Chapter 5: Discussion
The *Geminiviridae* family includes a large number of plant viruses that produce significant crop losses in economically important crops of both monocotyledonous and dicotyledonous plants (Moffat, 1999). Geminiviruses form one of the largest families of plant DNA viruses and are unique because they replicate via true DNA intermediates in the nucleus of infected cells as opposed to other viruses which mostly replicate via RNA intermediates. Besides being important plant pathogens, geminiviruses also possess the ability to be harnessed for useful applications. Their small, easily cloned, circular genomes are capable of being easily inoculated into plants by agroinfection or particle gun bombardment and replicate to high copy numbers in the plant nuclei, thus facilitating their use as vectors for gene expression, gene silencing and studying of transposition mechanisms (see Timmermans et al., 1994). Moreover, since geminiviruses usually infect terminally differentiated cells and encode only a few factors for their replication thus relying mostly on the plant DNA replication machinery, they have long been known to have the potential to shed light on the cell cycle controls and the biochemistry of replication in plants, about which limited information is available. The reasons for the lack of information regarding DNA replication in plant nuclei are many folds, including its complexity, the absence of defined templates, and difficulty in distinguishing it from organelle DNA replication. These problems have been overcome in animal and bacterial systems by using DNA viruses as models for cellular DNA replication (Kornberg and Baker, 1992). Geminiviruses possess the same potential for plants. In addition to these applications, understanding the biochemistry of geminiviral replication is essential towards evolving anti-viral strategies. Though many *in vivo* systems are available that support geminiviral replication, the establishment of a genetically tractable, easily culturable eukaryotic model system is required to pursue studies on geminivirus replication in a facile manner.

5.1 Establishment of the yeast model of geminivirus replication

Geminivirus replication has been studied over the years using a combination of *in vivo* systems that support geminiviral replication and a variety of *in vitro* experiments. Intact plants, leaf discs, cell suspension cultures as well as protoplasts have been known to support geminiviral replication and have contributed significantly to our understanding of geminivirus replication. Each system is associated with inherent limitations as has been discussed in detail in chapter 2, but taken in total, all the *in
vivo systems known so far have the major limitation of the lack of availability of mutants and the lack of genome information for all the plant hosts. 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 this organism an attractive model system to study viral replication. The ability of the budding yeast to support replication its endogenous L-A double-stranded RNA virus as well as of diverse RNA (FHV; Price et al., 1996, 2000 and BMV; Janda and Ahlquist, 1998) and DNA viruses (HPV; Angeletti et al. 2003 and BPV; Zhao and Frazer 2003) infecting both animals and plants is reported. Keeping in view the immense potential of yeast to support heterologous viral replication, we investigated if the budding yeast could support replication of the representative geminivirus, Indian mungbean yellow mosaic virus (IMYMV). For this purpose, a simple yeast transformation assay was developed by us to test the capability of the wild type and mutant A-genome to replicate in yeast. The IMYMV DNA-A carrying all the *cis* elements and trans acting factors required for geminiviral replication was cloned into the vector YCpO’ which was derived from the yeast shuttle vector, YCp50 (with URA marker) by removing the autonomous replicating sequence and hence was deficient in replication in yeast. The origin activity was measured as a function of the colony forming units (CFU) on ura’ selection medium when the ura auxotroph of yeast i.e. W303a strain was transformed with 6 μg (for YCpO’2A) or 715 fM (for others) plasmid DNA. Two A-genomes cloned in tandem in YCpO’ could recover the origin function to 34% of that of the ARS containing vector YCp50, thus indicating that in absence of the yeast origin, the geminivirus origin was capable of supporting replication in yeast though with less efficiency. This could reflect on the inefficient utilization of the host replication machinery by geminiviruses in yeast owing to the evolutionary divergence of the yeast replication machinery from that present in plants for which geminiviruses have adapted. Alternatively or additionally, it might also mean that the viral proteins function below the plant-level efficiency mark within the yeast hosts. There are similar reports on animal DNA viruses which have been shown to replicate in yeast, namely the human papilloma virus which was observed to replicate stably as episomes when linked to a yeast selectable marker (Angeletti et al., 2003) and the bovine papilloma virus which was shown to be capable of infecting *S. cerevisiae* protoplasts and could
replicate in the infected *S. cerevisiae* cells (Zhao and Frazer, 2003). However, our study is the first of its kind which reports the replication of a plant DNA virus in yeast.

