Discussion

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Geminiviruses replicate their genomes by rolling-circle (RCR) mechanism and recombination dependent (RDR) mode of replication is also implicated in certain geminiviruses. They replicate through DNA intermediates in the nucleus of the infected cell, form minichromosomes by association with histones and complete their replicative cycle with major input from cellular proteins. In addition, they interfere with cell cycle and growth regulatory pathways of the infected cell and induce a pseudo S-phase scenario for its replication. The nature of RCR of viral DNA and the recombinogenic property of the single-stranded viral DNA might contribute greatly to the cause of virus variability (Jeske *et al.*, 2001). The major player in the replication of geminiviruses is a viral encoded factor namely the replication initiator protein (Rep), which makes site specific nick in the conserved nonamer sequence TAATATT$^{\downarrow}$AC present in the common region (CR) of all geminiviruses and initiates RCR. In order to replicate their genome successfully, these viruses interact with and modulate number of host encoded factors involved not only in DNA replication but also in various cellular processes such as recombination, DNA damage response, repair, chromatin remodeling, etc. for their own benefits. Till date, very few host factors have been characterized in detail for their involvement in the geminiviral replication.

To understand the replication processes of the geminiviruses in greater details, we have chosen *Mungbean yellow mosaic India virus* (MYMIV) as the model system, which has single stranded bipartite genomes, namely DNA-A and DNA-B (Varma and Malathi, 2003). Rep, encoded by all geminiviruses, is a multifunctional protein, which is absolutely required for viral DNA replication and is also known to regulate its own transcription. MYMIV-A genome has Rep binding sites flanking the nonameric sequence and upon binding Rep helps in the formation of the stem-loop structure and thus RCR initiation (Singh *et al.*, 2008). MYMIV-Rep possesses ATP dependent topoisomerase I (Pant *et al.*, 2001), ATPase activities (Bagewadi *et al.*, 2004) and acts as a replicative helicase in the oligomeric form (~24mer). Rep is classified as a member of the SF3 superfamily (Choudhury *et al.*, 2006) that includes the NTP-binding pattern with conserved protein segments encoded by genomes of small DNA and RNA viruses (Gorbalenya *et al.*, 1990). In addition to its catalytic and DNA binding activities, Rep is involved in self oligomerization as mentioned above and interaction with other viral factors, *viz.*, viral replication enhancer, AC3 (REn,

Settlage *et al.*, 1996), AC4, AC5 (Raghavan *et al.*, 2004). Rep is also known to interact with various host factors such as Retinoblastoma protein (RBR1, Ach *et al.*, 1997; Arguello-Astorga *et al.*, 2004; Kong *et al.*, 2000; Xie *et al.*, 1996), PCNA (Bagewadi *et al.*, 2004, Kong *et al.*, 2002), RPA (Singh *et al.*, 2007), replication factor-C (RF-C, Luque *et al.*, 2002), histone H3, a serine/threonine kinase, a motor protein (Kong and Hanley-Bowdoin, 2002) and a host cell sumoylation enzyme (Castillo *et al.*, 2004).

The molecular mechanism of initiation of DNA replication in eukaryotic rolling-circle replicons is still poorly understood. DNA replication in eukaryotic cells initiates with the binding of a hetero-hexameric origin recognition complex (ORC1-6) to the origin sequences where pre-replication complexes (pre-RCs) can be assembled. The role of components of ORC has been shown in few animal DNA viruses (HPV, EBV etc.) but not explored in geminiviral context (Julien *et al.*, 2004, Dhar *et al.*, 2001). Since the activation of geminivirus origin of replication depends on the interaction of the Rep protein with the origin, we were interested in investigating the role of eukaryotic initiator proteins such as ORC or any of its components in the replication of the DNA-A genome via its interaction with Rep protein. In the present study, we assessed the interaction of ORC components with viral Rep protein.

In this study, we further screened for other probable Rep interacting host factors and identified a host recombination factor Rad54. The role of Rad54 has been characterized in greater details and its potential role in the geminiviral DNA replication has been emphasized. Rad54 is a member of the Snf2-family of DNA stimulated/dependent ATPases in the SF2 family of DNA helicases. In their core domain, all Rad54 proteins have seven conserved Snf2-specific motifs which were proposed to be diagnostic of DNA helicases (Gorbalenya and Koonin, 1993). However, Rad54 (and all other Snf2-related proteins) fail to catalyze strand displacement reactions typical for DNA helicases. Rad54 protein displays dsDNA-specific ATPase activity (Petukhova *et al.*, 1998). Rad54 is known to be involved in various cellular activities such as recombinational repair of double-strand breaks (Petukhova *et al.*, 1999), stimulates strand exchange by modifying the topology of double-stranded DNA (Bugreev *et al.*, 2007, Bianco *et al.*, 2007, Solinger *et al.*, 2001), genome stability (Schmuckli-Maurer, *et al.*, 2003) and chromatin remodeling (Kwon *et al.*, 2007, Alexiadis *et al.*, 2004 and Alexeev *et al.*, 2003). From all such evidences it has been proposed that RAD54 is recruited by

RAD51-ssDNA filament to the chromatin of the intact chromosome and that it remodels that chromatin to facilitate accessibility for strand exchange (Kwon *et al.*, 2007).

