Mechanism of Rolling Circle Replication in Geminivirus: Role of Replication Enhancer (REn/AC3/C3)

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by

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Certificate

This is to certify that the research work embodied in this thesis entitled **'Mechanism of Rolling Circle Replication in Geminivirus: Role of Replication Enhancer (REn/AC3/C3)'** has been carried out in the Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi. This work is original and no part of this thesis has been submitted for the award of any other degree or diploma to any other university.

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Declaration

I hereby declare that the research work embodied in this thesis entitled '**Mechanism of Rolling Circle Replication in Geminivirus: Role of Replication Enhancer (REn/AC3/C3)**' has been carried out by me under the supervision of Dr. Nirupam Roy Choudhury in Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi.

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Abbreviations

%	Percent
°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
β-ME	Beta-mercaptoethanol
6X-His	hexa histidine tag
ADK	Adenosine kinase
ARS	Autonomous replicating sequence
ATPase	Adenosine triphosphatase
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
C'-terminal	Carboxy-terminal
$CaCl_2$	Calcium choloride
cDNA	Complementary DNA
CEN	Centromere
CH_3COO^-	Acetate ions
CR	Common region
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
g	Gram
HCOOH	Formic acid
hr(s)	Hour(s)
IPTG	Isopropyl β-D-thio-galactopyranoside
K^+	Potassium ions
K_2HPO_4	Dipotassium phosphate
kb	Kilo base pairs
kDa	Kilo dalton
LB	Luria-Bertani medium
LiAc	Lithium acetate
M	Molar

MBP	Maltose binding protein
MCS	Multiple cloning site
$MgSO_4$	Magnesium sulphate
min	Minutes
mM	Millimolar
mRNA	Messenger RNA
N'-terminal	Amino terminal
NaCl	Sodium chloride
NaI	Sodium iodide
NaOH	Sodium hydroxide
Ni-NTA	Nickel nitrilotriacetic acid
nM	Nanomolar
NP-40	Nonidet P-40
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polythelene glycol
Pi	Inorganic phosphate
pmole	Pico mole
PMSF	Phenylmethylsulfonyl fluoride
PTGS	Post transcriptional gene silencing
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
ssDNA	Single stranded DNA
TE	Tris-EDTA
TEMED	N, N, N', N'-Tetramethylethylenediamine
TLC	Thin layer chromatography
U	Unit
YCp	Yeast centromeric plasmid
YPD	Yeast-peptone media with glucose

Abbreviations of Virus Species

AbMV	*Abutilon mosaic virus*
BCTV	*Beet curly top virus*
ChiLCV	*Chilli leaf curl virus*
ICMV	*Indian cassava mosaic virus*
MSV	*Maize streak virus*
MYMIV	*Mungbean yellow mosaic India virus*
MYMV	*Mungbean yellow mosaic virus*
PaLCuV	*Papaya leaf curl virus*
TGMV	*Tomato golden mosaic virus*
TLCV	*Tomato leaf curl virus*
ToLCBV	*Tomato leaf curl Bangalore virus*
ToLCGV	*Tomato leaf curl Gujarat virus*
ToLCKev	*Tomato leaf curl Kerala virus*
TYDV	*Tomato yellow Dwarf virus*
TYLCSV	*Tomato yellow leaf curl Sardinia virus*
TYLCV	*Tomato yellow leaf curl virus*
WDV	*Wheat dwarf virus*

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Review of Literature

1.1 History and Importance of Geminiviruses

Geminiviruses are regarded as one of the most destructive pests for vegetable, field and fiber crops that are either economically significant or are staple food (Moffat, 1999). First description of geminivirus infected plant (*Eupatorium makinoi*) was found in a poem written by Japanese empress Koken (752 AD) (Saunders et al., 2003), which describes the beautiful mosaic pattern of the leaves of geminivirus infected Abutilon plants.

Geminiviruses have attracted special interest due to their impact on crops such as sugar beet, beans, cassava, cotton, cucurbits, maize, pepper, tomato, cereals, soybean and tomato (Gilbertson & Rojas, 2004; Legg & Fauquet, 2004; Morales & Anderson, 2001). African cassava

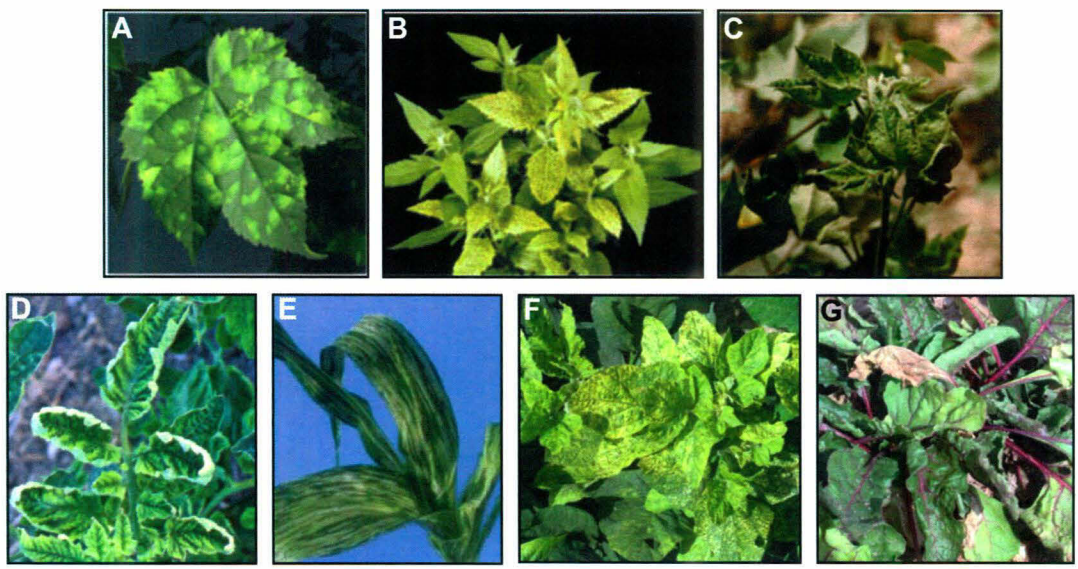


Figure 1.1. Symptoms of geminivirus infected plants. Geminivirus infections are prominently visible on the leaves of the infected plants. Geminiviruses are named on the basis of the characteristic symptom they cause in the infected plant. **(A)** Mosaic symptoms observed in abutilon plant leaves infected by *Abutilon mosaic virus* (adapted from Jeske 2009). **(B)** Mosaic pattern in eupatorium plant leaves infected by *Eupatorium yellow vein virus* (adapted from Saunders 2003). **(C)** Leaf curl symptoms caused by *Cotton leaf curl virus* in cotton plants (adapted from http://www.apsnet.org/pd/covers/1999/dap99cvr.htm). **(D)** Tomato leaves showing curly symptoms due to infection with *Tomato leaf curl virus* (adapted from http://www.ipm.ucdavis.edu/NEWS/IMAGES/tomatodisease.jpg). **(E)** *Maize streak virus* infection leads to formation of streaks on the maize leaves (adapted from http://www.ars.usda.gov/is/pr/2001/010420. mnsv.symptoms.jpg). **(F)** Yellow mosaic and curly symptoms observed in soybean infected with *Tomato leaf curl Karnataka virus* (adapted from http://www.bspp.org.uk/publications/new-disease-reports/ndr.php?id=013009#). **(G)** *Beet curly top virus* infection leads to curly leaves in sugar beet plants (adapted from http://www.coopext.colostate.edu/TRA/PLANTS/images/curlytop/beets.jpg).

mosaic disease is at pandemic levels on the African continent and is causing losses estimated as high as $2 billion annually (Legg & Fauquet, 2004). Maize streak disease is similarly a major constraint on corn production in Africa (Palmer & Rybicki, 1998). Beet curly top disease, which almost eliminated sugar beet production from the western United States in the early 1900s, continues to plague tomato and sugar beet production in this region (Soto & Gilbertson, 2003; Stenger & McMahon, 1997). Finally, tomato yellow leaf curl disease limits tomato production in many regions of the world (Salati et al., 2002) and bean golden mosaic disease is distributed throughout the Americas, where it can completely decimate common bean production (Morales & Anderson, 2001).

In basic research, geminiviruses have become useful models to analyse the molecular biology of plant gene regulation and cell-cell communication. The small size of DNA genome of geminiviruses possesses minimal coding capacity and replicates in the host cell nucleus with the help of host plant cellular machinery. Thus, studying geminiviral cellular processes also forms the best system in understanding the DNA replication, transcription, mRNA processing, protein expression and gene silencing in plants. A better knowledge of these cellular processes will help us in designing the antiviral strategies in plants.

1.2 Structure and Composition of Virion

Geminiviruses are non-enveloped viruses with incomplete icosahedral twinned virions. The virion dimensions of *Maize streak virus* are 38 nm long and 22 nm in diameter (Zhang et al., 2001). Purified virions appear as pair which was the reason for coining the name 'geminivirus' (Latin geminus = twin) and contain one circular ssDNA packaged in one geminate particle.

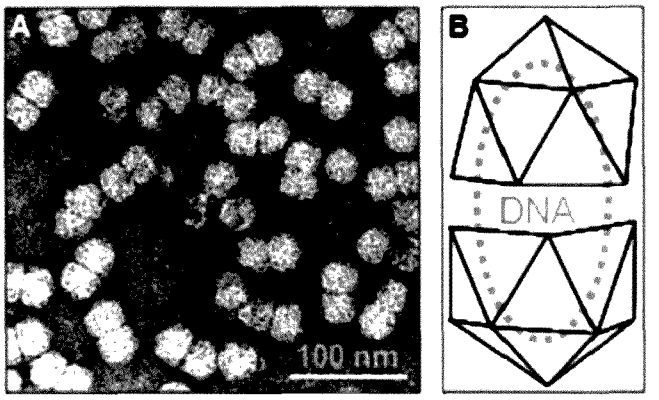


Figure 1.2. Structure and composition of virion. (A) Electron micrograph of *Tomato yellow leaf curl virus* showing the twinned structure of the virion (adapted from http://www.ncbi.nlm.nih.gov/ICTVdb/Images/Milne/gemini1.htm). **(B)** Picture shows the model of the how DNA fits into the virion. One virion can accommodate only one DNA ie., either DNA A or DNA B (adapted from Jeske, 2009).

1.3 Genome Organisation

Genome of geminiviruses comprises of a closed circular single stranded DNA in the range of 2.6 kb to 3.0 kb (Goodman, 1977). Open reading frames are oriented bidirectionally with an intergenic region (IR, common region CR, large intergenic region LIR) that contains promoters for the genes in either direction (Fontes *et al.*, 1994a; Frischmuth *et al.*, 1991; Hanley-Bowdoin *et al.*, 1990; Lazarowitz *et al.*, 1992; Morris-Krsinich *et al.*, 1985; Petty *et al.*, 1988; Shivaprasad *et al.*, 2005; Sunter & Bisaro, 1989; Sunter *et al.*, 1993; Townsend *et al.*, 1985). Intergenic region also contains a hairpin loop that is part of the viral origin of replication (Heyraud *et al.*, 1993a; Heyraud *et al.*, 1993b). The termination signals of the ORFs on either direction are located opposite to IR and are also shared between all the ORFs (Accotto *et al.*, 1989; Frischmuth *et al.*, 1991; Mullineaux *et al.*, 1993; Petty *et al.*, 1988; Shivaprasad *et al.*, 2005; Sunter & Bisaro, 1989; Sunter *et al.*, 1989; Townsend *et al.*, 1985). Genes responsible for regulation of replication and transcription are combined on the left side and those for packaging are on the right side with respect to the origin of the viral genome.

Some geminiviruses carry all the necessary genes required for full infection on one genome component and are termed monopartite where

as other geminiviruses have two genomes (DNA A, DNA B) and are termed bipartite (Hamilton et al., 1982). In bipartite viruses, DNA A carries all the ORFs required for replication of both the genomes and DNA B contains ORFs required for movement and infection. The viral genes were named according to their coding strand and genomic component. For example the ORF AC1 indicates a gene coded by DNA A component present on complementary strand. The genes were numbered as they were identified.

Monopartite geminivirus infected plants are often observed to contain another circular ssDNA genome with one ORF on the complementary strand which is completely dependent on the helper virus for replication. These genomes are approximately half the size of the helper virus genome and contain a similar hairpin loop elements that are found in the helper virus origin of replication. These are called satellite DNAs and are named DNA β or DNA 1. For some DNA β and helper virus combinations, the ORF βC1 is essential for symptom development.

1.4 Classification

The Geminiviridae family is classified into four subgroups with respect to insect vector, host range and genome organisation (Rybicki, 1994; Stanley *et al.*, 2005) as described in adjacent pages:

Subgroup I: Mastrevirus (type species: *Maize streak virus*, MSV) Mastreviruses are transmitted by leafhoppers, and have monopartite genomes. All members have narrow host ranges and, with the exception of TYDV (Tomato yellow dwarf virus) and BeYDV (Bean yellow dwarf virus), which infect dicotyledonous species, largely are limited to species in the Poaceae.

Subgroup II: Curtovirus (type species: *Beet curly top virus*, BCTV). Curtoviruses are transmitted by leafhoppers, and possess a monopartite

genome, which show fundamental differences in their organization from the mastrevirus genomes. The members have a very wide host ranges and primarily infect dicotyledonous species.

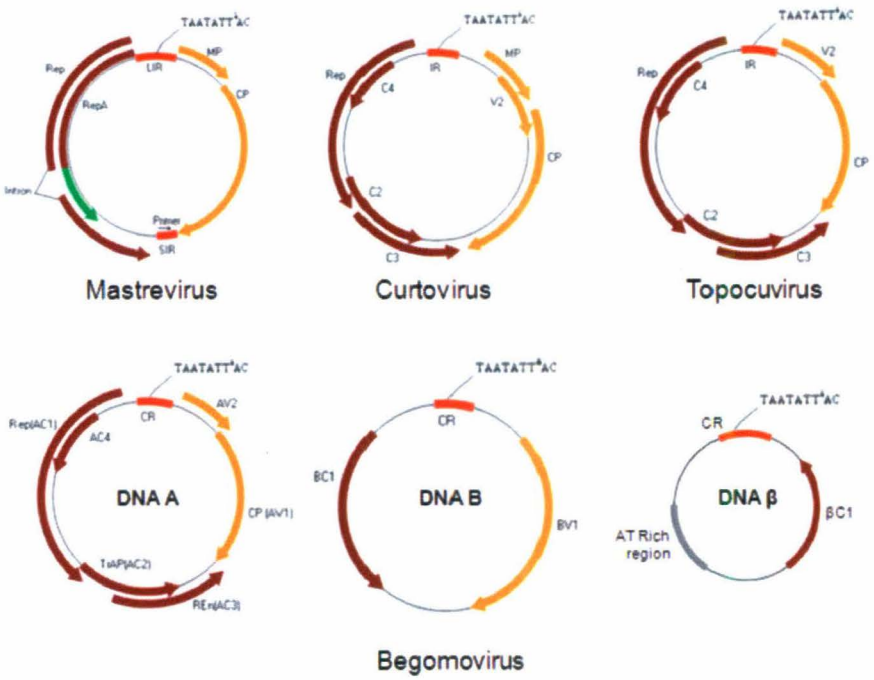


Figure 1.3. Genome organization of geminivirus subgroups. Maps of the type species of geminivirus subgroups *Maize streak virus* (Mastrevirus), *Beet curly top virus* (Curtovirus), *Tomato pseudo-curly top virus* (Topocuvirus). DNA A and DNA B are representative of *Bean golden mosaic virus* (Begomovirus). DNA β is the satellite DNA often associated with monopartite begomoviruses like *Tomato leaf curl virus*. Encoded proteins are: RepA/Rep/AC1/C1, replication initiation protein; TrAP/AC2/C2, transcription activator and/or called silencing suppressor; REn/AC3/C3, replication enhancer; MP, movement protein; CP, coat protein; BC1/MP, movement protein coded by DNA B, NSP/BV1, nuclear shuttle protein coded by DNA B, βC1 protein of DNA B performs the functions of DNA B proteins in monopartite begomoviruses.

Subgroup III: Topocuvirus (type species: *Tomato pseudo-curly top virus*, TPCTV) This is the least characterized genus that has been split off from the Curtovirus genus. It has similar genome organization to the curtoviruses but it is transmitted by the treehopper, *Mirculatis malleifera*.

Subgroup IV: Begomovirus (type species: *Bean golden mosaic virus*, BGMV) This is the largest geminivirus family, members of which have narrow host ranges among dicotyledonous species. They are transmitted by a single whitefly species, *Bemisia tabaci*. Most members of this genus are bipartite, though some members, such as tomato yellow leaf curl virus (TYLCV) are monopartite. Oganisation and functions of the

geminiviral genes are highly conserved. The following section briefs the function of various begomoviral genes.

1.5 Gene functions

1.5.1 Coat protein/AV1:

Coat protein is encoded by AV1 in begomoviruses. Coat protein form the twinned virion structure that encapsidates the viral genome. It is one of the major determinant in viral transmission within plants and among various plant hosts (Malik *et al.*, 2005; Qin & Petty, 2001; Wartig *et al.*, 1997). Systemic movement of the viruses is dependant completely on coat protein in case of monopartite begomoviruses where as it is dispensible for the systemic movement of bipartite viruses (Gafni & Epel, 2002).

1.5.2 Pre-coat protein/AV2:

Function of pre-coat protein was established by micro-injection studies. It is the pathogenecity determinant that acts through the regulation of double stranded and single stranded viral DNA levels. GFP fusion studies also suggested that pre-coat protein has an important role in cell to cell movement (Rojas et al., 2001).

1.5.3 Replication initiatior protein (Rep)/AC1:

The replication initiation protein is by far the most important protein. Rep is encoded by all geminiviruses and is variously named as C1:C2 in mastreviruses; C1 in curtoviruses, monopartite begomoviruses and AC1 in bipartite begomoviruses. Rep is a ~40 kDa multifunctional, highly conserved protein essential for replication of geminiviral genome (Elmer *et al.*, 1988; Schalk *et al.*, 1989). Rep confers virus-specific recognition of its cognate origin of replication (Fontes *et al.*, 1994a; Fontes *et al.*, 1992) and acts as an endonuclease by nicking at the viral *ori* to initiate the replication and ligates to terminate the rolling-circle replication (Laufs *et al.*, 1995b; Orozco & Hanley-Bowdoin, 1998). The Rep protein also has

an ATP-dependent topoisomerase activity (Pant et al., 2001) and helicase activity (Choudhury *et al.*, 2006; Clerot & Bernardi, 2006).

Rep is known to possess modular functions (Campos-Olivas *et al.*, 2002; Orozco *et al.*, 1997). The N-terminal part of Rep largely contains DNA-binding, nicking-ligation and oligomerisation domains, while the C-terminal half contains Walker A and Walker B motifs which contributes to its ATP binding and ATPase activity domains (Desbiez et al., 1995). Rep's ATP dependant helicase activity is located at its C-terminus. Rep regulates its own expression at transcriptional level and is also known to induce host replication machinery, presumably to enable the virus to replicate in differentiated cells (Egelkrout *et al.*, 2001; Kittelmann *et al.*, 2009; Nagar *et al.*, 1995). Rep modulates the host call cycle by interacting with various host proteins like retinoblastoma-like plant homolog (pRBR), PCNA, GRIK, GRAB etc. These interactions were shown to have strong implication on the replication of geminivirus. The functions ascribed to AC1 have been mapped to various regions of the Rep protein (Fig. 1. 4) (Orozco et al., 1997; Orozco and Hanley-Bowdoin, 1998; Kong et al., 2000). Various host proteins that interact with Rep are briefed in Table1.

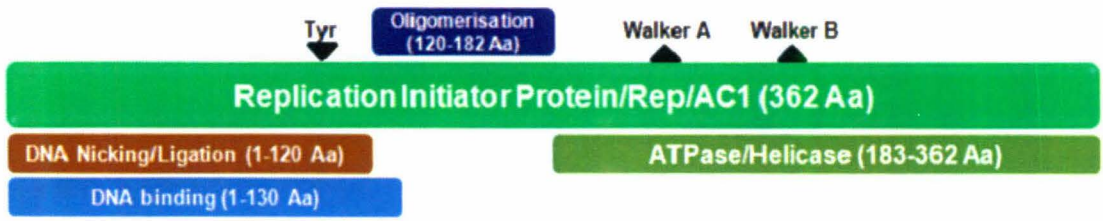


Figure 1.4. Modular organization of Replication Initiator protein/Rep. Rep/AC1 contains various activities and each of the activity has been characterised to a particular domain as shown in the picture. Numbers in the parenthesis represents the number of amino acids in that particular domain of the protein. 'Tyr' indicates the Tyrosine amino acid at position 103 in the protein. Tyrosine is the amino acid that catalyses the nicking and ligation of DNA. 'Walker A and Walker B' denotes the motifs that bind and hydrolyse ATP required for helicase activity (adapted from Choudhury et al., 2006).

1.5.4 Transcription Activator protein (TrAP)/AC2/C2:

Studies on the role of viral proteins on the viral gene expression established that AC2 functions as a transcriptional activator of virion sense genes: coat protein and BV1 (Haley *et al.*, 1992; Sunter & Bisaro,

1997). Recent studies have shown that the C' terminal acidic transcriptional activation domain of AC2 binds to the phloem specific CLE element and transactivates viral and host genes (Ruiz-Medrano et al., 1999) through its N' terminus zinc finger motif (Hartitz et al., 1999).

AC2 is also capable of suppressing host RNA silencing pathway (Voinnet et al., 1999) that gets activated upon viral entry into host cell. AC2 brings out suppression of PTGS by interacting with and inactivating kinases such as ADK and SNF1 kinase (Wang *et al.*, 2005; Wang *et al.*, 2003). Suppression of PTGS is thought to be brought out by a complex process involving activation of host genes that play role in endogenous network that controls silencing (Trinks et al., 2005). Recent observations from our lab suggested that transcription activation and silencing suppression functions of MYMIV AC2 can be separable (unpublished results from our lab).

1.5.5 Replication enhancer protein (REn)/AC3/C3:

AC3 is ~16 kDa protein that localises into the nuclei of infected plant cells at levels similar to the Rep protein (Nagar *et al.*, 1995; Pedersen & Hanley-Bowdoin, 1994). Transient replication studies have shown that a functional AC3 protein is required for enhanced viral DNA accumulation (Etessami *et al.*, 1991; Hormuzdi & Bisaro, 1995; Morris *et al.*, 1991; Stanley *et al.*, 1992; Sung & Coutts, 1995; Sunter *et al.*, 1990). Mutational studies with viruses expressing mutant AC3 protein exhibited delayed and greatly attenuated symptoms which results in reduced infectivity (Elmer *et al.*, 1988; Etessami *et al.*, 1991). It was also shown that mutation in AC3 reduces the replication of the virus in protoplast assays but doest abolish completely. This indicates the accessory role of AC3 in replication which is poorly understood at present.

Interaction studies with viral and host proteins established that AC3 oligomerises and interacts with AC1, PCNA, pRBR and SlNAC1 (Table 1). These interactions are thought to be playing important role in enhancing viral DNA accumulation. Recent work on AC3-SlNAC1 interaction

established that AC3 enhances SlNAC1 expression and constitutive expression of SlNAC1, in turn, enhanced viral replication (Selth et al., 2005).

Though there was consensus in the amelioration of the symptoms in the plants inoculated with AC3 mutant clones, the reports on the viral DNA accumulation varied with the species of the virus, strain of virus, host plant species under investigation and model system for the analysis of the replication. Replication of TGMV DNA A and DNA B was reduced by 50 fold upon mutation in TGMV AC3 in protoplast assays (Sunter et al., 1990). In another DNA replication assay in young leaves with mutated ACMV AC3 resulted in 5-10 fold reduction in DNA levels (Etessami et al., 1991). A contrasting observation was made in another experiment where there was no change in the replication of DNA A but replication of DNA B was drastically reduced (Morris et al., 1991). These varying and contrasting reports together with additional reports with BCTV suggested that the influence of AC3 on viral replication varies from virus to virus and also dependent upon the host plant under investigation.

1.5.6 AC4/C4:

Geminiviruses (except mastreviruses) encode a small protein AC4 that completely overlaps AC1 transcript. AC4 performs diverse functions that vary from virus to virus. AC4 plays important role in symptom formation of TLCV infection (Selth et al., 2004). Various studies on ACMV established that AC4 suppresses host cellular PTGS (Vanitharani et al., 2004) probably by its ability to bind miRNA (Chellappan et al., 2005). C4 of TYLCV impacts the movement of virus in the infected plant (Jupin *et al.*, 1994; Rojas *et al.*, 2001). Recent studies on BCTV C4 suggested that it is not required for symptom formation or movement (Etessami *et al.*, 1991; Piroux *et al.*, 2007; Stanley *et al.*, 1992) but is involved in signaling events in cell through brassinosteroid pathway by interacting with AtSketa kinase (Piroux et al., 2007).

