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Yeast as a model host to study replication and recombination of defective interfering RNA of *Tomato bushy stunt virus*

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Abstract

Defective interfering (DI) RNA associated with *Tomato bushy stunt virus* (TBSV), which is a plus-strand RNA virus, requires p33 and p92 proteins of TBSV or the related *Cucumber necrosis virus* (CNV), for replication in plants. To test if DI RNA can replicate in a model host, we coexpressed TBSV DI RNA and p33/p92 of CNV in yeast. We show evidence for replication of DI RNA in yeast, including (i) dependence on p33 and p92 for DI replication; (ii) presence of active CNV RNA-dependent RNA polymerase in isolated membrane-containing preparations; (iii) increasing amount of DI RNA(+) over time; (iv) accumulation of (−)stranded DI RNA; (v) presence of correct 5′ and 3′ ends in DI RNA; (vi) inhibition of replication by mutations in the replication enhancer; and (vii) evolution of DI RNA over time, as shown by sequence heterogeneity. We also produced evidence supporting the occurrence of DI RNA recombinants in yeast. In summary, development of yeast as a host for replication of TBSV DI RNA will facilitate studies on the roles of viral and host proteins in replication/recombination.

Keywords: *Saccharomyces cerevisiae*; Virus replication; Recombination junction; Replication enhancer; RNA heterogeneity; RdRp; Ribozyme

Introduction

Tomato bushy stunt virus (TBSV) and *Cucumber necrosis virus* (CNV) belong to Tombusviruses with a single-component (~4.8 kb) plus-strand RNA genome. The genomic RNA of these viruses is directly translated in infected cells to generate replicase proteins p33 and p92. The minor p92 protein is a translational readthrough product and carries the hallmark motifs of RNA-dependent RNA polymerases (RdRp) (O'Reilly and Kao, 1998; Scholthof et al., 1995). Previous in vivo data showed that both p33 and p92 are necessary for TBSV replication (Scholthof et al., 1995; Oster et al., 1998). Accordingly, both p33 and p92 have been found in functional partially purified replication complexes (J. Pogany, T. Panavas, and P.D. Nagy, unpublished data).

Tombusviruses are often associated with defective interfering (DI) RNAs that are derived from the viral genome. The prototypical DI RNAs, such as DI-72 associated with TBSV, contain four noncontiguous regions of the TBSV genomic RNA (Fig. 1A). DI-72 RNA do not code for any essential genes; therefore, it depends on the replication machinery of the parental TBSV for its own replication (*trans*-replication) (White and Morris, 1999).

Tractable viral hosts, such as yeast, are especially useful to define the roles of viral ancillary proteins and host factors in RNA virus replication, as shown elegantly with *Brome mosaic virus* (BMV) (Ahlquist, 2002; Diez et al., 2000; Noueiry et al., 2000; Schwartz et al., 2002). To develop a genetically tractable model host for studying tombusvirus DI RNA replication, we took advantage of the high *trans*-replication potential of DI-72 RNA and tested its ability to replicate in yeast (*Saccharomyces cerevisiae*). In this article, we demonstrate that DI-72 RNA can efficiently replicate in yeast in the presence of p33 and p92 replicase proteins. Development of the yeast-based host system for replication of the TBSV DI RNA will introduce new ways

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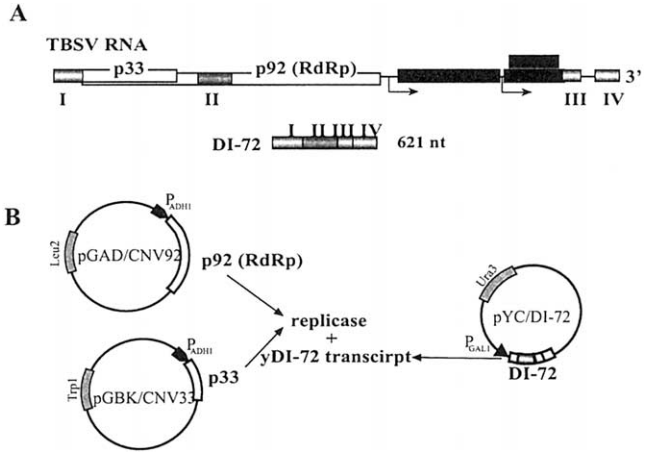


Fig. 1. Strategy of expression of the two CNV replicase genes and DI-72 RNA in *Saccharomyces cerevisiae*. (A) Schematic representation of RNA genome of a typical tombusvirus (TBSV) and the prototypical DI RNA, DI-72. The genomic RNA of TBSV contains five open reading frames. The replicase genes (open boxes) are directly expressed from the genomic RNA leading to major p33 and minor p92 production via a translational readthrough mechanism. Three other genes (coat protein, cell-to-cell movement, and suppressor of gene silencing, black boxes) are expressed from two subgenomic RNAs, depicted by arrows. The four noncontiguous regions in the genomic RNA, which are present in DI-72, are shown with gray boxes and roman numbers. (B) Schematic illustration of three plasmids used for simultaneous expression of p33 and p92 replicase proteins of CNV and DI-72 RNA in yeast cells. pGAD/CNV92 and pGBK/CNV33 are high copy number plasmids, while pYC/DI-72 is a low copy number plasmid. Constitutive expression of replicase proteins p92 and p33 (represented by open boxes) of CNV, which is closely related to TBSV, is driven by the ADH1 promoters from two separate plasmids. In contrast, DI-72 RNA is expressed under the inducible GAL1 promoter. The selectable marker genes, Leu2, Trp1, and Ura3, are depicted with gray boxes.

to study the mechanism of replication and the roles of host proteins in the replication process.

Results

Efficient replication of TBSV DI-72 RNA in the presence of CNV replicase proteins in yeast cells

To adopt yeast as a model host for studying tombusvirus replication, we tested the ability of DI-72 RNA, which is associated with TBSV infections, to replicate in yeast. Since previous studies in plant protoplasts defined that only p33 and p92 among the TBSV-coded proteins are required for *trans*-replication of DI-72 RNA (Scholthof et al., 1995; Oster et al., 1998), we coexpressed the replicase proteins with the DI-72 RNA in yeast. We have chosen the CNV p33 and p92 genes for these studies, since the CNV replicase proteins can support DI-72 RNA replication as efficiently as TBSV p33 and p92 can in plant protoplasts (White and Morris, 1995) and the partially purified CNV RdRp preparation obtained from plants can correctly recognize the *cis*-acting signals present in DI-72 RNA (Nagy and Pogany, 2000; Panavas and Nagy, 2003). However, functional CNV replicase proteins might be easier to purify from yeast than TBSV proteins, based on previous experience with purification of replicase complexes from plants (Nagy and Pogany, 2000).

The expression of both p33 and p92 proteins in yeast was based on a constitutive ADH1 promoter present in high copy plasmids (pGBKT7 and pGADT7, Invitrogen) to ensure the presence of high levels of these proteins for *trans*-replication (constructs pGBK/CNV33 and pGAD/CNV92, Fig. 1B). Indeed, using a polyclonal antiserum against p33 (generous gift of H. Scholthof), we detected both proteins in yeast by using Western blotting (not shown).

