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The motif V of plum pox potyvirus CI RNA helicase is involved in NTP hydrolysis and is essential for virus RNA replication

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ABSTRACT

The plum pox potyvirus (PPV) protein CI is an RNA helicase whose function in the viral life cycle is still unknown. The CI protein contains seven conserved sequence motifs typical of RNA helicases of the superfamily SF2. We have introduced several individual point mutations into the region coding for motif V of the PPV CI protein and expressed these proteins in *Escherichia coli* as maltose binding protein fusions. Mutations that abolished RNA helicase activity also disturbed NTP hydrolysis. No mutations affected the RNA binding capacity of the CI protein. These mutations were also introduced in the PPV genome making use of a full-length cDNA clone. Mutant viruses carrying CI proteins with reduced RNA helicase activity replicated very poorly in protoplasts and were unable to infect whole plants without rapid pseudoreversion to wild-type. These results indicate that motif V is involved in the NTP hydrolysis step required for potyvirus RNA helicase activity, and that this activity plays an essential role in virus RNA replication inside the infected cell.

INTRODUCTION

The unwinding of duplex RNA using NTP hydrolysis as an energy source is catalyzed by RNA helicases (for reviews, see 1–4). This reaction is required for several different biological processes such as gene transcription, RNA processing and translation, and often contributes to the regulation of cell growth and development.

In addition to the well characterized RNA helicases, many proteins are considered as putative RNA helicases on the basis of amino acid sequence analysis (2). Two sequence signatures termed motifs I and II, that correspond to the 'Walker box' purine NTP-binding sequence (5), are shared by all helicases and a wide variety of other NTP-utilizing proteins. Five other sequence motifs (Ia, III, IV, V and VI) are conserved in RNA and DNA helicases of the superfamilies SF1 and SF2 (2).

Both experimental evidence and sequence data analysis indicate the existence of sequences potentially encoding helicases in a large number of virus genomes (4). In particular, most of the plus strand RNA virus genomes encode at least one putative helicase protein. The NTPase activity expected for RNA helicases has been demonstrated for viral proteins belonging to each of the three superfamilies defined by Gorbalenya *et al*. (2), but RNA helicase activity has only been demonstrated for RNA virus proteins belonging to the SF2 superfamily (4). RNA helicase activity in an RNA virus protein was first demonstrated in the plum pox potyvirus (PPV) CI protein (6). This activity has also been found to be associated with the tamarillo mosaic potyvirus (TaMV) CI protein (7) and with the hepatitis C virus (8,9) and bovine diarrhea pestivirus (10) NS3 proteins, which also belong to the SF2 superfamily.

The genus *Potyvirus* is the largest group of plant viruses. The CI protein forms the cylindrical inclusion bodies typical of potyvirus infections and is synthesized as part of the single polyprotein encoded by the potyviral genome (11,12). The N-terminal half of the potyviral CI protein contains all of the typical domains of helicases of the superfamily SF2 (13). The CI C-terminal half shows no homology with RNA helicases, but small deletions in this region of a maltose binding protein (MBP)–PPV CI fusion synthesized in *Escherichia coli* abolished its RNA helicase activity (14).

Some data on the assignment of biochemical functions to the helicase conserved domains have been obtained in some SF2 superfamily proteins, such as the eukaryotic translation initiation factor 4A (eIF4A) and in the NTP phosphohydrolase II (NPH-II) of vaccinia virus (15–22). Much less information is available on the subgroup SF2 RNA helicases of viral RNA origin. Deletion mutagenesis has allowed us to localize two RNA binding domains in the PPV CI protein, which might be related to the helicase motifs Ia and VI (14,23). In this paper we report the use of point mutations to characterize the role of the motif V in the RNA helicase activity of the PPV CI protein using a variety of enzymatic assays *in vitro*. We also describe the effect of mutations in conserved residues of the PPV CI motif V on the viruses ability to replicate in isolated cells and to systemically infect plants.

MATERIALS AND METHODS

Construction of expression vectors

All recombinant DNA procedures were carried out by standard methods (24). *Escherichia coli* strains JM109 and DH5α were

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used for the cloning of the plasmids. pcNCI harbors the PPV CI coding sequence fused to the maltose binding protein gene of pMal-c (New England Biolabs) and was reported previously (14). G to A [nt 3696; numbering of nucleotides corresponds to the full-length sequence of PPV RNA (25)] and CT to TC (nt 5534, 5535) point mutations that create new *Cla*I and *Xba*I restriction sites in the CI cistron were produced by site-directed mutagenesis (26) of *Sac*I (vector)–*Sph*I (nt 4056) and *Sma*I (nt 4238)–*Pst*I (vector) fragments of pcNCI subcloned into a M13 vector. The mutated fragments were introduced into pcNCI to create pcNCIcx.

