THE ROLE OF TOMBUSVIRUS REPLICASE PROTEINS AND RNA IN REPLICASE ASSEMBLY, REPLICATION AND RECOMBINATION

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THE ROLE OF TOMBUSVIRUS REPLICASE PROTEINS AND RNA IN REPLICASE ASSEMBLY, REPLICATION AND RECOMBINATION

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Zivile Sliesaraviciute Panaviene

Lexington, Kentucky

Director: Dr. Peter D. Nagy, Associate Professor of Plant Pathology

Lexington, Kentucky

2004

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ABSTRACT OF DISSERTATION

THE ROLE OF TOMBUSVIRUS REPLICASE PROTEINS AND RNA IN REPLICASE ASSEMBLY, REPLICATION AND RECOMBINATION

Tombusviruses are single, positive strand RNA viruses of plants, often associated with parasitic defective interfering (DI) RNAs. Two viral-coded gene products, namely p33 and p92, are required for tombusvirus replication. The overlapping domains of p33 and p92 contain an arginine/proline-rich (RPR) RNA binding motif. In this study, the role of RPR motif and viral RNA in tombusvirus replication and recombination, as well as involvement of viral RNA in tombusvirus replicase assembly was examined.

Using site-directed mutagenesis I generated a series of RPR mutants of Cucumber necrosis tombusvirus (CNV). Analysis of RPR mutants defined that wild type RPR motif, especially two of the four arginines, were required for efficient RNA binding in vitro, for replication of tombusviruses, their associated DI RNAs, subgenomic (sg)RNA synthesis and DI RNA recombination in vivo. Experiments using a two-component tombusvirus replication system showed that RPR motif is critical for functions of both p33 and p92 in replication, but its role in these proteins might not be identical. Recombination studies using a novel tombusvirus three-component system revealed that mutations in RPR motif...
of p33 replicase protein resulted in an altered viral RNA recombination rate. Identified DI RNA recombinants were mostly imprecise, with recombination sites clustered around a replication enhancer and an additional putative cis-acting element that might facilitate the template switching events by the tombusvirus replicase.

To study the role of RNA during the assembly of functional tombusvirus replicase, recombinant CNV replicase that showed similar properties to plant-derived CNV replicase was purified from *Saccharomyces cerevisiae*. When in addition to p33 and p92 proteins DI RNA was co-expressed in yeast cells, the isolated replicase activity was increased ~40 fold. Further studies defined RNA motifs within two short DI RNA regions that enhanced active CNV replicase formation.

In summary, this study showed that the conserved RNA binding motif of the tombusvirus replicase proteins and viral RNA are involved in replicase assembly, viral RNA replication, subgenomic RNA synthesis and RNA recombination. This data shed new light on the complex roles of the viral elements in replication, and will help future studies aimed at interfering with viral infections.

**Keywords:** *Tomato Bushy Stunt Virus*, RNA dependent RNA polymerase; Defective Interfering RNA; Replication enhancer; Replicase assembly

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05/18/04
THE ROLE OF TOMBUSVIRUS REPLICASE PROTEINS AND RNA IN REPLICASE ASSEMBLY, REPLICATION AND RECOMBINATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

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ACKNOWLEDGMENTS

The following dissertation, while an individual work, benefited from insights and direction of several people. I am the most grateful to my advisor Dr. Peter D. Nagy for all support, understanding and patience during the graduate studies. Dr. Peter D. Nagy was always willing to answer the questions, gave very useful suggestions that helped to complete this work. Next, I would like to thank my Committee members Dr. Said A. Ghabrial, Dr. Michael Goodin and Arthur G. Hunt for their questions, ideas that expanded my knowledge, made me think about broader aspects of my study. I would like to thank my outsider examiner Dr. Theo Dreher for coming to the final exam, sharing his knowledge and giving suggestions how to improve the dissertation.

I am very grateful to Jannine Baker for all the help and technical assistance that allowed me to do this work. And I thank all formal and present Dr. Peter D. Nagy’s lab members for everyday advices, ideas, suggestions, and for being good friends. I am very grateful to other people in Plant Pathology department, especially graduate students for such a friendly atmosphere and support.

I could have not been able to go through graduate studies without my husband Tadas Panavas. I would like to say many thanks for his advices at work and all the help at home, especially for his patience and believe that this work could be done. I am thankful to our sons Karolis and Liudas for all the moments that helped me to forget about work and enjoy other things.

The huge support that kept me going and working during graduate study years came from my family and friends. I would like to say special thank you to my parents and grandmothers for their love and believe in me, for trying to help me in any possible way. I would like to thank my brother and his family, my mother in law and all the friends here and back at home for understanding and supporting me during these years.
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Chapter One

The overview of Tombusviruses

General properties

Tombusvirus is a genus of plant viruses in the Tombusviridae family. Tombusviruses have a single positive stranded RNA genome component about 4.7kb long (Russo et al., 1994). The virus genome is encapsidated by 180 subunits of 41kDa coat protein that form a 32-34 nm icosahedral particle (Russo et al., 1994).

Tombusviruses can infect plants from different groups (woody, perennial, ornamentals) although the host range of each individual tombusvirus is relatively narrow. An exception is the prototypical member of the family, Tomato bushy stunt virus (TBSV), which causes diseases of plants from wide ranging species, including tomato and other solanaceous plants, cherry, apple, plum, and grapevines (reviewed by T. J. Morris, 2001). Under experimental conditions, TBSV can infect plants from more than 100 species that belong to 20 families (Martelli et al., 1988)

Diseases caused by tombusviruses are soil borne, but from all studied tombusviruses only for Cucumber necrosis virus (CNV) has the vector, Chytrid fungus Olpidium bornovanus, been identified (Campbell, 1996). Most likely other tombusviruses can spread by soil water or by infected woody plant stocks (reviewed by T. J. Morris, 2001).

Molecular biology studies of tombusviruses have been done mainly using four members of the genus:TBSV, Cymbidium ringspot virus (CymRSV), Cucumber necrosis virus (CNV) and Carnation Italian ringspot virus (CIRV).

Genome organization of Tombusviruses

The plus-stranded genome of tombusviruses, like many other plant viruses (Dreher, T.W., 1999) lacks poly-A tail and 5’-cap (Russo et al., 1994), yet it is still translation competent and it encodes five functional open reading frames (ORFs) (Figure 1.1) between conserved 5’-AGAAAU… and …GCAGCCC-3’ sequences at the ends of untranslated regions (UTRs) (Russo et al., 1994). The replicase proteins of tombusviruses
p33 and p92 encoded by ORF1 and ORF2, respectively, are translated directly from gRNA (Figure 1.1). Tombusviruses use ribosomal readthrough mechanism of amber translation termination codon at the end of p33 to express p92 protein. Due to this translation strategy the, N-terminus of p92 contains the entire p33 sequence (Scholthof et al., 1995d).

The coat protein (p41) of tombusvirus, encoded by ORF3, is located downstream from the replicase protein genes (Figure 1.1). At the 3’ proximal end of genomic RNA are genes of cell-to-cell movement protein (p22) and suppressor of gene silencing (p19) (Figure 1.1). The sequences of the latter two proteins are overlapping, but they have distinct ORFs (ORF4 and ORF5). The leaky scanning translation mechanism allows production of both proteins from the same subgenomic (sg) RNA (Russo et al., 1994).

To express genes encoded by ORFs 3 through 5, tombusviruses use (sg) RNAs (Figure 1.1): sg RNA1 (~2.1kb) for ORF3 (p41), and sgRNA2 (~0.9kb) for ORF4 (p22) and ORF5 (p19). The transcription of sgRNA2 occurs earlier in virus infection cycle than transcription of sgRNA1 (Johnston and Rochon, 1995; Qiu and Scholthof, 2001; Tavazza et al., 1994; Zhang et al., 1999), suggesting possible regulation of protein expression time.

Proteins encoded by tombusviruses

Translation strategies of tombusviruses

It has been shown that for translation initiation in eukaryotic cells, 5' cap of mRNA bound by eukaryotic initiation factor eIF-4E and poly(A), bound by poly-A binding protein (PABP) are brought together via interaction with eIF4G forming "closed loop" structure, which promotes translation initiation by attracting the 40S ribosome subunit (reviewed in Bushell and Sarnow, 2002). Small ribosome subunit with attached methionyl- initiation tRNA scans mRNA in the 5' to 3' direction until it reaches an initiation codon, where 60S ribosome subunit binds and ribosome starts translation elongation process (reviewed in Bushell and Sarnow, 2002).

The 5’cap or 3’polyA tail structures are not present in tombusviruses. Nevertheless, these plant viruses are able to utilize eukaryotic cell translation machinery. The tombusvirus translation was studied using 3’end deletion mutants of TBSV. These studies revealed that 3’cap independent translation enhancer (3’CITE) was critical for the translation of tombusvirus
proteins (Wu and White, 1999). Interestingly, when mutant RNAs lacking 3’CITE were capped, protein translation occurred, suggesting, that 3’CITE can have similar function as 5’cap. In addition, it was found that for tombusvirus translation RNA-RNA interaction between 5’UTR and 3’CITE is necessary (M. Fabian and K.A. White, unpublished). It is possible that tombusvirus translation mechanism is similar to that reported for the luteovirus BYDV (Guo et al., 2001), where 3’translation enhancer interacts with 5’UTR via “kissing loop” mechanism. It was proposed that due to this interaction the circularization of viral RNA occurs that brings translation initiation factors and/or ribosomal subunits bound to 3’translation enhancer to the 5’end and promotes translation initiation (Guo et al., 2001).

To express proteins that are encoded by internally located ORFs tombusvirus replicase transcribes two sgRNAs. Several mechanisms for the synthesis of sgRNAs have been proposed (Miller and Koev, 2000). These include 1) internal initiation, when replicase initiates (+) sgRNA synthesis on the (-) strand gRNA; 2) premature termination, when replicase stops (-) strand synthesis due to secondary structure formed on the gRNA. These shorter (-) strands then are used to make (+) sgRNAs; 3) discontinuous transcription, which involves replicase jumping from termination site to the 5’end of gRNA during (-) strand synthesis. The findings that long distance interactions between tombusvirus (+) gRNA sequences can form secondary structures that might force the viral replicase to terminate (-) strand synthesis just upstream of sgRNA initiation sites (Choi et al., 2001; Choi and White, 2002) support the premature termination model.

To express p92 protein tombusviruses use translational readthrough of UAG termination codon. It is thought that a tyrosine is inserted at this position in p92 in vivo and accordingly, in vitro gRNA transcript that has tyrosine codon substitution in place of stop codon, has been shown to be able to complement tombusvirus replication (Oster et al., 1998).

To produce p19 protein from sgRNA2, tombusviruses use leaky scanning mechanism whereby some ribosomes do not initiate at the first start codon (for p22), but start at the second ORF start codon. It has been shown that amount of p22 and p19 depend on the length of 5’UTR in sgRNA2 and the sequence context around the start codon. When longer 5’UTR was constructed, more p22 was made, but when sequence context was made less favorable for p22 translation, the level of p19 increased (Johnston and Rochon, 1996).
**p33 and p92 are replicase proteins of tombusviruses**

The tombusvirus proteins p33 and p92, expressed directly from gRNA, are membrane associated (Rubino and Russo, 1998; Scholthof et al., 1995d). The p92 is a fusion protein made via ribosomal readthrough mechanism of p33 translation termination amber codon and it is 20 times less abundant than p33 in virus infected cells (Scholthof et al., 1995d). The tombusvirus replication studies using mutants of viral proteins showed that p92 and p33 are required for virus replication (Molinari et al., 1998; Oster et al., 1998; Scholthof et al., 1995d) and subgenomic RNA synthesis *in vivo* (Rubino et al., 1995; Scholthof et al., 1995d).

Amino acid comparison revealed the presence of GDD motif in p92 that is conserved among RNA polymerases (O'Reilly and Kao, 1998), suggesting that p92 functions as an RNA dependent RNA polymerase. The GDD motif is located in readthrough domain of p92. Mutations in GDD motif of Artichoke mottle crinkle virus (AMCV) p92 proteins abolished viral replication in protoplasts (Molinari et al., 1998) and supported the role of p92 as RNA-dependent RNA polymerase.

Comparative sequence analysis of p33 has not revealed any known motifs and the role of p33 in virus infection has not been defined, but it was shown that p33 was necessary for virus replication *in vivo* (Dalmay et al., 1993a; Kollar and Burgyan, 1994; Molinari et al., 1998; Oster et al., 1998; Scholthof et al., 1995b). To confirm that p33 was required for virus replication *in vivo*, the UAG (amber) stop codon at the end of p33 sequence was changed to UAC or UAU (encoding tyrosine). After this modification, only p92 was expressed from gRNA and virus replication was completely abolished in protoplasts (Oster et al., 1998; Scholthof et al., 1995d). These experiments showed that expression of p92 was not sufficient for virus replication, although p33 sequence is present in N terminus of p92. When p33 protein was expressed in trans from a defective interfering (DI) RNA in addition to p92, viral RNA was amplified (Oster et al., 1998). These results suggested that p33 was a part of viral replicase and that separate p33 expression was required for virus replication *in vivo*. Tombusvirus replicase purification studies supported the above data, showing that purified active replicase contained both proteins, p92 and p33 (Pogány and Nagy, unpublished).

The presence of membrane localization domain in the N terminus of p33 suggested that one of the roles of p33 in virus replication is membrane targeting of virus replication complex.
and formation of Multivesicular bodies (MVB) where virus replication likely occurs. It has been shown that p36 (CIRV) and p33 (CymRSV) proteins caused proliferation of mitochondrial or peroxisomal membranes, respectively (Rubino et al., 2000; Rubino and Russo, 1998a; Rubino et al., 2001; Weber-Lotfi, 2002). The role of p33 in RNA and possibly p92 targeting to the replication sites is supported by recent findings of RNA binding motif in p33 (Rajendran and Nagy, 2003) and p33:p92 interaction domain located at the C terminus of p33 (Rajendran and Nagy, in press).

**Coat protein, p41**

Tombusvirus coat protein sequence is located downstream from the ORFs coding for replicase proteins. Therefore, the coat protein is expressed from sgRNA1. A single coat protein is about 41kDa. Tombusvirus capsid is T=3 icosahedron composed of 180 coat protein molecules (Russo et al., 1994). It has been shown that tombusvirus coat protein is not required for virus replication and cell-to-cell movement (Dalmay et al., 1993a; McLean et al., 1994; Qiu and Scholthof, 2001), although mutations in coat protein delayed long distance movement and symptom development (Dalmay et al., 1992).

The studies of CNV coat protein revealed that it is important in virion binding to fungal vector *Olpidium bornovanus* zoospores, and it is involved in virus transmission (Kakani et al., 2003; Kakani et al., 2001; Robbins et al., 1997). When TBSV (closely related to CNV, but vector has not been found) CP was replaced with CNV CP, the chimeric virus was transmitted by *O. bornovanus* zoospores, while gCNV contained particles within TBSV CP were not (Robbins et al., 1997). This experiment showed that CP determines specificity of CNV and *O. bornovanus* interaction. Further experiments with CNV CP mutants determined the critical amino acid residues that were involved in virus binding to zoospores (Kakani et al., 2001). The searches of possible receptor on fungus zoospores revealed that binding of virus to the zoospores occurs via oligosaccharides containing mannose and/or fucose (Kakani et al., 2003).

**Movement protein, p22**

Tombusvirus movement protein p22, which is membrane associated, is encoded by ORF4 that is expressed from sgRNA2 (Scholthof et al., 1995b). The role of this protein is to facilitate virus cell-to-cell movement (Dalmay et al., 1993a; Scholthof et al., 1995b). It has been shown
that CyRSV mutants lacking movement protein were not detected in inoculated and systemic leaves (Dalmay et al., 1993a). When movement protein was deleted from TBSV genome, the virus replication in protoplasts was delayed by 1-2h when compared to wild type virus, but it did not abolished virus replication (Qiu and Scholthof, 2001). These findings suggest that p22 protein is not critical for replication, but is important for virus movement. The recent finding of a host factor from tobacco named Hfi22 that binds to p22 suggests that viral MP might interact with host proteins to ensure virus intercellular movement (Desvoyes et al., 2002).

**Suppressor of RNA silencing, p19**

The p19 protein is encoded by ORF 5, and is expressed from sgRNA2. Translation of this protein occurs via leaky scanning mechanism, allowing virus to use the same portion of the genome to encode two different proteins (p22 and p19).

The first studies of tombusviruses defined p19 as cytoplasmic protein, and virus symptom determinant (Chu et al., 2000; Dalmay et al., 1993b; Scholthof et al., 1995a; Scholthof et al., 1995b), because virus mutants lacking p19 induced milder symptoms in the plants in comparison to wild type.

As studies of post-transcriptional gene silencing (PTGS) advanced, it has been proposed that p19 functions as viral suppressor of gene silencing (Qiu et al., 2002; Qu and Morris, 2002; Voinnet et al., 1999). Further experiments (Silhavy et al., 2002) showed that p19 binds to siRNAs produced during RNA silencing process. These siRNAs work as RNA silencing induction signal and by binding to them p19 prevents the spread of the signal to the neighboring cells. The latest studies of viral RNA silencing suppressors from TBSV (Ye et al., 2003) and CIRV (Vargason et al., 2003) revealed the crystal structure of p19 protein bound to the 21nt long siRNA. The studies confirmed that p19 binds with the highest affinity to the 20-22nt long RNAs, but does not have sequence specificity. It has been proposed that the 21-22nt siRNAs are involved in gene silencing by triggering degradation of mRNA with complementary sequence (Vance and Vaucheret, 2001; Waterhouse et al., 2001). The viral protein’s ability to bind these RNAs supports p19 role in preventing degradation of viral RNA without interfering with cell processes that are regulated by longer microRNAs (Papp et al., 2003).
Defective interfering (DI) RNAs

DI RNAs are small RNA molecules derived from genomic RNA (White and Morris, 1999) (Figure 1.2). DI RNAs do not encode replicase proteins, but have cis acting RNA elements that are needed for replication. To be able to replicate they have to use the helper virus coded replicase in trans (White and Morris, 1999). It has been shown that DI RNAs are associated with several tombusviruses: TBSV(Hillman et al., 1987), CyRSV(Burgyan et al., 1991), CIRV (Rubino et al., 1995) and CNV (Rochon, 1991). De novo generation of tombusvirus DI RNAs accumulation was detected after passages at high multiplicity of infection (Burgyan et al., 1991; Knorr et al., 1991; Rochon, 1991). Using reverse transcriptase PCR, DI RNAs were detected in TBSV infected plants without passages (Law and Morris, 1994), suggesting that during passages DI RNAs are amplified. But in naturally occurring field infections DI RNAs have not yet been found (Celix et al., 1997).

It has been proposed that generation of DI RNAs occurs during virus replication via series of deletions made by viral replicase (White and Morris, 1994b). DI RNAs are able to replicate to high levels if introduced to the plant together with the helper virus. It was shown that many DI RNAs reduce accumulation of helper virus significantly and lead to attenuation of symptoms (Kollar et al., 1993; Rubio et al., 1999; Scholthof et al., 1995c).

DI RNAs associated with tombusviruses are about 400-600nt long RNA molecules (White and Morris, 1994b). Prototypical tombusvirus DI RNA, termed DI-72 is composed of four non-contiguous regions (I to IV) derived from genomic RNA (White and Morris, 1994b; White and Morris, 1999) (Figure 1.2). Regions I and IV are derived from 5’ and 3’ untranslated viral genome sequences, respectively. Region I also includes start codon for p33 and p92. Region II encompasses viral sequence just downstream from p33 stop codon within the p92 ORF. Region III represents sequences from the upstream portion of 3’ UTR plus 3’end of p22 and p19 (White and Morris, 1994b). The studies on the significance of each region showed that deletions of regions II and IV abolished DI RNA replication in protoplasts (Chang et al., 1995), deletions of region I reduced DI RNA accumulation about 90% (Wu and White, 1998). Although region III is not essential for TBSV DI RNA replication (Chang et al., 1995), deletion of this region reduced DI RNA accumulation about 10 fold (Ray and White, 1999). These studies suggested that cis acting elements in different regions of DI RNA might have different roles in DI RNA replication.
Studies on replication of DI RNAs revealed that promoter for (+) strand synthesis was localized to 3’end of RI (-). In addition, structural domains of RNA, named T-shaped domain (TDS) and downstream domain (DSD), separated by stem-loop (SL) 5 were defined within RI (+) (Ray et al., 2003). These RNA structures likely function in (+) strand and are important for DI RNA replication (Ray et al., 2003; Wu et al., 2001). When RI is deleted, DI RNA replication is not abolished most likely due to sequences in 5’of RII that resemble (+) strand promoter allowing low level of DI RNA replication (Wu and White, 1998). The role of RI (+) in virus replication has not been defined but it could be that this region is involved in protection of DI RNA (+) 5’end, interaction with viral and/or host proteins leading to replicase assembly, and/or initiation of (+) strand synthesis.

Sequence analysis of RII revealed that it could form an extended hairpin structure, which is critical for replication (Chang et al., 1995). The role of RII has not been determined, but it was shown that duplication of this region increases the level of DI RNA accumulation in protoplasts (White and Morris, 1994b). Recent studies indicate that RII (+) hairpin is involved in viral replicase binding, possibly leading to viral RNA recruitment and replicase complex assembly (Pogany and Nagy, unpublished).

The studies of RIII (-) revealed that it has two stem-loop structures separated by a single stranded region (Ray and White, 2003), can function in both orientations, preferably as a single copy, and that DI RNAs without RIII tend to form head –to- tail dimers (Ray and White, 1999). The extensive RIII studies in vivo (Ray and White, 1999; Ray and White, 2003) and in vitro (Panavas and Nagy, 2003a) suggested that RIII functions as replication enhancer on the minus strand. In addition it was shown that this region could be used by CNV replicase in recombination as donor or acceptor in in vitro assay (Cheng and Nagy, 2003).

The role of RIV in viral replication has been extensively studied in vivo and in vitro. It has been shown that region IV(+) of DI RNA has promoter (termed gPR) at the 3’end that is critical for (-) strand synthesis (Fabian et al., 2003; Panavas et al., 2002a). Solution structure mapping of secondary structures within RIV(+) revealed three stem- loop structures (SL1, SL2, SL3) and ssRNA region (Fabian et al., 2003). Further analysis in vivo and in vitro revealed that SL3 is critical for replication, and it contains a sequence that can interact with gPR and inhibit (-) strand synthesis (Pogany et al., 2003). The 5nt sequence in SL3 complementary to gPR, was named replication silencer (Pogany et al., 2003). While gPR includes SL1, which is important for
(-) strand initiation, the role of SL2 has not been defined, but mutational analysis in vivo suggested that it is important for virus replication (Fabian et al., 2003).

Overall, the ability of DI RNAs to replicate very efficiently, via utilizing helper virus replication machinery, makes them a very useful tool for studying viral RNA elements involved in replication and recombination.

Satellite (sat) RNAs

In addition to DI RNAs, tombusviruses can be associated with other type of subviral RNAs, termed satellite (sat) RNAs (Celix et al., 1997; Rubino et al., 1990), which are RNA molecules that require helper virus for their replication. In contrast to DI RNAs, satRNAs do not have significant sequence homology with the helper virus gRNA (reviewed by A.E. Simon, 1999). The first of a tombusvirus satellite RNA was found in association with CymRSV (Rubino et al., 1990), followed by identification of satRNA B1 (822nt) and B10 (612nt) in Nicotiana clevelandii after serial passages at high multiplicity of TBSV field isolates (Celix et al., 1997). It was shown that B10 satRNA attenuated symptom appearance in infected plants, while plants infected with B1 satRNA showed the same symptoms as infected with TBSV alone (Celix et al., 1997). Sequence comparison of satRNAs revealed that satRNAs associated with CymRSV and TBSV do not share significant sequence identity between each other (Celix et al., 1997), but there is an internal segment about 50nt long that is identical with the 3’portion of helper virus 5’ UTR. RNA structure mapping showed that 5’ends of sat RNAs can form structures similar to TSD found in RI (+) of DI RNAs and helper virus genomes (Wu et al., 2001). These features imply that these identical sequences and similar structures could be critical for viral RNA accumulation.

