Results and Discussion
4.1 AC3 Sequence analysis

Sequences of AC3 ORFs and respective AC3 proteins from various begomoviruses causing either leaf curl disease or mosaic leaf disease were aligned using ‘ClustalW’ algorithm. Sequence analysis indicated significant conservation at nucleotide and protein levels (Figs. 4.1, 4.2). Conservation was more prominent at the N’-terminus part of the protein. Though the middle and C’-terminus part of the protein exhibited little identity, there was substantial charge conservation in terms of amino acid side chains with similar properties.

A profound identity and charge conservation among amino acids observed at protein level suggests that all AC3 sequences were co-evolved and might perform similar functions in all viruses. Small variations in amino acids suggest the evolutionary pressure on the viral ORF to adapt for a successful infection in each host under different conditions.

4.2 Expression and Purification of AC3

4.2.1 Expression and Purification of GST fused AC3

AC3 ORFs from various begomovirus isolates (Table 3.2) were amplified with degenerate oligonucleotides: All_AC3_Fwd and All_AC3_Rev (Table 3.3) and ligated into pGEM-T Easy cloning vector. BamHI/XhoI digested AC3 ORFs from pGEM-T Easy clones were ligated into BamHI/XhoI digested pGEX-4T-1 expression vector. AC3 with GST fusion (GST-AC3) was expressed by inducing the E. coli [BL21(DE3)] cells with 0.1 mM IPTG (Fig. 4.3A). Upon lysis, most of the induced GST-AC3 protein from all viral isolates was observed in the pellet fraction of the sonicated bacterial cell lysate (Fig. 4.3B). Among the seven virus isolates, AC3 protein was purified from ToLCrKeV-[IN:Kerll:05] (henceforth referred to as ToLCrKeV) isolate. Purification of the ToLCrKeV GST-AC3 was carried out from the supernatant fraction of the sonicated cell lysate using glutathione
Figure 4.1. Multiple sequence alignment of few selected geminivirus AC3 coding regions. AC3 coding regions of various virus isolates that cause tomato leaf curl disease or mosaic leaf disease were aligned using 'ClustalW' programme. Nucleotides identical by more than 57% are highlighted by shading. The black bar indicates the highly AT rich region around the stop codon of AC3. The grey overlay region indicates the homologous sequence among the viruses that was used for designing the oligonucleotides for AC3 cloning. Consensus row indicates the nature of the nucleotides at that particular position in the column. '*' indicates that the nucleotides are 100% identical at that particular position. Details of the virus isolates are explained in the table 3.2.
Figure 4.2. Multiple sequence alignment of few selected geminivirus AC3 proteins. AC3 protein sequences of various virus isolates that cause tomato leaf curl disease or mosaic leaf disease were aligned using 'ClustalW' programme. Amino acids showing more than 57% identity in a column were highlighted by shading. Consensus row indicates the nature of the amino acids at that particular position in the column. '*' indicates that the amino acids are 100% identical at that particular position, ':' indicates that there is a replacement of amino acids but the nature of the amino acids is highly conserved, '.' indicates that there is a replacement of amino acid and the nature of the amino acids is weakly conserved. Details of the viral isolates are explained in table 3.2.
Figure 4.3. Expression and Purification of GST fused AC3. (A) GST-AC3 from selected viruses was induced with IPTG and visualised by Coomassie staining of the SDS-PAGE gels. The red arrow on the right indicates the induced GST-AC3 protein. 'V' and 'VI' indicates the uninduced vector and induced vector samples respectively. The induced viral GST-AC3s are from ToLCGV-[IN:ND:05] (ND1); ToLCKeV-[IN:KerI:05] (K2); ToLCBV-A[IN:KerIV:05] (K4); ChiLCV-IN[IN:PRM:Tom:05] (CL); MYMIV-[IN:ND:Bg3:91] (MY). (B) Most of the induced protein was observed in inclusion bodies. 'S' and 'P' indicates the soluble and pellet fraction respectively. (C) Purification of the K2-GST-AC3 protein from soluble fraction using Glutathione sepharose. 'I' indicates the induced cells, 'FT' indicates the flow through from the Glutathione resin and 'W' indicates the wash fraction to remove non-specific binding of proteins to resin. Numbers indicate the fraction number of the eluate collected. GST-AC3 gets purified along with another protein of ~60kDa.
Results and Discussion

sepharose. The profile of the purified protein indicated ~99% homogenous purification of GST-AC3 (Fig. 4.3C).

4.2.2 Expression and Purification of 6X-Histidine tagged AC3

In order to perform the in vitro pull down assay for analysing the oligomerisation status of AC3 there is a need for isolation and purification of AC3 with another tag/fusion other than GST. We have chosen 6X-His tag and MBP fusion to purify AC3 for this purpose. BamHI/XhoI digested AC3s (of the isolates mentioned above) from pGEM-T Easy vector were ligated into BamHI/XhoI digested pET28a expression vector. AC3 protein with 6X-His tag (6X-His-AC3) was induced with 0.1 mM IPTG. High expression of the 6X-His-AC3 was observed at ~18 kDa (Fig. 4.4A). Upon cell lysis, most of the induced 6X-His-AC3 protein was fractionated into pellet. Soluble fraction of bacterial cell lysate contained little amount of 6x-His-AC3 than the pellet fraction (Fig. 4.4B). Purification of ToLCKeV 6X-His-AC3 was observed to be very poor in quality. Most of the soluble protein did not bind to the Ni-NTA and appeared in the flow through fraction. Analysis of eluted fraction revealed the presence of other contaminating proteins co-purified with AC3 (Fig. 4.4C).

4.2.3 Expression and Purification of MBP fused AC3

Various AC3s cloned in pGEM-T Easy vector were digested with BamHI/Xhol and ligated into BamHI/SalI digested pMal-c2X expression vector. Induction was robust and the expression of MBP fused AC3 (MBP-AC3) protein was observed at ~58 kDa (Fig. 4.5A) with a significant amount of protein appearing in the soluble fraction of bacterial cell lysate (Fig. 4.5B). Elution of ToLCKeV MBP-AC3 with 10 mM maltose from amylose resin resulted in ~99% homogeneously purified protein (Fig. 4.5C).