Table 1.1. Geminiviral Protein Interactions related to Viral DNA Replication.

Sl.No	Interacting partner	Site(s) of interacting partner	Site(s) of Rep/REn	Role or significance of the interaction with Rep/REn
I. Rep interaction with geminiviral and host factors a) Geminiviral proteins				
1	Rep (self)	Aa 120-181	Aa 120-181	Oligomerisation, important for protein-protein interaction(Orozco et al., 1997)
2	REn	Aa 101-181	Aa 120-121	Increased accumulation of viral particles (Settlage et al., 1996)
3	CP	Not defined	Aa 120-181	Packaging of viral DNA (Malik et al., 2005)
4	RAD54			Increases Rep's helicase activity (unpublished results from our lab)
b) Host Proteins				
1	Rb	Not defined	Aa 120-181	Reprogramming of the cell cycle by freeing E2F (Arguello-Astorga *et al.*, 2004; Kong *et al.*, 2000; Xie *et al.*, 1995)
2	PCNA	Several residues across the protein	Aa 134-181	Elongation phase of DNA replication and possibly for viral DNA copy number (Bagewadi *et al.*, 2004; Castillo *et al.*, 2003)
3	RF C	RF C1 (p140)	Not defined	Loading of PCNA at the template–primer junction (Luque et al., 2002)
4	H3, GRIK & GRIMP	Not defined	Not defined	In viral replication and transcription (Kong & Hanley-Bowdoin, 2002)
5	SCE 1	Not defined	N'-terminus	Reduced DNA accumulation (Castillo et al., 2004)
6	GRAB 1 & 2	N' terminus	C'-terminal 37 Aa	GRAB expression inhibits WDV replication in culture cells (Xie et al., 1999)
7	RPA32	Not defined	Aa 184-362	Enhances ATPase and down regulates nicking and closing activities of MYMIV-Rep (Singh et al., 2007)
II. Interaction of REn with viral and host factors				
1	Rep	Aa 120-181	Aa 101-181	By interacting with REn, Rep may help in enhancing viral replication (Settlage et al., 1996)
2	Rb	Aa 132-187	Not defined	By interacting with REn, Rb may help in enhancing viral replication (Settlage et al., 2001)
3	PCNA	Aa 132-187	Not defined	Assembly of the plant replication complex to the viral *ori* (Castillo et al., 2003)
4	SlNAC1	Not defined	Aa 1-70	Interacting of NAC1 with REn helps it to act as enhancer of viral replication (Selth et al., 2005)

Abbreviations: **Rep:** Replication initiator protein; **REn:** Replication enhancer protein; **Rb:** Retinoblastoma protein; **PCNA:** Proliferating Cell Nuclear Antigen; **RF C:** Replication factor C; **GRIK:** Geminivirus Rep interacting kinase; **GRIMP:** Geminivirus Rep interacting movement protein; **GRAB:** Geminivirus RepA binding; **SCE:** SUMO-conjugating enzyme, homolog to yeast UBC9; **SlNAC**: *Solanum lycopersicum* NAC (for the no-apical meristem, A6TAF and C6UC2 genes domain); RPA32: Replication Protein A subunit 32kDa.

1.5.7 Nuclear shuttle protein (NSP)/BV1:

NSP functions in transport of DNA across the nuclear pore complex. BV1 performs this function by localizing in the nucleus and nucleolus of infected cells (Rojas et al., 2001) and binds DNA based on its size and form rather than sequence specificity. This interaction may be the limiting factor for size constraints associated with geminivirus replication (Rojas et al., 1998).

1.5.8 Movement Protein (MP)/BC1:

Movement protein binds to viral DNA in a sequence non-specific manner. Microinjection studies have shown that BC1 modifies host cell plasmodesmata and mediate the movement of viral DNA from cell to cell (Rojas et al., 1998). Additionally, BC1 is able to move from cell to cell, thus supporting its association with the long distance movement of viral ssDNA and dsDNA forms during systemic infections. Functional studies further suggest that BC1 may serve as a pathogenecity determinant capable of eliciting a non-host specific symptom phenotype (Pascal et al., 1993).

1.6 Geographic Differentiation

Geminiviruses show a geographic clustering upon sequence comparisons (Rybicki, 1994). New species can be easily assigned to North, Central or South America (New World), or to Europe, Africa, Asia (Old world). New world geminiviruses are always observed to be bipartite whereas the Old world viruses are monopartite with or without satellite DNAs as well as bipartite. New World viruses have lost the PCP (Pre Coat Protein) gene and have strict dependency on DNA B genes. Another character that is specific to the New World viruses is the faithfulness of DNA A to its cognate DNA B whereas the Old World viruses show promiscuity towards DNA B (Unseld *et al.*, 2000a; Unseld *et al.*, 2000b).

1.7. Taxonomy

Geminiviruses are named according to the plants they infect, the evident and prominent symptom and geographical origin. For example *Mungbean yellow mosaic India virus* indicates the identification of the virus in infected mungbean plant in India and causes yellow mosaic symptom. A threshold of 89% DNA sequence similarity was set for begomoviruses and 75% for mastreviruses by ICTV study group to differentiate various strains (Fauquet *et al.*, 2008; Fauquet & Stanley, 2005; Stanley *et al.*, 2005). DNAs β are named with reference to their helper begomovirus (Briddon et al., 2008).

1.8 Tissue Tropism of Geminiviruses

Geminiviruses are transferred from one plant to other by insect pests like white fly and leaf hopper. These insect pests are phloem-feeders. So, by default, geminiviruses enter and infect the phloem tissues. Phloem tissues contain nutrient rich continuous protoplasm and are connected directly to symplast of the whole plant via plasmodesmata. From the nutrition point of view of a parasite, there is obviously no need for the geminivirus to infect other tissues. Probably, for this reason geminivirus promoters are phloem-specific, that restricts their gene expression in other tissues thereby limiting their movement (Morra & Petty, 2000). Some geminiviruses are occasionally found in other tissues of leaf mesophyll (Rothenstein et al., 2007), as well as epidermis when a co-infection occurs with an unrelated RNA virus with a strong silencing suppressor (Wege & Siegmund, 2007). This suggests that gene silencing might be another phenomenon by which the plant restricts the geminiviruses in mesophyll cells and limit them to phloem tissues. Though most investigations were done on foliage leaves, these viruses are observed in roots, shoots, flowers and fruits but not in seeds (Wege, 2007).

1.9 Replication of Geminiviruses

Geminiviral biology involves a series of steps after the injection of viruses into the phloem tissue by insect vector: disassembly of the viral capsid, conversion of the ssDNA genome to dsDNA that forms the template for the transcription of viral genes and replication of viral genome. Most extensively studied part of the geminiviral biology is the replication (Gutierrez, 2002; Hanley-Bowdoin & Settlage, 2004; Hanley-Bowdoin *et al.*, 1999). Following sections brief the general replication strategy of the geminiviruses.

1.9.1 Complementary Strand Synthesis

Upon entry into plant tissues the first task of the geminiviruses is to synthesise the complementary strand in order to convert the ssDNA into a dsDNA that can be used as the template for replication and transcription. In subgroup I geminiviruses, the nucleo-capsid contains a small primer complementary to the short intergenic region that serves to initiate complementary strand synthesis with the help of host enzymes (Donson *et al.*, 1984; Hayes *et al.*, 1988). In other subgroup viruses the complementary strand synthesis needs de novo generation of RNA primers (Saunders et al., 1992) which are extended by DNA polymerase and are removed by RNase H, ligated by ligases and twisted by host's topoisomerases to wrap the dsDNA into minichromosomes (Pilartz & Jeske, 1992).

1.9.2 Plus strand synthesis

1.9.2.1 Origin of DNA replication

The plus-strand origin of all geminiviruses has been mapped to the 5'-intergenic region (Lazarowitz et al., 1992) which also contains the bidirectional promoter sequences (Lazarowitz *et al.*, 1992; Petty *et al.*, 1988; Sunter *et al.*, 1989; Sunter *et al.*, 1993). The plus-strand DNA replication origin has a modular architecture (Fontes *et al.*, 1994b; Sanz-Burgos & Gutierrez, 1998) and falls into two major categories: the Mastrevirus-type origin, which consists of a large cis-acting region where

the initiator Rep protein forms multiple complexes (Castellano *et al.*, 1999; Sanz-Burgos & Gutierrez, 1998) and the Begomovirus-type origin containing one binding site for Rep (Fontes *et al.*, 1994a; Lazarowitz *et al.*, 1992; Orozco & Hanley-Bowdoin, 1998). The cis elements that mediate viral replication are best characterized for the begomoviruses TGMV (Arguello-Astorga *et al.*, 1994; Fontes *et al.*, 1994a; Fontes *et al.*, 1994b; Lazarowitz *et al.*, 1992; Orozco *et al.*, 1998; Orozco & Hanley-Bowdoin, 1996; Revington *et al.*, 1989) and MYMIV (Singh et al., 2008). Comparison of the sequences of the TGMV plus-strand origin and 5′-intergenic regions of other geminiviruses uncovered similar elements in many Curto- and Begomoviruses.

The hairpin element is common to all geminivirus genomes (Arguello-Astorga et al., 1994) which contains a nonanucleotide sequence, 5′-TAATATTAC-3′ that is conserved among almost all geminivirus genomes and is found in the plus-strand origins of other rolling circle systems (Rogers et al., 1986). Other key elements in the geminiviral *ori* at 5' are: Rep binding GC rich directly repeated iterons (Fontes *et al.*, 1994a; Fontes *et al.*, 1992) and CA and AG motifs flanking the Rep binding site (Orozco et al., 1998).

The MYMIV origin contains four Rep- binding iterons (Pant *et al.*, 2001; Singh *et al.*, 2008). The presence of Rep-binding sites (CGGTGT) on both sides of the stem loop structure is a unique feature of MYMIV origin of replication. DNaseI foot-printing data revealed that four iteron sites are occupied first when the amount of protein is low and eventually the regions lying between four iterons are occupied in a cooperative manner with increasing concentrations of Rep. Binding of Rep leads to structural distortion of the CR region which is essential for the subsequent Rep mediated nicking, leading to the initiation of RCR (Singh et al., 2008). Similar processes might also be at work for replication of the genomes of other begomoviruses. It is also likely that some host factors participate in this initiation process to control the rate characteristic of the *in vivo* growth of viral DNA.

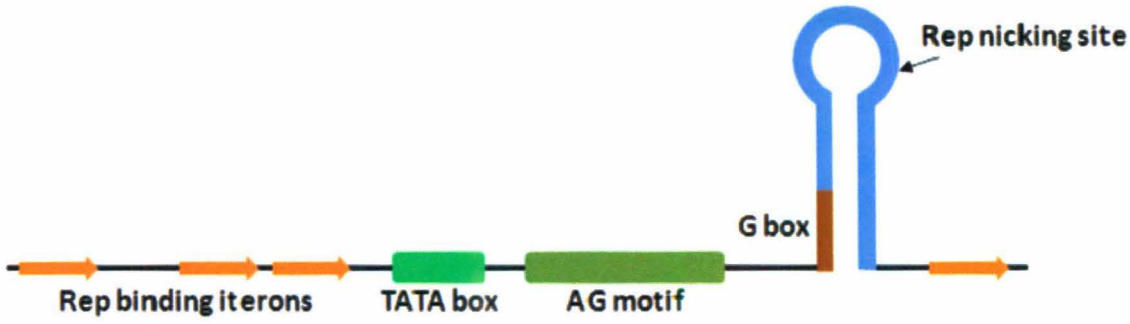


Figure 1.5. Modular organization of MYMIV-CR. Common Region of DNA A shows various replication and transcription regulatory elements. Stem-loop structure, essential for replication, contains conserved nonamer sequence 5′-TAATATTAC-3′ in the loop. Arrow indicates the site of initiation, where Rep mediated cleavage occurs. The Rep binding iteron sequences CGGTGT are present on both the sides of stem-loop structure. TATA box for AC1 transcription is present between Rep-binding sites and stem-loop structure. Other regulatory elements are AG-motif and G-box (adapted from Singh et al., 2008).

1.9.2.2 Initiation

Various experiments suggested that the geminiviral replication proceeds through Rolling Circle Replication. The two-dimensional gel electrophoresis (Saunders et al., 1991), followed by studies with transgenic plants with tandem repeats of viral genomes supported this observation (Heyraud *et al.*, 1993b; Stenger *et al.*, 1991). The priming of geminivirus plus-strand synthesis occurs with a site specific nick within the conserved nonamer TAATATT↓AC (indicated by an arrow) (Heyraud *et al.*, 1993a; Orozco & Hanley-Bowdoin, 1996) which provides the necessary 3′-OH, capable of being extended by cellular DNA polymerases, whereas the initiator protein remain covalently attached to the 5′ ends (Laufs et al., 1995a). The elongation and termination mechanisms of this rolling circle replication in geminiviruses remain obscure and await investigation.

1.9.2.3 Template for encapsidation

In the late replication cycle, the virion sense ssDNA produced by RCR starts accumulating, instead of being incorporated into the replication pool. Coat protein, in association with movement proteins has been implicated to have an important role to play in this step. Coat protein has the non-specific ssDNA binding property which is attributed for the sequestration of ssDNA for packaging into virions. Phosphorylated

movement protein is required for enhanced ssDNA accumulation (Kleinow et al., 2009)

1.9.3 Recombination-dependent replication

Recent experiments with two-dimensional gel electrophoresis showed that few of the DNA intermediates are not readily explained by rolling circle replication. Rather they fit into the recombination dependant replication (RDR) (Alberter *et al.*, 2005; Jeske *et al.*, 2001; Jovel *et al.*, 2007). RDR does not rely on an origin of replication, but rather can use any free 3' end of ssDNA or of ssDNA overhangs in a dsDNA to initiate replication in a homologous stretch of supercoiled master DNA (Fig. 1. 6). The role of viral proteins in the geminiviral replication process has been studied in detail for several aspects, but whether they are directly involved in a recombination dependant replication mechanism is unknown. However, the helicase activity described for TYLCSV Rep would fit into this scenario acting like RecBCD helicase in bacterial recombination (Clerot & Bernardi, 2006). Moreover, RDR may lead to recombination if two partially related viruses have entered the same nucleus, which helps to explain the emergence of recombinant geminiviruses.

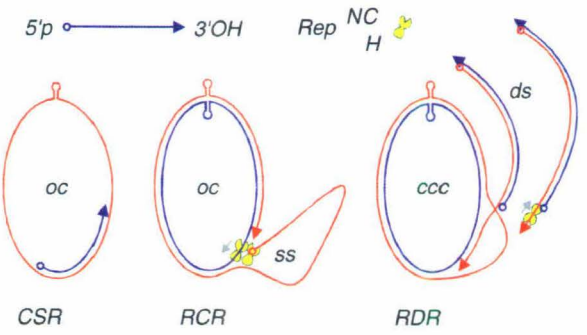


Figure 1.6. Mode of Replication in geminiviruses. Schematic planar representation of replication modes of geminiviruses with open circular (oc) or covalently closed circular (ccc) templates, and single-stranded (ss) or double-stranded (ds) products, for complementary strand replication (CSR), rolling circle replication (RCR) and recombination-dependent replication (RDR). The replication-associated protein (Rep) with its nicking-closing (NC) and helicase (H) domain is shown during different putative functions, and small arrows indicate the direction of movement (From Jeske, 2009).

1.10 Transcription

Geminiviruses employ the eukaryotic control elements such as TATA box, CAAT box as well as poly-adenylation signals for bidirectional transcription. There is also temporal regulation by expressing the early genes via complementary sense and late genes via viral sense transcripts (Shimada-Beltran & Rivera-Bustamante, 2007). The viral messenger RNAs have been characterised in a number of begomoviruses: ACMV (Townsend et al., 1985), TLCV (Mullineaux et al., 1993), MYMV (Shivaprasad et al., 2005), TGMV (Petty *et al.*, 1988; Ruiz-Medrano *et al.*, 1999; Shung & Sunter, 2007; Shung *et al.*, 2006; Sunter & Bisaro, 1989; Tu & Sunter, 2007) and ABMV (Frischmuth et al., 1991). Viral promoters are located within the LIR/IR/CR of geminiviruses overlapping with the origin of replication and the terminator sequences overlap at the opposite side. *In vitro* translation experiments confirmed that all the mRNAs of the geminiviruses are poly-cistronic (Thommes & Buck, 1994). However, the complementary mRNAs that code separately for C1 and C2 are spliced to form Rep in WDV, MSV, DSV and TYDV (Accotto *et al.*, 1989; Dekker *et al.*, 1991; Morris-Krsinich *et al.*, 1985; Wright *et al.*, 1997).

1.10.1 Transcription modulation of viral promoters by geminiviral proteins

Geminiviruses regulate their promoters with its own proteins as well as host transcription factors. Mastreviruses use RepA to transactivate viral sense transcripts (Horvath *et al.*, 1998; Munoz-Martin *et al.*, 2003) and begomoviruses use AC2 to promote transcription of AV1 and BV1 (Sunter & Bisaro, 1991; Sunter & Bisaro, 1992; Sunter & Bisaro, 1997; Sunter *et al.*, 1990). Recent reports suggest that viral sense transcript is synergistically activated by AC1 and AC2 (Shivaprasad et al., 2005) though a positional homologue in curtovirus, BCTV AC2 is not involved in transcriptional control. Complementary transcription is rather complex involving regulation by AC1 and AC4. Rep binds to the iterons in the *ori* and is responsible for the auto repression of AC1-4 (transcript that codes AC1 and AC4) transcription (Eagle *et al.*, 1994; Shivaprasad *et al.*,

2005; Sunter *et al.*, 1993). Another important feature of viral complementary sense transcription is the separate bicistronic transcript made for AC2 and AC3 with the regulatory elements located upstream of AC2 (Mullineaux *et al.*, 1993; Shivaprasad *et al.*, 2005; Sunter *et al.*, 1989). Interestingly, the repression of TGMV AC1 enhanced the promoter strength for the downstream transcript AC2-3 (transcript that codes AC2 and AC3) (Shung & Sunter, 2007). This bicistronic mRNA was shown to

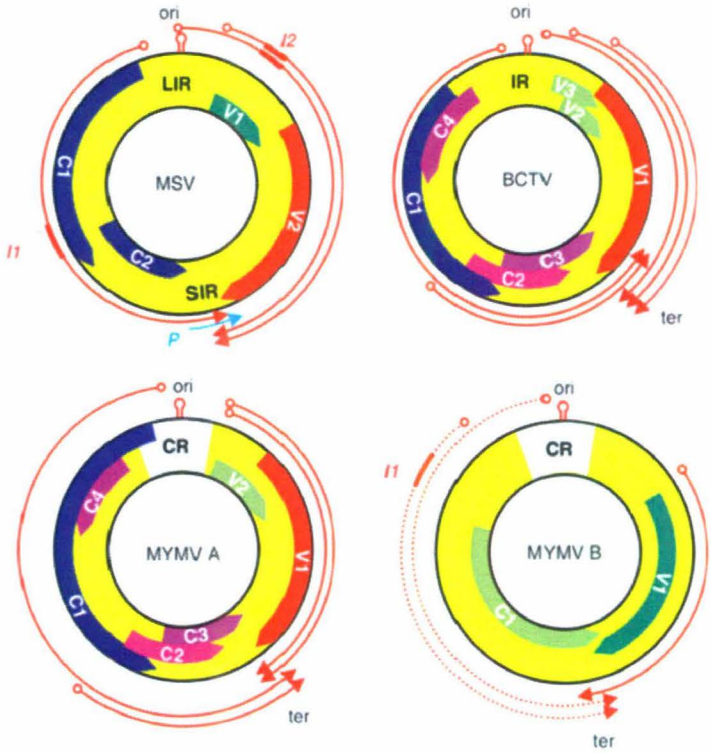


Figure 1.7. Transcript maps of representative geminiviruses. Thick arrows indicate ORFs, thin arrows represent transcripts with introns (Int), or primer (P), and hairpins origin of replication (ori). Intergenic regions (IR) are either named long (LIR) or short (SIR) for MSV and contain common regions (CR) for bipartite begomoviruses of the Old World (MYMV) and New World (AbMV), which are highly conserved in DNA A and DNA B of a given virus. Transcripts overlap in termination regions (ter) which harbour polyadenylation signals. Transcripts of MYMV B in complementary orientation are stippled because the termination could not be defined. Gene names are same as mentioned in Fig. 1. 3 (adapted from Jeske, 2009).

programme relatively low AC2 and high AC3 abundance, as a consequence of leaky ribosome scanning for the ATG of AC2 in a suboptimal Kozak context (Shung et al., 2006). In addition to AC1, AC4 of TGMV exerted a minor downregualtion of AC1-4 transcript by binding to DNA (Eagle & Hanley-Bowdoin, 1997; Groning *et al.*, 1994; Shung & Sunter, 2007). For the geminiviruses the chromatin structure supports the presence of the regulatory elements in the IR/CR and in regions upstream of AC2. Following complementary strand replication, the

geminiviral dsDNA is packaged into histones, thus forming a viral minichromosome (Abouzid *et al.*, 1988; Pilartz & Jeske, 1992; Pilartz & Jeske, 2003). One genomic component of 2600bp offers space for 13 nucleosomes, but minichromosomes with 11 and 12 nucleosomes have been detected as well. One nucleosome-free gap was located in the common region of the bipartite AbMV, another in the promoter region for the AC2-3 DNA A (Pilartz & Jeske, 2003). Positioning of nucleosomes may, therefore, exert transcriptional control at the structural level facilitating transcription factor binding.

1.11 Impact of geminivirus infection on host

Geminiviral DNA replication is coupled to a special state of the infected cell due to the absolute requirement for cellular factors. S-phase nuclei contain up to 20 times more abundant geminivirus dsDNA intermediates than in cells in other phases of the cell cycle (Accotto et al., 1989). This led to the conclusion that geminivirus DNA replication is somehow synchronized with the ability of the host cell to replicate its DNA during the S-phase. Further studies with transgenic plants provided evidence that geminivirus infection has a direct effect on cellular gene expression exemplified by the accumulation of proliferating cell nuclear antigen (PCNA) which occurs in TGMV-infected terminally differentiated cells, where PCNA is undetectable in the absence of TGMV (Nagar et al., 1995). Furthermore, it was demonstrated that the up-regulation of PCNA expression was dependent upon the presence of TGMV Rep (Nagar et al., 1995). It was also observed that BCTV-C4 is enough to induce tumourogenic growth indicating that this gene is enough in permissive cells to initiate cell division (Latham et al., 1997). Recent observation in yeast *S. pombe* suggested that ACMV Rep alone is sufficient to induce re-replication of yeast DNA (Kittelmann et al., 2009). In this way, one or more geminivirus proteins would be responsible for triggering a cellular environment favourable for viral multiplication within the infected cell. In

addition, induction of host functions and/or interference with the cell cycle control could have a potential effect on the fate of the infected cell. Rep and REn interact with the cellular proteins involved in development (GRAB), senescence (GRAB, SlNAC), pathogenecity (GRIMP, GRIK, GRAB), cell cycle regulation (Rb, PCNA), DNA replication and repair (H3, SUMO, RFC, PCNA), etc. The residues/domains and the role(s) of the interaction of Rep and REn with host factors are summarized in Table 1.

1.12 Cell cycle Modulation - Retinoblastoma protein (pRb) Pathway

In plants, DNA replication and the corresponding machinery are restricted to meristems, developing leaves and roots and the cambium. The observation that many geminiviruses replicate in differentiated cells that have exited the cell cycle (Nagar et al., 1995) suggests that these viruses have evolved strategies to activate specific sets of cellular genes required for their replication in non-proliferating cells.

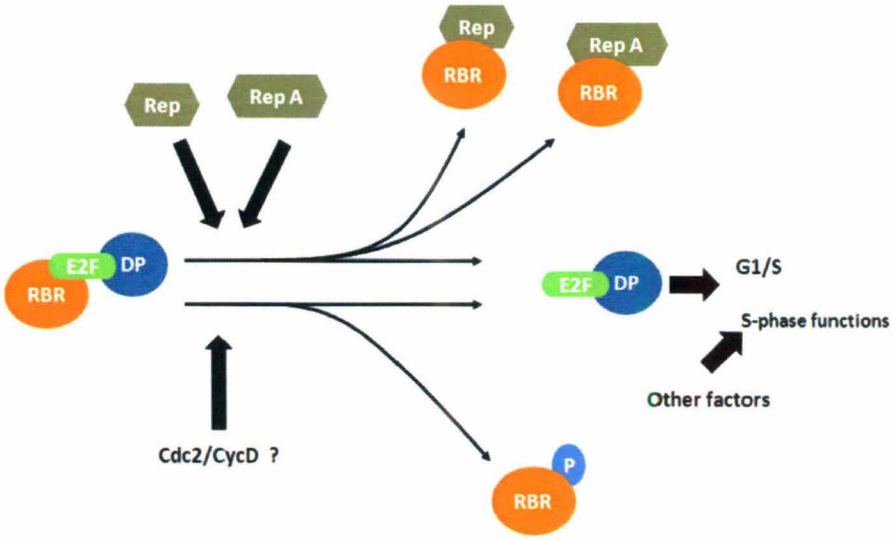
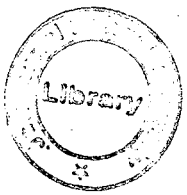


Figure 1.8. Model proposed for the interference of geminivirus proteins with the retinoblastoma-related (RBR) pathway. The G1-S transition in plants is controlled, most probably, by a pathway dependent on the retinoblastoma-related (RBR) protein which associates with the E2F-DP heterodimeric transcription factor(s). In cycling cells, phosphorylation of RBR by a CDK-cyclin complex (Nakagami et al., 1999) is thought to release active E2F-DP complexes required to activate G1-S transition and S-phase progression. S-phase-specific gene expression also occurs through other mechanisms. Geminivirus RBR-binding proteins (RepA and Rep) would bypass this control by sequestering RBR from the ternary RBR-E2F-DP complex (adapted from Gutierrez, 2000).