The yeast transformation assay was also used to validate the role of the *cis* elements and the other viral ORFs in geminiviral replication. If geminiviral rolling circle replication was active in yeast it would be expected that mutating the nonamer at the site of replication initiation would severely hamper replication in yeast. The observation that the YCpO'-OriM-2A replicated in yeast, at least 30 times less efficiently than the YCpO'-2A construct suggests rolling circle nature of replication initiation of plasmid YCpO'-2A within the yeast cells. The demonstration that oligonucleotides encompassing this mutation were refractory to cutting by Rep and also failed to compete out the cutting of Ori-Wt by Rep in *in vitro* assays gives support to this. Furthermore, mutation in the tyrosine residue (Y108F) of the replication initiation protein, AC1 which by alignment-based studies could be presumably responsible for the site-specific nicking-closing action drastically reduced YCpO'-2A replication within the yeast cells. Similar decrease was obtained on transformation of yeast with another construct carrying a mutation in AC1, which inserted a stop codon and frameshift after the second codon. The loss of site-specific activity of the recombinant Y108F-Rep in *in vitro* assays with Ori-Wt oligonucleotide correlated well with the loss of replication efficiency with the mutant YCpO'-2A plasmid (YCpO'-Y108F-2A). However, both the mutations failed to completely abolish replication as was expected considering the vital role the Rep protein plays in geminiviral replication. It could be possible that within yeast, a mechanism to by-pass the debilitating mutation in AC1 by internal initiation of translation within the AC1 ORF could be active, leading to a truncated Rep with significantly reduced activity. Similar results were also obtained with *rep* gene mutants in the agrobacterium model of TLCV replication (Rigden et al., 1996). Two lines of evidence from the yeast model validate the essential role played by the replication enhancer in geminiviral replication in yeast. One, the truncation of the AC3 ORF by the cloning site, *Hind* III drastically reduced the transformation efficiency of the YCpO'-A construct leading to the formation of very tiny colonies which did not grow further in liquid culture medium. This defect was recovered when two tandem copies of the A-genome were cloned thus restoring the full length AC3. Further support for this comes from the observation that monomers as well as dimers of the CR-AC3 construct, all of which
had full length AC3 resulted in similar relative transformation efficiencies when compared to the YCpO'-2A construct. Second, mutation in AC3 lead to at least 5 fold decrease in the transformation efficiency compared to the wild type. Preliminary observations from our laboratory that REn upregulates ATPase and possibly the helicase activity of Rep further establish the importance of REn as an accessory factor for geminiviral replication. Investigation of the role of the coat protein using our assay revealed that mutation in coat protein adversely affects YCpO'-2A replication in yeast indicating that at least in yeast CP has a role to play in geminiviral replication. This was in consistence with the earlier model proposed by Qin et al., 1998 which proposes that the CP (AR1) acts to signal the switch from viral dsDNA replication to the replication of viral ssDNA by a rolling circle mechanism. But at the same time it is in contrast to the reports which demonstrate that though CP is not required for replication of geminivirus in protoplasts or plants, mutations in CP lead to dramatic decrease the accumulation of ssDNA either without affecting the accumulation of dsDNA (Briddon et al., 1989; Woolston et al., 1989) or increasing dsDNA accumulation in case of TGMV (Sunter et al., 1990). Moreover, the popularity of coat protein replacement vectors for gene expression also proves that coat protein does not affect replication of the geminiviral genomes at least in whole plants and suspension cell cultures (see Timmermans et al., 1994). The possibility that any mutations in the geminiviral genome could be deleterious to geminiviral replication in yeast was ruled out by the ability of YCpO'-Ori M'-2A carrying a mutation in the origin, in which neither the DNA binding nor the specific cutting site of the recombinant IMYMV Rep protein was affected, to replicate to similar levels as the wild type YCpO'-2A. Moreover, the presence of viral factors was necessary since, the common region alone was not able to confer origin function to the YCpO' vector. Inclusion of the geminiviral factors, AC1 and AC3 along with the origin (CR) in the YCpO- vector (CR-AC3-YCpO') could support replication in yeast but with at least 20 fold reduction in transformation efficiency as compared to YCpO'-2A. Since the ORF AC2 partially overlapped both the AC1 and AC3, and the AC4 completely overlapped the AC1 ORF in different reading frames, both these ORFs were also included in the CR-AC3 construct. These results demonstrate that CR-AC3 region constituted the minimal requirement for supporting geminiviral replication in yeast. Taken together, these observations establish that budding yeast supports geminiviral replication and
that the plasmid YCpO'-2A replicates using the viral replication origin along with the virus-encoded factors. The viral factors were perhaps required for essential replication initiation whereas the yeast factors provided the replication elongation and maturation machinery. Similar partitioning of function might be required within the host plant itself for replication of the geminiviral genomes (Gutierrez, 2000). Given that the plants and yeasts (S. cerevisiae) are diverged in evolution, it is indeed remarkable to observe conservation in the mechanism and machinery of replication in these systems. However, it is possible that the reported model system may not exactly mimic the plant host with regard to the nature and copy numbers of the replicated products, the sub-cellular distributions and the activities of the viral proteins. Despite these shortcomings, development of the yeast model opens up avenues of exploration that were hitherto not possible with currently available protocols in plant virology.