We also attempted to purify ToLCKeV AC3 without a tag by cleaving MBP with Factor Xa. Though, the Factor Xa cleavage released AC3 (Fig. 4.5D)
Figure 4.4. Expression and Purification of 6X His tagged AC3. (A) Induction of Histidine tagged AC3 protein with IPTG. 'V' and 'VI' indicates the uninduced and induced pET28a vector sample, followed by AC3 samples from ToLCGV-[IN:ND:05] (ND1); PaLCuV-IN[IN:ND:Tom:05] (ND2); ToLCKeV-[IN:KerII:05] (K2); ToLCBV-A[IN:KerIV:05] (K4) and ICMV-IN[IN:Mah:88] (IC). The red arrow on the right indicates the induced 6X-His-AC3 protein. (B) Most of the induced 6X-His-AC3 was observed in inclusion bodies. 'S_Tx' indicates the soluble fraction from the bacterial cells treated with TritonX- 100 in sonication buffer, 'S' and 'P' indicates the soluble fraction and pellet fraction respectively from cells treated with normal sonication buffer. (C) Purification of the ToLCKeV-[IN:KerII:05] 6X-His-AC3 protein from soluble fraction using Ni-NTA resin. 6X-His-AC3 gets purified along with other bacterial proteins that bind to Ni-NTA.
Figure 4.5. Expression and Purification of MBP-AC3. (A) Induction of MBP fused AC3 with IPTG. Samples loaded are induced vector (VI), ToLCKeV-[IN:KerII:05] (K2); ToLCBV-A-[IN:KerIV:05] and ChiLCV-IN-IN:PRM:Tom:05] (CL). (B) Amount of MBP-AC3 was found to be high in soluble fraction. (C) Purification of the K2-MBP-AC3 protein from soluble fraction using amylose resin. K2-MBP-AC3 was purified to 99% homogeneity. (D) Factor Xa cleavage of the K2-MBP-AC3 releases AC3 from MBP. ‘UC’ indicates the uncleaved fusion protein and ‘PC’ indicates partially cleaved protein. (E) AC3 gets precipitated from the Factor Xa cleavage reaction mix upon complete cleavage. Absence of AC3 can be noticed in the soluble fraction of the completely cleaved (CC) fractions.
Results and Discussion

from MBP, the AC3 protein was not stable and got precipitated in the Factor Xa reaction mixture (Fig. 4.5E).

Kyte and Dolittle analysis for the hydrophobicity of ToLCKeV AC3 showed that the middle and C’-terminus portion of the protein has high composition of hydrophobic amino acids (68%). Presence of such a high percentage of hydrophobic residues was often observed in the inner core of proteins, transmembrane regions or at the interacting regions in an oligomer. The high composition of these hydrophobic residues in ToLCKeV AC3 might be a reason for the precipitation observed in the solution when expressed as a fusion with 6X-His tag or with GST and upon cleavage from MBP. Maltose binding protein being highly soluble might be helping the ToLCKeV AC3 to remain soluble as a fusion protein.

4.3 Oligomerisation studies

TGMV AC3 was reported to form a homo oligomer of ≥100 kDa with self (Settlage et al., 1996; Settlage et al., 2005) but the exact composition of its oligomeric form has not been investigated. Here, we examined the formation of an oligomer of ToLCKeV AC3 (referred as AC3 from now onwards) by in vitro GST pull down assay and ex vivo yeast two-hybrid assay. The status of oligomerisation of AC3 was further analysed by gel filtration as well as sucrose gradient ultracentrifugation.

GST pull down assay was performed by incubating the GST-AC3 and MBP-AC3 or MBP with glutathione resin in binding buffer. GST-AC3 bound to glutathione resin was stringently washed with 600 mM salt containing wash buffer to remove non-specifically bound proteins. Following washes, GST-AC3 bound proteins were visualised by western blotting with MBP-AC3 antibody (Fig. 4.6A). MBP-AC3 could be noticed with GST-AC3 in the bound fraction and MBP could not be detected in the bound fraction. This indicated that the interaction observed in case of MBP-AC3 was specific to AC3 and not because of any interaction between
MBP and AC3 or MBP and GST. Our results corroborated with earlier reports that AC3 forms an oligomer (Settlage et al., 1996). It should be noted that since the antibody used was raised against the MBP-AC3, the antibody was able to detect MBP and GST-AC3 as well.

In order to check the oligomerisation of AC3 ex vivo, we have utilised yeast two hybrid system. AC3 was digested by BamHI/Xhol from the pGEM-T Easy clone and ligated into BamHI/Sall digested vectors pGAD-C1 and pGBD-C1. Cloning was confirmed by colony PCR. AC3 cloned pGAD-C1 and pGBD-C1 constructs were co-transformed into AH109 yeast cells and plated on the minimal medium lacking leucine, tryptophan and histidine amino acids. We could not detect any growth of yeast cells on the selection medium after three days of incubation. Since, we could detect the oligomerisation in vitro, the absence of yeast growth on selection medium indicates the possibility that the ex vivo interaction between AC3 proteins is too weak to be observed in yeast. A similar observation was made in case of C3 protein of TYLCV (Settlage et al., 2005). In case of C3, authors designed various alanine scanning mutations in C3 ORF and checked for the oligomerisation of C3 protein which is necessary for replication enhancement activity of C3. Here, authors observed that in few cases where they could observe the replication enhancement in protoplasts, they could not detect the oligomerisation in yeast. Hence, it is possible that weak interactions could not be detected in yeast two-hybrid study.