The point mutations in the PPV CI motif V were created by site-directed mutagenesis (26) in the *Sma*I (nt 4238)–*Pst*I (vector) fragment of pcNCIcx subcloned in a M13 vector using the following mutator oligodeoxinucleotides: 5′-GTTGCAATCACG-AAATG-3′ (for V303I; the number corresponds to the amino acid sequence of PPV CI), 5′-TTGTTGCTGCCACGAAATG-3′ (for V303A), 5′-GTATTTGATGCAACC-3′ (for T305S), 5′-GTGACT-GA/TATTTTCAATG-3′ (for G311A and G311S), 5′-GTCCAAT-GAGACTCC-3′ (for T313S) and 5′-GTCCAATGCGACTCC-3′ (for T313A). The plasmids pcNCIcxV303I, pcNCIcxT305S, pcNCIcxG311A, pcNCIcxG311S and pcNCIcxT313S were obtained by replacing the *Eco*88I (nt 4238)–*Pst*I (vector) fragment from pcNCIcx with the corresponding ones from the recombinant M13 replicative forms that contained the different mutations. For the construction of pcNCIcxV303A and pcNCIcxT313A, the exchanged fragment was *Eco*88I (nt 4238)–*Apa*LI (nt 4875).

pGPPV carries a full-length cDNA copy of the PPV genomic RNA cloned downstream of a T7 RNA polymerase promoter (27). pGPPVx is a derivative of pGPPV that carries the CT to TC substitution (nt 5534 and 5535) that creates a *Xba*I restriction site (P.S and J.A.G., unpublished results). pGPPVxV303I was obtained by a triple ligation of the following restriction fragments: *Nco*I (nt 7678)–*Eco*88I (nt 4238) from pGPPV, *Xba*I (nt 5535)–*Nco*I (nt 7678) from pGPPVx and *Eco*88I (nt 4238)–*Xba*I (nt 5535) from pcNCIcxV303I. pGPPVxV303A, pGPPVxT305S and pGPPVxT313S were obtained by replacing the *Cfr*9I (nt 4238)–*Xba*I (nt 5535) of pGPPVxV303I by the corresponding ones of pcNCIcxV303A, pcNCIcxT305S and pcNCIcxT313S, respectively. In order to reconstruct pGPPVx from pGPPVxT313S, the *Cfr*9I (nt 4238)–*Xba*I (nt 5535) fragment from pGPPVxT313S was replaced by the corresponding one of pGPPVx.

Purification of the recombinant proteins

The expression of the recombinant plasmids and the partial purification of the corresponding MBP–CI fusion proteins were carried out essentially as previously described (14). After growing at 30°C in LB medium containing ampicillin (100 µg/ml) and induction with 50 µM IPTG, transformed JM109 cells were collected by centrifugation, and lysed by grinding with alumina. The crude extract was loaded onto an amylose resin column (New England Biolabs) equilibrated in 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 M NaCl. The non-retained proteins were successively washed with the same buffer containing 1, 0.5 and 0.2 M NaCl, and without NaCl, whereas the products specifically retained were eluted with buffer containing 10 mM maltose and no NaCl.

RNA helicase activity

Unwinding reaction mixtures contained 30 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 15 mM DTT, 30 µg/ml bovine serum albumin, 0.12 U/µl RNasin, 2 mM ATP, partially dsRNA substrate A (0.15 µM), prepared according to Laín *et al.* (6), and the indicated amount of the purified protein fractions. After 20 min of incubation at 25°C, the reactions were stopped by adding 3 µl of 0.5% SDS/40 mM EDTA. Samples were loaded in 8% polyacrylamide gels containing 0.1% SDS and 0.5× TBE buffer.

dATPase activity

The dATPase assays were carried out at 25°C in 20 µl reaction mixtures containing 15 mM HEPES–KOH (pH 7.5), 2.5 mM Mg $(CH_3CO_2)_2$, 1 mM DTT, 0.1 mM [α-^{32}P]dATP (~125 Ci/mol), 0.2 mM poly A and the indicated amount of the partially purified protein fractions. Reactions were stopped in ice after different incubation periods by adding EDTA to a concentration of 50 mM, and samples were analyzed by polyethylenimine cellulose thin-layer chromatography with 0.2 M potassium phosphate (pH 7.5) as the liquid phase. Hydrolysis activity was calculated by quantitating the radioactivity of the spots with a Storage Phosphor Imaging System (model GS-525, Bio-Rad).

Northwestern assay for RNA binding

The partially purified proteins were subjected to SDS–PAGE and transferred to nitrocellulose using a Trans-Blot apparatus (BioRad). The membrane was incubated at room temperature for 1 h in a renaturation solution containing 10 mM Tris–HCl (pH 7), 1 mM EDTA, 60 mM NaCl, 0.1% Triton X-100, 1× Denhardt's reagent. This step was repeated four times. The membrane was then incubated in the same buffer containing 6×10^4 c.p.m. of RNA labeled with [α-^{32}P]UTP by *in vitro* transcription of plasmid pT4ps2 digested with *Eco*RI and *Pvu*II, followed by removal of un-incorporated nucleotides by spun column centrifugation through Sephadex G-50 (6). After three washes (15 min each) in the renaturation buffer, the nitrocellulose membrane was air dried and exposed to X-ray film.

