Virology 447 (2013) 121–130

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The RNA-dependent RNA polymerase of *Citrus tristeza virus* forms oligomers

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ARTICLE INFO

Article history:
Received 4 June 2013
Returned to author for revisions
25 June 2013
Accepted 23 August 2013
Available online 23 September 2013

Keywords:
CTV
RdRp
Oligomerization
Protein–protein interaction
Epitope tagging
Coimmunoprecipitation
Yeast-two-hybrid system
Protein expression
Immunoblotting

ABSTRACT

The RNA-dependent RNA polymerases (RdRp) from *Citrus tristeza virus* (CTV) were tagged with HA and FLAG epitopes. Differentially tagged proteins were expressed either individually or concomitantly in *Escherichia coli*. Immunoprecipitation of the expressed proteins with anti-FLAG antibody followed by Western blot with anti-HA antibody demonstrated that molecules of RdRp from CTV interact to form oligomers. Yeast two-hybrid assays showed that molecules of RdRp interact in eukaryotic cells. Co-immunoprecipitation with anti-FLAG antibody of truncated HA-tagged RdRps (RdRpΔ1-166-HA, RdRpΔ1-390-HA, RdRp1-169-HA) co-expressed with full-length RdRp-FLAG showed that only RdRp1-169-HA interacted with the full-length FLAG-RdRp. Yeast two-hybrid assays with truncated RdRp constructs confirmed that the oligomerization site resides in the N-terminal region and that the first 169 aa of CTV RdRp are necessary and sufficient for oligomerization both in bacterial and yeast cells. Development of control strategies targeting viral RdRp oligomer formation may inhibit virus replication and prove useful in control of CTV.

Introduction

The RNA-dependent RNA polymerase (RdRp) catalyzes synthesis of RNA from an RNA template (Buck, 1996). RdRp is present in all RNA viruses and is responsible for transcription and replication of the genomes of these viruses (Goldbach and Wellink, 1988). The viral RdRps have a conserved "right handed" structure (Thompson and Peersen, 2004) with structurally and functionally conserved domains (Kamer and Agros, 1984; Poch et al., 1989; Koonin, 1991). During transcription and replication of viral genomes, molecules of the RdRp interact with each other and other viral and/or host proteins to form the replicase (Buck, 1996; Wang et al., 2000; Nagy et al., 2012). The homo-oligomerization of a viral RdRp was first demonstrated in poliovirus (Pata et al., 1995; Hansen et al., 1997), a positive-stranded RNA virus. The formation of oligomeric structure and the oligomerization sites are important for the functional activity of poliovirus RdRp (Hobson et al., 2001). The Hepatitis C virus (HCV) RdRp, NS5B, was shown to bind itself to form oligomers, with two amino acid residues critical for both catalytic activity and oligomerization identified (Qin et al., 2002; Wang, et al., 2002). The RdRp of *Sendai virus,* which is a negative-stranded RNA virus, was demonstrated to form oligomers, with the domain involved in oligomerization mapped to the N-terminus of the RdRp (Cevik et al., 2003, 2004a, 2007). It was later shown that the RdRps of other *Paramyxoviruses* (Smallwood et al., 2002; Cevik et al., 2004a, 2004b; Smallwood and Moyer, 2004) form oligomers in a similar manner. Oligomerization of the influenza virus polymerase complex was also reported in vivo (Jorba et al., 2008). These findings revealed that oligomerization is a common phenomenon among the RdRps of both negative- and positive-stranded RNA viruses of animals.

Although the oligomerization phenomenon of RdRps from plant viruses has not been studied as extensively as in animal viruses, self-interaction of the RdRp was reported for some plant RNA viruses in the *Potyvirus* genus. The RdRps from *Tobacco vein mottling virus* (TVMV; NIb protein; Hong et al., 1995), *Soybean mosaic virus* and *Shallot yellow stripe virus* (Lin et al., 2009), as well as *Plum pox virus* (PPV; Zilian and Maiss, 2011) were able to self-interact in different protein–protein binding studies, suggesting that plant virus-encoded RdRps may also form oligomers. On the other hand, the RdRp of *Potato virus A* (PVA) and *Pea seed-borne mosaic virus* (PSbMV) were unable to self-interact in the yeast two-hybrid system (Guo et al. 2001). The interactions of the RdRp with other viral proteins – including capsid protein (CP), movement protein or replication-associated proteins such as helicases – were shown in some plant viruses belonging to the genera *Potyvirus* (Hong et al., 1995), *Bromovirus* (O'Reilly et al., 1997), *Tombusvirus* (Rajendran and Nagy, 2004) and *Cucumovirus* (Hwang et al., 2005; 2007).

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http://dx.doi.org/10.1016/j.virol.2013.08.029

Furthermore, the helicase of *Tobacco mosaic virus* was shown to self interact in order to form a hexamer-like structure (Watanabe et al., 1999; Goregaoker et al., 2001; Goregaoker and Culver, 2003). These findings indicated that oligomerization of the RdRp and other replication-associated proteins is a common phenomenon among plant RNA viruses as well.

Citrus tristeza virus (CTV) is one of the most economically important plant viruses distributed worldwide (Moreno et al., 2008). It has a ~20 kb, single-stranded, positive-sense RNA genome encapsidated with two CPs (Febres et al., 1996) into a long thread-like, flexuous, filamentous particle of about 2000 nm by 11 nm (Bar-Joseph et al., 1979). It belongs to the *Closterovirus* genus in the *Closteroviridae* family (Bar-Joseph and Lee, 1990). The genome is organized into 12 open reading frames (ORF), potentially encoding 17 protein products and the 3′ and 5′ untranslated regions (UTR) (Pappu et al., 1994; Karasev et al., 1995). Sequence analysis of the CTV genome showed that a 56-kDa protein encoded by ORF1b contained sequences similar to the typically conserved motifs of the RdRp of positive-stranded RNA viruses (Karasev et al., 1995). The CTV RdRp is expressed by a +1 translational frameshift at the carboxyl (C) terminus of CTV polyprotein (Cevik et al., 2008). Replication studies revealed that ORFs 1a and 1b as well as the 5′ and 3′ UTRs are necessary and sufficient for replication of the CTV genome in *Nicotiana benthamiana* protoplasts (Satyanarayana et al., 1999) and in citrus (Satyanarayana et al., 2001).

The RdRp was detected independent of other replication-associated proteins in CTV-infected tissue and was localized in the cytoplasmic and membrane fractions of infected citrus plants (Cevik et al., 2008). These results suggested that the RdRp of CTV is expressed as an individual protein. However, neither formation of a functional RdRp nor its interaction with itself and/or other replication-associated proteins has been explored. Therefore, the nature of the functional RdRp of CTV remains unknown. In order to understand the structure of the RdRp, self-interaction of CTV RdRp was analyzed using both co-immunoprecipitation and yeast two-hybrid assays. This study demonstrated that molecules of the CTV RdRp interact to form oligomers and further mapped the binding site to the amino (N)-terminal region.

