CHAPTER 7

Studies on GRF1
Studies on GRF1: Results and Discussion

GRF1 Results

GRF1, the general regulatory factor 1, belongs to the 14-3-3 family of proteins and is characterized as 14-3-3 homolog isoform chi. The biological functions of this protein are to interact physically with specific client proteins and thereby effect a change in the client. GRF1 is known to regulate diverse processes via binding phosphorylated target proteins in all eukaryotes (Jones et al., 1995; Palmgren et al., 1998; Finnie et al., 1999; Roberts 2000; Fu et al., 2000). Moreover it is involved in a vast array of processes such as the response to stress, cell-cycle control, apoptosis, serving as adapters, activators, and repressors and is characterized by the presence of conserved 14-3-3 domain.

It is known that geminivirus dsDNA replicative forms are supercoiled and exist as minichromosomes which forms the stem-loop structure required for RCR initiation (Pilartz and Jeske, 1992). Stem loop structures are akin to cruciform structures which are secondary DNA structures which serve as recognition signals at or near eukaryotic (yeast and mammalian) origins of DNA replication. GRF1 protein belongs to the family of cruciform-binding protein (14-3-3 protein family) which binds to origins of DNA replication in a cell cycle-dependent manner (Zannis-Hadjopoulos et al., 2008 and Yahyaoui et al., 2007) and thus raises the possibility of involvement of GRF1 in geminiviral DNA replication.

Among the various replication and replication-related factors identified, we focused on investigating GRF1 due to the following reasons.

1. The representation of the peptide matching with GRF1 in the phage display library screening,

2. GRF1 protein has several biochemical functions related to response to stress, cell-cycle control, genome maintenance and apoptosis.

3. A yeast based geminiviral replication assay established in our laboratory (Raghavan et al., 2004) revealed that the mutants of 14-3-3 homolog in yeast (BMH1 and BMH2) affect viral replication efficiency, thereby hinted on the probable role of theses host factors in geminiviral replication., however no association of GRF1 or its homolog in geminiviral replication has been reported till now.

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Based on the above mentioned reasons we explored its role, if any, in geminiviral DNA replication.

7.1 Selection of GRF1 as one of the potential host factor interacting with Rep protein
Among the various peptides identified to interact with Rep protein, one of the peptide sequences in the screening matched with *A. thaliana* GRF1 (NP_567344.1) region 167-PTHPIRLGLAL-178. The AtGRF1 showed 68% identity and 74% similarity among plant species at protein level (Table 7.1).

Table 7.1. The Rep interacting peptides obtained from phage display peptide library screening, showing the frequency of occurrence in the screening and the BLAST hit against GRF1.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Frequency</th>
<th>Source</th>
<th>Start</th>
<th>Alignment</th>
<th>End</th>
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<tbody>
<tr>
<td>PTHPIRLGLAL</td>
<td>3</td>
<td>AtGRF1</td>
<td>1</td>
<td>PTHPIRLGLAL</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>167</td>
<td>PTHPIRLGLAL</td>
<td>178</td>
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7.2 Homology modeling of AtGrf11, ScGrf1 against DrGrf11 3-D structure
The three-dimensional structure of zebrafish GRF1, AtGRF1 and ScGRF1 were obtained from I-TASSER and these structures were superimposed using Swiss protein database (SPDB) software, to locate the position of the peptide region identified from phage display screening on the superimposed structures. The structural alignment of zebrafish GRF1 with AtGRF1 model and ScGRF1 model was performed using Clustal-W software. The peptide region 167-PTHPIRLGLAL-178 of AtGRF1 (Table 7.1) shares significant homology across species with zebrafish, and yeast (Fig. 7.1A) and this region forms the part of a helix and a loop region as revealed by the three dimensional structure of AtGRF1 (Fig. 7.1B). The extent of exposed surface is more prominent in AtGRF1 and thus can be predicted to be involved in protein-protein interaction. The model suggests that the peptide region PTHPIRLGLAL might act as an epitope and contribute to the interaction with Rep protein.