Formation of an oligomer results in increase of molecular mass of the complex. A careful analysis of the mass of the complex helped us in analysing the oligomeric status of the AC3. Gel filtration data suggested that the MBP-AC3 eluted as an oligomer with molecular mass of ~700 kDa (Fig. 4.6B). The same was confirmed by sucrose gradient ultracentrifugation which clearly showed that the mass of MBP-AC3 complex was in line with the gel filtration experiment (Fig. 4.6C). In addition, another faint peak of MBP-AC3 was observed in the eleventh fraction that corresponds to ~100 kDa. Thus, sucrose gradient ultra
Figure 4.6. ToLCKeV AC3 forms an oligomer. (A) Western blotting of GST pull down assay by MBP-AC3 antibody. Fractions corresponding to 'input' represent the protein composition of the total reaction mix for protein-protein interactions. Fractions corresponding to 'bound' represent the proteins that are interacting with GST-AC3 bound to GST sepharose. Presence of MBP-AC3 in the bound fraction indicates the formation of oligomer. (B) Gel filtration with Superdex-200 5/150 coloumn shows the elution of various proteins. MBP-AC3 elutes between the Dextran (2000kDa) and Thyroglobulin (669kDa) (C) [i] Protein distribution pattern for the MBP-AC3 after sucrose gradient ultra centrifugation was visualised by Comassie blue staining. MBP-AC3 forms faint peak at 11th fraction and a prominent peak at 32nd fraction as indicated by '*'. Arrows indicates the peak formation of molecular weight standard proteins: Aldolase (158kDa) at 17th fraction Ferritin (449kDa) at 26th fraction and Thyroglobulin (669kDa) at 30th fraction. [ii] MBP (43kDa) does not form an oligomer and peaks in the 5th fraction.
Results and Discussion

centrifugation suggested the formation of a dimer, albeit in very low proportion compared to the higher order oligomer.

Multitasking capability of viral proteins is often brought out by the ability of the protein to form oligomers. Multimerisation leads to the assembly of large multicomponent complexes with different activities dependent upon composition (Marianayagam et al., 2004). In case of geminiviral proteins, AC1 is known to form higher order oligomer in the range of 24 mer (MYMIV AC1) and hexamer (TYLCSV AC1). Both of these proteins are able to nick, ligate and bind DNA as monomers. However, helicase activity is observed only when they could form oligomers (Choudhury et al., 2006; Clerot & Bernardi, 2006). In a similar way, monomeric AC2 was able to act as RNAi suppressor silencing whereas it could transactivate coat protein promoter only in an oligomeric state (Yang et al., 2007). Here, our in vitro results suggested that AC3 exists in two states: a higher order oligomer (complex of 12-14 molecules) and a dimer in very low proportion (2-3% of higher order oligomer). Existence of two types of oligomers in case of AC3 indicates that it is likely to have more than one function as observed in case of AC1 (AC1 has site specific DNA nicking and ligation activity, helicase activity) and AC2 (AC2 functions as a transcription activator and silencing suppressor). Earlier observations that the requirement of oligomeric form of TYLCV AC3 for replication enhancement as well as PCNA interaction and the ability of monomeric TGMV AC3 to interact with its cognate AC1 supports the existence of multimeric forms of AC3 (Settlage et al., 2001; Settlage et al., 2005).

4.4 AC3 Interacting Host Factors

Existence of oligomeric AC3 provides multiple sites for protein binding. Hence, we hypothesised that AC3 might be involved in multi protein interactions within the host cell. In order to find the AC3 interacting host proteins, we have performed an exhaustive search by phage display analysis. Random peptide displaying M13 phage library with a 12 amino
acid peptide on the coat protein of M13 phage was utilised in the phage display. MBP-AC3/MBP protein was coated on the wells of the 96 well plate and phage library was used for panning the immobilised proteins. Phages that are strongly interacting with the protein were selected by stringent washes and amplified. The procedure was repeated for three pannings. DNA was isolated and sequenced from individual plaques obtained from the third panning. Sequences that were common with MBP-AC3 and MBP were excluded from further analysis as they represent the peptides interacting with MBP and not with AC3. The remaining unique peptide sequences were subjected to BLAST (blastp programme) analysis for the identification of proteins that contain peptide sequences homologous to phage display identified peptides. BLAST analysis was performed against the *A. thaliana* non-redundant protein database with the default parameters optimised for the small peptide sequences (Fig. 4.7A).

Proteins from various metabolisms were observed to contain the homologous peptide sequences from phage display (Fig. 4.7B). The list of proteins include various transcription factors, DNA polymerases, RNA polymerases, RNAi components, helicases involved in DNA repair and/or recombination, various cell cycle regulatory proteins and signalling proteins (Figs. 4.8, 4.9). In some cases multiple phage display peptides were found to be homologous to the peptides of the same protein at various positions indicating that the hits are highly significant (RPA1, RecQ, DCL2, AGO7 etc.). Presence of known AC1 and AC3 interacting proteins like pRBR, NAC domain containing proteins and GRIK1 protein indicated that the other proteins identified as putative interacting partners in our study are reliable (Selth et al., 2005; Settlage et al., 2001; Shen & Hanley-Bowdoin, 2006; Xie et al., 1999; Xie et al., 1995).