The involvement of DNA repair machinery in viral replication would be possible in view of the fact that most virus infected cells in a plant are differentiated and no longer contain detectable levels of replication enzymes (Rushing *et al.*, 1987; Coello *et al.*, 1992; Nagar *et al.*, 1995), though the involvement of host DNA synthesis enzymes associated with cell cycle activity is supported by the presence of condensed chromatin in infected nuclei (Bass *et al.*, 2000). Interestingly, both homologous (HR) and non-homologous end joining (NHEJ) recombination events have been observed in geminiviruses (Bisaro, 1994) and these reports along with the existence of RDR mode of replication (Jeske *et al.*, 2001) re-emphasizes the involvement of recombination and repair machinery in geminiviral setting. Moreover, the first line of evidence connecting the recombination and repair factors, such as Rad51 and Rad52 with MYMIV DNA replication has been shown using the yeast model system developed by Raghavan *et al.* (Raghavan, V., Ph. D. Thesis, ICGEB, 2004). The findings of this work would thus help in understanding the role of the recombination factor, Rad54, in the RCR of geminiviral replication.

ORC has marginal role on geminiviral DNA replication

Initiation of Rolling Circle Replication (RCR) of geminiviral DNA occurs by formation of stem-loop structure and nicking at the specific nonameric sequence present in the loop. MYMIV-Rep, the sole geminiviral protein involved in RCR, binds specifically to four sites (iterons) within in the geminiviral origin and initiates RCR (Singh *et al.*, 2008); however the viral replication initiation is almost entirely dependent upon cellular factors other than viral Rep protein. The involvement of eukaryotic replication initiation complex in the replication of the MYMIV DNA-A genome is verified by studying the interaction of Rep with the yeast ORC subunits ORC2 and ORC5. The interaction studies by yeast two hybrid analyses revealed that both ORC2 and ORC5 displayed weak interaction with geminiviral Rep protein indicating that the individual ORC subunits may not be sufficient in establishing an interaction with Rep. However, since ORC functions at the pre-RC as a hetero-hexameric unit, the complete elucidation of the function of ORC complex would require analyses with temperature sensitive (*ts*) mutants of individual subunits. Earlier reports from our laboratory revealed that a plasmid harboring geminiviral origin of replication (YCpO^{-}-2A) could replicate

efficiently in budding yeast (Raghavan *et al.*, 2004). Hence, we used the yeast model system to study the role(s) of ORC component(s) and in view of availability of '*ts*' mutants we chose ORC2 and ORC5 to elucidate the functional role of ORC in geminiviral DNA replication. The results obtained from our preliminary replication assays at the sub-lethal temperature (34°C) using the ORC2 and ORC5 *ts* mutants of yeast suggested that the subunits play roles in the replication of both the control plasmid (YCp50) containing yeast ARS and the plasmid YCpO^{-}-2A harboring geminiviral replication origin (Fig. 1). However, the low level of interaction between ORC subunits with viral Rep indicates that the role(s) of ORC subunits in geminiviral replication could perhaps only be marginal. More experiments are needed to evaluate the involvement of ORC, as a whole complex, in geminiviral DNA replication.

Since ORC showed weak interaction with viral Rep and marginal role in geminiviral replication, we proceeded to search for host factor(s) interacting with geminiviral Rep protein. In order to search for the host factor(s) interacting with Rep, phage display library screening method was employed in this study. Further, we also established yeast nuclear extract based *in vitro* reconstitution assays which would facilitate the study of geminiviral DNA replication.

Screening for MYMIV-Rep interacting partners revealed one of the host factors, Rad54

As mentioned earlier, geminiviruses codes for few proteins and depends on host cellular machineries for various viral functions. Till date, only few host factors has been identified to have role(s) in viral replication. With a view to identify host factor(s) essential for geminiviral replication, we screened for peptides from a randomized phage display peptide (12-mer) library interacting with the Rep protein which is the sole viral protein known to be involved in geminiviral replication. We obtained 38 peptides interacting with Rep and the BLAST search with the identified peptide sequences against *Arabidopsis* database revealed that Rep was capable of interacting with ~250 candidate host proteins, which might regulate the viral DNA replication directly or indirectly. The interacting proteins identified were classified in fifteen different categories according to their functions, such as replication, Chromatin remodeling, RNA interference, kinases, kinesins, etc. For example, 29 of them were found to belong to plant replication and repair factors, such as TopoII, DNA Polymerase ε, Polymerase ζ (REV3), E2F, Rad51, Rad54, Rad23, certain helicases etc., 4 of them are chromatin remodeling factors such as HAF2, HAT etc. and 4 of them are gene-silencing

factors such as RDR1, XRN2, SDE3 etc. Few proteins known to interact with viral Rep or REn proteins or speculated to have a role in geminivirus replication, e.g. retinoblastoma like proteins (Ach *et al.*, 1997; Arguello-Astorga *et al.*, 2004; Kong *et al.*, 2000; Xie *et al.*, 1996), NAC domain containing protein (Selth *et al.*, 2004), E2F (Gutierrez, 2000) and kinases (Kong & Hanely-Bowdoin, 2002) were also revealed suggesting that the phage display library screening method is efficient in elucidating the Rep interacting partners.