In contrast to the expression strategy used for p33 and p92, the DI-72 RNA was expressed under tight regulation and at a relatively low level. This was achieved by using a low copy plasmid (pYC2/CT, Invitrogen) and by positioning the cDNA of DI-72 behind the GAL1 promoter (P-GAL1), which has a very tight regulation of expression depending on the sugar supplied in the medium (construct pYC/DI-72, Fig. 1B). This expression strategy allowed us to induce transcription of DI-72 RNA (named yDI-72 to indicate the presence of 5′ and 3′ plasmid-derived sequences flanking DI-72 sequence, Fig. 2A) by culturing yeast in medium containing galactose. After yDI-72 transcripts reached high levels (12 h), we suppressed transcription from plasmid pYC/DI-72 by transferring the yeast cells to a glucose-containing medium (see below).

Transcription of yDI-72 does not yield the wt DI-72 RNA (Fig. 2A), because extended plasmid-derived sequences are present at both the 5′ and the 3′ ends and the yDI-72 transcripts are capped and polyadenylated, while the wt DI-72 RNA lacks these features (Russo et al., 1994). Since the wt 3′ end should be an advantage for replication of DI-72 RNAs, we placed a ribozyme derived from the antigenome of *Hepatitis delta virus* (HDV) (Pattnaik et al., 1992) at the 3′ end of the DI-72 sequence (Fig. 2A). Self-cleavage of yDI-72 transcripts by this ribozyme is expected to generate the wt 3′ end in DI-72 RNA (Fig. 2A), which contains the 19 nt 3′-terminal genomic promoter (gPR) (Panavas et al., 2002a, 2002b) for minus-strand synthesis. We expected that the replication of yDI-72 transcripts may correct the 5′ end, which carried ~110-nt 5′ leader sequence derived from the plasmid (shown as a straight line in Fig. 2A). Accordingly, our previous in vitro data with a partially purified CNV RdRp preparation demonstrated the ability of CNV RdRp to support efficient internal initiation from the 11-nt plus-strand initiation promoter present at the 3′ end of minus-stranded DI RNAs (Panavas et al., 2002a).

Total RNA was extracted from yeast cells coexpressing the p33 and p92 proteins and yDI-72 RNA and was used for Northern blot analysis with a DI-72-specific probe (Fig. 2B). Based on the size of yDI-72 transcripts (Fig. 2B, lane 1), we conclude that ribozyme cleavage of the primary transcripts were not efficient and the majority of yDI-72 transcripts contained both 5′ and 3′ plasmid-derived se-

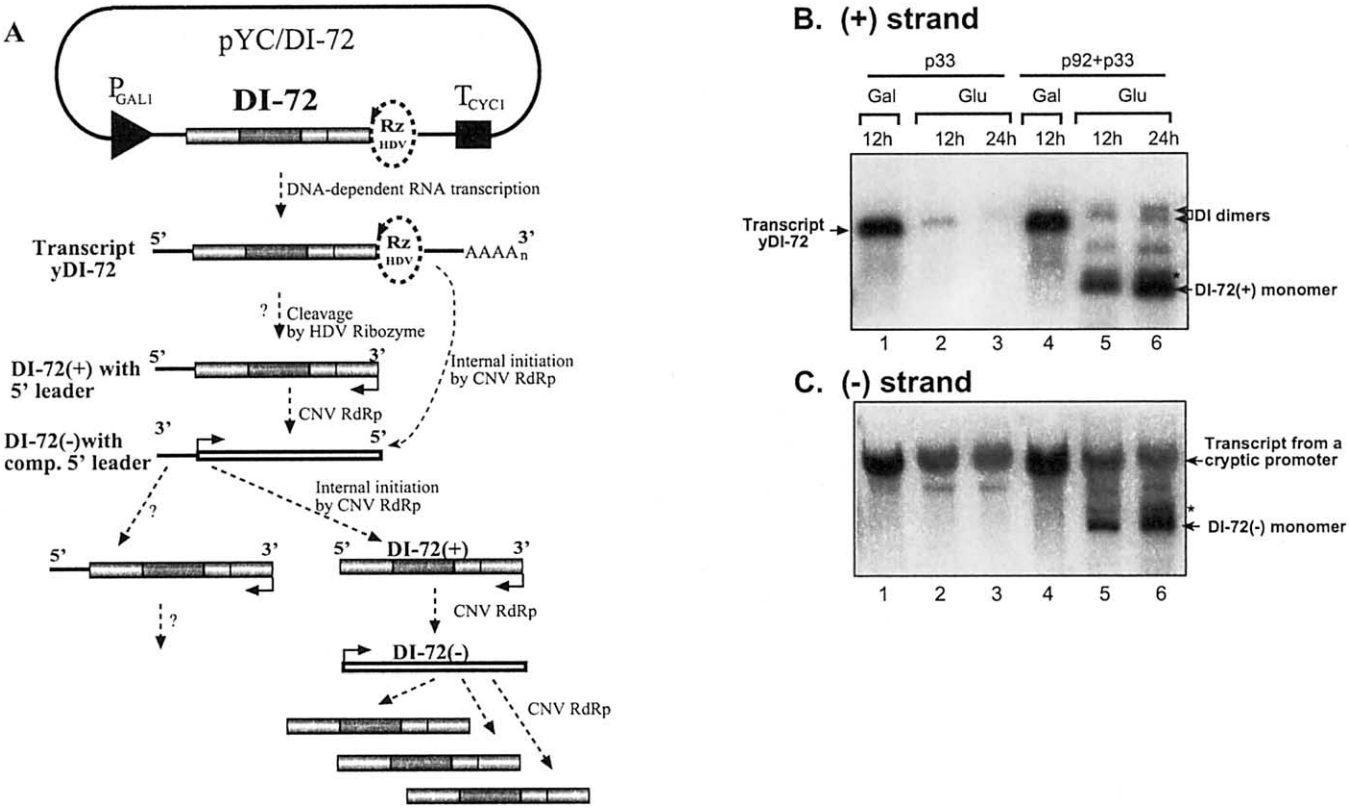


Fig. 2. Replication of DI-72 RNA in yeast cells expressing the CNV replicase proteins. (A) A schematic representation of the production of various DI-72 containing RNAs in yeast. DI-72 sequence is shown with four joined boxes that represent the four noncontiguous regions (Fig. 1A). The HDV antigenomic ribozyme at the 3′ end of DI-72 sequence is shown with a punctuated circle, while the GAL1 promoter and the CYC1 terminator are represented by closed triangle and rectangle, respectively. In yeast cells grown in galactose medium, yDI-72 transcripts, which have a plasmid-derived 5′ leader (straight line), DI-72, the ribozyme, and a 3′ tail with poly(A), is produced. After self-cleavage by the ribozyme, the authentic 3′ end of DI-72 is formed that contains the minus-strand initiation promoter (gPR) (Panavas et al., 2002a, 2002b) for the CNV replicase. Alternatively, internal initiation from the gPR promoter (3′ end of DI-72) may take place on the complete yDI-72 transcripts. The resulting (−)-stranded DI-72 RNA intermediate (still containing the complementary sequence of the 5′ leader) will be used by the CNV RdRp to produce (+)-stranded DI-72. Initiation of plus-strand synthesis may take place from two different locations: either from the 3′ end, producing DI-72(+) with the 5′ leader (shown on the left), or from the internal promoter (Panavas et al., 2002a), resulting in DI-72(+) RNA without extra sequences (shown on the right). Subsequent RNA replication cycles may produce more wt DI-72 progenies. (B) Northern blot analysis of accumulation of RNAs containing DI-72(+) sequence in yeast. Yeast was cotransformed with pYC/DI-72 along with either (i) pGBK/CNV33 and pGADT7 (shown as p33 on top); or (ii) pGAD/CNV92 and pGBK/CNV33 (p92+p33). Gal (galactose) and Glu (glucose) indicate the sugar used in the medium. Yeast cells were grown for 12 or 24 h (see Materials and methods for further details) before isolation of total RNA, gel electrophoresis, and blotting. Based on size estimation, the unit-length DI-72 RNA is shown as monomer, and the double-length DI-72 RNAs marked as dimer (lanes 5–6). The asterisk depicts the RNA that is ~100 nt longer than the unit-length DI-72(+) and potentially represents the DI-72(+) with the 5′ leader. (C) Northern blot analysis of accumulation of RNAs containing DI-72(−) sequence in yeast. The total RNA samples were the same as in (B). The unexpected (−)RNA transcripts produced from a cryptic promoter are indicated with an arrow. See (B) for further details.

