Chapter 6: Summary and Conclusion
Geminiviruses form a unique class of plant viruses which replicate via true DNA intermediates, interfering with the host cell cycle controls and relying extensively on the replication machinery of the plants. In addition, they cause major crop losses in the tropical and sub-tropical parts of the world. Therefore, understanding their replication mechanism may possess dual advantage of understanding the cell cycle controls and the replication machinery of plants as well as to evolve antiviral strategies. So far an easy model system has been lacking to facilitate such studies. The unicellular eukaryote, *Saccharomyces cerevisiae*, offers several advantages in this regard since it is genetically tractable, offers ease of manipulation and a variety of commercially available mutants to facilitate identification of host factors required for viral replication. Moreover, it is known to act as a heterologous host for a variety of RNA as well as DNA viruses and its powerful genetic tools have been used to identify host factors required for replication of RNA viruses such as the BMV and FHV. The budding yeast also offers the potential of development of an *in vitro* replication model which has so far been lacking for geminiviruses. Keeping the above-mentioned points in mind we investigated the potential of the budding yeast to act as a model system for geminivirus replication and our results based on these studies are summarized below.

*Saccharomyces cerevisiae* supports replication of two tandem copies of the viral genome-A of IMYMV.

The ARS element of yeast centromeric plasmid YCp50 (with ura3 marker) was removed by restriction digestion. The ΔARS YCpO− thus created was used for cloning in single copy, and also as dimer, of the whole IMYMV-Bg A-genome which contained the origin of replication as well as ORFs coding for replication and transcription functions. Transformation efficiency was taken as an assay for origin activity. The Gemini-recombinant plasmid (YCpO−-2A) was able to recover origin activity to 34% compared to the ARS-containing YCp50.

The replication of the Gemini-recombinant plasmid DNA in the model system relies specifically on the virus-derived elements and factors.

To determine the virus specificity of replication of the recombinant plasmid YCpO−-2A, a battery of site-specific mutants of DNA-A component was constructed and the origin activities of the plasmids bearing two tandem copies of
the mutant ‘A’ component were assayed. As expected, mutations in the invariant nonamer TAATATTAC where the geminiviral rolling circle replication (RCR) is initiated was lethal whereas mutations in AC1 (replication initiator protein) and AC3 (replication enhancer) greatly inhibited replication of the Gemini-construct. Mutation in coat protein also adversely affected replication of the geminiviral-recombinant construct in yeast. This assay also revealed the role of at least two hitherto unknown viral factors viz. AC4 and AC5 in the viral DNA replication.

Possible role of AC4 and AC5 in geminiviral replication

To determine if AC4 and AC5 exerted an effect on geminiviral replication via interaction with a viral protein essential for initiation of DNA replication, we investigated interaction of both these proteins with the Rep protein, both in vitro using pull down assays as well as in vivo using yeast two-hybrid. Both AC4 and AC5 displayed interaction with AC1 in the pull down assays but the yeast two hybrid analysis revealed that this interaction was weak as growth of the co-transformants carrying AC1 along with either AC4 or AC5 with corresponding AD or BD fusions, on the leu’ trp’ his’ medium was inhibited by concentrations of 3-AT, a competitive inhibitor of histidine (the reporter of interaction) as low as 2.5mM. AC4 and AC5 also interacted with each other withstanding 3-AT concentrations upto 5mM. AC5 was also capable of interaction with itself and could withstand 3-AT concentrations upto 5mM. These results taken together suggest that AC4 and AC5 might be involved at the replication initiation along with the Rep protein forming a hetero-oligomeric complex at the replication initiation site in the geminiviral origin. Moreover, we also detected a ~60 kDa from the yeast cell extract interacting with purified MBP-AC5 in pull down assays, suggesting that AC5 might also have a role in recruitment of certain host factors to geminiviral origin.

The plasmid cdc6-YCp0-2A complements the host cdc6 defect

Complementation of a temperature sensitive mutant of cdc6 with cdc6 gene expressed under its own promoter, carried by the ARS-deficient recombinant geminiviral construct (YCp0-2A-cdc6) further confirmed the integrity of this recombinant construct as an independently replicating vector in yeast.

The common region, AC1, AC2, AC3 and AC4 constitute the minimum requirement for supporting geminiviral replication in yeast.
The region encompassing the CR and the AC1, AC2, AC3 and AC4 was PCR amplified and cloned as a monomer or dimer in either direction in the YCpO' vector. On transforming W303a, all four constructs showed colony forming ability on Ura'selection medium, though with a reduced efficiency compared to YCpO'-2A, indicating that this construct contained the minimum cis elements and viral factors required for supporting geminiviral replication in yeast. The decrease in efficiency however indicates that CP and AC5, which are lacking in this construct, might be contributing to the efficiency of geminiviral replication.

The characteristics of episomal RCR products of YCpO'-2A:
Before proceeding for further characterization of the replicative intermediates in yeast, the presence of the YCpO'-2A DNA in yeast was demonstrated by dot blot analysis using E. coli-derived YCpO'-2A as probe and also by PCR using a combination of gene specific and vector specific primers. To reveal some of the episomal characteristics, YCpO'-2A plasmid DNA, isolated from the E. coli (Cairn's theta mode of replication) as well as yeast (RCR mode) sources were compared by Southern Blotting. Since the presence of single strand in the replicated DNA is the hallmark of rolling circle replication, the aspects of single-strandedness were examined. The isolated DNA products were chromatographed through a hydroxy apatite (HAP)-column, step-eluted using the defined concentration of phosphates to isolate the fraction containing single stranded DNA and further tested for sensitivity to S1 nuclease. About 10-15% of the yeast derived products were mostly single-stranded and were partially digestible with S1 nuclease treatment.