Inoculation of plants and protoplasts with PPV RNA synthesized *in vitro*

pGPPV and its derivatives were linearized with *Pvu*II and *Pst*I, that cut upstream of the T7 promoter and immediately downstream the poly(A) tail at the 3′-end of the PPV sequence, respectively. Capped full-length transcripts were synthesized from these constructs using T7mMESSAGEmMACHINE (Ambion) or T7 Cap-Scribe (Boehringer) kits. The yield and integrity of the transcripts were analyzed by agarose gel electrophoresis.

Three *Nicotiana clevelandii* primary leaves were dusted with Carborundum and mechanically inoculated with 2 µl (1 mg/ml of transcript) of the transcription reaction diluted 1:1 in 5 mM sodium phosphate buffer (pH 7.2). Virus infection was assessed by visual inspection of symptoms and by western blot analysis of PPV capsid protein accumulated in the inoculated plants. RT-PCR amplified fragments which included the PPV CI motif V were sequenced to determine if the mutations of the viral transcripts used as inocula were maintained in viral progeny.

Protoplasts were isolated from *N.clevelandii* leaves as described previously (28). Samples of 8×10^5 protoplasts resuspended in

0.8 ml of electroporation buffer were exposed together with 20 µl of *in vitro* transcription reaction mixture to an electroporation pulse (110 V, 1000 µF, 20–25 ms) in an Electro Cell Manipulator 600 apparatus (BTX). After a 15 min recovery period on ice, the protoplasts were washed, resuspended in 1.2 ml culture medium and incubated under diffuse light at 24°C for 30 h. To assess virus multiplication in the protoplasts, PPV RNA was detected by northern blot analysis and PPV virions by RT-PCR preceded by immunocapture PCR (IC-PCR).

For northern blot analysis, protoplasts (2×10^5) were collected by centrifugation, resuspended in 200 µl of extraction buffer [100 mM Tris–HCl (pH 9), 300 mM NaCl, 20 mM EDTA, 2% SDS, 0.25 mg/ml proteinase K and 0.5% heparin] and incubated at 37°C for 30 min. After phenol extraction, RNA was precipitated in the presence of 2 M LiCl, washed by ethanol precipitation, and resuspended in water. RNA was electrophoresed in 1.2% agarose–formaldehide gels, transferred to Zeta-probe membranes (BioRad) and hybridized with a ^{32}P-labeled riboprobe specific of the PPV NIb cistron, synthesized by *in vitro* transcription using a MAXIscript kit (Ambion).

For IC-PCR assays, the centrifugation pellet of protoplast cultures (2×10^5) was resuspended in 50 µl of immunocapture buffer (PBS, 0.05% Tween-20, 2% polyvinylpyrrolidone K25). This extract was incubated for 2 h in the presence of RNase (2.5 µg) and DNase (0.25 U) in tubes previously coated with rabbit anti-PPV IgGs. The immunoretained material was used as template for RT-PCR amplification of a DNA fragment corresponding to PPV nt 8839–8900. The reaction products were characterized by Southern blot analysis. They were electrophoresed in 1.2% agarose gels, transferred to Zeta-probe membranes (BioRad) and hybridized with a probe consisting of a PCR-synthesized PPV cDNA fragment (nt 8389–8900) ^{32}P-labeled by the random primer technique using a DECAprime kit (Ambion).

RESULTS

Conservation of motif V in RNA helicase-like proteins

The level of conservation of motif V is very high among all the sequenced members of the genus *Potyvirus* (Fig. 1). Indeed, there is a stretch of 11 amino acids (from Val 303 to Thr 313) that is strictly conserved in all cases (Fig. 1B). Figure 1C shows the comparison of the potyvirus motif V consensus sequence with the motif V of different members of the DExH and DEAD groups of the SF2 superfamily of helicases. The extent of amino acid similarity is striking not only within the RNA virus subgroup, but also between helicase-like proteins of this subgroup and other members of the DExH group and of the more distantly related DEAD group (Fig. 1C). The most frequently conserved residue is Thr 305 which is invariant in all of the proteins compared. A hydrophobic residue is always present in positions 302 and 319, while all the DExH proteins have an Asp in position 321, and all the RNA virus proteins have a Gly in position 311 (Fig. 1C).

RNA helicase activity of PPV CI proteins mutated at motif V

In order to elucidate the role that motif V plays in the activity of the PPV CI protein, several individual residues in the segment conserved in all the potyviruses were modified by site directed mutagenesis. Both Thr 305 and Gly 311 which are highly conserved in SF2 RNA helicases, and Val 303 and Thr 313 which seem to accept some sequence variations, were replaced either by a closely related amino acid or by Ala, a neutral residue that usually does not disturb the polypeptide chain.

The mutations were introduced into pcNCIcx, which contains the coding sequence of a modified CI fused to the MBP. This CI sequence contained two mutations, which were introduced to facilitate the cloning procedures. One of the mutations gives rise to a conservative Val to Ile change in position 16 of the protein, and the other is a silent mutation. The enzymatic activities of MBP–CI and MBP–CIcx were indistinguishable (data not shown).