5.2 Novel roles of the AC4 and AC5 proteins in geminiviral replication as revealed by the yeast model

AC4 and AC5 have no previously documented roles in geminiviral replication. The yeast transformation assay has been able to bring forth their roles as important contributors to geminiviral replication. Mutations in both AC4 and AC5 greatly debilitated the transformation efficiencies of their corresponding YCpO'-2A construct, with AC4 having a more severe effect. The AC4 ORF completely overlaps the AC1 ORF but in a different reading frame and hence any mutation in AC4 also leads to a mutation in AC1. Although this mutation in AC4 resulted in a leucine to isoleucine mutation at position 62 in the Rep protein, it did not affect the site-specific nicking activity of the recombinant L62I Rep. However, since Rep is a multifunctional protein the possibility that some other essential function of Rep is disrupted by this mutation cannot be completely ruled out. The demonstration by yeast two hybrid technique that AC4 interacts with Rep, albeit weakly suggests the possible role of AC4 as an accessory factor for geminiviral replication.

The role of AC5 in replication as revealed by the adverse effect of its mutation on YCpO'-2A replication in yeast was an interesting find since no role has so far been assigned to AC5 ORF and its presence as a valid ORF encoded by the geminivirus has been questioned. This result was supported by the observation that the complete absence of the AC5 along with the CP in the CR-AC3-YCpO' synergistically affected the transformation efficiency causing at least a 3-4.5 fold decrease in transformation
efficiency in comparison to the relative transformation values obtained when either of the two ORFs was individually mutated in the YCpO'-2A construct. Many eukaryotic DNA viruses are known to encode proteins which are not required for replication but strongly impact replication efficiency (DePamphilis, 1988; Guo and DePamphilis, 1992). These accessory factors influence replication directly as components of the replication apparatus (Li and Botchan, 1994; Liptak et al., 1996) and/or indirectly as host modulators that alter the cellular environment to favor viral DNA replication (Jansen-Durr, 1996). In both cases, their activities frequently depend on interactions with other viral proteins or host proteins (Frattini and Laimins, 1994; Nevins, 1992). To ascertain if AC5 also impacted geminiviral replication in a similar fashion its interaction with other viral proteins was tested using yeast two hybrid analysis. AC5 was found to interact weakly with Rep since the interactions were abolished by 3-AT concentrations as low as 2.5 mM as opposed to the positive control SNF1/SNF4 which could withstand concentrations upto 50 mM and the AC1/AC1 self interaction which could withstand concentrations upto 20 mM. The strength of the interactions could not be quantitated since they were beyond the sensitivity of the substrate, i.e. ONPG, used for the assay. The interactions were also validated \textit{in vitro} by pull-down assays using bacterially expressed recombinant proteins. These results indicate that geminiviral replication initiation by Rep is assisted by many accessory factors. The observation, that the interactions of AC1 with both AC4 and AC5 were weak, could indicate the transient nature of these interactions \textit{in vivo}. Both AC4 and AC5 also interacted with each other in the yeast two hybrid system with moderate strength, withstanding the inhibitor i.e. 3-AT concentrations upto 5 mM. Moreover, AC5 also demonstrated interaction with itself indicating that it could possibly be acting as an oligomer \textit{in vivo}. There has been previous report of \textit{Begomovirus} AC3 being capable of oligomerization as well as interaction with Rep (Settlage et al., 1996). Taking our results together with this report, we propose that a complex interplay of the geminiviral proteins AC1, AC3, AC4 and AC5 occurs at the geminiviral replication origin to initiate rolling circle replication, contrary to the earlier model which has implicated only the Rep and the REn protein in this function (see Hanley-Bowdoin, 1999). Using pull-down assay with yeast whole cell extract we have been able to demonstrate that AC5 in addition to its interaction with the Rep protein, also interacts with a \textasciitilde{}60 kDa protein of the host. The interaction was quite strong and specific since it could not be washed out even with high molar excesses of salt (upto 800 mM salt).
KCl). It is possible that this protein could be a part of the yeast replication machinery, which is recruited by the AC5 protein to the replication complex in addition to its interaction with AC1. Considering the weak nature of the interaction between Rep and AC5, it could be possible that this interaction is transient but sufficient to recruit the host factor to the replication complex to initiate rolling circle replication. In this way, AC5 could be similar to the AC3 protein which has been shown to interact with both the Rep and the host accessory factor of replication, PCNA (Castillo et al., 2002), and thus suggested to help recruitment of the host replication machinery essential to replicate the viral DNA.

