



Replication of *Tomato bushy stunt virus* RNA in a plant *in vitro* system

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ARTICLE INFO

Article history:

Received 2 March 2009

Returned to author for revision

28 March 2009

Accepted 13 May 2009

Available online 10 June 2009

Keywords:

RNA virus

BY-2

TBSV

In vitro replication

In vitro translation

Replicase

ABSTRACT

An ideal system to investigate individual determinants of the replication process of (+)-strand RNA viruses is a cell-free extract that supports viral protein and RNA synthesis in a synchronized manner. Here, we applied a translation/replication system based on cytoplasmic extracts of *Nicotiana tabacum* cells to *Tomato bushy stunt virus* (TBSV) RNA. *In vitro* translated TBSV proteins p33 and p92 form viral replicase, which, in the same reaction, accomplishes the entire replication cycle on exogenous TBSV DI or full-length RNA. Tests of mutant TBSV RNAs confirmed the template specificity of the *in vitro* replication reaction. Complementation experiments ascertained the significance of an earlier identified TBSV host factor. Interestingly, formation of the viral replicase occurs also in the absence of concurrent protein synthesis demonstrating that translation and RNA replication are not functionally linked in this system. Our studies with cell-free extracts of a plant host thus confirmed earlier findings and enabled novel insights into the TBSV RNA replication process.

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Introduction

The genomes of (+)-strand RNA viruses have two major functions in the cytoplasm of the infected host cell. Initially, they serve as mRNAs of the cellular translation machinery generating viral proteins. Viral RNAs, viral proteins and cell-encoded factors subsequently form membrane-associated replication complexes that catalyze the transcription of (–)-strand RNA intermediates. The (–)-strand intermediates, in turn, act as templates for the synthesis of progeny (+)-strand RNA molecules and, with certain virus families, also as templates for the synthesis of subgenomic (sg) mRNAs that encode additional viral proteins (Ahlquist et al., 2003; Buck, 1996; Schwartz et al., 2002). Important objectives involve the characterization of the molecular mechanisms that trigger the dynamic interactions between viral replication proteins, viral RNA, and membranes, and the identification of participating host factors. The experimental system ought to be supreme to address these tasks is a cell-free cytoplasmic extract that reproduces viral protein translation and RNA replication with added *in vitro* transcribed viral RNA. In the plant system, Komoda et al. recently established extracts of evacuated BY-2 protoplasts of *Nicotiana tabacum* that were shown to support translation/replication of *Tomato mosaic virus* (ToMV), *Brome mosaic virus* (BMV), and *Turnip crinkle virus* (TCV), respectively (Komoda et al., 2004).

An intensively studied (+)-strand RNA plant virus is *Tomato bushy stunt virus* (TBSV), the prototype member of the genus *Tombusvirus* in the *Tombusviridae* family (reviewed by White and Nagy, 2004). The

TBSV genome (gRNA) is a non-segmented (+)-strand RNA of ca. 4.8 kilobases (kb) (Hearne et al., 1990). The gRNA includes an open reading frame (ORF) encoding the components of the viral replicase, p33 and p92. p33 is a multifunctional protein that plays a role in template selection and enrolment of the viral RNA in the replication complex (Pogany et al., 2005; Rajendran and Nagy, 2003). p92, the viral RNA-dependent RNA polymerase (RdRp), is generated by translational read-through of the p33 stop-codon (Oster et al., 1998; Panaviene et al., 2003; Scholthof et al., 1995b; see Fig. 1). Two subgenomic RNAs (sg RNAs), which are transcribed by the viral replicase from (–)-strand derivatives (Lin and White, 2004; Lin et al., 2007), enclose three other ORFs that encode a coat protein (p41), a movement protein (p22), and a suppressor of gene silencing (p19), respectively (Hearne et al., 1990; Qiu and Scholthof, 2001; Qu and Morris, 2002; Scholthof et al., 1995a; reviewed by White and Nagy, 2004; Fig. 1).

Replication studies of tombusviruses have been considerably facilitated by the availability of small defective interfering (DI) viral RNAs (Knorr et al., 1991; White and Morris, 1999). The DI RNAs do not encode viral proteins but contain *cis*-acting RNA elements (subsequently termed as “replication elements”) that are essential for the RNA replication process. These elements were defined and functionally characterized in studies of tombusvirus replication in plants and plant protoplasts (Fabian et al., 2003; Monkewich et al., 2005; Park et al., 2002; Pogany et al., 2003; Qiu et al., 2001; Ray and White, 1999, 2003) as well as in *in vitro* assays with pre-assembled, fractionated tombusvirus replicase, which, besides p33 and p92, included a yet uncertain number of host factors (Nagy and Pogany, 2000; Panavas and Nagy, 2003; Panavas et al., 2002a, 2002b, 2003; Panaviene et al., 2004, 2005; Pogany et al., 2003, 2005). The promoter of (–)-strand

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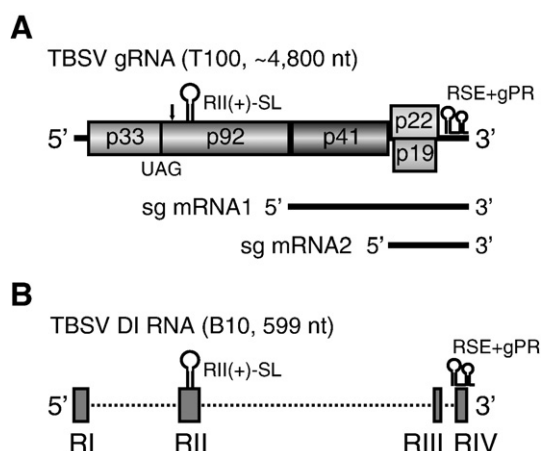


Fig. 1. Schematic representations of the *Tomato bushy stunt virus* (TBSV) genome and of a prototype defective interfering (DI) RNA. (A) The wildtype genome is depicted as a line, the coding regions are indicated as boxes. Note that p92 is expressed by translational read-through of the p33 stop-codon UAG. The two subgenomic (sg) mRNAs that are expressed in the course of the TBSV life cycle are indicated. The position of a frame-shift mutation (gRNA mutant 1) which was generated in the course of the present study is indicated by an arrow. (B) The composition of the DI RNA B10 that was used in this study is depicted. Boxes represent the genomic segments (RI–RIV) that are maintained in the DI RNA; dotted lines indicate the genomic segments that are absent in the DI. The *cis*-acting replication elements RII(+)-SL (containing the p33RE element), RSE and gPR that were mutated in the course of this study are shown schematically in the genomic as well as in the DI RNA (not to scale).

initiation (gPR) was shown to involve a hairpin structure and a short sequence-stretch at the immediate genomic 3'-end (Fabian et al., 2003; Panavas et al., 2002b), while the (+)-strand initiation promoter (cPR) consists of a 11 nucleotide (nt) single-stranded region at the 3'-end of the (–)-strand intermediate (Panavas et al., 2002a). Two replication elements, RE and RSE, were indicated to up- or down-regulate initiation from the promoters, respectively (Panavas and Nagy, 2003; Pogany et al., 2003; Ray and White, 2003). Experiments with fractionated replicase suggested that the RSE down-regulates (–)-strand synthesis by hybridizing to the 3'-terminus of gPR (Na and White, 2006; Na et al., 2006; Panaviene et al., 2005; Pogany et al., 2003; see Fig. 6). The p33 protein binds to another regulatory element, p33RE, which is located in a stem-loop structure named RII(+)-SL in the (+)-strand RNA. Association of p33 to p33RE was demonstrated to be important for the specific recruitment of the viral RNA to the replication process (Pogany et al., 2005). Several lines of evidence imply that p33RE, RSE, and gPR are acting cooperatively during initiation of the first replication step (Panaviene et al., 2005; Fig. 1).

For the first-line identification of host factors, it has been particularly valuable that the tombusvirus replicase assembles and replicates viral RNA in *Saccharomyces cerevisiae*. By yeast genetics, ca. 100 host genes were indicated to participate in the TBSV replication process (Jiang et al., 2006; Panavas et al., 2005). Further studies showed that the yeast homologue of the constitutively expressed Hsp70 protein (Hsc70) stimulates TBSV RNA replication and that the yeast homologue of GAPDH essentially adjusts the ratio of the (–)- and (+)-strand replication products (Serva and Nagy, 2006; Wang and Nagy, 2008). A cell-free yeast extract was recently established that supports assembly of the TBSV replicase and replication of TBSV RNA *in vitro* (Pogany and Nagy, 2008; Pogany et al., 2008). Nevertheless, exclusive use of the yeast model to study TBSV replication entails the risk to neglect factors and processes that are crucial in *planta* and during viral pathogenesis.