quences in addition to the DI-72 sequence. As expected, yDI-72 transcripts were degraded in those yeast cells, which expressed only p33 (from pGBK/CNV33 and pGADT7, Fig. 2B, lanes 1–3), while grown in glucose-containing medium for 24 h. The same result was obtained when p92 was expressed instead of p33 (data not shown). These results demonstrated that yDI-72 transcripts could not replicate in yeast cells in the absence of either p33 or p92.

Coexpression of p33 and p92 in yeast, however, resulted in accumulation of new DI-72-like RNA products after suppression of the synthesis of yDI-72 transcripts (lanes 5–6, Fig. 2B). The fast migrating major RNA band comigrated with the in vitro transcribed wt DI-72 RNA marker (we call this product DI-72 RNA monomer in Fig. 2B). Importantly, the amount of DI-72 RNA monomer increased during culturing the yeast cells for 12 and 24 h (compare lanes 5 and 6, Fig. 2B), suggesting that this RNA likely replicates in yeast cells. In this experiment, we also observed at least three additional minor RNA products, which migrated slower than the DI-72 RNA monomer. One minor RNA band (marked with an asterisk in Fig. 2B) right above the DI-72 monomer might represent a DI-72 RNA still carrying the entire or partial 5′ leader sequence, but lacking the 3′ tail present in yDI-72 (see below). The slowest migrating RNAs were predicted, based on size estimation, to be head-to-tail dimers formed during DI-72 RNA replication (see below).

To obtain further evidence for replication of DI-72 RNA in yeast, we tested the total RNA extracts for the presence

of minus strands of DI-72 RNA using Northern blot with a minus-strand-specific DI-72 RNA probe. Importantly, we detected two fast migrating RNA bands only in those samples that had both replicase proteins expressed (Fig. 2C, lanes 5 and 6). Based on size estimation, the faster migrating band corresponded to DI-72(−) RNA, thus confirming replication of DI-72 RNA via a minus-strand intermediate. As expected, the amount of this RNA is increased over time, as was also the case for the corresponding plus-strand DI-72 RNA. We estimated that the amount of DI-72 minus strand in yeast is ~200-fold less abundant than the positive-strand. We also estimated a similar 1:200 ratio between minus and plus strands in plant protoplasts (*N. benthamiana*) (J. Pogany and P.D. Nagy, unpublished data). The second fast-migrating RNA (marked with an asterisk in Fig. 2C, lanes 5 and 6) was ~100 nt longer than DI-72(−) and its amount also increased over time. This RNA may represent a DI-72(−) RNA, which still contains plasmid-derived sequences (i.e., the complementary sequence of the 5′ leader, see above). This observation suggests that the DI-72 RNA with the 5′ leader sequence can produce minus-strand RNA and it might replicate at a limited extent in yeast cells.

Unexpectedly, we also detected a major minus-strand RNA in all samples that derived from yeast growing on glucose-containing medium, regardless whether both p33 and p92 or only p33 were expressed (lanes 2–3 and 5–6, Fig. 2C). Since this minus-strand RNA was much larger than DI-72(−) RNA marker and it was present in samples lacking p92 (therefore no replication should take place), we propose that this major RNA is a transcript generated via RNA Polymerase II transcription from a cryptic promoter located 3′ from DI-72 sequence. A similar phenomenon was observed from a vector expressing *Flock house virus* in yeast (Price et al., 2002).

Unlike during the natural tombusvirus infections of plants, where the p92 protein is expressed via a ribosomal readthrough of the stop codon at the 3′ end of the p33 gene (Fig. 1A), the yeast cells in the above experiments expressed the two replicase proteins from separate plasmids (instead of the wt p92 gene, we used p92Y gene that carried a tyrosine codon in place of the p33 stop codon) (Panaviene et al., 2003). To test if the two replicase proteins can be expressed in yeast via the readthrough strategy used by tombusviruses in plant infections (Scholthof et al., 1995; Oster et al., 1998), we constructed plasmid pGBK/CNV33/92. This plasmid carried the wt p92 gene, including the stop codon at the 3′ end of the p33 gene (as shown in Fig. 1A); thus the production of wt p92 protein depends on the ability of the yeast strain to readthrough the stop codon during translation. Since p92 is essential for DI RNA replication in yeast (see above), we used the DI replication assay (Fig. 2) that includes pYC/DI-72 to test if p92 is expressed from plasmid pGBK/CNV33/92. Northern blot analysis of the total RNA extract obtained from yeast (cotransformed with pGBK/CNV33/92 and pYC/DI-72 and grown at 20°C) revealed that replication of DI-72 RNA took place, albeit inefficiently (Fig. 3, lanes 2 and 3). We estimated that replication of DI-72 RNA was supported by the readthrough construct (pGBK/CNV33/92) at ~12% of the level obtained with the yeast strain expressing p33 and p92Y from separate plasmids (not shown). This suggested that the production of p92 via the readthrough mechanism is inefficient in yeast. Indeed, when the yeast strain was cotransformed with pGBK/CNV33/92 and pGAD/CNV92, which expressed p92Y independently of p33, then we found ~50-fold increase in DI-72 replication (Fig. 3, compare lanes 2–3 and 5–6).

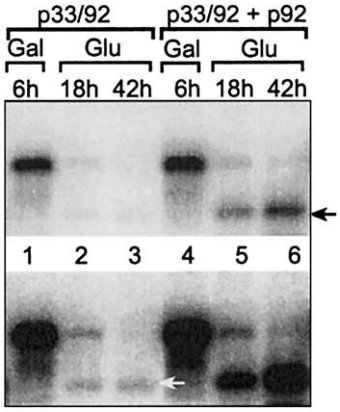


Fig. 3. Inefficient replication of DI-72 RNA when the replicase proteins were expressed via a translational readthrough mechanism in yeast. Northern blot analysis of total RNA extracts obtained from yeast carrying plasmids with the wild-type p33/92 gene alone or together with a p92 gene (p92Y, carrying a tyrosine in the place of p33 stop codon; Panaviene et al., 2003) along with expression of yDI-72 transcripts. The bands representing the replicating DI-72 are marked with arrows. The two images represent the same experiment, except the bottom image was obtained by longer exposure to visualize faints bands.