Results

Epitope tagging and expression of CTV RdRp in E. coli

FLAG- and HA-tagged RdRps were expressed in *E. coli*. The expression of the tagged proteins was optimized under a range of conditions: five different concentrations (0.1, 0.25, 0.5 and 1 M) of IPTG, varied durations (0, 0.5 1, 2, 3, 4 h) of induction, three media (LB, LB+0.5% glucose and 2XYT) and two growth temperatures (32 and 37 °C). Analysis of the total protein extracted by SDS-PAGE followed by Coomassie staining showed that a ~60 kDa protein corresponding to the expected sizes of the tagged RdRps was produced (Fig. 1). The optimum expression levels for both tagged RdRps were obtained in JM109 cells grown in LB+0.5% glucose medium at 37 °C by induction with 0.1 M IPTG for 4 h. Analysis of the expressed proteins by Western blot using anti-FLAG and anti-HA antibodies confirmed that the tagged proteins were expressed in cytoplasm or periplasmic space, based on the expression vector used. Furthermore, the antibodies specifically reacted with the corresponding epitope without any cross-reaction (Fig. 1 A and B). Similar results were obtained with bacterial cells expressing the RdRp in cytoplasm or periplasmic space. Therefore, further experiments were conducted using bacterial cells expressing the RdRp in cytoplasm unless indicated otherwise.

The RdRp of CTV forms oligomers in E. coli

To test self-interaction of the RdRp, differentially tagged, full-length RdRp-FLAG and RdRp-HA proteins were expressed individually and together in *E. coli*. Western blot analysis of the total protein extracts from bacterial cells by anti-FLAG antibody showed that the RdRp-FLAG was detected when it was expressed alone or together with RdRp-HA, whereas the RdRp-HA, expressed alone, did not react with anti-FLAG antibody (Fig. 1A). Similarly, analysis of total protein of the same samples by Western blot with anti-HA antibody detected RdRp-HA but not the RdRp-FLAG protein (Fig. 1B). These results confirmed that the antibodies were specific for the correspondingly tagged proteins.

Immunoprecipitation with anti-FLAG antibody of total protein extract from bacterial cells expressing both RdRp-FLAG and RdRp-HA followed by Western blot with anti-HA antibody revealed that the RdRp-HA was co-immunoprecipitated with the RdRp-FLAG (Fig. 1C). The data indicated that the RdRp-FLAG binds to the RdRp-HA to form higher ordered molecules. Since the number of RdRp proteins interacting was not known, molecules formed by the self-interaction of the RdRp were referred to as oligomers.

Oligomerization of the RdRp is not co-translational

To determine whether the self-interaction of the RdRp proteins requires co-translation, differentially tagged RdRp-FLAG and RdRp-HA proteins were expressed either together in the same cells (co-expressed Fig. 2 Lanes 3) or individually in different cells that were grown together in same tube (co-cultured Fig. 2 Lanes 4). Western blot analysis of total protein extracts with both anti-FLAG and anti-HA antibodies showed that the tagged proteins were expressed when co-expressed or co-cultivated (Fig. 2A and B). Additionally, total protein extracts of cells expressing the RdRp-FLAG and RdRp-HA alone (Fig. 2 Lanes 1 and 2) were mixed equally to obtain a mixture of RdRp-HA and RdRp-FLAG proteins expressed in separate cultures (mixed, Fig. 2 Lanes 5). All samples were immunoprecipitated with anti-FLAG antibody (Fig. 2C). The precipitated proteins were analyzed by Western blot with anti-HA-antibody. Analysis of immunoprecipitated samples by Western blot using anti-HA antibody demonstrated that the RdRp-FLAG proteins bound to RdRp-HA whether co-expressed in the same cell, co-cultivated in different cells, or synthesized separately and then mixed with RdRp-FLAG (Fig. 2C, Lanes 3–5). These results confirmed that RdRp-FLAG and RdRp-HA complex formation occurs and further showed that co-translation of the RdRp-FLAG and RdRp-HA proteins is not required for binding of CTV RdRp to each other when expressed in *E. coli*.

Binding site of the RdRp is located in the N-terminal region

To map the binding site of the RdRp, two N-terminal deletions – RdRpΔ1-166 and RdRpΔ1-390 – tagged with HA epitopes at the C-termini were constructed (Fig. 3A). The full-length and truncated RdRp-HA proteins were expressed alone or with the full-length RdRp-FLAG protein to test for self-interaction. Western blot analyses of the total protein extracts with either anti-FLAG or anti-HA monoclonal antibodies showed that all proteins were expressed in bacterial cells (Fig. 3B and C). When the total protein extracts of cells expressing singular RdRp proteins (either RdRp-FLAG or the full-length or truncated RdRp-HA proteins) were immunoprecipitated with anti-FLAG antibody, neither the full-length RdRp-HA nor the truncated RdRp-HA proteins were immunoprecipitated by anti-FLAG antibody (Fig. 3D Lane 2–4). When the full-length RdRp-FLAG was co-expressed with either the full-length or one of the N-terminal truncated RdRp-HA proteins, only the full-length RdRp-HA was co-immunoprecipitated with the

