Activities associated with the putative replication initiation 
protein of Coconut foliar decay virus, a tentative member of 
the genus Nanovirus

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Introduction

Coconut foliar decay virus (CFDV) is a small isometric plant 
virus with a single-stranded DNA genome (Randles et al., 1986). The only known genomic component of CFDV consists of 1291 nucleotides and has been proposed to contain up to six 
open reading frames in both orientations (Rohde et al., 1990). The largest, called ORF1, potentially encodes a 33 kDa protein 
possessing motifs typical for the replication initiation protein 
(Rep) of gemini- and nanoviruses (Gorbalenya et al., 1990; Boevink et al., 1995; Katul et al., 1997). This gene is 
expressed from a CFDV promoter which has been shown to be 
phloem-specific (Rohde et al., 1995) and to include the cis- 
acting elements involved in phloem-specificity (Hehn & Rohde, 1998). RNA transcript(s) which correspond to the entire 
coding sequence of CFDV Rep are polyadenylated at a 
position six bases downstream of the termination codon of this 
gene (Merits et al., 1995). The nucleotide sequence of CFDV 
DNA is most closely related to the Rep-encoding components 
of the nanoviruses Subterranean clover stunt virus (SCSV) (Boevink et al., 1995), Banana bunchy top virus (BBTV) (Burns et 
al., 1995), Milk vetch dwarf virus (MDV) (Sano et al., 1998) and 
Faba bean necrotic yellows virus (FBNYV) (Katul et al., 1997, 1998). The five principal DNA components of nanoviruses code for five or six proteins which have homologues present in 
all nanoviruses studied so far. One particular DNA component 
encodes the ‘master’ Rep that is responsible for replication of 
all other non-Rep DNAs – the conserved genes for coat 
protein, putative hydrophobic movement protein, potential 
nuclear shuttle protein, a cell cycle-linked protein (Clink) and 
several as yet unidentified genes (Aronson et al., 1999; Wanitchakorn et al., 2000). In total, up to 11 genomic 
components have been found in some nanoviruses where several additional Rep components are capable of independent 
replication but cannot support replication of non-Rep DNAs (Katul et al., 1997, 1998; Sano et al., 1998; Beetham et al., 1999; Timchenko et al., 1999). CFDV DNA differs from the Rep 
components of nanoviruses in its slightly larger size (common 
DNA size for nanoviruses is about 1 kb). In addition, CFDV 
is transmitted by the planthopper Myndus taffani, whereas typical 
nanoviruses are persistently transmitted by aphids (Randles et al., 1986; Franz et al., 1999).

Rep of CFDV and nanoviruses contains several sequence 
motifs similar to those in Reps of plant ssDNA viruses 
belonging to the family Geminiviridae (Boevink et al., 1995),
which are essential for DNA replication (Laufs et al., 1995; Palmer & Rybicki, 1998). Rep catalyses multiple reactions during the replicative cycle of the virus. So far, for nanoviruses only in vitro DNA nicking and joining activity of the BBTV Rep have been described (Hafner et al., 1997). In contrast, much more is known about Reps of geminiviruses [for recent review, see Palmer & Rybicki (1998)]. Geminivirus Rep is a sequence-specific DNA-binding protein (Fontes et al., 1992; Behjatnia et al., 1998; Castellano et al., 1999) and has an ATPase activity which is required for its helicase function and for viral DNA replication (Desbiez et al., 1995). Geminivirus Rep catalyses DNA cleavage and ligation of ssDNA during rolling circle replication. In solution, Rep forms oligomeric complexes and the self-association capability of geminivirus Rep is regulated by ATP (Jupin et al., 1995; Palmer & Rybicki, 1998). Rep catalyses DNA cleavage and primer 5'–3', representing positive-polarity ssDNA containing the stem–loop region of the CFDV genome, Xhol-linearized plasmid, containing full-length CFDV DNA (Chernov et al., 1992), and primer 5' CAATTATGAACTGAGTTATGCGGCGGCCTGCCC 3' were used (the Apal site from the CFDV genome is underlined). To obtain ssDNA(+) and ssDNA(−), three different templates with corresponding primers were used. For ssDNA(+), representing positive-polarity ssDNA containing the stem–loop region of the CFDV genome, Xhol-linearized plasmid, containing full-length CFDV DNA (Chernov et al., 1992), and primer 5' CAATTATGAACTGAGTTATGCGGCGGCCTGCCC 3' were used (the Apal site from the CFDV genome is underlined). To obtain ssDNA(−), complementary to the same sequence, CFDV was linearized by Apal cleavage and primer 5' CAGGACCGAGCGACTGCTGCGAG 3' was used (the Xhol site from the CFDV genome is underlined). To obtain the control, ssDNA(2), plasmid pGEM-3Z (Promega) was linearized by AatII cleavage and primer 5' ATTTAGGTGACACTATAGAATAC 3' (corresponding to the Sp6 promoter sequence in the plasmid) was used. ssDNA was synthesized in a reaction mixture consisting of 1 μg of linearized plasmid and 50 pmol of the corresponding primer, 0.25 mM of each dNTP and 5 units of DyNAzyme DNA polymerase (Finnzymes) in DyNAzyme reaction buffer. PCR reactions (25 cycles: 95 °C 30 s, 54 °C 30 s, 72 °C 60 s) were carried out in a thermal cycler, reactivation products purified using the QIAquick PCR purification kit (Qiagen), and labelled and purified as described above.