7.3 Interaction of AtGRF1 with Rep
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As the geminiviral Rep is an essential protein required for its replication, in order to study the involvement of GRF1 in geminiviral replication, it is important to examine its interaction with Rep. Moreover, since the peptide phage display library hinted upon the possible interaction of these two proteins, we aimed at validating the interaction of MYMIV-Rep with GRF1 as follows.

7.3.1 Ex vivo yeast two hybrid interactions between ScGRF1 and MYMIV-Rep protein

As a first step towards understanding the role of GRF1 in geminiviral DNA replication, we aimed at confirming its interaction with Rep protein using yeast two hybrid assay. In this assay, AH109 strain was used as a reporter strain, in which the expression of His and LacZ is driven by GAL responsive promoters. Interaction of the two protein partners fused to the DNA binding domain (BD) and the activation domain (AD) reconstitutes the GAL4 transcription factor and activates the expression of GAL4 responsive genes. Thus, expression of both His and LacZ could be used as reporters of protein-protein interaction.

The full length AtGRF1 and MYMIV-Rep genes were separately cloned in frame with both yeast GAL4 activation domain (pGAD) and GAL4 DNA binding domain (pGBD) vectors (Fig. 7.2B and C). The constructs were co-transformed in different combinations in the yeast AH109 strain and the co-transformants were selected on synthetic media lacking leucine and tryptophan. The strength of the interaction was assessed by the ability of the transformants to grow on four dropout plate (Leu" Trp" His" Ade") (Fig. 7.3A) and triple dropout plate (Leu" Trp" His") containing the histidine analog 3-AT (Fig. 7.3B). The results showed that the transformants were able to grow up to a concentration of 15mM 3-AT indicating strong interaction between AtGRF1 and MYMIV-Rep protein. The colony-filter lift assay for β-galactosidase resulted in blue color formation (Fig. 7.3C) re-emphasizing the strong interaction between MYMIV-Rep and GRF1. The MYMIV-Rep self interaction was taken as a positive control for this assay (Malik et al., 2005). When the empty vector (either pGAD or pGBD) was co-transformed with the vectors encoding in-frame fusion proteins, the yeast transformants were unable to grow on four dropout media suggesting specific interactions between two subunits and rejected the possibility of false positives (Fig. 7.3A). These results showed that the full length AtGRF1 interacted with full length MYMIV-Rep in the yeast cell.

With the aim of mapping the interaction domain of MYMIV-Rep, the Rep truncations Rep1-183, Rep184-362, Rep120-362 Rep1-133 were cloned in frame with the pGAD and pGBD vectors (as described in the Materials and Methods section) and the yeast two hybrid analyses
were performed with the full length AtGRF1 as before. The constructs were co-transformed in different combinations in AH109 strain (Fig. 7.4A, B) and the growth of the transformants in four dropout plate (Leu" Trp" His" Ade") revealed that the full length GRF1 interacts with the Rep truncations Rep1-183, Rep120-362 and unable to interact with Rep184-362, Rep1-133 (Fig. 7.4C). Taken together, these results demonstrate that the region responsible for binding to GRF1 lies between the amino acid residues 120 and 182, which constitute the oligomerization domain of Rep.

7.3.2 In vitro pull down assays

To further confirm the result of the yeast two hybrid interaction studies, the interaction of GRF1 and MYMIV-Rep were examined in vitro. For this purpose full length AtGrf1 was cloned in pGEX-4T2 vector for the expression of GST tagged recombinant fusion protein (Fig. 7.2D).

GST tagged full length AtGRF1 protein was purified to near homogeneity by glutathione-Sepharose and Q-sepharose chromatographies (Fig. 7.5 A, B and C). Similarly GST protein was purified (data not shown) and used as control for in vitro pull down assays. The recombinant His-Rep protein was purified by Ni-NTA and Q-sepharose chromatographies (Fig. 4.1) and was further subjected to heparin-sepharose CL-6B chromatography to remove the traces of contaminating proteins including nuclease.