DI-72 RNA replicating in yeast cells contains authentic 3′ and 5′ ends

To test if the replicating DI-72-like RNA in yeast (lanes 5 and 6, Fig. 2B) has authentic 5′ and 3′ ends (generated via deletion of the plasmid-derived sequences from yDI-72 RNA transcripts by the CNV RdRp), we determined the sequences present at both ends of the RNAs. First, to analyze the 3′ termini of the positive-sense DI RNAs, we adopted the 3′ RACE method. Briefly, the total RNA was extracted from yeast with either nonreplicating (lanes 1–3, Fig. 2B) or replicating DI RNAs and were subjected to 3′RACE. We detected the predicted-sized 3′RACE product in the samples obtained from both replication-incompetent (due to lack of p92 expression) and replication-competent yeast cells (Fig. 4A). The amount of the 3′RACE product was more in the replication-competent sample, suggesting that replication likely increased the amount of DI RNA with corrected 3′ ends. The minor 3′ RACE product in the replication-incompetent sample (lane 1 in Fig. 4A) was likely due to DI RNA generated from yDI-72 transcripts via

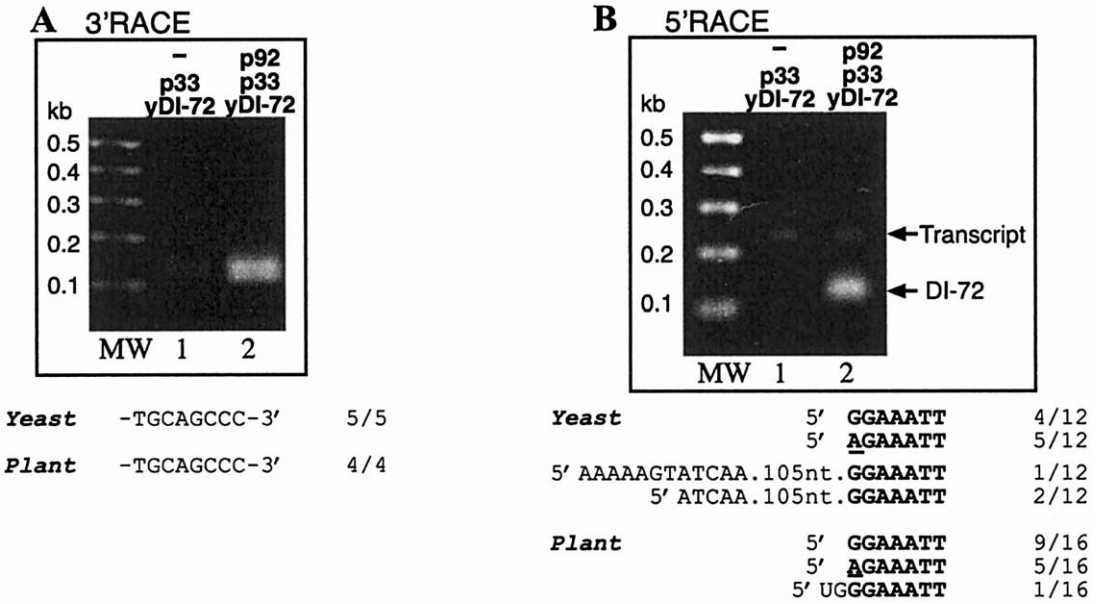


Fig. 4. Analysis of the 3′ and 5′ terminal sequences in DI-72 isolated from yeast and plant. (A) Agarose gel electrophoresis of RT-PCR products obtained by 3′ RACE. The gel was stained with ethidium bromide. The molecular weight standards are shown in the left lane. Lanes 1 and 2 contain the 3′ RACE products obtained from total RNA samples of yeast expressing p33/yDI-72 and p92/p33/yDI-72. The actual 3′ end of DI-72(+) RNA was determined by cloning and sequencing of the 3′ RACE product (lane 2). The number of independent clones used for sequencing is shown on the right. To allow comparison between DI RNA isolated from yeast and plant, 3′ RACE, cloning and sequencing was also done on RNA samples obtained from *N. benthamiana* protoplasts (Panaviene et al., 2003), electroporated with in vitro generated DI-72 and gCNV transcripts. (B) Agarose gel electrophoresis of RT-PCR products obtained by 5′ RACE. See (A) for details. 5′ sequences were determined for 12 and 16 clones obtained by 5′ RACE from total RNA extracts of yeast (lane 2) and plant protoplast (not shown), respectively. Bold and plain letters indicate DI-72 and 5′ plasmid-derived sequences (only the 5′ portion is shown), respectively, while underlined letters show the wt 5′-AG sequence.

ribozyme cleavage (Fig. 2A). The 3′RACE product from the replication-competent sample was gel isolated, cloned, and sequenced. Five random clones chosen for sequencing had authentic 3′ end sequences when compared to wt DI-72 derived from *N. benthamiana* protoplasts coinoculated with CNV genomic and DI-72 RNA transcripts (Fig. 4A). Due to the 3′ RACE methodology, we cannot exclude if an additional adenylate was present in the 3′ end of these DI RNAs. Based on these data, we conclude that at least the majority of replicating DI RNA molecules in yeast cells has wt 3′ ends.

To identify the 5′ end sequences in the replicating DI RNAs, we used a standard 5′ RACE method (Invitrogen). Two 5′ RACE products were detected in the replication competent samples: a short, major 5′ RACE product and an ~110 bp longer minor product (Fig. 4B, lane 2). The minor 5′ RACE product was also detected in the replication-incompetent sample (Fig. 4B, lane 1), suggesting that this product was derived from yDI-72 transcripts. Sequence analysis of the cloned 5′ RACE products revealed that 25% of the clones (3 of 12) contained 5′ sequences predicted to be present in yDI-72 transcripts (i.e., the ~110-nt 5′ leader sequence due to transcription starting from the GAL1 promoter) (Fig. 4B). The majority of the clones (9 of 12), however, contained "corrected" 5′ sequences, which were similar to sequences present in DI-72 RNA obtained from *N. benthamiana* protoplasts (Fig. 4B). Interestingly, we found that the actual 5′ sequences in the replicating DI RNAs contained sequence heterogeneity in both yeast and plant protoplast samples. The heterogeneity frequently included the 5′-GG sequence derived from the transcripts and the 5′-AG sequence, which may represent the wt 5′ end (Szittya et al., 2000). The overall similarity between the 5′ ends of DI RNAs replicating in yeast and plant protoplasts suggests that the CNV replicase supported replication of DI-72 RNA in yeast cells mimics DI RNA replication in the natural plant host.

Effect of 3′ nonviral sequences on DI RNA replication

To test if the HDV ribozyme located at the 3′ end of yDI-72 RNA increased the efficiency of DI RNA replication by generating the correct 3′ end, we compared the replication of DI-72 RNA carrying or lacking ribozymes at the 3′ end of the yDI-72 transcripts (Fig. 5A). yDI-72 transcript with 3′ extra nonviral sequences that lacked a ribozyme replicated inefficiently in yeast coexpressing p33 and p92 (Fig. 5A, lanes 1–3). In contrast, yDI-72 RNA transcript with the HDV ribozyme at the 3′ end, which can generate the wt-like 3′ end (CCC-3′) after self-cleavage, resulted in a 37-fold higher level of DI-72 replication than that obtained with yDI-72 lacking ribozyme (Fig. 5A, lanes 4–6).