**Methods**

### Cloning of the 6 × His-fused CFDV ORF1

The region of the CFDV genome corresponding to the Rep gene was amplified by PCR using P.tau-Turbo DNA polymerase (Stratagene) with oligonucleotides 5' GCGGATCCAAATGGGTTCCTCCATTCGCCG 3' and 5' GC-GTCGACTTATAATTTACAGTTTTATTCTGTCCC 3' (restriction sites for BamHI and SalI are underlined) as primers, and a cloned tandem dimer of the full-length CFDV DNA as a template (Chernov et al., 1992). The resulting PCR product was cloned into BamHI/SalI-digested plasmid pSK(+) (Stratagene) to give pSK.Rep, and sequenced. To obtain a clone for expression of the 6 × His fusion protein Rep (6 × His–Rep) in *E. coli*, the BamHI–SalI fragment from pSK.Rep was cloned into BamHI/SalI-digested vector pQE32 (Qiagen) to obtain clone pQE.Rep. This plasmid was used to transform *E. coli* strain M15 (Stratagene). Expression, purification and refolding of the 6 × His–Rep protein was carried out according to the Stratagene protocol.

### Expression of 6 × His–Rep in recombinant baculovirus

To obtain recombinant baculovirus expressing the 6 × His fusion protein of CFDV Rep, the EcoRI–SalI fragment from pQE.Rep was cloned into EcoRI/SalI-digested vector pFastBac1 (Gibco BRL) to obtain clone pFastBac.6 × His.Rep. This clone was used for obtaining recombinant baculovirus, named 6 × His.Rep-Bac, using the Bac-to-Bac system (Gibco BRL) to obtain clone pQE.Rep. This plasmid was used to transform *E. coli* strain M15 (Stratagene). Expression, purification and refolding of the 6 × His–Rep protein was carried out according to the Stratagene protocol.

### Nucleic acid binding assay

The purified recombinant CFDV 6 × His–Rep and control proteins [BSA and mallof-binding protein fusion with the x fragment of β-galactosidase (MBP)] were electrophoretically onto an Immobilon-P filter (Millipore) membrane after SDS–PAGE in 12% gels. Membranes were blocked and membrane-bound proteins denatured and renatured as described previously (Merits et al., 1998). Membranes were incubated for 2 h at room temperature with 10 μg/ml of labelled DNA or RNA (5 × 10⁶ c.p.m./ml) in 10 ml of nucleic acid-binding buffer (20 mM Hepes, 6 mM Tris–HCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, pH 7.0) with or without addition of NaCl. NaCl concentrations of 25
100, 300 and 500 mM were used to estimate the strength of protein–nucleic acid interactions. Membranes were washed with nucleic acid-binding buffer (with or without addition of NaCl) four times for 30 min, dried, and autoradiographed.