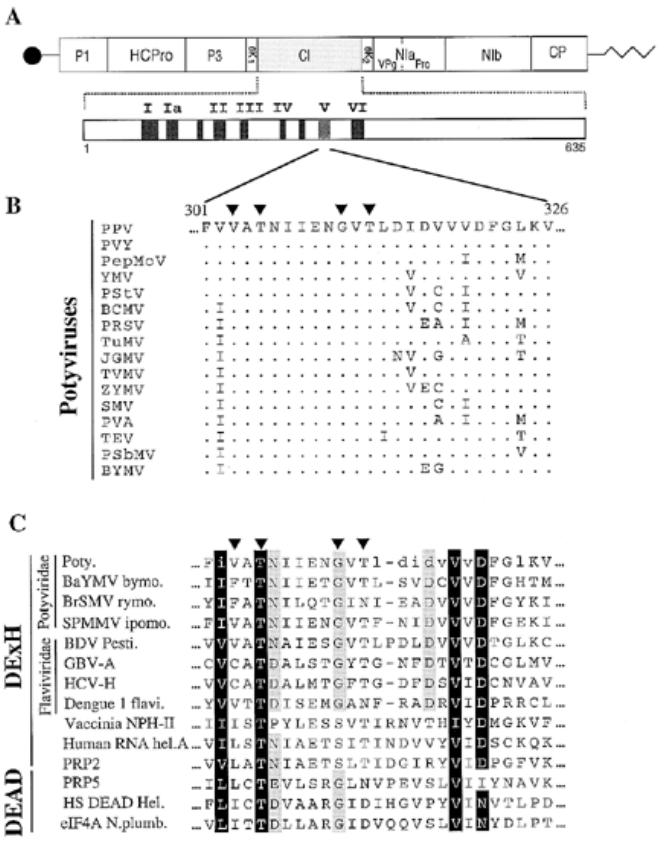


Figure 1. Sequence comparison of motifs V from different RNA helicase-like proteins. (**A**) Position of the CI cistron in the PPV genome. Motifs conserved among RNA helicases are shown as dark areas. (**B**) Conservation of the motif V sequence in the CI proteins from different potyviruses: PPV, plum pox virus; PVY, potato virus Y; PepMoV, pepper mottle virus; YMV, yam mosaic virus; PStV, peanut stripe virus; BCMV, bean common mosaic virus; PRSV, papaya ringspot virus; TuMV, turnip mosaic virus; JGMV, johnsongrass mosaic virus; TVMV, tobacco vein mottling virus; ZYMV, zucchini yellow mosaic virus; SMV, soybean mosaic virus; PVA, potato virus A; TEV, tobacco etch virus; PSbMV, pea seedborne mosaic virus; BYMV, bean yellow mosaic virus. Points indicate residues identical to those of PPV CI. (**C**) Conservation of the motif V sequence in representative members of different groups of the RNA helicase superfamily SF2: Poty., potyvirus consensus (lower case letters indicate residues not strictly conserved in all sequenced potyviruses); BaYMV bymo., barley yellow mosaic bymovirus; BrSMV rymo., brome streak mosaic rymovirus; SPMMV ipomo, sweet potato mild mottle ipomovirus; BDV pesti., bovine diarrhea pestivirus; GBV-A, GB virus A; HCV-K3a, strain K3a of hepatitis virus C; Dengue 1 flavi., Dengue 1 flavivirus; Human RNA hel. A, human RNA helicase A; HS DEAD Hel., DEAD helicase from *Homo sapiens*; eIF4A N.plumb., eIF4A from *Nicotiana plumbaginifolia*. All sequences were obtained from the GenEMBL database. Residues of the PPV CI protein modified by site directed mutagenesis are marked by ▼.