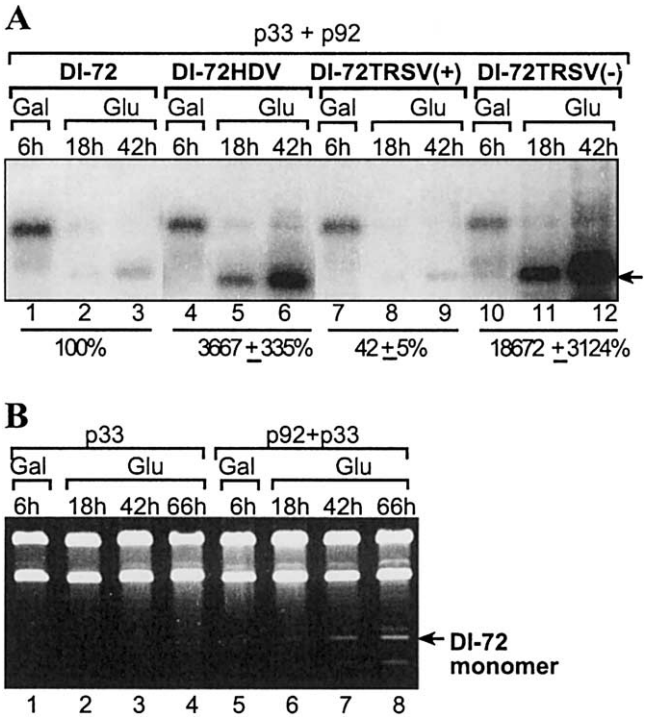


Fig. 5. Influence of various ribozymes at the 3′ end of yDI-72 transcripts on replication of DI-72 RNA in yeast. (A) Northern blot analysis of replication of yDI-72 RNA without a ribozyme at the 3′ end or with HDV, TRSV satellite (+) [TRSV(+)], or TRSV satellite (−) [TRSV(−)] ribozymes at the 3′ end. After induction in galactose-containing medium, yeast were transferred and cultured at 20°C in glucose-containing medium for the number of hours indicated above the lanes. The cultures were diluted 10 times with fresh medium at 0- and 18-h time points. The numbers below the gel show quantification of the relative accumulation of DI-72 RNA at the 42 h time point. The band representing the replicating DI-72 RNA is marked with an arrow. See other details in the legend to Fig. 2. (B) Total RNA extracts obtained from yeast were analyzed by agarose gel electrophoresis and ethidium bromide staining. The band representing the replicating DI-72 RNA is marked with an arrow. Note that the additional bands represent replication of yDI-72-derived RNAs either carrying 5′ extra nonviral sequences (slow migrating band) or lacking the region I sequence of DI-72 (fast migrating band).

In contrast, the presence of a ribozyme from *Tobacco ringspot virus* (TRSV) satellite RNA [TRSV(+), Ishikawa et al., 1997] in yDI-72 that could generate *CCCGUC* end (the wt sequence is underlined, while the nonviral sequence is italicized) did not increase the efficiency of DI-72 RNA replication (Fig. 5A, lanes 7–9). The last construct tested carried a ribozyme from TRSV satellite (−) [TRSV(−), Buzayan et al., 1986], which generated wt 3′ end, and supported DI-72 RNA replication by ~190-fold higher efficiency than the ribozyme-less construct in yeast (Fig. 5A, lanes 10–12). Interestingly, the replicating DI-72 RNAs were visible on ethidium bromide stained gels (Fig. 5B, lanes 6–8). When compared to the total RNA, we found that the level of DI-72 accumulation reached ~10% of that observed in plant protoplasts (not shown). Overall, we conclude that the correct 3′ end is not necessary, but certainly helpful for efficient DI-72 replication in yeast.

The role of region III replication enhancer in DI RNA replication in yeast

To further investigate the similarity between replication of DI-72 in yeast and in plant, we analyzed the role of a *cis*-acting element, namely the region III replication enhancer (Panavas and Nagy, 2003; Ray and White, 1999, 2003). First, we expressed construct yDI70F-MD1, which carried two mutations that disrupted the secondary structure in minus-stranded region III and caused fivefold reduction in DI-72 accumulation in cucumber protoplasts (Ray and White, 2003). The second mutant expressed in yeast was yDI-72SXPΔIII, which lacked the entire region III replication enhancer and showed 10-fold reduction in replication in protoplasts (Ray and White, 1999). We found that the coexpression of these mutants with p33 and p92 resulted in a significantly reduced level of DI RNA accumulation in yeast (5.4 and 0.2% of that of wt, Fig. 6). Thus, similar to the findings in plant protoplasts, region III was able to regulate the level of DI RNA replication in yeast. Note that the above experiments in yeast were conducted at 20°C, since replication of yDI-72SXPΔIII was not readily detectable in yeast grown at 30°C (not shown). This may be due to reduced replication rate for TBSV DI RNAs at higher temperature, which was indeed observed when TBSV DI RNAs replicated above 27°C in plant cells (Jones et al., 1990). Overall, the significant role of region III replication enhancer in DI RNA accumulation in yeast supports evidence that DI-72 replication in yeast is regulated by *cis*-acting elements in a manner similar to plant cells.

Evidence for recombination of DI-72 RNA in yeast

DI RNAs associated with various tombusviruses are known to undergo frequent recombination in plant cells by

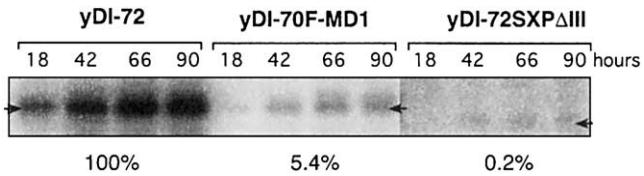
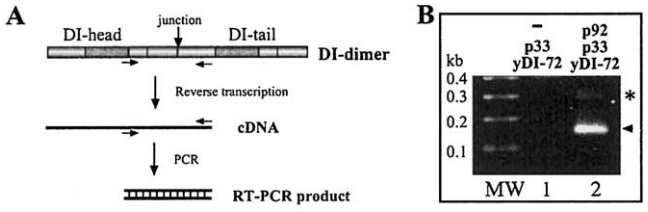


Fig. 6. Replication of DI-72 mutants in yeast cells expressing the CNV replicase proteins. Northern blot analysis was done as described in the legend to Fig. 2. The RNA transcripts produced in yeast cells were yDI-72 and its derivatives, which had either mutations within the region III replication enhancer (yDI-70F-MD1) (Ray and White, 2003) or lacked the entire region III sequence (yDI-72SXPΔIII) (Ray and White, 1999). The yeast cells were cotransformed with one of the above constructs along with pGAD/CNV92 and pGBK/CNV33. After induction in galactose-containing medium, yeast were transferred and cultured at 20°C in glucose-containing medium for the number of hours indicated above the lanes. The cultures were diluted 10 times with fresh medium at 0-, 18-, 42-, and 66-h time points. Isolation of total RNA, gel electrophoresis, and blotting were done as in Fig. 2. Arrows show the expected migration of the replicating DI RNA products. The percentages below the gel show a PhosphorImager-based quantification of the replicating DI RNAs (level of wt DI-72 = 100%).