What the thesis will show

The objectives of this work were to study the roles of a previously defined RNA binding motif of the replicase proteins of tombusvirus (CNV) and regions within DI RNA in replication and recombination of tombusviruses, as well as viral components necessary for active CNV replicase formation.
Chapter 2 will provide background information on tombusvirus replication, followed by experimental results defining the role of an RNA binding motif of CNV replicase proteins in RNA binding, virus replication and subgenomic RNA synthesis. Chapter 3 will describe recombination studies in protoplasts, which were used for revealing the role of the RNA binding motif in recombination of tombusvirus RNA. Chapter 4 will introduce tombusvirus replication system in yeast, a model host, followed by description of functional CNV replicase isolation procedure that was used to define viral elements required for enhancement of replicase activity. At the end of the thesis (chapter 5), I will discuss the possible impact of these studies on revealing the roles of replicase proteins and the viral RNA in tombusvirus replication, recombination and active replicase assembly.
Figure 1.1: Genome organization of Tomato bushy stunt virus (TBSV). Coding sequencies are shown as boxes. Expressed ORFs are represented as white boxes with protein (p) molecular mass (in kDa) and functions written in the box (RdRp- RNA dependent RNA polymerase; CP-coat protein; RSS- RNA silencing suppressor; MP- movement protein); non-expressed ORFs are shown as black boxes. Bellow genomic (g)RNA are shown TBSV subgenomic (sg)RNAs. The length of all three RNAs is in kilobases (kb).
Figure 1.2: Genome representation of TBSV genomic (g)RNA and DI RNA. The boxes in the gRNA represent coding sequences; the lines – non-coding RNA sequences. The sequences of gRNA present in DI RNA (regions RI through RIV) are enlarged and shown as boxes in DI RNA scheme.
Chapter Two

The overlapping RNA-binding domains of p33 and p92 replicase proteins are essential for tombusvirus replication

Introduction

Replication of RNA viruses in infected cells is performed by the viral replicase in a multi-step process. During the first step viral replicase proteins are translated from genomic RNA. Then replicase complex has to form to allow efficient viral RNA amplification. The replicase complex includes the viral-coded RNA-dependent RNA polymerase (RdRp), additional viral replicase protein(s) and host factors (Ahlquist et al., 2003; Buck, 1996; Buck, 1999; Diez et al., 2000; Lai, 1998; Noueiry et al., 2000; Quadt et al., 1993). Viral replicase complex has to be recruited to the site of replication, possibly forming membranous multivesicular bodies (MVB). In addition, the viral genomic RNA must also be recruited to the viral replicase complex (Ahlquist, 2002; Buck, 1996; Buck, 1999). After the assembly of the replicase complex and the recruitment of the viral genomic RNA, a complementary (minus-strand) RNA is made. This is followed by the synthesis of many plus-strand RNAs utilizing the minus-strand intermediates with (+) strands accumulation reaching up to 100 times higher level than (-) strand (Panavas T., PoganyJ. and P.D. Nagy, unpublished).

Interestingly, tombusviruses are frequently associated with defective interfering (DI) RNAs that are derived entirely from the genomic RNA. The most frequently occurring DI RNAs contain three or four short noncontiguous segments of the genomic RNA (Burgyan et al., 1991; Chang et al., 1995; Finnen and Rochon, 1993; Hillman et al., 1987; White and Morris, 1999) (Figure 1.2). DI RNAs replicate efficiently with the help of the tombusvirus replicase, therefore they are useful to study replication in vivo and in vitro (Nagy and Pogany, 2000a; Panavas et al., 2002a; White and Morris, 1999).

The replication of tombusviruses is a very efficient process that requires interaction between replicase proteins p33 and p92, as well as viral RNA and most likely host proteins. The finding that both p33 and p92 are required for virus replication suggests that these proteins
might interact between themselves. In addition, the ability of replicase proteins to replicate viral RNA implies that these proteins have to bind the RNA as well.

Recently it has been shown using purified recombinant TBSV replicase proteins in surface plasmon resonance (SPR) assay that p33 can interact with p92 as well as with other p33 molecules, but not with maltose binding protein (MBP) (Rajendran and Nagy, in press). More detailed studies revealed that protein–protein interaction domain is localized in the C-terminus of p33 (Rajendran and Nagy, in press). The analysis of the partially purified CNV replicase from plants supports this data, because in purified active replicase both p33 and p92 proteins were detected (Pogany and Nagy, unpublished)

The ability of the replicase proteins to bind to tombusvirus RNA was studied using gel mobility-shift assay and SPR (Rajendran and Nagy, 2003). These experiments revealed that: 1) both p33 and p92 could bind to tombusvirus RNAs in vitro; 2) p33 binds RNA in a cooperative manner (Rajendran and Nagy, 2003). Further studies performed with deletion derivatives of p33 expressed in E. coli as MBP fusion revealed that an arginine/proline-rich (the sequence in RPRRRP) motif (termed RPR motif), which is conserved among tombusviruses and related carmoviruses, is critical for efficient RNA binding (Rajendran and Nagy, 2003). The corresponding region in the overlapping domain of p92 may also bind to RNA, although this has not been confirmed yet. This is because p92 has two more RNA-binding domains within its unique C-terminal region (Rajendran and Nagy, 2003) therefore mutations within the RPR motif in p92 did not abolish RNA binding.

In this chapter I demonstrate that the RPR motif is essential for tombusvirus replication in vivo. Detailed site-specific mutagenesis of the RPR motif revealed that two of the four arginines within the RPR are critical for virus replication in protoplasts and for RNA binding in vitro.I also demonstrate that the RPR motif is essential for the replication function of both p33 and p92. This was achieved by using a two-component system, in which p92 was expressed from the viral genomic RNA and p33 was expressed from a DI RNA (Oster et al., 1998). I found that mutations within the RPR in either p33 or p92 inhibited replication in the above two-component system. The non-replicating DI RNAs carrying mutated p33 could be rendered replication-competent in the presence of the wild type (WT) tombusvirus RNA, excluding the possibility that the mutations debilitated the ability of the DI RNA to replicate. Overall, these
experiments supply direct evidence that functional RPR motif in both p33 and p92 is essential for replication.

**Materials and methods**

**Site directed mutagenesis of CNV (pK2M5)**

A full-length cDNA clone of CNV, named pK2M5 (Rochon and Johnston, 1991), was used to generate the RPR motif mutants of p33/p92 listed in Figure 2.2.A. Mutagenesis was performed by PCR using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). The PCR reactions included the *Pfu Turbo* DNA polymerase, different set of primer pairs designed for each mutant (see Table 2.1) and 40 ng of pK2M5 DNA as a template. PCR products were digested with *DpnI*, to remove template DNA before transformation into *E. coli* (DH5α). The presence of the desired mutations was confirmed by sequencing with primer 27 (5’-GTATTTCACACCAAGGGAC-3’).

The series of p92Y constructs, containing mutated or WT RPR motif (Figure 2.7), were obtained by replacing the *EagI* to *NsiI* (positions 998 and 1650 in the p92 ORF) fragment with the corresponding mutated fragment of Mal/p92 (Rajendran and Nagy, 2003), containing a stop codon to tyrosine codon mutation at the end of the p33 gene. The presence of tyrosine mutation was confirmed in each clone by sequencing, using primer 631 (5’-GAGGAATTCAAGGTAATTGCGTCCACA-3’). For the construction of DI-p33 series of mutants (Figure 2.6), we used construct DI-83CNV (a generous gift of Andy White) (Oster et al., 1998). Each full-length RPR-motif mutant of CNV was digested with *Stul* and *SphI* and the DNA fragment containing the p33 sequence was used to replace the corresponding sequence in DI-83CNV.

For the gel shift experiments, we used either full-length or a truncated version of the p33 protein that were fused to MBP (vector pMal-c2X, New England Biolabs). To make the p33 expression constructs, containing WT or each of the mutated RPR motif shown in Figure 2.2.A, we amplified a 90 nt-long fragment of the p33 gene (positions 616-705 in the p92 ORF) that represent a 30 amino acid long region including the RPR-motif by PCR using primers 631 (5’-GAGGAATTCAAGGTAATTGCGTCCACA-3’) and 632 (5’-GAGTCTA
GACTACTTCAGGTAACCCACCTT-3’) with Deep Vent polymerase (New England Biolabs). The templates for the PCR were WT or mutated pK2M5 DNA (see above). The PCR fragments were digested with EcoRI and XbaI, gel isolated and ligated into the similarly treated pMal-c2X vector.

To generate constructs expressing full-length p33 fused with MBP (Figure 2.1 and 2.3), the WT or mutant p33 sequences were PCR amplified with primers #25 (5’-GGA GTC TAG AGA TAC CAT CAA GAG GAT G-3’) and #992B (5’-GAGCTGCAAGCTATTTCACACCAAGGGA-3’) using Deep Vent polymerase (see above). The PCR products were digested with XbaI and PstI and cloned into pMAL-c2 vector to generate in frame MBP-p33 fusion. To obtain the expression construct [RPR (Figure 2.1), we amplified the 5’ portion and the 3’ portion of the CNV p33 gene separately using either primer pairs #25 (above) and #994B (5’-CCGCGCTAGCTCCTGTGGACGCAATTACCT-3’) or #993B (5’-GCGGGCTAGCTATGCAGCTAAGATTGCACA-3’) and #992 (above). The PCR product representing the 5’ portion of the p33 gene was digested with XbaI and Nhel, while the PCR product representing the 3’ portion of the p33 gene was digested with Nhel and PstI. Both PCR products were cloned into pMAL-c2, which had been digested with PstI and XbaI, in a three-piece ligation reaction. The clones were sequenced to validate their correctness.

**Preparation of RNA transcripts**

RNA transcripts were obtained using SmaI linearized WT pK2M5, mutated pK2M5, DI-72, DI-73 and DI-p33 (original name is DI-83CNV, (Oster et al., 1998) clones in a standard transcription reaction with T7 RNA polymerase (Nagy et al., 1999). Template DNA was removed by DNase I, followed by purification of the RNA transcript with phenol/chloroform extraction and 95% ethanol precipitation. The pellet was washed three times with 70% ethanol to remove residual salts. The RNA transcripts were quantified by UV spectrophotometer (Beckman), followed by 1% agarose gel electrophoresis.

**Purification of full-length and truncated p33-MBP fusion protein from E. coli**

The protein expression for each p33 RPR-motif mutant as an MBP fusion protein (Figure 2.2.A) was induced at 37 °C by IPTG in Epicurian BL21-Codon plus (DE3)-RIL cells (Stratagene) as recommended by the supplier. After 2 hours of induction, the cells were
harvested and processed as described by (Rajendran et al., 2002). Briefly, the bacterial cells were resuspended in column buffer (20mM Tris-HCl, pH8.0; 25mM NaCl; 1mM EDTA, pH8.0; 10mM [ -mercaptoethanol), sonicated and after centrifugation the supernatant was applied to equilibrated amylose column (New England Biolabs). Following the wash with column buffer, the proteins were eluted from the amylose column with column buffer containing 10mM maltose. All steps were carried out on ice or in the cold room. The quality of the proteins obtained was checked by 10% SDS-PAGE analysis and their amounts were measured as described by (Rajendran et al., 2002). The RNA-binding studies (see below) were done with the fusion proteins.

**In vitro** RNA binding studies

The RNA probe in the gel shift experiments was the 169 nt region I (+) of DI-72 (White and Morris, 1994), which was labeled with ³²P UTP using T7 RNA polymerase *in vitro* reaction. The amount of ³²P UTP was 100 fold less than unlabeled UTP (Rajendran et al., 2002). The gel shift experiments were performed according to Rajendran et al., (2002). Briefly, ~2 ng of ³²P UTP labeled RNA was mixed with 1 µg of the p33-MBP preparation for 15 min at 25 °C in the presence of 50 mM Tris-HCl, pH 8.2, 10 mM MgCl₂, and 10 mM DTT, 10% glycerol, 2.4U RNase inhibitor and 100ng tRNA. The samples were analyzed by electrophoresis on native 4% polyacrylamide gels run at 200V for 50 min at 4 °C in TAE buffer. Dried gels were analyzed using a phosphorimager.

**Preparation of protoplasts**

*N. benthamiana* protoplasts were prepared from callus cells by treatment with 0.5 g cellulysin and 0.1g macerase (Calbiochem) in 50 ml protoplast incubation media (Kong et al., 1997; Nagy et al., 1999; Nagy et al., 2001) for 4.5h with gentle shaking at 25 °C. After incubation, the protoplasts were filtrated through sieve set and a sterile nylon cloth. The protoplasts were washed twice with 0.5M mannitol and once with the electroporation buffer (10 mM HEPES, 10 mM NaCl, 120 mM KCl, 4 mM CaCl₂, 200 mM mannitol). 5x10⁵ protoplasts were electroporated with either 2µg *in vitro* transcribed gCNV RNA or the combination of 2µg gCNV RNA and 1µg DI-72 RNA. In the two-component studies (Figs. 2.6-2.7), we used 4µg of p92Y and 2µg of DI-p33 RNA transcripts. Electroporation was done using Gene pulser II
(Biorad, the settings were at 0.2kV voltage and 0.5µF capacity). After electroporation, samples were kept on ice for 30 min, followed by adding 1.8 ml protoplast culture medium (Kong et al., 1997) to each sample. Protoplasts were incubated in 35[×]10 mm petri dishes in the dark for 24-48h at 22 °C.

**Total RNA extraction from protoplasts and RNA analysis**

Total RNA was extracted using the phenol/chloroform-based method of Kong et al., (1997) and Nagy et al., (2001). For Northern blot analysis, the total RNA was treated with formamide at 85°C, followed by agarose gel electrophoresis (0.8% for gCNV RNA and 1.2% for DI RNA). After electrophoresis, the RNA was transferred by electrotransfer to Hybond-XL membrane (Amersham-Pharmacia) and hybridized with gCNV- or DI-72 specific probes in ULTRAhyb hybridization buffer at 68°C using the conditions recommended by the supplier (Ambion). The probes were prepared by T7 RNA polymerase in an in vitro transcription reaction in the presence of [³²P]UTP using PCR amplified DNA templates that were obtained by using primers 16 (5’-GTAATA CGACTC ACTATAGGGCTGCATTCTGCAATGTTC-3’) and 312 (5’-GCTGTC AGTGCTAGTGGGA-3’) for the gCNV and primers 15 (5’-GTAATAC GACTC ACTATAGGGCATGTCGCTTGTTGTTTGTG-3’) and 20 (5’-GGAAATT CTCCAGGATT TCTC-3’) for DI-72 RNA. These probes are complementary to the 3’-terminal 200 nt of gCNV RNA and the 5’ 169 nt of DI-72 RNA, respectively.

**RT-PCR analysis of gCNV RNA from protoplasts**

We tested the stability of the RPR-motif mutants of gCNV during virus replication in protoplasts by sequencing the RT-PCR products obtained from total RNA samples using primers 27 (5’-GTATTTTACACACCAAGGGAC-3’) for RT and primers 213 (5’-GAGGATGAAAGGGAATTCACGGAATTCCAGGATT GTTTGG-3’) and 27 for the PCR reaction. The RT-PCR products were gel purified before sequencing, which was performed by using primer 27 (see above).
Results

Essential role for the arginines of the arginine/prolin-rich motif in RNA-binding in vitro.

The RPR motif present in both p33 and p92 of Tomato bushy stunt virus (TBSV) and Cucumber necrosis virus (CNV) (Figure 2.1A) contains two prolines and four arginines that are the primary candidates to bind to the negatively charged viral RNA (the actual sequence of the RPR motif is R<sub>637</sub>P<sub>640</sub>R<sub>643</sub>R<sub>646</sub>R<sub>649</sub>P<sub>652</sub>, (Rajendran and Nagy, 2003)). Accordingly, deletion of the entire RPR motif from the full-length p33 of CNV (see construct ΔRPR, Figure 2.1) inhibited the in vitro RNA binding activity of ΔRPR (expressed and purified from E. coli, see below) by ~80% when compared to the full-length WT p33 protein (Figure 2.1C). Since the level of expression of the WT p33 is low in E. coli and it also shows poor solubility (not shown), we expressed a 30 amino acid-long region of p33 (which was fused to the maltose-binding protein, MBP), including the RPR motif in E. coli. This 30 amino acid-long region of p33 was demonstrated earlier to bind RNA efficiently (Figure 2.2 and (Rajendran and Nagy, 2003)). To test which amino acids within the RPR motif play an important role in RNA-binding, we have generated 19 mutants of the CNV p33 that had one or more altered amino acids within the RPR motif (see Figure 2.2A). One set of mutants contained one, two or three alanine mutations replacing either arginines or prolines. The second set of mutants contained arginine to lysine mutations, which maintained the positive charge of the RPR motif. The third set of mutants had one, two or three of the arginines deleted. The fourth set of mutants contained one or two extra arginines that increased the overall positive charge of the RPR domain. The purified truncated p33-MBP fusion proteins carrying one of the 19 mutations or the WT sequence within the RPR motif were used in similar amounts in gel mobility-shift assays in combination with the 5’ 169 nt segment of DI-72 RNA, a prototypical DI RNA (White and Morris, 1994b). These experiments demonstrated that replacing the first proline (P<sub>640</sub> in construct P<sub>640</sub>-A, lane 2, Figure 2.2A) or the second proline (P<sub>652</sub> in construct P<sub>652</sub>-A, lane 3, Figure 2.2A) with alanines did not alter the ability of the truncated p33 to bind RNA by more than 10% when compared to the truncated p33 carrying the WT RPR motif (construct WT, Figure 2.2B-C). Substitution of alanines for both prolines (P<sub>640</sub> and P<sub>652</sub> in construct P<sub>640-652</sub>-A, lane 4, Figure 2.2A), however, decreased RNA-binding by ~30%. In contrast, replacing either or both of the prolines with
arginine(s) increased RNA-binding by 2.5 to 3-fold when compared to WT (constructs P_{640-}{R}, P_{652-}{R} and P_{640-652-}{R}, lanes 5-7, Figure 2.2A-C).

Separate substitutions of each arginine with alanines in the RPR motif resulted in 30-50% decrease in RNA-binding when compared to WT (constructs R_{637-}{A}, R_{643-}{A}, R_{646-}{A} and R_{649-}{A}, lanes 1, 8, 10, 12 in Figure 2.2A). Combined replacement of three of the arginines (second, third and the fourth arginines in the RPR motif, i.e., R_{643}, R_{646}, and R_{649}) with alanines decreased RNA-binding by 42% (construct R_{643-649-}{A}, lane 19 in Figure 2.2A-C). Similarly, deletion of one, two or three of the arginines reduced RNA-binding significantly (by ~40 to 60%, see constructs [R, 2R and 3R, lanes 16-18 in Figure 2.2A-C). On the contrary, single lysine substitutions for the second or fourth arginines within the RPR motif (mutants R_{643-}{K} and R_{649-}{K}, lanes 9 and 13 in Figure 2.2A-C) increased RNA-binding by 32 and 280%, while similar substitution at the third arginine (mutant R_{646-}{K}, lane 11 in Figure 2.2A-C) actually decreased RNA-binding by ~40%. This suggests that the third arginine (R_{646}) within the RPR motif is important for RNA-binding and even the positively charged lysine is detrimental in that location. Insertion of one or two arginines into the RPR motif did not increase RNA-binding by more than 10-20% (mutants R_{643+}{R} and R_{643+2}{R}, lanes 14 and 15 in Figure 2.2A-C). Overall, these in vitro experiments suggest that the arginines in the RPR motif play important roles in RNA-binding.

Since the above RNA binding studies included a 30 amino acid fragment of p33, it is possible that the RPR motif mutations (Figure 2.2A-C) may have different roles and/or structures when present in the full-length p33. Therefore, I expressed three RPR motif mutants, namely P_{652-}{A}, R_{649-}{K} and R_{643-649-}{A} (see constructs # 3, 13 and 19 in Figure 2.2A), as full-length p33 proteins fused to the MBP domain (Figure 2.3). Due to the difficulty of obtaining large amounts of purified full-length proteins (unlike in the case of the truncated p33), I used reduced but comparable amounts of these proteins and the WT p33 in the gel shift experiments (Figure 2.3). Quantification of the bound RNA to the full-length p33 revealed that mutation P_{652-}{A} showed activity that was comparable to that of the full-length WT p33, while R_{649-}{K} bound 65% better and R_{643-649-}{A} bound only at 45% reduced level of the WT p33 (Figure 2.3A-B). Comparison of the data obtained with the RPR motif mutations present either in the full-length or the truncated p33 showed a similar trend in the overall effect on the RNA binding activities of
these RPR motif mutations (Figure 2.2 and 2.3). Therefore, I suggest that the sequences within the RPR motif in p33 play major roles in RNA binding.

The arginine/proline-rich RNA-binding motif is essential for tombusvirus replication in protoplast.

To test whether the RPR motif plays an essential role during tombusvirus replication, I introduced the above RPR motif mutations into the full-length genomic CNV (gCNV) RNA (see Materials and Methods). Note that due to the expression strategy of CNV, both p33 and p92 should carry the same RPR motif mutation during infection (see below). First, tombusvirus replication was tested in cis (i.e., the mutations were present on the replicating gCNV RNA) in Nicotiana benthamiana protoplasts after electroporation of the in vitro transcribed full-length gCNV RNA transcripts. Second, I also tested the ability of the RPR motif mutants of CNV to support trans-replication (i.e., the replication of DI-72 RNA that must use p33/p92 in trans for its replication) by using co-electroporation of the mutated gCNV RNA transcript and the WT DI-72 RNA. It is well established that the TBSV-associated DI-72 RNA can use the replicase proteins generated by the CNV helper virus for replication (Oster et al., 1998; White and Morris, 1994b). The accumulation of gCNV RNA and DI-72 RNA in protoplasts was studied using Northern blot with CNV-specific and DI RNA-specific probes, respectively. The amounts of total RNA loaded on gels were adjusted based on the host ribosomal RNA (Figures 2.4-2.5A).

The protoplasts experiments revealed that seven of the nineteen RPR mutants supported gCNV RNA accumulation in cis and DI RNA replication in trans at 50 to 95% of the WT level (constructs R637-A, P640-A, P652-A, P640-652-A, P640-R, R649-A and R649-K, lanes 1-5 and 12-13, Figures 2.4A-C and 2.5). Interestingly, several of the above mutants supported trans-replication of the DI RNA with 10-to 20% higher efficiency than the level of cis-replication of the corresponding mutant gCNV. These data suggest that none of these four amino acids (i.e., R637, P640, R649 and P652) within the RPR motif is essential for cis- or trans-replication of tombusviruses in protoplast. In contrast, replacing the third arginines (R646) with alanine reduced gCNV and DI-72 RNA replication by over 90% (mutant R646-A, lane 10 in Figures 2.4A-C and 2.5), suggesting that this arginine is important for tombusvirus replication. Replacement of the second arginine (R643) with alanine reduced gCNV RNA replication by 80%, but affected DI
RNA replication by 10% only (mutant R643-A, lane 8 in Figures 2.4A-C and 2.5), suggesting that the second arginine is more important for cis-replication than for trans-replication.

The essential role for the arginines within the RPR motif for tombusvirus replication was further supported by the observation that combined replacement of three of the arginines (the second, third and the fourth, i.e., R643, R646 and R649, see mutant R643-649-A, lane 19 in Fig. 4A-C and 5) with alanines completely abolished tombusvirus and DI RNA replication. Interestingly, replacing separately the two important arginines (the second and third arginines, mutants R643-K and R646-K, lanes 9 and 11, Figures 2.4A-C and 2.5) with lysines decreased tombusvirus or DI RNA replication by 95 to 98%. This is somewhat surprising since both lysine and arginine are positively charged. In contrast, the lysine for arginine substitution at the fourth arginine position in the RPR motif had smaller effect on virus replication (50% of that of WT, see construct R649-K, lane 13, Figure 2.4A-C), further indicating that this arginine may not be essential for tombusvirus and DI RNA replication.

To test if the number of arginines within the RPR motif is important for tombusvirus replication, first I used insertion mutants that contained one or two extra arginines between the two prolines in the RPR motif (see mutants R643+R and R643+2R in Figure 2.2A). Mutant R643+R with one extra arginine supported gCNV and DI-72 RNA replication only at a reduced efficiency (~5%, lane 14, Figures 2.4A-C and 2.5), while addition of two extra arginines in mutant R643+2R abolished tombusvirus or DI RNA accumulation completely (Figures 2.4A-C and 2.5, lanes 14 and 15). The second set of constructs had deletion of one, two or three arginines in the RPR motif that resulted in complete inhibition of tombusvirus replication (Figures 2.4A-C and 2.5, mutants □R, □2R and □3R, lanes 16-18). The third set of mutants carried proline to arginine mutations that did not alter the length of the RPR motif (see mutants P640-R, P652-R and P640-652-R, Figure 2.2A). Interestingly, one of the mutants supported gCNV replication at 60% of WT level, although the replication of DI-72 RNA was somewhat less efficient (Figures 2.4A-B and 2.5A-B, lane 5, 20% of WT level). The two other mutants replicated poorly in protoplast (5-10%)(Figures 2.4 and 2.5A-B, lanes 6-7). The data obtained with these deletion, insertion and replacement mutants indicate that, in general, the number of arginines in the WT RPR motif is optimal for tombusvirus replication.