The in vitro interaction study was carried out by following the Ni-NTA pull-down assay as depicted in the flow chart (Fig. 7.6A). The two proteins were mixed along with the Ni-NTA agarose beads and incubated in a binding buffer followed by gradual washing with increasing salt concentration (100-600mM NaCl). In principle, the interacting protein would co-elute along with the protein bound to its affinity matrix (bound fractions) while the non-interacting protein would not appear in unbound fractions. The bound and unbound fractions were analyzed on 10% SDS-PAGE. Two bands corresponding to GST-GRF1 and His-Rep were observed in the bound fraction even after 600mM NaCl wash indicating a strong interaction between the two proteins (Fig. 7.6B, lanes 8-10). As a control, His-Rep was incubated with purified recombinant GST protein (Fig. 7.6B) and the absence of any band corresponding to His-Rep in the bound fraction revealed that the GST tag did not have any effect on the interaction (Fig. 7.6B, compare lanes 2 and 7).

7.4 Elucidation of the role of AtGRF1 in geminiviral replication using yeast model system
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Budding yeast is conveniently used as a model system to elucidate the role of GRF1 in geminiviral DNA replication. Earlier studies had shown that budding yeast supported geminiviral DNA replication and that the plasmid YCpO"-2A, harboring two tandem copies of DNA-A component of MYMIV bipartite genome, replicated in yeast by using specifically the viral replication origin and virus encoded factors (Raghavan et al., 2004). The vector construct has been represented schematically as a vector map in Fig. 5.5B. Briefly, the vector YCpO"-2A was constructed by removing the ARS sequence (Autonomously Replicating Sequence) from the yeast shuttle vector YCpSO (Fig. 5.5A) by digestion with Xhol and BgII which yielded YCpO− vector and the two tandem copies of MYMIV-A genome was cloned in the YCpO− vector at the HindIII site, yielding the plasmid YCpO"-2A. The plasmid YCpO"-2A and control vector YCp50 were used to transform the wild type as well as GRF1 deletion mutant (grf1A) yeast strains and the efficiency of transformation were calculated by their colony forming units.

7.4.1 Replication efficiency assay of YCpO"-2A in yeast GRF1 deletion mutant (grf1A)

The wild-type (WT) yeast BY4742 and the grf1A strains were transformed with the control plasmid YCp50 and the YCpO"-2A plasmid and the relative transformation efficiency was calculated by taking the CFU of the control plasmid YCp50 in the WT strain as 100%. The result showed that the replication efficiency of the grf1A strain harboring YCpO"-2A plasmid was reduced to 45% while the YCp50 plasmid was ~85% (Fig. 7.7A). This demonstrates that in absence of GRF1, the replication efficiency of the plasmid harboring geminiviral origin is severely compromised as compared to that of ARS containing plasmid, indicating that GRF1 is required for efficient replication of geminiviral DNA in yeast.

7.4.2 Yeast replication restoration assay in grf1A mutant by complementation

In order to further substantiate our above finding, the grf1A mutant yeast was complemented with wild type A1Grf1 gene and transformed with YCpO"-2A plasmid. The replication efficiency of YCpO"-2A in grf1A mutant yeast (having empty vector pGBDC1) decreased immensely (~34%) while there was a complete restoration of replication (~100%) when complemented with a GRF1 expressing plasmid (pGBD-Grf1) (Fig. 7.7B and C). Together, these results suggested that GRF1 is critical for replication of plasmid DNA having the geminiviral origin of replication compared to that of plasmids with yeast ARS. This is the first report showing that GRF1 plays an active role in geminiviral DNA replication.
7.5 Effect of AtGRF1 on various biochemical properties of MYMIV-Rep

In order to understand how the interaction of GRF1 with MYMIV-Rep supports viral replication, we investigated the modulation of various biochemical activities of MYMIV-Rep in presence of AtGRF1. The role of MYMIV-Rep protein during initiation of geminiviral DNA replication has been implicated by its nicking activity (Pant et al., 2001). Further, MYMIV-Rep has been demonstrated to be involved in the elongation process as well by its intrinsic ATPase and helicase activities (Choudhury et al., 2006). Understanding how these biochemical functions of MYMIV-Rep are modulated by GRF1 would shed light on the complicated mechanism of viral DNA replication. With this view, the following biochemical assays were carried out with purified GST-GRF1 and His-Rep proteins. The assays were also performed with GST protein used as a control.