**Yeast two-hybrid system (YTHS).** This was used essentially as described by Voječek et al. (1993) and Hollenberg et al. (1995) with modifications described by Guo et al. (1999). To obtain the yeast expression constructs pLexA.Rep and pVP16.Rep, the BamHI–SalI fragment from pSK.Rep was cloned into vectors pLexA and pVP16 (Hollenberg et al., 1995). pLexA encodes the DNA-binding protein LexA and contains a selection marker for trp1 auxotrophy, whereas pVP16 encodes transcription activation domains and contains a selection marker for ura3 auxotrophy. Yeast strain L40 [MATα his3Δ200 trp1-901 leu2-3,112 a ade2 lys2-801am URA3::(lexAOp)–uro2] was used for hybrid protein expression. Yeast transformation was performed by the lithium acetate method (Schiestl & Gietz, 1989). Transformants were selected by plating on minimal media lacking leucine and tryptophan. Expression of the β-galactosidase reporter gene was evaluated by freezing colony filter lifts in liquid nitrogen and subsequently staining the filter with X-Gal in Z buffer (60 mM NaH₂PO₄, 40 mM NaH₂PO₄, pH 7.0; 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol and 0.3 mg/ml X-Gal). Quantitative β-galactosidase activity assays were performed as described by Tr avrilck et al. (1989).

**Transient expression of CFDV Rep in Nicotiana benthamiana cells.** For transient expression in N. benthamiana cells the BamHI–SalI fragment from pSK.Rep was cloned as an N-terminal fusion with the green fluorescent protein gene GFP5 (Haseloff et al., 1997) placed under control of the CaMV 35S RNA promoter. The resulting construct, named p35S.GFP5.Rep, was used in particle bombardment experiments. Particle bombardment was performed using the smoking disk method with a high-pressure helium-based apparatus PDS-1000 (Bio-Rad) as described in Morozov et al. (1997). GFP fluorescence was detected by a Zeiss-Axiovert Bio-Rad MRC 1024 confocal laser scanning microscope using a 15 mW krypton–argon excitation laser with excitation light of 488 nm.

### Results and Discussion

#### Expression and purification of recombinant Rep protein of CFDV

Repeated attempts to express 6 × His–Rep in E. coli resulted in the purification of approximately equal amounts of two proteins with apparent molecular masses of 33 and 29 kDa (Fig. 1). The 33 kDa protein had the expected molecular mass of 6 × His–Rep and represented the full-length product of pQE.REP. Since both proteins bound to Ni–NTA resin and reacted with a monoclonal antibody against the N-terminal epitope of the recombinant protein [RGS(H)₃ epitope], we suggest that the 29 kDa protein represents a product of premature termination, possibly due to unfavourable codon usage in E. coli at the C-terminal region of CFDV Rep (Fig. 1).

When CFDV 6 × His–Rep was expressed and purified from recombinant baculovirus, only one main protein band with an apparent molecular mass of 36 kDa was obtained (Fig. 1). This molecular mass is bigger than that calculated for 6 × His–Rep or observed for the protein expressed in E. coli. Similar to the proteins expressed in E. coli, this recombinant baculovirus-expressed protein also reacted with a monoclonal antibody against the RGS(H)₃ epitope (Fig. 1). Reasons for the observed mass increase remain to be determined, but one possible reason might be post-translational modification of the protein in insect cells, but not in E. coli cells. Both E. coli- and baculovirus-expressed proteins were used for further *in vitro* experiments.

#### Localization of CFDV Rep in insect cells and in N. benthamiana cells

The processing and subcellular localization of recombinant eukaryotic proteins in baculovirus-infected insect cells can be very similar to their localization in the original (or host) cells (Pascal et al., 1994; Patterson et al., 1996). The use of different monoclonal antibodies against N-terminal epitopes of recombinant protein [(H)₅, (H)₆, (H)₇, and RGS(H)₂] to immunostain 6 × His–Rep expressed in infected Sf9 or High Five cells did not produce consistent results. Typically, fluorescence was close to background levels and showed no specific distribution in the cells (not shown). It is possible that the N-terminal 6 × His tag epitope is hidden inside the protein and/or between different subunits of oligomerized protein. In attempts to overcome these difficulties, the infected High Five cells (48 h post-infection) were fractionated and analysed by SDS–PAGE and Western blotting. In these experiments most of the expressed recombinant protein (about 70%) was found in the...
Fig. 2. Distribution of CFDV 6×His–Rep in recombinant baculovirus-infected High Five cell fractions. Each lane corresponds to 15 μg of total protein, subjected to SDS–PAGE in 12% gels and Western blotted. Proteins were detected using monoclonal antibody against the RGS(H)3 epitope and the ECL procedure. Lane 1 contains the nuclear fraction, lane 2 contains the post-mitochondrial pellet fraction and lane 3 contains the post-mitochondrial supernatant fraction. New England Biolabs prestained molecular mass standards were used (mol. mass in kDa is indicated on the left).