I also tested if the relative accumulation of subgenomic (sg)RNAs (i.e., the ratio between the sg1 and sg2 RNAs versus gCNV) was different with all the above mutants in comparison
with the WT in protoplast. Four mutants, including R_{643}-A, R_{643}-K, R_{646}-K and R_{643}+R, supported the relative accumulation sg2 RNA at 4 to 20-fold higher level than the WT did (Figure 2.4C, lanes 8, 9, 11, 14). The increase in the relative accumulation of sg1 was much less pronounced (between 2 to 4-fold, Figure 2.4C, lanes 8, 9, 11, 14) with these four mutants. In contrast, mutant P_{640-652}-A generated sg2 RNA less efficiently (drop of 40% in relative accumulation) than the WT did (Figure 2.4A-C, lane 4). The difference in the ratio of sg2/gCNV between P_{640-652}-A and WT is significant after both 24 and 48 hours incubation and we confirmed this by repeating the experiment two times (not shown). Overall, this observation suggests that mutations within the RPR motif can affect subgenomic RNA synthesis/accumulation.

It is possible that unwanted mutations are introduced during the in vitro mutagenesis, in spite of the use of the high fidelity Pfu Turbo DNA polymerase to introduce the site-directed mutations (see Materials and Methods). However, sequencing of a 500-600 nt region around the targeted RPR motif mutations did not reveal any mutated positions, except the target ones (not shown). Also, we used two independently generated clones for P_{652}-R, R_{643-649}-A and 3R in the protoplast experiments, which in each case, gave results comparable to that shown in Figure 2.4A (lanes 6, 18 and 19), i.e. poor accumulation for P_{652}-R, and undetectable level of accumulation in case of R_{643-649}-A and 3R (not shown). Since it is very unlikely that the proven mutagenesis method used in this work (see Materials and Methods) would introduce the same second-site mutation for two separate clones, we conclude that the effect on the reduction of CNV RNA accumulation is due to specific targeted mutations introduced into the RPR motif.

Since RNA viruses can accumulate mutations quickly during infection, we have tested stability of the mutated gCNV RNA for the twelve constructs that supported gCNV replication at detectable levels. This was done by RT-PCR, followed by sequencing (in the vicinity of the RPR motif) of the progeny gCNV RNA obtained from protoplasts 48 hours after electroporation. These experiments confirmed that the original mutations in the RPR motif were stably maintained during the course of infection (not shown). This observation suggests that the original mutated p33/p92 proteins and not putative revertants or WT contaminants were responsible for supporting tombusvirus replication in protoplast (Figures 2.4A-C and 2.5).
Essential role for the RPR motif in p33 during tombusvirus replication in protoplast.

Although the above experiments demonstrated that the RPR motif plays an essential role in tombusvirus replication, due to the overlapping domains in p33 and p92, they could not answer whether the RPR motif is important for the function(s) of p33, p92 or both. To test this question, we used a two-component, complementation-based tombusvirus replication system developed by Oster et al., (1998). Briefly, in the two-component system, the p92 is expressed from the gCNV RNA that carries a tyrosine mutation eliminating the p33 termination codon (Figure 2.6A, construct p92Y). Since it cannot express p33, construct p92Y alone is not able to replicate in protoplast, confirming that p33 is not expressed (Figure 2.6B, lane 20, and Oster et al., 1998). The function of the second RNA, which is based on DI RNA, is to supply the p33 in the two-component system (Figure 2.6A). This RNA, which we name DI-p33 RNA, is not able to replicate in protoplast when present alone (Figure 2.6B, lane 21, and Oster et al., 1998). Co-infection of p92Y and DI-p33 RNA into protoplast, however, leads to replication of DI-p33 RNA, while construct p92Y can only replicate poorly (at a level close to the detection limit, Figure 2.6B, lane WT, and Oster et al., 1998), but apparently it can supply enough p92 to support the efficient replication of DI-p33 RNA in trans.

To test the role of the RPR motif in p33 in tombusvirus replication, we made a series of DI-p33 RNA mutants that included nine of the above RPR mutants (Figure 2.2A and Figure 2.6B). After co-electroporation of N. benthamiana protoplasts with construct p92Y and each of the DI-p33 RNA mutants, we tested the level of accumulation of DI-p33 RNA by Northern blotting (Figure 2.6B). The accumulation of three of the DI-p33 RNA mutants was not detected in this assay. These were the mutants with the third arginine being replaced with either alanine or lysine (Figure 2.6B, lanes 11-12 and 17-18, mutants R646-A and R646-K,) and the combined replacement mutant that carried three alanines in the place of arginines (Figure 2.6B, lanes 13-14, construct R643.649-A). The DI-p33 RNA mutant with the two prolines within the RPR motif being replaced with alanines (Figure 2.6B, lanes 7-8, construct P640-652-A) accumulated poorly (10% of WT level) in protoplast. In contrast, mutants R637-A, P652-A, P640-R and P640-A (Figure 2.6B-C, lanes 1-4, 9-10 and 15-16) accumulated to ~50% of WT level, while mutant R649-K (Figure 2.6B-C, lanes 5-6) reached accumulation level comparable to WT (Figure 2.6B-C, lanes
Overall, these experiments strongly support that the RPR motif in p33 is essential for replication of tombusviruses.

To rule out that the above RPR motif mutants in DI-p33 have a defect in RNA replication due to the altered RNA sequence within the RPR motif, we conducted complementation studies in protoplasts after co-electroporation of the mutated DI-p33 RNA and the full-length gCNV helper, which expressed both WT p33 and WT p92. For the complementation studies, we selected mutants, P_{640-652}-A, R_{646}-A, R_{646}-K and R_{643-649}-A, which did not replicate or replicated poorly and mutant R_{649}-K that replicated efficiently in the DI-p33/p92Y-based two-component system (Figure 2.6B-C). Northern blot analysis of the total RNA extract of the co-inoculated protoplasts revealed that all the 5 tested DI-p33 mutants replicated at comparable levels to the WT DI-p33 in the presence of the WT gCNV helper virus after 48 hours of incubation in protoplast (compare lanes 1-10 versus lane WT in Figure 2.6D). These data demonstrate that the DI-p33 mutants can be complemented in trans by the WT p33 expressed from the gCNV RNA. Therefore, it is unlikely that the RNA genomes of the DI-p33 mutants tested in this work have a cis-acting replication defect due to the altered sequence in the RPR motif.

**The RPR motif of p92 affects trans-replication of DI RNA in protoplast.**

To test the role of the RPR motif in p92 in tombusvirus replication, we also used the p92Y/DI-p33 complementation system (Figure 2.7A). For these experiments, we made a series of p92Y RNA mutants (Figure 2.7B), similar to the ones tested for p33 in DI-p33 (Figure 2.6B). After co-electroporation of *N. benthamiana* protoplasts with each of the p92Y RPR motif mutants and the WT DI-p33 RNA, we tested the level of accumulation of DI-p33 RNA by Northern blotting (Figure 2.7B). These experiments revealed that one of the RPR mutants of p92Y, namely construct R_{643-649}-A with three arginine-to-alanine changes (Figure 2.7B-C, lanes 13-14), did not support the replication of DI-p33 RNA at a detectable level. Another mutant, namely construct P_{640-652}-A with the two prolines being replaced with alanines (Figure 2.7B-C, lanes 7-8), supported tombusvirus accumulation at a very low level (2.5% of WT). Substitution of alanine for arginine in construct R_{646}-A also reduced tombusvirus accumulation by 70% (Figure 2.7B-C, lanes 11-12). The other six mutants of p92Y tested supported tombusvirus accumulation efficiently (75-85% of WT, Figure 2.7B-C). Overall, the observation that one of
the tested p92Y mutants did not support tombusvirus replication confirms that the RPR motif in
p92 is essential for tombusvirus replication. The data also suggest that the role of the RPR motif
is different in p33 than in p92, since two p33 mutants carrying either alanine or lysine
substitutions at position R\textsubscript{646} (namely DI-p33/R\textsubscript{646}-A and R\textsubscript{646}-K) did not support tombusvirus
replication at detectable levels (Figure 2.6B-C, lanes 11-12 and 17-18), while the corresponding
p92Y mutants did support DI-p33 replication (Figure 2.7B-C, lanes 11-12 and 17-18).

**Discussion**

The replication process of tombusviruses is currently not yet fully understood. The p92
protein that is expressed from the genomic RNA by a ribosomal readthrough mechanism due to
leaky termination at the end of the p33 ORF (Scholthof et al., 1995d), contains the signature
motifs of viral RdRp within its unique C-terminal region. In contrast, the role of the essential
p33 protein in tombusvirus replication is currently unknown. We have recently demonstrated
that the C-terminal portion of p33 and the corresponding region of p92 contain an RPR motif
that is involved in RNA-binding *in vitro* (Figure 2.1; (Rajendran and Nagy, 2003)). In this paper,
we further delineated the role of particular amino acids within the RPR motif in RNA-binding *in
vitro* and tombusvirus and DI RNA replication in protoplast.

The *in vitro* RNA-binding studies revealed that none of the changes within the RPR
motif that included one, two and three amino acid alterations, deletions or insertions abolished
completely the ability of p33 to bind to tombusvirus RNA (Figure 2.2A-C). In contrast, deletion
of the entire RPR motif did result in 80% loss in RNA-binding (Fig. 1, (Rajendran and Nagy,
2003), suggesting that the entire RPR motif contributes to the strength of RNA-binding. Among
the individual amino acids in the RPR motif (the sequence is R\textsubscript{637}P\textsubscript{640}R\textsubscript{643}R\textsubscript{646}R\textsubscript{649}P\textsubscript{652}, the third
arginine is underlined), the third arginine (R\textsubscript{646}, and to a less extent, the second arginine (R\textsubscript{643})
play the most significant roles in RNA-binding *in vitro*. Replacing these amino acids with
alanine resulted in 40 to 50% reduction in RNA-binding *in vitro* (Figure 2.2A-C). Replacing
the third arginine with lysine, a positively charged amino acid, also reduced RNA-binding by 44%
in *vitro*. Interestingly, similar replacement of either the second or the fourth arginines with
lysines increased the efficiency of RNA-binding. These data suggest that positively charged
amino acids should be present at the positions of the second and the fourth arginines, while the
function of the third arginine (R₆₄₆) depends not only on its charge, but possibly on its structure as well.

Combined deletion of the second, third and fourth arginines in the RPR motif reduced RNA-binding by 62%, but the fact that it did not reduce RNA-binding in vitro as much as the deletion of the motif (Figure 2.1, mutant []RPR) suggests that the first arginine also plays a role in RNA-binding. This is also supported by the observation that alanine substitution for the first arginine (R₆₃⁷) reduced RNA-binding by 33%. In contrast to the arginines, the direct role of the two prolines in RNA-binding is less significant in vitro. For example, replacing either of the prolines with alanines resulted in RNA-binding similar to WT level (Figure 2.2A-C, construct P₆₄₀⁻⁶₅₂-A). On the contrary, replacing both prolines with alanines resulted in 30% reduction in RNA-binding (Figure 2.2A-C, construct P₆₄₀⁻₆₅₂⁻⁶₅₂-A). This suggests that the prolines likely play a structural role that may facilitate the correct positioning of arginines for RNA binding. Interestingly, replacing one or both prolines with arginines (Figure 2.2A-C constructs P₆₄₀⁻⁶₅₂⁻R, P₆₅₂⁻R and P₆₄₀⁻₆₅₂⁻R) increased RNA-binding by 2.5-3-fold, indicating that the WT RPR motif is not the most efficient sequence in RNA-binding. Importantly, however, these mutants supported cis- or trans-replication inefficiently (Figures 2.4 and 2.5A-B), suggesting that the ability of p33 and/or p92 to bind strongly to RNA is actually detrimental for replication.

Detailed testing of the RPR motif mutants in protoplasts revealed that this motif is essential for tombusvirus replication. For example, 10 of the 19 RPR mutants tested replicated poorly in protoplast (Figure 2.4A-B). These mutants also supported trans-replication of DI-72 RNA poorly (Figure 2.5A-B), suggesting that the reduction in the level of tombusvirus RNA replication is likely due to the mutations that render p33 and/or p92 trans-acting factors less efficient.

The use of a complementation-based two-component system, which included the tombusvirus genomic RNA expressing only p92 (but not the p33 protein due to the elimination of the leaky stop codon in the p33 ORF) and a DI RNA producing p33, defined that mutations within the RPR motif of both p33 and p92 can debilitate tombusvirus replication. These data (Figures 2.6-2.7) confirmed that the RPR motif, and therefore likely the ability to bind RNA, is important for the function of both p33 and p92 during tombusvirus infection. Interestingly, however, we observed significant differences among particular mutations depending whether they were located in p33 or p92. For example, mutating the third arginine in the RPR motif in
p33 to alanine or lysine (constructs R_{646}-A and R_{646}-K) reduced tombusvirus replication below the level of detection. The same mutations when present in p92, however, only reduced the level of RNA accumulation to 30 to 90% of the WT level (Figures 2.6 and 2.7A-B). The ability of the same mutants of p33 and p92 to support tombusvirus replication at different levels suggests that the function/structure of the RPR motif is different when present in p33 or p92. It is also possible that the above differences may reflect quantitative differences between p33 and p92 during infection. Indeed, p33 is expressed approximately 20-fold higher levels in plants than p92 (Scholthof et al., 1995d), suggesting that more p33 may be needed for replication than p92. Therefore, reducing the ability of p33 to bind RNA may affect replication more than that of p92 that has a second RNA-binding region (Rajendran and Nagy, 2003). Alternatively, the RPR motif may overlap with another, yet unknown, domain in either p33 or p92 that is also involved in tombusvirus replication. If this is the case, then mutations within the RPR motif may also affect the function of the second putative domain in either p33 or p92, resulting in differences in RNA replication in the two-component system.

Comparison of the ability of p33 to bind RNA *in vitro* and the efficiency of tombusvirus replication in protoplast revealed three groups of mutants. The first group includes seven RPR motif mutants that bound to RNA by less than 60% of WT level *in vitro*. Six of these mutants supported dramatically decreased level of gCNV accumulation (10% or less than the WT level) in protoplast (Figures 2.2 and 2.4-2.5, constructs R_{646}-A, R_{646}-K, 4R, 2R, 3R, and R_{643-649}-A). These observations are valid for both *cis*-replication (i.e., for gCNV RNA accumulation) and *trans*-replication (i.e., for DI-72 RNA accumulation). The only somewhat unusual mutant in this group is R_{643}-A that supported gCNV accumulation at 20% of the WT level, but DI-72 RNA was replicated by over 80% of WT level. Overall, the results obtained with this group of mutants suggest that the ability to bind to RNA efficiently (more than 60% of WT level) may be important for the function of p33 and/or p92.

The second group of mutants includes four members (R_{637}-A, P_{652}-A, P_{640-652}-A and R_{649}-A) that are similar to the first group (i) in their ability to bind RNA less efficiently *in vitro* than WT (between 5 to 30% reduction) and (ii) they also support tombusvirus replication less efficiently than WT (between 5-60% reduction), but they are different from the first group because the effects on RNA binding and replication are not as dramatic as in the case of the first group.
The third group of mutants includes eight members that are able to bind RNA more efficiently than the WT protein (increase between 10 to 200%, Figure 2.2). Five of these mutants support tombusvirus replication at a very low level (10% or less) (Figures 2.4-2.5, mutants P$^{652}$-R, P$^{640-652}$-R, R$^{643}$-K, R$^{643}$+R and R$^{643}$+2R). The remaining three of these mutants also show reduced level of replication, but the reduction is less pronounced (between 25-50%, see constructs P$^{640}$-A, P$^{640}$-R and R$^{649}$-K). Overall, these results suggest that the ability to bind RNA stronger than WT, like the ability to bind weakly, is also detrimental for tombusvirus replication. This suggests that the WT p33 and p92 replicase proteins of tombusviruses contain the sequence that may be optimal for both binding and release of RNA templates during replication. In contrast, it is possible that those mutants that can bind RNA either too weakly or too strongly are deficient in replication.

Comparison of the RPR mutants for their abilities to support viral replication revealed that the third arginine (R$^{646}$), and to a less extent, the second arginine (R$^{643}$) affected tombusvirus replication to the largest extent among the single alanine mutants. Importantly, these mutations also inhibited *in vitro* RNA binding to the highest extent (Figure 2.2). Replacement of the first and the fourth arginines with alanines affected tombusvirus replication and RNA-binding to a lesser extent than the second and the third arginines did. Deletion of one, two or three of the arginines in the RPR motif reduced *cis*-replication below the level of detection, while the accumulation of the DI RNA (in the *trans*-replication system) was also reduced to 0 to 1% of WT level *in vivo*. Similarly, replacing three of the arginines with alanines resulted in tombusvirus accumulation below the level of detection. These data confirm that the arginines of the RPR motif are essential for tombusvirus replication. In contrast to the arginine mutations, replacement of the first, the second or both prolines with alanines affected the level of tombusvirus accumulation only by 30 to 60% in protoplast. Overall, the fact that several alanine mutants can support tombusvirus replication rather efficiently suggest that p33 and/or p92 may tolerate some loss in the effectiveness of RNA-binding without major detrimental effects on their functions under the conditions used *in vivo*. The possibility may exist that these mutants may have lost fitness that would only be obvious under competitive conditions (e. g., in the presence of WT p33/p92).

The interpretation of *in vitro* RNA binding data and the *in vivo* RNA replication data obtained with p33/p92 mutants carrying arginine or lysine substitutions or insertions is certainly
more complex than the corresponding alanine mutants. For example, separate replacement of the second and third arginines with lysines resulted in 130% and 55% RNA-binding, respectively, while both mutants replicated only at ~5% level of the WT. Lysine substitution for the fourth arginine in the RPR motif increased RNA-binding to 285%, and it reduced the level of replication to 50% when compared to WT. Similar to the above lysine mutants, the interpretation of data obtained with the proline to arginine mutants and for mutants carrying extra arginines within the RPR motif is not straightforward. For example, addition of one or two extra arginines increased the ability of p33 to bind RNA 20 to 30% more efficiently, but it inhibited replication dramatically (down to 0 to 10% of WT). In addition, our data show that the two prolines in the RPR motif can be replaced with small neutral amino acids (such as alanine), but not with the large positively charged arginine(s) without debilitating cis- or trans-replication of tombusviruses. To explain all these data, we propose that for the proper functions of p33 and p92, it may not only be important to bind RNA, but it is also critical to form the “right” structure with the viral RNA. For example, it is possible that the RPR motif in p33 and/or p92 is needed for proper presentation of the RNA in the viral replicase complex during replication. Mutations within the RPR motif might change the positioning of the RNA within p33 and/or p92 that could affect the efficiency of replication. In addition to the proper presentation of the RNA, it is also likely that the release of the RNA after replication is important and those features of the p33/p92 proteins are expected to affect the in vivo replication results but these features have not been measured with the gel mobility shift assay. Overall, the observed differences between the in vitro RNA binding and the in vivo replication results may reflect the multiple functions of the RNA binding domain in p33/p92, such as binding, positioning and releasing the RNA, all of which might be critical for RNA replication in the infected cells.

Interestingly, most of the RPR mutants tested supported cis-replication of gCNV and trans-replication of DI-72 RNA at somewhat similar relative levels when compared to WT gCNV and WT DI-72 RNA. The two notable exceptions are (i) P640-R that supported gCNV replication efficiently (60% of WT, Figure 2.4B) and DI-72 RNA replication inefficiently (20% of WT, Figure 2.5B); and (ii) R643-A that supported gCNV replication inefficiently (20% of WT), while it supported DI-72 RNA replication at 85% of the WT level (Figure 2.5B). Although the reason for the above differences is not yet known, they do suggest that the function(s) of the RPR motif in p33 and/or p92 is not completely the same in cis- versus in trans-replication.
Another interesting finding in this work is the altered ratio between genomic and subgenomic RNAs for several mutants when compared to WT. For example, the ratio between sg2 and gCNV was ~7-fold higher in case of R_{643}^+R than for WT CNV, while P_{640-652}^-A generated sg2 RNA 40% less efficiently than WT CNV did (Figure 2.4C). The currently popular model of subgenomic RNA synthesis for tombusviruses predicts that the viral replicase terminates prematurely during the minus-strand synthesis in the vicinity of the subgenomic promoter (Choi et al., 2001). The truncated minus-stranded RNA then serves as a template for the synthesis of plus-stranded subgenomic RNAs from the subgenomic promoter. It is possible that the above RPR mutants may have either higher (for example, R_{643}^+R) or lower (P_{640-652}^-A) termination rates in the vicinity of the subgenomic promoter than WT, which then could lead to altered sg2 and, to a lesser extent, sg1 RNA synthesis. Further in vitro experiments with purified p33/p92 mutants will be needed to address this question.