5.3 Characteristics of YCpO'-2A replication in yeast

Before proceeding for characterization of the replicative intermediates in yeast, we validated both the presence of the transforming YCpO'-2A construct in yeast as well as its ability to act as an independently replicating vector in yeast. Dot blot analysis of the replicative intermediates using YCpO'-2A as a probe as well as PCR analysis using a combination of vector backbone specific (YCpFwd) and gene specific primers, conclusively demonstrated the presence as well as integrity of the geminivirus-recombinant construct in yeast. Complementation of an essential gene of yeast, namely CDC6 in cdc6ts mutants with the CDC6 gene carried by the YCpO'-2A suggested that this plasmid could act as an independently replicating vector in yeast. Plasmid DNA isolated from YCpO'-2A transformed yeast was subjected to restriction digestion with unique cutters and analyzed by Southern hybridization using separately, the A-genome and the vector YCpO' as probe, with the corresponding control of E. coli-derived plasmid for comparison. The digestion pattern of both yeast-derived and E. coli-derived plasmids was comparable indicating that no major deletion occurred in the YCpO'-2A during replication in yeast. The presence of CEN4, which is functional in yeast and not in E. coli, was probably responsible for keeping the copy number of the YCpO'-2A plasmid low in yeast, leading to relatively low yields of the plasmid. The difference in the resolving patterns of the undigested plasmid from yeast and bacteria was also not unexpected as the modes of replication of the YCpO'-2A plasmid in both these cases was different, with the Cairn's theta mode of replication active in E. coli and possibly the rolling circle geminiviral mode of replication active in yeast. The presence of single-stranded DNA in the replicated DNA is a hallmark of rolling circle replication, and the observation that at least 10-
15% of the yeast-derived YCpO'-2A DNA was sensitive to the single-strand specific S1 nuclease and eluted in phosphate concentrations preferable for single-strand elution during Hydroxyapatite chromatography, was an indication that the RCR mode of replication was active in yeast.

However, Southern blotting experiments failed to detect the release of a 2.7 kb DNA-A circle in the replicative intermediates which we would expect if both the origins that were present in the YCpO'-2A were simultaneously active in yeast. Such a replicational release of the geminiviral DNA had been observed in the BCTV genome, agroinoculated in *N. benthamiana* as a tandem construct (Stenger et al. 1991) and also in the case of TLCV, TYLCV and ACMV replication within *Agrobacterium* (Rigden et al., 1996; Seith et al., 2002) itself.