C

3'of DI-head	Junction	5'of DI-tail	Frequency
AAATGCAGCCC		-GAAATTCTCCA	1/35
AAATGCAGCCC		GGAAATTCTCCA	6/35
AAATGCAGCCC	T	GGAAATTCTCCA	3/35
AAATGCAGCCC	**AA**	-GAAATTCTCCA	1/35
AAATGCAGCCC	**CA**	GTAAATTCTCCA	1/35
AAATGCAGCCC	**CT**	GGAAATTCTCCA	1/35
AAATGCAGCCC	**CAT**	GGAAATTCTCCA	1/35
AAATGCAGCCC	CTT	GGAAATTCTCCA	2/35
AAATGCAGCCC	**GGT**	GGAAATTCTCCA	6/35
AAATGCAGCCC	GCTT	GGAAATTCTCCA	5/35
AAATGCAGCCC	**GTGGA**	-GAAATTCTCCA	1/35
AAATGCAGCCC**C**---------ATCA	105	GGAAATTCTCCA	4/35
AAATGCAGCCC**CA**--AAAAGTATCA	105	GGAAATTCTCCA	2/35
AAATGCAGCCC**CAATAAAAGTATCA**	105	GGAAATTCTCCA	1/35

Fig. 7. Analysis of head-to-tail dimers formed during DI-72 RNA replication in yeast. (A) Schematic representation of the RT-PCR product generated through the junction in the DI-72 dimer. Arrows represent the two primers used. (B) Ethidium bromide stained agarose gel of RT-PCR products. The molecular weight standards are shown in the left lane. Lanes 1 and 2 contain the RT-PCR products obtained from total RNA samples of yeast expressing p33/yDI-72 and p92/p33/yDI-72 (see Fig. 2), respectively. The arrowhead points to the expected RT-PCR product generated from DI-72 dimer, while an asterisk marks a product ~110 bp longer than that obtained with the expected head-to-tail DI-72 dimer. (C) The actual junction sequences in head-to-tail DI-72 dimers were determined by cloning and sequencing of the RT-PCR products (lane 2, Fig. 7A). The number of independent clones used for sequencing is shown on the right. Deleted sequences are indicated with -, while the extra (nontemplated) sequences found at the junctions are shown with boldface letters. The plasmid-derived sequences (derived from the 5′ leader sequence, Fig. 2A) at the junctions are shown with plain letters. The number indicates the presence of 105 nt not shown plasmid-derived sequence.

forming head-to-tail dimers and multimers (the latter with lower frequencies) (Finnen and Rochon, 1995; Dalmay et al., 1995). Interestingly, we also observed dimer-sized DI-72 RNAs on the Northern blot in those yeast samples, in which DI-72 replicated efficiently (Fig. 2B, lanes 5 and 6). To test if these RNAs indeed represented head-to-tail DI-72 dimers, we performed an RT-PCR assay with a primer pair that is designed to allow amplification through the junction site (Fig. 7A) (Finnen and Rochon, 1995). We used total RNA extracts prepared from yeast expressing only p33 (predicted to be replication and recombination incompetent) as a control. Accordingly, we obtained a large amount of RT-PCR products only with total RNA samples from replication-competent yeast cells (Fig. 7B, lane 2), but not from the replication-incompetent yeast cells (Fig. 7B, lane 1). The size of the major RT-PCR product was consistent with the predicted size based on DI-72 dimers. A second fainter RT-PCR product, which was ~110 bp larger than the major RT-PCR product, was also generated in the case of the replication-competent sample. Based on the predicted size of this RT-PCR product, we assumed that this product also represents a DI-72 dimer, but containing the plasmid-derived ~110-nt leader sequence in addition to the duplicated DI-72 sequences. Indeed, sequencing of the cloned RT-PCR products from the replication-competent samples confirmed that two types of dimers were generated in these yeast cells. The majority of the clones (28 of 35) had the "standard" head-to-tail DI-72 dimers with the 5′ end sequence of one copy of DI-72 fused to the 3′ end of a second copy, while the rest of the clones contained the 5′ plasmid-derived leader sequence between the duplicated DI-72 sequences (Fig. 7C). Interestingly, 51% of the clones contained one to five extra nucleotides between the duplicated DI-72 sequences (Fig. 7C). In summary, the formation of DI-72 RNA dimers through RNA recombination only in those yeast cells that coexpresses p33 and p92 supplies evidence that DI-72 recombination, similar to replication, is driven by the tombusvirus replicase in yeast.

Isolation of active CNV RdRp complexes from yeast

Since CNV and TBSV RdRp complexes are actively involved in virus and DI RNA replication in plants and these complexes can be purified in active forms from tombusvirus-infected plants (Nagy and Pogany, 2000), we tested if similar complexes might also be formed in yeast cells coexpressing the replicase proteins (p33 and p92) plus yDI-72 RNA. To isolate the CNV RdRp complex, we used yeast cells, in which DI-72 RNA replication took place, while transcription was suppressed (cells were grown for 66 h in glucose-containing media). Similar to the RdRp purification procedure developed for plants, we separated the membrane-containing fraction from the soluble fraction by centrifugation. Both fractions were then used separately in standard RdRp reactions that included [^{32}P]UTP (Nagy and Pogany, 2000). As expected, the membrane fraction obtained from yeast coexpressing p33 and p92 contained the functional RdRp complex with the endogenous templates (Fig. 8). In addition, these experiments revealed that no active RdRp complex was obtained from yeast cells that expressed only the p33 and yDI-72 (Fig. 8). A majority of the RdRp products after the in vitro RdRp reaction was double-stranded (S1 nuclease-resistant). One band represented the wt DI-72-sized product, demonstrating that the isolated RdRp complexes were involved in synthesizing DI RNAs at the time of sample preparation. A slower migrating double-stranded RdRp product might represent DI-72 containing the ~110-nt nonviral extra nucleotides at the 5′ end, while the fast migrating double-stranded RdRp product represents a truncated version of DI-72 RNA lacking region I sequence (Fig. 1), as demonstrated by Northern blot analysis (not shown). These types of truncated DI RNAs have also been detected in plant protoplasts before (Wu and White, 1998).

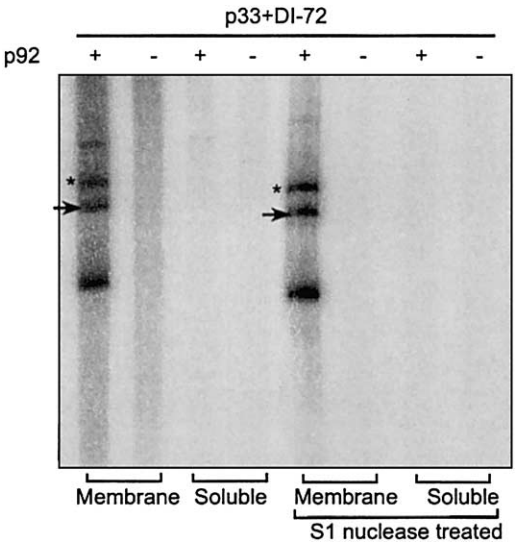


Fig. 8. Presence of active RdRp in yeast expressing p33 and p92 replicase genes. Membrane-containing or soluble extracts were prepared from yeast grown at 20°C after suppression of yDI-72 expression. Standard in vitro RdRp assays were performed in the presence of [^{32}P]UTP without added RNA templates. Half of each RdRp product was S1 nuclease treated. The obtained RdRp products were separated on 5% polyacrylamide gel. The band representing double-stranded DI-72-sized RNA is marked with an arrow.