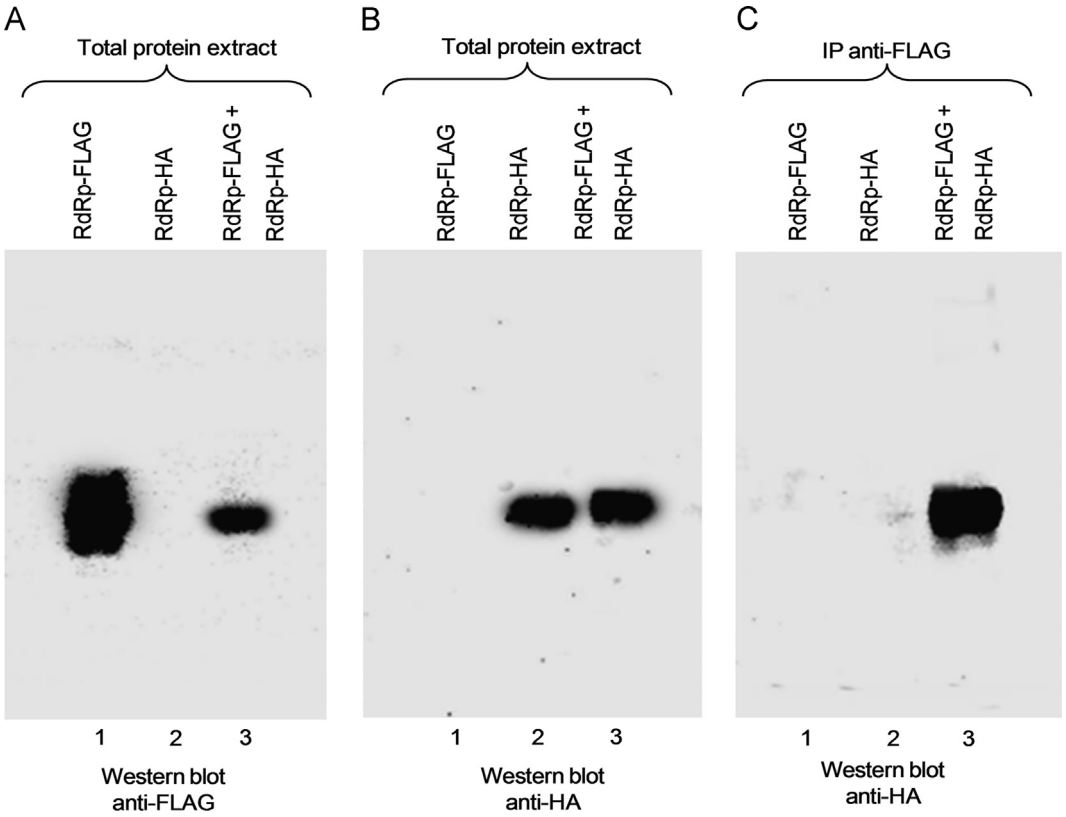

Fig. 1. Differentially tagged RNA-dependent RNA polymerase (RdRp) molecules expressed in *E. coli* bind each other. (A) Western blot of the full-length RdRp-FLAG protein expressed individually and co-expressed with the full-length RdRp-HA protein. Total protein extracts from *E. coli* expressing the full-length RdRp-HA and RdRp-FLAG proteins individually or together were separated by SDS-PAGE, blotted to membrane and probed using anti-FLAG antibody (B) Western blot of the full-length RdRp-HA protein expressed individually or co-expressed with full-length RdRp-FLAG protein. Total protein extracts from *E. coli* expressing the full-length RdRp-HA and RdRp-FLAG proteins individually or together were separated by SDS-PAGE, blotted to membrane and probed using anti-HA antibody (C) Co-immunoprecipitation of individually co-expressed full-length RdRp-HA and RdRp-FLAG proteins by anti-FLAG antibody. Total protein extracts were first immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitated proteins were then analyzed by western blot using anti-HA antibody.

RdRp-FLAG protein by anti-FLAG antibody (Fig. 3D Lane 5–7). Since neither of the truncated RdRp-HA proteins was co-immunoprecipitated by anti-FLAG antibody, it could be concluded that the RdRpΔ1-166-HA and RdRpΔ1-390-HA proteins were unable to bind the full-length RdRp-FLAG protein (Fig. 3D, lanes 6 and 7). The results indicated that the binding site is located in the N-terminal portion of the RdRp.

To confirm that the N-terminal portion of the RdRp protein plays a role in oligomerization, a C-terminal truncation of the RdRp containing only the first 500 bp, designated as RdRp1-169-HA, was constructed (Fig. 4A). To test the specificity of the RdRp binding, the major coat protein (CP) was also tagged with HA (CP-HA) and used as control. The C-terminally truncated RdRp-HA protein, the CP-HA protein and the full-length RdRp-FLAG proteins (Fig. 4A) were expressed alone or together with the full-length RdRp-FLAG. Western blot analysis of the total protein extracts of these bacterial cells with anti-FLAG and anti-HA antibodies showed that all proteins of expected sizes were expressed and that no cross-reaction with non-specific antibodies was observed (Fig. 4B and C). When the individually and co-expressed proteins were immunoprecipitated with anti-FLAG antibody and then immunobotted with anti-HA antibody, the individually expressed HA-tagged proteins were not immunoprecipitated. When the CP-HA was co-expressed with the full-length RdRp-FLAG (in either the cytoplasm or the periplasmic space), it was not immunoprecipitated by anti-FLAG antibody. However, when the RdRp1-169-HA protein was co-expressed with the full-length RdRp-FLAG (in either the cytoplasm or the periplasmic space), it was co-immunoprecipitated by anti-FLAG antibody, demonstrating that the RdRp1-169-HA contained the binding site. The result confirmed that the N-terminal domain is needed for the self-interaction of CTV RdRp.

CTV RdRp molecules interact in yeast

Since all binding assays were done in a prokaryotic system, the self-interaction of the RdRp was tested in a eukaryotic system using a LexA-based yeast two-hybrid system with GFP as reporter. The RdRp gene was cloned into both the pEG202 (prey) and pJG4-5 (bait) plasmids. Self-interactions were tested by induction of the GFP reporter gene expression. No GFP expression was observed in yeast cells transformed with negative control plasmids (pEG202-RdRp+pJG4-5 or pEG202+pJG4-5-RdRp), indicating that no interaction occurred (Fig. 5A sections 3 and 4). However, yeast cells co-transformed with either the positive control plasmids (pEG202-p53+pJG4-5-LTA) or the pEG202-RdRp and pJG4-5RdRp plasmids showed high levels of GFP expression (Fig. 5A, sections 1 and 2). The result showed that RdRp molecules are able to interact in the yeast two-hybrid system. These findings not only confirmed that individual RdRp molecules bind each other, but also showed that binding occurs in an eukaryotic system.

To test the effects of the N-terminal and C-terminal truncations on self-interaction of the RdRp in a eukaryote, the same truncations described above (RdRpΔ1-166, RdRpΔ1-390, RdRp1-169) were constructed (Figs. 3A and 4A) and cloned into pJG4-5 to generate pJG4-5-RdRpΔ1-166, pJG4-5-RdRpΔ1-390 and pJG4-5-RdRp1-169 bait plasmids. Yeast cells transformed with pEG202-p53 and pJG4-5 or pEG202-RdRp and pJG4-5-RdRp expressed GFP, indicating binding (Fig. 5B sections 1 and 2). In contrast, little yeast