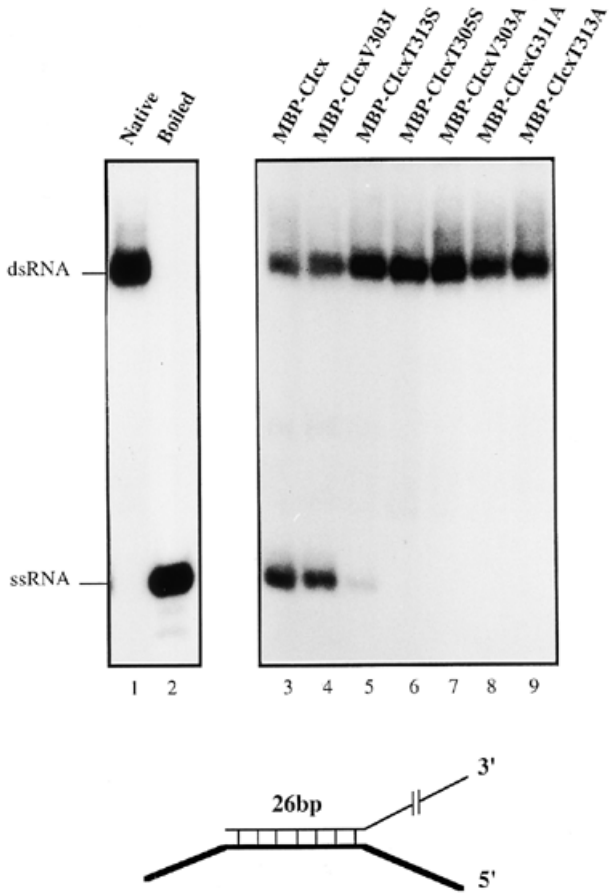


Figure 2. RNA helicase activity of MBP–CI recombinant proteins harbouring point mutations in the motif V sequence. Lane 1, heat denatured substrate; lane 2, native substrate incubated alone in the reaction conditions; lanes 3–9, native substrate incubated with 0.5 µg (lanes 3–5) or 1 µg (lanes 6–9) of the proteins indicated on the top of the lanes. The RNA substrate employed in the experiment is schematically depicted at the bottom of the figure. It has a 26 bp long ds region with 3′ and 5′ overhanging ss ends of 18, 18, 2 and ~200 nt. The small RNA strand, drawn with a bold line, was labeled with [^{32}P]UMP. The positions of the native partially ds substrate (dsRNA) and of the denatured [^{32}P]ssRNA (ssRNA) are indicated beside the panel.

MBP–CIcxV303I and MBP–CIcxT313S showed detectable RNA helicase activity (Fig. 2). Whereas the activity of MBP–CIcxV303I was similar to that of MBP–CIcx (lanes 3 and 4), that of MBP–CIcxT313S was clearly lower (lane 5). Less conservative substitutions in the same positions (V303A and T313A) lead to products without detectable helicase activity (lanes 7 and 9). On the other hand, mutations in the most conserved residues, even though they produced very conservative changes (T305S, G311A), caused the complete loss of RNA helicase activity (lanes 6 and 8). The same result was observed with a G311S mutation (results not shown).

RNA binding

In order to ascertain whether the motif V mutations affected the ability of PPV CI to interact with RNA, the MBP–CI mutant proteins were transferred to nitrocellulose and incubated with a ^{32}P-labeled RNA probe (northwestern assay). Although this assay does not allow precise quantitative estimations of the RNA binding, all the motif V mutant CI proteins showed efficient interaction with RNA (Fig. 3).

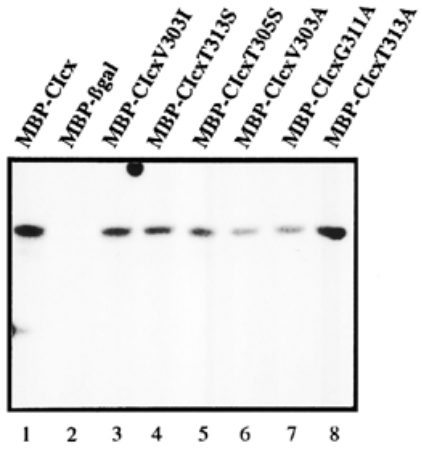


Figure 3. Northwestern assay of RNA binding by MBP–CI recombinant proteins. Approximately 10 µg (lanes 1 and 3–8) or 6 µg (lane 2) of the proteins indicated on the top of each lane were subjected to 10% SDS–PAGE, transferred to nitrocellulose and incubated with a [^{32}P]RNA probe. Binding of RNA was detected by autoradiography.

NTPase activity

All eight common nucleoside triphosphates support CI helicase activity (6). We assayed NTPase activity by measuring dATP hydrolysis because background dATPase activity is known to be very low in maltose-eluted fractions from cells harboring control plasmids (14). As expected from its RNA helicase activity, MBP–CIcxV303I had a dATPase activity in the presence of poly A similar to that of the MBP–CIcx control (Fig. 4). Likewise, MBP–CIcxT313S, which showed a reduced helicase activity, had a lower dATPase activity than those of MBP–CIcx and MBP–CIcxV303I (Fig. 4). Even though MBP–CIcxV303A and MBP–CIcxT305S did not show RNA helicase activity (Fig. 2, lanes 5 and 6), they did demonstrate a weak dATPase activity at high protein concentrations (Fig. 4). No detectable dATPase activity was found associated with the MBP–CIcxG311A and MBP–CIcxT313A proteins (Fig. 4). No significant differences in the rate of stimulation by poly A were observed between the active mutant proteins and the wild-type CI (results not shown).

Infectivity in plants of PPV harboring mutations in the CI motif V

In order to investigate the potential effects of the enzymatic dysfunctions caused by the CI motif V modifications on the viral replication cycle, the mutations which did not completely abolish the enzymatic activities analyzed *in vitro* (V303I, V303A, T305S and T313S) were introduced into the full-length PPV clone pGPPVx. Protoplasts and intact plants were inoculated with RNA transcripts synthesized *in vitro* from the resulting plasmids.