Here we report that cytoplasmic extracts of BY-2 cells of *N. tabacum*, a susceptible host of TBSV, can be applied as a robust, plant-derived *in vitro* translation/replication (t/r) system for TBSV RNA. The t/r system was tested in genetic, complementation and protein–protein interaction studies. Besides confirming and extending

earlier findings, new insights were obtained concerning the linkage of viral protein and RNA synthesis. The BY-2 t/r system was thus established as an important tool, particularly for the functional characterization of plant host factors involved in viral RNA replication.

Results

Cytoplasmic extracts of plant cells efficiently translate full-length TBSV RNA

To establish the *N. tabacum* BY-2 t/r system for TBSV, we followed a modified protocol of Komoda et al. (2004; see Materials and methods for details). Briefly, suspension cultures of BY-2 cells were grown for 4 days and the cell wall removed by cellulase/pectolyase treatment. The protoplasts were evacuated by centrifugation on a continuous/discontinuous percoll-gradient, and cytoplasmic extracts (final protein concentrations 7–12 mg/ml) prepared by douncing and removal of the nuclei. Different from the original protocol, the extracts were treated with micrococcal nuclease (Nuclease S7, MN) to remove internal mRNA.

First, to estimate the relative translation capacity of the evacuated BY-2 lysate (in the following referred to as BYL), we performed an *in vitro* translation reaction with the BYL and a reporter mRNA encoding firefly luciferase for 1 h at 25 °C. Side-by-side, the translation reaction was carried out with a commercial wheat germ translation extract (WGE, Promega). To allow a direct comparison of the translation activity of both extracts, the detected luciferase activity was calculated in relation to the extract's protein concentrations. The experiment showed that the BYL translated the reporter at a comparable efficiency as the commercial WGE (Fig. 2A). Importantly, mRNA depletion by MN treatment which left the integrity of the 18 and 28S ribosomal RNAs mainly unaffected (see Fig. 2B) markedly increased the translation efficiency for exogenous mRNA of the BYL up to 200 fold.

Prior to testing viral RNA in a translation reaction, we examined the relative stabilities of TBSV gRNA (T100) and TBSV DI RNA (DI B10) in the BYL. Thus, *in vitro* transcripts of both RNAs were added at identical molar amounts to the extracts and their integrity analyzed at different incubation times at 25 °C (Fig. 2B) by gel electrophoresis. While not determining exact turn-over rates at this point, both RNAs were estimated to exhibit a half-life of ca. 30 min, which revealed that despite of the evacuation procedure the BYL still contained considerable nuclease activity. Interestingly, in the course of further optimizations, the stability of the DI RNA could be significantly increased by adjustment of the final EDTA concentration to 1 mM. We applied this modification to all subsequent studies (see Discussion).

Next, we performed translation reactions with TBSV gRNA molecules. The BYL were supplemented with [³⁵S]-labeled methionine, and *de novo* synthesized proteins were detected by SDS-PAGE and autoradiography. Besides the wt TBSV gRNA, we applied a gRNA version as a negative-control, which contained a frame-shift mutation at position 1190/1 in the p33/p92 ORF that prohibited the translation of p92 (mutant 1, see Fig. 1A). As a further control in this and subsequently performed translation/replication experiments, we used a mutant gRNA that encoded a defective p92 RdRp where the catalytic GDD motif of the polymerase was changed to GDA (mutant 2; see Materials and methods and below). Translation assays with the wt TBSV gRNA generated two dominant protein bands with molecular weights of 33 and 92 kDa that correspond with the replicase components p33 and p92 (Fig. 2C). In accord with this assumption, translation of the frame-shift mutant RNA yielded exclusively a 33 kDa band, whereas translation of the RdRp-defective gRNA gave rise to the same protein pattern as the wt gRNA. In accord with earlier reports, where TBSV gRNA was translated in plant protoplasts (Scholthof et al., 1995b), the amount of the p92 translational read-through product was found to be significantly lower than that of p33.

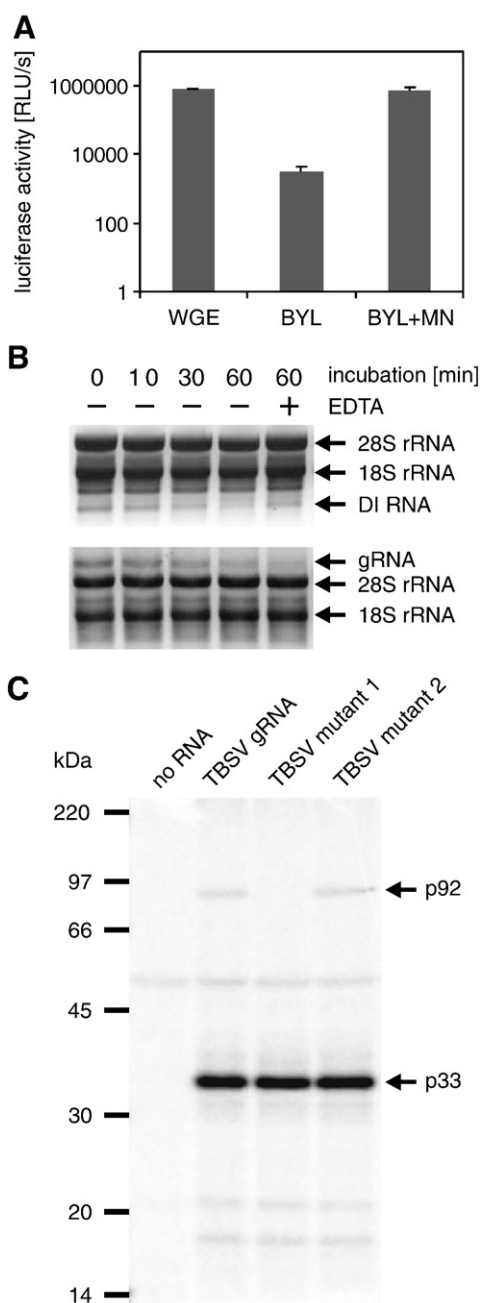


Fig. 2. Translation activity of the cell-free BY-2 lysate (BYL). (A) Translation activities of commercial wheat germ extract (WGE), untreated BYL and BYL treated with micrococcal nuclease (MN), respectively. Identical amounts of firefly luciferase mRNA were translated as described in [Materials and methods](#). Bars indicate the mean values and standard deviations of three experiments using independent batches of BYL. The enzymatic activities were normalized to protein concentrations (μ g of total protein in the reaction mixture). (B) Stability analysis of gRNA and DI RNA in BYL. Similar molar amounts of gRNA (8 μ g) and DI RNA (1 μ g) were incubated in the BYL under translation conditions. If indicated, EDTA was added at a concentration of 1 mM. Following the indicated time intervals, total RNA was isolated, separated on denaturing agarose gels and visualized by ethidium bromide staining. The positions of the cellular rRNAs and of the viral RNAs in the gels are marked. (C) PAGE analysis of [35 S]-labeled proteins that were generated by *in vitro* translation of TBSV gRNA in BYL. Three *in vitro* transcribed gRNA variants (wt, mutant 1, mutant 2) were translated in the presence of [35 S]-methionine; mutant 1 denotes a gRNA variant with a frame-shift mutation within the p92 ORF; in mutant 2, the GDD motif of the RdRp was replaced by GDA (see [Materials and methods](#)). Arrows indicate the migration positions of proteins of the expected sizes (33.3 kDa for p33, 92.1 kDa for p92).