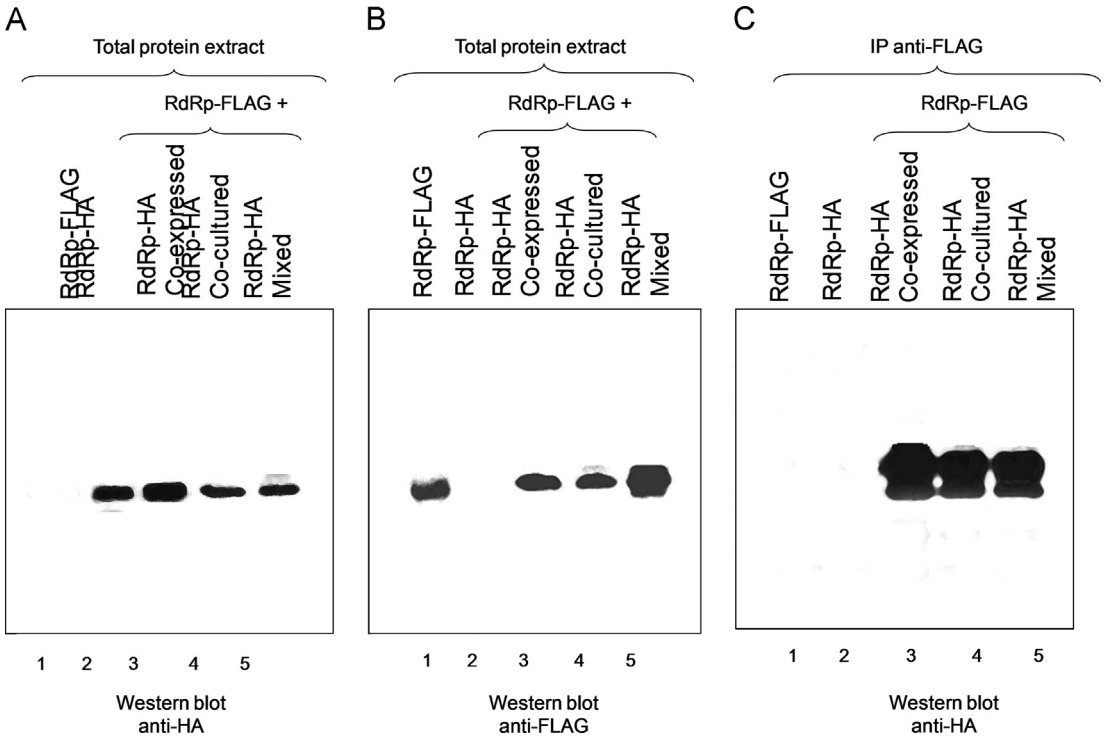

Fig. 2. Oligomerization of differentially tagged RNA-dependent RNA polymerases (RdRps) expressed in *E. coli* is not co-translational. (A) Protein extracts of the full-length RdRp-FLAG protein expressed individually, co-expressed, co-cultured or mixed with the full-length RdRp-HA protein were separated by SDS-PAGE and probed with anti-HA antibody. (B) Protein extracts of the full-length RdRp-HA protein expressed individually, co-expressed, co-cultured or mixed with full-length RdRp-FLAG protein were separated by SDS-PAGE and probed with anti-FLAG antibody. (C) Co-immunoprecipitation of individually, co-expressed, co-cultured or mixed full-length RdRp-HA and RdRp-FLAG proteins by anti-FLAG antibody. Total protein extracts were first immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitated proteins were analyzed by Western blot using anti-HA antibody.

growth or GFP expression was detected in cells expressing the full-length RdRp and the N-terminally truncated RdRpΔ1-166 or RdRpΔ1-390 proteins (Fig. 5B sections 3 and 4), confirming that the N-terminal region is important for binding in yeast as well. On the other hand, the C-terminally truncated RdRp1–169, containing only the first 169 amino acids of the RdRp and which binds to the full-length RdRp in *E. coli* (Fig. 4), was able to bind the full-length RdRp in yeast (Fig. 5B section 5), demonstrating that the RdRp binding site is located within this region. All data obtained from the yeast two-hybrid system confirmed that molecules of the RdRp interact to form oligomers and that the binding site is located in the first 169 aa of the CTV RdRp.

Discussion

Viral RdRps interact with themselves and other viral and host proteins to form functional replicases required for transcription and replication of viral genomes (Buck, 1996; Wang et al., 2000; Nagy et al., 2012). Since the first demonstration of self-oligomerization of a viral RdRp in poliovirus (Pata et al., 1995; Hansen et al. 1997), self-interaction and/or oligomerization of the RdRp from other animal RNA viruses, as shown by co-immunoprecipitation assays, suggested that oligomerization of the RdRp is a common feature in both positive- and negative-stranded RNA viruses (Smallwood et al., 2002; Cevik et al., 2003, 2004a, 2007; Smallwood and Moyer, 2004; Jorba et al., 2008; Zamoto-Niikura et al., 2009). Self-interaction of the RdRps from some *Potyviruses* (Hong et al., 1995; Lin et al., 2009; Zilian and Maiss, 2011; Guo et al. 2001) and oligomerization of the helicase from TMV (Goregaoker et al., 2001) indicated that the RdRp and other replication-associated proteins of plant RNA viruses may also form oligomers. Considering the functional and structural similarity of the RdRp among RNA viruses, the RdRp of the economically damaging citrus virus CTV was tested for oligomerization by immunoprecipitation of differentially tagged proteins expressed in *E. coli* as well as in a yeast two-hybrid binding assay.

Co-immunoprecipitation of differentially tagged RdRp proteins expressed in *E. coli* followed by Western blot analysis demonstrated that RdRp molecules from CTV were able to interact (Fig. 1). The data in this study showed that the RdRp of CTV was able to form dimers or even higher ordered oligomers. Future experiments with native protein gels or other methods may provide data as to how many RdRp molecules complex together. The finding further confirms that oligomerization of the RdRp is a common phenomenon in plant RNA viruses. While oligomerization of RdRps of some animal viruses has been demonstrated in eukaryotic expression systems such as animal cell culture, other viral RdRps were produced in a bacterial expression system for structural and functional studies due to either the absence of a eukaryotic expression system or to versatility of bacterial expression systems (Rothstein et al., 1988; Lohmann et al., 1997; Li et al., 1998; Högbom et al., 2009). CTV only replicates well in a citrus host, and to some extent in tobacco protoplast. Furthermore, there is no efficient protein expression system available for CTV in plants. Since previous studies showed stable expression of CTV RdRp in *E. coli* (Cevik et al., 2004, 2008), the expression and binding studies of CTV RdRp were first conducted in an *E. coli*-based bacterial expression system. Once self-oligomerization of CTV RdRp was established in *E. coli*, binding was confirmed in a yeast two-hybrid system, which has been effectively used for determination of protein–protein interactions and self-interaction of *Potyvirus* RdRps (Hong et al., 1995; Guo et al. 2001; Lin et al., 2009). Demonstration of RdRp binding in the eukaryotic yeast two-hybrid system confirmed that CTV RdRp molecules interact in yeast, suggesting that the RdRp is also able to interact in plant cells.

Oligomerization of the RdRp of other viruses has been shown to occur translationally, suggesting that a functional RdRp complex is