The protein hits obtained from BLAST search against yeast database (Table 4) also revealed various important replication factors such as CDC6, REV1, DNA ligase I etc., as well as DNA damage and repair related factors such as, Rad54, Rad2, Rad6, SRS2, ICL-recognition protein etc. Since geminiviral replication origin is functional in yeast system, the proteins identified from yeast database are likely to be of great significance in geminiviral DNA replication.

Rad54 is a highly conserved multifunctional protein having potential in geminiviral replication: Among the various replication and replication-related factors identified from the phage display analysis, we focused on investigating Rad54 due to the over-representation of the peptide (P-I-6) showing match with *At*Rad54 in the screening and another peptide P14 with *Sc*Rad54 (Table 5). Rad54, part of the Rad52 epistatis group, is known to be involved in various activities such as recombinational repair of double-strand breaks (Petukhova *et al.*, 1999), stimulation of strand exchange by modifying the topology of double-stranded DNA (Bugreev *et al.*, 2007, Bianco *et al.*, 2007, Solinger *et al.*, 2001), genome stability (Schmuckli-Maurer, *et al.*, 2003) and chromatin remodeling (Kwon *et al.*, 2007, Alexiadis *et al.*, 2004 and Alexeev *et al.*, 2003). Moreover it possesses DNA-dependent ATPase and DNA topoisomerase activities and is characterized by the presence of conserved seven helicase domains (Fig. 5) with no associated helicase activity. Rad54 interacts and functions along with the eukaryotic recombinase Rad51 during homologous recombination and shows enhanced chromatin remodeling in presence of Rad51.

The evidences such as geminivirus dsDNA replicative forms are supercoiled and exist as minichromosomes (Pilartz and Jeske, 1992); the Rep protein of *Cabbage leaf curl virus* (CbLCV) interacts with Histone H3 (Kong and Hanley-Bowdoin, 2002); and the existence of recombination dependent replication intermdediates in AbMV DNA replication (Jeske *et al.*, 2001) raises the possibility of involvement of important machineries including chromatin remodeling, DNA damage

response, recombination and various repair mechanisms in geminiviral DNA replication, other than those involved in host replication machinery. Based on the above arguments we envisaged that Rad54 could have some role in the geminiviral DNA replication processes.

With the availability of budding yeast system where MYMIV DNA-A replication is modeled, we analyzed the *Sc*Rad54 and *At*RAD54 sequence homology which showed 38% identity and 54% similarity. *Sc*Rad54 and *At*Rad54 shared some degree of functional conservation in plant and yeast in cross-species complementation studies (Klutstein *et al.*, 2008 and Shaked *et al.*, 2005). As *S. cerevisiae* supports the geminiviral DNA replication (Raghavan *et al.*, 2004) and *Sc*Rad54 functionally complements *At*RAD54 in *Arabidopsis*, we used *Sc*Rad54 conveniently to investigate its role, if any, in geminiviral DNA replication.

In absence of any X-ray crystallographic data, it is difficult to visualize the regions of interactions on the Rad54 protein structure. Recently, a partial crystal structure for zebrafish Rad54 from residues 91 – 738 was solved (Thoma *et al.*, 2005). The homology modeling studies of *At*Rad54 and *Sc*Rad54 with zebrafish Rad54 crystal structure revealed that the peptide region 286-FTRPR-290 found in *At*Rad54 (Table 5) shares significant homology across species such as *S. cerevisiae* and zebrafish and this peptide is forming part of a helix and a loop region and is exposed on the surface of the protein (Fig. 7). The difference in the protein surface at this region is due to the deletion of 3 amino acids in *At*Rad54 loop compared to zebrafish and yeast and the extent of exposed surface is more prominent in *At*Rad54 (Fig. 8). From these observations we speculate that this peptide region of *At*Rad54 might act as an epitope and contribute to the interaction with Rep protein. The peptide region of *Sc*Rad54 '90-YNSQDISF-97' (Table 5) falls in the N-terminal region which is unique and not found in other Rad54 proteins. Moreover, the X-ray crystallographic data for full length Rad54 protein is not available till date and thus locating this peptide region (90-97) of *Sc*Rad54 in the model structure is not possible. In our present study, yeast two-hybrid and *in vitro* pull down assays were employed to map the interacting domains for *Sc*Rad54 and Rep proteins.

N-terminal of ScRad54 interacts with the oligomerization domain of MYMIV-Rep

Yeast two-hybrid and *in vitro* interaction studies of Rep with *Sc*Rad54 revealed that the oligomerization domain of Rep (120-183 aa) is responsible for interacting with *Sc*Rad54 (Fig. 16). In the yeast two hybrid analyses the full length Rad54 showed interaction with the larger segments of the N-terminal (Rep_{1-183}) and the C-terminal ($Rep_{120-362}$) regions and unable to interact with the smaller N- and C- terminal segments ($Rep_{184-362}$, Rep_{1-133} respectively) (Fig. 9D). This suggests that the oligomerization domain of Rep is essential for its interaction with *Sc*Rad54. This is significant since most of the host proteins e.g. PCNA (Bagewadi *et al.*, 2004), coat protein (Malik *et al.*, 2005), pRBR (Arguello-Astorga *et al.*, 2004), GRIK and GRIMP (Kong and Hanley-Bowdoin, 2002) etc. interact with Rep through the oligomerization domain of the Rep and only a few proteins, viz., GRAB1 and GRAB2 (Xie *et al.*, 1999) and RPA32 (Singh *et al.*, 2007) are known to interact with C-terminal domain Rep. It is also important to note that the oligomerization status (~24-mer) of MYMIV Rep protein is crucial for its helicase function (Choudhury *et al.*, 2006). Similarly, with few exceptions, all the proteins including those involved in DNA metabolism (Kelman, 1997) function in oligomeric form e. g., helicases, helicase loaders, DNA clamps and clamp loaders etc. The oligomerization status is known to be essential for few helicases such as the hexameric helicases, *viz.*, DnaB of *Escherichia coli*, T4 and T7 DNA helicases, human MCM proteins etc. (Patel and Picha, 2000) and the double hexameric SV-40 large T-antigen of simian virus (Konieczny, 2003; Alexandrov *et al.*, 2002). Thus the oligomerization status of Rep protein influences interactions with host factors as well as geminiviral DNA replication.