Table 2.1: List of primer used for site directed mutagenesis of CNV(pK2M5)

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*aThe mutated sequences are underlined. bThe nucleotide positions within p92 ORF are indicated (Rochon and Johnson, 1991)*
Figure 2.1: The RPR motif in the CNV p33 is essential for RNA binding in vitro. (A) Schematic representation of the gCNV RNA (shown in 5’ to 3’ orientation). Among the five genes, p33 and p92, which are essential for tombusvirus replication, are directly expressed from the genomic RNA via a translational readthrough mechanism. The other three genes are the coat protein, the movement protein and the suppressor of gene silencing (shown with black boxes) that are expressed from two subgenomic RNAs (not shown). The previously defined RPR-motif in the overlapping region of p33 and p92 (Rajendran and Nagy, 2003) is shown with a gray box and the actual amino acids are specified. We used the following symbols for the amino acids: P for proline, R for arginine, A for alanine and S for serine. The deleted sequence in construct RPR is shown with (-). (B) A representative gel-shift analysis of protein/RNA complexes. The RNA probe (lane p, containing the probe only) was the 5’ 169 nt segment of DI-72(+) RNA, which was 32P-labeled using in vitro transcription. The same amount of RNA probe (2 ng) was added to each lane in the gel, while the WT and RPR proteins were added (0.5 µg each) to the lanes as indicated below the gel. (C) The relative RNA binding efficiencies of the p33-RPR mutant is shown compared to WT (100%) from three separate experiments. The amounts of free probes in each lane were measured using a phosphorimager.
A

WT
1. R637-A A
2. P640-A R
3. P652-A P
4. P640-652-A R
5. P640-R R
6. P652-R R
7. P640-652-R R
8. R643-A R
9. R643-K K
10. R646-A R
11. R646-K K
12. R649-A R
13. R649-K K
14. R643+R R
15. R643+2R R
16. R R R
17. R2 R R R
18. R3 R R R
19. R643-649A R

B

RNA/Protein complex
Free RNA probe

Bar graph showing the relative abundance of RNA/protein complexes for each sample with error bars representing standard deviation.
Figure 2.2: Defining the amino acids within the RPR motif in CNV p33 that are essential for RNA binding \textit{in vitro}. (A) The name and amino acid sequences of the 19 RPR motif mutants tested in this work. The mutated amino acids are bold-faced, while the deleted sequences are shown with (-). We used the following symbols for the amino acids: A for alanine, P for proline, R for arginine and K for lysine. (B) A representative gel-shift analysis of protein/RNA complexes. The RNA probe (lane p, containing the probe only) was prepared as described in the legend to Figure 2.1. The same amount of RNA probe (2 ng) was added to each lane in the gel, while the proteins (1 µg each) were different as indicated below the gel. Lane mb indicates the sample containing the maltose-binding protein, while lane WT depicts the truncated p33 with the WT RPR-motif, which was expressed and purified from \textit{E. coli} as a 30 amino acid fragment of p33 fused to MBP. Lanes 1-19 depict the truncated p33 with the RPR-motif in the order as shown in panel A. The migration of the free probe and the RNA/protein complex is indicated on the left. Note that the migration of the RNA/protein complex can be different in case of particular p33 mutants when compared to WT, probably due to the changes in the charge of the protein. (C) The relative RNA binding efficiencies of the given truncated p33 mutants are shown compared to WT (100\%) from three separate experiments. The amounts of free probes in each lane were measured using a phosphorimager. Light gray bars represent those mutants that showed decreased efficiencies in RNA binding.
Figure 2.3: Effects of amino acid changes within the RPR motif on RNA binding \textit{in vitro} when present in the full-length p33. (A) The names of the RPR motif mutants and the corresponding amino acid changes are the same as in Figure 2.2. Two separate representative gel-shift analyses of protein/RNA complexes are shown. The RNA probe was prepared as described in the legend to Figure 2.1. The same amount of RNA probe (2 ng) was added to each lane in the gels, while the proteins (0.5 µg each) were different as indicated below the gels. (B) The relative RNA binding efficiencies of the particular p33 mutants are shown in comparison with WT (100%). The amounts of free probes in each lane were measured using a phosphorimager.
A

RPR mutant number

B

C

WT 1  2    3    4    5    6    7    8    9   10  11  12  13  14  15  16  17  18  19

wt1  2    3    4    5    6    7    8    9   10  11  12  13  14  15  16  17  18  19
Figure 2.4: Relative *in vivo* accumulation of gCNV RNA carrying mutations within the RPR-motif. (A) The full-length, infectious gCNV RNA (2 µg) carrying a given mutation (the numbers of constructs corresepond to the mutations in Figure 2.2 (A)) within the RPR-motif was electroporated into *N. benthamiana* protoplasts (5x10^5 cells per experiment). Total RNA was isolated, electrophoresed and blotted onto a membrane, followed by probing with 32P-labeled RNA specific for gCNV (see Materials and Methods). The same amount of total RNA was used for loading onto the gels based on the estimation of the host ribosomal RNA in ethidium bromide-stained gels (not shown, see Figure 2.6 as example). The positions of the gCNV (g), subgenomic RNA1 (sg1) and subgenomic RNA2 (sg2) are depicted on the left. The samples were taken 24 (top) and 48 hours (bottom) after electroporation. The experiment was repeated three times. (B) The relative accumulation of gCNV RNA for the WT and the p33/p92 mutants are shown (WT was chosen as 100%). The amounts of gCNV in each lane were measured in the samples taken after 48 hours of incubation using a phosphorimager. (C) The percentage of sg1 RNA (dark bar) and sg2 RNA (gray bar on the right) accumulation was calculated based on the accumulation of the corresponding gCNV RNA for the WT and the p33/p92 mutants. The data represent the samples taken after 48 hours of incubation.
Figure 2.5: Relative trans-replication efficiency of DI-72 RNA by the gCNV helper carrying mutations within the RPR-motif. (A) The full-length DI-72 RNA (1 µg) (White and Morris, 1994a) was co-electroporated with the infectious gCNV RNA (2 µg) carrying a given mutation within the RPR-motif (the numbers of constructs corresponed to the mutations in Figure 2.2 (A)) into *N. benthamiana* protoplasts (5x10⁵ cells per experiment). Sample preparation and Northern-blotting was done as described in the legend to Figure 2.4, except the probing was done with ³²P-labeled RNA specific for DI-72 RNA (see Materials and Methods). The position of the DI-72 RNA is depicted on the left. The samples were taken 24 (top) and 48 hours after electroporation. The experiment was repeated three times. (B) The relative trans-replication of DI-72 RNA in the presence of the WT helper or one of the p33/p92 mutants are shown (WT was chosen as 100%). The amounts of DI-72 RNA in each lane were measured in the samples taken after 24 hours (left bars) and 48 hours (right bars) of incubation using a phosphorimager.
Figure 2.6: Testing the role of the RPR-motif in p33 in tombusvirus replication using a two-component system. (A). Schematic representation of the two constructs used. The stop codon at the end of the p33 gene was mutated to a tyrosine codon (represented by letter Y in the resulting construct, p92Y) in gCNV RNA to prevent the production of p33 from this RNA. The second RNA (DI-p33) was derived from a DI RNA that carried a translation competent p33 gene (Oster et al., 1998). The RPR-motif within the p33, shown with a gray box in DI-p33, was mutated (marked with an asterisk). (B) Northern blot analysis of replication of DI-p33 mutants in the two-component system. The p92Y RNA (2 μg) (Oster et al., 1998) was co-electroporated with DI-p33 (2 μg) carrying a given mutation within the RPR-motif into N. benthamiana protoplasts (5x10⁵ cells per experiment). Sample preparation and Northern-blotting was done as described in the legend to Fig. 4, except the probing was done with ³²P-labeled RNA specific for DI-p33 RNA (see Materials and Methods). The same amount of total RNA was used for loading onto the gels based on the estimation of the host ribosomal RNA in ethidium bromide-stained gels (see the gel at the bottom of panel B). The positions of the DI-p33 and p92Y RNAs are depicted on the left. Note that p92Y replicates poorly in protoplast (undetectable with this probe), while the control gCNV replicates to high level and the gCNV RNA can be detected with this probe (due to weak cross-hybridization). The names of the DI-p33 mutants are shown on the top. The samples were taken 48 hours after electroporation. The experiment was repeated three times. (C) The relative replication efficiency of DI-p33 RNA in the presence of the p92Y helper (WT was chosen as 100%). See legend to Figure 2.4 for details. (D) Complementation of replication of DI-p33 mutants by the WT gCNV helper RNA. The Northern blot was performed as described in panel B.
A

\[ p92Y \text{ (mutated)} \]

\[ \text{p92Y} \quad \text{p33 (WT)} \]

B

\[ \text{p92Y mutants + DI-p33} \]

C

\[ \text{WT} \quad \text{WT1} \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8 \quad 9 \quad 10 \quad 11 \quad 12 \quad 13 \quad 14 \quad 15 \quad 16 \quad 17 \quad 18 \]
Figure 2.7: Testing the role of the RPR-motif in p92 in tombusvirus replication using a two-component system. (A). Schematic representation of the two constructs used. See legend to Figure 2.6 for further details. The RPR-motif within the p92, shown with a gray box, was mutated (marked with an asterisk). (B) Northern blot analysis of replication of WT DI-p33 in the two-component system. The DI-p33 RNA (2 μg) was co-electroporated with p92Y RNA (4 μg) carrying a given mutation within the RPR-motif into N. benthamiana protoplasts (5x10^5 cells per experiment). Sample preparation and Northern-blotting was done as described in the legend to Figure 2.4, except the probing was done with ^32P-labeled RNA specific for DI-p33 RNA. The names of the p92Y mutants are shown on the top. The samples were taken 48 hours after electroporation. The experiment was repeated three times. See Figure 2.4 for further details. (C) The relative replication efficiency of WT DI-p33 RNA in the presence of the WT or mutated p92Y helper. See legend to Figure 2.4 for details.
Chapter Three

Mutations in the RNA-binding domains of tombusvirus replicase proteins affect RNA recombination in vivo

Introduction

Errors have been proposed to occur during viral RNA replication often leading to RNA recombination, which is a process that joins RNA sequences that were either present on separate molecules or they were noncontiguous when present on the same molecule (Jarvis and Kirkegaard, 1991; Lai, 1992; Nagy and Simon, 1997). RNA recombination has been documented for a growing number of bacterial (Mindich et al., 1992; Palasingam and Shaklee, 1992), fungal (Shapira et al., 1991), plant (Allison et al., 1990; Ayllon et al., 1999; Borja et al., 1999; Cascone et al., 1993; Nagy and Bujarski, 1993; Rao and Hall, 1993; White and Morris, 1999), animal and human viruses (Banner et al., 1990; Becher et al., 1999; Furuya et al., 1993; Hajjou et al., 1996; Keck et al., 1988; Khatchikian et al., 1989; King et al., 1982; Kirkegaard and Baltimore, 1986; Li and Ball, 1993; Molenkamp et al., 2001; Suzuki et al., 1998; Vezza et al., 1980; Walter et al., 2001; Weiss and Schlesinger, 1991; Worobey et al., 1999; Wu et al., 1999).

Depending on the sequences brought into proximity by RNA recombination, the changes in the viral genomes can be dramatic or minor. Due to the formation of viral genomes with novel traits, RNA recombination has been proposed to be a major driving force in viral evolution (Aranda et al., 1997; Fernandez-Cuartero et al., 1994; Gibbs, 1987; Lai, 1992; Strauss and Strauss, 1988; Worobey and Holmes, 1999). In addition, it has been shown that recombination process is involved in viral genome repair (White and Morris, 1994b) and DI RNA formation (White, 1996; White and Morris, 1994a).

There were proposed three models of viral RNA recombination (Nagy and Simon, 1997): 1) replicase driven template switching; 2) breakage and ligation and 3) breakage induced template switching (breakage in RNA molecule leads to template switching process).

The model most supported by recombination studies is the template switching model, which proposes that viral replicase (due to yet incompletely understood reasons) makes significant errors during the replication process, leading to template switching during
complementary RNA synthesis (Jarvis and Kirkegaard, 1991; Lai, 1992; Nagy and Simon, 1997). After the switch from the donor to the acceptor RNA, the viral replicase can resume RNA synthesis using the nascent RNA (made on the donor template) as a primer on the acceptor RNA. This model assumes relationship between viral replication and recombination processes in infected cells.

The breakage and ligation model has been proposed for recombinant Qb bacteriophage RNA formation and was based on findings that changes in 3’ end OH group of 5’ template influenced recombination (Chetverin, 1997). The fact that the recombination process occurred only in the presence of viral replicase does not allow to rule out the possibility that recombination might have occurred via template switching mechanism (Nagy and Simon, 1997).

Breakage - induced template switching model suggests that the breakage in RNA can stop replicase, and that would facilitate template switch. It has been shown that modifying 5’end of donor RNA to mimic cleaved RNA can lead to increased recombination in tombusviruses (White and Morris, 1995). Involvement of RNA cleavage in recombination was reported for HIV-1 reverse transcriptase (Peliska and Benkovic, 1992; Peliska and Benkovic, 1994).

The most of the tombusvirus recombination studies favor template switching mechanism that can be divided into three steps: 1) the pausing of replicase on the donor template, 2) release of donor RNA and binding to the acceptor RNA molecule, 3) resumption of nascent RNA synthesis by replicase on the acceptor RNA (Nagy and Simon, 1997). The major components facilitating this process are viral RNAs and replicase proteins, possibly with host proteins involvement.

There were extensive studies done to find out what RNA features are influencing recombination. It was shown that the first recombination step, replicase pausing, can occur due to donor template 5’end degradation (White and Morris, 1995) or shorter 5’ends caused by internal replication initiation (Panavas et al., 2002b), strong secondary structure formation (hairpins) (Carpenter et al., 1995; Cascone et al., 1990; Cascone et al., 1993; White and Morris, 1995) or heteroduplexes (Nagy and Bujarski, 1993; Nagy et al., 1995)), or AU rich regions in donor template (Nagy and Bujarski, 1995; Nagy and Bujarski, 1996; Shapka and Nagy, 2004).

It has been suggested that acceptor RNA template is important for replicase binding and facilitating 3’end extension on the nascent strand. The studies with Turnip crinkle virus (TCV)
revealed that the motif1-hairpin of satC RNA (-) of acceptor template was important for replicase binding, while the sequence around the replicase binding site was critical for nascent strand binding and for primer extension (Nagy et al., 1998). In addition to its role in recombination, the motif1-hairpin also functions as an enhancer for satC replication. Interestingly, it has been shown that RIII (-) functions as replication enhancer in tombusvirus DI-72 (Panavas and Nagy, 2003a; Ray and White, 1999; Ray and White, 2003) and it can bind to the replicase efficiently (Panavas and Nagy, unpublished) and promote recombination in in vitro assay using partially purified CNV replicase (Cheng and Nagy, 2003).

For the late step in recombination (nascent strand extension) complementarity between these two RNAs is important (Li and Ball, 1993b; Nagy and Bujarski, 1995; Olsthoorn and vanDuin, 1996; Pogany et al., 1995; Qiao et al., 1997; Raffo and Dawson, 1991; White and Morris, 1994a; White and Morris, 1994b; White and Morris, 1995). Further studies of primer extension in in vitro system using partially purified CNV replicase, revealed that the most effective primer extension occurred with primers (primers in this study resemble nascent strand) allowing 4-5nt base-pairing with the acceptor template (Cheng et al., 2002).

In summary, previous studies on the role of RNA in recombination revealed that abnormal 5’ ends of donor template as well as strong secondary structures could trigger replicase pausing and release from donor strand. This step is followed by replicase binding to acceptor RNA, facilitated by certain RNA structures on the acceptor RNA, and then by nascent strand extension supported by complementarity between nascent and acceptor RNAs.

The requirement of replicase activity during recombination implies that replicase proteins play an important role in this process as well. The above mentioned studies of the ability of replicase to bind RNA regions involved in recombination support this idea. In addition, the detection of recombinant RNAs in in vitro experiments using TCV replicase purified from E.coli and CNV replicase partially purified from N.benthamiana plants strongly suggest a critical role for replicase in viral RNA recombination. Taking all these findings together, it is clear that recombination and replication are closely interconnected processes.

To understand the relationship between replication and recombination, I was studying tombusviruses, which are single-component plus-stranded RNA viruses of plants. Two closely related tombusviruses, namely Tomato bushy stunt virus (TBSV) and Cucumber necrosis virus (CNV) are known to support RNA recombination at high frequencies (Borja et al., 1999; White
and Morris, 1994a; 1994b; 1995; Rochon, 1991). In addition to the genomic RNAs of tombusviruses, defective interfering (DI) RNAs associated with these viruses are also frequently used templates for RNA recombination. The major advantages of using DI RNAs for both replication and recombination studies are that (i) DI RNAs are involved in RNA recombination with high frequencies (White and Morris, 1999), (ii) they do not contribute essential protein factors to replication, and thus having higher genetic plasticity than the viral genomic RNA; and (iii) they use the same replication/recombination machinery as the genomic RNAs. The tombusvirus DI RNAs are mosaic types that are derived from the genomic RNA via two or three large sequence deletions (White and Morris, 1999; Figure 3.1A). The sequence deletions during the DI RNA formation are thought to be the consequence of viral replicase jumping on the template and the deletions may occur in a step-wise manner (White and Morris, 1999).

The TBSV and CNV replicases contain two essential viral proteins (i.e., p33 and p92) and possibly host factors (Scholthof et al., 1995; Oster et al., 1998; Nagy and Pogany, 2000). The p92 protein contains the signature motifs of RNA-dependent RNA polymerases (RdRp), while the function of p33 is currently not known. Both proteins bind to single-stranded RNA, including the plus-stranded DI RNA (Rajendran and Nagy, 2003). Due to the overlapping expression strategy for these proteins, p33 and p92 contain the same RNA-binding region that includes an arginine/proline-rich sequence (termed the RPR motif, Rajendran and Nagy, 2003; Panaviene et al., 2003). Many mutations within the RPR motif of p33 and p92 were detrimental to virus and DI RNA replication. Several mutations in the RPR motif affected the function of p33 differently than that of p92, suggesting that the RPR motif plays somewhat different roles in these proteins (Panaviene et al., 2003). Interestingly, few mutations in the RPR motif of p33 could also affect the level of subgenomic RNA relative to genomic RNA synthesis (Panaviene et al., 2003). These observations demonstrated that p33 and p92 and their RPR motifs play major roles in tombusvirus RNA replication. Therefore, due to the proposed interrelationship between replication and recombination, many of the mutations might also affect RNA recombination as well. This assumption was tested in this work using a single cell (protoplasts) system, which supports efficient recombinant of DI RNAs when the wild type (wt) p33 and p92 proteins are present. I found that mutations in the RPR motif in p33/p92 proteins resulted in either “fast”, wt-like, or “slow” recombinant formation. The altered recombination efficiency in these RPR motif mutants was mapped to the p33 protein. I also found that wt or selected replicase mutants with
altered recombination frequencies supported the relative accumulation of new recombinants versus the parental DI RNAs to similar extent, suggesting that post-recombinational selection is unlikely the reason for the observed differences among the mutants and the wt virus. Overall, this chapter provides evidence that the replicase proteins of a tombusvirus, particularly the RNA-binding region, are involved in RNA recombination.

**Materials and methods**

**Plasmid construction**

For construction of cDNA clones of DI-33 and DI-333 DI RNA, we PCR-amplified RIII of DI-72 using primers #355 (5’-GGACGCTGCAGGGCCCAGAAAGCGAGTAA GACAG-3’) and #356 (5’-GACGCTGCAGACGCGTGGGGGCGAGATCTCCAGAA CCCAAC-3’) and template DI-72XPXN (Shapka and Nagy, unpublished). DI-72XPXN, which contains Xho I and Neo I sites between RIII and RIV, is a derivative of DI-72SXP obtained from Andy White (White and Morris, 1994a). The PCR product was digested with Pst I, gel purified and cloned into DI-72XPXN linearized with Pst I. We selected the appropriate DI-33 and DI-333 clones, which contained either one RIII copy or two RIII copies, respectively, in 5’-3’ orientation by sequencing.

For construction of DI-2233 clone, RII of DI-72SXP was amplified by PCR using primers #386 (5’-GGACGGGGCCCATCGATAGAAACGGGAAGCTCGC-3’) and #387 (5’-GGACGGGGGCCCCATCGATAGAAACGGGAAGCTCGC-3’) and DI-72SXP template. The PCR product was digested with Apal, gel purified and ligated into DI-33 cDNA treated with Apal. Additional copy of RII was inserted between the original RII and RIII in 5’-3’ orientation.

To make DI-2323 clone, RII of DI-72 was PCR-amplified using primers #388 (5’-GGACGAGATCTGGGTGGATCGATAGAAACGGGAAGCTCGC-3’) and #389 (5’-GGACGACGCGTGGGTGGGATATCCTGCTTTTACGAAGGTA-3’), and template DI-72SXP RNA. The PCR fragment was digested with BglII and MluI, followed by gel purification and ligation into DI-33 treated with BglII and MluI.
Construction of RPR motif mutants

Site directed mutagenesis within the RPR motif of gCNV (Panaviene et al., 2003; Rajendran and Nagy, 2003) was performed with Quick Change XL Site Directed Mutagenesis Kit (Stratagene). The primer pairs used for each mutant are shown in the Table 3.1, while the template was pK2M5 (Rochon and Johnston, 1991), encoding a full-length cDNA clone for CNV. Before transformation into E.coli (DH5::), the PCR products were digested with DpnI. The clones for each mutant were confirmed by sequencing using primer #27 (5’-GTATTTTCACACCAAGGGAC-3’).

We made derivatives of gCNV-Y (expressing protein p92-Y) and DI-p33, carrying selected mutations in the RPR motif (Table 3.2) as described in Panaviene et al., 2003(Chapter 2). To confirm the presence of the RPR mutation and that the termination codon was replaced with tyrosine codon in derivatives of gCNV-Y, each clone was sequenced using primer #631 (5’-GAGGAATTCAAGGTAATTGCGTCCACA-3’).

Cloning recombinant DI RNAs

To obtain cDNA clones for a representative number of recombinant DI RNAs, we performed reverse transcriptase (RT) reaction with primer #41 (5’-GGACGAATTCGGGCTGCATTTCTGCAATGTTCC-3’) and 2% (10%) of total RNA obtained from protoplast. The RT products were amplified using PCR with primers #41 (5’-GGACGAATTCGGGCTGCATTTCTGCAATGTTCC-3’) and #40 (5’-GGACAAGCTTGGCTACCGTTGCTTTTG-3’). The RT-PCR products were digested with XbaI and EcoRI, gel isolated and ligated into similarly treated pUC19 vector. The clones for sequencing were selected after restriction digestion with Xbal and EcoRI. The sequencing was done using primer #157 (5’-GGGCTTGGCATTTTCTGCAATGTTCC-3’). Note that cDNA clones with identical recombination junction sites were counted only once if they were derived from the same protoplast samples.

To generate full-length cDNA clones for selected recombinant DI RNAs (see control experiment in Figure 3.6), selected partial cDNA clones, which were used for sequencing, were digested with Xbal and XhoI. This is followed by gel purification of the appropriate fragments and cloning into similarly treated DI-2323 (Figure 3.1A).
Preparation of RNA transcripts

To generate RNA transcripts, all pK2M5 and DI-72XPXN-derived plasmids were digested with *SmaI*, and they were used in an *in vitro* transcription reaction with T7 RNA polymerase (Nagy et al., 1999). The obtained DI RNA transcripts used in recombination studies were gel purified from 1% agarose gel, followed by phenol/chloroform extraction, precipitation in 95% ethanol and three times repeated wash with 70% ethanol to remove residual salts. For the rest of the *in vitro* RNA transcripts, DNA templates were removed by *DNase* I treatment, followed by phenol/chloroform extraction, precipitation and washing as described above. The RNA transcripts were electrophoresed on 1% agarose gels and quantified by UV spectrophotometer (Beckman).

Preparation and electroporation of protoplasts

*N. benthamiana* protoplasts were prepared as described Panaviene et al., 2003 (Chapter 2). At the end of the procedure, we resuspended 5×10⁵ protoplasts in the electroporation buffer (10 mM HEPES, 10 mM NaCl, 120 mM KCl, 4 mM CaCl₂, 200 mM mannitol). For electroporation, we used the following amounts of RNA transcripts: 2 µg gCNV RNA and 1 µg gel-purified DI RNAs (unless specified otherwise in the figure legends); After electroporation, the samples were incubated in 2 ml of protoplast culture medium (Kong et al., 1997) in the dark for 24-48h at 22⁰C.

Total RNA extraction from protoplasts and RNA analysis

Total RNA was extracted from protoplast using a standard phenol/chloroform method (Kong et al., 1997; Nagy et al., 2001). Aliquots of total RNA were analyzed on 1 or 1.2% agarose or 4% polyacrylamide/8M urea gels. RNA samples were treated with formamide at 85⁰C right before loading on the gels. For Northern blot analysis, the RNA was transferred from the gels to Hybond XL membrane (Amersham-Pharmacia) by electrotransfer and hybridized with DI-72 (+) or DI-72 (-) specific probes (Panaviene et al., 2003; chapter 2). Hybridization was done in ULTRAhyb hybridization buffer at 68⁰C using supplier (Ambion) recommended conditions. The probes were made in an *in vitro* transcription reaction with T7 RNA polymerase in the presence of a [³²P]UTP and DNA templates representing the 169 bp RI (Figure 3.1A). These templates were generated by PCR using primers #15 (5’-
GTAATACGACTCATAAGGCAATGTCGCTTGTGTGGTG-3’) and #20 (5’-GGAAATTCTCCAGGATTTTC-3’) for detection of (+) strands, and primers #31 (5’-GTAATACGACTCATAAGGCAATTCTCCAGGATTTC-3’) and #678 (5’-GGGCTGCATTTCTGCAATGGTCGCTTGTGTGGAG-3’) for (-) strand detection.

Results

Rationale: To study the role of replicase proteins in RNA recombination in tombusviruses, first I developed an efficient recombination system based on a prototypical DI RNA (i.e., DI-72 that is derived from TBSV via three deletions, Figure 3.1A). Since I wanted to test the effect of the RNA-binding domains of the replicase proteins on recombination, we chose DI RNA sequences, namely, region II [RII, (Shapka and Nagy, 2004)] and region III [RIII, Panavas and Nagy, unpublished], which, as minus-stranded sequences, are known to bind to the replicase proteins with high affinity in vitro. While the function of RII(-) is currently unknown, RIII(-) has recently been defined as a strong replication enhancer (Panavas and Nagy, 2003a; Ray and White, 1999; Ray and White, 2003). It has also been demonstrated recently that RIII(-) promotes template switching by the CNV RdRp preparation in vitro (Cheng and Nagy, 2003). Interestingly, RIII(-) served both as a donor and acceptor sites during the recombination events in vitro. Therefore, I reasoned that due to their binding to the viral replicase proteins, RIII(-) and possibly RII(-) sequences might constitute recombination hot spots in vivo as well. This hypothesis was tested in protoplasts below.

Replication and recombination of TBSV DI RNAs carrying duplicated RII and RIII sequences.

To test the effect of RII and RIII sequences on the ability of DI RNA to replicate and to recombine, I made four constructs as shown in Figure 3.1A. Namely, DI-33 and DI-333 carried two and three copies of RIII sequences, while constructs DI-2233 contained two copies of RII and RIII sequences. The fourth construct, DI-2323 carried a tandem repeat of RII and RIII (Figure 3.1A).