Discussion

The genetically tractable yeast is an excellent model host to study the mechanism of plus-strand RNA virus replication, as demonstrated for BMV (Ahlquist, 2002; Diez et al., 2000; Noueiry et al., 2000; Schwartz et al., 2002). The data presented in this article demonstrate that the TBSV-associated DI-72 RNA (a prototypical DI, Fig. 1, White and Morris, 1999) replicates efficiently in yeast cells coexpressing both p33 and p92 replicase proteins of the closely related CNV (Fig. 2B). Evidence for DI-72 replication in yeast includes the following: (i) DI-72 RNA did not replicate when only one of the replicase proteins was expressed in yeast (Fig. 2B and not shown). Thus, similar to plant protoplast infections, DI-72 RNA requires both p33 and p92 for its replication. (ii) In addition, we found that active RdRp preparation involved in DI-72 RNA synthesis could be obtained only from those yeast cells that coexpressed p33 and p92 proteins. (iii) As expected for replication, the amount of DI-72 RNA increased over time in yeast cells grown under conditions suppressing yDI-72 RNA transcription. (iv) Also, minus-strand DI-72 RNAs, which are replication intermediates, were present only in those cells coexpressing p33 and p92 (Fig. 2C). (v) Furthermore, sequence analyses of DI-72 progeny revealed that both the 5′ and the 3′ ends contained authentic (correctly processed) sequences, despite the presence of extra plasmid-derived sequences in the yDI-72 transcripts. This can be explained by the correct recognition of the terminal promoters (present in plus- and minus-strand DI RNA) (Panavas et al., 2002a, 2002b) during the replication process conducted by the CNV replicase. Non-replication-based processing of terminal sequences is likely only for the 3′ end sequence due to the presence of the HDV antiribozyme in the yDI-72 transcripts, but it is unlikely in the case of the 5′ end sequence. Indeed, the possibility of correct recognition of internally located promoter sequences by the CNV RdRp has been demonstrated before using a partially purified CNV replicase (RdRp) preparation (Panavas et al., 2002b). (vi) We also demonstrated that DI-72 RNA with mutated region III replication enhancer replicated poorly in yeast (Fig. 6). Deletion of region III (in the minus-strand) also resulted in reduced RNA synthesis in vitro by our CNV RdRp preparation (Panavas and Nagy, 2003). (vii) Accumulation of DI-72 dimers in yeast cells expressing p33 and p92 also supports the active CNV replicase-driven mechanism, since both p33 and p92 were required for the generation of these products (Fig. 7).

Comparison of replication of DI-72 RNA in plant protoplasts (Jones et al., 1990; Scholthof et al., 1995; White and Morris, 1994) and in yeast cells reveals many common trends, including the asymmetrical replication of plus-versus minus-strand DI RNAs (~200:1 ratio); the evolution of DI-72 RNAs toward the 5′-AG sequence, although the DI-72 transcripts contained the 5′-GG sequence; the dependence of DI RNA replication on the presence of *trans*-acting p33 and p92 replicase proteins and *cis*-acting regulatory sequences, such as the region III replication enhancer, and the presence of RdRp activity. In summary, these similarities between the plant and yeast hosts in DI-72 replication indicate that yeast can be a useful model host for studies on RNA replication.

Finding of head-to-tail dimers of DI-72 RNAs (Figs. 2B and 7) in yeast cells expressing p33 and p92 suggests that RNA recombination is rather frequent under the appropriate growing conditions. RT-PCR analysis, followed by cloning and sequencing around the junction sites, revealed that recombination is likely driven by the CNV replicase via template-switching mechanism. The supporting evidence includes the requirement for both p33 and p92 for dimer formation (Fig. 2B) and the presence of one to five nontemplated nucleotides at many junction sites (Fig. 7). In addition, the presence of 5′-GG sequence and the absence of 5′-AG within the junction sites in the dimers suggests that recombination occurred relatively early during replication, before the evolution of DI-72 RNA resulted in the accumulation of DI-72 RNAs carrying the wt 5′-AG sequence (Fig. 4). Interestingly, dimer formation is also common in protoplasts infected with various tombusviruses and DI RNAs. Many of these DI dimers derived from plants also contain nontemplated nucleotides at the junction region (Finnen and Rochon, 1995; Dalmay et al., 1995), suggesting that DI dimer formation is similar in plant and yeast cells. Even more surprisingly, DI-72 RNAs carrying the plasmid-derived 5′ leader sequence also participated in the recombination events. This observation in combination with data on

minus-strand RNA accumulation (see minus-strand RNA corresponding in size with DI-72 containing the 5′ leader sequence, Fig. 2C) suggest that DI-72 RNA with nonviral 5′ sequences is competent for replication and recombination, albeit with reduced efficiency when compared to the processed wt DI-72 RNA. Inefficient initiation of RNA synthesis from non-TBSV sequences has also been observed in the CNV RdRp assay in vitro (Panavas et al., 2002b), supporting the above model. Alternatively, DI-72 RNA with 5′ plasmid-derived sequence could only produce its complete minus-strand RNA copy (but not complete plus-strand copies, Fig. 2A), which might be involved in dimer formation/recombination in yeast cells.

In conclusion, the many observed similarities between plant and yeast hosts in supporting replication and recombination of TBSV DI RNA demonstrate the usefulness of the yeast system for future studies. Indeed, a work performed independently and in parallel to ours has demonstrated *trans*-replication of DI RNA in yeast by *Carnation Italian ringspot virus*, another tombusvirus (Pantaleo et al., 2003). These authors demonstrated that the yeast cells expressing the p36 and p95 replicase proteins of CIRV can correctly recognize the terminal sequences in a DI RNA and the replication likely takes place on the outer membranes of the mitochondria. Comparison of the level of plus strands and minus strands suggests that the replication process is more robust in the above CNV-based system than in the CIRV-based system. Overall, these developments will likely contribute to progress in studies of host–virus interactions in tombusviruses.

Materials and methods

Plasmid construction

Three expression plasmids were constructed in yeast (Fig. 1B). Plasmid pGAD/CNV92 was constructed by placing the CNV p92 gene behind the ADH1 promoter in pGADT7, which carries LEU2 selectable marker (Clontech). The pGADT7 plasmid was digested with *Hin*dIII followed by blunting with *Klenow* (NEB). After phenol/chloroform extraction, the plasmid was cut with *Xho*I, gel isolated, and used for ligation with the CNV p92 gene, which was amplified using CNV with the stop codon at the end of p33 gene replaced with the tyrosin codon (p92Y) (Panaviene et al., 2003) as a template. PCR amplification was carried with DeepVent DNA polymerase using primers 951 (5′-AAAGATGGATACCATCAAGAGGATGCTGT) and 952 (5′-CCCGCTCGAGTCATGCTACGGCGGAGTCAAGGA). After phosphorylation with T4 kinase (NEB) and digestion with *Xho*I, the fragment was cloned into the above pGADT7. To generate pGAD/CNV33/92 construct, we followed the same method as for pGAD/CNV92, except that the wild-type gCNV clone was used as a template for PCR.

The second plasmid was pGBK/CNV33, carrying the wild-type CNV p33 gene inserted behind an ADH1 promoter present in plasmid pGBKT7 with a TRP1 selectable marker (Clontech). pGBKT7 was digested with *Bsp*1407I (MBI Fermentas) and *Nco*I (NEB), followed by gel purification. We used two separate PCR fragments for simultaneous insertions into the above plasmid. One PCR product was generated using pGBKT7 as a template with primers 953 (5′-GATCCTTTTGTTGTTTCCGGGTGTACAATA) and 954 (5′-CCCGCTCGAGTTGGAGTTGATTGTATGCTT). This PCR product was digested with *Bsp*1407I and *Xho*I. The second PCR product included the p33 gene. It was obtained by using primers 955 (5′-CCCGCTCGAGAAAGATGGATACCATCAAGAGGATGCT) and 956 (5′-GCCCACCATGG-CTATTTCACACCAAGGGACTCA) and pK2M5 (Rochon and Johnston, 1991) as template. The PCR product was subsequently digested with *Xho*I and *Nco*I.