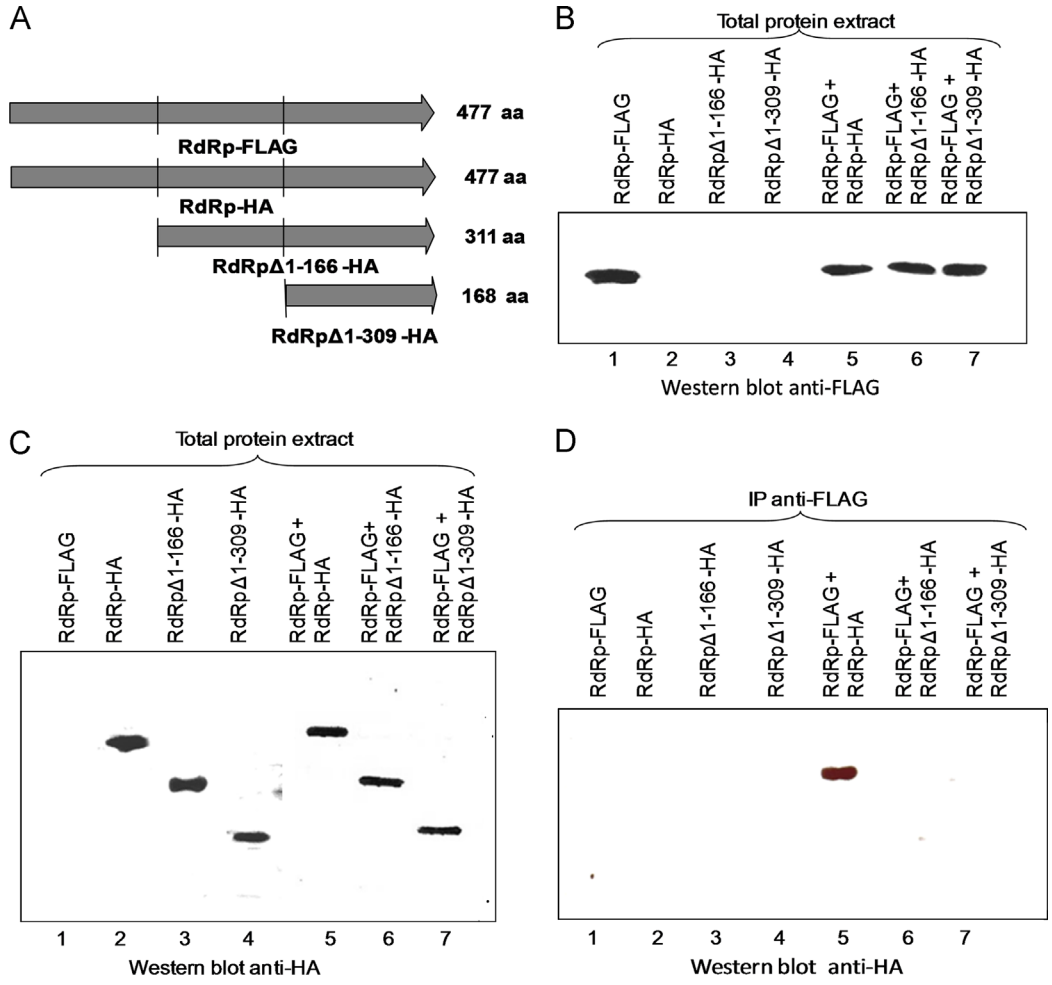

Fig. 3. N-terminal truncations abolish oligomerization of the RNA-dependent RNA polymerases (RdRps) (A) A schematic representation of the full-length and N-terminally truncated RdRp showing the sizes of the proteins on the right. (B) Total protein extracts from cells expressing the full-length RdRp-FLAG protein individually and co-expressed with full-length or N-terminally truncated RdRpΔ1-166-HA or RdRpΔ1-390-HA proteins were separated by SDS-PAGE and analyzed by Western blot using anti-FLAG antibody. (C) Total protein extracts from cells expressing the full-length RdRp-FLAG protein individually and co-expressed with N-terminally truncated RdRpΔ1-166-HA or RdRpΔ1-390-HA proteins were separated by SDS-PAGE and analyzed by Western blot analysis using anti-HA antibody. (D) Co-immunoprecipitation of individually and co-expressed full-length or N-terminally truncated RdRpΔ1-166-HA or RdRpΔ1-390-H with RdRp-FLAG proteins by anti-FLAG antibody. Total protein extracts were first immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitated proteins were then analyzed by Western blot using anti-HA antibody.

formed during translation of these proteins (Smallwood et al., 2002). However, results obtained through co-expressing or co-culturing RdRp showed that oligomerization of CTV RdRp does not require co-translation. The indication that oligomerization of CTV RdRp occurs post-translationally is consistent with the fact that the RdRp of CTV is expressed via a translational frameshift in a polyprotein and released by proteolytic processing (Cevik et al., 2008).

Viral RdRps are subdivided into functional domains conserved in different groups of viruses (Kamer and Argos, 1984; Poch et al., 1989; Koonin, 1991). Some of the conserved domains are functionally characterized, with their role in specific processes determined (O'Reilly and Kao, 1998). Although regions responsible for self-oligomerization of the RdRp were identified in some viruses, the binding sites do not seem to be conserved as a functional domain and show virus to virus variation. While in some cases the oligomerization is effected by a few amino acids (Qin et al., 2002; Clemente-Casares et al., 2011), in other cases a portion of the protein is required for oligomerization (Cevik et al., 2003, 2004a, 2007; Smallwood and Moyer, 2004). To determine the oligomerization site in CTV RdRp, several deletion mutants were generated and tested for their ability to bind to the full-length RdRp. The result of co-immunoprecipitation as well as yeast two-hybrid assays demonstrated that the amino acids or regions involved in self-interaction of the CTV RdRp is located in the N-terminal portion of the protein. The oligomerization domains of RdRps from negative-stranded viruses in the *Paramyxovirus* group are also located in the N-terminal region, with the 174-aa region necessary and sufficient for oligomerization (Cevik et al., 2004a, 2007). The results obtained in this study revealed that the 169 aa in the N-terminal region is necessary and sufficient for the self-interaction of CTV RdRp, suggesting that the binding site is located within the first 169 aa in the N-terminal region of CTV RdRp. Site directed-mutagenesis in that region of *Sendai virus* L RNA polymerase protein did not abolish self-binding, suggesting that the oligomerization was mediated by two independent regions in the N-terminal portion of the L protein rather than individual amino acids (Cevik et al., 2007). On the other hand, the oligomerization domain of HCV was mapped to two amino acids in the RdRp protein, of which site-directed mutagenesis abolished both the activity and oligomerization (Qin et al., 2002). Additional deletion constructs and site-directed mutagenesis in the N-terminal region of CTV RdRp are needed to further delineate the shorter regions and/or individual amino acids involved in the self-binding.

Co-immunoprecipitation analysis of differentially tagged proteins and protein- protein interaction as determined by the yeast two-hybrid system showed self-interaction of the RdRp; however,