Since the disruption of the origin sequence would be the first suspect for this lack of replicational release, we first confirmed the integrity of both the origins in the A-genomes cloned in tandem in YCpO'-2A. For this purpose, we resorted to amplifying the tandem copies of the A-genome from YCpO'-2A using Taq: Pfu and primers flanking the *Hind* III cloning site in YCpO'-2A. The 5.6 kb 2A amplified fragment was digested with *BamHI* which separated the two origins in fragments of different sizes. The demonstration that the complete 229 bp CR sequence as well as partial CR sequences encompassing regions both 5' and 3' from the nonamer sequence could be amplified from both the origins present in YCpO'-2A, similar to those obtained from the *E. coli* derived template control, indicated that there was no apparent deletion in either of the origin sequences that could hamper their activity. Comparison of sequences obtained after sequencing of the CR sequences amplified from both the origins as well as that obtained from the *E. coli* control DNA confirmed the integrity of both the origins after replication in yeast. Thus, loss of integrity of either of the origin sequences after replication in yeast was not the cause of failure to observe replicational release from the tandem A-construct. In *Agrobacterium*, it has been shown by quantitative GUS assay, that all the promoters of the TLCV genome are active, but with varying efficiencies with the C1 promoter showing maximum activity (Selth et al., 2002). Under these conditions, replicational release of the 2.7 kb viral DNA has been observed in agrobacterium. It could be possible that expression of Rep to similar high levels is not obtained in yeast, which could be a probable cause for inability of both the origins to be active for rolling circle replication at the same time. It is also possible that the replicational release occurs at very low levels in yeast and
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the lack of any selection marker to enrich these intermediates could be the cause for lack of detection of replicational release of the 2.7 kb viral DNA.

We also attempted to directly visualize the replication intermediates in yeast by electron microscopy in order to prove conclusively the existence of rolling circle mode of replication in yeast, for which we have so far only indirect evidences. 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, which 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 a 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, which were demonstrated by Southern blotting experiments to be present in low quantities. We were able to observe some structures with a beads-on-strings appearance in the electron micrographs (data not shown) which could possibly represent nucleosome like particles of the geminiviral DNA but at this stage we are unable to say conclusively if these structure represent true nucleosome-like geminiviral particles. The possibility that such structures could be present as a consequence of geminiviral replication in yeast is not very remote, since AbMV is shown to form nucleosome-like structures in AbMV infected plants (Abouzid et al., 1988) and a direct interaction of histone H3 cDNA with CbLCV Rep (Kong and Hanley-Bowdoin, 2002) has also been demonstrated using yeast two-hybrid analysis. This interaction was proposed to be important to displace nucleosome assembly of the double-stranded form of geminivirus DNA allowing access of the replication and transcription machinery.

5.4 Viral gene expression in yeast

Since the presence of active viral factors is a prerequisite to geminiviral replication, we sought to prove that viral transcription and translation was active in yeast. Reverse-transcription (RT) PCR data obtained with the total RNA from yeasts harboring the appropriate plasmids, revealed the presence of viral transcripts
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corresponding to Rep, REN and CP. Southern hybridization using the yeast actin gene (which is constitutively expressed) was used as an internal control. Northern blot analysis with total RNA isolated from YCpO'-2A transformed yeast revealed the presence of a 1.8 kb transcript for Rep, which was absent from YCp50 transformed yeast. Observation of similar sized transcript from IMYMV infected French bean plant indicates that the transcription of viral genes in both plants and yeast was equivalent.

At the protein level, we examined the expression of the coat protein, in view of its importance as single stranded DNA binding protein during geminiviral replication and the availability of heterologous ICMV-CP antibody. Western bolt analysis revealed that CP of expected size (~28 kDa) was expressed in cells bearing the YCpO'-2A plasmid. Our confocal imaging studies revealed that IMYMV-CP was present in the yeast nucleus as well as the cytosol, but not in the large vacuolar region, partitioning evenly in the budding cells. This was in contrast to the observation from N. benthamiana var. Xanthi protoplasts, in which the CP of a member of the Begomovirus family, Squash leaf curl virus was found to localize in the nucleus (Qin et al., 1998), suggesting that the subcellular localization of viral proteins in a heterologous host could be very different from that found in its natural host, even though they might be performing similar functions.