7.5.1 Modulation of binding activity of MYMIV-Rep to the origin DNA ‘CR’ by AtGRF1

The DNA binding activity of MYMIV-Rep to the common region (MYMIV-CR, the origin of RCR) in presence of GST-GRF1 was analyzed in the filter binding assay (Fig. 7.8). The MYMIV-Rep possesses sequence specific binding activity to the CR region (Singh et al., 2008). The 5'-labeled 260 bp common region (CR) was incubated in presence of MYMIV-Rep in absence and presence of various concentrations of GST-GRF1 and the DNA binding ability was analyzed through scintillation counting. In a parallel experiment, the binding activity of GST-GRF1 to the ‘CR’ region was also checked in absence as well as in presence of various concentrations of MYMIV His-Rep. The results showed that GST-GRF1 binds to ‘CR’ and it also enhances the binding activity of Rep to ‘CR’ DNA. The binding of MYMIV His-Rep (475nM) to ‘CR’ increases two fold in presence of GST-GRF1 (30nM) compared to that in absence of GST-GRF1 (Fig. 7.8A). Similar results were also observed when GST-GRF1 binding activity was studied in presence of MYMIV His-Rep, where it was observed that there was 1.5 fold increase in the GST-GRF1 (675nM) binding ability to ‘CR’ in presence of MYMIV His-Rep (50nM) (Fig. 7.8B). The specific binding nature of MYMIV-Rep and GST-GRF1 to ‘CR’ also studied by comparing the binding abilities of these proteins to non-CR region (as mentioned in the Materials and Methods section) and we observed that GST-GRF1 bound to non-CR region, although the binding affinity was lower as compared to binding to CR region (Fig. 7.8C) however Rep did not show any binding to
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Thus, we can conclude that GST-GRF1 has DNA binding activity and it also enhances the DNA binding activity of Rep to 'CR' DNA.

7.5.2 Study of oligomeric status GST-GRF1 in vitro

GST-GRF1 forms homo or hetero dimer in solution. It will be interesting to see the oligomeric status of GST-GRF1 in solution. For this purpose, GST-GRF1 were subjected to sucrose density gradient centrifugation as described in the 'Materials and Methods' section. Different molecular mass markers were also used for centrifugation under the same experimental conditions. Several fractions were collected for each molecular mass marker and peak fraction was identified (shown as table, Fig. 7.9A). A standard curve was plotted using the log fraction number (peak fraction) against molecular mass of the standard proteins (Fig. 7.9B). Subsequently, the peak fraction of GST-GRF1 was identified by SDS-PAGE and coomassie staining of the gel containing different fractions following centrifugation (Fig. 7.9C). The log of the fraction number 9 was then extrapolated on the standard curve and the molecular mass of native GST-GRF1 (60 kDa) was estimated to be ~130 kDa. The apparent difference in the calculated molecular masses between the GST-GRF1 proteins could be due to the presence and some extra amino acid residues in GST-GRF1 contributed by the expression vector.

Comparison of the calculated molecular mass of monomeric form of GST-GRF1 (60 kDa) with the estimated molecular mass obtained from sucrose gradient experiments revealed that the GST-GRF1 would be in dimeric form. Thus, GRF1 forms homo-dimer in its native form.