It is interesting to note that the *in vitro* pull down experiments indicate that the N-terminal region of *Sc*Rad54 (1-107) is sufficient to establish the interaction with the full length Rep as well as with its truncations Rep_{1-183}, $Rep_{120-362}$ (Fig. 15). The Rad54 truncations ($Rad54_{114-898}$ and $Rad54_{130-898}$), where the first 107 residues are absent, failed to interact with Rep protein (Fig. 14). These results clearly indicate that the residues 1-107 of *Sc*Rad54 are crucial for its interaction with MYMIV-Rep protein. Another identification that lends further support to our conclusion is that the peptide P14 identified from phage display library screening spans the region from amino acids 90-97 of *Sc*Rad54 (Table 5) is found to be within this Rep interacting domain of *Sc*Rad54 (1-107). It is

noteworthy that Rad54 has been shown to physically associate with Rad51, the eukaryotic recombinase both *in vitro* and *in vitro* (Raschle *et al.*, 2004, Petukhova *et al.*, 1998, Clever *et al.*, 1997) and that the Rad51 binding motif is mapped to the N-terminal portion of Rad54 from residue 1 to 107 and the presence of other epitopes that contribute to Rad51 interaction other than the N-terminal portion also has been reported (Raschle *et al.*, 2004). Rad54 is the target for the binding of several proteins involved in DNA damage response, repair and cell cycle regulation. It also binds to proteins involved in DNA replication such as TOP3, PCNA, subunits of Replication protein A (1 & 2), CDC45, subunits of RFC (4 & 5), subunit of DNA polymerase δ (POL32), etc. Thus the binding of Rad51 with Rad54 during geminiviral DNA replication can not be ruled out.

Till date, the host factors known to interact with viral Rep protein are involved in replication, transcription as well as of cell cycle machinery. To our knowledge, other than PCNA (which participates in damaged DNA repair mechanism), *Sc*Rad54 is the first repair and recombination protein to be interacting with a geminiviral protein. In turn, the Rep interacting domain overlaps with the Rad51-interacting domain of *Sc*Rad54; the viral Rep might be modulating the host recombination and repair mechanisms for its replication and/or its genome integrity.

ScRad54 is essential for the replication of plasmid bearing geminiviral origin in budding yeast

Evidences of both homologous and non-homologous recombination events in geminiviruses are well documented (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 *Saccharomyces cerevisiae*, mechanisms of homologous recombination are dependent on a set of genes known as the *RAD52* epistasis group of which Rad54 is also a member.

If RDR 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 in *RAD54* gene was specific to geminiviral replication rather than a result of general effect on yeast replication, we transformed both the ARS-containing YCp50 and the ARS-deficient YCpO$^-$-2A in the wild type and the mutant yeast cells and did a comparative study of the effect of the mutation on the replication of both transformed plasmids. Relative to the wild type strain BY4742, a deletion mutation in *RAD54* affected the transformation efficiency of YCpO$^-$-2A to a greater extent than that of YCp50, giving relative values of 27% and 65% respectively (Fig. 18). Thus, it is apparent that Rad54 could be playing a significant and specific role in geminiviral replication in yeast, and hence its absence in the deletion mutant was inhibitory to YCpO$^-$-2A than YCp50.

The above results were further corroborated by the complementation of *rad54Δ* mutant yeast with WT *RAD54* gene and transformed with YCpO$^-$-2A plasmid. The replication efficiency of YCpO$^-$-2A in *rad54Δ* mutant yeast decreased immensely (~24%) which was restored completely (~100%) on complementation with a Rad54 expressing plasmid (Fig. 19). Together, these results suggested that Rad54 is critical for replication of plasmid DNA harboring geminiviral origin of replication compared to that of plasmids with yeast ARS. This is the first report showing that Rad54 plays an active role in geminiviral DNA replication.

Earlier in our lab, among other Rad52 epistasis group of proteins, Mre11, Rad51and Rad52 were checked for their role in geminiviral replication and the transformation efficiency results revealed that Rad51 and Rad52 deletion mutants were more inhibitory to YCpO$^-$-2A than YCp50 and could be playing some specific role in geminiviral replication in yeast (Raghavan, V., Ph. D. thesis, ICGEB, 2004). From these observations, we speculate that the Rad52 epistasis group is playing a crucial role in geminiviral replication in yeast.