Both the origins of YCpO'-2A retain integrity in yeast
Since we were unable to detect replicational release of the 2.7 kb viral DNA in our Southern blotting experiments, we sought to determine if both the origins of YCpO'-2A are intact in transformed yeast. The 5.4 kb 2A region was amplified from yeast-derived plasmid DNA using primers flanking the Hind III cloning site in YCpO'. The origins of both the A-genomes present were separated onto different sized fragment by BamH I digestion and gel purified. These purified fragments were then used as templates for PCR amplification using combination of primers specific for the CR and the conserved nonamer sequence. The amplification pattern did not reveal any major deletion in the origin. Further, sequencing of the amplified products revealed that not a single base change was
apparent, thus proving that disruption of one of the origins in the YCpO'-2A was not responsible for the failure to observe replicational release in yeast.

**The expression of viral genes from the plasmid YCpO'-2A:**
To visualize the presence and level of viral transcripts and proteins, a few candidate viral targets, namely the AC1, AC3 and CP genes, were chosen. The RT-PCR data, obtained with the total RNA from yeasts harboring the appropriate plasmids, revealed the presence of viral transcripts of expected sizes and such transcripts were not found in absence of the viral genome. The isolated RNA samples were also examined by Northern analysis using the radio labeled Rep-DNA which revealed the presence of a 1.8 kb Rep mRNA in the yeast harboring the YCpO'-2A plasmid. Rep-specific mRNA of same size was also detected in the leaves of IMYMV-infected French-bean plants. Hence, it seems that the viral transcripts produced in yeast and plants were equivalent. The expression of coat protein was confirmed by Western analysis and confocal imaging. The confocal imaging studies revealed that IMYMV-CP was present at the yeast nucleus as well as the cytosol sparing the large vacuolar region.

**Visualization of geminiviral replication intermediates in yeast by electron microscopy**
Since geminivirus replicates via rolling circle replication, formation of single-stranded intermediates is expected. For direct visualization of such intermediates, Electron Microscopy is a method of choice. Before proceeding for the electron microscopy studies, the yeast strain was cured of the intrinsic 2 micron circle, which replicates via recombination events forming partial lariat structures and circles of sizes similar to the YCpO'-2A that could cause problems in distinguishing geminiviral replication intermediates. The loss of two micron circle was confirmed by Southern hybridization with the 2 micron based YEp352-GAL-FLP vector as probe. These cured yeast cells were then transformed with YCpO'-2A and the plasmid preparation from such transformed cells were processed for electron microscopy studies. However, extensive Proteinase K and phenol treatment as well as the careful handling could not remove the protein contaminants and the chromosomal debris from the plasmid preparation which masked the single-stranded replicative intermediates.
Efficiency of yeast deletion mutants to support replication of the ARS-containing plasmid YCp50 and the ARS deficient, geminivirus-recombinant construct YCpO-2A

In order to identify host factors required for geminiviral replication, we screened a few representative mutants for their efficiency to support replication of YCpO-2A in comparison to the control plasmid YCp50. Since recombination-dependent replication has also been implicated as a mode of geminiviral replication, a few recombination mutants namely, mre11, rad51, rad52 and dmcl were chosen for the study. Since the formation of a cruciform structure is prerequisite to rolling circle replication, we also chose mutants of the two isoforms of the cruciform binding protein, bmh1 and bmh2, which have been known to play a role in replication in yeast.

The mre11 mutant strain had the most severe effect on replication of YCpO-2A but also similarly affected replication of YCp50 with transformation efficiency values of 8.2% and 8.4% respectively compared to wild type cells, indicating an essential role of Mre11 in yeast survival. In silico analysis revealed that the MRE11 homologue in Arabidopsis thaliana (At) showed 31% homology to ScMRE11 whereas no homologue of MRE11 was found in the monocot Oryza sativa (Os).

The rad51 mutant also had a similar but less severe effect on transformation efficiency of both YCp50 and YCpO-2A with the relative transformation efficiency values of 37.3% and 39.9% respectively. In the dmcl mutant a decrease in the relative transformation efficiency was observed with the ARS-containing YCp50 (78.1%) but showed an increase in the transformation efficiency with ARS-deficient geminiviral recombinant construct YCpO' 2A (125%). In silico analysis of RAD51 and DMC1 from both Arabidopsis and Oryza sativa revealed percentage homologies of 53% and 50% respectively with respect to those in S. cerevisiae. Though the two plant proteins differed to a similar extent from the S. cerevisiae homologue; within the two plant species, the two proteins had a greater degree of conservation with protein identities of 84% in both the cases.

The rad52 mutant differentially affected transformation efficiency of YCp50 and YCpO' 2A giving values of 89.9% and 53.9% respectively compared to wild type cells. However, a search for homologues of RAD52 in both Arabidopsis and
Oryza sativa was unsuccessful and no homologue was detected in any other plant species as well.

The bmh1 mutant adversely affected the transformation efficiency of both YCp50 and YCpO'-2A but with varying severity. The effect on YCp50 was less severe with relative transformation efficiency of 58.5 % compared to wild type cells, whereas the YCpO'-2A was more drastically affected with the value coming further down to 35.6 % indicating the involvement of Bmh1 in the replication of both the yeast ARS1 and the geminivirus origin. The bmh2 mutant did not affect replication of either YCp50 or YCpO'-2A yielding relative transformation efficiency values of 113% and 102% respectively.