Oligomerisation of a protein provides multiple sites for ligand binding when two or more ligand binding sites overlap each other. Studies of AC3 interacting host proteins indicated that the PCNA and AC1 interacting sites in AC3 were overlapping (Settlage et al., 2005). In such a case, the
Figure 4.7. Phage display analysis of AC3 protein. (A) Representative peptides that are interacting with AC3 are shown in a table. DNA sequence of the M13 phage was translated with unique M13 phage genetic code. Peptides that were common in MBP and MBP-AC3 were excluded from the further analysis as they represent the peptides interacting with MBP. Twelve amino acid peptides thus obtained were searched for homology against A.thaliana protein database using 'blastp' programme with parameters adjusted for small peptide sequences. (B) Proteins that contain atleast five contiguous amino acids identical to the 12mer peptide obtained from phage display are listed. The list includes various proteins from signaling, cell cycle, transcription activators, RNA and DNA polymerases and RNA silencing machinery.
<table>
<thead>
<tr>
<th>Replication protein A1 (RPA1)</th>
<th>211 WWKRFYP</th>
<th>219</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geminivirus Rep interacting kinase 1 (GRIK1)</td>
<td>164 NKMK</td>
<td>167</td>
</tr>
<tr>
<td>Retinoblastoma like protein (pRBR)</td>
<td>317 HKIY</td>
<td>320</td>
</tr>
<tr>
<td>Anti silencing function 1b (ASF 1b)</td>
<td>43 IYVG5</td>
<td>47</td>
</tr>
<tr>
<td>RecQ Helicase</td>
<td>678 FHKSPLLILAARDSH</td>
<td>692</td>
</tr>
<tr>
<td>Werner Helicase</td>
<td>801 LLHHK</td>
<td>806</td>
</tr>
<tr>
<td>RAD1</td>
<td>436 ITTNP</td>
<td>440</td>
</tr>
<tr>
<td>RAD5</td>
<td>42 NIFDTP</td>
<td>48</td>
</tr>
<tr>
<td>RAD4</td>
<td>586 SHEY</td>
<td>580</td>
</tr>
<tr>
<td>RAD23-3</td>
<td>119 APRPTPA</td>
<td>129</td>
</tr>
<tr>
<td>RAD60</td>
<td>306 KEWRT+FYQRR</td>
<td>315</td>
</tr>
<tr>
<td>H3-K9 Methyltransferase</td>
<td>87 PPLRS</td>
<td>91</td>
</tr>
<tr>
<td>Histone Methyltransferase</td>
<td>340 KTPVRR</td>
<td>345</td>
</tr>
<tr>
<td>Histone acetyl transferase</td>
<td>562 QWPS</td>
<td>566</td>
</tr>
<tr>
<td>Decreased DNA methylation (DDM1), SNF2</td>
<td>986 AMYY</td>
<td>989</td>
</tr>
<tr>
<td>Variant in methylation 2 (VIM2), VIM4, VIM5</td>
<td>294 KHMP</td>
<td>297</td>
</tr>
<tr>
<td>Increase in Bonsai methylation 1</td>
<td>448 PRPLPNVP</td>
<td>455</td>
</tr>
<tr>
<td>Maintenance of methylation</td>
<td>556 HVQH</td>
<td>559</td>
</tr>
<tr>
<td>Decreased methylation to DNA (MET1)</td>
<td>394 P+PSG</td>
<td>399</td>
</tr>
<tr>
<td>MET3</td>
<td>1588 PSGS</td>
<td>1591</td>
</tr>
</tbody>
</table>

**Figure 4.8. Selected list of proteins identified in phage display.** (A) Table lists various proteins that have helicase activity or play an important role in replication. GRIK1 and pRBR are known to interact with TGMV Rep. (B) Table lists various proteins and enzymes that are involved in modification of histones. Numbers flanking the amino acid residues in the table represents the coordinates of the amino acids in the respective proteins showing identity with peptides identified in phage display. Residues in bold are identical (or similar in few cases) to the residues in phage display identified peptides.
**Figure 4.9. Selected list of proteins identified in phage display.** (A) Table lists various proteins involved in RNAi. (B) List of various polymerases that might interact with AC3 through the residues identified in phage display. Residues in bold are the putative amino acids that might be at the interface of interaction with AC3. Numbers flanking the residues are the co-ordinates of the amino acids in the proteins that are identical (or similar in few cases) to the peptides identified in phage display.
interaction was mutually exclusive when AC3 exists as a monomer. However, oligomer formation of AC3 provides multiple sites for protein binding. This would enable AC3 to bridge interaction with two or more proteins at a time. Thus, it is possible that AC3 in co-operation with host interacting proteins will help the sustenance of the viral infection. Identification of various putative AC3 interacting proteins is indicative of the role of AC3 in various cellular pathways that would enhance the viral load in the host cell.

4.5 Expression and Purification of AC1

4.5.1 Expression and Purification of His-GST-AC1
AC1 from two closely related begomovirus isolates (Table 3.2) causing tomato leaf curl disease was amplified with degenerate oligonucleotides, *viz.*, ToLCV_AC1_Fwd and ToLCV_AC1_Rev (Table 3.3) and ligated into pJET1/blunt cloning vector (Fig. 4.10A). Ncol/XhoI digested AC1 fragment from pJET1/blunt vector were ligated into Ncol/XhoI digested pETM30 expression vector (Fig. 4.10B). ToLCKeV His-GST-AC1 was expressed by inducing the *E.coli* [BL21(DE3)] cells with IPTG (Fig. 4.11A). Most of the expressed protein was observed in the pellet fraction of the sonicated bacterial cell lysate (Fig. 4.11B). Purification of the ToLCKeV His-GST-AC3 protein from the supernatant of the sonicated cell lysate with Ni-NTA or glutathione sepharose indicated that the protein binds poorly to the resin (Fig. 4.11C, 4.11D). Hence, we tried purifying AC1 fused to MBP.

4.5.2 Expression and Purification of MBP-AC1
BamHI/XhoI digested AC1 from pJET1/blunt vector was ligated into BamHI/SalI digested pMal-c2X expression vector. ToLCKeV MBP-AC1 was expressed by inducing the *E.coli* [BL21(DE3)] cells with IPTG (Fig. 4.12A). ToLCKeV MBP-AC1 was highly soluble and most of the expressed protein was observed in the supernatant fraction of the sonicated bacterial cell lysate after centrifugation (Fig. 4.12A). ToLCKeV MBP-AC1
Figure 4.10. Cloning of AC1 into pJET1 and pETM30. (A) AC1 coding regions from ToLCGV and ToLCKeV was PCR amplified, blunt ended and cloned into pJET1/blunt. (B) Cloning into pJET1/blunt was confirmed by digestion with Ncol and Xhol. (C) Schematic map of pETM30, an expression vector used to express AC1. The schematic below vector shows the orientation of three protein tags available in the vector for easy purification of the recombinant protein. (D) Cloning of AC1 in pETM30 was confirmed by digestion with Ncol and Xhol.
Figure 4.11. Expression and purification of His-GST-AC1. (A) Expression of ToLCGV (ND1) and ToLCKeV (K2) AC1 proteins in pETM 30. (B) Most of the induced protein was observed in the pellet fraction after sonication with a small amount of the protein in supernatant. (C) Purification of K2-His-GST-AC1 by Ni-NTA. Binding of the fusion protein was found to be very weak as most of the protein was observed in the flow through (FT). (D) K2-His-GST-AC1 was purified by glutathione resin. Fusion protein bound poorly to the glutathione resin.
Figure 4.12. Expression and purification of MBP-AC1. (A) Expression of K2-AC1 as a fusion protein with Maltose binding protein. A clear thick band is visible in the induced sample (I). Significant amount of soluble K2-MBP-AC1 was visible in the supernatant fraction (S) of the induced bacterial cell lysate. (B) Purification of K2-MBP-AC1 by amylose resin. A series of truncated products of MBP-AC1 including MBP were observed in the 10 mM maltose eluted samples. Arrow shows the position of full length MBP-AC1.
Results and Discussion

from the supernatant of the sonicated cell lysate was purified using
amylose resin. Purification pattern indicated that the protein was highly
expressed in the soluble fraction and most of the protein got truncated in
the AC1 region. Elution fractions contained full MBP-AC1 as well as
truncated MBP-AC1 (Fig. 4.12B). MBP fusion containing AC1 and
truncated AC1 protein elutions were pooled and subjected to ion
exchange chromatography. MBP AC1 was thus purified to 80%
homogeneity (data not shown).