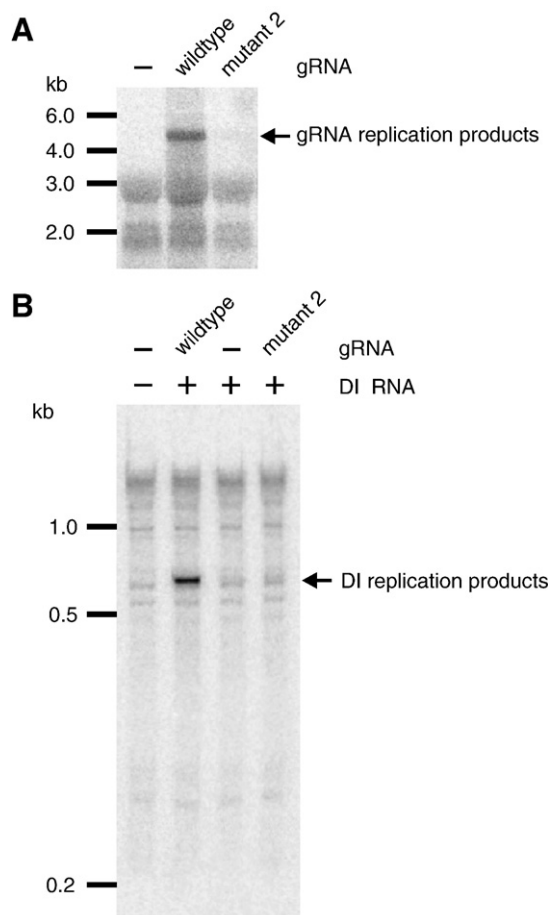


Fig. 3. Replication of TBSV RNAs in BYL. (A) A translation reaction was performed without exogenous RNA, wt TBSV gRNA and mutant 2 gRNA. The subsequent replication reaction was started by the addition of replication mix that included [α^{32} P]-labeled CTP and an additional aliquot of the respective gRNA (see [Materials and methods](#)). Total RNA isolated from the replication reaction was separated on a denaturing agarose gel and analyzed by autoradiography. (B) Translation was performed with wt gRNA or mutant 2 gRNA in the presence of DI RNA. Another aliquot of DI RNA was added to the subsequent replication reaction. Replication products were separated by denaturing PAGE and analyzed by autoradiography.

In vitro replication of TBSV RNAs in BYL

The fact that the p92/p33 replicase components were efficiently translated from the TBSV gRNA in BYL encouraged us to test the extracts next in translation/replication experiments. For this purpose, we first performed the translation reactions with wt gRNA and RdRp-defective gRNA (negative-control) as explained but using unlabeled amino acids in the reaction. Subsequently, the assay mixtures were supplemented essentially with actinomycin D, nucleotides (one species [32 P]-labeled), and a high concentration of magnesium-ions ([Komoda et al., 2004](#); see [Materials and methods](#) and [Discussion](#)). Following another 1–3 h of incubation at 25 °C, the reaction was stopped, the total amounts of nucleic acids extracted and prospective [32 P]-labeled replication products analyzed on denaturing gels. As the *in vitro* translated p33 and p92 proteins were expected to assemble into replicase complexes that act *in cis* as well as *in trans*, the replication reaction was performed with gRNA template alone and with a combination of gRNA and DI RNA templates, respectively. Thus, in the experiments that applied solely the gRNA, we detected radio-labeled RNA products that had the length of the input wt gRNA and that were not present in the control experiments with RdRp-defective gRNA (mutant 2; see [Fig. 3A](#)). In the experiments that applied a combination of gRNA and DI RNA templates, we also obtained labeled nucleic acid products. These had the same size as the original (input)

DI transcript, and no comparable products were detectable in analogous translation/replication assays that applied a combination of RdRp-defective gRNA and DI RNA (Fig. 3B). As the labeled products were sensitive to RNase treatment (Fig. 4 and data not shown), these

data, in sum, revealed that the labeled products came about by *de novo* RNA synthesis and not by end-labeling of the input RNA substrate, for example, by nucleotidyl-transferases that are present in the BYL.

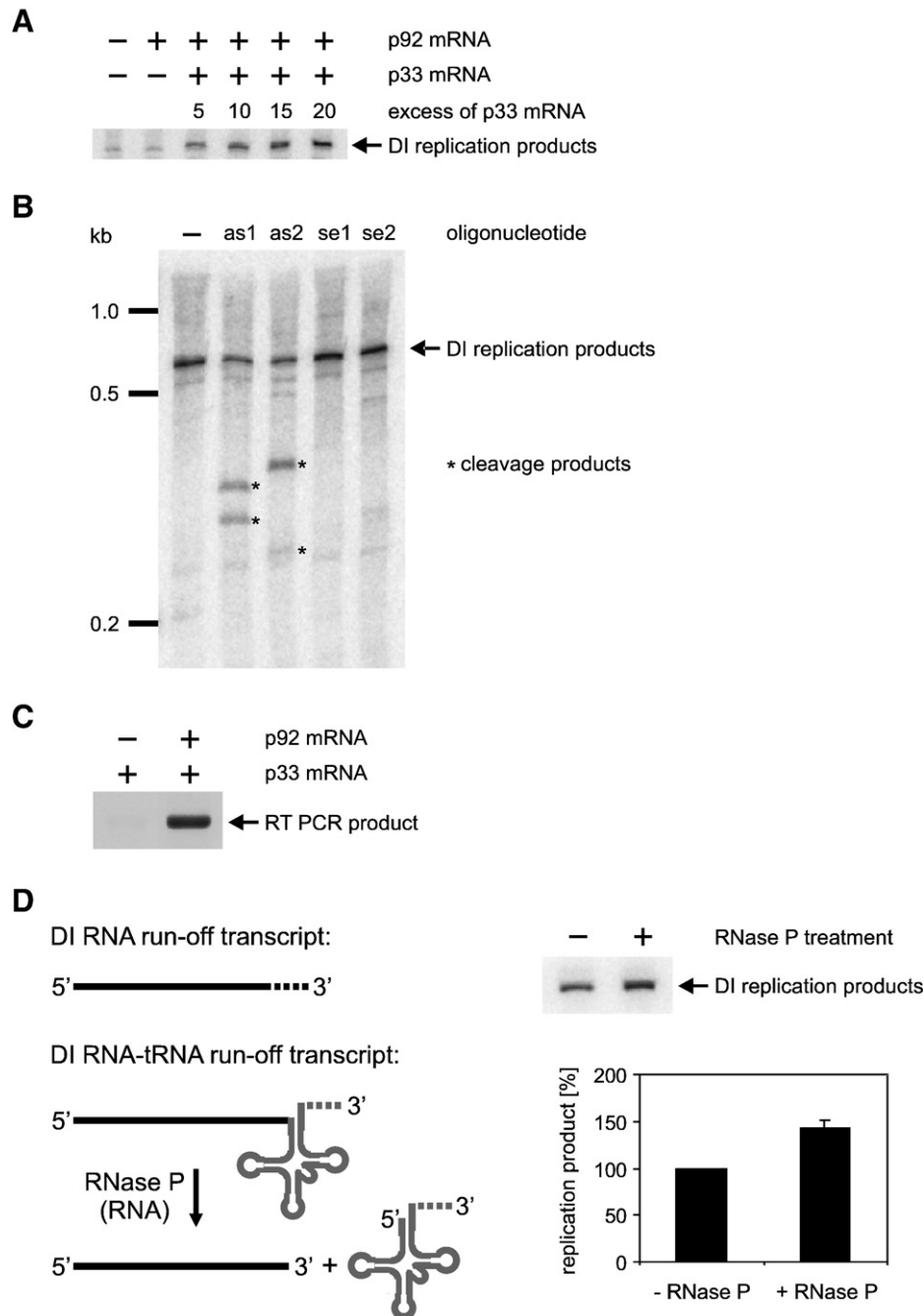


Fig. 4. TBSV replicase that is assembled of individually expressed p33 and p92 is capable of performing the entire replication cycle. (A) Replication of DI RNA by individually expressed p33 and p92. Translation and replication reactions as well as the analysis of replication products were performed essentially as described earlier (Fig. 3B), except for that gRNA was replaced by p92 mRNA and p33 mRNA (at the indicated molar excess with respect to the p92 mRNA). (B) Characterization of the replication products. Total RNA purified from a replication reaction was treated with RNase H in the presence of different antisense (as) or sense (se) oligonucleotides (see Materials and methods). The RNase digestion products (indicated by stars) were separated by denaturing PAGE and analyzed by autoradiography. (C) Detection of (–)-strand replication intermediates. Replication reactions were performed with DI RNA in the absence or presence of p92 RNA. Total RNA isolated from the reaction was subjected to an RNase protection procedure to remove the excess of (+)-strand RNA. This was followed by RT-PCR using in the reverse transcription reaction an oligonucleotide primer which is complementary to the (–)-strand RNA. (D) RNase P processing of DI RNA enhances replication efficiency. (Left) It has been described that run-off transcription with T7 RNA polymerase generates the correct transcript as well as a variety of products of different length, including premature termination products as well as transcripts with extensions (indicated schematically by a dashed line). To generate RNA transcript molecules with homogeneous 3' ends, cDNAs were generated that encoded TBSV DI RNA fused to a downstream tRNA sequence. Endonucleolytic cleavage by the RNase P ribozyme then yields RNA molecules with uniform 3' termini. (Right) Replication was performed with DI RNAs which were either synthesized by run-off transcription using a *Sma*-linearized template or generated by cleaving a primary DI RNA-tRNA transcript with RNase P RNA (see Materials and methods). The diagram represents the mean values and standard deviations of three separate experiments.