ScRad54 is vital for initiation and elongation stages of RCR by modulating the functions of MYMIV-Rep

The rolling-circle replication (RCR) of geminivirus is responsible for the massive production of ssDNA. RCR of viral genome is extremely dependent on the site-specific nicking and ATPase activities as well as helicase function of Rep protein. In presence of Rad54, the nicking activity of Rep was enhanced ~10 folds (Fig. 20) suggesting that even at low concentrations of Rep inside the host cell, the nicking activity required for RCR can be enhanced several fold in presence of host

Rad54. Earlier reports from our lab suggested that PCNA, as well as coat protein (CP) have inhibitory effect on the nicking activities of Rep (Bagewadi *et al.*, 2004; Malik *et al.*, 2005) and are possibly involved in controlling viral copy number. Unlike PCNA and CP, Rad54 enhances the nicking activity of Rep, though the oligomerization domain of Rep is involved in interaction with Rad54. As discussed earlier, the oligomerization of Rep is crucial for its helicase activity and thus plays a significant role in post-initiation steps of RCR. These results suggested that Rad54 is likely to play a direct role in initiation of RCR of the geminiviral DNA and enhance viral amplification.

Our studies on the ATPase activity revealed that the Rep-Rad54 interaction also led to the enhancement of the ATPase activity of Rep upto ~2 folds (Fig. 21). In view of the fact that Rad54 also possesses characteristic dsDNA dependent ATPase activity (Fig. 23), the reactions were performed in absence of DNA. Unlike typical helicases, it has been reported that Rad54 protein uses the energy of ATP hydrolysis to translocate on dsDNA inducing topological changes (Heyer *et al.*, 2006). In geminiviral RCR context, our findings are in line with the fact that Rad54 might assist Rep protein in its post-initiation activities of viral DNA replication where the ATPase activities are essential for energy-requiring extensive unwinding of the nicked origin. As there are evidences that geminiviruses also replicate via recombination dependent mechanism, we speculate that the ATPase activity of Rad54 might also assist in translocating ssDNA fragments onto the homologous ccc-dsDNA intermediate in order to complete the broken viral ssDNA replication (Jeske *et al.*, 2001).

The interaction between Rep and Rad54 also led to the enhancement of the intrinsic helicase activity of Rep to several (~6) folds (Fig. 22, panel D). From these results it is thus clear that similar to nicking and ATPase activities, Rad54 also enhances the helicase activity to several folds even with minimum amount of Rep used. These results together reinforce the role of Rad54 in both initiation and elongation steps of RCR of geminivirus and also the ability of viral Rep to utilize the host factors efficiently for its own replication.

Rad54 possesses the characteristic conserved seven helicase domains but its involvement in a typical strand displacement function has not been demonstrated till now. To our surprise, we found that the recombinant fusion protein Trx-Rad54 showed a maximum of 61% unwinding activity with increasing amounts (0.3-6.0 pmoles) of Trx-Rad54 (Fig. 22, Panel B) whereas the Rad54 protein (where the tag is cleaved off) did not exhibit any helicase activity (Fig. 22, Panel C). These data

could be explained in light of Rad54 protein with the thioredoxin-His tag might assume a conformational and structural advantage over the Rad54 without tag and hence renders Trx-Rad54 fusion protein the ability to unwind the annealed oligonucleotide. (Several site-directed mutageneses experiments are needed to further validate the observed Trx-dependent helicase activity of Rad54). As a result of Rep-Rad54 interaction, various intrinsic activities of Rep have been enhanced several folds in presence of Rad54 *in vitro* thus revealing essential roles for Rad54 in both initiation and post-initiation stages of geminiviral RCR.

Yeast nuclear extract based *in vitro* replication system supports the replication of geminiviral origin bearing plasmid

Yeast genetics, the study of SV40 viral replication, and an *in vitro* replication assay based on Xenopus oocyte extracts have led to a significant progress in identifying the factors that catalyze eukaryotic DNA replication (Pasero *et al.*, 1999). Nonetheless, to understand the regulation and molecular mechanisms of site specific initiation of DNA replication, it will be essential to have efficient systems that reconstitute DNA replication *in vitro*. The budding yeast, *Saccharomyces cerevisiae*, has been used to complement the powerful replication assay from Xenopus oocytes, as it has a normal mitotic cell cycle and is amenable to both molecular and classic genetic techniques (Pasero and Gasser, 1998). In addition, yeast provides many advantages for the establishment of a replication assay, such as well-defined origins, convenient synchronization protocols, and a large number of conditional mutations in components of the replication machinery. Moreover, yeast is suitable for techniques that allow precise mapping of sites at which DNA replication initiates (Gerbi and Bielinsky, 1997). It is thus apparent that the availability of yeast cell extract (Mitkova *et al.*, 2005; Seki and Diffley, 2000; Pasero *et al.*, 1997) would pave the way for *in vitro* reconstitution of the replication of viral DNA.