Each of the gel-isolated TBSV DI RNA transcripts obtained after T7 RNA polymerase transcription was co-electroporated with genomic CNV RNAs (gCNV) into N. benthamiana protoplasts as described earlier (Panaviene et al., 2003; chapter 2). I used the heterologous
gCNV, which is closely related to TBSV, as the helper virus to exclude those DI RNAs that might be generated spontaneously from the helper RNA during infection, a process known as de novo DI RNA formation (White and Morris, 1999). I followed the accumulation of plus- and minus-stranded DI RNAs by using Northern blotting with a DI RNA-specific probe [RI(+) or RI(-), Figure 3.1B-C]. These experiments detected only the parental (input)-sized (+) and (-) DI RNAs even after 48 hours of incubation (termed zero passage), suggesting that (i) these DI RNAs can replicate in protoplasts; and (ii) they do not support recombinant formation/accumulation at a detectable extent (Figure 3.1B). Interestingly, none of the DI RNAs with repeated RII or RIII sequences replicated as efficiently as the wt DI RNA (lowest and the highest accumulation was ~25 and 60% of wt for DI-333 and DI-33, respectively, Figure 3.1B). Also, the amounts of plus-strands were decreased significantly more than the amounts of minus-strands for all four constructs tested (Figure 3.1B-C, especially at the 24 hr time point).

It is possible that the lack of recombinant accumulation with these mutants in the zero passage protoplasts is due to the limited time available for replication of the putative recombinants after their formation during protoplast incubation (viability of the protoplast cells sharply declined after 48 hours under the test conditions, not shown). Therefore, to increase the chance for the formation/accumulation of recombinants, I made a passage with the total RNA obtained after 48 hours of incubation in protoplasts via electroporation into a new batch of protoplasts (termed first passage). Then the total RNA from the first passage (after incubation for 48 hours) was used for electroporation into yet another batch of protoplasts (termed second passage) and so on (Figure 3.2). Northern blot analysis of the total RNA extracts obtained from the various protoplast samples after the 1st passage revealed the occurrence of novel, ~100-250 bp shorter than input, recombinant-like RNAs for constructs DI-33, DI-333 and DI-2233 in addition to the parental-sized DI RNAs (Figure 3.2A, 1st passage). Interestingly, many of the samples contained at least two different-sized recombinant-like DI RNAs, suggesting that more than one recombination events took place in cells. Total RNA from the 2nd passage showed similar pattern to that from the 1st passage, except the input-sized DI RNAs became hardly detectable (Figure 3.2A). Overall, recombinant-like DI RNAs were observed in 100% of the protoplasts experiments (1st and 2nd passages) with constructs DI-33, DI-333 and DI-2233, based on Northern blot (Figure 3.2A) and RT-PCR (Figure 3.2B) analyses.
In contrast to the above three constructs, DI-2323 supported recombinant formation/accumulation less efficiently. The first recombinants were observed (at hardly detectable level) in 50% of the samples after the second passage (Figure 3.2A). Half of the samples still did not contain recombinants after the third passage, suggesting that this construct could support recombinant formation/accumulation relatively inefficiently.

The observed novel DI RNAs in protoplasts appear to be true recombinants, since they can be detected in the total RNA extracts using either ethidium bromide-stained gels (not shown), Northern blotting (Figure 3.2A), or RT-PCR analysis (Figure 3.2B). Control RT-PCR reactions performed on gel isolated DI RNAs (the same preparations that were used for electroporation to protoplasts) did not detect recombinant-sized DI RNAs for DI-33 and DI-333 constructs (Figure 3.2B). In contrast, recombinant-sized RT-PCR products were observed in the control RT-PCR reactions in case of DI-2233 and DI-2323, because of the presence of the 239 nt RII repeat in these constructs. These were precise recombinants between the duplicated RII sequences (leading to deletion of one of the duplicated copies, not shown), and we did not count them in our analysis (Figure 3.3). Nevertheless, DI-2233 and DI-2323 generated unique-sized recombinants that were not present in the control RT-PCR reactions. These recombinants were imprecise and they were included in our analysis (see below).

Cloning and sequencing of the above putative recombinants demonstrated that (i) they were indeed recombinants, derived from the input DI RNAs via single deletions; and (ii) the recombination junction sites were varied in the recombinants (imprecise recombination) (Figure 3.3). The fact that many of the recombinants obtained with DI-33 and DI-333 had unique and imprecise junction sites (Figure 3.3A-B) shows the genome plasticity of the DI RNA. The occurrence of precise recombinants obtained with DI-33 and the lack of these recombinants for DI-333 indicates that the same sequences might support different recombinants depending on the neighboring sequences. Many recombinants obtained with constructs DI-2323 and DI-2233 had the junction sites within the first copy of RII and the second copy of RIII, albeit other recombinants still carried portions of duplicated sequences (Figure 3.3C-D). Note that I did not study precise recombination with constructs DI-2323 and DI-2233 due the artifactual products in the control RT-PCR reactions (see above and Figure 3.2B). Overall, I found that the majority of the recombinants lacked most of the inserted (duplicated) sequences, and they frequently contained further deletions in RII and RIII. The observed variability among the obtained DI
RNA recombinants (Figure 3.3) in junction site pattern is similar to previous findings with *de novo* generated recombinants in tombusvirus populations, which also contained unique deletion sites (White and Morris, 1999).

Testing DI RNA replication and recombination supported by CNV with mutations in the RPR motif of the replicase proteins

I have previously tested 19 RPR motif mutants that contained either deletions, insertions of extra arginines or substitutions (arginine/proline to alanine or lysine) in protoplasts (Panaviene et al., 2003, chapter 2). Among these mutants, 14 supported gCNV and DI-72 accumulation at detectable levels (Panaviene et al., 2003, chapter 2). To increase the number of viable mutants with possibly altered properties in replication/recombination, we generated a new set of RPR motif mutants (total of nine) that had arginine to histidine, lysine, glutamine or alanine changes (Table 3.2, single and double mutants, R<sub>637</sub>-K, R<sub>637</sub>-H, R<sub>637</sub>-Q, R<sub>637</sub>-A/R<sub>643</sub>-H, R<sub>637</sub>-A/R<sub>646</sub>-H, R<sub>637</sub>-A/R<sub>649</sub>-H). Additional mutants carried proline to glycine or tyrosine mutations (Table 3.2, constructs P<sub>640</sub>-Y, P<sub>652</sub>-Y, P<sub>640-652</sub>-G). Testing the ability of these mutants to support DI-333 RNA replication in protoplasts revealed that seven of them were 75-95% as efficient as the wt CNV (Figure 3.4 and Table 3.2). Two double mutants, namely R<sub>637</sub>-A/R<sub>643</sub>-H, R<sub>637</sub>-A/R<sub>646</sub>-H, which carried arginine to histidine mutations, were able to support DI RNA replication only inefficiently (1-2% of wt level, Table 3.2).

To test the ability of the RPR motif mutants to support recombination in protoplast, I used a total of 17 mutants and the wt virus in a recombination assay with DI-333. When tested after the first passage, ten of the RPR motif mutants supported the formation/accumulation of recombinant DI RNAs with comparable efficiency to the wt CNV (Figure 3.5A). Five mutants, P<sub>640</sub>-A, P<sub>640</sub>-Y, P<sub>652</sub>-A, and P<sub>652</sub>-Y and P<sub>640</sub>-A/P<sub>652</sub>-A, however, supported the formation/accumulation of recombinant DI RNAs inefficiently (~15-40% of that of wt, Figure 3.5B). I found that the low recombination efficiency for the above proline mutants was repeatable in three separate experiments (not shown). In those samples, which accumulated recombinant DI RNAs, the relative level of DI RNA accumulation (compared to the level of the parental DI-333 RNA) was low (~5%). These observations suggest that the recombinant DI RNAs are formed inefficiently or their formation is greatly delayed in the presence of the helper virus carrying the above proline mutations in the RPR motif (“slow mutants”).
On the contrary, two RPR motif mutants (R\textsubscript{646}-A and R\textsubscript{643}+R) accumulated recombinant DI RNAs apparently faster than the wt helper did when tested with DI-333 RNA (not shown). To further test this observation, I used DI-2323, which can support recombination less efficiently than DI-333 in the presence of the wt helper (Figure 3.2A). I found that in the presence of mutants R\textsubscript{646}-A or R\textsubscript{643}+R, DI-2323 RNA supported recombinant DI RNA accumulation in 82-100% of samples, while the corresponding value is ~18% in the presence of the wt helper (Figure 3.5C) after the second passage. The relative amount of the recombinant DI RNAs (in comparison with the parental DI-2323 RNA) was also higher in samples obtained with R\textsubscript{646}-A and R\textsubscript{643}+R (“fast mutants”) than with the wt. As an additional control, I also used mutant R\textsubscript{643}-A (which supported wt level of recombination with DI-333, Figure 3.5A) in combination with DI-2323. Importantly, I found that R\textsubscript{643}-A supported low, (~17%) wt-level of recombination with DI-2323 after the second passage (Figure 3.5C). These observations suggest that the recombinant DI RNAs might be formed more efficiently and/or earlier during infections in the presence of the helper virus carrying the R\textsubscript{646}-A and R\textsubscript{643}+R mutations than in the presence of wt or R\textsubscript{643}-A.

The observed differences among the wt gCNV and several mutants in supporting the occurrence of novel recombinant DI RNAs might be due to (i) differences in the frequency of recombinant generation. In other words, it is possible that the mutated replicases might have reduced or increased capacity to generate recombinants (i.e., slow or fast mutants, Figure 3.5B-C). (ii) It is also possible that there are differences in replication levels of the new recombinants and the parental DI RNAs (i.e., the difference is at the level of post-recombinational amplification). To test if those RPR motif mutants, which generated recombinants slower than wt (e.g., P\textsubscript{640}-A, P\textsubscript{640}-Y, P\textsubscript{652}-A, P\textsubscript{652}-Y and P\textsubscript{640}-A/P\textsubscript{652}-A), might show reduced relative replication levels for the recombinant DI RNAs (compared to the parental DI RNAs), I performed template competition experiments between the parental DI-333 RNA and two different recombinant DI RNAs (derived from experiments shown in Figures 3.2-3.3) in the presence of wt gCNV or the above mutants. One recombinant, namely #13, had the larger deletion that extended 51 nt into RII in DI-333 (Figure 3.3B), while recombinant #6 had only 6 nt deleted from RII and it contained a 5 nt stretch of the marker sequence (Figure 3.3B). I found that after 48 hours of incubation, both recombinant DI RNAs replicated ~15 to 22-fold better than the parental DI-333 RNA in the template competition experiments. More importantly, these recombinant DI RNAs showed comparable level of advantage over the parental DI RNA regardless of wt or mutant
gCNV background (Figure 3.6A-B). These data argue that, after their formation, the recombinant DI RNAs should be replicated by either the wt or the mutant gCNV to levels higher than that of the parental DI-333. Since I detected the parental DI RNAs readily, but not the recombinants (Figure 3.5B), in the presence of slow mutants (i.e., P_{640}^-A, P_{640}^-Y, P_{652}^-A, P_{652}^-Y and P_{640}^-A/P_{652}^-A), it is unlikely that the differences between the wt and the slow mutants are due to differences in supporting the accumulation of recombinant DI RNAs over the parental DI RNA. Instead, it is more likely that the difference is due to the altered efficiency of generation of recombinant DI RNAs by the wt and the selected mutants (see Discussion).

I also tested the relative accumulation levels of recombinant DI RNAs in a competition assay with DI-2323 parental RNA for the “fast” mutants and the wt (Figure 3.6C). Similar to the above observations, the two fast mutants tested (Figure 3.6C, R_{646}^-A and R_{643}^+R) as well as wt gCNV supported the recombinant DI RNAs at comparable relative levels in the competition assay after 48 hours of incubation (Figure 3.6C). Based on this data, I conclude that the “fast” mutants do not support the recombinant DI RNAs over the parental DI RNA better than the wt gCNV. This observation further supports that the observed differences in frequency of recombinant DI RNA occurrence are unlikely due to differences between the wt and the mutants in post-recombinational selection of recombinant DI RNAs over the parental DI RNAs (see Discussion).

To test if the mutations in the replicase genes of the tested mutants were stably maintained during infection, I RT-PCR amplified, cloned and sequenced the replicase genes from total RNA extracts derived either from the first (for constructs P_{640}^-A, P_{652}^-A, R_{637}^-A, R_{649}^-A and R_{649}^-K) or the second passage (R_{643}^+R, R_{646}^-A, R_{643}^-A and R_{643}^-K) as described earlier (Panaviene et al, 2003, chapter 2). I found that the site-specific mutations were stably maintained in the replicase genes of all these mutants (not shown). This indicates that the mutated replicase proteins were present in protoplasts at the end of the experiments.

**Mutations within the RPR motif of p33 affect RNA recombination**

Due to the overlapping expression strategy of p33/p92 genes in tombusviruses, the RPR motif is present in both replicase proteins. Therefore, it is possible that the observed differences among the RPR motif mutants in supporting formation of DI RNAs were influenced by mutations present in both replicase proteins. To test the involvement of these proteins in RNA
recombination separately, I have developed a three-component, complementation-based system, based on expression of the p33 protein from a DI RNA (DI-p33, Fig. 7A, Oster et al., 1998; Panaviene et al., 2003) and the p92 from the mutated gCNV RNA that carries a tyrosine mutation eliminating the p33 stop codon (construct gCNV-Y, Figure 3.7A, Panaviene et al., 2003, chapter 2). The third component was DI-333, which can be replicated in trans by p33 and p92 expressed separately from DI-p33 and gCNV-Y (not shown).

To test the role of the RPR motif mutations in p33 on RNA recombination, I constructed four DI-p33 mutants, namely P_{640}-A, P_{640}-Y, P_{652}-A, and P_{652}-Y, which can express the p33 protein with the given RPR motif mutation. I co-electroporated N. benthamiana protoplasts with one of the DI-p33 mutants, the unmodified gCNV-Y and DI-333, followed by passaging the total RNA extracts obtained after 24 hours incubation to new batch of protoplasts as described in the Materials and Methods. Total RNA extracts from the samples of the first passage were analyzed by Northern blotting to detect putative DI-333-derived recombinants (Figure 3.7B). The control three-component system that included the wt p33 (expressed from WT DI-p33, Figure 3.7B) supported formation of DI RNA recombinants efficiently (85% of samples contained recombinants), demonstrating that the three-component system is suitable for recombination studies. Similar experiments, which included one of the mutated DI-p33, instead of the wt DI-p33, in the three-component system, gave lower frequency level of DI recombinant formation: 45% for P_{640}-A, 31% for P_{640}-Y, 47% for P_{652}-A, and 29% for P_{652}-Y (Figure 3.7B). Moreover, the relative levels of DI recombinants were significantly lower than those observed in case of the wt p33 (Figure 3.7B). This data suggests that mutations within the RPR motif in p33 can affect RNA recombination (see Discussion).

**Mutations within the RPR motif of p92 have no major effect on RNA recombination**

To test the role of the RPR motif mutations in p92 on RNA recombination, I constructed four gCNV-Y mutants, namely P_{640}-A, P_{640}-Y, P_{652}-A, and P_{652}-Y, which can express the p92Y protein with the given RPR motif mutation, and used them in the three-component RNA recombination assay described above (the other components were wt DI-p33 and DI-333). Interestingly, all four gCNV-Y mutants supported DI recombination as efficiently as the wt gCNV-Y did in protoplasts when tested after the first passage (Figure 3.7C). The relative amounts of recombinant versus parental DI-333 were also comparable for these gCNV-Y
mutants and the wt gCNV-Y (Figure 3.7B). Based on these results, I propose that the RPR motif in p92 (at least the particular mutations tested) might have lesser effect on RNA recombination than the corresponding mutations in p33.

**Discussion**

**The RPR motif in the p33 replicase protein is involved in RNA recombination**

The most popular models of viral RNA recombination predict a close relationship between replication and recombination. This is because recombination is thought to be an error made by the viral replicase during the replication process. Therefore, it is possible that replication and recombination might share the same viral replicase complex and the viral RNA templates, although the final RNA products generated by replication and recombination could be different. Indeed, there is a unique template switching step performed by the viral replicase during recombination that is not needed (based on current models) during replication. The template switching is proposed to occur from the donor to the acceptor RNAs during complementary RNA synthesis on the donor template (or from the donor site to the acceptor sites if they are on the same RNA molecule). Accordingly, *in vitro* experiments with the Q phage (Biebricher and Luce, 1992), *poliovirus* (Arnold et al., 1999; Tang et al., 1997), *Brome mosaic virus* (BMV) (Dzianott et al., 2001; Kim and Kao, 2001), *Bovine viral diarrhea virus* (Kim and Kao, 2001), *Turnip crinkle virus* (TCV) (Nagy et al., 1998) and CNV RdRps (Cheng and Nagy, 2003) demonstrated that template switching by these enzymes is possible and it occurs at a high frequency. If template switching also takes place *in vivo*, then mutations in the viral replicase proteins should affect not only replication, but recombination as well in *in vivo* experiments. Indeed, the data obtained in this work demonstrates that several mutations within the RPR motif of p33/p92 replicase proteins affected RNA recombination. For example, a group of p33/p92 mutants of CNV that carried alterations of either or both prolines in the RPR motif (e.g., $P_{640}$, $P_{640}$, $P_{652}$, $P_{652}$ and $P_{640}/P_{652}$), supported recombination at reduced efficiency (frequency) when compared to wt (Figure 3.5B). On the contrary, two of the arginine mutants (e.g., $R_{646}$ and $R_{643}$, Figure 3.5C) supported recombinant formation more efficiently than wt did. The observed differences among the RPR motif mutants and wt are likely due to altered efficiency in recombinant formation (frequency of recombination) driven by the mutated
replicase proteins. This is because these RPR motif mutants supported replication of selected recombinants at relative levels (when compared to the level of replication of the parental DI RNA) similar to that observed with wt CNV helper virus in a template competition experiment in protoplasts (Figure 3.6). In other words, the above template competition experiments suggest that, after the RNA recombination step, a particular recombinant (such as the recombinants shown in Figure 3.6) is predicted to have comparable chance to out compete the parental DI RNA in both the mutant and wt p33/p92 background in protoplasts. Therefore, I suggest that the p33/p92 replicase proteins of CNV are directly involved in recombination. The direct involvement of the replicase proteins in recombination is also supported by in vitro data that demonstrated the ability of a partially-purified CNV RdRp preparation to support recombinant formation (Cheng and Nagy, 2003).

Interestingly, previous works with the BMV (which belongs to a different supergroup than Tombusviruses) 1a and 2a proteins also suggested the role of the viral replicase proteins in recombination (Figlerowicz et al., 1997; Figlerowicz et al., 1998; Nagy et al., 1995). Also, the polymerase acidic protein subunit, a possible elongation factor, of the RdRp of influenza A virus has been proposed to affect DI RNA formation (Fodor et al., 2003). These findings are in agreement with the template switching based recombination models.

Development of a three-component complementation-based recombination system that contained tombusvirus RNAs expressing p33 and p92 proteins separately allowed us to study the role of the RPR motif mutations in p33 and p92 independently. I found that selected RPR motif mutations, which affected recombination frequency in the two-component system where both p33 and p92 were simultaneously mutated, were capable of affecting RNA recombination when present in p33 alone. On the contrary, their effects were less obvious on DI RNA recombination when present in p92. This data indicates that the RPR motif in p33 is involved in recombinant formation. This further supports the model that RNA replication and RNA recombination might depend on similar factors and elements (also, see below the discussion on the role of the RNA template). The actual function of p33 in tombusvirus replication/recombination will need further studies.
Is there a relationship between recombination, replication or subgenomic RNA synthesis?

Comparison of the abilities of the RPR motif mutants to support replication and recombination revealed the lack of correlation between the extent of replication and the efficiency of recombination. For example, mutants $R_{646}$-A and $R_{643}$+R supported replication poorly (Table 3.2), but they showed accelerated rate of recombination (Figure 3.5C). On the other hand, mutants $P_{640}$-A, $P_{640}$-Y, $P_{652}$-A, and $P_{652}$-Y, which supported recombination inefficiently (Figure 3.5B), were capable of supporting replication at levels (58-87%) similar to that obtained with mutants $R_{637}$-K, $R_{637}$-Q, $P_{640}$-$652$-G, and $R_{637}$-A/ $R_{649}$-H, etc. (Table 3.2), which could support ~wt-level of recombinants. The difference in the effect of particular mutant on the efficiency of replication and recombination suggests that the RPR motif in p33 (and possibly in p92) plays different functions during replication and template switching. Similar observation was also made for BMV 2a mutants that affected the levels of replication and recombination differently (Figlerowicz et al., 1997; Figlerowicz et al., 1998). These observations are not surprising in that sense that protein factors and RNA elements (see below), which are involved in replication and recombination, are likely utilized somewhat differently by the replicase proteins.

One of the surprising observations of this work is that the two RPR motif mutants, namely $R_{646}$-A and $R_{643}$+R (Figure 3.5C), which accelerated RNA recombination, also increased the relative amount of subgenomic RNAs (by ~2-20-fold when the levels of subgenomic RNAs were compared to the level of genomic RNA) in protoplast (Panaviene et al., 2003, chapter 2). This observation suggests that there might be a common step in RNA recombination and subgenomic RNA synthesis (such as termination of RNA synthesis?), which is stimulated by these RPR motif mutants. Future experiments will address this possibility.

The role of cis-acting elements in tombusvirus recombination

Based on in vitro studies with the partially purified CNV RdRp, we predicted that the RIII sequence, which has been shown to be a strong replication enhancer in vitro (Panavas and Nagy, 2003a) and in vivo (Ray and White, 1999a; Ray and White, 2003a), should be active in promoting RNA recombination. Accordingly, I found that DI RNAs carrying two or three copies of RIII recombined rapidly in protoplasts. Although the recombination junctions were mainly imprecise, they clustered within the RIII sequence, suggesting that RIII is a recombination hot spot sequence. I show similar data for RII (construct DI-2233 and DI-2323 in Figure 3.3C-D),
which is a putative cis-acting element (albeit its function is currently unknown, (Shapka and Nagy, 2004) ) that can bind to the replicase proteins in vitro. It has been shown previously that insertions of short sequences (~110-150 nt) derived from GFP or barstar genes into DI RNA did not promote recombination (Shapka and Nagy, 2004) under the same conditions used in this work.

Interestingly, multiple copies of regions II and III elements have not increased the replication potential of the prototypical DI RNA. Instead, I observed (i) a moderate level of interference with replication (Figure 3.1); and (ii) reduced competition against the recombinant DI RNAs (Figure 3.6). The interference might take place during plus-strand synthesis since the amounts of plus-strands for DI RNAs with multiple copies of regions II and III decreased significantly more (at the 24 hours time point) than the amount of minus-strands for all four constructs. Note that, similar to our observation, Ray and White (1999) also observed the lack of additional stimulation of DI RNA replication due to the duplicated RIII replication enhancer sequence in the presence of the TBSV helper virus. Also, a duplicated copy of the motif1 replication enhancer of satC, a satellite (chimeric) RNA associated with TCV infections (Nagy et al., 1999; Nagy et al., 2001) did not further enhance the replication of satC RNA when compared to satC carrying a single motif1 replication enhancer. It is possible that one copy of the replication enhancer is optimal for replication and additional copies might only disturb the replication process, thus leading to replicase errors, such as RNA recombination.

In summary, this is the first report on the role of a tombusvirus replicase protein (namely p33) in RNA recombination. This and a previous work (Panaviene et al., 2003, chapter 2) firmly establish that the RNA-binding domains in the replicase proteins are involved in RNA replication, subgenomic RNA synthesis and RNA recombination. Additional structure/function studies will be helpful in understanding the mechanism of these processes that are vital for tombusvirus infection and evolution.

Table 3.1: List of primers used for site-directed mutagenesis of CNV (pK2M5)

<table>
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<tr>
<th>Mutant</th>
<th>Primer pair</th>
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<th>Position&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> The mutated sequences are underlined.