Individually expressed p33 and p92 form replicase complexes with TBSV DI RNA that are capable to perform a complete replication cycle

In the above experiments, p33 and p92 were translated from the TBSV gRNA, and the proteins apparently assembled to active viral replicase in the BYL that was capable of *de novo* RNA synthesis on a DI RNA template *in trans*. The next aim was to establish a better defined assay that applied solely the individually expressed p33 and p92 proteins and DI RNA as a template. For this purpose, the genetic units coding for p33 and p92, the p92 ORF without internal translational stop-codon (UAG replaced by UAU coding for tyrosine), were cloned downstream of an SP6 promotor, and the individual mRNAs were generated by *in vitro* transcription. Different ratios of the p33 and p92 mRNAs then were supplied to a t/r reaction with DI RNA in BYL, and resultant replication products analyzed in the described manner (Fig. 4A). These experiments yielded two important results. First, also with the individually translated p33 and p92 efficient replication of TBSV DI RNA could be detected in the BYL. Functional viral replicase was hence shown to assemble in the presence of the DI RNA template. Second, the application of individual mRNA transcripts allowed a straight adjustment of the ratio of p33/p92: a 20:1 ratio of p33/p92 transcripts was accordingly found to support TBSV DI RNA replication most efficiently. In sum, the BYL-based *in vitro* t/r assay could be considerably simplified by using defined amounts of essentially three RNA transcripts, namely the p33 and p92 mRNAs, and the TBSV DI RNA.

Applying these conditions to all subsequent tests, it was next important to determine if both replication steps, i.e. up to the synthesis of progeny (+)-strand RNA molecules, occurred in the BYL *in vitro* system. For this purpose, the products of a DI RNA replication assay were hybridized to different sets of sense and antisense (DNA) oligonucleotides, respectively, and the resulting DNA/RNA hybrids digested with RNase H (Fig. 4B). Thus, with the antisense oligonucleotides, we observed the generation of two RNase H cleavage products in comparison to untreated replication products. Primer as1 generated fragments of ca. 0.27 kb and 0.33 kb in length, primer as2 generated fragments of ca. 0.24 kb and 0.36 kb, which, in total, corresponded to the size of the full-length (ca. 0.6 kb) [³²P]-labeled (+)-strand DI RNA. When applying sense oligonucleotides and RNase H, no cleavage products were detected suggesting that the level of (–)-strand RNA was below the detection limit of the RNase H approach. Nevertheless, the synthesis of (–)-strand RNA intermediate in the BYL t/r assay could be clearly demonstrated by RT-PCR and qRT-PCR procedures, where the (–)-strand RNA was detected after RNase protection (Fig. 4C; see Materials and methods and below). Along with this procedure, we could estimate the ratio of (–)-strand intermediate versus progeny (+)-strand RNA molecules to be in the range of 1:200 (data not shown). It is known that during *in vitro* transcription reactions T7 RNA polymerase produces premature termination products, transcripts with extensions, as well as aberrant products that are complementary to the correct transcript (Schenborn and Mierendorf, 1985; Triana-Alonso et al., 1995). Hence, to prohibit the detection of false-positive (–)-strand RNA by RT-PCR, we used in these (and all following) experiments DI RNA templates that were generated from a larger DI RNA-tRNA primary transcript by RNase P cleavage (Ziehler and Engelke, 1996) and that were subsequently purified by PAGE (see Materials and methods). The application of RNA template molecules that displayed homogeneous, RNase P-generated 3'-ends was found to have a second positive effect as it significantly stimulated the RNA replication reaction (see Fig. 4D and Discussion). In sum, these data demonstrated that the TBSV replicase that assembled under the conditions of the “three RNA approach” in the BYL performed the complete viral RNA replication cycle.

TBSV RNA replication in BYL is not functionally coupled with translation

In the earlier experiments, the TBSV RNA template was already included in the translation process. While switching the assay to RNA

replication by supplementing with actinomycin, nucleotides, and magnesium, we added viral RNA once again (see Materials and methods) in order to enable replicase assembly in the translation as well as in the replication reaction. However, it was unclear at this stage whether pre-assembly of the replicase during translation was actually necessary for replication. Although high magnesium concentrations are not supportive for translation, it was also uncertain whether formation of the active replicase was functionally linked to the translation process of p33 and p92. To address these questions, we performed the following experiments. First, translation of p33 and p92 was carried out in the presence as well as in the absence of TBSV DI RNA. Then, when switching the assay to replication, viral RNA was added in each case and the reaction continued in the absence and presence of puromycin, respectively. Using the sensitive luciferase-based translation assay (see Fig. 2), the applied puromycin concentration was prior assured to fully inhibit protein synthesis (Fig. 5A). Interestingly, as shown in Fig. 5B, RNA replication was found to occur at the same level, irrespective of whether the viral RNA template was

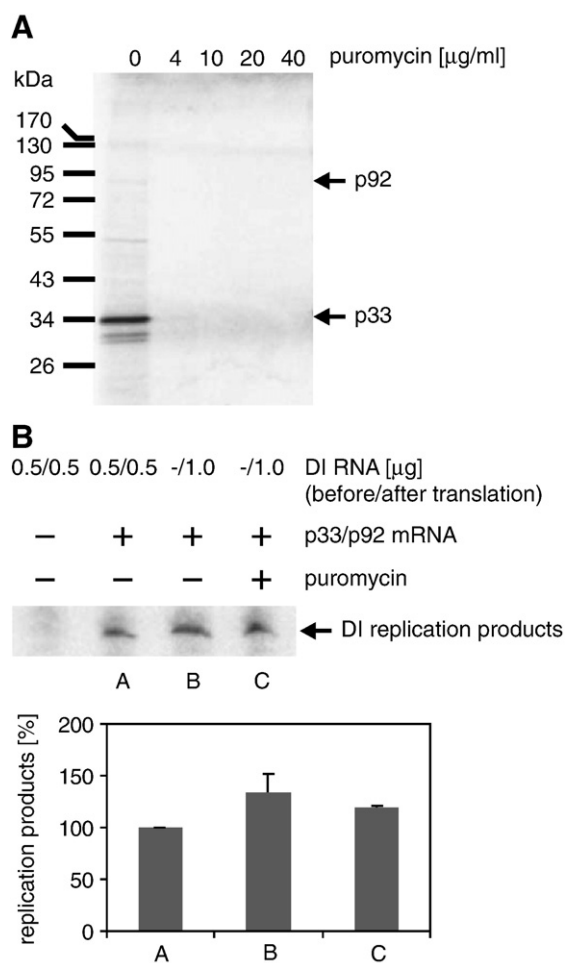


Fig. 5. TBSV RNA replication in BYL is not functionally coupled with translation. (A) Inhibition of translation by puromycin. PAGE analysis of [³⁵S]-labeled proteins that were obtained by translation of p33 and p92 mRNAs in the presence of the indicated amounts of puromycin. (B) Translation/replication experiments. These were carried out in the presence and absence of DI RNA in the translation reaction, and in the presence of puromycin, respectively. Translation and replication reactions were performed as described using p33 mRNA, p92 mRNA and a total of 1 μg of TBSV DI RNA. As in the earlier experiments shown in Figs. 3 and 4, the viral RNA was added at 0.5 μg portions to the translation and replication reactions, respectively. Alternatively, 1 μg of DI RNA was added solely to the replication reaction. Where it is indicated, the reaction mixture was adjusted to a final concentration of 10 μg/ml puromycin before the replication reaction was started. This puromycin concentration totally inhibited translation of the viral proteins (see A). The diagram shows mean values and standard deviations of three separate experiments.

already present during translation or whether it was added when replication was initiated. Most interestingly, RNA replication was observed to proceed without restrictions also in the presence of puromycin.

Hence, we concluded that TBSV RNA replication in the BYL system does not require pre-assembly of the viral proteins and viral RNA during translation. Moreover, these data revealed that RNA replication occurred independently of concurrent translation (see Discussion).