The *in vitro* system used in our study for the dissection of DNA synthesis requires nuclear protein extract from budding yeast *S. cerevisiae*, synchronized to the S phase (Braguglia *et al.*, 1998; Mitkova *et al.*, 2002). DNA synthesis in this system is bidirectional, aphidicolin-sensitive, semi-conservative, and requires supercoiled plasmid as DNA template (Braguglia *et al.*, 1998). Bona fide DNA replication should also exhibit a requirement for correct cell cycle coordination (Diffley, 1996) and be dependent on the origin recognition complex (ORC), Cdc6p, and MCM proteins for origin-

specific initiation (Diffley, 1996; Dutta and Bell, 1997). Our results on replication assay using yeast nuclear extract demonstrated that the replication of the plasmid bearing the geminiviral origin is cell cycle dependent; resulting in much greater levels of replication in S-phase extracts than in G1-phase extracts (Fig. 27B). The assay also revealed the requirement of the viral origin of replication for efficient replication of the YCpO$^-$-2A plasmid. The mutant viral origin bearing plasmid OriM-YCpO$^-$-2A showed very less accumulation of RIs (6.51%) as compared to the wild type origin containing plasmid (Fig. 28) thus emphasizing the requirement of viral origin of replication *in vitro*.

Since the YCpO$^-$-2A plasmid does not contain an ARS element, it ought to replicate using the viral origin of replication which is recognized by the viral replication initiator protein; Rep. The *in vitro* replication assay demonstrated that the viral Rep is critical for geminiviral replication. The replication assay carried out with S-phase (NE_S) nuclear extract, in presence (2A+Rep) and absence (2A–Rep) of Rep protein did not show any significant radiolabel incorporation while pre-incubation of the DNA with Rep protein (2A*Rep) resulted in a significant incorporation of radiolabel (Fig. 26A). This result clearly points to the fact that Rep protein is involved in the primary step during the replication initiation. This result is further corroborated by analyzing the replication intermediates (RIs), digested with *Hind*III, on an agarose gel and autoradiography. With increasing amounts of NE_S, the accumulation of replication intermediates (in the size range of 2.7-7.2 kb) were enhanced (Fig. 26B) while no RI was observed with reactions in presence or absence of Rep and/or DNA. These results provide evidence that the appearance of the RIs observed in case of S-phase nuclear extract are dependent on the nicking activity of viral Rep protein and the template DNA. The fact that the replication is inhibited if DNA template is not pre-incubated in presence of Rep, but added along with the nuclear extract further reveals that Rep protein is most likely quenched by the host factors present in the nuclear extract, making it inaccessible to carryout the nicking activity.

Further, our observation that ~18% of the *in vitro* replication is contributed by viral factors other than Rep protein, whereas Rep alone exhibited a significant (~82%) replication efficiency (Fig. 27A) reveals that Rep is the major viral factor essential for the initiation of geminiviral replication

observed *in vitro*. In this system the YCpO$^-$-2A template shows a cell cycle-dependent competence for initiation and the subsequent initiation step is both viral origin specific and Rep dependent.

Elsewhere, we demonstrated that yeast supports the formation of ssDNA during the replication of YCpO$^-$-2A plasmid, which is a characteristic feature of RCR (Raghavan *et al.*, 2004). In the present study the *in vitro* replication products were only partially digested by S1 nuclease (Fig. 29A, lanes 3 & 4), suggesting the presence of both ds and ssDNA forms. This data further lends support to the RCR mode of replication.

As the hemi-methylated DNA is resistant to *Dpn*I digestion, *de novo* replication of DNA *in vitro* by either semi-conservative or RCR mode of replication was verified by studying the sensitivity of the replicated products to *Dpn*I digestion. The differential sensitivity of the input (*E. coli* replicated) vs. the *in vitro* replicated DNA to the *Dpn*I digestion supports the occurrence of *de novo* DNA synthesis *in vitro* (Fig. 29A, lanes 5-7) and thus differentiates from the occurrence of any repair DNA synthesis. Moreover, this yeast nuclear extract based *in vitro* assay system is characterized to be dependent on the replicative DNA polymerases, viz., pol δ and, in particular, pol α, which is the only polymerase uniquely implicated in the *de novo* initiation of DNA replication (Mitkova *et al.*, 2005).

The appearance of high molecular weight intermediates with a diffuse smear till the well of the gel in the undigested reaction product points to the presence of both highly structured and unstructured RIs in the *in vitro* replication (Fig. 28, lane 6). All our observations of *in vitro* replication studies collectively indicate that the *in vitro* DNA replication in yeast nuclear extracts support YCpO$^-$-2A plasmid replication which is dependent on various factors such as Rep and other viral proteins, the presence of viral origin of replication and on the host cell cycle (S-phase). In addition, analyses of the nature of the replication products confirm that the *in vitro* system supports *de novo* complementary strand synthesis and point towards the possibility of rolling-circle replication. With the above characterization it is evident that this *in vitro* replication system can be conveniently used to further assess the role played by host factor(s) in geminiviral DNA replication.

ScRad54 affects the replication of YCpO-2A plasmid *in vitro*

As discussed above, our *in vitro* assay system supports the replication of YCpO$^-$-2A plasmid which can be conveniently used for validating the involvement of various viral and host factors. The amount of replication products formed in the *in vitro* replication reactions performed with the wild type (WT) and the *rad54* mutant yeast nuclear extracts in our study clearly demonstrate that the WT extracts were at least 4 folds more efficient in replicating the YCpO$^-$-2A DNA than the rad54Δ extract (Fig. 30). It is evident from these results that the replication of YCpO$^-$-2A plasmid *in vitro* is inhibited in absence of Rad54 protein and re-emphasizes that Rad54 plays an essential role in geminiviral replication.