5.5 Yeast model versus Agrobacterium model of geminivirus replication: A comparison

Previously it has been reported that A. tumifaciens containing tandem repeats of TLCV DNA in the plasmid pBin19 can support processes resembling viral DNA replication in plants. The TLCV DNAs were produced in the bacterium only when the constructs contained two copies of the viral ori and a wild-type Rep gene. Our initial obeservation that YCpO'-A carrying only a single copy of the origin was unable to replicate in yeast whereas a YCpO'-2A with tandem repeat of the A-genome could replicate, suggested that two copies of the origin might be necessary for efficient viral replication in yeast as well (Rigden et al., 1996). However, in addition to two copies of the origin, this construct also had a functional full-length AC3 which was absent in the monomer construct. When the restriction site that truncated the AC3 was mutated and the CR-AC3 construct with full-length AC3 was cloned as monomer or dimer in either orientations in YCpO', there was no significant difference in the relative
transformation efficiencies with all of these constructs, indicating that neither the number of origins nor the orientation of the genome in the construct is an absolute determinant of the origin activity in yeast. But it is possible that these parameters control the efficiency of origin activity.

The observations that TLCV, ACMV and TYLCV DNA replication was supported by the bacterial cellular machinery provided the first experimental information supporting the hypothesis that geminiviruses may have evolved from pokaryotic episomal replicons (Rigden et al., 1996; Selth et al., 2002). Further evidence for this was provided when it was shown by quantitative GUS assay that various viral promoters were active in Agrobacterium. The finding of TLCV DNA replication in E. coli, although not to level of A. tumefaciens as well as active expression from two viral promoters, namely C1 and C2, raises the possibility that geminiviruses may also replicate in other bacterial species (Selth et al., 2002).

However, several features of geminiviruses are unique to eukaryotes and consequently, it is proposed that geminiviruses have adapted to the peculiar environment of the eukaryotic cell, including advantages and restrictions by gaining functions which are exclusive of eukaryotic replicons. This is supported by our observation that replication of a tandem copy of IMYMV A-genome carried by an ARS-deficient vector can be supported by the eukaryotic, unicellular model host, Saccharomyces cerevisiae. This finding could have immense impact on investigations into varied aspects of geminiviral replication.

Though, both the bacterial as well as the yeast system raise the possibility of development of an in vitro replication system for geminiviruses using cell extracts supplemented by viral Rep, yeast definitely has the advantage of having a replication machinery more close to the eukaryotic plant hosts than the more distant prokaryotic bacteria. Thus an in vitro system developed using the model would prove more useful than the agrobacterial model as a tool for further characterization of geminivirus replication as well as for identification of putative host factors from plants. The added advantage of the budding yeast is the knowledge of the complete genome sequence and the availability of a huge repertoire of replication mutants for screening purposes.

5.6 Potential host factors screened using the yeast transformation assay

The budding yeast S. cerevisiae poses several advantages in identification of host factors as currently there is a wide repertoire of yeast mutants available commercially.
that can be effectively used for screening, utilizing the simple transformation assay developed by us.

Evidences of both homologous and non-homologous recombination events in geminiviruses are well documented (see Bisaro, 1994). Moreover, recombination dependent repair has been proposed to be a major pathway of geminiviral replication (Jeske et al., 2000; Preiss and Jeske, 2003). These processes normally involve proteins of host or viral origin, which work in a Rec-A like manner (Kowalczykowski and Eggleston, 1994). In \textit{Saccharomyces cerevisiae}, mechanisms of homologous recombination are dependent on a set of genes known as the \textit{RAD52} epistasis group for which deletion mutants are available commercially from EUROSCARF.

If recombination dependent replication of geminiviruses is functional in yeasts as well, it would be expected that mutations of the Rec-A like genes would have an effect on geminiviral replication. However, recombination mechanisms are also integral to replication checkpoints in yeast and therefore mutations in genes involved in recombination could also affect yeast replication in general. To rule out the possibility that the effect of mutation were specific to geminiviral replication rather than a result of general effect on yeast replication, we transformed both the ARS-containing \textit{YCp50} and the ARS-deficient \textit{YCpO'-2A} in the wild type cells and the mutants and did a comparative study of the effect of the mutation on the replication of both transformed plasmids.