<sup>b</sup> The nucleotide positions within the p33/p92 ORF of CNV are indicated (Rochon and Johnston, 1991).
Table 3.2: The level of accumulation of DI RNAs in the presence of gCNV carrying mutations in the RPR motif

<table>
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<th>Mutants</th>
<th>RPR motif</th>
<th>DI-RNA replication (%)</th>
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A  TBSV RNA

B (+) strand

C (-) strand

Replication (%)
Figure 3.1: Replication of DI RNAs carrying duplicated copies of known and putative cis-acting elements in protoplasts. (A) Schematic representation of TBSV genomic and DI RNAs used in these experiments. The genomic RNA is shown at the top with five genes (indicated with white and black boxes), which include the p33 and p92 replicase proteins (they overlap in sequence within the p33 gene). Note that CNV has similar replicase genes to those of TBSV. The prototypical DI RNA, DI-72, contains four conserved segments derived from the TBSV genomic RNA, termed RI to RIV, shown as grey boxes. The shown DI RNA constructs carry one or two copies of the 82 nt RIII, a known replication enhancer. In addition, DI-2323 and DI-2233 also contain two copies of the 239 nt RII sequence (as shown, framed boxes represent inserted copies of RII and/or RIII), which is a putative cis-acting replication element. Note that TBSV DI RNAs can only replicate in the presence of a helper virus, such as TBSV or the closely related CNV (not shown). (B) Accumulation of (+)-strands of DI-72 RNA and its derivatives in N. benthamiana protoplast in the presence CNV helper RNA. DI RNAs (1μg) were co-electroporated with CNV RNA (2μg) into protoplasts (5 ¥ 10⁵ cells per experiment). The names of the DI RNA constructs are shown on the top. Samples were taken 24 (top) and 48 (bottom) hours after electroporation. DI RNAs were detected using 32P-labeled RNA specific for RI(+). Ethidium bromide-stained ribosomal RNA represents loading control. The relative accumulation of DI RNAs, compared with DI-72 RNA, is shown based on quantification of Northern blots with a phosphorimager. The data was adjusted according to loading control. Gray bars represent samples after 24h and black bars represent samples taken after 48h. The experiments were repeated four times. (C) Accumulation of (-) strands of DI-72 DI-RNA and its derivatives in protoplasts. The same total RNA samples were analyzed as in panel B. The total RNA was electrophorized in 4% denaturing acrylamide gel before transfer to the membrane. The labeled RNA probe was specific for RI(-). See other details in panel B.
Figure 3.2: Generation of DI RNA recombinants in *N. benthamiana* protoplasts. (A) Northern blot analysis of DI RNAs accumulating in protoplasts that were co-electroporated with the shown DI RNAs and gCNV helper as described in Fig. 1B. Note that the DI RNA transcripts (1\(\mu\)g) were gel isolated prior to electroporation into zero passage (primary-infected) protoplasts. Comparable amounts of the total RNA extracts from the zero passage protoplasts were used for electroporation to a new batch of protoplasts (first passage), and so on. Names of DI RNAs are shown on the top. An arrow points to the input-sized DI RNA (also marked with an asterisk since the sizes are variable based on the DI constructs), while the recombinant-like DI RNAs are bracketed. (B) Agarose gel analysis of RT-PCR products (using primers specific for the 5’ and 3’ ends of DI-72 RNA and its derivatives) obtained from total RNA (first or third passage as shown). The RT-PCR products obtained with four independent samples are marked as R1 to R4. Letter C (control) represents the RT-PCR products obtained using the gel purified *in vitro* transcripts of the corresponding DI RNAs. Note that, in case of DI-2233 and DI-2323, the RT-PCR resulted in recombinant products, which were precise (homologous) based on sequence determination (not shown). These artifactual products were excluded from analysis (Figure 3.3).
Figure 3.3: Distribution of recombination sites in DI RNAs. (A-D) The sequences around the recombination junctions are shown in plus-strand orientation with 5’ sequences on the left. Various regions of the parental DI RNAs (see Figure 3.1A for the schematic representation of the full-length constructs) are shown on the top, while the marker mutations between the regions are underlined. The sequences above and below the asterisk represent the parental and the recombinant sequences, respectively. Nucleotides that are deleted in the recombinants are indicated with blank spaces corresponding to the parental sequence. Not shown sequences are indicated with //, and their length is specified in parenthesis. The frequency (number of recombinant/total number of sequenced recombinants) is shown on the right. Note that due to sequence identity in the duplicated regions, the actual recombination sites cannot be determined in the precise recombinants (the crossover region is boxed).
Figure 3.4: Effect of mutations in the RPR motif on the accumulation of DI-333 RNA in protoplasts. (A) Schematic representation of the location of the RPR motif (a grey box) in gCNV and in the p33 and p92 proteins. The actual amino acids and their positions in the p33/p92 gene are specified. (B) Northern blot analysis of total RNA extracts obtained with DI-333 in the presence of either wild type gCNV or one of the RPR motif mutants in protoplasts. Further details are as shown in the legend to Figure 3.1.
A  WT-like

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<td>9/10</td>
<td>10/11</td>
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B  “Slow”

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C  “Fast”

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Figure 3.5: Effect of RPR motif mutations on recombination frequency of DI RNAs. (A-B) Northern blot analysis of total RNA extracts (first passage) obtained from protoplasts that contained DI-333 and either wt gCNV or one of the RPR motif mutants. Protoplasts (zero passage) were co-electroporated with 1μg of gCNV (carrying wt or mutated RPR motif) and 0.15 μg of gel purified DI-333 transcript. For “passaging” the viral RNA, protoplasts were co-electroporated with the same amounts (5μl each) of total RNA extracts obtained from zero passage protoplast 48h after electroporation. The frequency of recombinant DI RNA accumulation (number of recombinants/total number of first passage protoplast samples) is shown on the bottom. Further details are as shown in the legend to Figure 3.1. (C) RPR motif mutants that increased recombination frequency in the initial tests (performed with DI-333, not shown) were tested in the presence of DI-2323 that recombines inefficiently (Figure 3.2). Protoplasts (zero passage) were co-electroporated with 0.4μg of wt gCNV and 1μg of gel purified in vitro transcript of DI-2323 RNA or 5μg of the given RPR motif mutant RNA and 2μg of DI-2323 RNA, respectively. The higher amounts of RNA were used for the mutants because of the low replication level of DI RNAs when these mutants were used as helpers (Table 3.1, and Panaviene et al., 2003, chapter 2). For “passaging” the viral RNA, protoplasts were co-electroporated with the same amounts (5μl each) of total RNA extracts obtained from zero passage protoplast 48h after electroporation, plus additional 0.4 μg wt or 5 μg of the given RPR motif mutant gCNV transcripts. Total RNA extract after the second passage was analyzed by Northern blot as described in panel A. The bottom panel includes an additional control, mutant R_{643}-A, which supports wt level recombination with both DI-2323 and with DI-333 (see panel B). Note that the amount and conditions for R_{643}-A was the same as for R_{646}-A.
Figure 3.6: Similar levels of competitiveness of recombinant DI RNAs for replication in mutant and wt p33/p92 backgrounds in vivo. (A) Replication of two of the cloned recombinant DI RNAs (recombinant #13, Figure 3.3B and recombinant #6, Figure 3.3B) were tested in the presence of DI-333 and wt or mutated gCNV (as shown). Protoplasts were co-electroporated with 1μg of wt or mutated gCNV, 0.5μg of recombinant DI RNA transcripts and 1μg of DI-333 RNA transcripts. Total RNA extracts (48h after electroporation) were analyzed by Northern blot as shown in Figure 3.1. The input- and recombinant-sized RNAs are depicted with arrowheads. (B). The relative accumulation level of the two recombinant DI RNAs (compared to the level of DI-333 RNA in each sample) in the presence of wt or mutated CNV, as shown. Light grey and dark grey bars represent the data obtained with recombinant #13 and #6 DI RNA (Figure 3.3D), respectively. Experiments were repeated four times. (C) Left and right panels: Competition experiments between recombinant #29 (Figure 3.3D) and DI-2323 RNA in the wt or P646-A gCNV background; and between recombinant #12 (Fig. 3D) and DI-2323 RNA in the wt or P643+R gCNV background, respectively. Relative accumulation levels of recombinant DI RNAs (recombinant/DI-2323) are shown at the bottom. Experiments were repeated four times.
Figure 3.7: Characterization of the role of the RPR motifs in p33 and p92 proteins in DI RNA recombination. (A) Schematic representation of the complementation-based, three-component system. The p33 stop codon in gCNV has been changed to tyrosine to express only the p92 protein from gCNV-Y. The p33 protein was expressed from a translation competent DI RNA, termed DI-p33. The RPR motif is shown as a grey box in both constructs. Note that the RPR motif mutations were introduced separately into either gCNV-Y or DI-p33. The third component is DI-333 (Figure 3.1A), which is expected to support recombination, as shown in Fig. 2. (B) Northern blot analysis of total RNA extracts obtained from the first passage with RPR motif mutations present in p33 only (as indicated). Protoplasts were co-electroporated with 5µg gCNV-Y, 2µg wt or RPR motif mutants of DI-p33 and 1µg of gel purified DI-333 RNA. The total RNA (5 µl, obtained 24h after electroporation of protoplasts) was used for the passage in addition to 5µg of gCNV-Y and 2µg of wt or RPR motif mutant DI-p33 (the extra amount was needed since gCNV-Y and the mutated DI-p33 replicated inefficiently, but they were needed for DI-333 replication/recombination to supply the replicase proteins in trans). See further details in the legend to Figure 3.5. (C) These experiments were performed with the p92 RPR motif mutants using the conditions described in panel B.
Chapter Four

Purification of the *Cucumber necrosis virus* replicase from yeast: The role of co-expressed viral RNA in stimulation of replicase activity

Introduction

Plus-stranded RNA viruses, which constitute the largest group among plant and animal viruses, replicate in the infected cells by using the viral replicase complex. The replicase complex consists of viral-coded proteins, such as the RNA-dependent RNA polymerase (RdRp), auxiliary proteins and possibly host-derived proteins and the RNA template (Ahlquist et al., 2003; Buck, 1996; Buck, 1999; Kao et al., 1999; Osman and Buck, 1997). To study the mechanism of viral RNA replication, functional replicases are purified from virus-infected hosts (Bates et al., 1995; Cheng et al., 2001; Gal-On et al., 2000; Hayes and Buck, 1990; Nagy and Pogany, 2000; Osman and Buck, 1996; Plante et al., 2000; Quadt and Jaspars, 1990; Quadt et al., 1991; Song and Simon, 1994; Watanabe et al., 1999) or from heterologous systems, including *E. coli* (Hong and Hunt, 1996; Kao et al., 1999; Lai et al., 1999; Neufeld et al., 1991a; Rajendran et al., 2002; Ranjith-Kumar et al., 2003), yeast (Quadt et al., 1995), insect (Lohmann et al., 1997; Neufeld et al., 1991; You and Padmanabhan, 1999), Xenopus (Gamarnik and Andino, 1996), and mammalian cells (Gosert et al., 2003; Neufeld et al., 1991). The advantage of the heterologous systems is that expression of the replicase proteins can be achieved without the dependence on virus replication, thus facilitating mutational analysis of the replicase genes. In summary, viral replicase systems, which are very useful to dissect the protein (trans-acting) and RNA (cis-acting) factors that control virus replication, have only been developed for a limited number of plus-stranded RNA viruses.

Tombusviruses are small plus-stranded viruses that belong to supergroup 2 viruses, such as *Hepatitis C virus*, flaviviruses and pestiviruses, based on the similarity among their RdRp sequences (O'Reilly and Kao, 1998; Russo et al., 1994). The genome of Tombusviruses codes for five genes: involved in replication (termed p33 and p92, Figure 4.1), cell-to-cell movement (p22), encapsidation (p41) and suppression of gene silencing (p19, Figure 4.1A, reviewed in (Russo et al., 1994) and in White and Nagy (in press). The two overlapping replicase genes are
essential for replication of the genomic (g)RNA in plant cells (Oster et al., 1998; Panaviene et al., 2003; Scholthof et al., 1995d). While the p92 has the RdRp signature motifs in its unique C-terminus, the function(s) of p33 is currently unknown. Mutagenesis of the RNA-binding site (an arginine-proline-rich motif, termed RPR-motif (Rajendran and Nagy, 2003)) in p33 was found to affect gRNA replication (Panaviene et al., 2003, chapter 2), subgenomic (sg)RNA synthesis and RNA recombination (Panaviene and Nagy, 2003, chapter 3), suggesting that p33 is a multifunctional protein. Similar mutations within the RPR-motif in p92 also affected RNA replication, suggesting that the overlapping (pre-readthrough) domain in p92 is essential for its function in infected cells (Panaviene et al., 2003).

Tombusviruses are popular model viruses to study virus replication due to the presence of small defective interfering (DI) RNAs, which are deletion derivatives of the viral gRNA (Figure 4.1A). These DI RNAs do not code for essential genes, allowing for convenient testing of the role of essential and/or regulatory cis-acting elements, such as promoters (Havelda and Burgyan, 1995; Panavas et al., 2002a; Panavas et al., 2002b) replication enhancers (Panavas and Nagy, 2003a; Panavas et al., 2003; Ray and White, 2003) and a replication silencer (Pogany et al., 2003).

Efficient assays based on partially-purified RdRp have been developed for TBSV and CNV, which were obtained from infected plants (Nagy and Pogany, 2000). These preparations were then used to confirm the role of cis-acting sequences and structures in TBSV DI RNA replication and recombination in vitro (Cheng and Nagy, 2003; Panavas and Nagy, 2003a; Panavas et al., 2003; Panavas et al., 2002a; Panavas et al., 2002b; Pogany et al., 2003). The disadvantage of the above preparations is that replication of Tombusviruses is necessary to obtain enough RdRp activity for biochemical characterization.

The possibilities to use tombusvirus in broader replication studies increased with development of DI RNA replication systems in yeast for TBSV and CIRV DI RNAs (Panavas and Nagy, 2003b; Pantaleo et al., 2003). For example, expression of CNV replicase proteins was achieved from separate plasmids under control of constitutive ADH1 promoter, while DI RNAs were expressed via galactose inducible (Gal 1) promoter. This study showed that in the presence of replicase proteins TBSV DI RNA replicated in yeast cells (Panavas and Nagy, 2003b). The replication of DI RNA was confirmed by: 1) detection of (-) strand DI RNA, 2) accumulation of (+) strand DI RNA over period of time, 3) generation of the
5' and 3' ends that are found in DI RNA replicating in plants and protoplasts, 4) dependence of DI 72 replication on the presence of cis acting element (RIII (-) enhancer for TBSV DI RNA; 5) generation of dimer RNAs that suggests possible recombination events during DI RNA replication in yeast, 6) recovery of CNV replicase activity from yeast cells, which replicated DI RNA (Panavas and Nagy, 2003b).

In addition to studies of DI RNA replication in vivo, development of a heterologous system to express and purify highly active recombinant tombusvirus replicase would be useful to define the role of the replication proteins and RNA templates in replication.

To obtain such a system, in this study, I used yeast cells, which co-expressed p33 and p92, as well as the DI RNA template from plasmids (modified system described in Panavas and Nagy, 2003b). Characterization of the obtained CNV replicase preparation from yeast revealed that it had similar properties when compared with the plant-derived preparation. The yeast-derived preparation was capable of de novo initiation on exogenous plus- and minus-stranded DI RNA templates.

The previous studies of replicase activity in vitro suggested that there were differences in viral components required for replicase activity in vitro. It was reported that the RdRp of several viruses, including Turnip crinkle virus, Tobacco etch virus, Bamboo mosaic virus, Hepatitis C virus, Bovine viral diarrhea virus (Hong and Hunt, 1996; Kao et al., 1999; Kao et al., 2001; Lai et al., 1999; Lohmann et al., 1997; Rajendran et al., 2002; Ranjith-Kumar et al., 2003), are active when expressed without other viral-coded auxiliary proteins. On the contrary, RdRps for several other viruses, such as Brome mosaic virus (BMV) and Alfalfa mosaic virus (AMV), required the presence of several factors, such as the RdRp, a viral auxiliary protein and the viral RNA in order to be functional in vitro (Quadt et al., 1995; Vlot et al., 2001). It was established that 3’UTR and intracistronic region in RNA3 are required for active BMV replicase formation. The study showed that there was correlation between amount of (-) strand RNA3 and purified BMV replicase activity in vitro. The similar requirements for 3’UTR were reported for AMV replicase activity in vitro.

My experiments with CNV replicase purified from yeast showed that the generation of highly active replicase in yeast required the co-expression of a DI RNA template together with the essential p33 and p92 replicase proteins. The more detailed studies of DI RNA elements
revealed that strong hairpin in RII (RIISL) and ~80 3’nt of RIV were required for active CNV replicase formation in yeast cells.

Materials and methods

Construction of expression plasmids in yeast

To express the CNV p92 gene with an N-terminal 6xHis-tag, we generated pGAD-His92(Figure 4.1B). This was done by: (i) eliminating the HindIII site in pGADT7 (position 2280, BD Biosciences) by partial digestion with HindIII, blunting the ends with Klenow polymerase and religation. This modification yielded pGAD(H-) vector. (ii) The CNV p92 gene was amplified by PCR using primers #424 (5’-CGACGGATCCGATACCATCAAGAGGATGCTGTG) and #952 (5’-CCCGCTCGAGTCATGCTACGGCGGAGTCAAGGA) from p92Y, in which stop codon of the p33 gene was replaced with a tyrosine codon (Oster et al., 1998; Scholthof et al., 1995d). The obtained 2.5 kb PCR fragment was digested with BamHI and XhoI. (iii) In addition, we generated the 116 bp HindIII–BamHI fragment encoding a 6x His tag at the 5’ end (the actual sequence is: 5’-CTGGTTGACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGGTACCCGGA TCC) from pYES2/NT-C vector (Invitrogen). (iv) Finally, the 2.5 kb and the 116 bp fragments were cloned simultaneously into HindIII-XhoI digested pGAD(H-) vector to generate pGAD-His92.

For the construction of pGBK-His33 (Figure 4.1B) to express the CNV p33 gene with an N-terminal 6xHis-tag, we gel isolated the 1.5 kb HindIII (position 6544) – HindIII (position 738) fragment from HindIII digested pGBK7 plasmid (BD Biosciences). The CNV p33 sequence was amplified with PCR using primers #424 and #992B (5’-GAGCTGCAGCTATTTCACACCAAGGGA). The obtained 0.9 kb PCR fragment was digested with BamHI and PstI and gel isolated. In addition, the 116 bp HindIII-BamHI encoding a 6xHis tag fragment was obtained as described above. The generated 1.5 kb, 0.9 kb and 116 bp fragments were ligated together, followed by re-amplification of the ligated product with PCR using primers #953 (5’-GATCCTTTTTGTTTGGTACCAATA) and #992B. The
obtained PCR product was digested with Bsp1407I and PstI and inserted into Bsp1407I and PstI digested pGBK7 vector.

To modify the original RNA transcription vector (Panavas and Nagy, 2003) that produced yDI-72 RNA with a ~120 nucleotide 5' leader, which is plasmid-borne nonviral sequence, we constructed a new expression vector based on the high copy number pYES2/NT-A plasmid carrying a URA3 selectable marker (Invitrogen) and the GAL1 promoter. First, pYC2/CT plasmid (Invitrogen), which was originally used to express yDI-72 RNA in yeast (Panavas and Nagy, 2003), was modified to remove most of the plasmid-borne leader sequence, leaving only 4 bp between the start site of transcription and a HindIII site in pYC2/CTm. This was done using primers #1080 5’CAGGCAAGCGATCCGTCGCCGCGGAACGT) and #1145 (5’-CCCGAAGCTTACTTTTAATTACATTTGAATAAGTAAT) in a PCR reaction in the presence of pYC2/CT template. The obtained PCR product was digested with NaeI and HindIII enzymes, gel purified and ligated into pYC2/CT between NaeI (position 4479) and HindIII (position 501) sites, resulting in the pYC2/CTm plasmid. After the above modification of the expression vector, we inserted DI-72 sequence in order to facilitate the expression of plus and minus-stranded DI-72 RNA. This was made by a two-step PCR reaction. First, I used primers #542 (5’-GCCCGAAGCTTGGAAATTCTCCAGGATTTC) and #157 (5’-GGGCTGCATTTCTCAATGTTTC) for plus-stranded DI-72, and primers #719 (5’-GCCCGAAGCTTGGGCTGCATTTCTCAATGTTTC) and #20 (5’-GGAAATTCTCCAGGATTTC) for minus-stranded DI-72 in the presence of DI-72SXP template (White and Morris, 1994b). The obtained PCR products were separately gel isolated and ligated with gel isolated PCR products that included the ribozyme sequence from the minus-stranded satellite of Tobacco ringspot virus (TRSV) (Buzayan et al., 1988; Buzayan et al., 1986) obtained with primers #1067 (5’-AGTCTCGTTTCTTGGCACAACAGAGAGGGCACCAGAGAAA) and #1068 (5’-GGTAATATACCAACACGTTGTGTGTCTCTGTTGCCCCTTCTC). The ligation products were PCR amplified with primers #542 and #1069 (5’-CCGGTGCAGCTCTACCAGGTAATATACCACAACGTTGTGT) for plus-stranded DI-72, and with #719 and #1069 for minus-stranded DI-72. The obtained PCR products were digested with HindIII and ScaI enzymes, gel isolated and ligated into the similarly treated pYC2/Ctm
plasmid. Finally, to transfer the cloned plus-and minus-stranded DI-72 sequences to the high copy plasmid pYES/NT-A, I treated the above plasmids with *NgoMIV* and *EcoRI* and then the gel isolated fragments were inserted into *NgoMIV* and *EcoRI*-treated pYES/NT-A plasmid. The obtained plasmids were termed as pYES-DI-72(+)Rz (Fig. 1B) and pYES-DI-72(-)Rz.

DI-72 region deletion derivatives were obtained using PCR with following primer pairs and templates: for RII/IV primers #709 (5'- GCCGAAGCTT AGAAACGGAAGGCTCGC) and #157 with template DI-72 XPXN [RIII; for RI/II/III primers #542 and #454A (5'- GACCCAACAAGGATACCTGTACCTATGCTATGC) with template DI-72 SXP; for RI/II/IV and RI/III/IV primers #542 and #157 with DI-72 XPXN [RIII and DI-72 XPXN [RII, respectively; for RI/III/IV primers #709 and #157 with template DI-72SXP. The PCR products were gel isolated, ligated to ribozyme as described above, and secondary PCR was performed using forward primers #542 (RI/II/III, RI/II/IV, RI/III/IV) and #709 (RI/II/III, RI/IV) with reverse primer #1069 using as templates ligation products. The obtained PCR products were cloned into pYES/NT-A, using the same procedure as described for pYES-DI-72(+)Rz.

Construction of RII/IV deletion mutants are described below. RII/gPR was made first amplifying RII by PCR with primers #709 and #1314 (5'- CCCGGCATGCTTTCTGCTTTTACGAAGTAG) and gPR with ribozyme sequence using primers #1317 (5'- CCCGGCATGCCATTGCAGAAATGCAGCCC) and #1069 (template was RII/IV construct), followed by *SphI* digestion, gel isolation, ligation and 2nd PCR with primers #709 and #1069. The obtained PCR product was digested with *HindIII* and *SacI*, gel isolated and ligated into similarly treated pYES-DI-72 (+ Rz that was used as the vector. RII/RIV[ss4] was made ligating RII PCR product as for RII/gPR construct and PCR product obtained with primers #1318 (5'- CCCGGCATGCGACACGTTGATCTCACCCCTC) and #1069 (template RII/IV), digested with *SphI*, followed by cloning steps used for RII/gPR construction. The first step in RII[ss]SL/RIV construction was making two PCR products: (1)- primers #709 and #1315 (5'- CCCGGGATCCGGTTTCTGTAACGCGGAAG); (2)- primers #1316 (5'- CCCGGGATCCGGAGAGTCTGATATACAC) and #1314 using as template RII/RIV construct. These PCR products were digested with *BamHI*, gel isolated, ligated and used in the
2nd PCR reaction with primers #709 and #1314. The RIV with ribozyme sequence was amplified using primer #1309 (5’- CCCGGCATGCATTCTGTTTACGAAAGTTAGG) and #1069 from RII/IV construct. Then both PCR products were digested with SphI and following cloning steps were the same as for RII/gPR. To make RIIISL/RIV/ss4 construct, first I amplified from RII/IV construct RIISL sequence by PCR using primers #709 and #1308 (5’- CCCGGCATGCAGCTGCTTCCAC) and digested PCR product with SphI enzymes, gel isolated and ligated into RII/RIV/ss4 replacing RII sequence in this plasmid. For construction of RII/RIV∆SL3 we did PCR using template RII/IV and primers #709 and #1319 (5’- CCCGGCATGCATCTGCTGGTCCGCTTCCAC) for 5’ fragment and #1320 (5’- CCCGGCATGCAAGCACTACCGGACAACCGG) and #1069 for 3’ fragment. The PCR products were digested with SphI, gel purified, ligated and amplified with primers #709 and #1069 and cloned into pYES/NT-A as described above.

\textbf{Yeast transformation and growth}

\textit{S. cerevisiae} strain INVSc1 (Invitrogen) was used in these studies (Panavas and Nagy, 2003b). The yeast cells were co-transformed with all three plasmids (i.e., pGAD-His92, pGBK-His33 and pYES-DI-72(+)Rz) using the LiAc/ssDNA/PEG method (Panavas and Nagy, 2003b). In control experiments, we replaced one of the three plasmids with the original plasmid lacking viral sequences. After transformation, yeast cells were plated on selective SC medium without uracil, leucine and tryptophan (SC-ULT []).

