Introduction

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The molecular mechanism of DNA replication in eukaryotic rolling-circle replicons is still poorly understood. Geminiviruses constitute a large family of plant viruses and contain small, single-stranded DNA genomes that replicate via combination of rolling circle (RCR) and/or recombination-mediated replication (RDR). Their replicative cycle consists of two distinct stages: first, conversion of the genomic ssDNA into double-stranded DNA (dsDNA) intermediates, a process carried out entirely by cellular factors and then formation of new dsDNA replicative intermediates and mature ssDNA genomes by a rolling circle mechanism (Saunders et al., 1991; Stenger et al., 1991) where viral proteins other than host cellular factors are involved. All these processes occur in the nucleus of the infected cell and the replicative intermediates form minichromosomes by association with histones (Pilartz and Jeske, 1992; Kong and Hanley-Bowdoin, 2002) completing their replicative cycle with major input from cellular proteins. In addition, they interfere with cell cycle and growth regulatory pathways of the infected cell (Gutierrez, 1999; Gutierrez, 2000, Gutierrez, 2002). Since geminiviruses code for just a few proteins, interaction between the viral proteins and a large number of host factors becomes an obvious mechanism for virus replication.

In recent years, some progress has been achieved in our understanding of the mechanisms by which geminivirus proteins may impinge on plant cell cycle regulatory proteins, in particular with the retinoblastoma related (RBR) protein. During early G1 phase, pRBR remains bound to one of the important transcription factors, E2F, which controls the transcription of many genes involved in G1/S phase transition and S phase progression. Once pRBR gets phosphorylated at the late G1 phase, it releases the E2F factor, which might turn on the expression of many genes (Gutierrez, 2000). The binding of geminiviral Rep protein to pRBR also releases the E2F factor and might force the cell to enter into S phase like scenario, a condition prevalent also in many animal cells infected with tumour viruses. Among the 180 potential E2F-regulated targets identified in *Arabidopsis*, few components of the replication machinery such as CDC6, ORC1, ORC3, RPA1, MCM, etc. have been shown to be the targets of E2F factor (Ramirez-Parra et al., 2003).

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). The replication origins of all geminiviruses include an invariant nonamer sequence (TAATATT$^{\downarrow}$AC), which is site-specifically nicked and ligated (at the position of the arrow) by Rep, to initiate, and terminate RCR. Rep (or AC1, the replication initiator protein), encoded by all geminiviruses, is a multifunctional protein, which is absolutely required for viral DNA replication and regulates 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 has 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 also involved in several protein-protein interactions, including oligomerization as mentioned above, binding to viral replication enhancer, AC3 (REn, Settlage et al., 1996), AC4, AC5 (Raghavan et al., 2004) and interaction 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), 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 Rep protein is also reported to activate host gene expression such as proliferating cell nuclear antigen (PCNA) (Bagewadi et al., 2004, Kong et al., 2002). PCNA functions in both replicative and repair DNA synthesis (Bravo et al., 1987; Kelman, 1997), its induction and interaction with viral Rep protein does not directly reveal the nature of its role in viral replication though it has been proposed to have a putative role in initiation and control of viral DNA replication (Bagewadi et al., 2004). 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 Mre11, Rad51, DMC1 etc., with MYMIV DNA replication has been shown in our lab using the yeast model system (Raghavan, V., Ph. D. Thesis, ICGEB, 2004).

Till now, there is no report showing the involvement of any viral protein in the conversion of the genomic ssDNA in to dsDNA and amplification of the dsDNA intermediates. In eukaryotic cells, DNA replication begins with the binding of a six subunit 'origin recognition complex' (ORC) to the origin sequences where pre-replication complexes (pre-RCs) can be assembled. ORCs or part of the ORC complex also help in replication of many viral DNA of animal origin (e.g., HPV, EBV etc.) though the viral DNAs lack the obvious binding elements for the ORC subunits (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 are 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 order to replicate their genome successfully, geminiviruses interact with and modulate number of host encoded factors involved not only in DNA replication but also in various cellular processes for their own benefits. Development of an antiviral strategy also necessitates proper understanding of the DNA replication principles of the viral genomes so that the steps could be interfered with. Till date, very few host factors have been characterized in detail for their involvement in the geminiviral replication. Identifying various probable host factors which might have either direct or indirect role in viral DNA replication will give more insight in establishing the mechanism of replication.

Our initial studies on screening of MYMIV Rep interacting proteins revealed one important host factor, *viz.* Rad54 to considerably influence the geminiviral replication efficiency in the yeast model system as well as in *in vitro* and *in planta* studies. 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 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) fail to catalyze strand displacement reactions typical for DNA helicases. Rad54 protein displays dsDNA-specific ATPase activity (Heyer et al., 2006). Typical DNA helicases display ssDNA-dependent/enhanced ATPase activity and use the energy of ATP hydrolysis to translocate on ssDNA (Singleton and Wigley, 2002). On the contrary, Rad54 protein has been demonstrated to use the energy of ATP hydrolysis to translocate on dsDNA inducing topological changes. On circular duplex DNA, Rad54 introduces unconstrained positive and negative supercoils and displaces a triplex-forming oligonucleotide, typical for a translocating motor protein (Heyer et al., 2006). Recently, evidences of supercoiled domains anchored by Rad54 protein (Ristic et al., 2001) and direct visualization of Rad54 translocation on dsDNA (Amitani et al., 2006) provide further evidence for a translocation model.

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). Rad54 interacts with the eukaryotic recombinase Rad51, stabilizes the Rad51-ssDNA filament, shows enhanced chromatin remodeling in presence of Rad51-ssDNA complex (Kwon et al., 2007) and thus plays important roles in homologous recombination. 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).

In order to study the effect of host factors known to play a role in animal viruses such as ORC and to identify other host factors involved in geminivirus DNA replication, the present study was undertaken with the following objectives:

1. To check for the importance of ORC in geminiviral DNA replication via yeast two hybrid interaction with Rep and temperature sensitive (*ts*) mutant studies.
2. To screen for probable Rep-interacting motif(s) using a random peptide phage display library,

3. To investigate the essentiality of the identified host factors in geminiviral DNA replication using the yeast model system and the yeast mutant(s) of the identified host factor(s).

4. To study the *in vivo* and *in vitro* interactions between MYMIV-Rep and the chosen host factor and map their interaction domains.

5. To examine the effect of host factor(s) on the biochemical characteristics of Rep.

6. To study the implication of the host factor in viral DNA replication using geminiviral based viral amplicon (VA) in plant.

7. To develop an *in vitro* replication assay and to apply it in validating the role of the identified host factor(s) in MYMIV DNA replication.