Summary

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Geminiviruses replicate their genomes by rolling-circle replication (RCR) and also by recombination mediated replication (RDR) in the nucleus of the infected plant cell and complete their replicative cycle with major input from cellular proteins. The major player in the replication of geminiviruses is a viral encoded factor namely the replication initiator protein (Rep), which makes site specific nick and initiates RCR. In order to replicate their genome successfully, these viruses interact with and modulate a number of host factors for their own benefits. Till date, very few host factors have been characterized in detail for their involvement in the geminiviral replication.

The molecular mechanism of initiation of DNA replication in eukaryotic rolling-circle replicons is still poorly understood. DNA replication in eukaryotic cells begins 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 (Bell and Dutta, 2002). ORC or part of the ORC complex also helps in replication of many viral DNA of animal origin (HPV, EBV etc.) though the viral DNAs lack the obvious binding elements for the ORC species (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, which has a characteristic modular structure, we proceeded to investigate 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 the viral initiator Rep protein. In the present study, we assessed the interaction of ORC components with viral Rep protein and further screened for other putative 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.

In spite of harboring seven conserved helicase domains, Rad54 (a member of the SF2 family of DNA helicases) fail to catalyze DNA unwinding (Gorbalenya and Koonin, 1993). 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).

The involvement of DNA repair machinery in geminiviral replication appears to be a distinct possibility 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). The findings of this work helps in understanding the role of the recombination factor Rad54, from *S. cerevisiae*, in the rolling-circle replication of MYMIV-DNA 'A' component. The salient findings of our present study are summarized below:

◆ **ORC has marginal role on geminiviral DNA replication**

In order to study the interaction of ORC subunits with Rep, ScORC2 and ORC5 subunits were PCR amplified from their respective parent plasmids and cloned in to the yeast two hybrid vectors. The yeast two hybrid analyses showed weak interaction of ORC2 and ORC5 proteins with the MYMIV-Rep protein. Using the yeast model system, our preliminary studies with the *orc2-1* and *orc5-1 ts* mutants suggested that ORC2 and ORC5 subunits play roles in DNA replication of both the ARS- and geminiviral origin- containing plasmids. 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.

◆ **Screening revealed one of the host factors, Rad54 as MYMIV Rep interacting partner**

With a view to identify host factor(s) involved during geminiviral DNA replication, we employed the 12-mer peptide phage display library to screen for probable host factors interacting with MYMIV-Rep protein. 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 geminiviral DNA replication directly or indirectly. As one of the peptides corresponding to *At*Rad54 was over-represented and another peptide matched with *Sc*Rad54, in our present study, we focused on investigating the role of Rad54 in geminiviral replication.

Since *Sc*Rad54 and *At*RAD54 share sequence homology of 38% identity and 54% similarity and the *Sc*Rad54 functionally complements *At*RAD54 in *Arabidopsis*, we preferred to investigate

*Sc*Rad54 for its role, if any, in geminiviral DNA replication. 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 shares significant homology across species and this peptide is forming part of a helix and a loop region and is exposed on the surface of the protein. 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.

◆ **N-terminal of *Sc*Rad54 interacts with the oligomerization domain of MYMIV-Rep**

Yeast two-hybrid and *in vitro* pull down assays were used to map the domains of interaction between Rep and *Sc*Rad54. In 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. However, it failed to interact with the smaller N- and C- terminal segments ($Rep_{184-362}$, Rep_{1-133} respectively). This clearly indicates that the oligomerization domain of Rep is essential for its interaction with *Sc*Rad54. The *in vitro* pull down experiments demonstrated 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} and $Rep_{120-362}$. These results clearly denote 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, identified from phage display library screening that showed match with the yeast Rad54, spanning the region from amino acids 90-97 is found to be present within this Rep interacting domain of *Sc*Rad54 (1-107). This implicates that Rep interacts with Rad54 via its oligomerization domain and thus the oligomerization status of Rep holds definite biological significance. In turn, the Rep interacting domain overlaps with the Rad51- and Histone H3- interacting domains of Rad54 thereby indicate that the viral Rep might be modulating the host recombination and chromatin remodeling machineries for its replication and/or its genome integrity.

◆ **ScRad54 is essential for the replication of plasmid harboring geminiviral origin in budding yeast**

In the yeast model studies, compared to the wild type strain, a deletion mutant of Rad54 affected the transformation efficiency of YCpO-2A plasmid (27%) to a greater extent than that of YCp50 (65%), indicating that the absence of Rad54 was inhibitory to YCpO^{-}-2A than YCp50. Also complementation of the *rad54Δ* mutant with wild type Rad54 expressing plasmid resulted in

complete restoration of the replication suggesting that Rad54 is critical for replication of geminiviral origin bearing plasmids.

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

In order to study the effect of interaction of Rad54 with MYMIV Rep, the various intrinsic biochemical functions of Rep were examined in presence of Rad54. The nicking, ATPase and helicase activities of Rep were enhanced several folds in presence of Rad54. These results clearly show that Rad54 is involved in initiation and elongation stages of geminiviral rolling-circle replication. The oligomerization status of Rep has been demonstrated to be crucial for its helicase activities and these functions are enhanced in presence of Rad54, signifying a biological role for Rad54 in the Rep mediated RCR of geminiviruses.

The helicase assay performed with the recombinant purified Rad54 proteins showed that the thioredoxin tagged Rad54 exhibited DNA unwinding activity, while the Rad54 (without fusion tag) did not show any unwinding activity. This led us to the speculation that the Rad54 protein with the thioredoxin-His tag could assume a conformational and structural advantage over the Rad54 without tag and hence able to catalyze DNA unwinding.

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

An *in vitro* replication assay based on yeast nuclear extract was developed in the present study using the nuclear extract of S-phase synchronized yeast cells and supercoiled plasmid templates. By employing such *in vitro* assay, the YCpO^{-}-2A plasmid replication was characterized and observed to be dependent on the following:

(a) Viral origin of replication
(b) Viral Rep protein
(c) Other viral factors
(d) S-phase (cell-cycle oriented)

The replication products were analyzed for the presence of ssDNA forms and *de novo* synthesis by restricted S1-nuclease digestion and *Dpn*I digestion. *In vitro* replication products were only partially digested by S1-nuclease and *Dpn*I, suggesting the presence of ds as well as ssDNA forms and newly synthesized DNA molecules. These data lend support to the RCR mode of replication and occurrence of *de novo* DNA synthesis *in vitro*.

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

The soluble replication system, as developed above, was used to elucidate the role of Rad54 *in vitro*. The results depicted that the nuclear extract obtained from *rad54Δ* yeast did not support DNA synthesis, but upon reconstituting the reaction with purified Rad54 protein resulted DNA synthesis. These results further supported the notion that Rad54 plays a crucial role in geminiviral replication.

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

The role of Rad54 in geminiviral replication *in planta* 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* whereas in the Rad54 mutant *Arabidposis* line, the ToLCV based amplicon failed to replicate suggesting that Rad54 is critical for the replication of geminiviral based amplicon in the plant system.

In our 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 in this study to be directly involved in geminiviral DNA replication. Transient replication studies with Rad54 mutants of the model plant *Arabidopsis* and ToLCV based viral amplicon further validated and established a crucial role for AtRad54 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.