For CNV replicase studies, yeast was grown in SC-ULT [] medium containing 2% galactose for 24 h at 30°C. Then the cultures were diluted 10-fold with fresh SC-ULT [] medium with 2% galactose and grown under the same condition until 0.6-0.7 OD_{600} (approximately 24 h). Yeast cells were then harvested by centrifugation at 1100g for 5 min, followed by washing the pellet with 20mM Tris- HCl, pH 8.0 and centrifugation (same as above). The pelleted cells were re-suspended in a small amount (~1-2ml) of fresh buffer (20mM Tris-HCl, pH 8.0), aliquoted,
followed by centrifugation at 21,000g for 1 min and storage of the pellet at –80°C until further use.

For obtaining the enriched membrane fraction, yeast was grown at 30°C in SC-ULT with 2% glucose for 24 h, followed by dilution to 0.1 OD₆₀₀ in SC-ULT with 2% galactose. After further incubation at 23°C, yeast samples were taken at time points described in legend to Figure 4.2.

**Purification of the CNV replicase from S. cerevisiae cells co-expressing the replicase proteins and DI RNA**

Frozen yeast cells were homogenized in liquid nitrogen by grinding in a mortar, followed by transferring the obtained powder to a tube containing the extraction buffer (200mM sorbitol, 50mM Tris-HCl, pH7.5, 15mM MgCl₂, 10mM KCl, 10mM β-mercaptoethanol, yeast protease inhibitor mix, Sigma), which was applied in 10-fold excess over the fresh weight of yeast cells. Unbroken cells were removed by centrifugation at 100g for 1.5 min at 4°C. Supernatant was then transferred to a new tube and the enriched membrane fraction was pelleted via centrifugation at 21,000g for 15 min at 4°C. The obtained fraction was considered “enriched membrane fraction”, which was re-suspended in the extraction buffer and used in the in vitro replicase reactions as described below.

To further purify the CNV replicase preparation, the enriched membrane fraction was re-suspended in the extraction buffer containing 1.2M NaCl, followed by gentle rotation for 20 min at 4°C and centrifugation at 21,000g for 15 min at 4°C. The obtained pellet was re-suspended in the extraction buffer containing 1% Triton X-100 and 5% SB3-10 (caprylyl sulfobetaine) (Sigma) by gentle rotation for 1h at 4 °C, followed by centrifugation at 21,000g for 15 min at 4°C. The obtained supernatant was considered the “solubilized membrane fraction”. This preparation was tested in the in vitro replicase assay as described below.

To prepare the samples for the His-tag-based metal-affinity purification, I solubilized the enriched membrane fraction in the solubilization buffer (extraction buffer plus 1% Triton X-100, 5% SB3-10 and 0.5M KCl) as described above. After centrifugation, the supernatant was applied to a column containing ProBond resin (Invitrogen) equilibrated with the solubilization buffer. Then the column was rotated for 1 h, followed by washing with two column volumes of the solubilization buffer, followed by washing with the extraction buffer containing 1% Triton X-
100, 5% SB3-10, then a second wash with the extraction buffer containing 1% Triton-100, 5% SB3-10 and 2mM imidazole. The recombinant proteins were recovered from the column in the extraction buffer containing 150mM imidazole, 1% SB3-10 and 0.1% Triton X-100 in two-step elution (each in a half column volume). The purity of the obtained recombinant protein-containing preparations was tested with SDS-PAGE (Sambrook et al., 1989), while the amount of the recombinant proteins in various samples was compared by using Western blotting with monoclonal anti-His-tag antibody (Amersham).

**In vitro CNV replicase assay**

To test the activity of various preparations, I performed two types of assays. One tested the endogenous template activity present within the CNV replicase preparation, whereas the other assay examined the ability of the CNV replicase preparation to accept exogenous templates. The only difference between the two assays is whether extra templates were added to the reaction or not.

I used 0.5 µg RNA templates for the CNV replicase reactions. The reactions contained either 25 µl of recombinant or 25 µl of plant – derived CNV replicase as described previously (Nagy and Pogany, 2000). Briefly, RdRp reactions were done in the buffer containing 50mM Tris-HCl, pH8.2, 15mM MgCl₂, 10mM dithiothreitol, 20mM potassium glutamate, 1.0mM each ATP, GTP, CTP and 0.01mM UTP, 0.3µl [α⁻³²P] UTP, 4.8 U RNAse inhibitor (Pharmacia). Samples were kept at 25°C for 2h. After phenol : chlorophorm extraction RdRp products were precipitated with ammonium-acetated - isopropanol mixture and washed with 70% ethanol. Following heating with formamide at 85°C for 5 min. half of the RdRp products were analyzed under denaturing conditions (i. e., 5% PAGE containing 8M urea) (Nagy and Pogany, 2000).

**Preparation of protoplast membrane fraction for in vitro RdRp assay**

*Nicotiana benthamiana* protoplasts were prepared and electroporated as described (Panaviene et al., 2003, chapter 2). The enriched membrane fraction and the solubilized membrane fraction were prepared from pelleted protoplast cells using the method described earlier (Nagy and Pogany, 2000): to each protoplasts sample was added 500 µl of buffer A (50mM Tris-HCl, pH 8.0, 15mM MgCl₂, 10mM KCl, 2mM EDTA, 20% (v/v) glycerol, 10mM D-mercaptoethanol and 1x protease inhibitor (Sigma). Samples were shaken at 4°C for 10min,
and spun down at 1000 rpm for 1.5 min. Supernatant was transferred to the fresh eppendorf tubes and spun at 21,000g, for 10 min at 4°C. The pelleted membrane fraction was resuspended in 100 µl of buffer B (50mM Tris-HCl, pH 8.0, 10mM MgCl₂, 1mM EDTA, 6% (v/v) glycerol, 10mM D-mercaptoethanol and 1x protease inhibitor).

**Preparation of RNA transcripts**

RNA transcripts for the replicase assays and protoplasts experiments were prepared using standard *in vitro* T7 transcription reaction with various DNA templates as described (Panaviene et al., 2003). The DNA templates used in this work were prepared as described in (Panavas and Nagy, 2003a; Panavas et al., 2002b; Panavas et al., 2002d). Following the T7 RNA polymerase reaction, the RNA transcripts were isolated from 1% agarose gel for the RNA blot, followed by phenol/chloroform extraction, and precipitation in 95% ethanol. The RNA transcripts were dissolved in water.

**Total RNA extraction from yeast cells and RNA blot analysis**

To extract total RNA from yeast, equal volumes of buffer (50mM sodium acetate, pH 5.2, 10mM EDTA, 1% SDS) and water-saturated phenol were added to the pelleted cells (Panavas and Nagy, 2003b). Samples were vortexed, incubated for 4 min at 65°C, followed by incubation for 2 min on ice and centrifugation at 21,000g for 5 min at room temperature. Total RNA was precipitated from the aqueous phase by adding 3 volumes of 95% ethanol with 0.1 volume of 3M sodium acetate, pH 5.2, and washed with 70% ethanol. RNA was dissolved in TE buffer and formamide (in 1:1 ratio). Total yeast RNA samples were heated for 5 min at 85°C, electrophoresed in 1% agarose gels, and transferred to Hybond XL membrane (Amersham). *In vitro* made RNA transcripts, following 5 min preincubation with formamide at 85°C, were pipetted to the Hybond XL membrane and crosslinked with UV (BioRad). Hybridization was done using ULTRAhyb solution (Ambion) at 68°C according to the supplier’s instructions. The ³²P-labeled replicase products were used as probes for hybridization.

**Western blot**

Aliquots of replicase proteins purified from yeast cells using Nickel-chelating affinity columns were mixed in 1:1 ratio with SDS-PAGE sample loading buffer (Sambrook et al.,
1989), heated for 5 min at 85°C, electrophoresed in 8% SDS-PAGE gels and electrotransfered to a PVDF membrane (BioRad). Non-specific binding sites on the membranes were blocked using 5% non-fat dry milk solution in TBS buffer (Sambrook et al., 1989) also containing 0.1% Tween 20, and the membranes were washed three times with TBS-Tween buffer and incubated with monoclonal anti-His antibodies (Amersham) for 1 h at room temperature. Following three 10-min washes with TBS-Tween buffer, membranes were incubated for 1 h at room temperature with secondary alkaline phosphatase-conjugated antibody (Sigma). After three washes of membranes with TTBS, protein bands were developed using BCIP and NBT (Sigma).

Results

Comparison of CNV replicase activity of the enriched membrane fraction obtained from yeast and plant protoplasts

To facilitate the detection and purification of CNV replicase proteins from yeast, I modified the yeast expression system described earlier (Panavas and Nagy, 2003b). Briefly, we added an N-terminal 6xHis-tag to both the p33 and p92 proteins, which were expressed constitutively from high copy number plasmids (Figure 4.1B). Co-expression of the p33 and p92 proteins together with the DI-72 RNA replicon, which is obtained from the galactose-inducible GAL1 promoter (Figure 4.1B), results in robust DI-72 RNA replication in yeast cells. The presence of His-tags in p33 and p92 does not appear to inhibit DI-72 RNA replication, because the amount of DI-72 plus-strand can reach ribosomal RNA levels in yeast, similar to that in plant cells (see below).

Enriched membrane preparations were obtained from yeast co-expressing p33, p92 and DI-72 as described in Materials and Methods. Briefly, yeast was grown on selective medium in the presence of galactose for 12 to 30 hours (in a time course experiment, Figure 4.2), followed by disruption of cells and differential centrifugation to obtain the crude, enriched membrane fraction. For comparison, I used N. benthamiana protoplasts electroporated with CNV gRNA transcripts (note that we did not use DI RNA template here, because the super-competitive DI-72 RNA would reduce gCNV replication, thus resulting in reduced amount of replicase in plant cells), followed by harvesting of cells at various time points (Figure 4.2A). Then the enriched membrane preparations were obtained from the CNV gRNA-transfected protoplasts using the
method described in (Nagy and Pogany, 2000). Subsequently, the obtained enriched membrane preparations from yeast and from plant protoplasts were tested in the presence of four ribonucleotides, including $^{32}$P-labeled UTP. Under these conditions, the CNV replicase complex is expected to complete RNA synthesis on the endogenous templates (i.e., the viral RNA that was co-purified with the replicase), which are part of the actively synthesizing replicase complex in cells. Since the replicase products became $^{32}$P-labeled during the in vitro reaction, they were analyzed in denaturing gels (Figure 4.2A-B). These experiments demonstrated that both the yeast- and plant protoplasts-derived CNV replicase complexes synthesized $^{32}$P-labeled products, which were either DI-72 RNA-sized (in yeast, Figure 4.2A) or gRNA-, sgRNA1- and sgRNA2-sized (in plant protoplast, Figure 4.2A). The amounts of these products increased over time in both hosts, suggesting that more replicase complexes were formed in the cells during longer incubation times. Accordingly, the amount of p33 increased continuously during incubation up to 24 hours in N. benthamiana protoplasts (not shown).

To test the nature of the in vitro synthesized replicase products, I recovered the $^{32}$P-labeled RNAs after the in vitro replicase reaction, and used them as probes in RNA blotting. I applied the same amounts of denatured plus- and minus-stranded DI-72 RNA for yeast samples and CNV gRNA for protoplast samples as target RNAs, which were fixed on nylon membranes (Figure 4.2C). After hybridization of the membranes with the $^{32}$P-labeled RNAs obtained from the in vitro replicase reactions, I measured the ratio of plus- versus minus-strand-specific signals on the RNA blots using a Phosphorimager (Nagy and Pogany, 2000). These experiments demonstrated that the enriched membrane preparations containing the CNV replicase from both yeast and plant protoplasts synthesized more minus-stranded than plus-stranded RNAs at the early time point, while the synthesis was gradually shifted toward plus-strands at later time points (Figure 4.2D, left panel). This finding was expected based on detection of more abundant plus-strands than minus-strands in plant protoplasts at late time points (Ray and White, 2003). Interestingly, I observed new minus-strand synthesis by the CNV replicase even at late time points in both preparations (Figure 4.2D, right panel), suggesting that the minus-strand synthesis had not been “shut down” in order to favor plus-strand synthesis (see Discussion).
Solubilized CNV replicase preparation from yeast is capable of using exogenous templates in vitro

Because the CNV replicase present in either the crude yeast extract or the enriched membrane fraction was unable to utilize exogenous template (i.e., template added for the in vitro reaction) (Figure 4.3A), I tried various detergents to solubilize the replicase complex from membranes (not shown). I found that after treatment with either 1% Triton X-100, the replicase complex remained active, because it could synthesize $^{32}$P-labeled RNA product using the endogenous template (see Figure 4.3A, lane 9). The same preparation, however, was also capable of complementary RNA synthesis on added (exogenous) template as well (Figure 4.3A, lane 9). In summary, I conclude that the solubilized preparation contains a highly active CNV replicase that can be programmed by added templates.

Affinity-based purification of CNV replicase from yeast

To purify the recombinant CNV replicase from the solubilized preparation, I utilized the engineered 6xHis-tag present at the N-termini of p33 and p92 for nickel-chelating affinity purification as described in Materials and Methods. Analysis of crude yeast proteins, and proteins eluted from the column samples on SDS/PAGE gel showed amount of p33 protein in comparison to yeast proteins. As a control were used proteins purified from yeast cells that did not express p33 protein (Figure 4.3, B). I found that the obtained purified CNV replicase preparation showed high polymerase activity (Figure 4.3A, lane 10). Characterization of the replicase product by PAGE analysis revealed that the recombinant CNV replicase was able to synthesize complementary RNA on added RI/III(-) template, which contains minus-stranded sequence of region I and region III of DI-72 (Figure 4.1A), in the presence of ribonucleotides and $^{32}$P-labeled UTP. Next, I wanted to know if the properties of the purified recombinant CNV replicase was comparable with that of the partially-purified CNV replicase (RdRp) preparation obtained from infected N. benthamiana plants (Nagy and Pogany, 2000). First, I tested plus- and minus-stranded DI-73 RNA templates in vitro. DI-73 RNA contains the same sequence as DI-72 (Figure 4.1A), plus a 167 nt long sequence between RIII and RIV, which is missing in DI-72 RNA (White and Morris, 1994b). Importantly, the larger size of DI-73 than that of DI-72 allowed me to distinguish between the added exogenous DI-73 RNA and the co-purified endogenous DI-72 templates after the in vitro reaction. I found that the yeast-derived
recombinant and the plant-derived CNV replicase produced comparable products with DI-73(+) and (-) templates (Figure 4.4, lanes 1-4). For example, both preparations used the minus-stranded template more efficiently than the plus-stranded DI-73. In addition, both replicase preparations synthesized three different types of RNA products on DI-73(-), such as (i) template-sized complementary RNA obtained by de novo initiation from the 3’-terminal promoter, (ii) several shorter-than-template-sized products, which were due to de novo initiation at internal positions (resembling promoter-like sequences (Panavas et al., 2002b)), and (iii) a longer-than-template-sized product, which was the result of primer extension from the 3’ end of the template (self-priming reaction). All these in vitro replicase products were characterized in detail before using the plant-derived preparation (Nagy and Pogany, 2000; Panavas et al., 2002a; Panavas et al., 2002b).

Additional templates tested in the recombinant CNV replicase assay were the CNV-derived DI-42, the related Turnip crinkle virus (TCV)-associated satD and satC (Song and Simon, 1994), the Q beta-associated satellite RNA (termed MDV, (Axelrod et al., 1991)) and yeast tRNA (Figure 4.4). All these viruses belong to “supergroup 2”, though TCV and CNV are more closely related (they belong to Tombusviridae family) than Q beta bacteriophage. The recombinant and the plant-derived CNV replicases were found to use DI-42(-) (lanes 15-16) and satC(-) (lanes 11-12) templates efficiently, while DI-42(+)(lanes 13-14), satC(+) (lanes 9-10), satD(+) (lanes 5-6) and satD(-) (lanes 7-8) RNAs were less efficient templates in vitro (Figure 4.4). In contrast, MDV(-) (lanes 17-18) and the yeast tRNA(lanes 23-24) were not recognized by the CNV replicase as templates (Figure 4.4). Overall, the template specificities of the recombinant and the plant-derived CNV replicases were very similar.

To test if the recombinant CNV replicase can recognize properly TBSV and CNV-derived cis-acting sequences, such as minus- or plus-strand initiation promoters, I used constructs MDV(-)/gPR and MDV(-)/cPR, which carried either the gPR promoter (i.e., the minimal minus-strand initiation sequence in TBSV and CNV, (Panavas et al., 2002a; Panavas et al., 2002b)) or the cPR promoter (i.e., the minimal plus-strand initiation sequence (Panavas et al., 2002a)) in addition to the MDV(-) sequences at the 5’ end. Analysis of the in vitro replicase products revealed that the recombinant yeast derived CNV replicase, similar to the plant-derived preparation, recognized these minimal promoter sequences correctly and efficiently (Figure 4.4, lanes 19-22).
Isolation of highly active CNV replicase complex requires co-expression of template RNA in yeast

To take advantage of the heterologous CNV replicase system, I co-expressed various combinations of p33, p92 and DI-72 RNA in yeast, followed by testing the replicase activity of the purified preparations in the presence of an added RNA template [RI/III(-)] as shown in Figure 4.5. These experiments demonstrated that neither p33 nor p92, when expressed separately in yeast, had detectable levels of replicase activity in vitro (Figure 4.5, lanes 1-2). Co-expression of p33 and p92 in the same yeast cells, however, resulted in detectable, albeit very low, level of replicase activity on the added template in vitro (Figure 4.5, lane 3). The observed polymerase activity seems to come from the CNV replicase, because it was capable of producing full-length products, which were single-strand-specific RNase-resistant (not shown), and these products were similar to those observed with the plant-derived CNV replicase preparation (Figure 4.3, lane 11).

Co-expression of p33, p92 and DI-72(+) RNA in the same yeast cells, however, led to the isolation of a CNV replicase preparation with the highest activity as shown in Figure 4.5 (lane 5). These observations suggest that the RNA template stimulates significantly (~40-fold increase) the formation of stable CNV replicase complex in vivo (see Discussion). In contrast, co-expression of DI-72(-) template with p33 and p92 resulted in basal level of CNV replicase activity (Figure 4.5, lane 4), suggesting that the minus-stranded RNA cannot stimulate the formation of the CNV replicase in vivo. Western-blot analysis of levels of p33 and the less abundant p92 in the purified replicase preparations revealed that the protein levels were comparable in the active (Figure 4.5C, lane 5), less active (lanes 3-4), and inactive preparations (lanes 1-2), suggesting that there is no correlation between the amount of the individual replicase proteins and the activity of the purified CNV replicase in vitro.

To examine if active CNV replicase can be assembled in vitro from separately expressed components, I used three types of mix-and-match experiments. In one type, I mixed in 1:1 ratio two separate enriched membrane fractions obtained from two yeast strains, which co-expressed p92/DI-72 RNA and p33/DI-72 RNA separately, prior to the replicase assay (Figure 4.6, lane 3). I did not observe endogenous (32P-labeled) DI-72 RNA product in this mix-and-match assay in contrast to the high template activity obtained with similar extract derived from yeast co-
expressing p33/p92/DI-72 RNA (Figure 4.6, lane 1). The second type of assay included the mixture of purified p33 and p92 components (expressed separately in two yeast strains) and exogenous RI/III(-) template obtained by in vitro transcription. Again, I did not observe detectable level of replicase activity in this in vitro mix-and-match experiment (Figure 4.6, lane 6). The control experiment, which included affinity-purified preparation obtained from yeast co-expressing p33/p92/DI-72 RNA, resulted in ³²P-labeled products from the endogenous and exogenous templates (Figure 4.6, lane 10).

Because it is possible that I might have lost one or more host-derived components from the CNV replicase during purification, thus resulting in inactive replicase in the above mix-and-match experiment, I also used non-purified solubilized membrane fractions for in vitro studies (Figure 4.6, lanes 11-17). However, mixing (prior to the replicase assay) solubilized membrane fractions obtained from two separate yeast strains, which co-expressed either p92/DI-72 or p33/DI-72, in 1:1 (Figure 4.6, lane 14) and 1:4 ratio (Figure 4.6, lane 15), respectively, did not give the predicted ³²P-labeled products in the in vitro assays. Based on these mix-and-match experiments with (i) the enriched membrane fractions, (ii) the solubilized membrane fractions, or (iii) the affinity-purified replicase proteins and RNA transcripts, I conclude that the in vitro conditions used did not favor the assembly of functional CNV replicase complex. This result is in contrast with the in vivo conditions in yeast co-expressing p33/p92/DI-72 RNA, which gave functional CNV replicase. Future studies will be aimed to test if cellular membranes or host factors, which might be missing in the above preparations, are needed for the assembly of the CNV replicase complex.

**Identification of DI RNA elements required for active CNV replicase assembly in vivo**

To identify DI RNA elements necessary for active CNV replicase formation in yeast cells, I made a series of DI RNA deletion constructs (Figure 4.7A) that were individually co-expressed with p92 and p33 proteins. Expression of the above deletion mutants of DI RNA was achieved by growing yeast cells in galactose containing media. The CNV replicase was isolated as described above and its activity was tested in in vitro reaction using exogenous template RI/III (-) (Figure 4.7B). The results of this experiment showed that when yeast cells co-expressed p92/p33 and one of DI RNA deletion mutants: namely RI/II/IV, RII/III/IV, RII/IV (Figure 4.7A), the activity of the purified CNV replicase was comparable to activity of replicase purified from
cells co-expressing full length DI-72 and replicase proteins (Figure 4.7B lanes 1, 2, 5 and 6). These results suggested that both RII and RIV are necessary for replicase activation. The requirement of RII/IV for active replicase formation in vivo was supported by experiments performed with DI RNA deletion mutants lacking one of these regions (Figure 4.7A, constructs RI/II/III and RI/III/IV). CNV replicase purified from yeast cells co-expressing one of these constructs and p92/p33 proteins had only basal level of activity (Figure 4.7B, lanes 3 and 4).

The total yeast RNA analysis by Northern blot using as a probe RI/III/IV (-) RNA showed that most of the DI RNA deletion mutants did not replicate at all or replicated very little (Figure 4.7C), with exception of construct RI/II/IV (Figure 4.7C, lane 6). I have not observed correlation between DI RNA replication in yeast cells and the activity of the purified replicase in vitro (constructs RII/III/IV and RI/II/IV (Figure 4.7B-C, lanes 5 and 6)). Both these constructs stimulated replicase activity, albeit construct RI/II/IV RNA is more abundant than RII/III/IV (Figure 4.7B-C, lanes 5 and 6). These results suggest that very limited amount of certain viral RNA sequences is sufficient for active replicase formation.

The aim of the next set of experiments was to identify RNA elements within RII/IV RNA that are required for CNV replicase activation. I made separate deletions in RII and RIV, eliminating predicted RNA structures (Figure 4.8A). These constructs were co-expressed with p92 and p33 in yeast cells, followed by replicase purification and in vitro reaction with exogenous template RI/III(-). The results showed that deletion of RII SL (Figure 4.8A, construct 2) resulted in low replicase activity (Figure 4.8B, lane 2). Combination of (-) strand initiation promoter (gPR) and RII (Figure 4.8A, construct 3) did not increase replicase activity (Figure 4.8B, lane 3). This observation suggested that RII is not sufficient for assembly of the active replicase, even in the presence of promoter. Separate deletions of SL3, SL2 and gPR in construct RII/IV resulted in reduced CNV replicase activity suggesting that each of these sequences is important in CNV replicase assembly (Figure 4.8A, constructs 5, 6, 7. In contrast, deletion of single stranded (ss) region from 5’end in RIV (Figure 4.8A, construct 4) did not affect replicase activity when compared to RII/IV (Figure 4.8B, lane 4 and 1). Additional experiments defined that RIISL/RIV[^ss4] (Figure 4.8A, construct 8) is the minimal construct, which still support efficient replicase assembly.
The future studies should further define structures and nucleotides within RIISL/RIV\[ss4 RNA that are critical for stimulation of CNV replicase and possibly will lead to better understanding the role of the viral RNA role in replicase assembly.