7.5.3 Modulation of the nicking activity of MYMIV-Rep by AtGRF1

In order to investigate the role of AtGRF1 in initiation of RCR, if any, we carried out the nicking assay of MYMIV-Rep in presence of GST-GRF1 protein. In the assay, the 5'-32P-labelled 26-mer nucleotide substrate was incubated with 230 n mole of Rep in presence or absence of GST-GRF1 and the 18-mer cleaved product was analyzed on 15% Urea-PAGE. Our results demonstrate that the nicking activity of Rep was enhanced ~4-5 fold in presence of increasing amounts of GST-GRF1 (160 to 800 nmol) (Fig. 7.10, lanes 2-7). GST-GRF1 and GST proteins did not show any endonucleolytic activity as revealed by the absence of any degradation products (Fig. 7.10, lanes 8 and 9). In presence of 650 n moles of GRF1, the nicking activity of 230 n mole of Rep was enhanced ~4.5 folds which is comparable to the amount of nicking reaction catalyzed by 950 n mole of Rep alone (Fig.
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7.10, compare lanes 2 and 6). This suggests that even at low concentrations of Rep inside the host cell, the initiation of RCR can be enhanced several fold in presence of host GRF1. These results suggested that GRF1 is likely to play a role in initiation of RCR of the geminiviral DNA.

7.5.4 Modulation of the ATPase activity of Rep by AtGRF1

After establishing a role of GRF1 in the initiation of geminiviral RCR, we questioned whether GRF1 has any role in replication elongation and proceeded to examine the effect of AtGRF1 on the ATPase activity of MYMIV-Rep in presence of GST-GRF1 protein. The ATPase experiments with Rep were carried out in absence and presence of GRF1 to assess the effect of GRF1 on the ATPase function of Rep. The intensity of ATPase activity was calculated from autoradiogram and free Pi intensities plotted as histograms. The results demonstrate that the ATPase activity of Rep showed ~2 fold increment in presence of increasing amounts of GST-GRF1 (75 to 315 nmole) (Fig. 7.11, lanes 2-5). As GST-GRF1 also possesses ATPase activity the amount of ATP hydrolysis by 315 nmole of GRF1 alone was taken as control for above calculations (Fig. 7.11, compare lanes 2, 5 and 7). These results suggest that GRF1 might help Rep protein in its post-initiation activities of viral DNA replication where the ATPase activities play crucial role.

7.5.5 Modulation of the helicase activity of Rep by AtGRF1

Since enhancement of the ATP hydrolysis in presence of GRF1 is indicative of its role in replication elongation step and the helicase function is known to be dependent on ATPase activity, we first checked whether GST-GRF1 alone promotes separation of 2 complementary strands of partial duplex DNA substrates. We used the 3 different helicase substrates as shown in Fig. 7.12A, B and C. One of the substrates had a flushed end (M13FWD23), while the others had 6-nt flaps at the 5'- and 3'-ends (HELFOK8 and HELFOK9), respectively (Choudhury et al., 2006). In all of these helicase reactions, no strand displacement was observed in the reactions even at a high concentration of GST-GRF1 (640 nmol). It is thus clear that GST-GRF1 alone is incapable to function as a helicase.

We further examined the effect of AtGRF1 on the helicase activity of MYMIV-Rep.. The substrate (31-mer oligonucleotide annealed to M13mp18 DNA) was incubated with increasing amounts of the proteins as indicated followed by autoradiography (Fig. 7.13A, B). It was observed that with increasing amounts of GST-GRF1 (80-640 nmoles) a
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maximum of 81% unwinding was observed (Fig. 7.13, lane 6), considering the unwinding of boiled substrate as 100%, whereas GST-GRF1 alone did not show any displacement (Fig. 7.13B, lane 7). The helicase activity of Rep in presence of GRF1 was found to be ~4-fold higher than that of Rep alone (Fig. 7.13B, lanes 1 and 6).

From these results it is thus clear that similar to nicking and ATPase activities, GRF1 also enhances the helicase activity several fold even with the minimum amount of Rep used. These results together reaffirm the role of GRF1 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.

