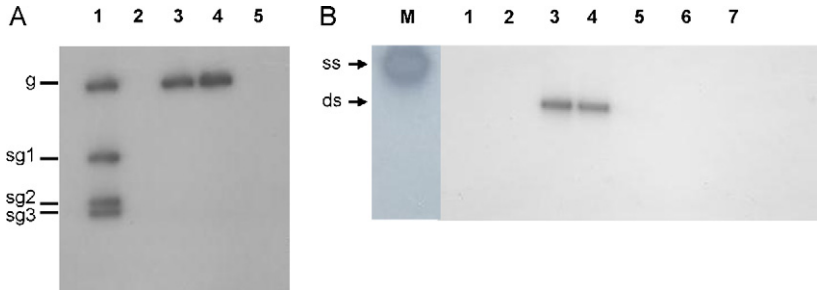

Fig. 3. PepMV template-specific transcription-active RdRp. (A) 8% urea polyacrylamide gel analysis of template-dependent ^{32}P-labelled RdRp transcription products using different templates: water (lane 2), *in vitro*-synthesized PepMV plus-strand RNA following digestion of pTOPO-T7 PepXL6 infectious clone with KpnI (lane 3), PepMV virion RNA (lane 4) and *in vitro*-synthesized ToMV plus-strand RNA (lane 5). Membrane-bound RdRp generated ^{32}P-labelled dsRNA replication products were loaded as markers (lane 1). (B) 8% urea polyacrylamide gel analysis of template-dependent ^{32}P-labelled RdRp transcription products using 300 nucleotides-long *in vitro*-synthesized PepMV RNA fragments from each strand and terminus: 5′-plus strand (lane 2), 3′-plus strand (lane 3), 3′-minus strand (lane 4) and 5′-minus strand (lane 5). In lanes 6 and 7, the 3′-termini of the plus and minus strand of ToMV RNA were used as templates, respectively. In lane 1, no RNA template was added into the reaction. M: ^{32}P-labelled ssRNA marker corresponding to the 300-nt 3′-terminal fragment of the PepMV plus strand RNA.

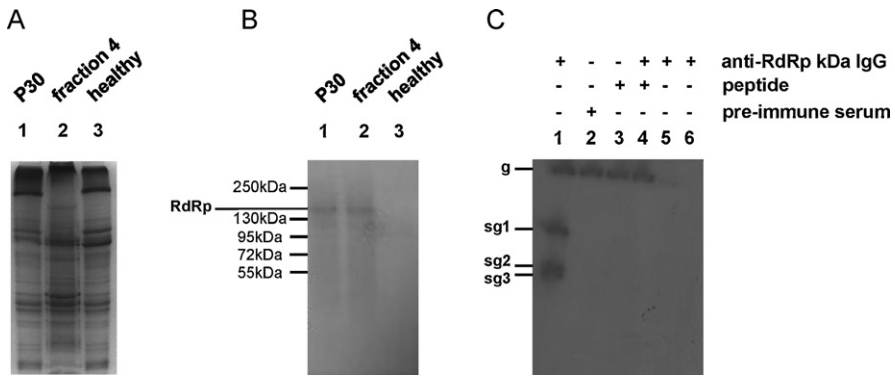

Fig. 4. Detection of PepMV RdRp and inhibition of PepMV template-dependent transcription by an anti-RdRp IgG. (A) Coomassie brilliant blue-stained 12% polyacrylamide protein gel showing total protein extracts from PepMV-infected (P30, lane 1), sucrose-gradient fraction 4 (lane 2) and mock-infected healthy (P30, lane 3) tomato plants. (B) Immunoblot replica of (A) for the identification of PepMV RdRp in P30 (lane 1) and sucrose-gradient fraction 4 (lane 2) using an anti-RdRp polyclonal IgG. (C) 8% urea polyacrylamide gel analysis of ^{32}P-labelled PepMV RNA products using template-dependent RdRp. Transcriptions were carried out using *in vitro*-transcribed, full length PepMV genomic RNA templates, in the absence (lanes 2 and 3) or presence (1 μg, 1 μg, 1 μg and 2 μg in lanes 1, 4, 5 and 6 respectively) of the anti-RdRp IgG in the presence of the homologous epitope (1 μg, for lanes 3, 4, respectively). In lane 2, pre-immune serum was included in the transcription reactions. Lane 1, membrane-bound PepMV RdRp-generated products from endogenous RNA templates.

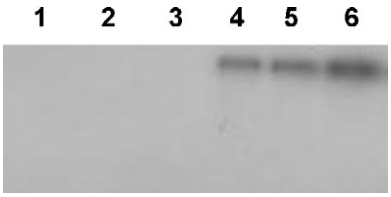

Fig. 5. Effect of different poly(A)-tail lengths on initiation of PepMV negative-strand RNA synthesis. 8% urea polyacrylamide gel analysis of ^{32}P-labelled template-dependent RdRp transcription products using the most 3′-terminal 300 nucleotides of the positive-sense RNA strand including a poly(A) tail of 10, 15, 20, 30, or 40 residues (lanes 2-6, respectively). No RNA template was used as a negative control (lane 1).

efficiency for the poly(A)-tail, possibly through the stabilization of a pseudoknot structure in the 3′-terminus of the plus strand of the RNA.

4. Discussion

Unlike the template-dependent RdRp, the extracts from the initial P30 pellet contained transcriptional activity that was unaffected by the presence of RdRp-specific IgGs possibly due to limited exposure to antibodies of the membrane-bound RdRp (Fig. 4C). The P30 preparation was shown to be capable of replicating the full-length PepMV genomic and three putative (Sempere et al., 2011) 3′-co-terminal subgenomic dsRNAs from the endogenous, partially synthesized RNA templates (Fig. 1). Similarly to reports concerning PVX and BaMV, both potexviruses (Plante et al., 2000; Cheng et al., 2002), the newly synthesized subgenomic dsRNA species comprise of equal plus/minus RNA strand ratios (Table 1; Fig. 2). Interestingly for the full genomic dsRNA species, significantly greater numbers of the newly synthesized RNA molecules were found to be positive-stranded (Fig. 2).

Conversion of the replication system to template-dependency resulted in the specific recognition of the promoters from the 3′-termini of both PepMV RNA plus- and minus-strands and thus may permit studies on the initiation of synthesis of both strands (Fig. 3). It is generally accepted that the initiation site of the minus-strand RNA synthesis is critical for the accurate generation of positive-sense progeny RNA (Kao et al., 2001) and for BaMV, the existence of a functional pseudoknot that includes part of the 3′-poly(A) tail has been suggested (Tsai et al., 1999), with the initiation of replication postulated to occur at several positions (Cheng et al., 2002). In the case of PepMV, our data indicate that the first twenty adenosine residues downstream of the 3′-untranslated region are adequate for the initiation of PepMV negative-strand RNA synthesis (Fig. 5). It appears that the presence of additional ten or twenty adenosine residues significantly increases the efficiency of replication. This observation might be explained by a possible role for the poly(A) tail in the formation and/or stabilization of a pseudoknot that would act as binding site for RdRp. GAPDH, an inhibitor of BaMV replication, is considered to bind competitively to the viral pseudoknot structure (Prasanth et al., 2011). The PepMV RdRp system described here can be utilized in the future in conjunction with the infectious PepMV clone expressing a GFP reporter gene (Sempere et al., 2011). These two systems could be used to identify *in vitro* and verify *in vivo* essential elements and co-factors for the initiation of PepMV negative-strand RNAs.

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