Discussion

TBSV and CNV replicases obtained from infected plants have been used extensively to characterize viral RNA elements that affect plus- and minus-strand synthesis, including promoter (initiation) elements (Panavas et al., 2002a; Panavas et al., 2002b), replication enhancers (Panavas and Nagy, 2003a; Panavas et al., 2003) and a replication silencer element (Pogany et al., 2003), and template-switching \textit{in vitro} (Cheng and Nagy, 2003; Cheng et al., 2002). These processes are important for replication and recombination (including DI RNA formation) of Tombusviruses. In spite of the usefulness of the above plant-derived CNV replicase preparations in providing \textit{in vitro} data that complement the wealth of \textit{in vivo} data on Tombusviruses (Fabian et al., 2003; Havelda and Burgyan, 1995; Park et al., 2002; Ray and White, 1999; Ray and White, 2003; Ray et al., 2003), there is one major drawback in obtaining such preparations: the production of the replicase proteins depends on virus replication. Therefore, the effect of detrimental mutations within the viral RNA or the replicase proteins on replication is difficult to study in the plant-derived \textit{in vitro} CNV replicase assay. The development of a heterologous expression system for the CNV replicase genes in yeast should however circumvent this problem. This is because the recombinant replicase proteins are produced from expression plasmids in yeast even in the absence of viral replication (Figure 4.5D). Accordingly, I succeeded to purify active CNV replicase complex from yeast in the absence of replicating CNV gRNA (Figure 4.5A). This recombinant preparation will complement the traditional plant-based CNV replicase assay.

Comparison of the recombinant CNV replicase from yeast and the partially-purified CNV replicase preparation from plants revealed that both were capable of complementary RNA synthesis on added templates. I observed three different types of replicase products when minus-stranded DI-72 RNA was used as a template: (i) production of full-length complementary RNA product (via \textit{de novo} initiation from the 3’-terminal cPR promoter (Panavas et al., 2002a)); (ii)
internal initiation from promoter-like sequences (also via de novo initiation (Panavas et al., 2002b)); and (iii) primer-based initiation (self-priming from the 3’ end) of complementary RNA synthesis (Cheng et al., 2002). In contrast to the minus-stranded templates, the plus-stranded DI-72 RNA was used inefficiently by both replicase preparations (Figure 4.4). Thus, in spite of (i) the different expression strategies for p33 and p92 in plant (CNV-infection-based) and in yeast (plasmid-based), (ii) the different purification methods (i.e., affinity-based purification from yeast and purification via chromatography from plants), and (iii) the different N-terminal sequences in p33 and p92 (i.e., wt p33/p92 in the plant-expressed and His-tagged p33/p92 in the yeast-expressed preparations), the properties of the purified recombinant CNV replicase preparations and the plant-derived preparations are comparable under the in vitro conditions used.

The results shown in this study confirm that the functional CNV replicase complex contains both p33 and p92 replicase proteins (Figure 4.5). This has been predicted earlier based on data obtained from biochemical and genetic experiments (Nagy and Pogány, 2000; Oster et al., 1998; Panaviene et al., 2003; Scholthof et al., 1995d). Yet, it is somewhat surprising that the purified recombinant p92, when expressed alone in yeast, was not a functional polymerase (RdRp) in vitro (Figure 4.5). This is because p92 contains the same sequence as p33 in its N-terminal overlapping (pre-readthrough) domain (Figure 4.1A). Apparently, the N-terminal region in p92 cannot fulfill the function(s) provided by the “free” p33 in the CNV replicase complex. In contrast to the CNV p92, which requires p33 in the functional CNV replicase, the similar TCV p88 RdRp protein or its N-terminally truncated version (when purified from E. coli) are highly active polymerases in vitro in the absence of the smaller replicase protein (i.e., p28 (Rajendran et al., 2002)). It is currently not known what is the reason for this difference between the CNV and TCV RdRp proteins in the requirement for the auxiliary viral protein.

In addition to the p33 and p92 replicase proteins, the endogenous DI RNA is also an important factor in the CNV replicase complex. Although the purified CNV replicase containing p92 and p33 was active in the in vitro assay, co-expression of DI-72 RNA in the same yeast cells resulted in a CNV replicase preparation with ~40-fold enhanced activity (Figure 4.5A). By using Western blotting, I excluded the possibility that the increase in replicase activity is due to increased amounts of the replicase proteins in the purified preparations obtained from yeast lacking or co-expressing DI-72 RNA (Figure 4.5D). I also demonstrated that the enhanced CNV
replicase activity requires the plus-stranded DI-72 RNA, while the minus-stranded DI-72 failed to achieve the same effect (Figure 4.5). The more detailed studies of DI RNA elements revealed that strong hairpin in RII (RIISL) and ~80 3’nt of RIV were required for active CNV replicase formation in yeast cells (Figure 4.8). Based on these observations, I propose that the role of the plus-stranded RNA template is to promote the assembly of the functional replicase in cells by possibly providing an “assembly platform”. A similar model on the “nontemplate” role of the RNA has also been proposed for the BMV and the Alfalfa mosaic virus (AMV) replicases, which are related to each other, but only very distantly related to the CNV replicase (Quadt et al., 1995; Vlot et al., 2001). A seemingly important difference between the BMV and AMV replicases versus the CNV replicase is that the latter is functional, albeit at a low level, in the absence of the RNA template (Figure 4.5).

Although the co-expression of DI-72 RNA is important for isolation of highly active CNV replicase from yeast, I found that, after purification of the replicase complex, the template RNA can be removed partially without the loss or large reduction in the activity of the CNV replicase (based on exogenous templates, Figure 4.4). This suggests that the CNV replicase, once it has been formed in cells, is a stable complex even in the absence (or in the presence of minute amount) of RNA.

The actual role(s) of the RNA template during the assembly of the CNV replicase complex or during other processes, such as recruitment of replicase proteins to the membranous structures (the proposed sites of Tombusvirus replication (Pantaleo et al., 2003; Rubino and Russo, 1998)), is currently unknown. My effort to assemble the CNV replicase in vitro from either purified components (p33/p92 and DI-72 RNA) or by mixing enriched or solubilized membrane fractions failed to yield functional preparation (Figure 4.6). It is possible that our in vitro preparations lacked host factors and/or particular membrane surface, which might be essential for the assembly of the functional CNV replicase complex in vivo.

Time-course experiments (Figure 4.2A-D) revealed that the CNV replicase present in the enriched membrane fractions obtained from yeast and N. benthamiana protoplasts synthesized both plus and minus-stranded products in vitro. As expected, the CNV replicase synthesized more plus-stranded products at the late time points than at the early time point (Figure 4.2D, left panel). However, we also observed new minus-strand synthesis taking place even at the late time points in the enriched membrane preparations obtained from yeast and plant protoplasts. Based
on this observation, I propose that the CNV replicase is involved in minus-strand synthesis during the entire replication process, thus there is no “shut down” of minus-strand synthesis in cells, a process that would favor plus-strand synthesis at the late time points, as suggested for the unrelated *Tobacco mosaic virus* (Ishikawa et al., 1991). This observation seems to be valid for both the yeast-based infection-independent and the protoplast-based infection-dependent systems (Figure 4.2C). I interpret these results that new CNV replicase complexes, which first synthesize minus-stranded RNA products, are continuously being formed in yeast cells and in plant protoplasts, even at the late time points. In agreement with this model, the amounts of plus- and minus-stranded TBSV (Ray and White, 2003) and CNV RNAs (not shown), both gRNA and DI RNA, increase continuously over 24-30 hours of infection in cucumber and *N. benthamiana* protoplasts. Also, I found that the amount of p33 increases over time (up to 24 hours) in plant protoplasts (not shown), which suggests continuous production of p33, and possibly p92, replicase protein in these cells.

The above model is based on the assumption that the obtained CNV replicase preparations can only complete RNA synthesis on the endogenous templates, thus the new $^{32}$P-labeled *in vitro* replicase products would only consist of the complementary strands of the co-purified RNAs. However, it is also possible that the CNV replicase present in the enriched membrane fraction can perform sequential plus- and minus-strand synthesis from the endogenous templates. This complete *in vitro* replication would result in new $^{32}$P-labeled replicase products consisting of both strands, even if the co-purified RNA was consisted of one strand. Although I cannot exclude this model at this time, I do not have any evidence supporting complete replication in our CNV replicase assays (based on either endogenous or exogenous templates). Future experiments will address this possibility.

In summary, in this work, I have developed three new *in vitro* assays with the recombinant CNV replicase. One assay is based on obtaining an enriched-membrane fraction, the second on solubilized membrane preparation, and the third on affinity-purified replicase preparation, which will be useful to further study the mechanism of Tombusvirus replication and recombination. In addition, I have demonstrated that the CNV replicase complex contains both p33 and p92 replicase proteins. Also, the activity of the CNV replicase obtained from yeast is greatly enhanced by co-expression of DI-72(+) strand, in particular RNA elements located in RII and RIV, suggesting that the viral RNA plays a role in assembly of the CNV replicase.
Figure 4.1: Expression of CNV replicase proteins in yeast. (A) Genome organization of CNV and the closely related TBSV RNA encoding five open reading frames, and TBSV-derived DI-72 RNA. The p33 and p92 replicase genes are shown as white boxes (“STOP” sign represents translational termination codon at the end of p33) and remaining three genes are shown as black boxes. Regions of TBSV present in DI-72 RNA are marked on the gRNA. (B) Plasmids used to express the CNV p33 and p92 replicase proteins and DI-72 RNA in yeast cells. pGBK- His33 and pGAD-His92 have constitutive P_{ADH} promoter, and DI-72 RNA is expressed from galactose inducible (P_{GAL1}) promoter. Black boxes in pGBK-His33 and pGAD-His92 constructs represent the 6xHis tag. The translation termination codon of p33 was replaced with a tyrosine (Y) codon allowing p92 expression from pGAD-His92 plasmid. There is a satTRSV(-) ribozyme (Rz sat) at the 3’end of DI-72.
A  
12h 18h 24h 30h

Yeast

DI-72

gCNV

N. benthamiana protoplasts

B

Relative activity (%)  

100

75

50

25

0

12 18 24 30

Time (h)

C

12h 18h 24h 30h

Yeast

N. benthamiana protoplasts

blotted RNA

+    -    +    -    +    -    +    -

Relative amount (%)  

100

75

50

25

0

12 18 24 30

Time (h)
Figure 4.2: Characterization of CNV replicase activity in enriched membrane fractions derived from yeast or N. benthamiana protoplasts. (A) PAGE analysis of the ($^{32}$P-labeled) *in vitro* replicase products on the endogenous templates, which are present in the enriched membrane fractions, obtained from yeast (top) and from protoplasts (bottom). Time of incubation, shown on the top of the gel in the time-course experiments, started after the addition of galactose to the media for yeast or with the addition of incubation media after electroporation for N. benthamiana protoplasts. Arrows indicate the bands that correspond to products obtained on the various endogenous RNAs. (B) Relative RdRp activity of enriched membrane fractions. Circles and triangles represent data obtained with CNV replicase derived from protoplasts and yeast, respectively. The CNV replicase activity at the 30 hr time point corresponds to 100%. (C) RNA blot showing (+) and (-) strand levels in the *in vitro* CNV replicase products on endogenous templates. Unlabeled *in vitro* transcripts of DI-72(+) and DI-72(-) (400 ng each) for yeast and gCNV(+) and gCNV(-) for protoplasts-derived samples were blotted on the membrane as shown below the blots. Time points for harvesting the yeast and protoplast samples for isolation of membrane fractions are shown on the top. The blotted RNAs were hybridized with denatured $^{32}$P-labeled RNA probes, which were generated by the CNV replicase *in vitro* on the endogenous templates present in the enriched membrane fractions from yeast or protoplast (see panel A). (D) Relative amounts of plus- and minus-stranded RNAs in the *in vitro* replicase products. Total replicase products at each time point were taken as 100% (left panel), or the amount of plus-stranded replicase product at the 30 hr time point was taken as 100% (right panel). Solid and dotted lines represent plus- and minus-stranded products, while circles and triangles represent data obtained with preparations from protoplasts and yeast, respectively.
Figure 4.3: Activity of CNV replicase in different preparations obtained from yeast. (A) Each preparation, as shown on the top, was tested in the absence or presence of exogenous RI/III(-) template, which contains the minus-stranded regions I and III of DI-72 (Figure 4.1A) in a standard CNV replicase assay. The positions of the exogenous RI/III (-), the endogenous DI-72 (for the yeast - derived samples), genomic (g) and subgenomic (sg1 and sg2) templates (plant), are shown by arrows and bracketed lines on the side of the gel. The internal initiation products and the primer extension product are shown with a bracket and an asterisk, respectively. “Sol” represents “solubilized membrane fraction”, while “Pur” indicates affinity-purified CNV replicase from yeast, while “Nb” represents the partially -purified CNV replicase obtained from N. benthamiana plants. The replicase products obtained from the endogenous templates representing gCNV, sgRNA1 and sgRNA2 are indicated as “g, sg1 and sg2”.

(B) Silver stained SDS-PAGE gel of protein samples from indicated replicase preprations used in (A).
Figure 4.4: Comparison of template usage by the CNV replicases obtained from yeast and plant in vitro. Radiolabeled replicase products were analyzed on denaturing 5% PAGE/8M urea gels. The exogenous RNA templates used in the in vitro replicase reaction are shown on the top of the gel. The products of the CNV replicase purified from yeast (odd lane numbers) and from plants (even lane numbers) are shown. RNA size markers obtained by T7 transcription with $[^{32}P]U$TP are shown on the left side of the gel. Template-sized products are marked with arrowheads, while the primer extension and internal initiation products are marked with asterisks and bracketed lines, respectively. The replicase products obtained from the endogenous templates representing gCNV, sgRNA1 and sgRNA2 are indicated as “g, sg1 and sg2”.
**A**

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**B**

**Northern**

- Transcript yDI-72
- DI-72

**Agarose gel**

- yeast rRNA
- DI-72

**C**

**Western**

- p92
- p33
Figure 4.5: Co-expression of DI-72 RNA in yeast stimulates the activity of the purified CNV replicase. (A) \(^{32}\)P-labeled RNA products obtained in the \textit{in vitro} CNV replicase assay with exogenous template [RI/III(-)] were analyzed on 5% PAGE/8M urea gel. The co-expressed p33, p92 and DI-72(+) or (-) RNAs in yeast cells are shown on the top as “+”, while yeast lacking one of these components is marked as “-“. See additional symbols in the legend to Fig. 4. (B) Northern blot of total yeast RNA (top) and corresponding ethidium-bromide stained agarose gel (bottom). The total RNA was extracted from the yeast cells co-expressing various components as shown in panel (A), analyzed in 1% agarose gel (bottom) and with Northern blotting (top) using \(^{32}\)P-labeled RI/III/IV(-) RNA probe. Transcripts of yDI-72-(before ribozyme cleavage) and DI-72-sized bands are indicated by arrows. (C). Western blot analysis of p33 and p92 proteins, which were affinity-purified from yeast cells co-expressing the shown viral components (see panel A), with anti-His antibodies.
Figure 4.6: Mix-and-match *in vitro* replicase assays. The CNV replicase preparations represent enriched membrane fractions (lanes 1-5), affinity purified proteins (lanes 6-10), solubilized membrane fractions (lanes 11 - 17) obtained from yeast, except lane 16 contains products, which were generated by CNV replicase obtained from *N. benthamiana* plants. The CNV replicase assay in lanes 6-17 included exogenous RI/III(-) templates. The co-expressed p33, p92 and DI-72 (+) RNA in yeast cells are shown on the top as “+”, while yeast lacking one of these components is marked as “-“. The boxed lanes represent the mixed preparations as follows: preparations containing either p33 or p92 (co-expressed with DI-72 RNA) were obtained from separate yeast lines, which were mixed in 1:1 ratio prior to the CNV replicase reaction (replicase products in lanes 3, 6 and 14-15 are from mixing preparations corresponding to yeast lines shown in lanes 4-5; 7-8 and 12-13, respectively). Note that the CNV replicase preparation in lane 15 was obtained by mixing p92 and p33 preparations in 1:4 ratio. The CNV replicase products generated on endogenous DI-72 or exogenous RI/III(-) templates are marked. Lane marked as “Nb” represents the replicase product obtained with the plant-derived CNV replicase preparation.
Figure 4.7: DI-72 regions necessary for purified CNV replicase activity stimulation. (A) Schematic representation of DI-72 deletion derivatives used in (B) and (C) analysis. Boxes represent DI-72 regions present in the construct (numbers indicated inside box), while thin bent lines show deleted regions. (B) \(^{32}\)P-labeled RNA products obtained in the \textit{in vitro} CNV replicase assay with exogenous template [RI/III(-)] were analyzed on 5% PAGE/8M urea gel. The co-expressed with p33 and p92 DI-72 deletion derivatives are indicated by numbers on the top of the gel. Template-sized products are marked with arrowheads, while the primer extension and internal initiation products are marked with asterisks and bracketed lines, respectively. (C) Northern blot of total yeast RNA. The total RNA was extracted from the yeast cells co-expressing various components as shown in panel (B), analyzed with Northern blotting using \(^{32}\)P-labeled RI/III/IV(-) RNA probe. Transcripts of yDI-72 constructs -(before ribozyme cleavage) and DI-72 constructs-sized bands are indicated by arrows.
Figure 4.8: Defining DI-72 regions II/IV (RII/IV) motifs involved in purified CNV replicase activity stimulation. (A) Schematic representation of RII/IV constructs used in (B). Boxes represent RII/IV motifs present in the construct (names marked inside box) and bent lines indicated deleted part of the region. RIISL-region II stem-loop; ss4- single stranded 4; SL3 -stem-loop 3 (includes silencer); SL2 - stem-loop 2; gPR - genomic promoter ((-)-strand initiation promoter). (B) $^{32}$P-labeled RNA products obtained in the in vitro CNV replicase assay with exogenous template [RI/III(-)] were analyzed on 5% PAGE/8M urea gel. The co-expressed with p33 and p92 RII/IV deletion derivatives are indicated by numbers on the top of the gel. Template-sized products are marked with arrowheads, while the primer extension and internal initiation products are marked with asterisks and bracketed lines, respectively.
Chapter Five

Summary

Tombusviruses are small positive-strand RNA viruses of plants. They are excellent model system that can help us understand replication and recombination of RNA viruses in general. Tombusviruses use only two viral coded proteins p33 and p92 for replication.

The purpose of this work was to study in detail the role of the common arginine/proline rich (RPR) RNA binding motif of p33 and p92 (Rajendran and Nagy, 2003) in tombusvirus replication, recombination and sgRNA synthesis. The first part of the study showed that RPR motif is critical for RNA binding and, thus, for replication. Mutational analysis revealed that the second and third arginines residues within the RPR (sequence RPRRRP) motif were required for virus replication in cis (gRNA) and in trans (DI RNA), and they are likely involved in RNA binding. I also showed that addition of one more arginine within the RPR motif increased sgRNA2 accumulation about 20 fold, compared to gRNA, while mutations of proline to alanines decreased sg RNA2 accumulation by ~ 40%. Separately introduced mutations to p33 or p92 showed that the third arginine mutations were critical for p33 function, while they had smaller effect when present in p92 for virus replication. This finding suggests that RPR motif might be more critical for function of p33 than for p92. This effect could be related to the requirement of higher concentration of p33 than p92, or RPR is required for p33, but is less important for p92 due to the presence of two more RNA binding motifs in p92 protein.

The second part of my study focused on the effects of RPR mutations on DI RNA based recombination. We developed a DI RNA based system that allowed us to test how RPR mutations in replicase proteins influenced recombination. I found that various DI RNAs replicated and recombined differently in the presence of wt replicase proteins, pointing to the role of RNA elements in recombination. The effects on recombination by introduction of the second copy of regions RII and RIII depended on their order in the DI RNA. Addition of one or two copies of RIII to DI-72 (DI-33, DI-333), or two RII copies in tandem followed by two copies of RIII (DI-2233) led to more efficient recombination compare to DI-2323 where RII and RIII were duplicated in head-to-tail position. It is possible that by changing the order of regions, I
created less optimal arrangement for \textit{cis}-acting sequences that resulted in enhanced recombination.

The RPR motif studies showed that the most of the mutations did not affect recombination, but proline mutations to alanine in replicase proteins slowed down recombinant DI RNA appearance, while additional arginine (the same mutation that increased sgRNA2 accumulation), or mutation of central arginine to alanine led to rapid recombination. The experiments with mutations introduced separately into p33 and p92 showed that RPR motif in p33 was important for DI RNA recombination. This finding suggests that the binding of p33 to viral RNA is a critical factor in recombination.

In the third part of the thesis I developed CNV replicase purification method from yeast cells. The data showed that expression of only p33 and p92 in the yeast cells led only to very low replicase activity. But co-expression of (+) strand DI RNA increased the replicase activity by $\sim 40$ fold (Panaviene et al., in press). In the next set of experiments we identified that co-expression of stem-loop (SL) structure in RII and SL3, SL2 and gPR ((-) strand initiation promoter) in RIV, were all necessary to obtain highly active CNV replicase. Taken together these findings suggest that viral replicase proteins p33 and p92, as well as certain viral RNA elements are required for active replicase formation.

Based on my results presented in this thesis, I would like to propose the following model to explain tombusvirus replicase assembly, virus replication and recombination.

When tombusvirus gRNA enters the cell, first replicase proteins are translated. When p33 is made, it binds to viral RNA via its RNA binding motif. The recent data suggest that p33 binds with high specificity to SL structure in region II (+) (Pogany and Nagy, unpublished data). This structure is present in p92 ORF as well. It is known that p33 binds to tombusvirus RNA in cooperative manner, possibly via its protein- protein interaction domain (Rajendra and Nagy, in press). It means that a few p33 molecules can bind to viral RNA, coat it and possibly interfere with ribosome movement and translation. The RNA binding motifs and protein-protein interaction domain are also present in p92 that functions as RdRp. Since p33 can interact with p92 (Rajendran and Nagy, in press), it is possible that p33 facilitates p92 targeting to replication site on the membrane. On the other hand, it is possible that p92 (it also has membrane localization domain) with or without RNA finds its way to the replication site, and interaction with p33 occurs later.

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The studies of replicase purified from yeast suggest that p33 and p92 without viral RNA can form replicase only with limited activity. It means that viral RNA likely serves not only as the genetic material, but it is also involved in the active replicase formation. It could be that binding to viral RNA sequences could lead to conformational changes in p33 and p92 that possibly would affect their interaction and/or attraction of host factors leading to active replicase formation.

The results showing that only RII SL (is bound specifically by p33 (Pogany and Nagy, unpublished), SL3 (silencer), SL2 and gPR (--) strand initiation promoter) from RIV (about 200nt in total) are necessary for active CNV replicase formation in yeast cells, suggest that these motifs have essential roles in replicase assembly. The finding that RII with gPR, is not sufficient to form active replicase, points to the conclusion, that having RII SL, involved in recruitment (Pogany and Nagy, unpublished), and promoter is not enough for replicase activation. It is likely that replicase formation is a more complex process. The requirement of RII SL and SL3, SL2 and gPR, suggests a possibility that these components can interact between themselves and form certain structures that in return would influence activity of replicase proteins.

It could be that binding of p33 to certain viral RNA structures ensures RNA specificity and integrity and then it can direct p92 for RNA synthesis. It is possible that p92 could only initiate RNA synthesis process, after p33 binds to gPR and destroys interaction between silencer and promoter. The finding that mutations in the RNA binding motif of p33 had stronger effect on virus replication and recombination than the same changes in p92, support the above idea.

It is also possible that p33 binding to tombusvirus RNA is important for later steps of replication (e.g.anchoring RNA in replication site) and sgRNA synthesis. There is a possibility that p33 could bind to secondary structures in the viral RNA that lead to termination of (--) strand synthesis by RdRp and production of sgRNA. Increased viral RNA binding by addition of arginine to RPR motif could make p33 an “obstacle” for RdRp leading to more frequent termination at the (--) strand synthesis step. Proline mutation might have an opposite effect that would allow RdRp to finish gRNA (--) strand synthesis without termination more frequently than in wt situation and reduce production of sgRNAs.

The observed effect of RPR motif mutations in p33 and p92 on tombusvirus recombination suggests that p33 binding to RNA is critical for recombinant RNA formation. The role of p33 in recombination might be similar to the one in sgRNA synthesis: p33 binds stronger
(for example, arginine mutations) or weaker (proline mutations) to viral RNA and interferes or makes it easier for RdRp to synthesize full length RNA. It is possible that RNA elements in DI RNA influence where p33 binds and where the recombination sites will be.

The experiments described in this study imply that all three viral components, p92, p33 and viral RNA, are critical for virus replicase assembly, replication and recombination. The RNA in these processes is an important factor influencing replicase assembly by “attracting”, changing not only replicase proteins but possibly host factors as well. The requirement of RNA binding motif of replicase proteins for p33 function in virus replication and recombination suggests that tombusvirus auxiliary protein and RNA interactions plays essential role in viral infection and evolution.
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