The percentage of *N.clevelandii* plants infected by inoculation with transcripts from pGPPVxV303I (tGPPVxV303I) was similar to that of wild-type tGPPV (20 plants infected out of 24 inoculated versus 19 out of 28, respectively, in three different experiments). Sequencing of a PPV cDNA RT-PCR fragment amplified from progeny virus of plants infected with tGPPVxV303I demonstrated that the V303I mutation was

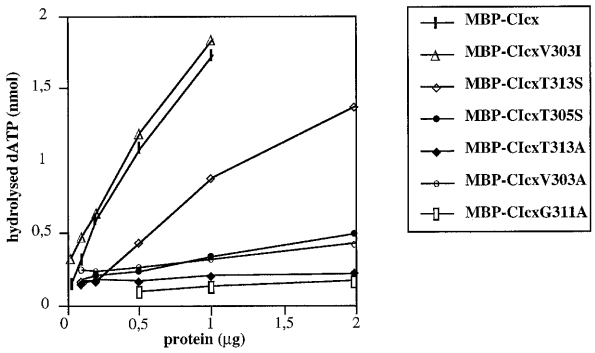


Figure 4. dATPase activity of MBP–CI recombinant proteins assayed in the presence of poly A (12 min reaction).

conserved during virus multiplication. No other mutations were detected in the sequenced region. These results corroborated those obtained *in vitro* and suggest that the V303I mutation in the CI protein is well tolerated.

None of the 14 plants inoculated with tGPPVxT305S (two independent experiments), nor any of the 28 plants inoculated with tGPPVxT313S (three experiments) were infected. In order to verify whether the lack of infectivity might be the result of unnoticed additional mutations introduced during the construction of the full-length cDNA clones, a restriction fragment of pGPPVxT313S that included the T313S mutation (and that did not contain unexpected mutations, as demonstrated by DNA sequencing) was replaced by the corresponding one from pGPPVx. The *in vitro* transcripts synthesized from the resulting plasmid were infectious and caused symptoms indistinguishable from those of wild-type PPV. This result demonstrated that there were no other introduced mutations in pGPPVxT313S that affect infectivity. Thus, the defects caused by the T305S and T313S mutations that abolished (T305S) or weakened (T313S) RNA helicase activity in *vitro* (Fig. 2, lanes 5 and 6), were also apparent *in vivo*, preventing virus propagation in the plant.

Interestingly tGPPVxV303A, which harbors a mutation that abolished *in vitro* RNA helicase activity (Fig. 2, lane 7), was able to infect plants (5 out of 8). The symptoms of plants infected with tGPPVxV303A were similar to those of plants infected with wild-type PPV, however the appearance of symptoms was delayed. Sequencing of a cDNA RT-PCR fragment amplified from progeny virus of plants infected with tGPPVxV303A indicated that a second mutation was introduced at the GCA triplet that codes for Ala 313, leading to GUA that codes for the wild-type Val 313. This residue is encoded in wild-type PPV by a GUU triplet. This rapid pseudoreversion demonstrated that, as expected from its *in vitro* effects, the V303A mutation was deleterious for PPV *in vivo*.

Replication in protoplasts of PPV harboring mutations in the CI motif V

In order to determine whether modifications in the CI motif V interfered with virus replication in isolated cells, protoplasts prepared from *N.clevelandii* leaves were inoculated with transcripts synthesized *in vitro* from PPV full-length cDNA clones harboring the different mutations.

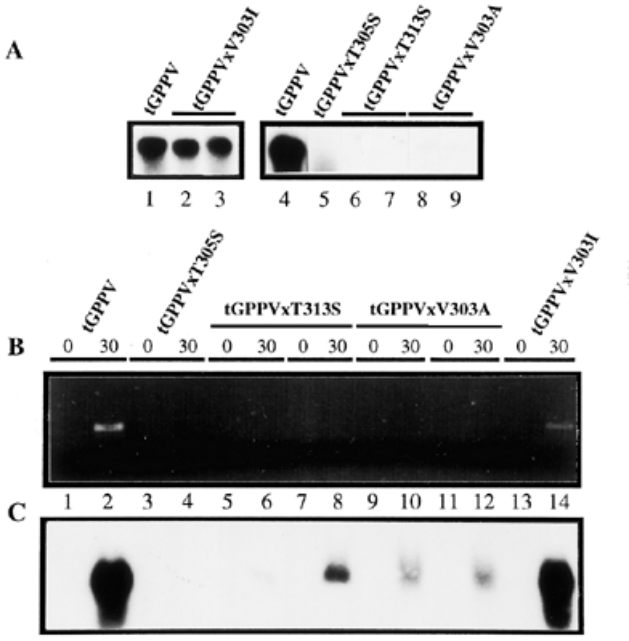
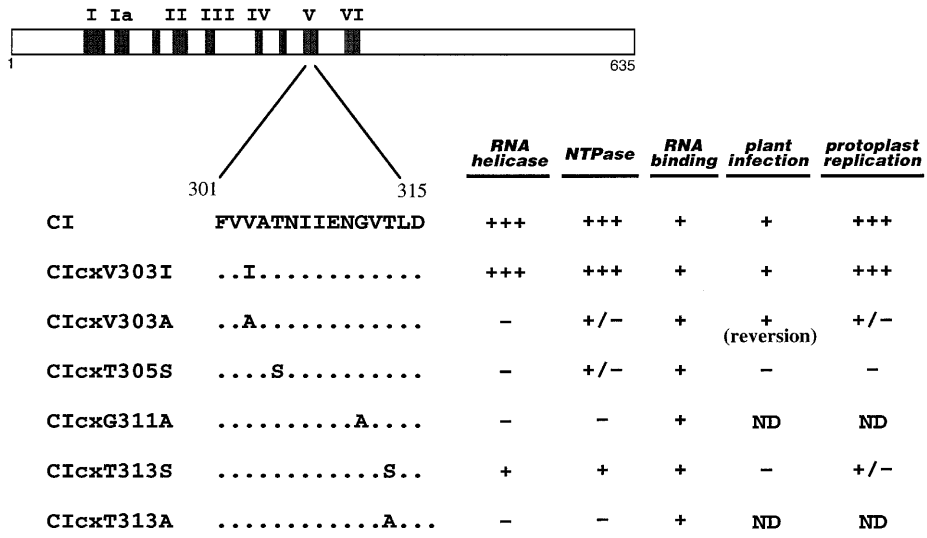