Table 1. Homotypic interactions of the CFDV Rep as detected by the yeast two-hybrid system

<table>
<thead>
<tr>
<th>pLexA</th>
<th>pVP16</th>
<th>Filter assay</th>
<th>Liquid assay</th>
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<tr>
<td>Rep</td>
<td>Rep</td>
<td>+</td>
<td>176 ± 8.6</td>
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<tr>
<td>Rep</td>
<td>Empty</td>
<td>-</td>
<td>&lt; 2</td>
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<tr>
<td>Empty</td>
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<tr>
<td>PVA HC</td>
<td>PVA HC</td>
<td>+</td>
<td>37 ± 2.4</td>
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Fig. 3. Fluorescence microscopy of transformed N. benthamiana epidermal cells transiently expressing GFP fusion of CFDV Rep. (A)–(D), association of fluorescence with the nucleus and elements of the cell endomembrane system including the nuclear envelope in cells expressing the GFP–Rep protein. (A, C), cell images reconstructed by superimposition of a series of confocal optical sections. (B), the central confocal optical section of the same cell as in (A), (D), the central confocal optical section of the same cell as in (A)., (D), image of a cell expressing free GFP. Scale bar in (B), (D) and (E) represents 50 μm.

nuclear fraction of infected cells (Fig. 2). Considerable amounts of 6×His–Rep protein were also found in the post-mitochondrial pellet fraction (membrane fraction, about 25% of the recombinant protein) and only small quantities (less than 5% of the recombinant protein) were found in the soluble protein fraction (Fig. 2). An alternative method, based on particle bombardment of plant leaves, was also used to study the subcellular distribution of CFDV Rep. When GFP–Rep fusion protein was transiently expressed in the epidermal cells of N. benthamiana leaves, fluorescence was detected predominantly in nuclei and at the cell periphery (Fig. 3A–D). Free GFP is known to be associated partially with the plant cell nucleus (Reichel et al., 1996). In control experiments with plants expressing non-fused GFP5 gene from the 35S promoter, the distribution of the fluorescence differed from that of GFP–Rep fluorescence (Fig. 3E). No specific targeting of fluorescence was observed and the distribution of fluorescence was typical for free GFP in plant cells (Reichel et al., 1996; Morozov et al., 1999). This allowed us to conclude that the distribution of GFP fluorescence, presented in Fig. 3 (A–D), reflected the distribution of Rep in the plant cell. Thus, the

Fig. 4. ATPase and GTPase activities of the E. coli-expressed CFDV 6×His–Rep and their dependence on the presence of divalent cations in the reaction. Reaction products were separated by TLC and the plate was exposed to X-ray film. Positions of NTP substrates and Pi are marked by arrows at the right. Lane 1 on both panels represents the products of a control reaction (no 6×His–Rep added); lane 2 represents products of reaction with 6×His–Rep without divalent cations; lanes 3, 4 and 5 represent products of reaction in the presence of 5 mM Mg2+, Ca2+ or Mn2+, respectively.
Activities of CFDV Rep protein

CFDV Rep protein is self-interacting in the yeast nucleus

To study the capability of CFDV Rep to form di- and oligomers, the corresponding gene was cloned in the YTHS vectors pLexA and pVP16. The β-galactosidase activity present in yeast transformants was used as an indicator of interactions between fusion protein partners. When both plasmids carrying CDFV Rep (pLexA and pVP16) were co-transformed into yeast cells, double transformants showed high β-galactosidase activity as indicated by the appearance of blue colonies. The colonies turned blue after staining for less than 2 h, indicating a strong interaction between the proteins. The strength of the CFDV Rep:Rep interaction was confirmed by quantitative β-galactosidase activity assay. No β-galactosidase activity was detected in yeast transformed with one plasmid only or with a plasmid containing CFDV Rep and an empty vector (Table 1). Thus we conclude that CFDV Rep is capable of di- or oligomer formation similar to Reps of geminiviruses, homo-oligomers of which are important for carrying out different functions in the regulation of virus replication and transcription (Jupin et al., 1995; Settlage et al., 1996; Orozco et al., 1997, 2000; Orozco & Hanley-Bowdoin, 1998; Horvath et al., 1998).