4.6 AC1 and AC3 Interaction Studies

AC1 is the most essential protein for viral replication in host plant. AC1
was also shown to induce the host cellular replication machinery. TGMV
AC3 was shown to interact with AC1 and enhance replication in
protoplasts by an unknown process. Here we tried to address the
interaction between ToLCKeV AC3 and AC1 in vitro and the effect of this
interaction on the biochemical activity of AC1.

GST-AC3 was incubated with MBP-AC1/MBP in the presence of
glutathione resin. The resin was subsequently subjected to stringent
washes to remove the non-specific interaction with GST-AC3. The bound
fractions with GST-AC3 contained only MBP-AC1 indicating that MBP-
AC1 interacted with GST-AC3. Presence of MBP-AC1 and not
MBP in the
bound fraction indicated that the interaction observed with MBP-AC1
was specific between AC1 and AC3 and not between MBP and AC3 (Fig.
4.13).

In order to check the AC1 interaction with AC3 ex vivo, we have utilised
yeast two-hybrid system. AC1 was digested with BamHI/XhoI from
pJET1/blunt vector and cloned into BamHI/Sall digested pGAD-C1.
BamHI/XhoI digested AC3 was ligated into BamHI/Sall pGBD-C1. AC1
and AC3 cloned plasmids were co-transformed into yeast strain AH109
and plated onto selection medium (minimal medium lacking leucine,
Figure 4.13. ToLCKeV AC3 and AC1 forms an oligomer. Coomassie staining of GST-AC3 and MBP-AC1 showing their interaction. Input fractions represent the total protein composition in the binding reaction mix. Bound fractions represent the proteins that were interacting with GST-AC3 bound to the GST sepharose. Presence of MBP-AC1 in the bound fraction indicates its interaction with AC3. Resin was thoroughly washed to remove the non-specific interactions among proteins.
histidine and tryptophan amino acids). Five days after transformation, colonies of yeast appeared to be very small indicating that growth of the yeast was severely hampered. Delayed growth phenotype is an indicator of the toxicity of the protein expressed in yeast. A similar observation of delayed growth was observed with Rep proteins from TYLCSV and TLCV suggesting that few AC1 proteins from geminiviruses are often toxic to yeast cells (Castillo et al., 2007; Selth et al., 2004).

We checked if the interaction between AC1 and AC3 has any affect on the ATPase activity of AC1. ATPase activity analysis indicated that AC3 enhances the activity of AC1 by 50% at 0.02 pM concentration and to a maximum of 80% at 0.2 pM concentration in the reaction mixture. Any further increase in the AC3 concentration beyond 0.2 pM in the reaction mixture dampened the ATPase activity of AC1 (Fig. 4.14). The concentration of AC1 was carefully selected and kept constant so that the ATPase activity of AC1 does not get saturated in the reaction mix. Thus, we were able to see the modulation in the ATPase activity. It may be noted that AC3 itself does not possess any ATPase activity (lanes 11-13, Fig. 4.14A). Our observation that AC3 enhances ATPase activity is significant as such activity is central to various biochemical properties viz., helicase activity, of Rep.

Interaction between AC1 and AC3 assumes significance in the view of earlier reports suggesting the interaction of AC3 with PCNA (the sliding clamp that binds to the DNA polymerase) and PCNA with AC1 (Castillo et al., 2003). Since, AC1 initiates replication by nicking at the viral origin of replication, one outcome of AC1/AC3 interaction that could be envisaged is AC3 might bring PCNA to the site of replication. Since, AC1 also interacts with PCNA the other possibility is that AC3 might increase the affinity of such interaction. In the view of this web of interactions, the influence of AC3 on AC1 assumes significance and needs to be further explored. WDV AC1 interaction with RFC-1, the largest subunit of PCNA clamp loader adds more importance to these interactions (Luque et al., 2002). Another important observation was made with respect to the AC1
Figure 4.14. AC3 modulates the ATPase activity of Rep: (A) Autoradiograph showing the ATPase activity of Rep in the absence and presence of AC3. AC3 increases the ATPase activity of Rep at low concentration (0.02-0.2 pm) by 50-80%. Composition of the proteins in the reaction mix is shown at the top of each lane in the autoradigraph. ATPase reaction was carried with a uniform concentration of Rep protein and varying concentrations of MBP-AC3 as denoted in the figure. MBP was used as a negative control. (B) Graphical representation of ATPase activity of Rep in the presence of MBP-AC3. ATPase activity in the reaction mix containing the Rep protein alone was arbitrarily assigned a value of 100% and activity in other lanes was calculated accordingly. Graph was plotted for the lanes 1-8 that correspond to the lanes of autoradiograph.
Results and Discussion

binding to DNA at the origin of replication. Loss of DNA binding capacity by TGMV AC1 to the 5’ iteron of AC1 binding motif in the common region was restored in the presence of AC3 suggesting that AC3 might enhance DNA binding activity of AC1 (Fontes et al., 1994).

4.7 Role of AC3 on Viral Replication

4.7.1 Role of AC3 on Viral Replication ex vivo

We have constructed the ex vivo replicon in yeast based on the fact that replication of ARS deficient yeast plasmid (YCPo-) can be restored when viral origin of replication is cloned into the plasmid along with the viral trans acting factors (protein coding ORFs that are required for viral replication) supplied in cis or trans (Fig. 4.15).