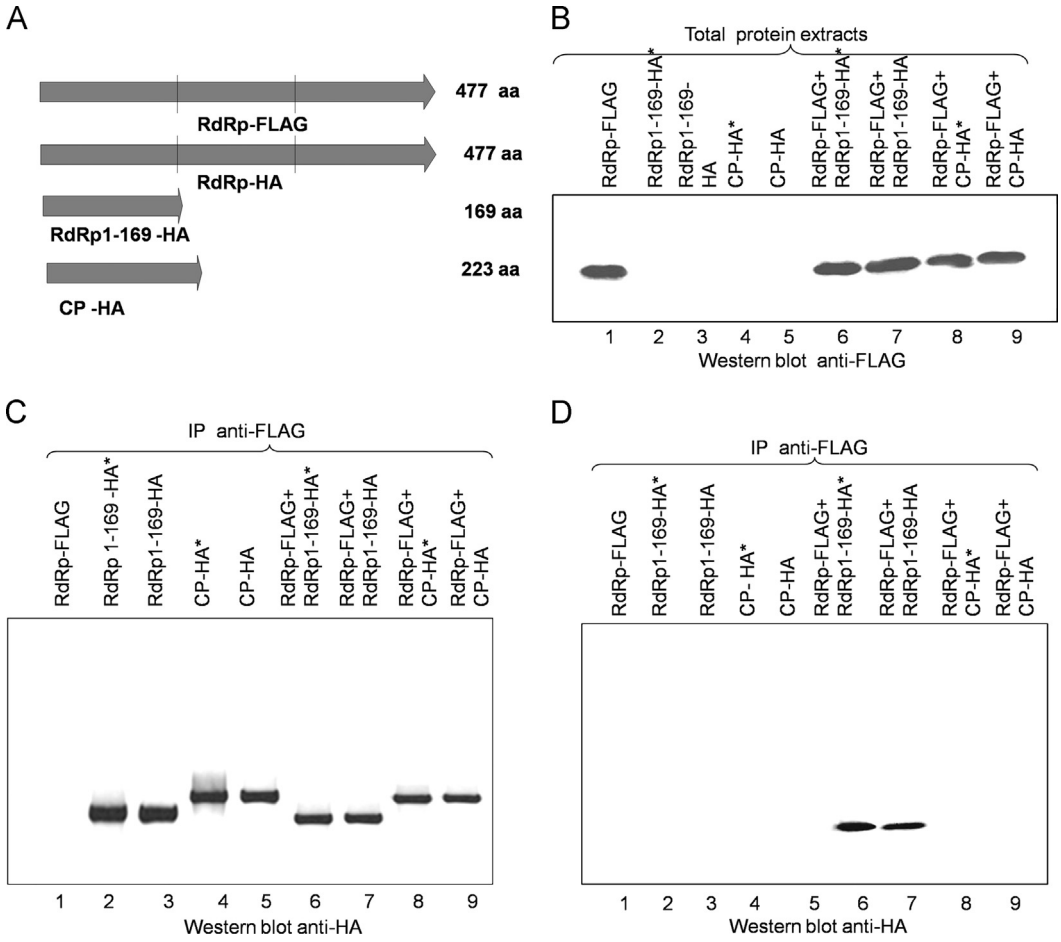

Fig. 4. Mapping of oligomerization site to the N-terminal region of the RNA-dependent RNA polymerase (RdRp). (A) A schematic representation of the full-length and C-terminally truncated RdRp and the full-length CP-HA showing the sizes of the proteins on the right. Expression cassettes were cloned into both cytoplasm- and periplasm-targeting plasmids. (B) Western blot of the full-length RdRp-FLAG protein expressed individually or co-expressed with RdRp1-169-HA or CP-HA proteins. Total protein extracts from *E. coli* expressing C-terminally truncated RdRp1-169-HA, CP-HA and RdRp-FLAG proteins individually or in combination were separated by SDS-PAGE and analyzed by Western blot using anti-FLAG antibody. (C) Western blot of the full-length RdRp-FLAG, C-terminally truncated RdRp1-169-HA, and CP-HA proteins expressed individually and in combination. Total protein extracts from *E. coli* expressing the C-terminally truncated RdRp1-169-HA,CP-HA and full-length RdRp-FLAG proteins individually or together were separated by SDS-PAGE, blotted to membrane and probed using anti-HA antibody. (D) Co-immunoprecipitation with anti-FLAG antibody of individually and co-expressed C-terminally truncated RdRp1-169-HA and CP-HA proteins with RdRp-FLAG proteins. Total protein extracts were first immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitated proteins were then analyzed by Western blot using anti-HA antibody. * indicates proteins expressed in periplasmic space.

the higher order structure cannot be determined by these assays. The number of RdRps that bind to form the final RdRp complex can be further analyzed by native gel analysis (Schägger et al., 1994), mass spectrometry (Heck and Van Den Heuvel, 2004), high-performance liquid chromatography (Folta-Stogniew, 2006) or fluorescence energy transfer (Li et al., 1999) methods. Since oligomerization of most viral RdRps has functional implications, the effect of oligomerization on the function of viral RdRp has been determined in replication and transcription assays for many animal viruses. Although the interaction between molecules of CTV RdRp was demonstrated herein, the functional role of RdRp oligomerization has yet to be determined. Further studies with additional approaches are needed for determination of the functional implications of dimerization and/or oligomerization of the RdRp for CTV replication and transcription.

In conclusion, oligomerization of the RdRp of CTV was demonstrated in both a prokaryotic and an eukaryotic system, showing that molecules of an RdRp of a plant RNA virus interact to form oligomers, as in many animal RNA viruses. In addition, the binding site was mapped to the N-terminal region of the RdRp, similar to those of some animal RNA viruses. A better understanding of RdRp oligomerization, its functional implications and fine mapping of the oligomerization site(s) could provide new targets for antivirals and enable the development of novel strategies for controlling this and other economically important RNA viruses in plants.

Experimental procedures

Virus isolate, cells, plasmids, and antibodies

The Iğdır isolate of CTV, originally obtained in the 1980s from a sweet orange tree grafted onto sour orange rootstock in a commercial orchard in the Eastern Mediterranean region of Turkey (Baloğlu, 1988), was used in this study. The isolate was maintained in Mexican lime as a reference isolate at the Western Mediterranean Agricultural Research Institute. *Escherichia coli* JM109 cells were used in all cloning and expression experiments. *Saccharomyces cerevisiae* strain EGY48 was used for all yeast two-hybrid experiments. Antibodies used for immunoprecipitation and Western blot assays were anti-FLAG M-2 (α-FLAG Sigma, USA) monoclonal antibody and anti-HA probe F-7 monoclonal antibody (α-HA, Sigma, USA). The plasmids, pGEM-Teasy RdRp-1 and pGEM-Teasy CP-1 containing the RdRp and CP gene of CTV, respectively,