7.6 Role of GRF1 in geminiviral replication in planta

The plant being the natural host of geminiviruses, it is important to investigate the role of GRF1 in viral replication in planta. In order to study the viral replication in host and non-host plants, a transient replication assay using a Tomato leaf curl virus (ToLCV) based viral replicon (termed as viral amplicon or VA) was constructed in our laboratory earlier (Pandey et al., 2009). The construct used is schematized in Fig. 6.6, where the minimal region of replication CR-AC3 and CR were cloned in pCAMBIA 1391Z binary vector. The construct also includes a 35S promoter and a polyA signal region. Studies indicate that when the tobacco leaves are agroinfiltrated with the agrobacterium harboring the binary construct (Cam/VA), a circular episome of 2.6 kb is released (Pandey et al., 2009) following Rep dependent site-specific nicking action at CR and subsequent DNA replication by RCR (Stenger et al., 1991).

We argued that if replication (and consequent formation of 2.6 kb episomal DNA) were dependent on GRF1, grf1 mutant Arabidopsis lines would render the line defective in replication and cause lower level (or zero level) of accumulation of the 2.6 kb episome compared to the WT Col-0 lines. In order to identify the replicated episomal DNA (2.6 kb), we employed PCR based method of detection as explained below. Upon release of the 2.6 kb episome, PCR using the 35S forward and Rep133 reverse primers would result in the amplification of a 1.4 kb fragment as schematized in Fig. 6.6. However, no PCR amplification would be expected with unreplicated Cam/VA (12.9 kb) DNA template under such conditions owing to the low processivity of the Taq Polymerase.

The agrobacterium LBA 4404 cultures harboring the Cam/VA plasmid were infiltrated into the leaves of wild type Arabidopsis thaliana Col-0 and a confirmed atgrf1 T-DNA insertion
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The atgrf1Δ mutant lines showed normal phenotypes as the wild type Col-0 plants (Fig. 7.14A, B). As negative control, agrobacterium LBA 4404 cultures harboring the vector pCAMBIA-13912 was also infiltrated into the wild type and atgrf1Δ mutant plants. After 4, 8 and 12 days post-infiltration the leaves were collected and total genomic DNA were isolated by standard (CTAB) method.

The total DNA isolated from the agro-infiltrated leaves of Col-0 and atgrf1Δ lines were used as template for the PCR. The PCR was limited to 26 cycles only to keep the PCR products to minimum to facilitate the comparative quantification between the lines. The Col-0 4, 8 and 12 dpi samples showed a gradual increase in the 1.4 kb amplified fragment while the atgrf1Δ samples showed no amplification except for the 4 dpi sample, which showed a significant amplification (Fig. 7.15A, lanes 2-4 and 6-8 respectively). In order to distinguish between the replicated episomal and unreplicated parental DNA, the PCR was carried out with the DpnI digested templates. The bands were quantified and normalized with respective actin controls and plotted as histogram (Figs. 7.15A and B). It is evident from the results that the atgrf1Δ lines did not support the replication of cam/VA indicating the role of AtGrf1 in geminiviral replication. The level of actin signal in the PCR with the actin specific primers, on the other hand, showed the uniformity in loading control (Fig. 7.15A). This transient replication assay strongly points towards a vital role played by GRF1 in viral DNA replication in planta.

7.7 Discussion

In this part of our study we have investigated the role of Arabidopsis Growth Regulatory Factor 1, a representative of the 14-3-3 family in geminivirus replication. It is known that geminiviruses require many host factors for genome replication, however, the roles of many of these are yet to be elucidated. The Arabidopsis GRF1 was chosen in this study, as a previous investigation of host factors involved in MYMIV replication (yeast based geminiviral replication assay) hinted on the Saccharomyces cerevisiae homolog of 14-3-3 (BMH1 and BMH2) as factors affecting viral replication (data not shown). A phage display based search of the host factors interacting with MYMIV-Rep protein identified the Arabidopsis thaliana homolog of 14-3-3 as a putative Rep interacting partner (data not shown).