This finding is further corroborated by the *in vitro* reconstitution of the purified recombinant Rad54 protein. It is observed that with increasing concentrations of the purified recombinant Rad54 protein a gradual increase in the formation of replication intermediates are obtained (Fig. 31). The highest concentration of Rad54 used in the assay (0.45 pmole) resulted in as high as 3 fold increase in the replication products. Our data clearly demonstrate that the defect in YCpO$^-$-2A plasmid replication due to the absence of Rad54 can be alleviated by the addition of recombinant Rad54 protein *in vitro*.

At*Rad54 is indispensable for the replication of ToLCV based amplicon *in planta

The plant being the natural host of geminiviruses, it is important to investigate the role of Rad54 in geminiviral replication *in planta*. Recent studies in our laboratory have showed that a *Tomato leaf curl virus* (ToLCV) based viral amplicon is competent to replicate in the host as well as non-host plants (Pandey *et al.*, unpublished data). Earlier reports also suggested that a binary construct bearing two copies of 'CR' releases an episomal DNA within the injected plant during rolling circle replication (Stenger, *et. al.*, 1991). Our studies indicate that when the tomato leaves are agro-infiltrated with the agrobacterium harboring the binary construct (Cam/VA), a circular episome of 2.6 kb is released (Pandey *et al.*, unpublished data) following Rep dependent site-specific nicking activity at CR and subsequent DNA replication by RCR (Stenger *et al.*, 1991).

At present, *in planta* based replication assays to investigate the role of Rad54 in geminiviral replication are difficult to carry out because of lack of availability of Rad54 mutants in the natural

hosts such as tomato or mungbean. However, *in planta* role of Rad54 in geminiviral replication was highlighted in the transient replication assay using the *Arabidopsis* T-DNA insertion mutant line of Rad54. Our data with the wild type *Arabidopsis* lines clearly indicate that the ToLCV based amplicon is efficient in replicating in the model plant *Arabidposis* (Fig. 34). The transient replication assay in *Arabidopsis* showed that in absence of Rad54 protein, the ToLCV based amplicon failed to replicate and thus the replicated bands could not be detected by divergent PCR (Fig. 34) suggesting that Rad54 is critical for the replication of geminiviral based amplicon in the plant system.

Role of Rad54 in geminiviral DNA replication

Rad54, a multifunctional protein, is a member of the Snf2-family of DNA stimulated/dependent ATPases in the SF2 family of DNA helicases. In their core domain, all Rad54 proteins have the seven conserved Snf2-specific motifs that were proposed to be diagnostic of DNA helicases (Gorbalenya and Koonin, 1993). However, Rad54 (and all other Snf2-related proteins) fails to catalyze strand displacement reactions typical for DNA helicases. Rad54 was found to be involved in various stages of the homologous recombination and functions in concert with Rad51 *in vitro*. Rad54 mediates formation or stabilization of Rad51 filaments on ssDNA, translocates the Rad51-ssDNA filament along duplex DNA. It induces strand separation through induction of topological change and exhibits chromatin remodeling activity that may clear nucleosomes or other proteins from the pairing site. Rad54 catalyzes heteroduplex extension (branch migration) and could dissociate the Rad51–dsDNA product complex, possibly to allow DNA polymerase access to the invading 3'-OH end to prime DNA synthesis. Rad54 is likely to act as an oligomer on DNA and an oligomeric Rad54 particle has been directly visualized at the terminus of Rad51–dsDNA filaments by electron microscopy (reviewed in Tan *et al.*, 2003 and Heyer *et al*, 2006).

The rolling-circle replication (RCR) of geminivirus is responsible for the massive production of ssDNA. The ssDNA forms are packaged into the twin icosahedral particles and are transmitted to healthy plants through insect vectors. RCR of viral genome is critically dependent on the site-specific nicking and ATPase as well as helicase activities of Rep protein. Earlier reports suggested that geminiviral Rep protein binds to the double stranded origin DNA in a sequence-specific manner (Fontes *et al.*, 1992; 1994a; 1994b; Singh *et. al.*, 2008). The binding of Rep to the origin

DNA leads to conformational changes in the origin DNA and cause limited local unwinding of the origin DNA, resulting in the extrusion of the stem loop structure due to torsional stress. The conserved nonamer sequence TAATAT↓AC (cleavage site of Rep) is present in the single stranded loop region. At this conserved site, Rep makes a nick and initiates RCR. The interaction of *Sc*Rad54 with Rep leads to the enhancement of the nicking activity of the Rep suggesting that Rad54 might be involved in initiation of RCR. Our earlier data also suggested that Rep acts as replicative helicase (Choudhury *et al.*, 2006). Our observation in the present study that the interaction of Rep with Rad54 enhances the ATPase and helicase activities of Rep suggests that Rad54 may assist Rep in extensive unwinding of the origin DNA and thus replication fork movement.