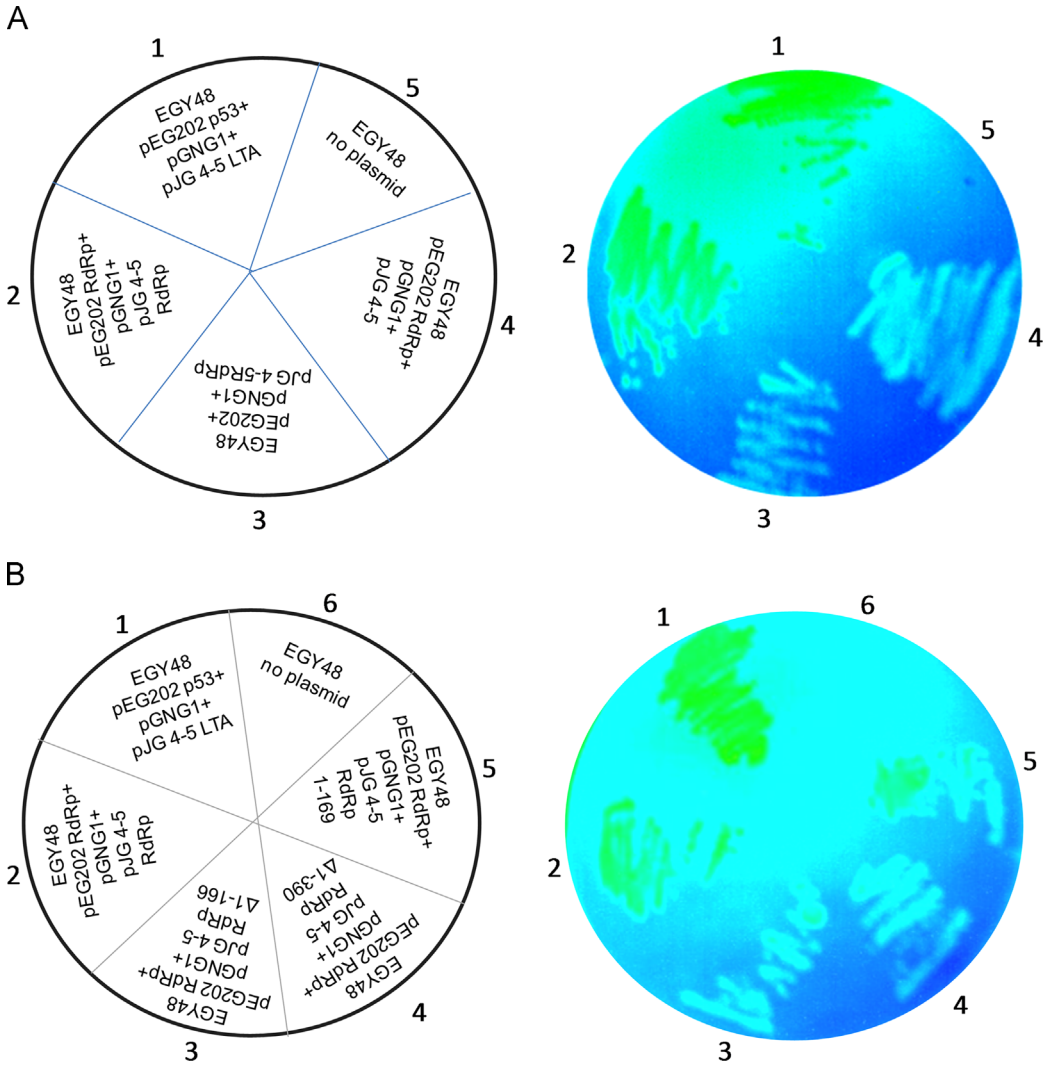

Fig. 5. The oligomerization of the RNA-dependent RNA polymerase (RdRp) and its oligomerization site were confirmed by a yeast two-hybrid assay in *Saccharomyces cerevisiae* strain EGY48. (A) Molecules of the full-length RdRp interact in the yeast two-hybrid assay. Self-interaction was tested by trans-activation of the GFP reporter in pGNG1 plasmid by the full-length RdRp cloned into both pEG202 and pJG4-5 plasmids. The format of the petri plate is shown on the left, the expression of the GFP reporter is shown on the right. (B) The binding of the truncated RdRp proteins to the full-length RdRp in the yeast two-hybrid assay. Binding was tested by trans-activation of the GFP reporter by the interaction of the truncated RdRps cloned into the pJG4-5 plasmid with the full-length RdRp cloned into the pEG202 plasmid. The format of the petri plate is shown on the left, the expression of the GFP reporter is shown on the right. The pEG202p53 and pJG4-5 LTA were used as positive interaction control.

are from Cevik et al. (2013). Primers are listed in Table 1. All PCR reactions used *Pfu* DNA polymerase (New England Biolab, USA).

Epitope tagging the full-length and truncated RdRp genes

The RdRp gene was amplified from pGEM-Teasy RdRp-1 plasmid (Cevik et al., 2013) by PCR using sense primer BC46 containing *Xho*I site at the 5′ end followed by 21 nt from the 5′ end of the RdRp gene and anti-sense primer BC48 with *Bgl*II site and 20 nt from the 3′ end of the RdRp gene without the stop codon. The resultant PCR-amplified RdRp fragment – without a stop codon and containing a 5′ *Xho*I and a 3′ *Bgl*II site – was gel-purified and digested with *Xho*I and *Bgl*II. Digested PCR products were cloned into *Xho*I and *Bgl*II sites in pFLAG CTC or pFLAG CTS plasmid vectors to express the C-terminally FLAG-tagged RdRp of CTV in the cytoplasm or periplasmic space, respectively. The resulting clone was ampicillin resistant and designated RdRp-FLAG.

The HA epitope was incorporated in-frame to the 3′end of the full-length RdRp gene of CTV by PCR using the anti-sense HA primer BC14, which contains a *Bgl*II site at the 5′ end following the stop codon, the HA epitope sequence, and 17 nt from the 3′ end of the RdRp gene. The PCR products were gel purified and digested with *Xho*I and *Bgl*II and cloned into those sites in pFLAG CTC or pFLAG CTS plasmids expressing the proteins in the cytoplasm or periplasmic space, respectively. The resulting clone was ampicillin resistant and designated RdRp-HA. JM109 cells expressing HA- and FLAG-tagged RdRp individually or together were selected by colony PCR using tag-specific primers.

To map the oligomerization site in the RdRp, two N-terminally truncated RdRp proteins with a C-terminal HA tag were generated by progressive deletion of 500 bp and 1000 bp from the 5′ end and addition of the HA tag at the 3′ end of the truncated CTV RdRp. The truncated RdRp genes were amplified from pGEM-Teasy RdRp-1 plasmid by PCR using primers BC53 and BC54 specific to corresponding 5′ regions of the RdRp gene with a *Xho*I site in combination with primer BC55 containing the HA epitope sequence followed by a *Bgl*II site. In addition, the major CP gene of CTV was also tagged with HA epitope by PCR. The CP gene was amplified from pGEM-Teasy CP-1 plasmid (Cevik et al., 2013) by PCR using primers BC71 – specific to the 5′ end of the CP gene with a *Xho*I site – in combination with primer BC70 – containing *Eco*RI and *Bam*HI sites followed by the HA epitope sequence and a *Bgl*II site. All resulting PCR products were gel

Table 1
Primers used for amplification and cloning of the constructs used for protein expression, immunoprecipitation and yeast-two-hybrid assays.