One strategy employed by geminiviruses is to modulate the cell cycle through Retinoblastoma. Both WDV RepA protein and TGMV Rep interact with pRBR proteins from humans (Collin *et al.*, 1996; Xie *et al.*, 1995) and maize (Xie *et al.*, 1996; Xie *et al.*, 1995).

Further, the TGMV replication accessory factor (REn) interacts with Rep as well as pRBR suggesting that it might play an important role in modifying plant cells, to create a replication-competent environment (Settlage et al., 2001). Schematic diagram of the model of interference of geminivirus RepA/Rep with the retinoblastoma related pathway is shown in Fig. 1. 8.



1.13 Vein swelling and hyperplasia

A question that still remains poorly understood is whether interference of geminiviruses with cell cycle regulators leads to an abnormal progression through the S-phase which, eventually, leads to cell division and an increase in cell number. In this context, TGMV infected cells in plants and ACMV Rep expressing yeast do not seem to be committed to divide (Kittelmann *et al.*, 2009; Nagar *et al.*, 1995). However, quite interestingly, infection by BCTV is associated with morphological changes, such as vein swelling (enations) and leaf curling (Latham et al., 1997), directly linked to abnormal cell cycle control and an increase in cell number. The molecular basis for this hyperplastic response resides in the viral protein C4, a protein known to be a determinant of symptom severity (Latham *et al.*, 1997; Stanley *et al.*, 1992). Mutants in BCTV C4 protein fail to induce vein swelling and upward leaf curling, although viral DNA accumulates to normal levels (Latham et al., 1997). A similar elongated cell phenotype was observed in yeast expressing ACMV Rep (Kittelmann et al., 2009). It is, thus, tempting to speculate that such tumor-like growth in the phloem in case of plant and in yeast is a consequence of the disruption of cell cycle control in the infected cells through specific interaction of the viral protein(s) with one (or more) cell growth regulatory pathways.

Further studies are needed to expand our knowledge of the molecular mechanism underlying the hyperplasic response of these geminiviruses.

1.14 Modulation of sumoylation system

Sumoylation is a posttranslational process that modifies function, activity, or localization of the target protein by the covalent attachment of an ubiquitin (Ub)-like polypeptide (Ubl) called SUMO (also known as sentrin, Smt3, UPL, and PIC1). The general consequence for SUMO modification varies depending on the target. SUMO conjugation has been implicated in cellular responses to environmental stress, subcellular protein translocation, nuclear body formation, centromere segregation, protection from ubiquitin-mediated proteolysis and regulation of transcriptional activity (Wilson & Rangasamy, 2001). In plants, sumoylation was shown to contribute to the regulation of ABA signaling that mediates plant responses to several environmental stresses (Lois et al., 2003) and may also play an important role in pathogen plant defense responses (Chosed et al., 2006). Screening for TYLCV Rep interacting host proteins identified NbSCE1, a SUMO-conjugating enzyme (Castillo et al., 2004). Transgenic plants expressing sense or antisense LeSUMO (tomato orthologue of AtSUMO) accumulated less amount of TGMV viral DNA upon infection with TGMV in comparison to infection in wild-type plants indicating that both positive as well as negative changes in SUMO levels disturb viral replication. The observation that some of the putative sumoylation sites are located within known functional domains of TGMV Rep including the DNA binding and cleavage domains, oligomerisation domain, RBR binding domain and the helicase domain indicates that a direct effect on Rep as a consequence of altered SUMO levels in plants cannot be ruled out.

1.15 Geminiviruses and host DNA repair machinery

During viral infection, host responds with various defence systems and viruses attempt to divert or decimate the activated defence mechanism. Hosts antiviral arsenal also includes the cellular DNA damage response machinery. Host cell DNA repair machinery can recognize viral genetic material as 'damage' (Weitzman et al., 2004). Attempts of host cellular machinery to process the viral genome are detrimental to the virus survival in some cases. So, some viruses have developed ways to inhibit or circumvent the host-cell responses. For other viruses, the cellular response seems to be beneficial which have evolved ways to steer cellular DNA repair proteins to aid their own replication (Lilley et al., 2007).

TGMV-infected cells accumulate proliferating cell nuclear antigen (PCNA), the processivity factor of DNA polymerase δ (Nagar et al., 1995). MYMIV AC1 and TYLCSV AC1 interact with arabidopsis PCNA (Bagewadi *et al.*, 2004; Castillo *et al.*, 2003). Since PCNA functions in both replicative and repair DNA syntheses (Bravo *et al.*, 1987; Kelman, 1997), its induction does not directly reveal the nature of the interaction between the host and the virus that leads to DNA synthesis. The involvement of DNA repair machinery would be consistent with the fact that most virus infected cells in a plant are differentiated and no longer contain detectable levels of replication enzymes (Coello *et al.*, 1992; Nagar *et al.*, 1995). However, the involvement of host DNA synthesis enzymes associated with cell cycle activity is supported by the finding that many infected nuclei contain condensed chromatin (Bass et al., 2000).

Homologous (HR) and non-homologous end joining (NHEJ) recombination events have been observed in geminiviruses (Bisaro, 1994). Moreover, few geminiviruses replicate their genome partly by employing the RDR mode of replication (Jeske et al., 2001). Hence the involvement of recombination and repair machinery in geminiviral replication cannot be completely ruled out. The involvement of replication and repair factors

such as RAD51, RAD52, MRE11, and DMC1 in MYMIV DNA replication has been validated using the yeast model system (Raghavan et al., 2004).

1.16 Methods to study geminiviral replication

Our understanding of several features of geminiviral replication has steadily increased over the past few years, though there are still several aspects that are poorly understood. Development of *in vivo* systems supporting geminiviral replication in combination with several *in vitro* experiments has contributed a great deal in understanding geminiviral replication. Because of the involvement of host systems and close integration with other stages of the infection cycle, it is generally believed that a full picture of the viral replication can only be obtained from *in vivo* systems. Consequently, studies on geminiviral DNA replication were performed in intact plants by mechanical inoculation (Hayes *et al.*, 1988; Stenger *et al.*, 1991). Simultaneously, assays based on transfected protoplasts and agroinoculated leaf discs were also developed to specifically examine DNA replication (Elmer *et al.*, 1988; Townsend *et al.*, 1986). However, owing to their complexity, *in vivo* systems are difficult to establish and many of the questions can only be addressed by *in vitro* systems. Both *in vitro* and *in vivo* methods used for studying geminiviral DNA replication are discussed below.

1.16.1 *In vivo* systems

1.16.1.1 *In planta*

Most geminiviruses, with few exceptions, are not mechanically transmissible as virions or as cloned DNA. The ability of *Agrobacterium tumefaciens* to be an alternative to mechanical transmission to introduce geminiviral DNA into intact plants was first revealed with *Maize streak virus* (Grimsley et al., 1987), and the technique was termed as "agroinfection" or "agroinoculation". They demonstrated that agrobacterium containing tandem repeats of MSV DNA carried on the Ti plasmid when inoculated into whole maize plants led to symptoms of

MSV infection. This was a major breakthrough in the study of the mechanically non-transmissible *mastreviruses* since it allowed for genetic manipulation of cloned viruses and reintroduction of these viruses into plants. This procedure was used to provide for a simple and efficient assay for TGMV replication (Elmer et al., 1988). The first evidence for geminiviruses utilizing rolling circle mechanism also came from studies with tandem repeats of BCTV genome utilising agroinfection of intact *N. benthamiana* plants (Stenger et al., 1991). The potential of this technique was utilized to demonstrate that mutations in the conserved nonamer sequence 'TAATATTAC' abolished WDV replication in agroinfected maize plants (Schneider et al., 1992). Agroinfection technique was extended to other genomes including *Digitaria streak virus* (Donson et al., 1988), MSV in various species of *Poacea* (Boulton et al., 1989), ACMV (Morris et al., 1991) and *Tomato yellow leaf curl China virus* (Yin et al., 2001), MYMIV (Singh et al., 2007) and many others.

The study of geminiviral replication in intact plants as a whole is limited by the complexity of the plant genome, difficulty in isolating replicative intermediates and the variability associated with usage of systemically infected leaves. Some of these difficulties can be overcome by using transient replication assay in discs of leaves instead of using the whole plant.

1.16.1.2 Leaf disc assays

Circular discs of approximately 1 cm in diameter are cut from intact leaves and can be used for various assays as they mimic the whole plant leaves. They can be used to monitor the response of plant to various biotic and abiotic stresses. Usage of leaf discs for transient replication assays reduces the complexity involved with intact plants and also has the advantage that pieces from many leaves can be combined in one sample to smooth out leaf-to-leaf variation. Mutational analysis of viral ORFs in petunia leaf discs were used in a transient assay to demonstrate that AL1 was essential for TGMV-A replication (Elmer et al., 1988). Analysis of the replication competency of chimeric BCTV genomes in

which portions of the origin of DNA replication (ori) were derived from heterologous BCTV strains, in tobacco leaf discs, permitted identification of an essential cis-acting element governing strain-specific replication in *curtoviruses* (Choi & Stenger, 1995; Choi & Stenger, 1996). Analysis of replicative intermediates isolated from AbMV infected leaves and leaf discs of *N. benthamiana* agroinfected with AbMV provided the first indication that the geminivirues replicate by RDR also (Jeske et al., 2001). Viral replication assay was improved for the cloned ACMV in cassava leaf discs using particle bombardment mediated delivery instead of the commonly used agrobacterium-mediated transfer (Zhang & Gruissem, 2003). The observation that viral DNA replication was detected only when hormones were present in a pre-culture medium inducing plant cells to divide actively, lent support to the theory that geminiviruses need S-phase like conditions in the host for their replication.

The leaf disc assay, though convenient to use, also has inherent disadvantages. The growth of microorganisms on the surface of the leaf discs with resulting in contamination of the medium and necessity to maintain the viability of the leaf disc may well alter the biochemical situation in the cells of interest.

1.16.1.3 Cell suspension cultures

Some of these disadvantages with leaf discs can be overcome by using suspensions of surviving but non-dividing cells. Cell suspension culture was first used in an experiment to introduce WDV vectors into cultured wheat cells with the aim to evaluate how origin structure and replicon size affected viral DNA replication (Suarez-Lopez & Gutierrez, 1997). Wheat suspension cells were also utilized to show the functional importance of the presence of an intact Rb binding motif, LxCxE during geminiviral replication (Xie et al., 1995). Plant host factor named GRAB (geminivirus RepA binding protein), a member of the NAC domain family, isolated by yeast two-hybrid method using WDV RepA as bait, was also tested for its effect on WDV replication *in vivo* in cultured wheat cells (Xie et al., 1999). However, the use of cell suspensions to study geminiviral

replication has so far been restricted to wheat and has not been extended to other systems possibly because of difficulties associated with its long-term maintenance.

1.16.1.4 Protoplasts

Protoplasts are isolated plant cells that lack the rigid cellulose walls found in intact tissue. Use of protoplasts offer several advantages over the intact plants including improved synchrony of infection, close control of experimental conditions, uniform sampling, relatively high efficiency of infection and better isolation of organelles such as nuclei.

Transient replication assays in protoplasts were first used to study *Cassava latent virus* replication (Townsend et al., 1986) in *Nicotiana plumbaginifolia* protoplasts. The use of tobacco protoplasts has been a significant contributing factor for delineating the important cis-acting elements involved in replication of begomoviruses (Chatterji *et al.*, 2000; Eagle & Hanley-Bowdoin, 1997; Fontes *et al.*, 1994a; Fontes *et al.*, 1994b; Gladfelter *et al.*, 1997; Orozco *et al.*, 1998) and mastreviruses (Kammann et al., 1991). Protoplasts have contributed a great deal to our understanding of geminiviral origin of replication and its interaction with Rep. They have also been excellent systems to characterize the effect of mutations in the viral genome on viral replication (Kong *et al.*, 2000; Liu *et al.*, 1998; Woolston *et al.*, 1989).

Though, protoplasts have provided an easy, rapid and efficient system to study geminiviral replication, a number of actual and potential limitations and difficulties are also associated with the use of protoplasts. Protoplasts are very fragile and survive only for 2-3 days after which they decline and die. Moreover, the isolation procedure and the medium in which they are maintained drastically affect the physiological state of the cells. As a consequence of these changes, protoplasts vary with time in many properties during the period they survive after isolation. It is also known that some features of virus replication differ in intact leaves and

in protoplasts thereby making the conclusion ambiguous (Hormuzdi & Bisaro, 1995; Hull, 2002).

1.16.2 *In vitro* systems

1.16.2.1 Two dimensional gel electrophoresis

Two-dimensional DNA electrophoresis has been useful in a variety of versions to analyze replicative intermediates of prokaryotic and eukaryotic organisms (Friedman & Brewer, 1995). The first detection of RCR intermediates for geminiviruses was based on two-dimensional analysis in a combination of neutral and alkaline gels (Saunders et al., 1991). An improved and more sensitive version of this technique for analyzing AbMV intermediates in agroinoculated leaf discs was developed by using a combination of neutral and chloroquine gels. Chloroquine intercalates into dsDNA and adds positive superhelical turns into negatively supercoiled circular DNA. Depending on the concentration of chloroquine and the size of the circular DNA, several topoisomers of covalently closed circular DNA could be visualized. This technique was used to identify additional forms of DNA, which were consistent with RDR mechanism of replication (Jeske et al., 2001).

1.16.2.2 Primer Extension

This technique is used to study the properties of replication complexes by adding nucleotide tri-phosphates under the appropriate conditions and assessing the resulting products from extension of primed strands on the existing template. The products can be analyzed either by incorporating a labeled nucleotide tri-phosphate or by probing the product with a labeled probe. Traditional primer extension techniques were improvised to study replicative intermediates of AbMV in agroinoculated leaf discs, by performing multiple primer extensions using a PCR based approach with divergent primers designed from the virion strand (Jeske et al., 2001). The resulting primer extension products were resolved on alkaline gels by electrophoresis, blotted using alkaline transfer solution and hybridized with digoxigenin labeled AbMV DNA A probes. Using this method, the

first strong evidence for a RDR strategy of replication was reported for geminiviral replication.

1.16.2.3 Electron microscopy

Electron microscopy is a highly useful technique to examine the replicative intermediates *in planta* and was used to give direct evidence for RCR mode of geminiviral replication (Jeske et al., 2001). Besides examining the replicative intermediates, electron microscopic studies were also performed to visualize DNA-Rep complexes obtained by binding experiments using purified WDV Rep MBP fusion protein and defined fragments of WDV LIR region (Sanz-Burgos & Gutierrez, 1998). The large apparent size of the complexes was consistent with the participation of several Rep monomers in complex formation. Increasing the DNA fragment length used for binding experiments to include the two regions identified by EMSA and DNA footprinting to contain two high-affinity Rep binding sites, allowed visualization of these Rep-DNA complexes by electron microscopy. This technique was also utilized in mapping of the location of these two complexes (Castellano et al., 1999).

1.16.2.4 DNA binding and Electrophoretic mobility shift assay

DNA binding assays provide a simple and efficient assay to detect interaction of proteins with nucleic acid. Essentially, immuno-protein complexes are incubated with labeled DNA fragments in the presence or absence of competitor DNA. The bound DNA is then extracted from the nucleoprotein complex and visualized on denaturing polyacrylamide gels. DNA binding studies have contributed to defining the binding domain of Rep to a directly repeated motif in the common region (Fontes *et al.*, 1994a; Fontes *et al.*, 1992). DNA binding assays of TGMV and BGMV Rep with exchanged Rep binding sites established the tight binding affinity of Rep for their cognate binding motifs *in vitro* (Fontes et al., 1994b). These experiments thus suggested that Rep confers virus-specific recognition of its cognate origin of replication, which was subsequently supported by transient replication assays.

The electrophoretic mobility shift assay (EMSA) using non-denaturing polyacrylamide gel electrophoresis provides a simple, rapid and sensitive method for detecting proteins that bind to nucleic acids and have contributed significantly to our understanding of nucleoprotein interactions at the geminiviral origin. The binding of the proteins retards the mobility of the labeled nucleic acid fragment on gel electrophoresis that can be detected by comparing the mobilities of treated and untreated nucleic acid fragments. Electrophoretic mobility shift assays with purified recombinant Rep protein, and probes and competitor DNAs encompassing different lengths of the large intergenic region (LIR) of WDV identified two high affinity and one low affinity DNA-protein complex at the WDV origin (Castellano et al., 1999). EMSA has also been used to determine properties of Rep/origin interaction such as affinity and binding specificities (Chatterji *et al.*, 2000; Lin *et al.*, 2003).

1.16.2.5 DNase footprinting

The basis of this assay is that bound protein protects DNA from DNase catalyzed hydrolysis. The protected DNA fragments are separated by denaturing gel electrophoresis and analyzed by techniques such as sequencing and autoradiography (Ausubel et al., 2002). The technique has been used to map the location of the Rep binding site in the LIR of WDV (Castellano et al., 1999), satellite DNA of TYLCV (Lin et al., 2003) and CR region in MYMIV (Singh et al., 2008).

1.16.2.6 Yeast two hybrid

This system utilizes the modular organization of transcriptional factors for investigating the interactions between two proteins. For this purpose the two proteins, one-termed the 'bait' is cloned so that it is fused to a DNA-binding domain and the other-termed the 'prey' is fused to an activation domain. The two clones are then introduced into a suitable yeast line and interaction between the bait and prey tether the DNA-binding domain to the activation domain, allowing the activation of a downstream reporter such as *LacZ*, *HIS3*, *ADE2* etc. This technique has

been extensively used to identify protein-protein interactions either among the geminiviral proteins or of geminiviral proteins with a host factor. Host factors with a probable role in geminivirus replication as a consequence of their interaction with the geminiviral replication initiation protein, were identified using Rep as bait to screen *Arabidopsis* cDNA library. Some of the host factors identified using this technique include the retinoblastoma like protein (Ach *et al.*, 1997; Xie *et al.*, 1995), GRAB protein (Xie et al., 1999), RF-C (Luque et al., 2002), PCNA (Bagewadi *et al.*, 2004; Castillo *et al.*, 2003), a motor protein (Geminivirus Rep interacting motor protein, GRIMP), a kinase (Geminivirus Rep interacting kinase, GRIK) and a partial clone of histone H3 (Kong & Hanley-Bowdoin, 2002), SlNAC1 (Selth et al., 2005), ADK (Wang et al., 2003). The interactions were confirmed by pull down assays or co-immunoprecipitation and the functional implications of some of these factors were tested either *in planta* or in transient replication assays.

1.16.2.7 Yeast as a model system to study viral replication

As discussed earlier, no method seems suitable for studies related to *in vivo* viral DNA replication. The existence of versatile and powerful genetics for *Saccharomyces cerevisiae,* availability of a large repertoire of deletion and conditional mutants, the easy handling and extensive manipulations possible in this simple organism makes it an attractive model system to study viral replication.

The initial studies examining the potential of yeast as a heterologous host supporting viral replication used a plant-infecting virus, Brome mosaic virus (BMV), a positive strand RNA virus (Janda & Ahlquist, 1993). Replication of human papillomavirus (HPV16) was the first DNA virus to be shown to replicate in *S. cerevisiae* in the absence of complementing ARS or CEN elements (Angeletti et al., 2002). Another study on Bovine papillomavirus (BPV-1) also showed that the viral episome could replicate in yeast (Zhao & Frazer, 2002). Another contemporary study with *Mungbean yellow mosaic india virus,* a begomovirus showed that it can replicate well from its origin of replication when the ARS and CEN were

mutated making the yeast specific origin non-functional in YCp plasmid. This study facilitated in narrowing down the minimal region that contributes to the replication of virus in host. With this model system, the authors were able to show the role of various host recombination proteins (MRE11, RAD51, RAD54) in viral replication (Raghavan et al., 2004).

Introduction

Tomato is an economically important crop often affected by a wide range of begomoviruses (a subgroup of geminiviruses). Database update of tomato infecting begomoviruses consists of 54 species of viruses (Fauquet et al., 2008). Begomoviruses infecting tomato cause a wide variety of diseases including leaf curl disease, yellow leaf curl disease, yellow spot disease, mottle disease, golden mosaic disease and severe rugose disease. Majority of these virus species cause leaf curl disease and are grouped into tomato leaf curl viruses.

Tomato leaf curl disease in tomato is characterised by stunted growth of the plant with short internodal length, internally curled leaves and downward bending. Leaves of the infected plants are thicker and leathery in texture with a purple tinge to the veins on the underside. Young leaves are slightly chlorotic with normal flowers but dry and small fruits.

In addition to natural host tomato plant, tomato leaf curl viruses infect a wide range of plants depending on the species of the virus and not all hosts show symptoms of infection. List of amenable hosts include *N. benthamiana* (wild tobacco), *N. tabacum* (tobacco), *P. hybrida* (petunia), *P. vulgaris* (french bean), *S. melongena* (egg plant), *S. tuberosum* (potato) and *A. thaliana.* The broad host range of these viruses and the severity of diseases pose a grave threat to economy as well as the food.

Various strategies have been developed to generate geminivirus resistant plant varieties. They include traditional breeding and transgenic approaches. Several transgenic approaches based on viral intergenic sequences (Abhary et al., 2006), mutant viral proteins (Chatterji et al., 2001; Hou et al., 2000; Lucioli et al., 2003; Noris et al., 1996; Shepherd et al., 2007; Shivaprasad et al., 2006), antisense RNAs (Asad et al., 2003; Bendahmane & Gronenborn, 1997), peptide aptamers (Lopez-Ochoa et al., 2006), recombinant zinc finger proteins (Sera, 2005) and RNAi constructs have been generated. Most of these approaches do not confer high levels of resistance or are limited to a narrow range of virus species.

Thus, there is a need for a better and consistent approach to generate resistant plants.

Development of an antiviral strategy to control tomato leaf curl viruses or geminiviruses in general involves proper understanding of the viral biology- DNA replication, transcription of viral genes and the viral protein functions, so that the steps could be interfered with.

Tomato leaf curl viruses are peculiar in their genomic content. They are either monopartite (Kheyr-Pour et al., 1991; Navot et al., 1991), monopartite with β DNA (Dry et al., 1997; Dry et al., 1993) or bipartite (Padidam et al., 1995; Rochester et al., 1994) depending upon the strain of the virus. Sequencing of the full length DNA A genome isolated from infected samples from Kerala: Tomato leaf curl Kerala virus-[India:Kerala II:2005], accession no. DQ852623 showed presence of ORFs represented in the following genome map which is common to all the tomato leaf curl viruses.

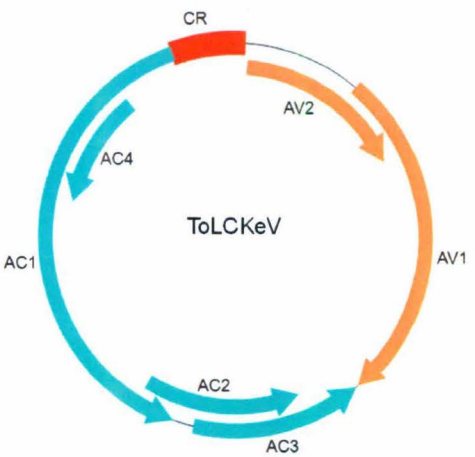


Figure 2.1. DNA A genome of *Tomato leaf curl Kerala virus*. Genome organization of DNA A of the isolate Tomato leaf curl Kerala virus-[India:Kerala II:2005], accession no. DQ852623. ORFs coding from complementary strand are denoted by AC1, AC2, AC3, AC4, and genes on the sense strand are denoted by AV1, AV2. Intergenic region is denoted by CR.

Of all the proteins encoded by geminiviruses, Rep is the essential viral protein required for replication (Elmer et al., 1988). AC3 is another viral protein that has been shown to act as an accessory protein which enhances replication by an unknown mechanism (Sunter et al., 1990). AC3 is a very unique protein in terms of its characteristics. It does not have any known DNA binding motif or any enzymatic motif that can be

attributed to its replication enhancement activity. AC3 does not have any other homologous protein except in geminiviruses making the characterisation of the viral protein very difficult.