Figure 5. PPV RNA replication in protoplast cultures. Protoplasts were inoculated with *in vitro* transcripts synthesized from full-length wild-type cDNA clone or those harboring point mutations in the CI motif V coding sequence as indicated on the top of each lane (some mutant transcripts were assayed in duplicate, using protoplast samples prepared from different plants). (**A**) Northern analysis of RNA purified from protoplasts collected 30 h post inoculation. (**B**) Ethidium bromide staining of a PPV genomic fragment (nt 8839–8900) amplified by IC-PCR from extracts of protoplasts collected shortly (0) or 30 h (30) after inoculation. (**C**) Hybridization of the amplification products shown in (B) with a PPV-specific ^{32}P-labeled probe.

Northern blot analysis of two samples of protoplasts inoculated with tGPPVxV303I showed levels of virus RNA similar to those of protoplasts inoculated with wild-type tGPPV(Fig. 5A, lanes 1–3). Viral RNA accumulation could not be clearly detected by northern blot analysis in protoplasts inoculated with tGPPVxT305S (Fig. 5A, lane 5), tGPPVxT313S (Fig. 5A lanes 6 and 7) or tGPPVxV303A (Fig. 5A, lanes 8 and 9), although in the last case very faint hybridizing bands were observed. This result indicates that CI motif V mutations hampered virus replication in isolated cells.

RT-PCR preceded by IC-PCR is more sensitive that northern blot, but only detects encapsidated virus and it is not strictly quantitative. IC-PCR amplified bands specific for PPV RNA were detected by ethidium bromide staining of agarose gels only in protoplasts inoculated with tGPPV and with tGPPVxV303I (Fig. 5B, lanes 2 and 14). In addition, Southern blot analysis of IC-PCR amplified products showed trace amounts of PPV-specific bands in protoplasts inoculated with tGPPVxT313S and with tGPPVxV303A (Fig. 5C, lanes 6, 8, 10 and 12), but not in those inoculated with tGPPVxT305S (Fig. 5C, lane 4). PPV-specific bands were observed in IC-PCR products of protoplast samples collected after 30 h of incubation (Fig. 5B and C, even lanes), but not in samples collected shortly after the protoplasts were inoculated (Fig. 5B and C, odd lanes). Thus, these bands were not the result of amplification of inoculum remains, but rather the amplification products of PPV RNA synthesized in the protoplasts. This result indicates that, although MBP–CIcxT313S and



		RNA helicase	NTPase	RNA binding	plant infection	protoplast replication
CI	FVVATNIIENGVTLD	+++	+++	+	+	+++
CIcxV303I	..I.....	+++	+++	+	+	+++
CIcxV303A	..A.....	–	+/–	+	+ (reversion)	+/–
CIcxT305S	....S.....	–	+/–	+	–	–
CIcxG311A	.....A....	–	–	+	ND	ND
CIcxT313S	.....S..	+	+	+	–	+/–
CIcxT313A	.....A...	–	–	+	ND	ND

Figure 6. Summary of the effects caused by motif V point mutations on the *in vitro* activities of the PPV CI protein and on PPV multiplication in protoplasts and in whole plants. Differences in activity levels are indicated by different number of + symbols and by the use of the +/– symbol. ND, not determined.

MBP–CIcxV303A have reduced and undetectable *in vitro* RNA helicase activity, respectively (Fig. 2, lanes 5 and 7), these mutations do not completely abolish viral replication.

DISCUSSION

The high level of conservation of the motif V observed among the potyvirus CI proteins (Fig. 1) suggests that it might play an important role in the protein function. The results reported in this paper indicate that small modifications in this region, which are not expected to disturb the overall conformation of the protein, have profound effects on the enzymatic activities of the PPV CI protein (Fig. 6). In the most invariant positions (305 and 311), even very conservative substitutions (Thr to Ser or Gly to Ala) abolished the RNA helicase activity (Fig. 2). The functional requirements at positions 303 and 313 seem to be less strict since MBP–CI fusion products harboring the V303I or T313S mutations still displayed RNA helicase activity. Actually, MBP–CIcxT313S showed low helicase activity relative to that of wild-type CI. More drastic substitutions (V303A and T313A) also reduced RNA helicase activity to undetectable levels *in vitro*.