Primer code	Construct(s)	Sequence (5′–3′)	Orientation
BC46	pFLAG CTC RdRp-FLAG	CCGCTCGAGGGCGTGGTAAGGTCGCAAGC	sense
BC48		GAAGATCTGCCGGTCACTAAGTCGTCCG	antisense
BC47	pFLAG CTS RdRp-FLAG	CCGCTCGAGGCGTGGTAAGGTCGCAAGC	sense
BC48		GAAGATCTGCCGGTCACTAAGTCGTCCG	antisense
BC46	pFLAG CTC RdRp-HA	CCGCTCGAGGGCGTGGTAAGGTCGCAAGC	sense
BC14		CGTAGATCTTCATGCGTAATCAGGGACGTCGTAAGGATAGCCGGTTGC TAATTCACCCGC	antisense
BC47	pFLAG CTS RdRp-HA	CCGCTCGAGGCGTGGTAAGGTCGCAAGC	sense
BC14		CGTAGATCTTCATGCGTAATCAGGGACGTCGTAAGGATAGCCGGTTGCTAATTCACCCGC	antisense
BC53	pFLAG CTC RdRpΔ1-166-HA	CCGCTCGAGAGATGACGAACGCTGGGTTGGCG	sense
BC55		CGTAGATCTTTCATGCGTAATCAGGGACGTCGTAAGGATAGCCGGTCACTAAGTCGTCCGC	antisense
BC54	pFLAG CTC RdRpΔ1-390-HA	CCGCTCGAGAAACTAAATTTATGTCTCCTAGC	sense
BC55		CGTAGATCTTTCATGCGTAATCAGGGACGTCGTAAGGATAGCCGGTCACTAAGTCGTCCGC	antisense
BC59	pEG202 RdRp, pJG4-5 RdRp	CGGAATTCGGCGTGGTAAGGTCGCAAGC	sense
BC60		CTCGAGGCCGGTCACTAAGTCGTCCGC	antisense
BC59	pJG4-5 RdRp1-169	CGGAATTCGGCGTGGTAAGGTCGCAAGC	sense
BC69		GACTCGAGTCACACTATATTACCGTTTAG	antisense
BC71	pFLAG CTC CP-HA	CTTCTCGAGATGGACGACGAAACAAAGAAATTG	sense
BC70		CGTAGATCTTTCATGCGTAATCAGGGACGTCGTAAGGATATATGAATTCGGATCCACGTGTGTTGAATTTCCCAAG	antisense
BC72	pFLAG CTS CP-HA	CCGCTCGAGGATGGACGACGAAACAAAG	sense
BC70		CGTAGATCTTTCATGCGTAATCAGGGACGTCGTAAGGATATATGAATTCGGATCCACGTGTGTTGAATTTCCCAAG	antisense
BC46	pFLAG CTC RdRp1-169-HA	CCGCTCGAGGGCGTGGTAAGGTCGCAAGC	sense
BC73		ATGAATTCGGATCCCACTATATTACCGTTTAG	antisense
BC47	pFLAG CTS RdRp1-169-HA	CCGCTCGAGGCGTGGTAAGGTCGCAAGC	sense
BC73		ATGAATTCGGATCCCACTATATTACCGTTTAG	antisense
BC59	pJG4-5 RdRpΔ1-166	CGGAATTCGGCGTGGTAAGGTCGCAAGC	sense
BC74		CGGAATTCTTCACCGAGATGACGAACGCT	antisense
BC59	pJG4-5 RdRpΔ1-390	CGGAATTCGGCGTGGTAAGGTCGCAAGC	sense
BC75		CGGAATTCACTAAATTTATGTCTCCTAGC	antisense

purified, digested with *Xho*I and *Bgl*II and cloned into those sites in pFLAG CTC or pFLAG CTS plasmids. Resulting clones were designated as RdRpΔ1-166-HA, RdRpΔ1-390-HA and CP-HA.

A construct containing only the first 500 bp of the CTV RdRp gene was generated and tagged with the HA epitope. The first 500 nt of RdRp were amplified from pGEM-Teasy RdRp-1plasmid by PCR using *Pfu* DNA polymerase and primers BC47 or BC48 specific to the 5′ end of the RdRp gene with an *Xho*I site in combination with primer BC73 specific to 500 bp region of the RdRp containing a stop codon followed by *Eco*RI and *Bam*HI sites. The PCR product were gel-purified, digested with *Xho*I and *EcoRI* and cloned into those sites in pFLAG CTC and/or CTS plasmids. Resulting clones producing the C-terminal 169 aa of RdRp were designated as RdRp1-169-HA. All clones were sequenced to confirm the integrity of the cloned regions.

Protein expression and extraction

Escherichia coli JM109 cells were transformed with plasmids containing the full-length and truncated epitope-tagged RdRps. Cells transformed with appropriate plasmid(s) were grown overnight in LB medium supplemented with 0.5% glucose (LB+glu) and 100 μg/ml ampicillin at 37 °C with 220 rpm shaking. The overnight cultures were inoculated into fresh LB+glu medium with 100 μg/ml ampicillin and grown at 37 °C and 220 rpm until reaching OD_{600} 0.2. The expression was induced by addition of 0.5 M isopropyl-beta-D-thiogalactopyranoside (IPTG), after which the cells were grown at 37 °C and 220 rpm for 4 h or until the OD_{600} reached 1.5–2.0. Total protein was extracted from bacterial cells using CelLytic B buffer (Sigma, USA) supplemented with lysozyme (0.4 mg/ml), protease inhibitor cocktail (2 mg/ml) and benzonase (50 units/ml). The cell lysate was used for immunoprecipitation and immunoblotting.

Immunoprecipitation

Total protein extracts were immunoprecipitated with about 1 μg anti-FLAG M2 monoclonal antibody. For this, 1 ml of total protein extract was incubated with 50 μl of EZview Red Affinity Gel covalently linked to anti-FLAG M2 monoclonal antibody at 4 °C on ice with gentle agitation for 16 h. The mixture was centrifuged at 13,000 rpm for 1 min, after which the supernatant was removed by pipette. The agarose beads were washed with 500 ml of TBS for 5 min at least three times by gentle mixing at 4 °C.

Immunoblotting

The total and immunoprecipitated samples were denatured and separated by 10-12% SDS-PAGE and electroblotted onto PVDF membrane (Osmonics). The blots were first incubated with blocking buffer containing 3% non-fat dry milk and 1% BSA at RT for 1 h. The blots were then incubated with anti-HA (0.4 μg/ml) or anti-FLAG (4 μg/ml) antibodies conjugated to horse radish peroxidase (HRP) in blocking buffer (Sigma, USA). The blots were finally treated with SuperSignal WestDura substrate mixture for HRP (Pierce, USA). Reactions were visualized by the ChemiDoc-It chemiluminesent imaging system (UVP, England) and analyzed by LabWorks image analysis software (UVP, England)

Yeast two-hybrid assay

A LexA-based GFP yeast two-hybrid system (Grow'n'Glow system; MoBiTec, Germany) was used. For this, the full-length and truncated RdRP genes as well as the full-length CP gene used as control were amplified from pGEM-Teasy RdRp-1 or pGEM-Teasy-CP plasmids by PCR using primers listed in Table 1. *Xho*I and *EcoR*I restriction sites were incorporated into the 5′ and 3′ ends of all constructs, respectively. While the full-length RdRp was cloned into *Xho* I and *EcoR*I sites of pEG202 prey and pJG4-5 bait plasmids, the truncated RdRps and CP was cloned only into the pJG4-5 bait plasmid using the same restriction sites. Resulting plasmids are designated as pEG202-RdRp, pJG4-5-RdRp, pJG4-5-RdRp1-169, pJG4-5-RdRpΔ1-166, pJG4-5-RdRpΔ1-390 and pJG4-5-CP.

Saccharomyces cerevisiae strain EGY48 were transformed with the pGNG1 GFP reporter plasmid, the pEG202-RdRp prey plasmid along with one of the bait plasmids using the Grow'n'Glow Fast and Easy yeast transformation kit according to the manufacturer's protocol. All plasmid combinations were co-transformed with the plasmid containing the GFP reporter gene. Transformed cells were grown on [DOBA (gal/raf)–HIS–URA–TRP–LEU] selective media (MoBiTec, Germany) to observe interaction under UV light. Yeast colonies with GFP were selected, streaked and grown onto the same DOBA media for further observations.

Acknowledgments

This study was financially supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK), Grant number 104O584.

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