In planta studies with mutated AC3 ORF in different viruses resulted in delayed and attenuated symptoms (Elmer et al., 1988) with decrease in viral load and systemic movement of viruses (Etessami et al., 1991; Stanley et al., 1992). Moreover, viral infections with truncated AC3 resulted in enhanced recovery of infected plants indicating that importance of AC3 in efficient viral infection (Etessami et al., 1991; Hormuzdi & Bisaro, 1995; Stanley et al., 1992). Mutation in TGMV AC3 ORF also resulted in reduction of TGMV DNA A and DNA B accumulation by 50 fold in tobacco protoplasts (Sunter et al., 1990). Other reports on replication studies indicated that mutation in ACMV AC3 down regulates the replication of DNA B drastically without any effect on DNA A (Morris et al., 1991). In another case, Logan strain of BCTV was tolerant to C3 mutation in tobacco plants whereas its replication was down regulated in case of sugar beet plants. The same BCTV strain was unable to replicate when the replication was analysed in tobacco protoplasts (Hormuzdi & Bisaro, 1995). These contrasting reports regarding the role of AC3 in replication leaves a question if it really affects the replication of virus in general or it depends on the strain of the virus, type of host plant or the experimental approach. In any case, one can not negate the role of all these factors playing together. These experiments, though carried out on young plant leaves, leaf discs or protoplasts with mutated AC3s leaves another question unanswered: is this observation (reduction in DNA replication wherein cases observed) really due to loss of function or due to a dominant mutant protein [in all these mutations the AC3 was truncated either by deletion of C-terminal part after synthesising a minimum of 80 amino acids (Sunter et al., 1990) or a frame shift after synthesising 110 amino acids (Hormuzdi & Bisaro, 1995; Morris et al., 1991; Sung & Coutts, 1995)] affecting the replication of the virus?

The evolutionarily conserved nature of AC3 ORF across all the curtoviruses, begomoviruses and topocuviruses indicates that AC3 protein performs unique but yet uncharacterised functions for the successful viral infection. Above reports on the role of AC3 in viral replication suggests that it has an indispensable role in viral replication and affects systemic movement of viruses; but the mechanism by which AC3 affects these important events is yet to be deciphered.

In this study we aim to characterise the functions of AC3 protein from *Tomato leaf curl virus* with the following objectives:

- To express and purify the AC3 protein
- To analyse the formation of oligomer and oligomeric composition of AC3, if any
- To examine the interaction of AC3 with the most important viral protein, Rep and the effect of this interaction on the biochemical activity of Rep
- To perform exhaustive search for various AC3 interacting host proteins by phage display and yeast two hybrid library screening
- To construct viral replicon vectors and analyse the role of AC3 in the replication of the constructed viral replicons *in planta* and *ex vivo* in yeast
- To analyse the role of AC3 in other aspects of viral biology especially RNAi.

Materials and Methods

3.1 Materials

Table 3.1. List of various materials, consumables used in this study

Material	Source
Plants	
Tomato plants	Green House, ICGEB, New Delhi
Tobacco plants	Green House, ICGEB, New Delhi
Bacterial and Yeast strains	
Escherichia coli (DH5α)	Invitrogen life technologies, Carlsbad, USA
Escherichia coli [BL21(DE3)]	Novagen, Germany
Escherichia coli (ER2738)	New England Biolabs Inc. MA, USA
Saccharomyces cerevisiae (AH109)	Clontech, Palo Alto, California, USA
Saccharomyces cerevisiae (BY4741)	EUROSCARF, Frankfurt, Germany
Agrobacterium tumefaciens (LBA4404)	ICGEB, New Delhi
Mice	
Balb/c	ICGEB, New Delhi
Vectors	
pGEM-T Easy	Promega Life Science, Madison, WI, USA
pJET1	Fermentas International Inc., Ontario, Canada
pET-28a	Novagen, Germany
pMal-c2X	New England Biolabs Inc. MA, USA
pGEX-4T-1	GE Healthcare, Chalfont St. Giles, United Kingdom
pETM30	Dr. Amit Sharma, ICGEB, New Delhi
pCAMBIA1391Z	CAMBIA, Australia
YCp50	Dr. Nilanjan Roy Choudhury, Ohio State University, USA
YCpO$^-$	ICGEB, New Delhi
pGAD-C1	Clontech, Palo Alto, California, USA
pGBD-C1	Clontech, Palo Alto, California, USA
Markers and Ladders	
1 Kb Ladder	Fermentas International Inc, Ontario, Canada

Protein marker	GE Healthcare, Chalfont St. Giles, United Kingdom
Protein marker	Fermentas International Inc, Ontario, Canada
Prestained marker	Fermentas International Inc, Ontario, Canada
Antibodies	
Secondary antibody (anti-mice IgG)	Sigma, St. Louis, USA
Membranes and filter papers	
Nitrocellulose transfer membrane	Advanced Microdevices Pvt. Ltd., Ambala Cantt, India
Radioisotopes	
[γ^{32}P] ATP	NEN, Perkin-Elmer Life Sciences Inc., Boston, USA
Restriction endonucleases and DNA modifying enzymes	
T4 Polynucleotide kinase	New England Biolabs Inc. MA, USA
FastDigest restriction enzymes	Fermentas International Inc, Ontario, Canada
T4 DNA ligase	Fermentas International Inc, Ontario, Canada
FastAP Alkaline phosphatase	Fermentas International Inc, Ontario, Canada
DNA Polymerases	
Taq DNA polymerase	New England Biolabs Inc. MA, USA
Phusion DNA polymerase	Finnzymes Oy, Finland
Yeast medium components	
Yeast nitrogen base	Difco, Becton Dickinson Company, Maryland, USA
Yeast nutrient drop out mix	Clontech , Palo Alto, California, USA
Amino acids, Adenine	Sigma Chemical Company, St. Louis, USA
General chemicals	
Sigma Chemical Company, St. Louis, USA; Serva, Heidelberg, Germany; Buckinghamshire, UK; GE Healthcare, Chalfont St. Giles, United Kingdom; BIO-RAD Labs, Hercules, CA, USA; Boehringer Manheim, GmbH, Germany; Promega Life Science, Madison, WI, USA, Fermentas, Ontario, Candada.	

Table 3.2. List of various virus isolates used in this study. Virus isolates are named according to the Eighth report of ICTV nomenclature (Stanley et al., 2005)

Virus Isolate	Short Description	Accession No.
Tomato leaf curl Gujarat virus-[India:New Delhi:2005]	ToLCGV-[IN:ND:05]	DQ629101
Papaya leaf curl virus-India-[India:New Delhi:Tomato:2005]	PaLCuV-IN [IN:ND:Tom:05]	DQ629102
Tomato leaf curl Kerala virus-[India:Kerala II:2005]	ToLCKeV-[IN:KerII:05]	DQ852623
Tomato leaf curl Bangalore virus-A [India:Kerala IV:2005]	ToLCBV-A [IN:KerIV:05]	DQ887537
Indian cassava mosaic virus-India [India:Maharashtra:1988]	ICMV-IN [IN:Mah:88]	AJ314739
Chilli leaf curl virus-India [India:PRM:Tomato:2005]	ChiLCV-IN [IN:PRM:Tom:05]	DQ629103
Mungbean yellow mosaic India virus-[India:New Delhi:Blackgram 3:1991]	MYMIV-[IN:ND:Bg3:91]	AF126406

3.2 Growth Conditions

3.2.1 Bacteria

Bacteria were grown in LB broth (1% Tryptone, 0.5% Yeast extract, 1% NaCl in water, pH 7.0±0.2) at 37°C with 180-200 rpm shaking for 12-16 hr. LB-Agar (Luria Bertani medium components + 1.5% Agar) plates were incubated in a 37°C incubator 12-16 hr.

3.2.2 Agrobacterium

Agrobacterium tumefaciens was grown in YEM (0.04% yeast extract, 1% D-Mannitol, 0.01% NaCl, 0.02% $MgSO_4$, 0.05% K_2HPO_4) liquid culture. To isolate single colonies of agrobacterium, YEM agar (YEM + 1.5% agar) plates were incubated at 28°C-30°C for a minimum of three days.

3.2.3 Yeast

Saccharomyces cerevisiae was grown in YPD (1% Yeast extract, 2% Peptone, 2% Dextrose)/minimal medium at 30°C with 200 rpm for 16-18 hr. To isolate single colonies of yeast, YPD agar (YPD + 1.5% Agar) plates were incubated at 30°C for 3-4 days.

3.2.4 Plant

Tomato, seeds were germinated in small pots containing vermiculite. Fifteen days post germination the plantlets were transferred to big pots with nutrient rich soil. Agrobacterium infiltration was carried out in the young leaves 18 days post germination for the gene silencing experiments. Transient replication studies were carried out in the mature leaves of 25-30 day old plants. Throughout experiments tomato plants were maintained in green house at 24°C and 14 hr light cycle.

3.3 Methods

All the molecular biology protocols were essentially followed as reviewed in *Molecular Biology Protocols: A Laboratory Manual (3rd Edition)* (Sambrook & Russel, 2001) with slight modifications.

3.3.1 DNA Isolation

3.3.1.1 Plasmid Isolation from Bacteria

Bacterial plasmid was isolated by alkaline lysis method. Protocol for the isolation of plasmid from 100 ml culture is briefed here

- Single colony of bacteria was inoculated in 100 ml LB with appropriate antibiotic and grown for 16-18 hr.
- Bacteria were then harvested by centrifugation at 6000×g for 10 min at 4°C.
- The cells were suspended in 2 ml of Solution I [50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA] with RNase (100 µg/ml) and incubated on ice for 10 min.
- Bacterial cells were then lysed by addition of 4 ml Solution II (0.2 N NaOH, 1% SDS) and mixing gently by inversion. Cells were kept at room temperature for 5 min.
- The lysed bacterial cell suspension was neutralised with 3 ml of Solution III (3 M K^+ ions and 5 M CH_3COO^- ions) and incubated on ice for 10 min.

- The mixture was centrifuged at 9000×g for 10 min and the supernatant was collected by filtering through cheese cloth.
- Plasmid in the filtrate was precipitated by addition of 0.6 volumes of Isopropanol and centrifuging at high speed for 10 min.
- The supernatant was discarded and the pellet was washed with 70% ethanol.
- The air dried pellet was dissolved in 0.5 ml TE [10 mM Tris-Cl (pH 8.0) and 1 mM EDTA (pH 8.0)] and treated with RNase to remove traces of RNA.
- The RNA free solution was extracted with equal volume of Phenol:Chloroform:Isoamylalcohol (25:24:1 v/v) to remove the RNase protein contamination.
- The aqueous phase was treated again with Chloroform:Isoamylalcohol (24:1 v/v) to remove traces of phenol. The protein free DNA solution was once again precipitated by isopropanol and washed with 70% alcohol, air dried and dissolved in 0.3-0.4 ml TE.

3.3.1.2 Genomic DNA Isolation from Plant Leaves

- Plant tissue (2 g) was ground in liquid nitrogen. To the ground tissue 5 ml of homogenization buffer (5 M NaCl and 2% N-Lauroyl sarcosine) was added and allowed the buffer to thaw in the mortar.
- The homogenized tissue is then transferred to a 15 ml centrifuge tube and the cell debris was pelletted at 6800×g for 10 min at room temperature.
- Supernatant was transferred into another tube and mixed with 2 volumes extraction buffer [100 mM Tris-Cl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% CTAB and 0.2% β-ME]. This mixture was incubated at 65°C for 30 min with gentle mixing by inverting the tubes.
- To this mixture, equal volume of Chloroform:Isoamylalcohol solution was added and mixed by inversion. The whole mixture was centrifuged at 10,000×g for 5 min at room temperature and

the upper aqueous phase was transferred to a fresh tube. This was repeated till the chlorophyll colour disappeared completely.

- DNA was precipitated by the addition of 0.6 volume of isopropanol and was collected by centrifugation at 10,000×g for 15 min at room temperature. The pellet was rinsed with 70% ethanol and air dried.
- Dried pellet was dissolved in 500 µl TE and treated with RNase. DNA was then extracted twice with phenol:chloroform:isoamylalcohol and the aqueous phase was transferred to a fresh tube. The DNA was precipitated by adding one-tenth volume of 3 M Sodium acetate, pH 5.2 and two volumes of ethanol and collected by centrifugation at 10,000×g for 10 min at 4°C. The DNA pellet was washed with 70% ethanol, air dried and dissolved in 300 µl TE.

3.3.1.3 DNA Isolation from M13 Phage

DNA from M13 phage was isolated from the phage cultured on *E.coli* (ER2738). The isolation procedure is described below:

- Stationary phase culture of *E.coli* was used as inoculum in sterile LB broth. Five hundred microlitre of stationary phase culture was mixed well with 50 ml of fresh LB broth and dispensed as 2 ml aliquot into each culture tube.
- Each M13 plaque from LB agar plate was picked with a sterile toothpick and inoculated in the culture tube. Bacterial growth and lysis was allowed for a maximum of 5 hr at 37°C with vigorous shaking at 200 rpm.
- 1 ml of lysed cell culture was centrifuged at 10,000×g for 1 min at 4°C. Three fouth of supernatant was taken and mixed with 0.3 ml of PEG/NaCl (20% PEG-8000 and 2.5 M NaCl). This mixture was kept at 4°C for 10-15 min and centrifuged at high speed for 10 min at 4°C. Supernatant was discarded completely.
- The pellet was dissolved in 0.1 ml of Iodide buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 M NaI] followed by addition of 0.25 ml of ethanol and incubated for 10 min at room temperature. The solution is then centrifuged at 10,000×g for 10 min and

supernatant was discarded. Pellet was washed with 70% ethanol, air dried and dissolved in 30 µl of TE.

3.3.2 DNA quantification

Quantity and quality of nucleic acids in solution was determined by measuring the absorbance at 260 and 280 nm. Concentration of DNA was calculated by taking 1 OD_{260}=50 µg/ml for DNA, 40 µg/ml for RNA and 33 µg/ml for single stranded oligonucleotides. The purity of nucleic acid solution was checked by taking the A_{260}/A_{280} ratio.

3.3.3 DNA Restriction Digestion

1 Unit of FastDigest enzyme was used to digest 1 µg of DNA in 5 min at 37°C in a volume of 20 µl reaction containing FastDigest buffer that is common to all FastDigest enzymes.

3.3.4 DNA Modifications

3.3.4.1 End Lablelling by T4 Polynucleotide Kinase

T4 Polynucleotide kinase was used to add γP^{32}phosphate at the 5' –OH of the DNA or oligo. The kinasing reaction was done with one Richardson unit enzyme to catalyse the incorporation of 1nmol of acid-insoluble (^{32}P) in a total reaction volume of 50 µl in 30 min at 37°C in T4 Polynucleotide kinase reaction Buffer with 66 µM ATP (γP^{32}) (5×10^6 cpm/µmol) and 0.26 mM 5'-hydroxyl-terminated DNA or oligo.

3.3.4.2 Ligation by T4 Ligase

T4 DNA ligase was used to ligate the recombinant gene into a cloning or expression vector. One unit of the enzyme was used to catalyze the conversion of 1 nM of (^{32}PPi) into Norit-adsorbable form in 20 min at 37°C

(Weiss unit). Our ligation reactions worked perfectly with the same amount of DNA and ligase at varying temperatures: 4°C (overnight incubation), 12°C (overnight incubation), 16°C (overnight incubation), 25°C (4 hr incubation) and 37°C (1 hr incubation).

3.3.4.3 Removal 5' Phosphate by FastAP Alkaline Phosphatase

FastAP Alkaline phosphatase was used to remove the 5' –Phosphate from the linearised vectors to prevent self ligation. One unit of FastAP Alkaline phosphatase was used to dephosphorylate 1 µg of linearised vector in 10 min in FastAP buffer at 37°C.

3.3.5 DNA Purification

DNA samples were resolved by electrophoresis on 0.8% agarose gel. The desired fragment was identified using standard molecular weight marker (1 kb ladder) and purified using the following techniques.

3.3.5.1 Purification of Digested Products from Agarose gel

Excised agarose gel pieces containing the DNA band were macerated by passing though a needle attached to a syringe. To this an equal volume of Tris-Cl (pH 8.0) saturated phenol was added, vortexed and frozen at minus 80°C for 1 hr. The samples were then centrifuged at 13,000 rpm for 5 min at room temperature. The upper aqueous phase was collected and subjected to two rounds of Chloroform:Isoamylalcohol extraction. The DNA fragment was then precipitated by adding one-tenth volume of 3 M Sodium acetate (pH 5.2), 2.5 volumes of ethanol and glycogen (40 ng/µl) and kept for 1 hr at –80°C. The DNA was collected by centrifugation at 14,000 rpm for 15 min at 4°C, washed with 70% alcohol, air dried and dissolved in TE.

3.3.5.2 Purification of End Labelled products by Sephadex G-50

The bottom of a 1 ml syringe was plugged with autoclaved glass wool using a plunger. The syringe was placed in a 14 ml culture tube

containing a decapped micro-centrifuge tube at its bottom. The syringe was filled with Sephadex G-50 beads till the column volume was around 0.9 ml and then centrifuged at 2,000 rpm for 2 min. The packed column was washed with 100 µl of TE buffer pH 8.0 and centrifuged at 2,000 rpm for 2 min. This procedure was repeated until the volume of eluent was equal to the volume of TE loaded (100 µl). Radiolabelled DNA was loaded onto the spin column and centrifuged at 2,000 rpm for 2 min. The radiolabelled DNA probe was eluted out from spun column whereas the free radioactive label was retained in the spun column.

3.3.6 DNA Amplification

Rapid amplification of the DNA fragments was done using either Taq DNA polymerase or Phusion High-Fidelity DNA polymerase and a set of convergent primers. Each primer (0.16 µM) along with dNTP (200 µM of each) and Taq DNA polymerase (0.5 U) or Phusion High-Fidelity DNA polymerase (0.5 U) was used for PCR reaction of 50 µl. The reaction conditions are tabulated as follows:

Step	Taq polymerase	Phusion polymerase
Initial denaturation	94°C/2 min	98°C/30 sec
Cyclic denaturation	94°C/30 sec	98°C/10 sec
Annealing	52-58°C/30 sec	-
Extension	72°C/1 min for 1000bp	72°C/30 sec for 1000bp
Final Extension	72°C/5-10 min	72°C/5-10 min

3.3.7 Oligonucleotides

Oligos in this study were designed with various factors in consideration: %GC content, melting temperature for the primer set, formation of hairpin loops and dimerisation of oligos. We have used 'OligoAnalyzer' tool and 'Bioedit' for the analyses (Hall, 1999; Owczarzy et al., 2008).

Various oligonucleotides used in this study are listed below in 5'→3' direction.

Table 3.3. List of oligonucleotides used in this study

CR_K2_Fwd_HindIII	AGCAAGCTTGCTTTCAGGATGAC
CR_AC3_K2_Rev_HindIII	CCAAAGCTTTGGCATGTACTCATGC
CR_K2_Fwd_EcoRI	AGCGAATTCGCTTTCAGGATGAC
CR_K2_Rev_EcoRI	ATGGAATTCCATATTGAATTGGTC
ToLCV_ AC1_Fwd	CATGGATCCATGGCHVCYCCMAAWCG
ToLCKeV_AC1_Rev38	GTGCCTCTTCTTTAGTTAGAGAGC
ToLCKeV_AC1_Rev119	AGCTCGAGCTAATCGACTTGGAAAAC
ToLCV_AC1_Rev	TGACTCGAGTCAACYCGWCGACGHCTGG
All_AC3_Fwd	CATGAGCTCGGATCCATGGATTCACGCACAGGG
All_AC3_Rev	CCATCTAGACTCGAGTGGCRTGTACTCAYGCCTCTAAYCC
M_K2AC3_Fwd	GTTCTGCAACGTGCACGGATTCACGCACAGG
M_K2AC3_Rev	CCTGTGCGTGAATCCGTGCACGTTGCAGAAC
M21_K2AC3_Fwd	GGCGTGTTTATCTAGTAAATTCAAAATCCC
M21_K2AC3_Rev	GGGATTTTGAATTTACTAGATAAACACGCC
Actin_fwd	ATGCCATTCTCCGTCTTGACTTG
Actin_rev	GAGTTGTATGTAGTCTCGTGGATT
PolyA_Fwd	GTGTGAGTAGTTCCCAGATAAGG
YCP50_Fwd	CACATTTCCCCGAAAAGTGC
YCP50_Rev	GTTAGATTTCATACACGG
PCNA300_Fwd	ACGGATCCGTTCTAGAATCGATTAAGGATCTGG
PCNA300_Rev	GGGGATCCCCATTAGCTTCATCTCAAAATCAG

3.3.8 Site Directed Mutagenesis

Site directed mutagenesis was carried out in a three step protocol as briefed here:

- Forward and reverse oligos were designed to accommodate the mutation and the gene along with whole vector was amplified using Phusion High-Fidelity DNA Polymerase.
- The amplified product was treated with DpnI restriction enzyme to remove the original DNA template (optional)
- The amplified vector was phophorylated and ligated with T4 Polynucleotide kinase and T4 Ligase in the same reaction supplemented with T4 Ligase buffer.

- Kinased and ligated DNA was transformed into *E.coli* (DH5α) and plated onto LB agar with appropriate selection.
- Five colonies were chosen at random plasmid was isolated and confirmed the mutation by DNA sequencing.

3.3.9 Competent Cell Preparation and Transformation

3.3.9.1 Bacteria

E. coli (DH5α) cells were grown in 100 ml LB medium at 37°C with 200 rpm shaking till the OD_{600} ≈ 0.4-0.5. Cells were harvested by centrifugation at 3000×g for 10 min at 4°C. The pellet was suspended in 20 ml of 50 mM $CaCl_2$ and incubated on ice for 1 hr. Cells were collected by centrifugation at 3000×g for 10 min at 4°C and resuspended in 4 ml of 50 mM $CaCl_2$ (with 25% glycerol), and incubated at 4°C for overnight. Cell suspension was dispensed as 100 µl aliquots into micro-centrifuge tubes and stored at -80°C.

Plasmid DNA (~100 ng) was added to the competent cells, gently mixed and incubated on ice for 10 min. Cells were subjected to heat shock at 42°C for 90 sec and chilled immediately on ice for 10 min. The cells were allowed to recover by adding 1 ml of LB medium to the tube and incubated at 37°C for 1 hr with 150 rpm shaking. Different volumes of transformed competent cells were plated on LB plates containing appropriate antibiotic and incubated overnight at 37°C.

3.3.9.2 Agrobacterium

A. tumefaciens strain LBA4404 was grown in YEM medium till mid log phase (OD_{600} ≈ 0.5) and chilled on ice for 10 min. Cells were harvested at 2800×g for 20 min at 4°C. The cell pellet was gently suspended in ice-cold, sterile 10 mM $CaCl_2$ and incubated for 2 hr in ice. Calcium chloride treated cells were harvested again by centrifugation at 2800×g and suspended in 10 mM $CaCl_2$ and dispensed as aliquots into sterile micro-centrifuge tubes after adding 80-100% glycerol and stored at -70°C.

For transformation, 1 µg of binary plasmid DNA was added to the ice thawed agrobacterium competent cells (100 µl) and incubated on ice for 30 min. Cells were freezed by immersing the micro-centrifuge tube in liquid nitrogen followed by heat shock at 37°C for 5 min. Sterile YEM medium (850 µl) was added to the micro-centrifuge tube and was incubated at 28°C for 3-5 hr at 200 rpm. The transformation mix was plated on YEM-agar medium containing the appropriate antibiotic(s) and incubated at 28°C for 48 hr to allow for the colonies to appear.

3.3.9.3 Yeast

Small-scale transformation of yeast for complementation, replication assays as well as for yeast two-hybrid analysis was performed using the protocol described in yeast protocol handbook (BD Clontech, USA)

- Primary culture was prepared by inoculating a single colony of yeast in 50 ml YPD broth and incubated for 16-18 hr at 30°C with 200 rpm shaking.
- Secondary culture was prepared by inoculating 300 ml YPD with the primary culture to bring the $OD_{600} \approx 0.2$-0.3 and incubated at 30°C with 200 rpm shaking till the $OD_{600} \approx 0.4$-0.6.
- Cells were harvested by centrifuging at 5000×g for 10 min. Harvested cells were washed with 30 ml sterile water.
- Washed cells were then suspended in TE/LiAc [10 mM Tris-HCl (pH7.5), 1 mM EDTA and 100 mM LiAc] solution to make up to 1 ml and the cell were dispensed into micro-centrifuge tubes (~100 µl each).
- For each transformation, 500 ng of salmon sperm DNA and 1 µg of desired DNA construct were added and vortexed to mix well.
- Six hundred microlitre of PEG/TE/LiAc [40% Polythelene glycol–4000, 10 mM Tris-HCl (pH 7.5), 100 mM Litium acetate] solution was added to the yeast cell mixture and vortexed well.
- This cell suspension was incubated in a shaker at 30°C for 30 min at 200 rpm.