The 14-3-3s play a central role in many cellular processes that involve regulation by phosphorylation. They are involved in the control of cell cycle, in differentiation, in apoptosis, in targeting of proteins to different cellular locations and in the coordination of
multiple signal transduction pathways (Palmgren et al., 1998; Finnie et al., 1999; Roberts 2000). The common feature of 14-3-3s is their ability to bind target proteins, and their diverse functions are always ascribable to this property. Thus, considering GRF1 protein as a representative of 14-3-3 family (which is conserved across the plant kingdom) the protein is studied in detail with regard to MYMIV and ToLCV genome replication. It has been suggested that this family of proteins has an indirect role in DNA replication. However, no report is available in the literature to indicate if GRF1 is involved in the establishment or elongation of the replication fork.

Because the replication of begomoviruses is entirely dependent on the virus-encoded replication initiator protein Rep, the host factors that interact with Rep is likely to contribute to RCR in Begomovirus genomes. Employing phage display technique we observed that the peptide 'PTHPIRLGLAL', which interacts with MYMIV-Rep, shares sequence homology with yeast, Arabidopsis and zebrafish GRF1, as shown in blue in Fig. 7.1B. The superimposed 3D structural model revealed that the above-mentioned peptide region in Arabidopsis, yeast and zebrafish are exposed on the surface, which renders the possibility for this peptide region to directly interact with MYMIV-Rep. However, a structure for the Arabidopsis GRF1 and MYMIV-Rep complex is required to reveal the precise role of the 'PTHPIRLGLAL' peptide.

Using different interaction assays, viz. the yeast two-hybrid (Fig. 7.3) and in vitro binding assays (Fig. 7.6) we showed that MYMIV-Rep and AtGRF1 interact with each other. The yeast two hybrid results also demonstrate that the region responsible for binding to GRF1 lies between the amino acid residues 120 and 182, which constitute the oligomerization domain of Rep (Fig. 7.4). Because Rep interacted with AtGRF1 via its oligomerization domain, the oligomeric status of Rep might be important for this interaction with GRF1 protein. However, as the GRF1 contain only one known domain (the 14-3-3 domain) which covers the major part of the gene, it was difficult to carry out similar domain interaction studies of AtGRF1.

**Yeast supports GRF1-dependent geminivirus DNA replication in vivo**

Developing the model systems, especially a yeast model to gain information on biological processes has been a standard and long-standing goal of molecular biologists, as evidenced from a large number of reports in the literature (Longo and Fabrizio, 2002; Perego et al., 2000; Simon and Bedalov, 2004; Foury and Kucej, 2002; Siggers and Lesser, 2008; Replansky et al., 2008; Zhu, 2001; Alves-Rodrigues et al., 2006; Ahlquist, 2006;
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Nagy, 2008). An earlier study from our laboratory revealed that a plasmid vector harboring geminiviral origin of replication could replicate efficiently in budding yeast (Raghavan et al., 2004). Hence, we used the yeast model system to elucidate the role of GRF1 in geminiviral DNA replication. Our data demonstrated that GRF1-deficient (grf1A) yeast do not support the viral DNA replication well. Further, the replication complementation assay showed that when the grf1A yeast strains were complemented with Atgrf1 gene, the replication efficiency was restored to the wild type value (Fig. 7.7B, C). This clearly indicates that AtGRF1 functionally complements ScGRF1. Interestingly, AtGRF1 interacted with the oligomerization domain of the MYMIV-Rep protein. This is significant since most of the host proteins e.g., PCNA (Bagewadi et al., RAD54 (Kaliappan et al., 2011), pRBR (Xie et al., 1996), GRIK, and GRIMP (Kong and Hanley-Bowdoin, 2002) etc. interact with Rep through the oligomerization domain of the Rep and only a few proteins, namely, GRAB1 and GRAB2, are known to interact with the C-terminal domain of Rep.