Significance of RII(+)-SL and RSE for TBSV RNA replication in BYL

The following aim was to determine the role of the RII(+)-SL and RSE elements in the TBSV RNA replication process in BYL. The functions of these elements were extensively investigated in the past with either pre-assembled and fractionated replicase complexes *in vitro* or in gRNA and DI RNA transfected plant protoplasts *in vivo* (see

Introduction and Discussion). Nevertheless, we considered this “re-evaluation” of RII(+)-SL and RSE as important to understand whether the BYL t/r assay was generally applicable to genetic studies and whether the obtained data were comparable to those of earlier reports that were carried out with other experimental systems. For this purpose, we generated a gRNA as well as a DI mutant that had a single nucleotide exchange in the RII(+)-SL element (mutant A; see Fig. 6A); this mutation was earlier shown to prohibit the binding of p33 to p33RE (Pogany et al., 2005). Moreover, two mutations were introduced into the DI where the RSE–gPR interaction should be disturbed by single nucleotide exchanges in the RSE or in the gPR sequence (mutants B, C). In a fourth DI variant (mutant D), the RSE–gPR interaction was restored, though by a modified sequence in RSE and gPR, respectively (Pogany et al., 2003; Fig. 6A). When measuring the overall levels of RNA replication of these mutants, we found that mutation A inhibited RNA replication completely. Conversely,

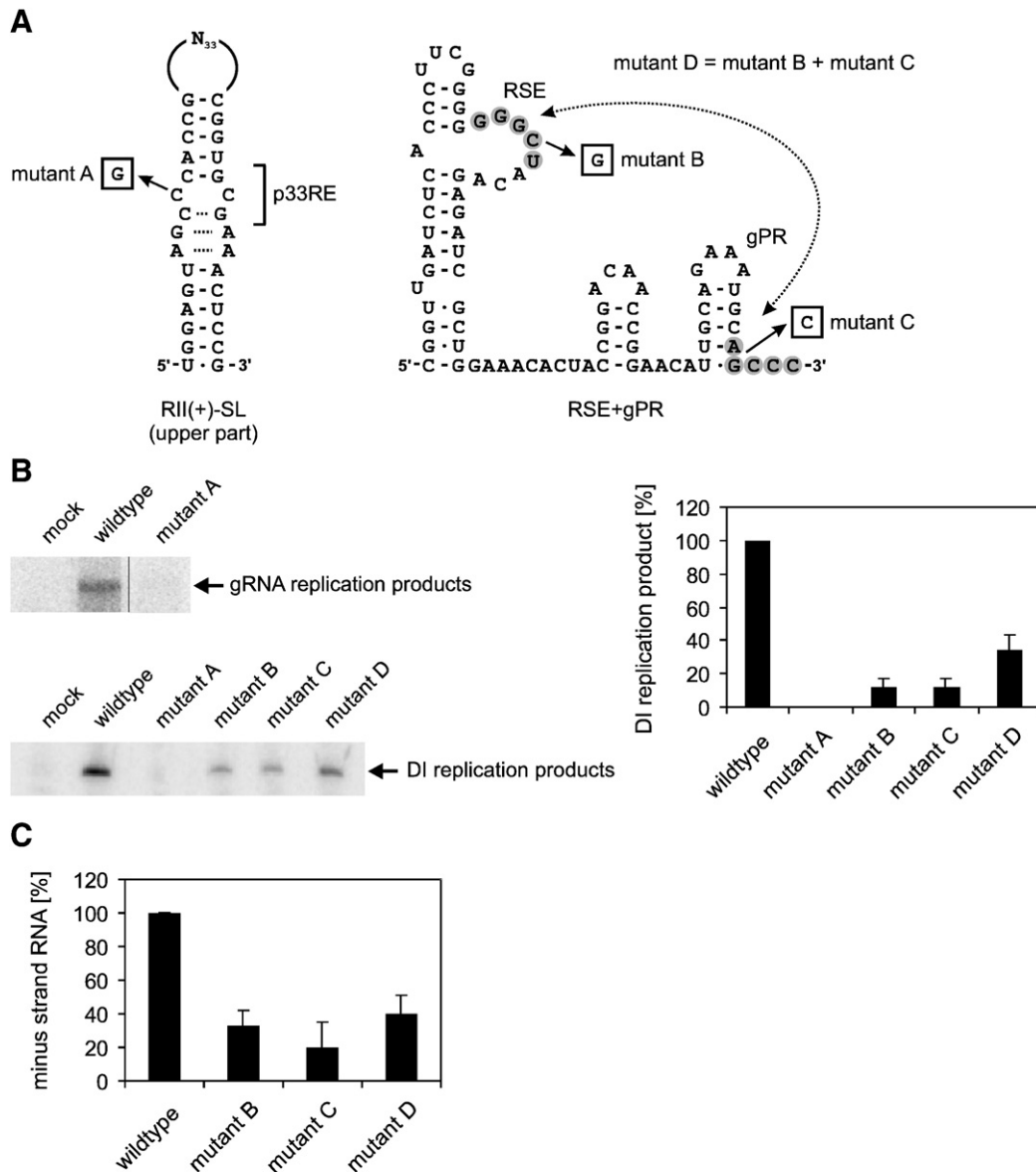


Fig. 6. Effect of mutations in replication elements on TBSV RNA replication in BYL. (A) RNA structures of the wildtype and mutant cis-acting replication elements RII(+)-SL, RSE and gPR. The postulated interaction between RSE and gPR in the wildtype sequence is indicated by a dotted line, involved nucleotides are highlighted. The introduced mutations are indicated by arrows. (B) Relative replication efficiency of the different mutants. Translation and replication reactions were performed as described using wt or mutant gRNA (top) or p33 mRNA, p92 mRNA, and the respective DI variants (bottom). The diagram represents the mean values and standard deviations of three separate replication experiments using DI RNA (wt RNA replication was set 100%). (C) Relative amount of (–)-strand RNA generated during replication of the different DI mutants. (–)-strand RNA was detected after RNase protection and reverse transcription (see Fig. 4C) by qRT-PCR (wt RNA replication was set 100%). The diagram shows the mean values and standard deviations of four independent experiments.

mutations B and C reduced the level of DI replication to about 10–12% of the wt-level, while mutant D displayed a replication level that corresponded ca. 35% of that of the wt RNA (Fig. 6B). Measuring the amounts of (–)-strand RNAs with the aforementioned qRT-PCR procedure, we found that with the replicating mutants B and C, the level of intermediate was somewhat increased with respect to the total amount of replication products (mutant B: ca. 33% wt-level; mutant C: ca. 20% wt-level; mutant D: ca. 40% wt-level).

These results underlined the essential role of the p33RE element for TBSV replication and confirmed the template specificity of the RNA replication reaction in the BYL. With the RSE-gPR mutants, our data revealed analogies as well as discrepancies with earlier reported transfection/replication experiments in protoplasts (see Discussion).

Evaluating the role of a TBSV host factor in the BYL *t/r* system

An *in vitro* system should enable straight-forward depletion and/or complementation experiments. Accordingly, the final study of this report aimed at testing the BYL-based *t/r* assay in terms of its potential to determine the role of a complementing host factor. For this purpose, we decided to investigate the constitutively expressed heat shock protein Hsp70 (Hsc70), which was indicated to represent an important TBSV host factor. That is, the yeast Hsp70/Hsc70 homologue Ssa1p was shown to interact with p33 and to stimulate RNA replication (Serva and Nagy, 2006). Moreover, while this work was in progress, it was shown that Ssa1p supports the assembly of the p92/p33 replicase (Pogany et al., 2008).

Initially, we attempted to remove Hsp70/Hsc70 from the BYL by immunodepletion-chromatography with commercially available antibodies, which were raised against the human and rat Hsp70/Hsc70

proteins, respectively. This was considered reasonable, as the domains of the Hsp70/Hsc70 proteins that were recognized by the antibodies displayed significant homologies to the corresponding *N. tabacum* proteins (see Materials and methods). Nevertheless, the achieved levels of depletion corresponded only 10 to 20% of the total amount of Hsp70/Hsc70 contained in the BYL, and no effect (neither positive nor negative) on TBSV translation/replication was observed with such depleted extracts (data not shown). To perform the inverse experiment and to complement the BYL with additional Hsc70, two experimental lines were followed. First, we tried the complementation by titrating mRNA transcript of the cloned (human) Hsc70 gene to the translation/replication reaction with TBSV DI RNA substrate (see Fig. 7A): the human and the plant Hsc70 display a homology of ca. 75%. In the second approach, we added different amounts of commercially available recombinant (bovine) Hsc70 protein (the homology of bovine and plant Hsc70 also is ca. 75%) to the BYL prior to performing the TBSV translation/replication reaction. Importantly, in each case, we observed a significant increase, i.e. up to ca. 2.5 fold, in the replication efficiency of the TBSV DI RNA in the complemented extracts as compared to mock-complemented controls (Fig. 7A; see figure legend for experimental details).