NTPase activity of CFDV Rep

ATPase activity is an intrinsic property of geminivirus Reps (Desbiez et al., 1995). To examine further activities associated with CFDV Rep, an in vitro assay for NTPase activity was carried out using purified bacterially and baculovirus-expressed CFDV 6×His–Rep (Fig. 1). First, it was found that recombinant CFDV Rep is capable of ATP and GTP hydrolysis (Fig. 4). This activity was detected for both E. coli- and baculovirus-expressed proteins. These ATPase and GTPase activities were not stimulated by ssRNA, ssDNA and dsDNA (data not shown), but did require the presence of divalent cations in the reaction. Maximal stimulation of NTPase activity was observed in the presence of 5 mM Mg²⁺, and to a lesser extent by 5 mM Mn²⁺; 5 mM Ca²⁺ had only minor stimulatory effect (Fig. 4).

![Fig. 5. Autoradiography of the nucleic acid-binding blots of purified CFDV 6×His–Rep. The left half of each panel (A–H) represents binding in the presence of 25 mM NaCl; the right half represents binding in the presence of 300 mM NaCl. Lane 1 contains 1 µg BSA, lane 2 contains 1 µg MBP, putative Rep of CFDV is largely associated with the nucleus, the cell compartment where Reps normally exert their functions (Palmer & Rybicki, 1998).](image-url)
Nucleic acid-binding properties of CFDV Rep

In low salt conditions (up to 150 mM NaCl) CFDV ω × His–Rep was found to bind non-specifically to ssRNA, dsDNA and ssDNA (Fig. 5 A–H, left-hand panels). The dsDNA binding was observed only if the assay was carried out in low salt conditions (Fig. 5 B, D, left-hand panels). CFDV Rep complexes with dsDNA were dissociated almost completely in the presence of 300 mM NaCl whether or not the dsDNA contained the conserved stem–loop region (Fig. 5 B, D, right-hand panels). If the same dsDNA probes were denatured by boiling and then cooled on ice before adding to the binding reaction, the nucleic acid–protein complexes were stable in 0-3 M NaCl (Fig. 5 C, E, right-hand panels) and even in 0-5 M NaCl (data not shown). These data indicate that CFDV ω × His–Rep binding to ssDNA is significantly stronger than to dsDNA. To examine, whether ssDNA binding of CFDV Rep is strand- or sequence-specific, three ssDNA probes, ssDNA(+), ssDNA(−) and ssDNA(α), were tested in the same assay. No difference in complex formation activity or complex stability was observed, indicating that CFDV ω × His–Rep behaves as a sequence non-specific ssDNA-binding protein (Fig. 5 F, G, H). The RNA-binding ability of CFDV ω × His–Rep protein was found to be rather weak. CFDV ω × His–Rep bound unrelated RNA under low salt conditions (Fig. 5 A, left-hand panel) but ω × His–Rep:RNA complexes were completely dissociated in the presence of 0-3 M NaCl (Fig. 5 A, right-hand panel). Thus, CFDV ω × His–Rep binds different types of nucleic acids in a sequence non-specific manner, and complexes formed with ssDNA are much more stable than with dsDNA or RNA. No complex formation with any nucleic acid probes was detected for control proteins (BSA, MBP) or proteins from molecular mass standards under identical conditions (Fig. 5 A–H). Importantly, no significant differences in DNA-binding properties between baculovirus- and E. coli-expressed proteins were observed in these assays.

Recently, a novel Rep-encoding nanovirus-like DNA component, similar to CFDV in size and genetic organization, was found to be associated with plants infected with Cotton leaf curl virus (CLCuV), a member of the genus Begomovirus (Mansoor et al., 1999). This Rep and CFDV Rep are phylogenetically only distantly related to the nanovirus master Reps, which form a distinct cluster (H. J. Vetten, personal communication). A dimer of CLCuV-associated DNA, introduced biologically into tobacco plants, was shown to replicate autonomously (Mansoor et al., 1999). However, in the case of tandem dimers of CFDV DNA that were cloned previously (Chernov et al., 1992), we never observed replication in electroporated protoplasts or bombarded leaf cells of several non-host plants (A. Merits, unpublished data). These data suggested that the published CFDV DNA clone may contain a non-functional Rep pseudogene as was found in some BBTV Rep-related DNAs (Yeh et al., 1994; Merits et al., 1995). However, our findings on the subcellular distribution, oligomerization and in vitro biochemical activities allow us to conclude that CDFV ORF1 indeed encodes an enzymatically active Rep that shares some properties with to the Reps of geminiviruses and nanoviruses.

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