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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.

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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).

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