Transformation efficiency data revealed that the effects of mutations in Mre11 were the most drastic, but the mutations was equally debilitating to replication of \textit{YCp50} and \textit{YCpO'-2A}. Considering this observation together with the fact that Mre11 is involved in a number of important functions such as, the end-joining pathway of repair, telomere maintenance, in DNA replication-associated repair, and in the DNA damage checkpoint in mitotic cells (see Symington 2002) leads us to believe that the absence of Mre11 could be effecting genome stability as a result of interference in the DNA damage response. Thus, the equally low transformation efficiencies of both \textit{YCp50} and \textit{YCpO'-2A} could be due to interference with yeast survival as such and not specific for geminivirus replication. Furthermore, no homologue of Mre11 was found in the monocot \textit{Oryza sativa} and only one homologue was found in \textit{Arabidopsis}, which showed very less homology (37\%) with the \textit{S. cerevisiae} homologue. Similar low homology has been found between the \textit{Arabidopsis} and \textit{S.
homologues of Mre11 suggesting that this protein is not very well conserved between plants and fungi and may be performing different role in plants. Rad51 is conserved in all eukaryotes for which sequence identity is known. Comparison of the plant homologues, AtRad51 and OsRad51 with the ScRad51 reveals a significant homology of 53%. Mouse and human Rad51 proteins also display similar levels of homology with the Sc Mre11 with values upto 59%. Between the two plant homologues, however, the homology is relatively quite high (84%). Though Rad51 is present wherever DNA metabolism is active, whether in the S-phase of dividing cells or in recombinationally active cells that repair DNA damage or initiate meiosis its absence in various systems has varied effects. Yeast rad51 null mutants are viable but show high sensitivity to ionising radiation and meiotic inviability, whereas in vertebrates its absence could be lethal (Lim and Hasty, 1996; Tsuzuki et al., 1996; Sonoda et al., 1998). These observations suggest that by-pass pathways for Rad51 function exist and may be different in different organisms. With Rad51 mutant also, a decrease in transformation efficiency values for both YCp50 and YCpO'-2A (37.3% and 39.9% respectively) was observed, though with less drastic effect than the Mre11 mutant. Thus, like Mre11, Rad51 can also be ruled out as a host factor specific for geminivirus replication.

The Dmc1 exhibits 45% homology to Rad51 at the amino acid level but differs from it in not forming a part of the RAD52 epistasis group as its mutants are resistant to ionizing radiation. Initially, the DMC1 gene characterised from yeast was thought to be associated with the repair of DSBs during meiotic recombination and hence its name deficient in meiotic control (Bishop et al., 1992). Dmc1, besides Rad51, is one of the two main eukaryotic homologs of RecA and forms complexes with DNA prior to meiotic chromosome synapsis. However, the observation that AtDMC1 was induced to a higher level in the exponentially growing cells from a suspension culture and that this expression correlated with the expression of H4, a histone gene specifically induced in early S phase (Doutriaux et al., 1998), suggested that Dmc1 could also be involved in mitosis. Our experiments with deletion mutant of DMC1 indicate that this protein affects YCp50 and YCpO'-2A replication differently. YCp50 replication was hindered in this mutant with approximately 20% reduction in the transformation efficiency as compared to that of the wild type. Interestingly, there was a 25% increase in the transformation efficiency of YCpO'-2A as compared to the wild type in the Dmc1 mutant, indicative of the fact that the absence of Dmc1 was
somehow beneficial to geminiviral replication. This was not a chance phenomenon since the results were consistent in three independent experiments. It could be possible that Dmc1 interferes with the action of either Rep or any of the replication accessory proteins, either by direct interaction or by modulating their function. However, further biochemical characterization is required before we can conclusively comment on this aspect. Since homologues of this protein, exhibiting a significant 50% homology to ScDmc1 have been found in both Arabidopsis and rice, probing the mechanism by which this protein exerts its effect on geminivirus replication would be an interesting study.