Finally, considering again the report of Serva and Nagy (2006), we wanted to know whether also in the plant extracts the Hsp70/Hsc70 protein specifically interacts with the TBSV replicase. For this purpose, we expressed the individual p33 and p92 proteins by *in vitro* translation in BYL that was supplemented with [³⁵S]-labeled methionine. Subsequently, we performed an immunoprecipitation assay applying the anti rat Hsp70/Hsc70 antibody, which, in the previous depletion attempts turned out to precipitate the plant homologues at highest efficiency (see above). As shown in Fig. 7B, we observed an

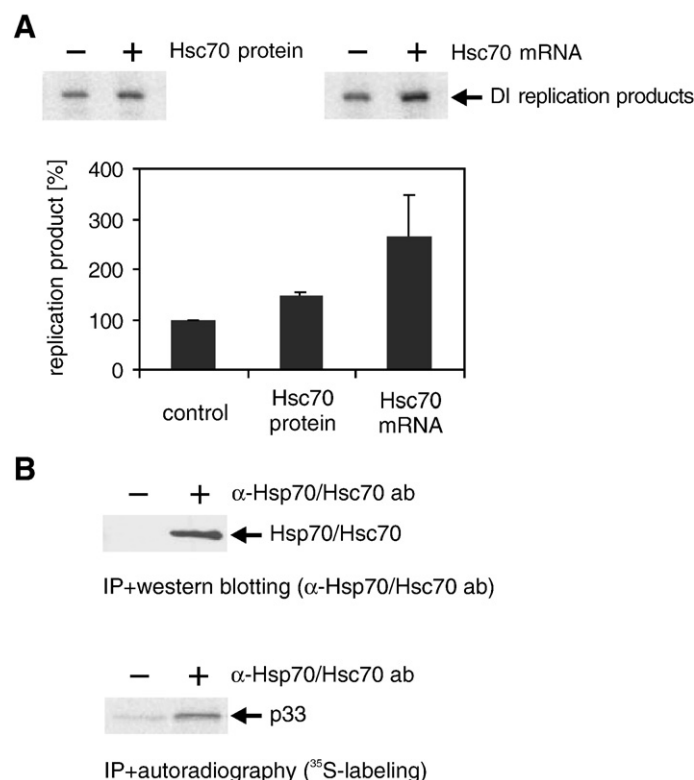


Fig. 7. The role of Hsp70/Hsc70 in TBSV RNA replication. (A) Complementation experiments with Hsc70 protein or Hsc70 mRNA. Translation and replication reactions were performed with p33 mRNA, p92 mRNA and TBSV DI RNA as described. Moreover, where it is indicated, the translation reaction was complemented with recombinant bovine Hsc70 protein (2 µg) or *in vitro* transcribed mRNA of the human Hsc70 (3 µg), respectively. As negative controls, buffer without protein or the same molar amounts of a firefly luciferase-coding mRNA were applied to the assay, respectively. The diagram represents the mean values and standard deviations of three separate experiments. (B) Co-immunoprecipitation of p33 and Hsp70/Hsc70. A standard translation reaction was performed with p33 RNA and p92 RNA in the presence of [³⁵S]-methionine. Subsequently, immunoprecipitation was carried out with a polyclonal anti rat Hsp70/Hsc70 antibody and the resulting precipitate split. One half of the precipitate was analyzed by PAGE and western blotting with a monoclonal anti Hsp70/Hsc70 antibody (see Materials and methods). The other half of the precipitate was analyzed by PAGE and autoradiography (detecting precipitated p33; see also Figs. 2 and 5).

evident and specific co-precipitation of the labeled p33 subunit of the replicase with the anti Hsp70/Hsc70 antibody confirming that also in the plant system Hsp70/Hsc70 interacts with the replicase component. Notably, this interaction was shown to occur even at high salt concentrations (i.e., up to 500 mM) supporting the view that the interaction of Hsp70/Hsc70 and p33 is specific and stable (Fig. 7B). In sum, these experiments confirmed the important role of Hsp70/Hsc70 for TBSV replication.

Discussion

The aim of this study was to establish an authentic, plant-derived *in vitro* t/r system for Tomato bushy stunt virus (TBSV) and to determine the suitability of this system for its use in genetic and host factor studies. For this purpose, we employed and further developed the methodology of the Ishikawa lab generating cytoplasmic extracts from evacuated protoplasts of *N. tabacum* BY-2 cells (Komoda et al., 2004). Following the removal of internal mRNAs by micrococcal nuclease treatment, the BY-2 extracts (BYL) translate exogenous, *in vitro* transcribed mRNA at similar efficiencies as commercial wheat germ extracts (Fig. 2). Most importantly, in the BYL, the *in vitro* translated TBSV p33 and p92 proteins assemble to viral replicase complexes that catalyze the complete replication cycle of TBSV DI RNA in the same reaction tube (Figs. 3 and 4; see below for a further discussion).

The BYL system was reliable in our hands. That is, ca. 95% of the total number of extracts that were prepared in the course of these studies were translationally active, and ca. 60% showed *in vitro* replication of TBSV DI RNA as well. Likewise, the efficiency of the BYL could be enhanced by specific measures. Thus, EDTA supplemented at a concentration of 1 mM markedly increased the stability of the TBSV DI RNA in the extracts (Fig. 2B) and raised the rate of DI replication by more than 3 fold in comparison with non-supplemented extracts. The BYL t/r assay was further improved by using DI RNA templates with homogeneous RNase P processed 3'-termini (Fig. 4D). While replication of both, TBSV DI RNA and TBSV gRNA was clearly detectable, replication of the full-length RNA was generally lower as already reported by Jones et al., 1990 (Figs. 3 and 6). With gRNA, it will be interesting to test next for the formation of progeny virus particles in the plant cell extracts.

In vitro replication of TBSV RNA was accomplished in several ways in the BYL. Initially, the viral proteins p33 and p92 were generated from the full-length TBSV gRNA, and assembled replicase then was active in *cis* and on DI RNA *in trans* (Fig. 3). Later on, we could show that p33 and p92 that had been translated from individual mRNAs in the BYL were also capable to form active replicase (Fig. 4A). It was apparent that under the latter conditions where the BYL were programmed essentially with three RNAs, i.e., p33 mRNA, p92 mRNA and TBSV DI RNA, the replicase assembled in the extract and was active on the DI RNA. Most interestingly, we could demonstrate that once p33, p92 and the viral RNA are present in the extract, replicase assembly and RNA replication occurs independently of translation (Fig. 5). The “three RNA approach” thus offers several advantages. First, it enables variations and optimizations of the ratio of the viral replicase proteins and of the viral template (Fig. 4A). Moreover, in future studies the synchronized t/r experiments with BYL will allow to precisely dissect molecular determinants of the TBSV RNA replication process from elements and factors involved in other stages of the viral life cycle.

To further evaluate the experimental potential and properties of the BYL system, we introduced a set of mutations into the RII(+)-SL, RSE and gPR elements of the TBSV DI RNA (Fig. 6A). These mutations were already characterized in other systems and shown to yield a profound effect on viral RNA replication. Mutation A, which destroyed a critical C-C bulge in RII(+)-SL, was known to prohibit selective binding of p33 to the p33RE. RNA binding of p33, in turn, was

demonstrated to be crucial for recruitment of the viral RNA to the replication complex in plant and yeast cells (Monkewich et al., 2005; Pogany et al., 2005). As expected, mutation A inhibited RNA replication of TBSV gRNA and TBSV DI RNA also in the BYL (Fig. 6B), which emphasized the essential role of RII(+)-SL/p33RE and unambiguously confirmed the template specificity of the BYL-based TBSV translation/replication reaction. Concerning the RSE and gPR elements, two applied mutations (B + C) were known to disturb the formation of an RNA pseudoknot structure that involves both elements, while a third mutation (D) allowed formation of the pseudoknot with a different sequence composition (Fig. 6A). These mutants were described in a detailed study of Pogany et al. (2003) and tested *in vitro* with fractionated replicase as well as *in vivo* with protoplasts that were co-transfected with TBSV gRNA and the mutant DI RNAs, respectively. Thus, in the *in vitro* replicase assays, mutations B and C were found to considerably accumulate (–)-strand intermediate. In the *in vivo* experiments, mutations B + C caused a lethal phenotype, while mutation D allowed replication at an intermediate rate. The RSE-gPR pseudoknot was hence proposed to act as a ‘replication silencer’ that keeps replication initiation at a low level, and this silencing was suggested to be a key regulator of several stages of the viral life cycle (Pogany et al., 2003). In general accord with the data of Pogany et al., we observed that mutant D RNAs replicated at a lower level than the wt RNAs (Fig. 6B) but considerably better than mutant B and C RNAs. This confirmed that the sequence composition as well as the correct formation of the gPR-RSE pseudoknot is an important determinant of TBSV RNA replication. However, different from the observations of Pogany et al., mutant B and C RNAs were clearly replication competent in the BYL, though at low levels (Fig. 6B). Speculating about the reasons for these discrepancies, it is important to note that in the protoplast studies of Pogany et al., the replicase was supplied by a “helper” gRNA to become active on the co-transfected mutant DI RNAs *in trans*. In contrast, in the here-applied “three RNA” BYL approach, the replicase was encoded by two mRNAs and assembled either during translation or in the presence of the viral RNA (see above). Another major difference concerns that the here-performed *in vitro* assays were analyzed within 1 h and accordingly considered to be more sensitive than transfection studies with protoplasts that were carried out for several hours. A higher sensitivity of the assay hence may have permitted to measure residual activities of the mutant RNAs. Another significant difference to the studies of Pogany et al. concerned our observation that the amounts of (–)-strand intermediate (in relation to total replication product) that were generated during replication of mutants B and C were only slightly increased in comparison to the situation with the wt and mutant D RNAs (Fig. 6C). Accordingly, our data question the essential role of RSE-gPR for RNA replication as well as its role as a ‘silencer’. Rather, they support the view that RSE and gPR form a common structure motif, perhaps together with p33RE (Panaviene et al. 2005), which is important for efficient assembly of the viral replicase.