Our data on interaction study combined with that obtained through the ex vivo replication assay clearly suggest the importance of the GRF1-Rep interaction in viral DNA replication.

Importance of AtGRF1 in geminivirus replication

As evident from our study, recombinant AtGRF1 alone has no role in site-specific nicking or helicase activity. Thus, the role of this protein in either RCR initiation or elongation can be ruled out. However, its association with MYMIV-Rep enhances the nicking and helicase activities of Rep. It is possible that Rep undergo an allosteric change following complex formation with AtGRF1 such that the region surrounding Y103 is more exposed and enhances nicking near the RCR initiation site. Additionally, the AtGRF1-Rep complex is also shown to enhance the CR-specific DNA binding, which increases RCR initiation. GRF1 also possesses an ATPase motif; thus, its association with Rep may increase Rep-mediated ATPase activity. Such increased ATPase activity, in turn, might enhance Rep-mediated helicase activity.

GRF1 dependence of geminiviral RCR in planta

To examine if the conclusions derived based on our in vitro and ex vivo models that GRF1 protein is required for geminiviral RCR hold true for in planta studies as well, we employed a viral amplicon (VA) based on the CR-AC3 region of Tomato leaf curl virus (ToLCV-VA), developed earlier in our laboratory (Pandey et al., 2009). The genome structures are similar for both ToLCV and MYMIV-DNA-A, and both replicate through RCR mechanisms. The
ToLCV and MYMIV-Rep proteins have long stretches of amino acid identity. The Rep oligomerization domain required for GRF1 binding from both proteins exhibit 81% aa identity (Palmgren et al., 1998) Moreover, the ToLCV-VA can replicate in a wide variety of monocot and dicot plants using RCR principles (Pandey et al., 2009). Because the host deletion mutant is available only in Arabidopsis and the ToLCV replicon accumulates well in WT Arabidopsis, we tested the GRF1 requirement using an Arabidopsis host.

An additional consideration in choosing the ToLCV-VA was to test the generality of the GRF1 requirement, if any, in geminiviral DNA replication. The in planta model study offers a more convenient method to study geminiviral replication as in such study the replication of geminivirus can be directly monitored by the RCR release of viral ssDNA. Our results displayed no accumulation of ToLCV-VA in case of the Arabidopsis grf1A mutant until 12 d postinoculation, whereas a substantial accumulation of the amplicon was observed in WT Arabidopsis. This leads to the conclusion that GRF1 plays an important role in geminiviral DNA RCR.

Thus, GRF1 likely plays a significant role in Begomovirus DNA RCR. However, GRF1 can operate through multiple mechanisms in vivo. Its role in the coordination of multiple signal transduction pathways (Jones et al., 1995; Palmgren et al., 1998; Finnie et al., 1999; Roberts, 2000; Fu, H. et al., 2000) is not difficult to envisage.

Taken together, these results demonstrate that GRF1 plays essential roles in initiation as well as elongation steps of rolling-circle replication of geminiviral DNA and its role has been re-emphasized through ex vivo, in vitro and in planta assays. To the best of our knowledge this is the first report showing the role of a growth regulatory protein GRF1 in DNA replication thereby implicating on wider spectrum of biochemical activities of GRF1.

Thus in the present study, we show for the first time that AtGRF1 interacts with Begomovirus-Rep and increases Rep-mediated nicking and helicase activity, which implies that it has a role both in RCR initiation and replication fork progression. Also, the absence of host GRF1 down regulated viral DNA replication in yeast as well as in planta model. This is clear demonstration of a direct role of 14-3-3 GRF1 in begomoviral DNA replication RCR. However, whether such role of GRF1 in begomoviral RCR replication also holds true for other geminivirus genera, such as Mastrevirus, Curtovirus, and Topocuvirus, needs further investigation. If Rep-GRF1 interaction is found to be universal for all geminivirus and host combinations, then a suitable antigeminivirus strategy could be conveniently evolved.