Rad52 in S. cerevisiae is involved in many processes of recombination and mutants show the most severe recombination defects of all the rad52 group mutants. Rad52 is nuclear and forms discrete foci in response to IR and during S phase of unirradiated cells (Lisby et al., 2001). Relative to the wild type strain BY4742, a deletion mutation in RAD52 affected the transformation efficiency of YCpO'–2A to a greater extent than that of YCp50, giving relative values of 53.9 and 89.9 respectively. Thus, it is apparent that Rad52 could be playing some specific role in geminiviral replication in yeast, and hence its absence in the deletion mutant was more inhibitory to YCpO'–2A than YCp50. Genetic studies with yeast have suggested the presence of a RAD52-dependent, Rad51-independent pathway for strand invasion, and recent studies have shown the formation of D-loops by HsRad52 (Kagawa et al., 2001). The formation of D-loops by rad52 probably occurs by annealing between the incoming ssDNA and transiently ssDNA present in the supercoiled plasmid. Similar mechanism has been proposed for the recovery of incomplete DNA molecules produced as a result of hindrance to geminiviral replication and conversion to full-size genomic DNA (Jeske et al., 2001). Thus it is possible that Rad52 functions at this stage of geminiviral replication. However, the absence of any homologues of Rad52 in plants raises the possibility that this function in plants is carried out by some other protein which is functionally similar to the yeast Rad52. Our yeast transformation assays do not conclusively support the presence of a RDR-like mechanism for geminiviral replication. But, a large number of recombination genes are known in yeast and plants and therefore, no conclusive statement can be drawn for this aspect unless all of them are screened in the same manner.

14-3-3 proteins are known to be involved in a multitude of biological processes such as apoptosis, signal transduction and cell cycle progression (Fu et al., 2000). In
addition to these functions, the role of 14-3-3 proteins as cruciform binding proteins (CBP) involved in DNA replication in both mammalian cells as well as yeast (Novac et al., 2002; Callejo et al., 2002; Zannis-Hadjopoulos, 2002) has been of special interest to us since, formation of cruciform structures in geminiviral (+) strand replication origins is prerequisite to initiation of rolling circle replication. Transiently forming cruciforms have long been demonstrated to form at origins of DNA replication and serve as regulatory signal for the initiation of DNA replication (Pearson et al., 1996). Thus it is possible that CBPs either form an important part of the geminivirus replication machinery or may be involved in the regulation of geminiviral replication. Two isoforms of this protein are found in S. cerevisiae, namely, the major isoform, Bmh1 and the minor isoform, Bmh2. It has been demonstrated that, though disruption of the BMH1 or the BMH2 gene alone hardly had any effect (van Heusden et al., 1992; 1995), disruption of both genes simultaneously is a lethal event (van Heusden et al., 1995). This lethal mutation could be complemented by four Arabidopsis homologues that were capable of being expressed in yeast (van Heusden et al., 1996), thus suggesting at least some plant isoforms share similar function in yeast. In view of these observations we investigated the role of CBP in geminiviral replication by testing BMH1 and BMH2 deletion mutants in our yeast transformation assay. The bmh2 mutant could support replication of both YCp50 and YCpO'2A to same levels as the wild type cells, BY4742. However, bmh1 reduced the transformation efficiency of both YCp50 and YCpO'2A, though not very significant difference was found in both the values. This indicates the involvement of cruciform structures in replication of both ARS1 (carried by YCp50) and the geminiviral origin present in YCpO'2A. The fact that only bmh1 mutant exerts a negative effect on replication of both these plasmids whereas bmh2 mutant did not show any effect, indicating different functions of these two isoforms. This is consistent with the postulate proposed by Rosenquist et al. (2000) based on their study of Arabidopsis homologues, that different isoforms are associated with isoform-specific functions.

These results demonstrate the beginning of the wide applicability of this system to identify host factors required for geminiviral replication. Using the wide repertoire of yeast mutants available in the budding yeast coupled with our simple yeast transformation assay, can allow genome-wide screening of host factors required for geminiviral replication as has been possible for the RNA virus BMV (Kushner et al.,
2003). Moreover, Quadt et al. (1995) have shown that yeast expressing BMV replication proteins and replicating RNA derivatives could be extracted to yield BMV-specific template-dependent RdRP activity. The observations of Quadt et al. (1995) provide evidence that the well characterized and genetically tractable budding yeast could have potential for the reconstitution of a defined in vitro replication system for studying BMV replication. Our yeast model has the same potential for studying geminivirus replication. Thus, the development of the yeast model opens up avenues of exploration into the mechanisms and host factors involved in geminiviral replication, hitherto not possible with currently available protocols in plant virology. Moreover, our studies raise the possibility that the yeast system could be used as a host for other DNA viruses that are currently difficult to study in their natural hosts.