Moolenar *et al*. have described that a Gly to Ser mutation in the motif V of the *E.coli* UvrB DNA helicase, that belongs to the SF2 superfamily, had a rather small effect on the basal ATPase activity of the UvrA2B complex, but caused a severe reduction in ATP hydrolysis in the presence of damaged DNA and abolished the helicase activity (18). On the other hand, a similar mutation (Gly to Ala) in the motif V of the DNA helicase UL5 of the SF1 superfamily gave rise to an increase in the K_m for ssDNA and a decrease of the K_{cat} of the DNA-dependent ATPase activity of the herpes simplex virus type 1 UL5–52 subcomplex (29). These data are not directly comparable to those obtained for the PPV CI protein, first because they correspond to proteins with DNA rather than RNA helicase activity, and second because both UvrB and UL5 require the presence of accessory proteins (UvrA and UL52, respectively) for their helicase activity. The positive correlation of the deleterious effects on the RNA helicase and dATPase activities that we have observed for the different PPV CI motif V point mutations (Figs 2 and 4) indicates that this motif is involved in the NTP hydrolysis process required for the unwinding activity of the CI protein. The CI motif V does not seem to be directly involved in nucleic acid binding. The fact that point mutations in the PPV CI motif V did not substantially alter the RNA binding capacity of the MBP–CI fusion products in northwestern assays supports this conclusion (Fig. 3). We cannot discard the possibility however, that the mutated proteins might interact with the RNA through alternative nucleic acid binding domains (23). Further supporting the assumption that motif V does not interact with RNA, it has been previously reported that the RNA binding activity of a PPV CI fragment encompassing aa 339–402 (CI339–402) which lacks the motif V, was very similar to that of the CI262–402 fragment that contains both motifs V and VI, whereas the CI262–338 fragment that includes motif V but not motif VI, did not bind RNA at all (14). We found that motif V seems to participate directly in the NTPase activity rather than in the coupling between NTP hydrolysis and RNA binding since both the poly A-stimulated and the basal dATPase activities were drastically affected by CI motif V mutations, and no significant effects on the levels of poly A stimulation were observed (Fig. 4 and data not shown). Nevertheless, it is important to remark that the recent X-ray crystallographic three-dimensional structures of the *Bacillus stearothermophilus* PcrA DNA helicase (superfamily SF1) (30) and of the hepatitis C virus RNA helicase domain (superfamily SF2) (31) seem to indicate that the different conserved helicase motifs are closely connected in the tertiary structure of the protein, and that they may form a large functional domain, rather than seven individual ones with strictly independent functions.

Two functions in the replicative cycle of the potyviruses have been proposed for the CI protein. One hypothesis postulates that the replication of large positive polarity genomic RNAs requires the action of a helicase activity working in the 3′ to 5′ direction that would unwind the double-stranded replicative intermediate to allow for the progression of the replicative complex (6). Supporting this hypothesis, small insertions in the tobacco vein

mottling potyvirus CI protein abolished the replication of virus RNA in protoplasts (32). The second hypothesis argues that the cylindrical inclusions formed by the CI protein are structures involved in virus movement between adjacent cells (33,34). In this regard, recent experiments indicated that cylindrical inclusions are formed near plasmodesmata very early in the infection, apparently facilitating the passage of virions through them (35). In this report, we demonstrate that mutations in the motif V of PPV CI interfered not only with the establishment of infection in the whole plant, but also with viral RNA replication in isolated cells (Fig. 6). Mutations that caused moderate (T313S) or severe (V303A, T305S) disturbances of the *in vitro* RNA helicase activity of the PPV CI protein also lead to drastic reductions in viral RNA accumulation in protoplasts inoculated with the mutant virus (Fig. 5). The fact that PPVxV303A was able to establish a systemic infection in plants in spite of its very low levels of replication in protoplasts can be explained by its ability to pseudorevert to PPVx by a single C to T transition. PPVxT313S, which replicated in isolated cells at levels similar to those of PPVxV303A, would not be able to infect plants because it requires a more drastic T to A transversion in order to revert to wild-type, and the efficiency of replication of the mutant virus may be too low to establish a plant infection. However, we cannot rule out the possibility that T313S mutation, in addition to affecting virus replication, interferes with other virus fuctions required for movement in the plant. Both the accumulation of encapsidated RNA (IC-PCR assay) and that of total viral RNA (northern assay) were affected by the motif V mutations, suggesting that the step of the viral cycle altered by them was at the level of RNA replication.

It is important to remark that, although the data presented in this paper indicate that the helicase activity of the CI protein plays a role in the multiplication of PPV inside the cell, presumably in RNA replication, they do not exclude the possibility that CI might additionally be involved in a later step of the viral infection cycle, as has been discussed above on the effects of the T313S mutation. In fact, mutations of the tobacco etch potyvirus CI that affect cell to cell spread have been recently described (J.Carrington, personal communication). Thus, the CI protein seems to resemble other potyvirus proteins in being multifunctional. Further research is required to unravel the relationships among the different functions of the potyvirus CI protein as well as the role played in them by its enzymatic activities.

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