Viral sequence spanning the origin of replication (CR), AC1 and AC3 (referred as CR-AC3 here onwards) was amplified with oligonucleotides CR_K2_Fwd_HindIII, CR_AC3_K2_Rev_HindIII (Table 3.3) and digested with HindIII. Yeast replicon vector (YCP-CR-AC3) was constructed by cloning HindIII digested CR-AC3 fragment into HindIII digested YCPo-. To investigate the function of AC3 by reverse genetics approach, start codon of AC3 was mutated by site directed mutagenesis using the oligonucleotides M_K2AC3_Fwd and M_K2AC3_Rev (Table 3.3) that resulted in AC3 mutated yeast replicon (YCP-CR-AC3M). Start codon mutation converted methionine in AC3 to threonine without any change in the amino acid of overlapping AC2 protein sequence (Fig. 4.16). Efficiency of replication was analysed by transforming yeast cells with YCP-CR-AC3 or YCP-CR-AC3M along with controls YCP50 and YCPo-. Yeast transformed with YCP-CR-AC3 and YCP-CR-AC3M exhibited much delayed growth phenotype (0.25–0.5 mm diameter size colonies 5 days after transformation) indicating that the expression of viral ORFs are possibly toxic to cell growth. However, the growth of yeast transformed with control plasmids was normal (3–4 mm diameter size colonies 5 days after transformation). Similar delayed and reduced growth was observed
Figure 4.15: **Viral replicon construction in yeast.** Schematic diagram representing the construction of viral replicon in yeast. YCp50 is a binary plasmid that is capable of replication in bacteria and yeast. ARS and CEN4 sequences of the plasmid confer the ability to replicate in yeast. Removal of ARS fragment makes the plasmid unable to replicate in yeast (YCpO). CR-AC3 fragment of the begomovirus contains the cis-acting sequences (origin of replication) and trans-acting viral genes (AC1, AC3) required for viral replication. Cloning of CR-AC3 of MYMIV was reported to confer the ability to replicate YCpO' in yeast.
Figure 4.16. Mutation in ToLCKeV AC3 - Strategy I. (A) Schematic diagram of the CR-AC3 region of the replicon construct. The dashed arrow shows the mutation of start codon in AC3. (B) Sequence alignment of the mutated AC3 with the wild type AC3. The mutated base is shown against the white background at base number 2 in the start codon. Mutation was confirmed by sequencing.
in our experiments analysing the AC1/AC3 oligomerisation in yeast which indicated AC1 to be toxic to yeast cells.

4.7.2 Role of AC3 on Viral Replication in planta

We further proceeded to analyse the role of AC3 on viral replication in planta. The plant viral replicon was constructed based on the following facts: geminiviruses replicate by rolling circle replication; geminiviral Rep initiates rolling circle replication by nicking at the viral origin and ligates the viral strand at the viral origin into a covalently closed circular DNA upon sense strand synthesis. Supplying two viral origins of replication in the same orientation along with AC1 and AC3 generates an episome which is expected to replicate autonomously in planta (Fig. 4.17). Viral replicon (pCK2 replicon) was constructed as follows: ToLCKeV viral origin of replication (CR), was amplified by primers CR_K2_Fwd_EcoRI and CR_K2_Rev_EcoRI (Table 3.3) and digested with EcoRI. CR was cloned into EcoRI digested binary vector pCAMBIA1391Z (Fig. 4.18A). Direction of the CR was confirmed by digestion with NdeI which has multiple restriction sites in vector and one restriction site in CR (Fig. 4.18B, 4.18C). HindIII digested CR-AC3 fragment was then cloned into HindIII site of CR containing binary vector. Orientation of CR-AC3 relative to CR was confirmed with NdeI digestion (Fig. 4.19). To assess the role of AC3, HindIII digested CR-AC3 (AC3 mutated at the start codon) was also cloned in a similar way. Viral wild-type replicon (pCK2 replicon), and AC3 mutated viral replicon (pCK2M replicon) were transformed separately into agrobacterium and the agrobacterium cultures were infiltrated into leaves of separate young tobacco plants.

Formation pCK2 and pCK2M episomes and relative replication of the episomes was analysed by semiquantitative PCR using primers M_K2AC3_Fwd and ToLCKeV_AC1_Rev119 (Table 3.3) which amplifies a 1.4 kb fragment (Fig. 4.17) or using PolyA Fwd and ToLCKeV_AC1_Rev38 (Table 3.3) which amplifies a 500 bp fragment that are specific to the episome formed by rolling circle replication. (Hanson et al., 1995; Lopez-Ochoa et al., 2006; Singh et al., 2007).
Figure 4.17. Viral replicon construction design. (A) Common region (CR) denotes the origin of replication of the begomoviruses containing cis-acting regions required for replication. Blue bars indicate the iterons where Rep protein binds to the DNA. Green bar indicates the loop region of hairpin where Rep protein nicks DNA to start the replication. (B) Complete replicon construct design that contains the region spanning from CR to AC3 (CR-AC3) and CR. Presence of CR on either end in the same orientation enable the completion of rolling circle replication. Rolling circle replication releases the episome that contains only one complete CR and region spanning from AC1 to AC3. Red arrows indicate the nicking site of Rep protein in hairpin loop in either CRs and the black line represents the region of the vector that forms episome. Episome formation can be checked by the amplification with the oligonucleotides indicated by blue arrows. Internal primers were designed to amplify the DNA only from the episome under standardized PCR conditions.
Figure 4.18. Cloning of ToLCKeV-[IN:KerII:05] CR into pCAMBIA1391Z. (A) 232 bp CR region was released from the vector upon digestion with EcoRI as indicated by red arrow on left. (B) Fragments released from the vector upon digesting with NdeI. Vector has three sites for NdeI and CR has one at the 5' end. (C) Orientation of CR was confirmed by the NdeI restriction digestion pattern shown in the figure. Position of 35S promoter and CR are shown as arcs on the circle. Blue arcs with numbers inside the circle indicate the size of fragments released upon restriction digestion with NdeI. Restriction sites of NdeI are labelled along with corresponding nucleotide number.
Figure 4.19. Construction of pCK2 (wild type) Replicon. (A) 1849 bp CR-AC3 region cloned into pCAMBIA1391Z was released from the vector upon digestion with HindIII as indicated by red arrow on left. (B) Fragments released from the vector upon digestion with NdeI. pCAMBIA1391Z vector has three restriction sites for NdeI and viral DNA CR-AC3 has two restriction sites and one in another CR at the 5' end. (C) Orientation of CR-AC3 was confirmed as shown in the picture. Position of CR-AC3, 35S promoter and CR are shown as arcs on the circle. Blue arcs with numbers inside the circle indicates the size of fragments released upon digestion with NdeI. Restriction sites of NdeI are labelled along with corresponding nucleotide number.
Results and Discussion