- Dimethyl sulfoxide (70 µl) was added to the cell suspension and subjected to heat shock by incubating at 42°C for 15 min followed by incubating on ice for 1-2 min.
- Cells were collected by centrifugation at 9000×g for 5 sec at room temperature, supernatant was removed and pellet was suspended in 0.5 ml of sterile TE buffer.
- Selection of the transformed cells was carried out by spreading 100 µl of cell suspension uniformly on the minimal medium plates (Yeast drop out medium). Plates were incubated at 30°C until colonies appear (2-4 days).

3.3.10 Expression and Purification of Recombinant Protein

Expression of recombinant protein was carried in *E.coli* (BL21(DE3)]. All the proteins were expressed in this study were done in a similar way as mentioned below:

- Single colony was used as inoculum for the primary culture with appropriate antibiotic and cultured for 12-16 hr.
- Primary culture [0.1% (v/v)] was used as inoculum for the secondary culture and cells were grown at 37°C with shaking at 200 rpm till $OD_{600} \approx 0.4$-0.6.
- Cells were induced to express the recombinant protein with IPTG (final concentration of 0.1 mM) for 8-12 hr at 18°C.
- Cells were harvested by centrifugation at 4000 rpm for 10 min at 4°C.
- Bacterial cells were then suspended in native buffer [50 mM Tris-Cl (pH 8.0), 100 mM NaCl, 10% Glycerol] with protease inhibitors (1 mM PMSF and 1 mM Benzamidine).
- The cell suspension was sonicated to lyse the cells.
- The lysed suspension was centrifuged at high speed at 4°C for 15 min to pellet the cell debris and unlysed cells.

- Suitable resin depending upon the fusion tag was added to the supernatant containing the recombinant protein and purified through gravity column as follows:

3.3.10.1 His Tagged protein purification

Purification of 6X-His-tag containing protein was performed as per the protocol detailed in 'The QIAexpressionist' technical manual. In brief, steps were as follows:

- Nickel-nitrolotriacetic acid agarose (Ni-NTA Agarose, 1 ml resin per 1 L of bacterial culture) was added to the recombinant protein containing supernatant and kept for rocking at 4°C for 1 hr in a column sealed at the bottom.
- After 1 hr incubation with the resin, unbound protein was collected as flow through from the column.
- Bound protein was washed with native buffer containing 50 mM Imidazole to remove non-specific proteins interacting with Ni-NTA.
- Native buffer containing 300 mM Imidazole was used as eluent and collected as 0.5-1.0 ml aliquots.

3.3.10.2 GST fused protein purification

The protocol for purification of glutathione-s-transferase (GST) containing protein was adapted from GST gene fusion system handbook. In brief, steps were as follows:

- Glutathione sepharose 4B (0.5 ml resin per 1 L of bacterial culture) was added to the recombinant protein containing supernatant and kept for rocking at 4°C for 1 hr in a sealed column.
- After 1 hr incubation with the resin, unbound protein was collected as flow through from the column.
- Bound protein was washed with native buffer to remove non-specific proteins interacting with resin.
- Elution of bound protein was performed with native buffer containing 10 mM reduced glutathione and collected as 0.5-1.0 ml aliquots.

3.3.10.3.1 MBP fused protein purification

Maltose binding protein (MBP) fusion containing protein purification was followed as per the protocol detailed in technical manual of pMAL protein fusion and purification system. Purification procedure was briefed as follows:

- Amylose resin (1.0 ml resin per 1 L of bacterial culture) was added to the recombinant protein containing supernatant and kept for rocking at 4°C for 1 hr in a column sealed at the bottom.
- After 1 hr incubation with the resin, unbound protein was collected as flow through from the column.
- Bound protein was washed with native buffer to remove non-specific proteins interacting with resin.
- Elution of bound protein was done with 10 mM maltose containing native buffer and collected as 0.5-1.0 ml aliquots.

3.3.10.3.2 Factor Xa cleavage of MBP Fusion protein

1 µg of Factor Xa (New England Biolabs) was used to cleave 50 µg of MBP fusion protein test substrate, MBP-AC3 to 95% completion in a total reaction volume of 50 µl in 12-16 hours or less at 16°C in 20 mM Tris-HCl (pH 8.0) with 100 mM NaCl and 2 mM $CaCl_2$.

3.3.11 Protein-Protein Interaction studies

3.3.11.1 GST pull down

- Purified GST fusion protein was incubated with varied amounts of MBP fused protein in binding buffer [25 mM Tris (pH 8.0), 75 mM NaCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 2.5 mM DTT, 1% NP-40] at 37°C for 30 min.
- Glutathione sepharose 4B resin was equilibrated with binding buffer and 10 µl of resin was added to the incubated protein mixture and kept on nutator for 30 min.
- Unbound protein fraction was separated from the resin by centrifugation at 3,000×g for 3 min. Resin bound to the protein was washed with increasing concentrations of NaCl (100 mM to

400 mM) in binding buffer. Equal amount of 2X sample loading buffer [100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% Bromophenol blue] was then added to the resin, boiled for 5 min, centrifuged briefly and the supernatant was analyzed by SDS-PAGE. The protein bands were visualized by western blotting or coomassie blue staining.

3.3.11.2 Gel Filtration

Oligomerisation status of AC3 was analysed with gel filtration using Superdex 200 5/150 column in Acta Prime (GE Healthcare) having a bed volume of 3 ml and a void volume of 1.374 ml. Protein sample (100 µl) was injected and the flow rate of the column was maintained at 200 µl per minute all through the process. Dextran (2000 kDa), Thyroglobulin (669 kDa), Ferritin (449 kDa), Aldolase (158 kDa) and Aprotinin (9 kDa) were used as molecular weight standards under the same conditions.

3.3.11.3 Sucrose Gradient

We followed the protocol that was used for the analysis of oligomerisation of Rep (Choudhury et al., 2006). About 250 mg of each purified proteins was layered directly on a 10.5 ml of 10–40% (w/v) sucrose step gradient in a buffer containing 25 mM Tris (pH 8.0), 250 mM NaCl, 2 mM Sodium bisulphite and 0.05% Triton X-100. Gradients were centrifuged in a Beckman SW41Ti rotor at 35,000 rpm for 20 hr at 4°C. Fractions (250 µl) were collected and subjected to 10% SDS–PAGE. The protein bands were visualized by silver staining. Protein molecular mass markers were run in parallel gradients, viz., Aldolase (158 kDa), Ferritin (449 kDa), Thyroglobulin (669 kDa). Each fraction of 250 µl represented a sedimentation distance of 2.12 mM as an 11 ml solution filled up an axial length of 89 mM in the centrifuge tube. The sedimentation distance (y in mm) corresponding to a fraction 'f' was represented by the equation $y=67+2.12\times f$, where 67 is the distance from the axis of rotation to the top of the centrifuge tube. Regression analysis using the Microsoft Excel application program yielded the equation: $y=35.490+29.754\times\text{Log}(x)$; $R^2=0.997$, where y represents the sedimentation distance (in mm) and x

represents the molecular mass (in kDa). The sedimentation distance for MBP-AC3 was fitted into the standard curve and their native molecular mass was estimated.

3.3.11.4 Phage display

Phage display offers rapid screening for interacting peptides with the protein of choice. We have used the 'Ph.D–12 phage display library' kit for analysing the various peptides that interact with AC3 protein. The protocol was followed as per the technical bulletin of the kit. In brief, the analysis was carried as follows: panning was carried out by incubating a library of phage-displayed peptides with a plate coated with the MBP-AC3 or MBP, washing away the unbound phage, and eluting the specifically bound phage. The eluted phage is then amplified and taken through additional binding/amplification cycles to enrich the pool in favour of binding sequences. After three rounds, individual clones are characterized by DNA sequencing. The sequence of peptides was analysed by 'BioEdit' software and peptides common in MBP-AC3 and MBP interacting peptides were removed Each peptide sequence thus obtained was then analysed searched for homologous regions against the non-redundant protein database at NCBI through 'blastp' programme adjusted for small sequence analysis. Proteins showing a minimum of five continuous amino-acid matches were shortlisted as potential candidates for further analyses.

3.3.12 ATPase Assay

The ATPase reaction was carried out by incubating the radiolabelled ATP [10 μCi of (γ-^{32}P) ATP (6000 Ci/mmol) was diluted 50 fold with 5 mM ATP] with Rep and/or MBP-AC3 in buffered solution [20 mM Tris-Cl (pH 8.0), 1 mM $MgCl_2$, 100 mM KCl, 8 mM DTT, and 80 ng of BSA/μl] for 30 min at 37°C. After the reaction, 1 μl of the reaction mix was spotted on PEI-TLC plate. Plate was air-dried and chromatographed with a running solvent (0.5 M LiCl and 1 M HCOOH). Following completion of chromatography, TLC paper was dried and autoradiographed. The

relative intensities of the released Pi were estimated by densitometric scanning using Typhoon 9210 scanner and analyzed by ImageQuant TL software (GE Healthcare, UK).

3.3.13 Replication Effeciency Assays

3.3.13.1 Replication Assay in Yeast

Replication deficient yeast shuttle plasmid YCpO$^-$ was utilised for the replication assays in yeast (Raghavan et al., 2004). CR-AC3 region of ToLCV was amplified with the primers CR_K2_Fwd_HindIII, CR-AC3_K2_Rev_HindIII (Table 3.3). Amplified fragment was digested with HindIII and cloned into HindIII digested YCpO$^-$ plasmid. Simultaneously, another construct with AC3 mutated at the start codon by site-directed mutagenesis with the oligonucleotides M_K2AC3_Fwd and M_K2AC3_Rev (Table 2, p 34) was amplified and cloned into YCpO$^-$. Both plasmids in equimolar concentrations were transformed into yeast. Transformation efficiency was analysed by counting the colony forming units.

3.3.13.2 Transient Replication Assay in Plant Leaves

The binary plasmid containing normal ToLCV replicon (pCK2 replicon) or AC3 mutated ToLCV (pCK2^{M21} replicon) replicon containing agrobacterium was grown in YEM at 30°C till $OD_{600} \approx 1.0$-2.0. Cells were harvested and washed with sterile YEM to remove antibiotic. Agrobacterium cells were resuspended in YEM to an $OD_{600} \approx 1.0$-2.0 and then agroinfiltrated into tobacco or tomato leaves. Infiltrated leaves were collected at various intervals (1, 2, 3, 4, 8, 10, 12, 15, 18 days post inoculation) and genomic DNA was extracted. PCR was done with divergent primers: M_K2AC3_Fwd and ToLCKeV_AC1_Rev119 or PolyA_Fwd and ToLCV_AC1_Rev38 (Table 3.3) and the amplification which is part of the replicon was visualized by agarose gel electrophoresis.

3.3.14 Construction of Virus Induced Gene Silencing (VIGS) Vector for Tomato PCNA

Total RNA was extracted from of Tomato (variety Pusa Ruby) and first strand cDNA was synthesised using the *RevertAid H Minus First Strand cDNA Synthesis* kit (Fermentas Life Sciences, USA). Three hundred base pair PCNA fragment was amplified using the primers PCNA300_Fwd and PCNA300_Rev (Table 3.3) designed with reference to sequence AJ515747 submitted in Genbank, NCBI. The amplified fragment was cloned in pGEMT vector (Promega, USA). PCNA fragment was excised out using BamHI and cloned in the BamHI digested pCK2 replicon or $pCK2^{M21}$ replicon to generate pCK2-PCNA and $pCK2^{M21}$-PCNA constructs respectively.

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 ToLCKeV-[IN:KerII:05] (henceforth referred to as ToLCKeV) isolate. Purification of the ToLCKeV GST-AC3 was carried out from the supernatant fraction of the sonicated cell lysate using glutathione

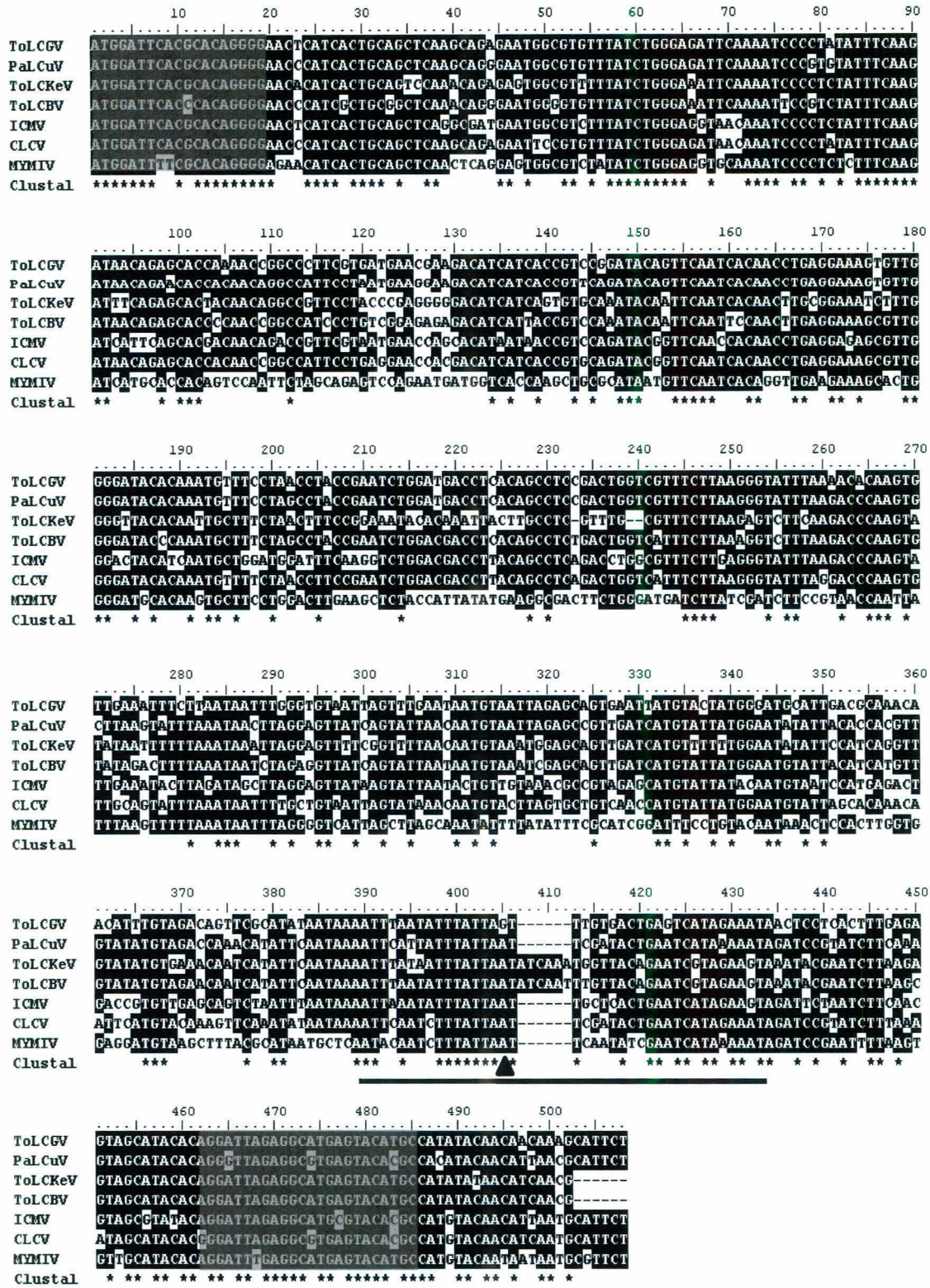


Figure 4.1. Multiple sequence alignment of few selected geminivirus AC3 coding regions. AC3 coding regions of various virus isolates that cause tomato leaf curl disease or mosaic leaf disease were aligned using 'ClustalW' programme. Nucleotides identical by more than 57% are highlighted by shading. The 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 coloumn . '*' indicates that the nucleotides are 100% identical at that particular position. Details of the virus isolates are explained in the table 3.2.

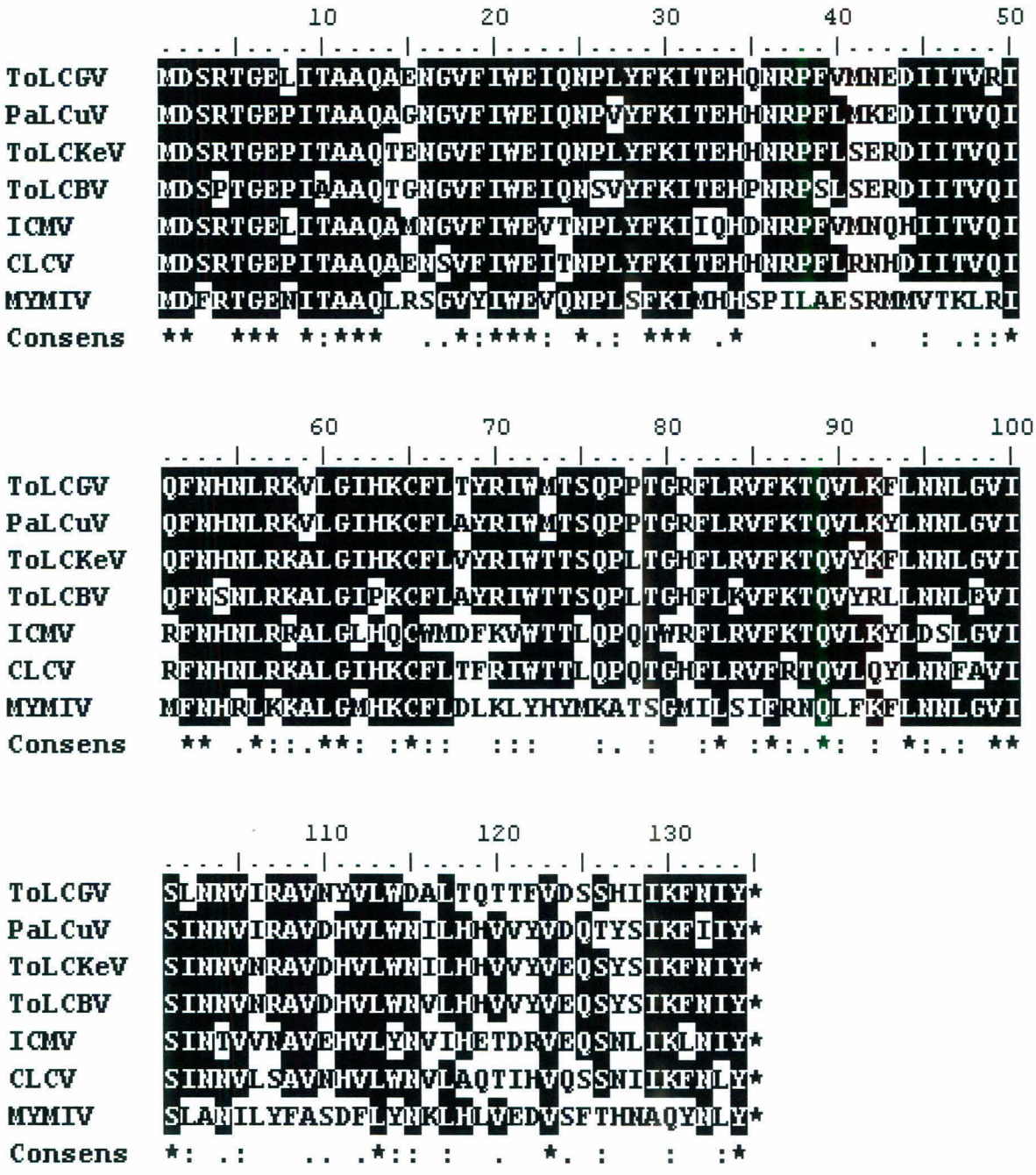


Figure 4.2. Multiple sequence alignment of few selected geminivirus AC3 proteins. AC3 protein sequences of various virus isolates that cause tomato leaf curl disease or mosaic leaf disease were aligned using 'ClustalW' programme. Amino acids showing more than 57% identity in a coloumn were highlighted by shading. Consensus row indicates the nature of the amino acids at that particular position in the coloumn. '*' 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.

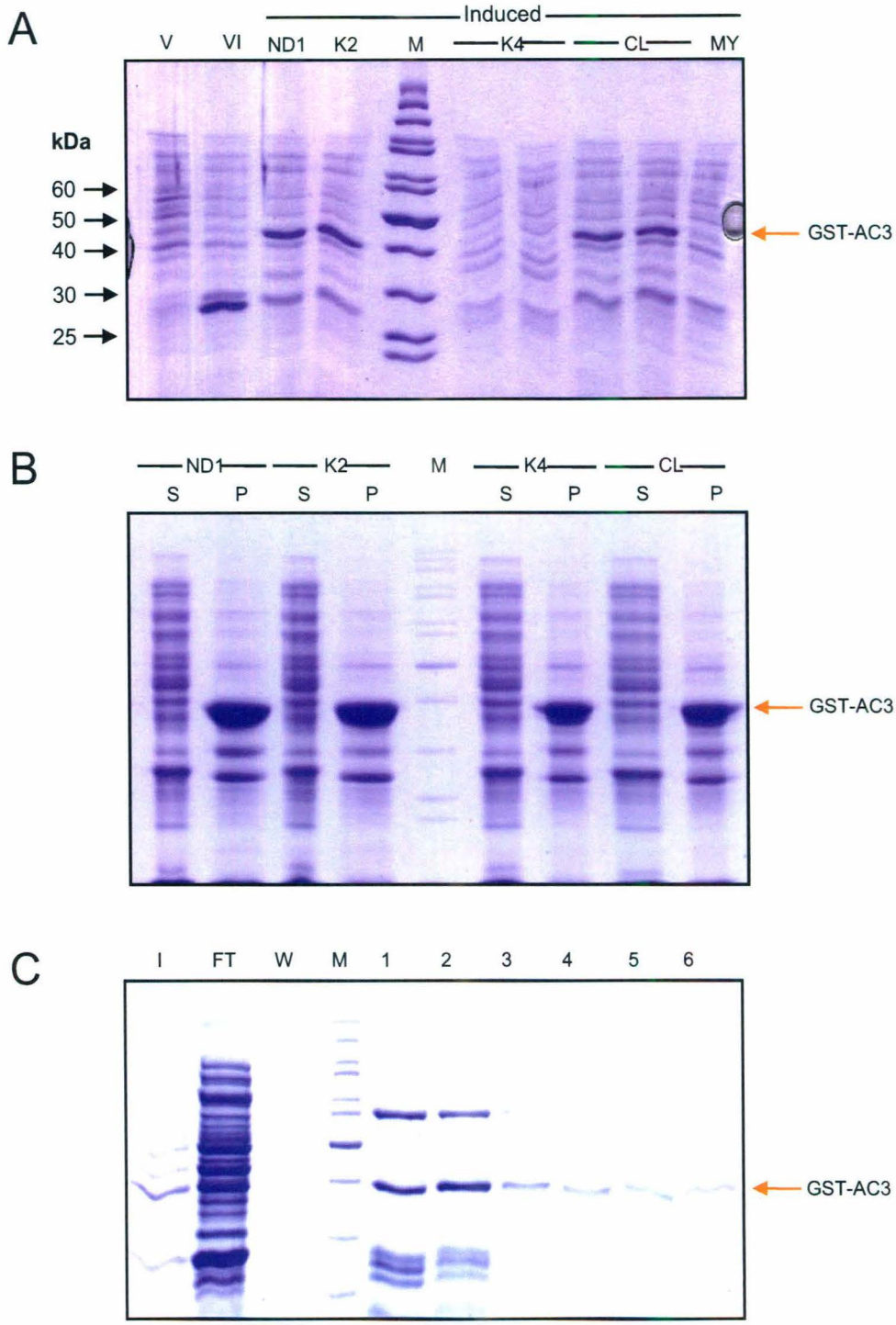


Figure 4.3. Expression and Purification of GST fused AC3. (A) GST-AC3 from selected viruses was induced with IPTG and visualised by Coomassie staining of the SDS-PAGE gels. The red arrow on the right indicates the induced GST-AC3 protein. 'V' and 'VI' indicates the uninduced vector and induced vector samples respectively. The induced viral GST-AC3s are from ToLCGV-[IN:ND:05] (ND1); ToLCKeV-[IN:KerII: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.