The last section of this study examined the applicability of the BYL *in vitro* t/r assay to explore the activity of viral host factors. While depletion experiments failed due to the lack of efficient antibodies that were directed against the plant Hsp70/Hsc70, complementation approaches clearly confirmed the earlier reported stimulating role of Hsc70 on TBSV RNA replication. Interestingly, co-translation of Hsc70 together with p33 and p92 turned out to be significantly more efficient than complementation with the purified protein (Fig. 7A). This observation may have simple technical reasons; it may also indicate that the Hsc70 host factor supports the assembly of the viral replicase most actively *in statu nascendi*. Along the same line it is remarkable that similar to the experiments in yeast, a surprisingly stable interaction of the plant Hsp70/Hsc70 was detectable with the TBSV p33 (Fig. 7B). This finding supports the idea that the cellular chaperon acts as a stabilizing and/or folding agent of the viral replicase.

In sum, these studies represent an important proof of principle, which emphasized the suitability of the BYL-based *in vitro* t/r assay for future studies that aim at understanding specific aspects of the replication process of tombusviruses *in planta*, particularly the function of host factors.

Materials and methods

Cells and cell culture

Suspension-cultured tobacco cells (*N. tabacum*, line BY-2: L.cv. Bright yellow 2) were kindly provided by Bettina Hause, Leibnitz Institute of Plant Biochemistry, Halle, Germany. The cells were sub-cultured weekly and maintained at 23 °C in the dark in Murashige–Skoog liquid medium (Murashige and Skoog Basal Salts, Duchefa Biochemie, Haarlem, The Netherlands), 3% (w/v) sucrose, 1 mg/l thiamine–HCl, 0.2 mg/l 2,4-D, 100 mg/l myo-inositol, 200 mg/l KH₂PO₄, pH 5.8.

Preparation of cytoplasmic BY-2 cell extract

Extract from evacuated BY-2 protoplasts was prepared as described by Komoda et al. (2004) with some significant modifications. To prepare protoplasts, cells from a 4 days old culture (40–50 ml packed cell volume) were treated with a solution of 0.32% Cellulase Onozuka RS and 0.1% Pectolyase Y-23 (Duchefa Biochemie) in 12.5 mM NaOAc, 5 mM CaCl₂, 0.37 M mannitol, pH 5.8, for 2–3 h. Subsequently, the protoplasts were overlaid on a discontinuous/continuous Percoll gradient, containing (from bottom to top) 70% (2 ml), 40% (2 ml) and 0–30% (6 ml) percoll (Sigma, Taufkirchen, Germany) in 0.7 M mannitol, 20 mM MgCl₂, and 5 mM Pipes–KOH (pH 7.0). After centrifugation at 10,000 g for 1 h at 25 °C in an SW 40 rotor (Beckman Coulter), the evacuated protoplasts were pooled from the 40–70% Percoll solution interface. The evacuated protoplasts were suspended in 3–4 volumes of TR buffer (30 mM Hepes–KOH, pH 7.4, 80 mM potassium acetate, 1.8 mM magnesium acetate, 2 mM DTT) supplemented with Complete Mini EDTA-free protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) and disrupted using a Dounce homogenizer (Wheaton Science Products, Millville, NJ). Nuclei and non-disrupted cells were removed by centrifugation for 10 min at 500 g at 4 °C. The supernatant was supplemented with 0.5 mM CaCl₂, treated with nuclease S7 (75 U/ml, Roche Diagnostics) for 15 min at 20 °C, adjusted to 2 mM EGTA and subsequently frozen in aliquots at –80 °C until use.

Plasmid constructs

The cDNA clones encoding the full-length TBSV genome (T100), and a 599 nt defective interfering (DI) RNA (B10) were kindly provided by Herman Scholthof (A&M University, Texas, USA) and described previously (Hearne et al., 1990; Knorr et al., 1991). The TBSV gRNA mutant 1 was generated by inserting a nucleotide (C) between positions 1190 and 1191 of the RNA, which caused a frame-shift within the p92 ORF downstream of the p33 stop-codon. TBSV gRNA mutant 2 was created by replacing nucleotides 2049 (A) and 2050 (T) in the cDNA by C and A, respectively. This way, the catalytic GDD motif of the RdRp was substituted by GDA. In each case, an additional restriction site was introduced into the respective cDNA constructs (mutant 1 *Pst*I, mutant 2 *Sph*I). To generate a substrate for RNase P cleavage, the *Thermus thermophilus* tRNA^{Gly} gene, followed by an *Eco*RI site, was positioned immediately downstream of the sequence encoding TBSV DI RNA, resulting in plasmid pTGCRC3. To produce the TBSV gRNA or DI RNA mutant A, we substituted C at position 1384 (gRNA) or 261 (DI RNA) by G, which modified the p33 recognition motif (p33 RE; see Figs. 1 and 6A). Likewise, DI RNA mutants B (in RSE) and C (in gPR) were generated by replacing C (543) and G (596) by G and C,

respectively. Mutant D combined both mutations at positions 543 and 596, respectively. To generate plasmids pSPTVP33 and pSPTVP92Y encoding the TBSV p33 and p92 mRNAs, the respective viral genes were amplified by PCR using the T100 cDNA clone as a template. After confirmation of the sequence of the PCR products, they were digested with *Nco*I and *Xba*I and inserted downstream of the SP6 promoter into vector pSP-luc+ (Promega, Madison, WI) digested with the same enzymes. Subsequently, the internal stop-codon TAG within the p92 ORF was replaced by a TAT triplet encoding tyrosine. pCITELuc and pCITEHsc70 were generated by cloning the PCR amplified genes of firefly luciferase (using pSP-luc+ as template) and Hsc70 (using a human cDNA library as template) via *Nco*I and *Not*I into pCITE2A (Novagen, Darmstadt). Details on further plasmid constructs will be given on request.

In vitro transcription

TBSV gRNA, TBSV DI RNA and transcripts coding for human Hsc70 were synthesized *in vitro* by transcription of *Sma*I- or *Xho*I-linearized template DNAs with T7 RNA polymerase (Stratagene) using standard procedures. DI RNA-tRNA primary transcripts were produced by transcription of the *Eco*RI-linearized template DNA with T7 RNA polymerase. *Escherichia coli* RNase P RNA was transcribed with T7 RNA polymerase from plasmid pDW98 linearized with *Bsa*AI (Heide et al., 1999). The mRNAs encoding TBSV p33 or p92 were transcribed from the *Xho*I-linearized templates using SP6 RNA polymerase (Roche Diagnostics). Transcripts coding for firefly luciferase were generated either by T7 or SP6 RNA polymerase using *Xho*I-linearized templates pCITELuc or pSP-luc+, respectively. Transcription was stopped by the addition of 500 U/ml DNase I (Roche Diagnostics) and further incubation for at least 90 min at 37 °C. The transcripts were subsequently extracted with phenol/chloroform/isoamylalcohol. Unincorporated nucleotides were removed by ammonium acetate/ethanol precipitation. The RNA pellet was washed with 70% ethanol, air-dried, and dissolved in water.