Time course analysis of the pCK2 and pCK2\textsuperscript{M} episome formation in tobacco plant leaves did not show any significant down-regulation in replication upon AC3 mutation (Fig. 4.20). We questioned if there was any reversion that restored the AC3 start codon. However, DNA sequencing of the episome confirmed the presence of the mutation indicating that there was no reversion of the mutation (Data not shown). The non-significant alteration in the replication efficiency might be due to various reasons: one being the minimal role of ToLCKeV AC3 in viral replication. Another possibility could be the role of AC3 in viral replication at a later stage requiring analysis of samples beyond 10 dpi. The other reason might the permissiveness of the tobacco plant for the viral replication that masked the role of AC3. Such a conjecture gets support from an observation made in case of BCTV (California strain). When BCTV C3 was mutated, BCTV genome replicated to almost wild-type levels in tobacco plant whereas the replication was reduced in natural host plant sugar beet (Stanley et al., 1992).

To exclude the possibility of permissiveness in tobacco, we have performed the agroinoculation experiment in the natural host tomato with an extra AC3 mutation. Mutation was done in AC3 with oligonucleotides M21\textsubscript{K2AC3} Fwd and M21\textsubscript{K2AC3} Rev (Table 3.3) with the pGEM-T Easy CR-AC3\textsuperscript{M} (AC3 mutated at start codon) as template. In this new mutation (CR-AC3\textsuperscript{M21}), AC3 has consecutive stop codons at amino acid positions 20 and 21 in addition to mutation at start codon (Fig. 4.21). Since, AC2 and AC3 ORFs overlap each other, we checked if these mutations have any effect on the AC2 protein sequence. Only the mutation at 21\textsuperscript{st} amino acid of AC3 confers a change in the overlapping AC2 (Glycine changed to Valine at 70\textsuperscript{th} amino acid of AC2). Since 70\textsuperscript{th} amino acid of AC2 does not lie in any of the domains (C'-terminal nuclear localisation signal, Zn finger motif and N'-terminus acidic transcription activation domain) required for silencing activity or transcription activation activity, we argued that there will be not be any effect on function of AC2. HindIII digested CR-AC3\textsuperscript{M21} was then cloned into binary vector to generate pCK2\textsuperscript{M21} replicon.
Figure 4.20. Semi-quantitative amplification of episomal DNA from wild-type and AC3 mutated replicon. Tobacco plant leaves were infiltrated with wild-type replicon (pCK2) and AC3 mutated replicon (pCK2M) separately. DNA from the infiltrated leaves was isolated at 5 and 10 days post infiltration. Equal quantities of DNA was then used to amplify episome or actin. PCR conditions were specific to amplify a part of replicon from the episome only. Amount of episome formed was almost equal in wild-type and mutated AC3 replicons.
Figure 4.21. Mutation in ToLCKeV AC3 - Strategy II. (A) Schematic diagram of the CR-AC3 region of the replicon construct. The dashed arrows shows the regions of mutation in AC3. (B) Nucleotide sequence alignment of the mutated AC3 with the wild type AC3. Mutated bases are shown against the white background at base no. 2, 62 and 64 in AC3. Two of these base changes cause silent mutations in AC2 (details are explained in the corresponding text). Mutations were confirmed by sequencing of DNA. (C) Translated protein sequence alignment of the AC3 from wild type and mutated replicons. The mutated AC3 has two stop codons at 21st and 22nd amino acid positions in addition to the start codon mutation.
Examination of the relative replication levels of the episome between pCK2 replicon and pCK2M21 replicon was carried out at various time intervals till 15 dpi (Fig. 4.22). Isolated plant genomic DNA was treated with DpnI to remove the episome replicated in agrobacterium, if any. Upon analysis, we noticed change in the relative levels of replication between the pCK2 replicon and pCK2M21 replicon. Within the first five days, there was no difference in the levels of replication. However, the relative change in replication was more pronounced at 10 dpi when the replication of wild-type replicon (pCK2) was 3-4 fold more than AC3 mutant replicon (pCK2M21). The difference in the relative level of replication diminished to 1.5-2 fold at 15 dpi.

Our observation suggested that AC3 enhances replication but is not essential for replication. This is in line with earlier observations. Role of AC3 was evident at 10-15 dpi. However, our results differed from published reports on the level of AC3 influence on viral replication. This might be due to various differences in the experimental design or the assay system. Earlier work on AC3’s role in replication was analysed by mutating AC3 after the AC2 stop codon. This resulted in truncated AC3 with 80 amino acids in case of TGMV AC3 and 110 amino acids in other viruses (Etessami et al., 1991; Hormuzdi & Bisaro, 1995; Morris et al., 1991; Stanley et al., 1992; Sung & Coutts, 1995; Sunter et al., 1990). In these studies it was possible that the truncated AC3 was not completely functional. It was also possible that truncated AC3 interfered the cellular pathways. With its N'-terminus and oligomeric middle region being normal, AC3 could titrate various proteins that interact with AC1 (like PCNA, pRBR). In such a case the signal received by N'-terminus of AC3 gets abruptly terminated being unable to relay the signal through a functional C'-terminus, thereby affecting replication. Our mutation strategy assured that AC3 is not expressed since we have mutated the start codon (In case it overcomes the start codon mutation, it encounters two consecutive stop codons at 20th and 21st amino acid positions that guarantees its termination). It is possible that in complete absence of AC3, another alternate pathway or protein might rescue the viral
Figure 4.22. Semi-quantitative amplification of episomal DNA from wild-type and AC3 mutated replicon. Tomato plant leaves were infiltrated with wild-type replicon (pCK2) and AC3 mutated replicon (pCK2M21) separately. DNA from the infiltrated leaves was isolated at 5, 10 and 15 days post infiltration and subjected to DpnI restriction digestion. Equal quantities of DNA was then used to amplify episome or actin. PCR conditions were specific to amplify a part of replicon from the episome only. Difference in the amount of replicon in wild-type and mutant was prominent in the 10 dpi sample (3-4 fold difference). By 15 dpi, the difference in the amount of episome was only 1.5-2 fold.
replication. This hypothesis gets considerable support from an experiment performed with transgenic plants (Hayes & Buck, 1989). In their work authors raised various transgenic plants expressing DNA A ORFs and tandem repeats of DNA B genome. Various DNA A ORFs expressing plants were crossed with tandem repeat DNA B (2DNA B) containing plants. When DNA from two such plants: AC1×2DNA B and AC1AC3×2DNA B was analysed, the difference in the replication of DNA B in the presence and absence of AC3 was observed to be less than 1.5 fold indicating that the replication was sustainable without AC3 in planta. Delay and amelioration of symptoms and reduced systemic movement of the virus in case of AC3 mutations observed in case of experiments in planta by agroinoculation experiments suggest that AC3 has a more important role in systemic spread. Role in replication, if any is an indirect effect rather than its direct involvement. Having a multitude of interacting partners that were discovered and are being discovered; large multimer forming ability that enables interaction with multiple partners indicate that AC3 is an important protein with multifunctional capability which needs further examination of its involvement in various cellular process of the virus in addition to replication.