As in case of PCNA and MYMIV-Coat protein (CP), *Sc*Rad54 also interacts with the oligomerization domain of Rep, but unlike PCNA and CP, *Sc*Rad54 enhances the nicking activity of Rep thereby positively contributing to viral replication. *Sc*Rad54 interacts with MYMIV-Rep through its N-terminal region and it is also known to interact with Rad51 and Histone H3 through the same N-terminal region (Raschle *et al.*, 2004; Kwon *et al.*, 2007). It is interesting to note that Rep of *Cabbage leaf curl virus* (CbLCV) interacts with Histone H3 and this interaction is proposed to be important to displace nucleosome assembly of the double-stranded form of geminivirus. DNA allowing access of the replication and transcription machinery (Kong and Hanley-Bowdoin, 2002). These evidences suggest that Rad54-mediated chromatin remodeling coincides with DNA homology search by the Rad51 pre-synaptic filament and that this process is facilitated by an interaction of Rad54 with histone H3 (Kwon *et al.*, 2007). Evidences suggest that distinct sub-domains in the N-terminus of Rad54 are likely mediating the interactions with Rad51 and histone H3 (Kwon *et al.*, 2007). This led us to speculate that Rep, through its interaction with Rad54, could modulate the chromatin remodeling or Rad54 and synaptic as well as post-synaptic events of homologous recombination (HR). Such events of HR are necessary and has been implicated in the RDR of *Abutilon mosaic virus* (*AbMV*) in order to complete the replication of incomplete DNA molecules produced as a result of Hindrance to viral replication (Jeske *et al.*, 2001). RDR normally involves proteins of host or viral origin, which work in a Rec-A like manner (Kowalczykowski and Eggleston, 1994). Invasion of disrupted strands into cccDNA and subsequent loop migration would increase the fidelity of repair by recruiting a full-length endless template and leave the master

sequence intact. Such interaction of viral Rep with three host protein network (Rad54 – Histone H3 – Rad51) suggests that geminiviruses could multitask in replication and reveals the complexity of interplay existing between various cellular machineries and the viral strategies.

In the present study, the significance of *Sc*Rad54 in geminiviral replication has been demonstrated in three different ways: the yeast model system, *in vitro* replication assay and *in planta* transient replication of viral amplicon. All these independent strategies clearly established that *Sc*Rad54 plays a critical role in replication of the geminiviral genome. Rad54 protein may thus be visualized to be acting as a landing pad and recruit various key proteins involved in DNA replication machinery such as PCNA TOP3, subunits of Replication protein A (1 & 2), CDC45, subunits of RFC (4 & 5), subunit of DNA polymerase δ (POL32) etc. at the initiation site and helps in the formation as well as progression of functional replication fork in geminivirus scenario.

Rad54 not only shows similarity to known chromatin remodeling factors but also exhibits chromatin remodeling activity *in vitro* (Jaskelioff *et al.*, 2003; Alexeev *et al.*, 2003; and Alexiadis and Kadonaga, 2003) and also interacts with the N-terminal tail of histone H3. Genome wide genetic screens have identified possible interactions between Rad54 and chromatin remodeling proteins such as ASF1, ESA1, CTF4, EAF1, HDA1, HOS2, ITC1, RTT109 and various other proteins (Tong *et al.*, 2001; Pan *et al.*, 2006). All such interactions support that Rad54 can function as a chromatin remodeling factor. The formation of chromatin prevents the access of transcription factors to promoters. Rb is known to interact with histone modifying enzymes and SWI/SNF-like nucleosome remodeling complexes and leads to either transcription repression or activation (Zhang and Dean, 2001). In geminiviral context, Rad54 along with other chromatin remodeling factors, listed above, might be involved in remodeling of geminiviral dsDNA mini-chromosome replicative forms by either direct or indirect interaction with Rep protein and could regulate the transcription of viral genes. Indirectly, Rad54, a SWI/SNF-like chromatin remodeling factor, might prevent Rb from its inhibitory effect on E2F by its chromatin remodeling activities and thus activates transcription of E2F regulated target genes and force the host cell to enter S-phase.

Rad54 interacts with Asf1 complex which is known to be involved in transition of G2/M to S phase (Tyler et al. 1999; Ramey et al. 2004). Earlier, it has been reported that RFC directly interacts with Asf1 and recruits Asf1 to DNA *in vitro* and promotes genome stability by maintaining replication elongation proteins at sites of stalled DNA synthesis (Franco et al., 2005). These reports led us to speculate that Rep might be modulating the differentiated plant cells to enter pseudo S-phase via its interplay with Rad54-Asf1 or any such interaction and could act as landing pad for host replication elongation factors at the site geminiviral origin of replication. Importantly, Rad54 is known to interact with pol32 (DNA polymerase δ -III subunit) and pol30 (PCNA) which are involved in DNA replication. Pol32 is involved in error-prone DNA synthesis upon DSB induced replication. Rad54 might as well load Pol32 at the site of geminivirus DNA replication initiation and cause error-prone DNA synthesis leading to the observed diversity of the viral genome.

In the present study, the role of Rad54 in geminiviral DNA replication has been elucidated in detail. This study has provided evidences for the different strategies by which plant DNA viruses adopt to interact with host proteins. Rad54 is the first host DNA repair machinery that has been shown to be directly involved in geminiviral DNA replication, in this study. Transient replication studies with Rad54 mutants of the model plant *Arabidopsis* and ToLCV based viral amplicon further verified and established a crucial role for AtRad54 in geminiviral DNA replication. Similar studies with other Rad52 epistasis group of proteins would shed more light on the involvement of host repair and recombination machinery in geminiviral DNA replication. As geminiviruses provide an excellent model for the study of plant DNA replication, the strategies used and mechanism proposed in the present study could be extrapolated to understand the intricate and complex mechanism of plant DNA replication.