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/XhoI 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)

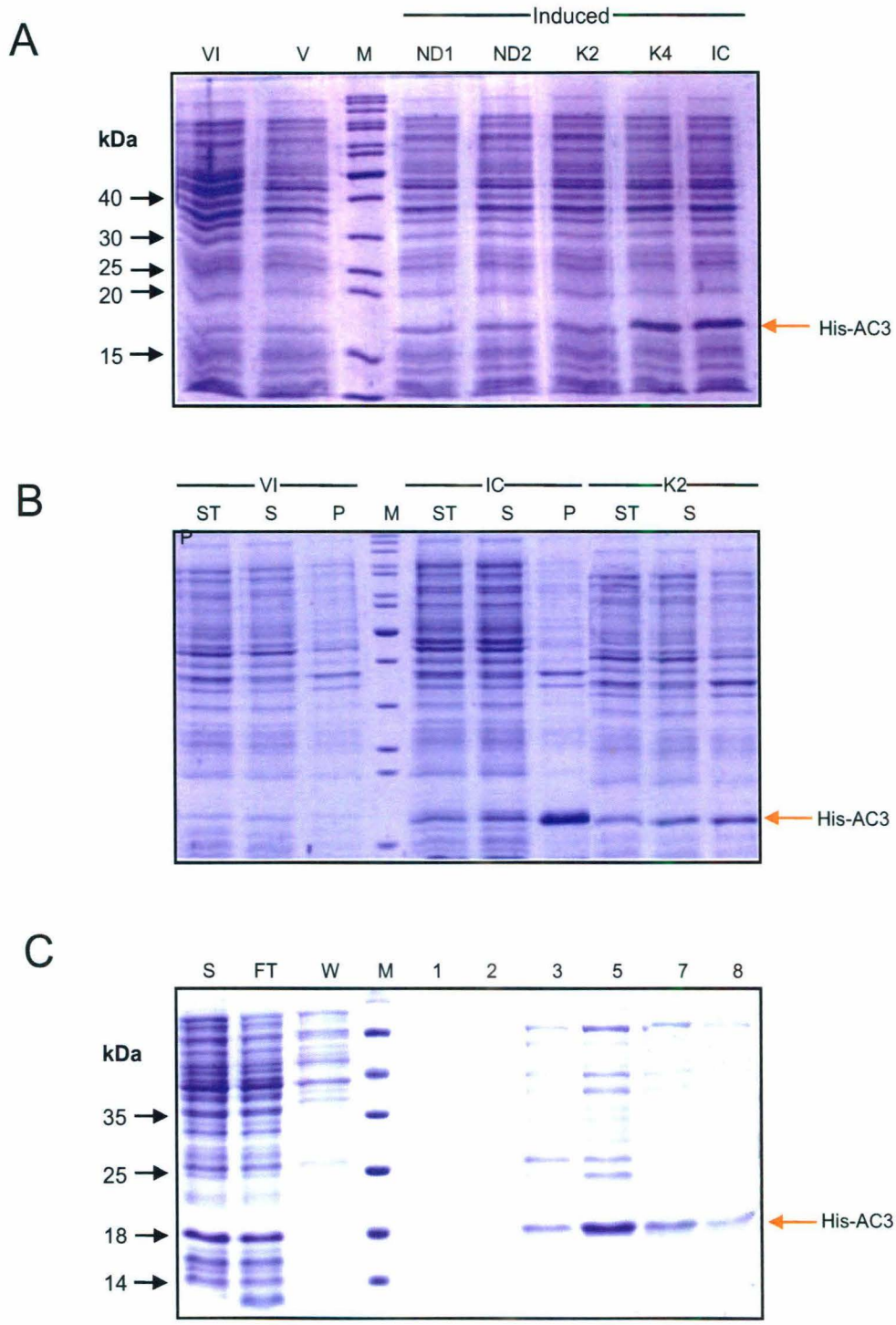


Figure 4.4. Expression and Purification of 6X His tagged AC3. (A) Induction of Histidine tagged AC3 protein with IPTG. 'V' and 'VI' indicates the uninduced and induced pET28a vector sample, followed by AC3 samples from ToLCGV-[IN:ND:05] (ND1); PaLCuV-IN[IN:ND:Tom:05] (ND2); ToLCKeV-[IN:KerII:05] (K2); 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.

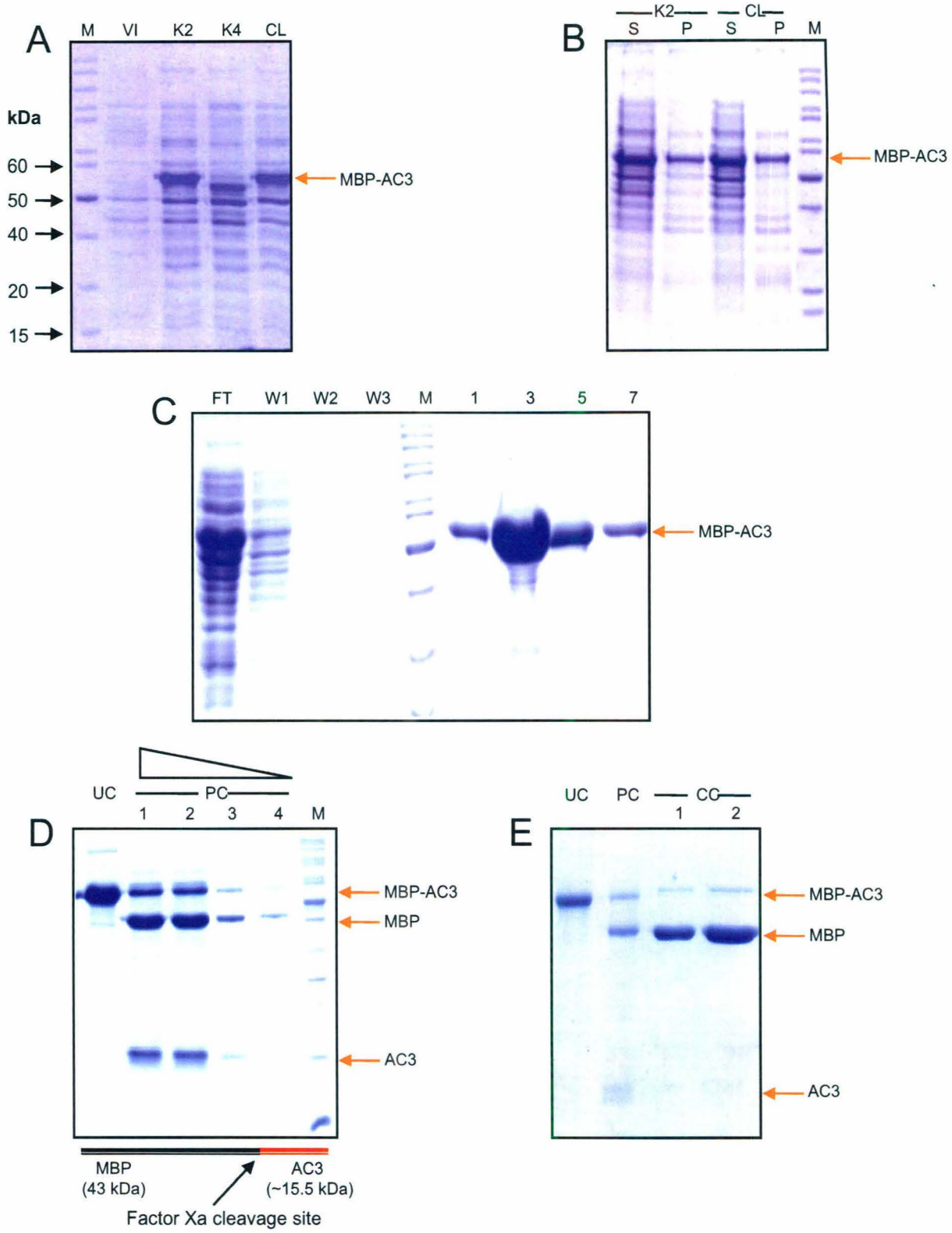


Figure 4.5. Expression and Purification of MBP-AC3. (A) Induction of MBP fused AC3 with IPTG. Samples loaded are induced vector (VI), ToLCKeV-[IN:KerII:05] (K2); ToLCBV-A[IN:KerIV:05] and ChiLCV-IN[IN:PRM:Tom:05] (CL). **(B)** Amount of MBP-AC3 was found to be high in soluble fraction. **(C)** Purification 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.

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/XhoI from the pGEM-T Easy clone and ligated into BamHI/SalI 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 oliogmerisation 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

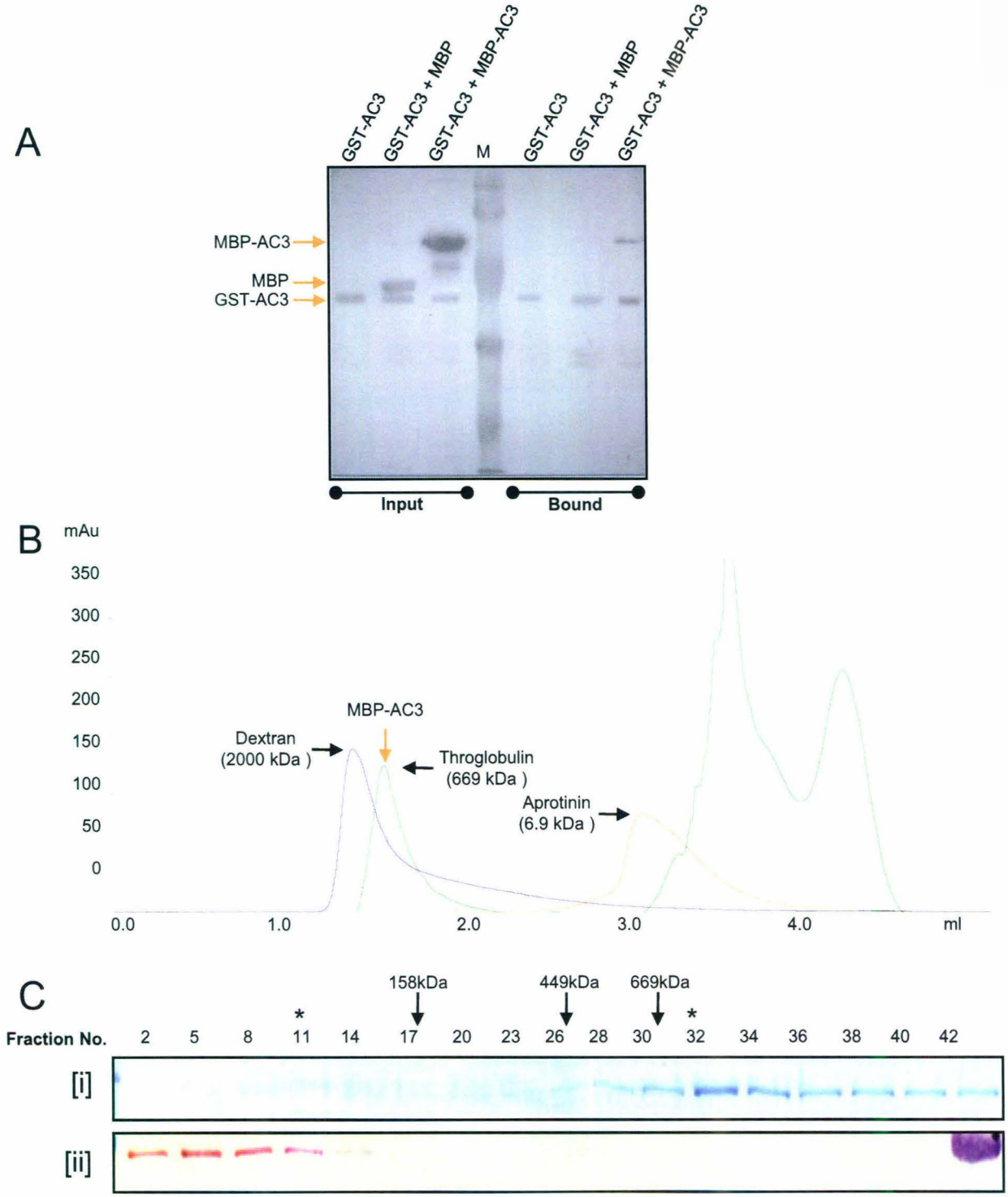


Figure 4.6. ToLCKeV AC3 forms an oligomer. (A) Western blotting of GST pull down assay by MBP-AC3 antibody. Fractions corresponding to 'input' represent the protein composition of the total reaction mix for protein-protein interactions. Fractions corresponding to 'bound' represent the proteins that are 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.

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

A

1	TLTWHTKTPVRP
2	LTYQPLFPTLVY
3	HFKYHHHMLRSP
4	SHEIYVGSDGFR
5	HISPISAYPWVS
6	HGSKHMPQQSTH
7	WHKHIPSPRASS
8	HFKHQHSYARPP
9	AMYYPLWPSLVY
10	FHKEWRTHFQQR
11	FPRAFHHHKIYK
12	LITNNPGRLPPQ
13	TCPRYICQAPHP
14	CPLPYPLCLPHG
15	WHQSWWAARLGQ
16	WHKHPHAVFNAR
17	LSPLYPQLLGLA

18	QTTTAPRHTLWL
19	HLPRHHWQWPSR
20	SSPFXWQSFSEV
21	HSFHSHVHLKNR
22	WHSSWKSRVVPT
23	SLVPSYHRSLST
24	FHKHSPRSPIFI
25	TNVPNPLQPNPR
26	GLLHHKHHRSPY
27	AYSPISTVTQPY
28	APGYARLPSLMS
29	SPRTPDLTSLLP
30	TTGLVQPTAIDP
31	YALKHLPESTIP
32	NVHIRQPLGASS
33	SHWWARVPFYPP
34	GSAVASTLPLGQ

35	LTHNKMHREQAS
36	SMMMPDQLSLGR
37	AVGGQTPIRAKI
38	LLHAPYDHSVSP
39	IPTMPHPSTARE
40	SPPTPHHPHPRL
41	SAPYKPLLHHFG
42	IQSGTPHPPLRS
43	QSMLGFISVSAK
44	HILPWKIPAHSA
45	HSYMPPLPPQLY
46	STAQPRFGPSSL
47	NISSIRPTLVEV
48	DAMIMKKHWHRF
49	YPTSNIIPSIWS
50	SMTHLYTDLWQP
51	YKAPRHLASHLF

52	SYSRTVPPAQWP
53	LEAPRPTPAVPM
54	NAISWFPMHLAH
55	GPLLVLNSHSFD
56	QNNLDYIGLYAR
57	DLQALLPNYPRI
58	FHKYPRPVAMTF
59	HFNHTIPLSANP
60	QHVQHQLASTGE
61	TTNIYFNTPAEV
62	HILSPSGSPRMS
63	ELTWPHVHRKPS
64	ERQGTPYSMYVL
65	IPHPHRWPLHSH
66	LHSSVQLTYPLP

B

- TAF2
- Histone Methyltransferase
- Histone Acetyltransferase
- Pectin Methyltransferase
- Pectin Methyltransferase inhibitor
- DNA and RNA helicases
- Brahma
- Kanadi
- Wox8
- Wuschel
- VQ motif
- MutS 2, 5, 7, 4
- Retinoblastoma like
- E2F
- CDCs
- CDK
- Rev1,3
- Rec A
- Nucleoid binding proteins
- Excision repair proteins
- Gyrase
- Primase
- ATM
- AT Hook DNA binding proteins
- Sumo Protease
- Regulator of chromosome condensation
- Variation in Methylation
- MCM8
- RNA lariat branching enzyme
- BRCA
- DNA polymerases
- RNA polymerases
- Rep protein A 1
- Argonaute 1
- Argonaute 7
- RAD 1, 4, 5, 23-3
- RAD 50
- DCL 1, 2
- Rec Q
- Werner Helicase
- Hua Enhancer 1, 4
- Suppressor of Gene Silencing 2, 3
- Repressor of Silencing 1
- NAC domain containing proteins
- Geminivirus Rep Interacting Kinase 1
- Anti Silencing Factor 1b

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.

A

Replication protein A1(RPA1)	211	WWKIRFYP	219
	219	PISTV	223
	273	HFKH	276
	285	WHTKMWPV	292
Geminivirus Rep interacting kinase 1 (GRIK1)	164	MIMK	167
Retinoblastoma like protein (pRBR)	317	HKIY	320
Anti silencing function 1b (ASF 1b)	43	IYVGS	47
RecQ Helicase	678	FHKSSPNTLAARSAI	692
	326	LKHLPSII	333
	697	HAPYE	701
	483	LTYPLP	488
	89	SVNNTY	94
	254	NPLKPN	259
Werner Helicase	501	LLHHK	505
RAD1	436	ITNNP	440
RAD5	42	NIIFDTP	48
	606	QNNLEDLY	613
RAD4	556	SHEIY	560
	655	PLCLP	659
RAD23-3	119	APRPTPA	129
	129	APAPTRPPPPA	135
RAD50	306	KEWRTHFQQR	315
	512	HEIY	515
	572	GRLPPE	577

B

H3-K9 Methyltransferase	87	PPLRS	91
	26	PLRS	29
Histone Methyltransferase	340	KTPVRP	345
Histone acetyl transferase	562	QWPS	565
	986	AMYY	989
Decreased DNA methylation (DDM1), SNF2	294	KHMP	297
Variant in methylation 2 (VIM2), VIM4, VIM5	448	PRPLPNVP	455
Increase in Bonsai methylation 1	556	HVQH	559
Maintanance of methylation	394	IPSPSG	399
	1588	PSGS	1591
	1821	SPSGAPR	1827
Decreased methylation to DNA (MET1)	113	LPLG	116
	392	AVRSTALG	399
	720	GSAV	723
MET3	250	STLPLPGQ	257

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.

A

Repressor of Silencing 1 (ROS1)	79	GQTPI	81
Suppressor of gene silencing 3 (SGS3)	237	AISWFPMHPLLAH	249
Hua Enhancer 1 (HEN1)	704	YALKHIRES	712
(HEN4)	403	AYGRPIETMTQ	413
	858	TVTRPY	863
Dicer-like 1 (DCL1)	687	LPSL	690
	948	PGTAR	952
	1329	RLPSIM	1334
Dicer-like 2 (DCL2)	29	HQYTDL	34
	246	IPSPKRAS	253
	1224	HKHI	1227
	1235	HKHI	1238
Argonaute1 (Ago1), Ago2	47	NVSVRQP	53
Ago1	129	VSS-QPTLSEV	138
	366	SIRPT	370
	649	SARPEQVE	656
Ago2	910	THYYT-LW	916
Ago7, Pinhead like protein, zippy	14	KHIPS	18
	25	LLHKPYHHHV	34
	75	HNSLPPPPP	83
	80	PPPPPHL	86
	91	PPLPPLL	91
	98	PLPP	101
	184	YNVEISP	190
	293	PLPPE	297

B

RNA dependant RNA polymerase (RDR1), RDR2	18	AARLGQ	23
RDRP	933	LYPQALAL	940
	1003	W-LHSH	1007
	1292	PHEWPL	1297
RDR6	55	YPNFEIADTSNI-PSI	69
	1033	DLIPEAW	1039
DNA pol γ2	17	HL SPSSS--WVS	26
DNA pol ε subunit	1855	FMDQHNYA	1862
DNA pol α subunit	115	TNKSQRLHP	123
	588	NPGRL	592
DNA pol η splice variant	157	MNRE	160
	213	HKNM	216
DNA pol ζ catalytic subunit	1460	HRIFNAR	1466
DNA pol λ	247	LKHLP	251
DNA pol δ small subunit	311	NFHSFD	316

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). NcoI/XhoI digested AC1 fragment from pJET1/blunt vector were ligated into NcoI/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

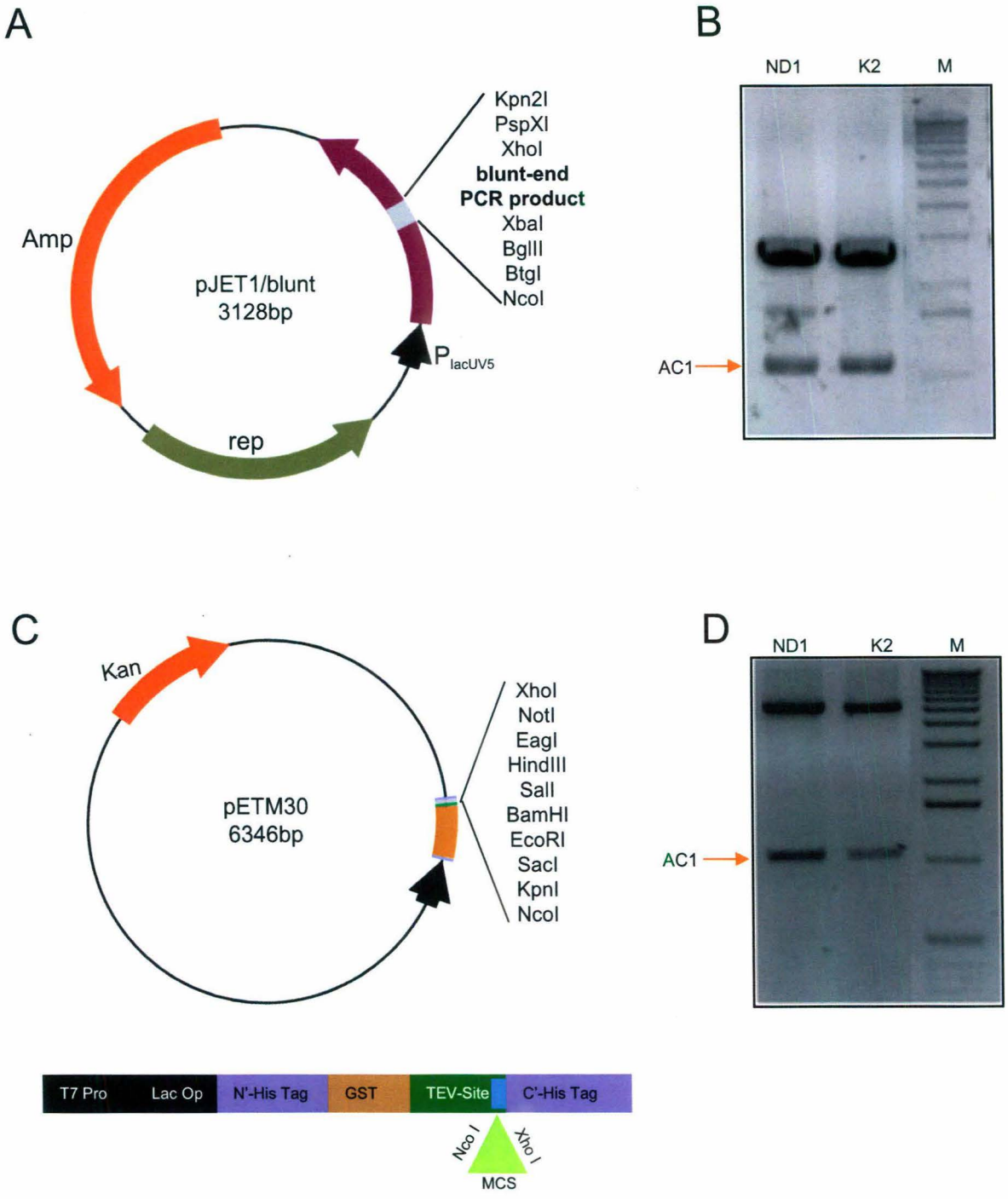


Figure 4.10. Cloning of AC1 into pJET1 and pETM30. (A) AC1 coding regions from ToLCGV and ToLCKeV was PCR amplified, blunt ended and cloned into pJET1/blunt. **(B)** Cloning into pJET1/blunt was confirmed by digestion with NcoI and XhoI. **(C)** Schematic map of pETM30, an expression vector used to express 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 NcoI and XhoI.