4.8 Role of AC3 in RNAi pathway

The phage display data show that various ToLCKeV AC3 interacting peptides are homologous to the protein sequences (Figs. 4.8B, 4.9A) of RNAi protein machinery. Interestingly, we found that few of these proteins (MOM1, MET1, DCL1, DCL2, AGO1, AGO2, AGO7, and HEN4) have multiple peptide sequences that are homologous to various peptides identified from phage display. We believe that multiple hits for a single protein indicate that the interaction between the AC3 and the proteins having the homologous sequence of phage display interacting peptides might be reliable (although not verified individually for any of these proteins identified here). Hence we investigated if AC3 could influence the RNAi pathway(s) upon viral infection. One way to examine the role of AC3
in RNAi is to study the silencing of an endogenous gene using the virus induced gene silencing mechanism (VIGS) with wildtype and mutant AC3.

Geminiviruses are inducers of RNA silencing and are also subjected to RNA silencing (Vanitharani et al., 2003). The first evidence of VIGS by geminivirus was observed when magnesium chelatase gene cloned as a part of replicating TGMV episome silenced the endogenous magnesium chelatase in tobacco (Kjemtrup et al., 1998). Later studies done with PCNA cloned into TGMV DNA A indicated that VIGS system can be used to silence homologous genes in the meristematic tissues where geminiviruses are generally not detectable (Peele et al., 2001). Recent studies carried out in our lab established that VIGS can be achieved successfully with the minimal region spanning CR-AC3 of ToLCV (unpublished results).

With the view that CR-AC3 is the minimal region required for successfull VIGS, we have utilised our pCK2 and pCK2M21 replicon constructs to silence the endogenous gene PCNA. A 300bp fragment of PCNA from tomato cDNA was amplified with oligonucleotides PCNA Fwd and PCNA Rev, digested with BamHI and cloned into the BamHI digested 35S promoter of the replicon. Agrobacterium containing the PCNA cloned replicons pCK2-PCNA and pCK2M21-PCNA (pCK2 with PCNA and pCK2M21 with PCNA) and control vector pC-PCNA (PCNA under 35S promoter without the viral sequences coding for viral ORFs in the same vector background) were infiltrated into leaves of tomato plants at 4 leaf stage. Growth of the plants was found to be normal and indistinguishable till 20 dpi. We noticed little retardation in the growth of the infiltrated plant at 30 dpi which was prominent by 45 dpi whereas the growth of the plant infiltrated with pCK2M21-PCNA and control was normal and similar to the plants without any agroinfiltration (Fig. 4.23A). Retardation in growth of pCK2-PCNA infiltrated plants was relieved by 60 dpi which was evident from the plant height. Growth retardation was accompanied with reduced flowering, internodal distance and absence of fruits at 45 dpi whereas formation of fruits and flowers were indistinguishable in plants infiltrated
with pCK2M21-PCNA, pC-PCNA and plants without any infiltration (Fig. 4.23B, 4.23C, 4.23D).

PCNA gene expressed in meristematic tissues that rapidly divide and is absent in the mature leaves (Kelman, 1997). So, silencing of endogenous PCNA can be easily visualised by the lowered growth of the meristematic tissues that ultimately results in retarded growth of the plant. In our case plant growth was severely retarded which was evident from the reduced plant height, flowering and absence of fruits. Another advantage of our VIGS construct is the absence of movement protein without which the virus cannot move from one plant cell to other plant cell. Thus, our VIGS vector is not able to induce the disease symptom which actually is the manifestation of the viral movement within the plant cells. This particular character enables us to differentiate if the observed deformities in the plant growth are due to tomato leaf curl disease or silencing of PCNA.

Growth retardation observed in our experiments in the presence of AC3 indicates that AC3 could have strong influence on virus induced gene silencing of endogenous gene PCNA in this experiment. However, it is difficult to ascertain the role of AC3 in RNAi with such experiment in isolation. Further work to check levels of siRNA, mRNA and protein levels of PCNA in the apical leaves needs to be carried out to establish the role of AC3 in RNAi.
Figure 4.23. Role of ToLCKeV AC3 on gene silencing. (A) High level transcription of a part of PCNA gene lead to the reduced growth of the plant. Retardation is observed in the growth of the plant agroinoculated with the wild type VIGS vector (wild type AC3) along with PCNA fragment under 35S promoter. Growth retardation is evident in this experiment by shortened height and decreased internodal distance between the stems of the tomato plant (plant on the left) and the plant agroinoculated with AC3 mutated VIGS vector (middle), control vector without any geminiviral DNA (right) and plant with out any agroinoculation (not shown). (B) Growth retardation was coupled with reduced flowering (circle) and no fruits. (C) Normal flowering (circles) and developing fruits (arrow) were observed in plant agroinoculated with AC3 mutated VIGS vector. (D) Leaf morphology was altered in the plants agroinoculated with wild type VIGS vector.