The third construct was pYC/DI-72, which was based on the low copy number pYC2/CT plasmid with URA3 selectable marker (Invitrogen). The sequences coding for DI-72 RNA plus the *Hepatitis delta virus* antigenomic ribozyme (Pattnaik et al., 1992; a generous gift of Andy Ball) were inserted behind the GAL1 promoter between the *Hin*dIII/*Sac*I sites in the polylinker of pYC2/CT. The DI-72 and the HDV insert was prepared in a two-step PCR reaction, where the first PCR was done using primers 542 (5′-GCCCGAAGCTTGGAAATTCTCCAGGATTTC) and 544 (5′-CCGCGAGGA-GGTGGAGATGCCATGCCGACCCGGGCTGCATTTCTGCAATG) and template DI-72SXP (White and Morris, 1994; a generous gift of Andy White). The second PCR was done with primers 542 and 545 (5′-GGCCGGAGCTCTCCCTTAGCCATCCGAGTGGACGACGTCCTCCTTCGGATGCCCAGGTCGGACCGCGAGGAGGTGGAGA) using the gel-isolated product from the first PCR reaction as template. The final PCR product was digested with *Hin*dIII and *Sac*I and inserted between the *Hin*dIII/*Sac*I sites in pYC2/CT.

The TRSV-satellite (+) (Ishikawa et al., 1997) and (−) ribozymes (Buzayan et al., 1986) [(TRSV(+) and TRSV(−)] were generated in sequential, two-step PCR reactions. The primers of the primary PCR for TRSV(+) were 1063 (GTCACCGGATGTGTTTTCCGGTCTGATGAGTCCGTGAGGA) and 1065 (GAGCTCGCATTCCAGTTTCGTCCTCACGGACTCATCAGAC). The PCR product was gel isolated and used in the secondary PCR with primers 1063 and 1066 (GACCGGTCGAGCTCGCATTCCAGTTTCGTCCTC). For TRSV(−), the primary oligo sets were 1067 (AGTCCTGTTTCTTGCCAAACAGAGAAGGGCACCAGAGAAA) and 1068 (GGTAATATACCACAACGTGTGTTTCTCTGGTGCCCTTCTC) and the secondary sets were 1067 and 1069 (CCGGTCGAGCTCTACCAGGTAATATACCACAACGTGTGT). The secondary PCR products were gel isolated and blunt-end ligated to the 3′ end of DI-72 followed by digestion of DI-72-Rz with *Hin*dIII and *Sac*I and ligated to pYC2/CT as described above.

Yeast transformation and induction of expression

S. cerevisiae strain INVSc1 was purchased from Invitrogen (Carlsbad, CA, USA). Yeast was transformed with plasmids using the LiAc/SS-DNA/PEG method according to Gietz and Woods (1998). Transformation was performed simultaneously with all three plasmids (pGAD/CNV92, pGBK/CNV33, and pYC/DI-72) and cells were plated on selective minimal SC medium lacking leucin, tryptophan, and uracil (SC-ULT). As a control, the original pGADT7 was used instead of pGAD/CNV92. First, we cultured the positive yeast transformants for 12 h in SC-ULT containing 2% glucose, followed by harvesting yeast cells by centrifugation and resuspension in SC-ULT medium containing 2% galactose for induction of transcription of yDI-72 RNA transcripts. After 12 h growth at 30°C, the yeast suspension was diluted 1:10 with the selective medium containing 2% glucose. Further incubation lasted for 12 h at 30°C (or for 24 h at 20°C in some experiments as specified in the figure legends). Second dilution of the cultured yeast cells with SC-ULT + 2% glucose, followed by incubation at 30 or 20°C, was as described above.

RNA extraction and Northern blot analysis

For extraction of total RNAs, yeast cells were broken by vigorous shaking for 10 min at 4°C with acid washed glass beads in RNA extraction buffer containing 50% phenol. The obtained total RNA (10 μg) was separated on a 1% agarose gel and the RNA was transferred to Hybond-XL membrane (Amersham Pharmacia) and hybridized with DI-72 specific probes (Nagy et al., 2001; Panaviene et al., 2003). The RNA probes were prepared by T7 RNA polymerase in an in vitro transcription reaction in the presence of [α-^{32}P]UTP using PCR-amplified DNA templates (Nagy et al., 1999, 2001). To detect positive-strand DI-72 RNA, we prepared the PCR product with primers 253 (5′-TTGGAAATTCTCCTTAGCGAGTAAGACAGACTC) and 22 (5′-GTAATACGACTCACTATAGGGCTGCATTTCTGCAATGTTCC) using DI-72SXP as template. To detect the minus strands (Nagy et al., 2001), we generated the PCR product with primers 18 (5′-GTAATACGACTCACTATAGGAGAAAGCGAGTAAGACAG) and 21 (5′-GGGCTGCATTTCTGCAATGTTCC) using DI-72SXP as template. For minus-strand detection, total RNA obtained from yeast was separated on a denaturing 5% polyacrylamide/8 M urea gel as described previously (Panavas et al., 2002a).

Cloning and sequencing of DI RNAs from yeast

The 5′ and 3′ ends of DI-72(+) was determined using 5′ RACE and 3′ RACE kits (Gibco-BRL, Rockville, MD, USA) according to manufacturer's instruction. The total RNA was polyadenylated with poly(A) polymerase prior to the 3′ RACE. The two nested gene-specific primers used for 3′ RACE were 1004 (5′-AGGTGTCACTTGTGGAAGCGGA) and 1050 (5′-GCGCCAAGCTTGCTATAGAGATCGCTGGAAGCACT). The PCR product was cloned at the *Hin*dIII/*Sal*I sites in pUC19 vector. For the 5′ RACE, the two sequence-specific primers were 15 (5′-GTAATACGACTCACTATAGGGCATGTCGCTTGTTTGTTGG) and 1049 (GCGCCGAATTCGCAACGGTAGCGCAAGTCACCAGA). The PCR product was cloned into the *Eco*RI/*Sal*I sites of pUC19 vector. The PCR amplification through the junction site in the DI dimers was made with primers 1049 and 1050 after the reverse transcription was performed with primer 15 (Finnen and Rochon, 1995).

In vitro RdRp assay

Yeast cells expressing CNV p92 and p33 together with yDI-72 or only p33 and yDI-72 were harvested by centrifugation and homogenized in liquid nitrogen by grinding with mortal and pestle. The powder was then suspended in buffer containing 50 mM Tris–HCl, pH 8.0, 15 mM $MgCl_2$, 10 mM KCl, 10 mM β-mercapto-ethanol, and 200 mM sorbitol. The intact cells were removed by centrifugation for 3 min at 100 *g*. The supernatant was then centrifuged at 21,000 *g* for 15 min. The pellet was considered "membranous fraction" and the supernatant was the "soluble fraction." The RdRp assay with the internal DI-72 template (endogenous template) was performed as described elsewhere (Nagy and Pogany, 2000). S1 nuclease digestion was performed as described earlier (Nagy and Pogany, 2000). The RdRp products were separated on 5% acrylamide gel.

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