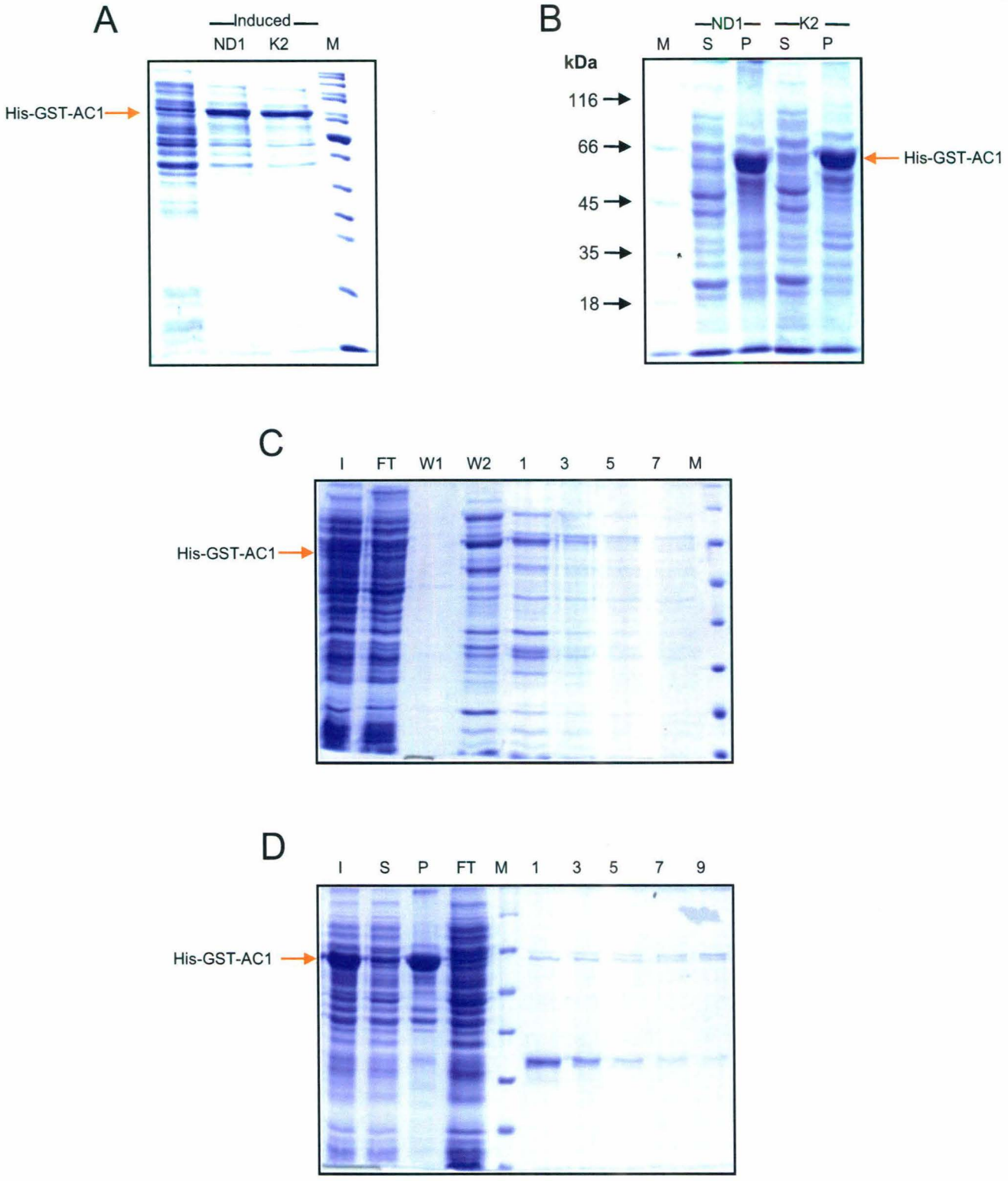


Figure 4.11. Expression and purification of His-GST-AC1. (A) Expression of ToLCGV (ND1) and ToLCKeV (K2) AC1 proteins in pETM 30. **(B)** Most of the induced protein was observed in the pellet fraction after sonication with a small amount of the protein in supernatant. **(C)** Purification of K2-His-GST-AC1 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.

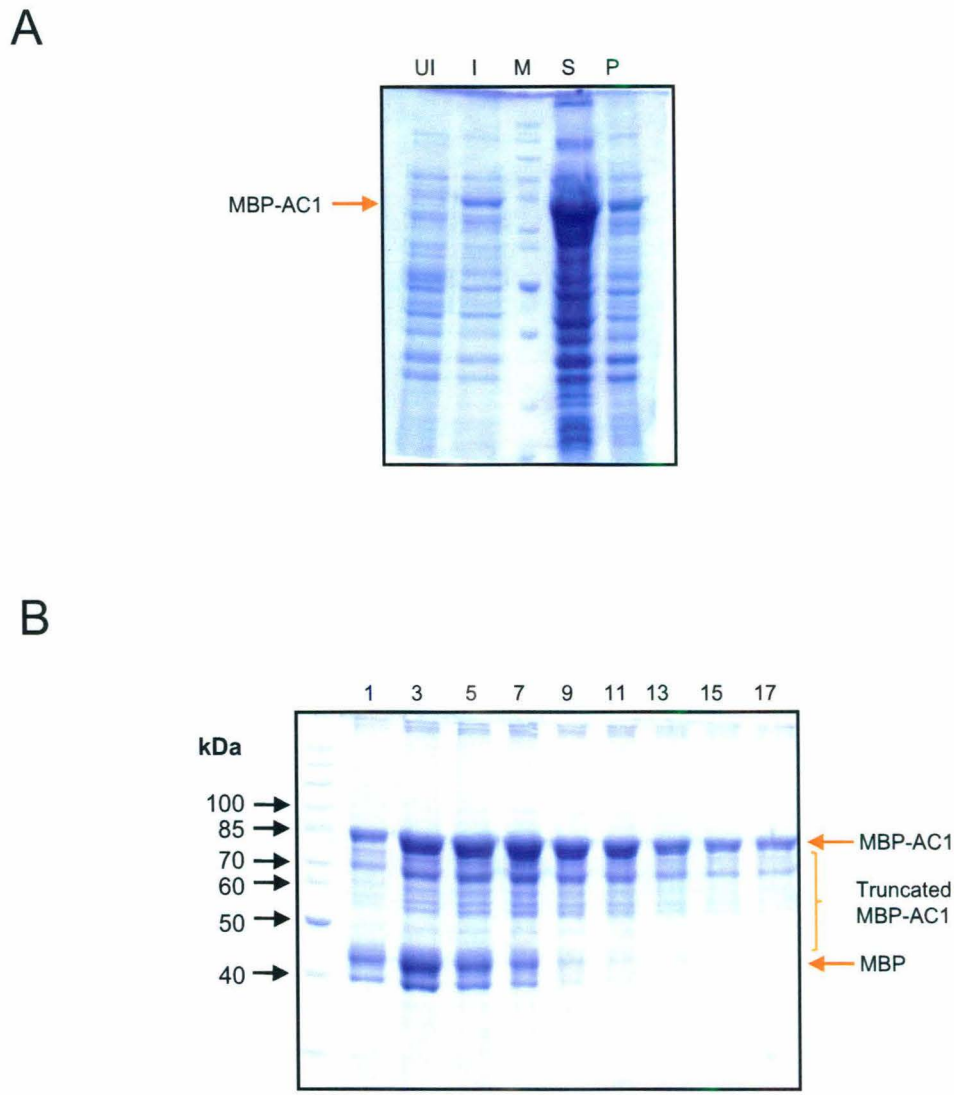


Figure 4.12. Expression and purification of MBP-AC1. (A) Expression of K2-AC1 as a fusion protein with Maltose binding protein. A clear thick band is visible in the induced sample (I). Significant amount of soluble K2-MBP-AC1 was visible in the supernatant fraction (S) of the induced bacterial cell lysate. **(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.

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/SalI digested pGAD-C1. BamHI/XhoI digested AC3 was ligated into BamHI/SalI pGBD-C1. AC1 and AC3 cloned plasmids were co-transformed into yeast strain AH109 and plated onto selection medium (minimal medium lacking leucine,

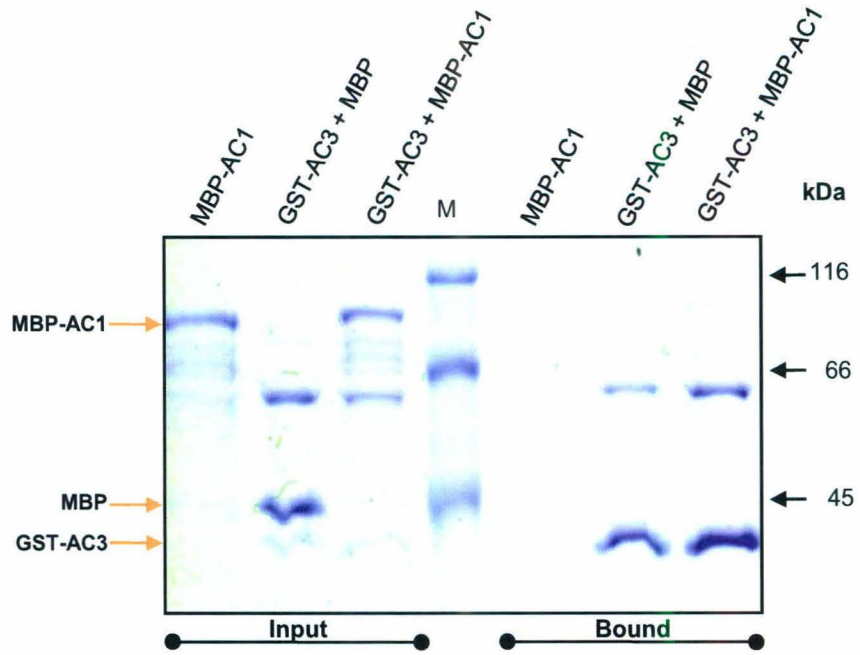


Figure 4.13. ToLCKeV AC3 and AC1 forms an oligomer. Coomassie staining of GST-AC3 and MBP-AC1 showing their interaction. Input fractions represent the total protein composition in the binding reaction mix. Bound fractions represent the proteins that were interacting with GST-AC3 bound to the GST sepharose. 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 trptophan 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 acitivity 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

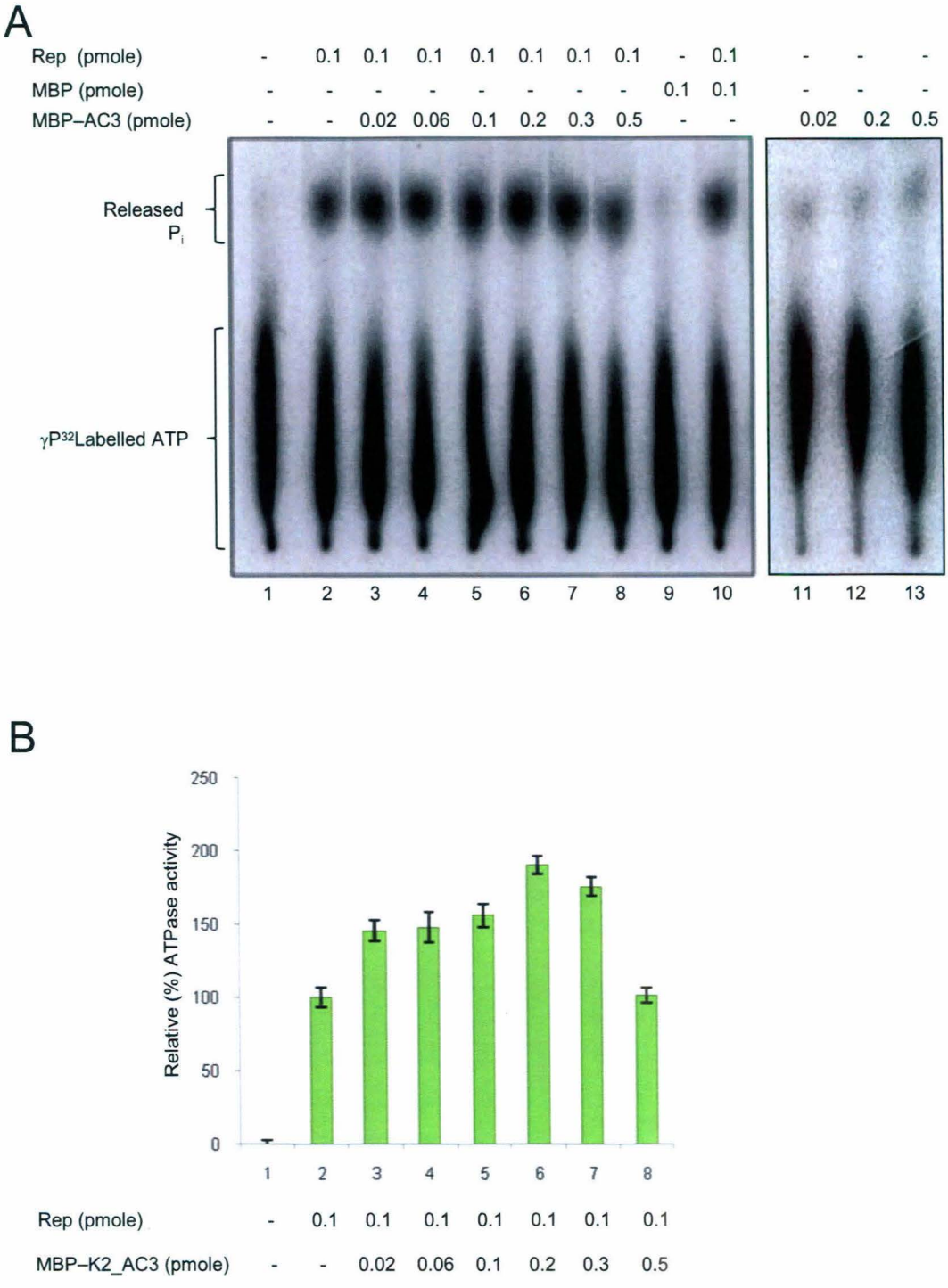


Figure 4.14. AC3 modulates the ATPase activity of Rep: (A) Autoradiograph showing the ATPase activity of Rep in the absence and presence of AC3. AC3 increases the ATPase activity of Rep at low concentration (0.02-0.2 pM) by 50-80%. Composition of the proteins in the reaction mix is shown at the top of 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.

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 ($YCp\text{-}CR\text{-}AC3^M$). Start codon mutation converted methinonine in AC3 to threoinine without any change in the amino acid of overlapping AC2 protein sequence (Fig. 4.16). Effeciency of replication was analysed by transforming yeast cells with YCp-CR-AC3 or $YCp\text{-}CR\text{-}AC3^M$ along with controls YCp50 and $YCpO^-$. Yeast transformed with YCp-CR-AC3 and $YCp\text{-}CR\text{-}AC3^M$ 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

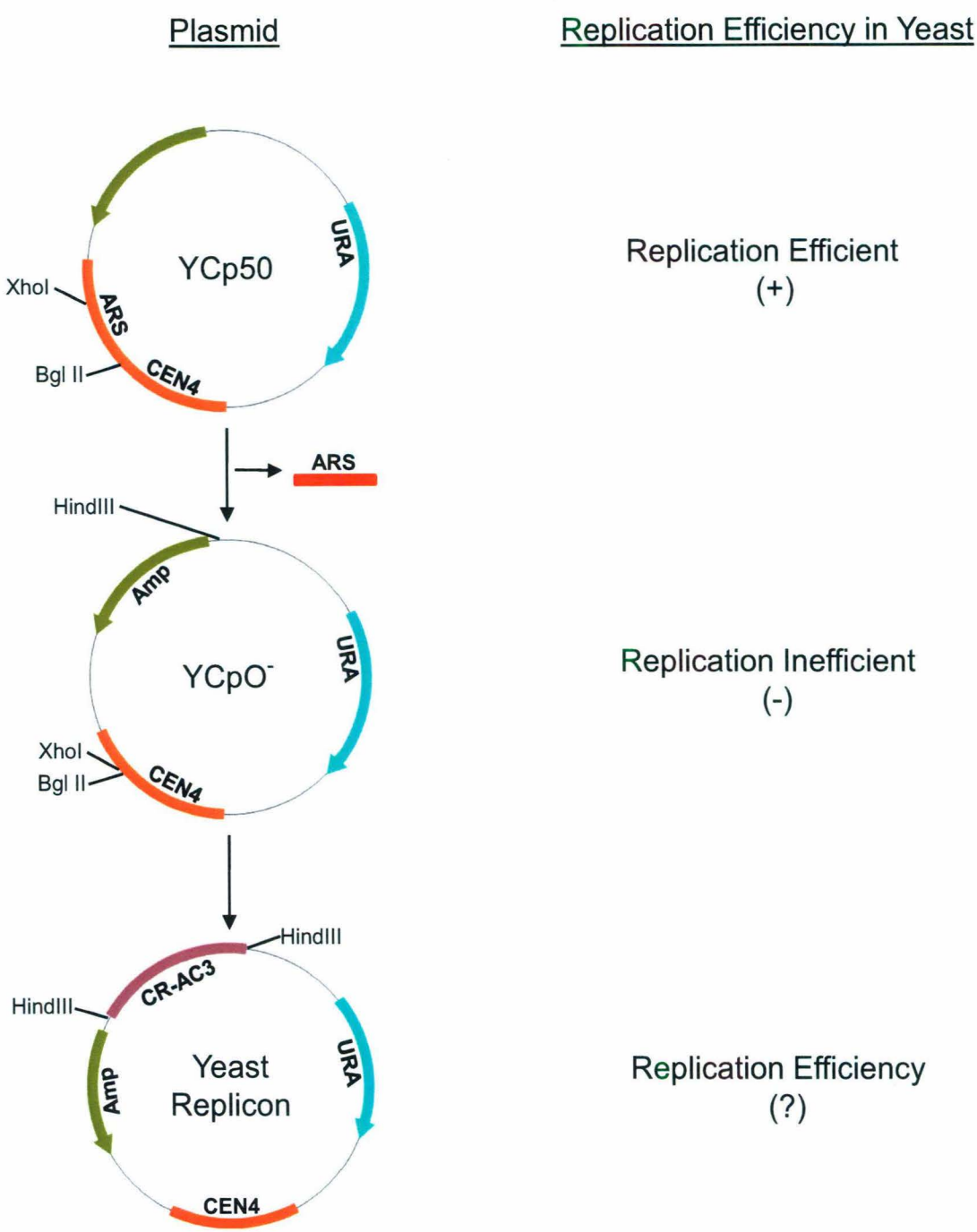
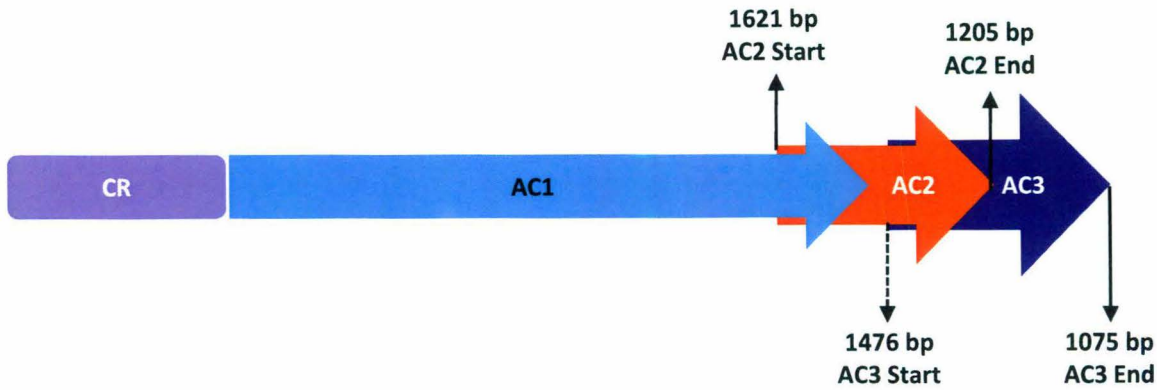


Figure 4.15: Viral replicon construction in yeast. Schematic diagram representing the construction of viral replicon in yeast. YCp50 is a binary plasmid that is capable of replication in bacteria and yeast. ARS and CEN4 sequences of the plasmid confer the ability to replicate in yeast. Removal of ARS 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.

A



B

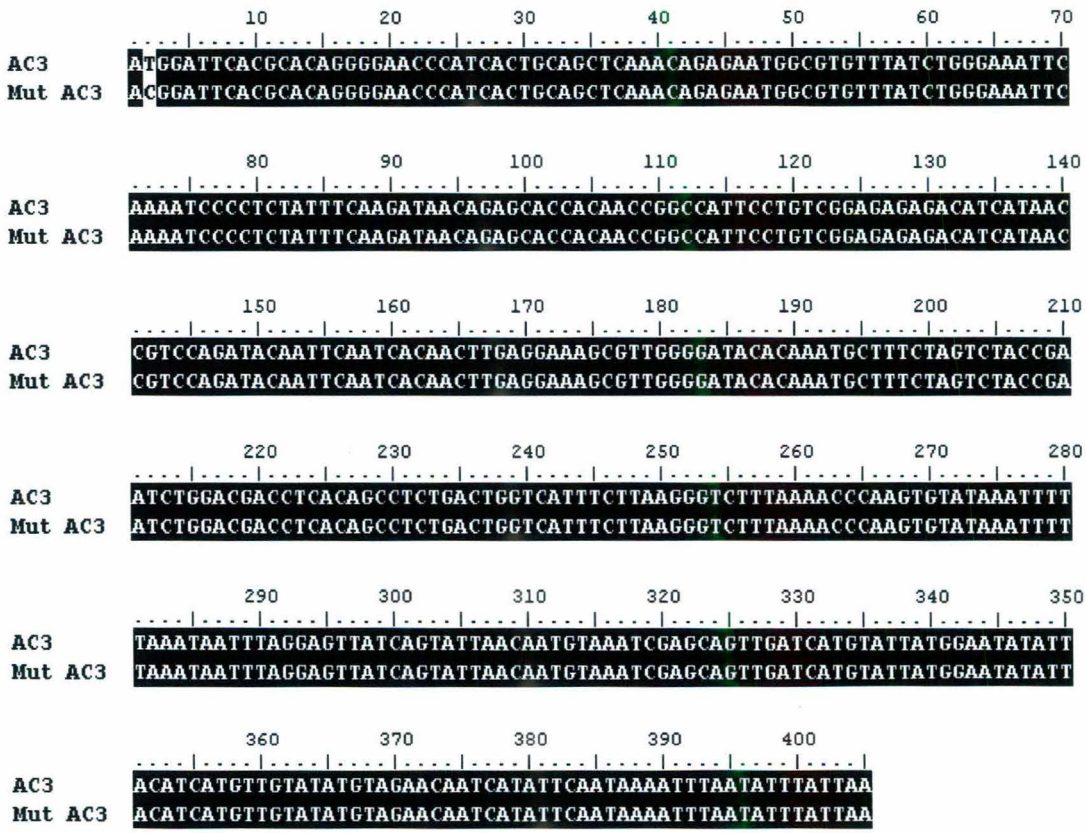


Figure 4.16. Mutation in ToLCKeV AC3 - Strategy I. (A) Schematic diagram of the CR-AC3 region of the replicon construct. The dashed arrow shows the mutation of start codon in AC3. **(B)** Sequence alignment of the mutated AC3 with the wild type AC3. The mutated base is shown against the white background 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 initates rolling circle replication by nicking at the viral origin and ligates the viral strand at the viral origin into a covalently closed circlular 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^{M} (AC3 mutated at the start codon) was also cloned in a similar way. Viral wild-type replicon (pCK2 replicon), and AC3 mutated viral replicon (pCK2^{M} replicon) were transformed separately into agrobacterium and the agrobacterium cultures were infiltrated into leaves of separate young tobacco plants.

Formation pCK2 and pCK2^{M} 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).

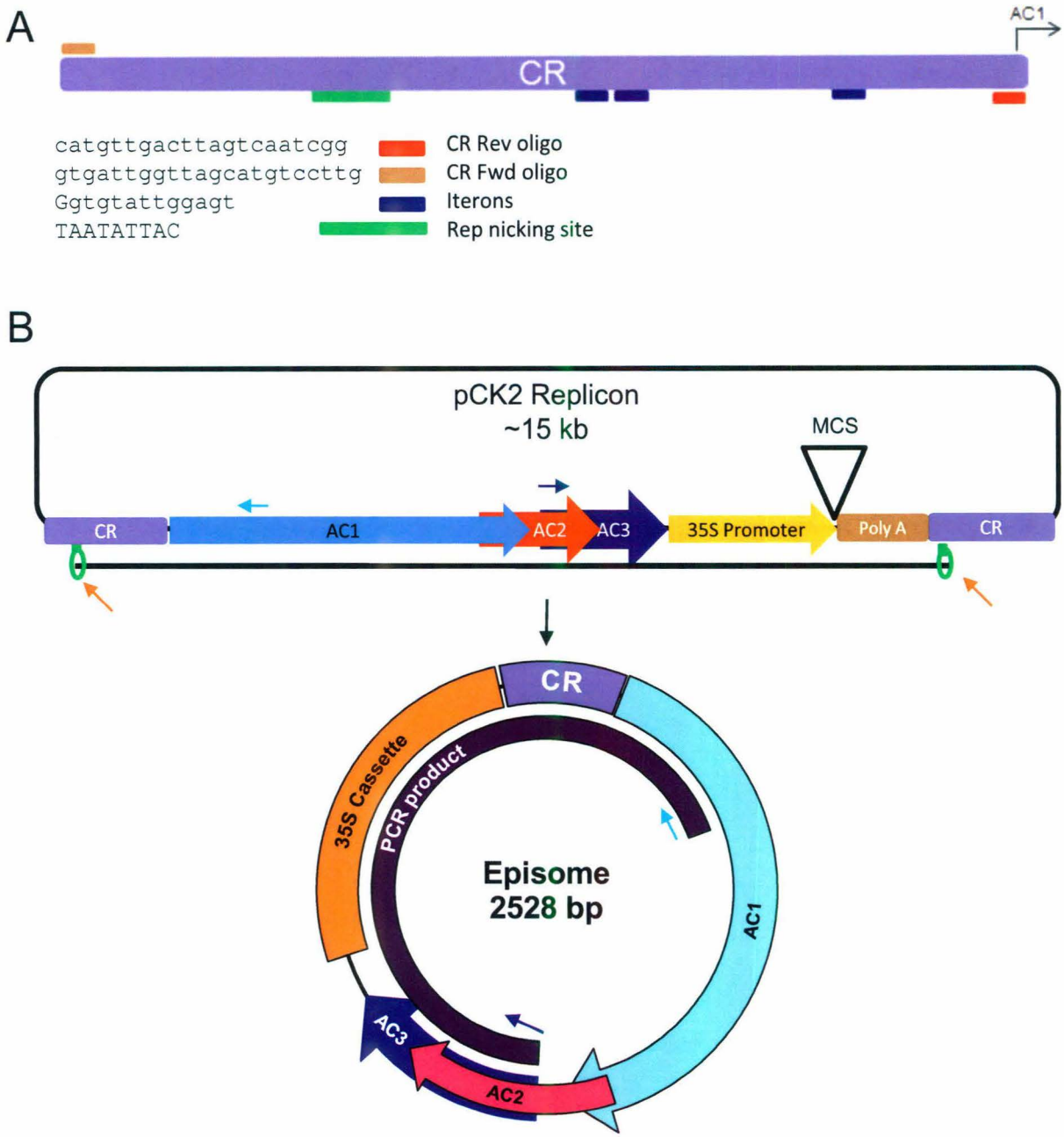


Figure 4.17. Viral replicon construction design. (A) Common region (CR) denotes the origin of replication of the begomoviruses containing cis-acting regions required for replication. Blue bars indicates the iterons where Rep protein binds to the DNA. Green bar indicates the loop region of hairpin where 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.