TBSV DI RNA transcripts were further purified by separation on a 4.5% nondenaturing Tris–Borate polyacrylamide gel, eluted in TNEs buffer (10 mM Tris–HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 0.1% SDS) and extracted with phenol/chloroform/isoamylalcohol. The RNA was desalted by gel filtration using Sephadex G-50 and precipitated with ethanol.

Transcript concentrations were determined spectrophotometrically, and the RNA integrity was verified by using denaturing agarose gel electrophoresis.

RNase P cleavage of transcripts

Cleavage reactions were performed with 20 µg DI RNA-tRNA primary transcript and 25 µg RNase P RNA for 2 h at 37 °C in 100 µl cleavage buffer (50 mM Tris–HCl, pH 7.4, 5% (w/v) PEG 6000, 0.1 mM EDTA, 100 mM ammonium acetate, 100 mM magnesium acetate). The desired cleavage product was purified by PAGE as described above.

In vitro translation and replication reactions

Translation mixtures using BY-2 cytoplasmic extract contained 50% (v/v) BYL, 30% (v/v) TR buffer, 0.75 mM ATP, 0.1 mM GTP, 25 mM creatine phosphate, 50 µM of each amino acid, 80 µM spermine, 0.2 mg/ml creatine phosphokinase (Roche Diagnostics), 800 U/ml of RiboLock Ribonuclease Inhibitor (Fermentas, St. Leon-Rot, Germany). Unless differently stated in the Results section or figure legends, 1 µg of gRNA, or 1.5 µg of p33 mRNA and 200 ng of p92 mRNA were used per 50 µl total reaction volume. For subsequent replication assays with the TBSV genome, the amount of gRNA in the translation reaction was raised to 4 µg, in case of DI replication assays, 0.5 µg of DI RNA were included already in the translation reaction. For luciferase activity

assays, 100 ng of luciferase mRNA were used per 50 μ l of final reaction volume. The translation reactions were commonly performed for 60 min at 25 °C. To visualize the translation products, 4 μ Ci of [35 S]-methionine (1000 Ci/mmol, GE Healthcare, Buckinghamshire, UK) were added per 10 μ l reaction mixture and unlabeled methionine was omitted from the amino acid mixture. Translation reactions using wheat germ extract (WGE, Promega) were performed according to the manufacturer's instructions. To perform replication, the translation reaction was adjusted to 1.25 mM EDTA and mixed with 5 \times RdRp buffer (50 mM DTT, 500 μ g/ml actinomycin D, 17 mM magnesium acetate, 5 mM each of ATP, GTP, and UTP, 0.125 mM CTP containing 20 μ Ci of [α - 32 P] CTP [3000 Ci/mmol, GE Healthcare]). Unless differently stated another 4 μ g of gRNA or 0.5 μ g of DI RNA were added and the reaction performed for 3 h (gRNA) or 1 h (DI RNA) at 25 °C.

Protein and RNA analysis

Firefly luciferase activity was measured with the Luciferase Assay System (Promega) and a SIRIUS luminometer (Berthold detection systems, Pforzheim, Germany). Total RNA was purified from *in vitro* replication reactions with the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. [32 P]-labeled RNA products were electrophoretically separated either on 4% Tris–Borate polyacrylamide gels containing 8M urea (DI RNA) or on 1.5% formaldehyde agarose gels (gRNA) and visualized by phosphor-imaging (Storm 860, Molecular Dynamics). Quantification was carried out with the corresponding software ImageQuant Tools. [35 S]-labeled translation products were separated on 12% SDS-polyacrylamide gels and detected by phosphor-imaging.

Prior to detecting (–)-strand DI RNA by RT-PCR, the excess of (+)-strand RNAs was removed by an RNase protection procedure (Novak and Kirkegaard, 1991). Briefly, 15 μ g total RNA isolated from an *in vitro* replication reaction were precipitated with ethanol and the pellet resuspended in 20 μ l of hybridization buffer (40 mM Pipes, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% (v/v) formamide). The samples were denatured for 3 min at 92 °C and overnight hybridized at 45 °C. After adding 200 μ l RNase mixture (10 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 5 mM EDTA, 40 μ g/ml RNase A, 100 U/ml RNase T1), RNase digestion was performed for 2 h at 37 °C. In the presence of 0.5% SDS, the samples were treated with proteinase K (0.3 mg/ml) for 30 min at 37 °C. RNA was isolated as described above and treated with RNase-free DNase I (Roche Diagnostics) to remove residual amounts of plasmid DNA. Reverse transcription was performed using 10 pmol of oligonucleotide RTP3s (GGAAATCTCCAGGATTCTCG) which is complementary to the 3' end of (–)-strand DI RNA and M-MuLV reverse transcriptase (Fermentas) according to the instructions of the manufacturer. PCR was performed with GoTaq DNA polymerase (Promega) and oligonucleotides RTP1s (TATCTGGTGACTTGCGCTACC, sense) and RTP4a (GGGCTGCATTTCTGCAATG, antisense), respectively. Real-time PCR was performed with the QuantiTect SYBR Green PCR System (Qiagen) according to instructions of the manufacturer, using oligonucleotides RTP3s (sense) and RTP1a (CAGAAACCCATCATACC-CAAG antisense).

RNase H digest

10–15 μ g of total RNA isolated from a replication reaction were mixed with 50 pmol oligonucleotide (as1: CAGAAACCCATCATACC-CAAG, as2: CTACTCCAACCTCCTCACAAC, se1: TGGGTATGATGG-GTTTCTGTC, se2: GGTTTGTGAGGAGTTGGAGTAG) heated for 3 min at 95 °C and placed on ice. Then, the mixture was incubated with 3 U of RNase H (Promega) for 2 h at 37 °C in 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, and 10 mM DTT. The reactions were extracted with chloroform and the nucleic acids analyzed on a 4% Tris–Borate polyacrylamide gel containing 8 M urea.

Antibodies and western blotting

Two commercial antibodies against the human and rat Hsp70/Hsc70 proteins were applied for immunodepletion and immunoprecipitation experiments, respectively. The anti human Hsp70/Hsc70 antibody was a mouse monoclonal (Abcam, Cambridge, UK), the anti rat Hsp70/Hsc70 antibody was a rabbit polyclonal antiserum (Stressgen, Victoria, BC, Canada). While the protein domain (amino acids 122–264 of Hsp70/Hsc70) that is recognized by the mAb against the human proteins has ca. 85% homology with *N. tabacum* Hsp70, the conservation was lower with the protein domain recognized by the anti rat Hsp70/Hsc70 antibody (amino acids 446–641; ca. 60% homology). Western blotting was performed by using standard procedures. The anti rat Hsp70/Hsc70 polyclonal antibody was used at a 1:1000 dilution, the anti human Hsp70/Hsc70 monoclonal antibody was used at a 1:5000 dilution. Antibodies were detected by horseradish peroxidase-linked anti-rabbit IgG from donkey or anti-mouse IgG from sheep (both GE Healthcare) and chemiluminescence staining using the SuperSignal West Pico Substrate (Pierce, Rockford, IL).

Immunoprecipitation

Translation of the viral proteins were performed in a 100 μ l reactions with BYL in the presence of [35 S]-methionine (see above) and subsequently mixed with 4 μ g rabbit anti-Hsc70/Hsc70 polyclonal antibody that was previously coupled to Protein A Sepharose CL-4B (GE Healthcare). Immunoprecipitation was performed for 5 h at 4 °C in 30 mM Hepes–KOH, pH 7.4, 80 mM potassium acetate, 1.8 mM magnesium acetate, 0.7 mM DTT, 1% NP40, 0.5% caprylyl sulfobetaine and 10% glycerol supplemented with Complete Mini EDTA-free protease inhibitor mixture (Roche Diagnostics). The beads were washed five times with 0.5 ml precipitation buffer containing additional 300 mM KCl, and the proteins were eluted with 2 \times SDS-PAGE loading buffer. Samples were separated on a 12% SDS-polyacrylamide gel and analyzed by western blotting or phosphor-imaging.

Acknowledgments

This work was supported by the Sonderforschungsbereich SFB 648 (project A7) of the Deutsche Forschungsgemeinschaft (DFG) at the Martin-Luther-University Halle-Wittenberg. We are particularly grateful to the speaker, Ulla Bonas, as well as to all members of the SFB648 for continuous support, discussions and reagents. Particular thanks also to Dr. Ishikawa for sending us a detailed protocol on the preparation of evacuated BY-2 cells. Dr. Scholthof is gratefully acknowledged for supplying us the cDNA constructs of TBSV gRNA and DI. Thanks to Bettina Hause for BY-2 cells and valuable tips on cultivation and handling.

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