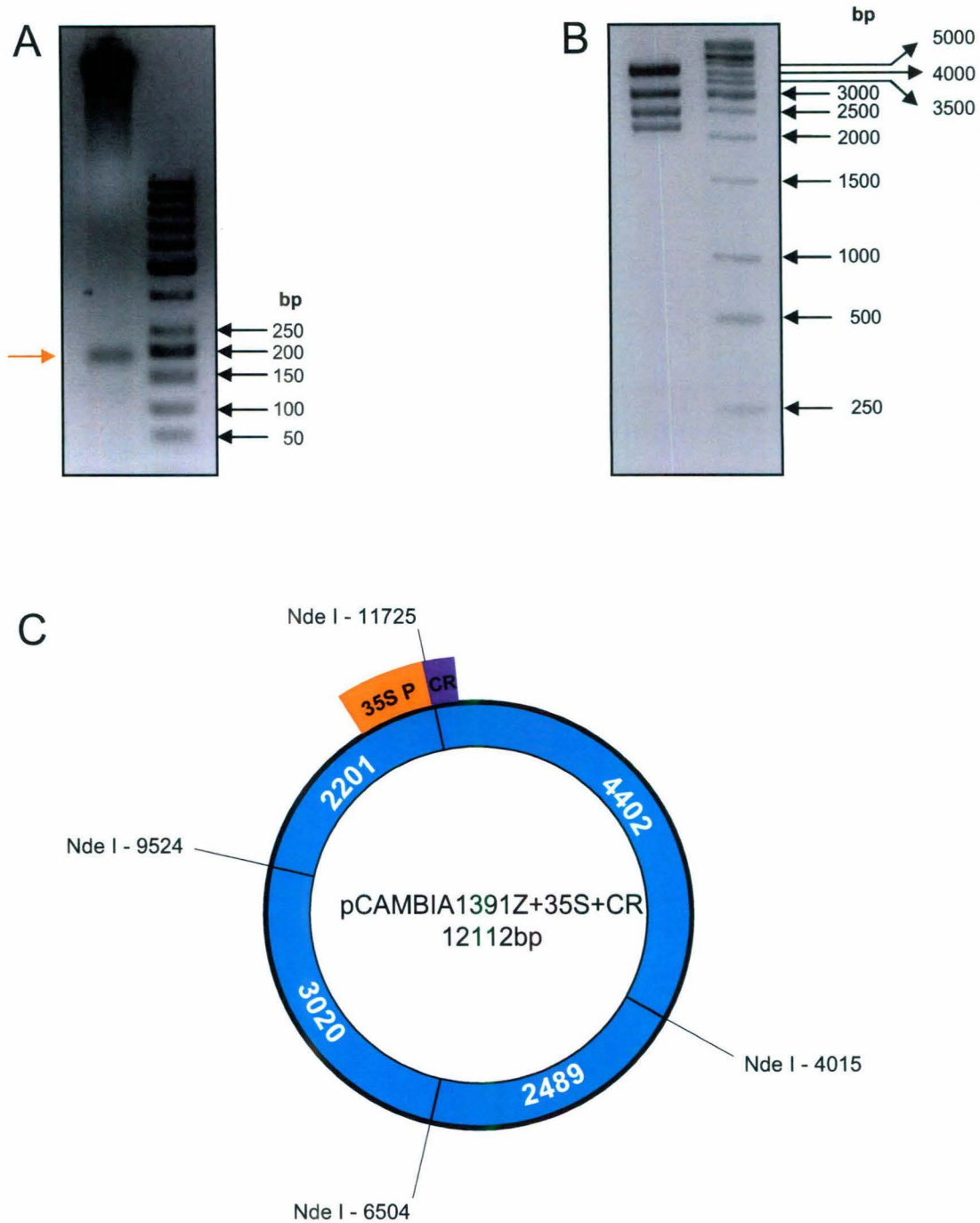


Figure 4.18. Cloning of ToLCKeV-[IN:KerII:05] CR into pCAMBIA1391Z. (A) 232 bp CR region was released from the vector upon digestion with EcoRI as indicated by red arrow on left. **(B)** Fragments released from the vector upon digesting with NdeI. Vector has three sites for NdeI and CR has one at the 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.

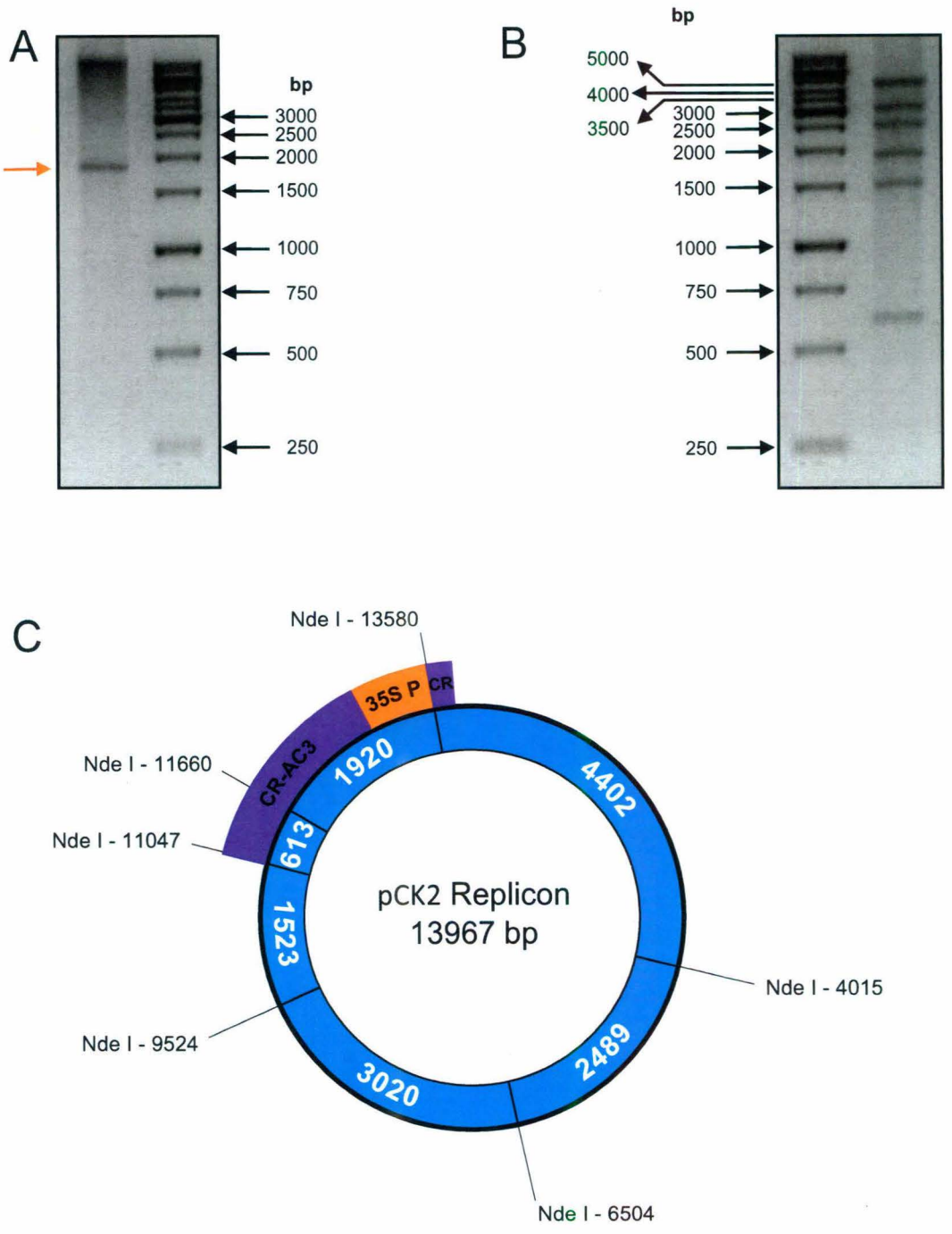


Figure 4.19. Construction of pCK2 (wild type) Replicon. (A) 1849 bp CR-AC3 region cloned into pCAMBIA1391Z was released from the vector upon digestion with HindIII as indicated by red arrow on left. **(B)** Fragments released from the vector upon digestion with NdeI. pCAMBIA1391Z vector has three restriction 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.

Time course analysis of the pCK2 and pCK2^{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_K2AC3_Fwd and M21_K2AC3_Rev (Table 3.3) with the pGEM-T Easy CR-AC3^{M} (AC3 mutated at start codon) as template. In this new mutation (CR-AC3^{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 21st amino acid of AC3 confers a change in the overlapping AC2 (Glycine changed to Valine at 70th amino acid of AC2). Since 70th 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^{M21} was then cloned into binary vector to generate pCK2^{M21} replicon.

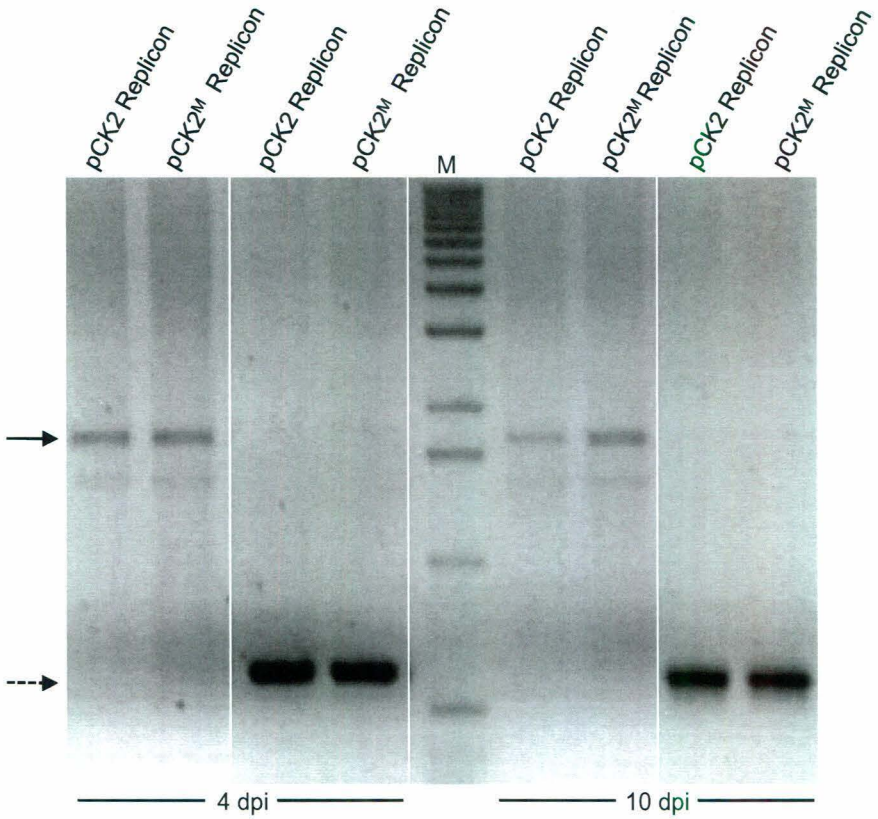


Figure 4.20. Semi-quantitative amplification of episomal DNA from wild-type and AC3 mutated replicon. Tobacco plant leaves were infiltrated with wild-type replicon (pCK2) and AC3 mutated replicon ($pCK2^M$) separately. DNA from the infiltrated leaves was isolated at 5 and 10 days post infiltration. Equal 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.

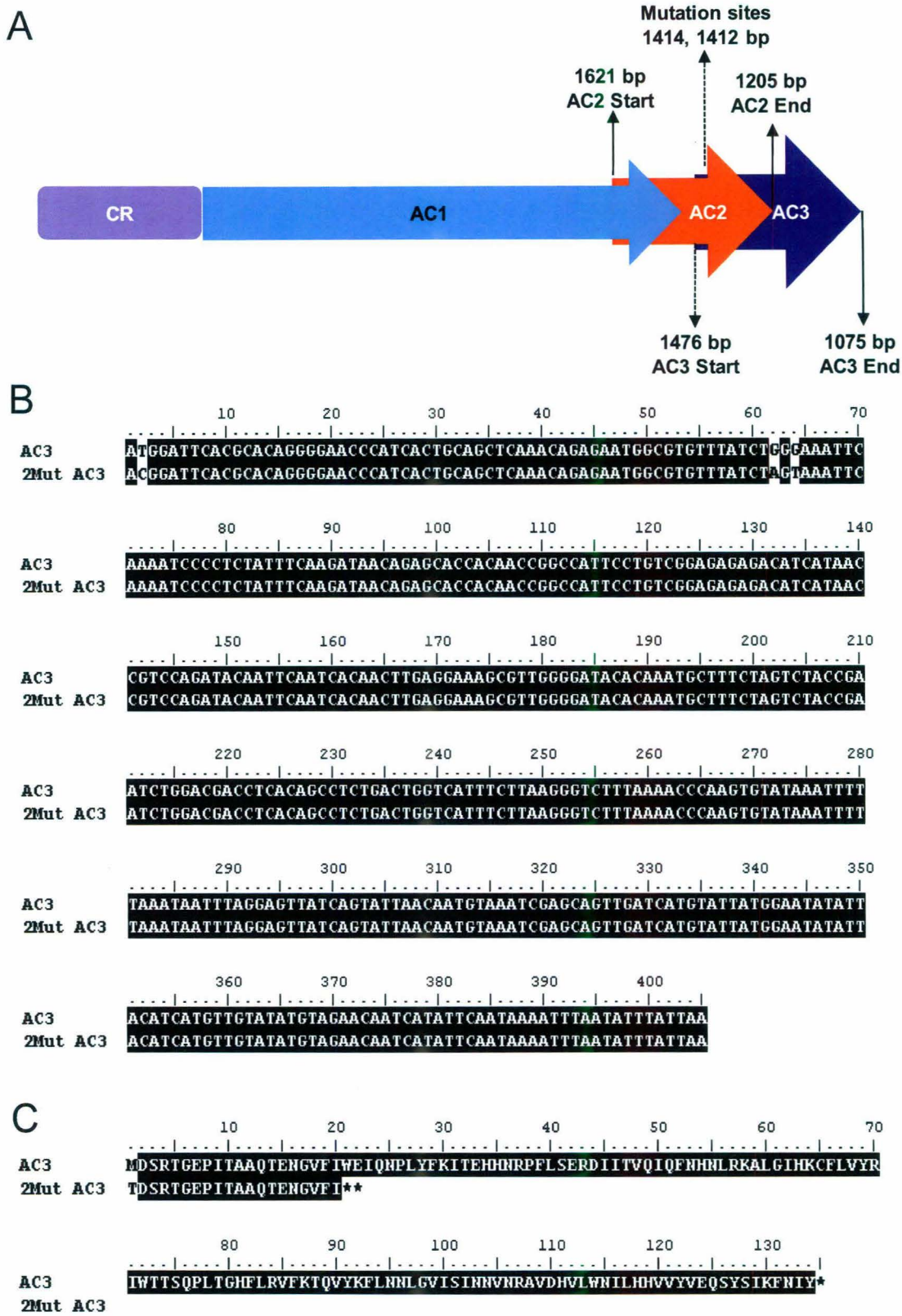


Figure 4.21. Mutation in ToLCKeV AC3 - Strategy II. (A) Schematic diagram of the CR-AC3 region of the replicon construct. The dashed arrows shows the regions of mutation in AC3. **(B)** Nucleotide sequence alignment of the mutated AC3 with the wild type AC3. Mutated bases are shown against the white 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 pCK2^{M21} 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 pCK2^{M21} 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 (pCK2^{M21}). 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

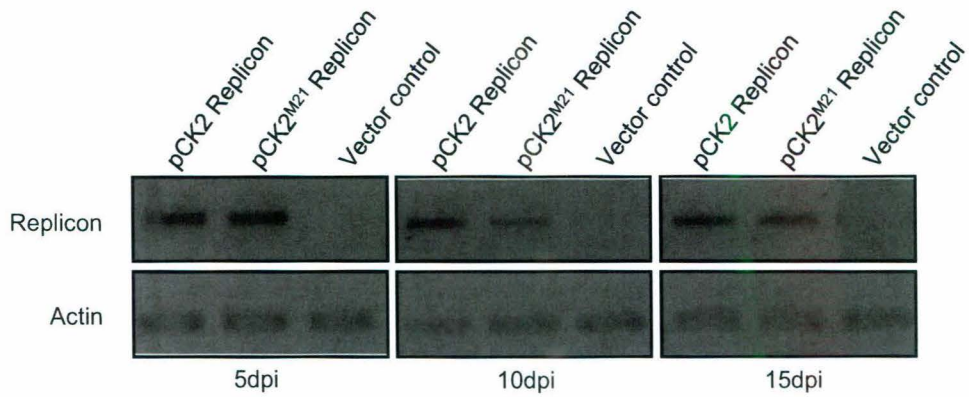


Figure 4.22. Semi-quantitative amplification of episomal DNA from wild-type and AC3 mutated replicon. Tomato plant leaves were infiltrated with wild-type replicon (pCK2) and AC3 mutated replicon ($pCK2^{M21}$) separately. DNA from the infiltrated leaves was isolated at 5, 10 and 15 days post infiltration 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 multi-functional 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 pCK2^{M21} 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 pCK2^{M21}-PCNA (pCK2 with PCNA and pCK2^{M21} 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 pCK2^{M21}-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 pCK2^{M21}-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.

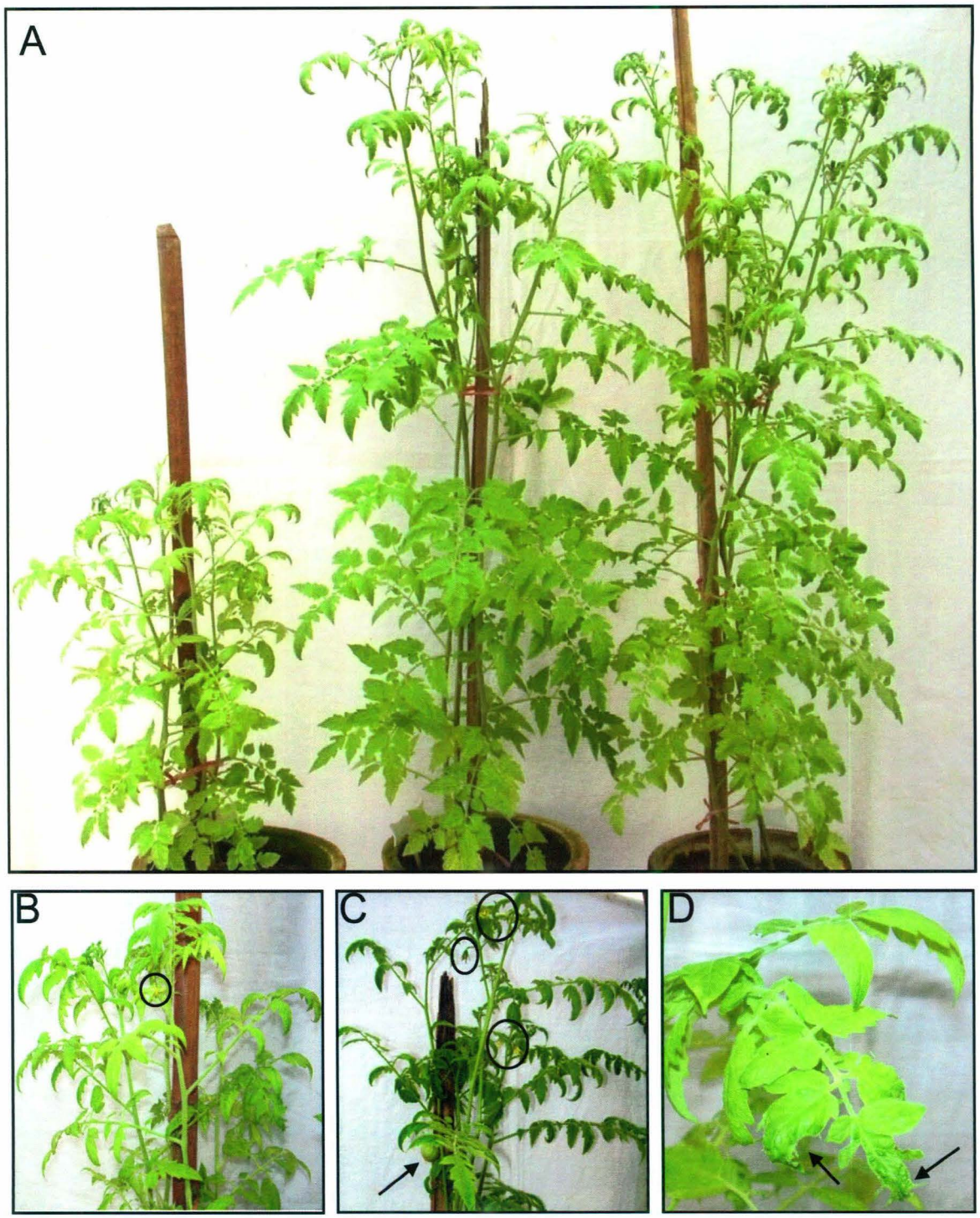


Figure 4.23. Role of ToLCKeV AC3 on gene silencing. (A) High level transcription of a part of PCNA gene lead to the reduced growth of the plant. Retardation is observed in the growth of the plant agroinoculated with the wild type VIGS vector (wild type AC3) along with PCNA fragment under 35S promoter. 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.

Summary

Geminiviral AC3 is an important protein which has been implicated for the successfull viral infection (Elmer et al., 1988). Viruses with truncated AC3s resulted in severe impairment of the viral infection in terms of systemic movement of viral DNA (Etessami et al., 1991; Stanley et al., 1992). DNA replication of the viruses was also affected in protoplast assays of bipartite as well as monopartite viruses (Hormuzdi & Bisaro, 1995; Sunter et al., 1990). However, the unique sequence of the AC3 protein which does not share any homology with any of the characterised proteins and enzymes involved in DNA replication or any of the known metabolic pathways rendered the characterisation of the viral protein very difficult.

In this study an effort has been made to understand the role of the ToLCKeV AC3 which is representative to the geminiviral AC3/C3 protein. The summary of the results is briefed as follows:

- Recombinant ToLCKeV AC3 protein was cloned and expressed in the soluble fraction with GST and MBP fusion tags. The MBP-AC3 protein was purified to homogeneity of ~99%.
- Oligomerisation studies performed with GST pull down assay indicated that the AC3 protein forms a homo oligomer. Multimerisation studies through sucrose gradient ultracentrifugation and gel filtration suggested that AC3 exists as a higher order multimer comprising of 12-14 molecules of AC3 and as a dimer.
- Exhaustive study to identify the AC3 interacting host proteins through phage display indicated that AC3 might interact with proteins from various metabolic pathways including DNA methylation, DNA replication, DNA & histone modification and RNAi pathway.
- AC3 interaction study with AC1 suggested that both the proteins interact with each other. The preliminary assay on

the effect of this interaction suggested that AC3 enhances the ATPase activity of AC1 to an extent of 50–80%.

- *Ex vivo* and *in planta* vector systems were developed to study the role of viral replication. Semiquantitative *in planta* viral replicon assay suggested that AC3 enhances viral replication in the range of 2–3 fold in the agroinfiltrated leaves at 10 dpi.
- We have utilised our *in planta* replicon vector to successfully silence an endogeneous gene PCNA through virus induced gene silencing mechanism. Silencing phenotype of PCNA was more prominent in the presence of wild-type AC3 containing VIGS vector.

The higher order oligomer of AC3 offers a unique ability to interact and bridge the protein-protein interactions in host. The identification of various host proteins involved in the DNA modification and DNA synthesis pathways suggest that AC3 modulates various DNA metabolic pathways. Enhancement of PCNA gene silencing through VIGS indicates the role of AC3 in RNAi pathway(s). Further work regarding the modulation of the biochemical properties of AC1, analysis of generation of siRNA and protein levels of PCNA needs to be done to elucidate the mode of AC3 role in geminivirus.

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