Analysis of tombusvirus revertants to identify essential amino acid residues within RNA-dependent RNA polymerase motifs

K. Boonrod,1 S. Chotewutmontri,1 D. Galetzka2 and G. Krczal1

1Centrum Grüne Gentechnik, Dienstleistungszentrum Ländlicher Raum Rhein-Pfalz, Breitenweg 71, D-67435 Neustadt, Germany
2Klinikum der Johannes Gutenberg Universität Mainz, Institut für Humangenetik, Langenbeckstr. 1, D-55101 Mainz, Germany

The RNA-dependent RNA polymerase (RdRp) of Tomato bushy stunt virus (TBSV) contains an arginine- and proline-rich (RPR) motif. This motif functions as an RNA-binding domain and is essential for tombusvirus replication. A mutant carrying three arginine substitutions in this motif rendered the virus unable to replicate in Nicotiana benthamiana plants and protoplasts. When the replicase function was provided in trans, by expressing the TBSV RdRp in N. benthamiana plants, an infectious variant could be isolated. Sequence analysis showed that only the substituted glycine residue (position 216) had reverted to arginine; all other substitutions remained unchanged. This finding suggested that strong selection pressure is active to maintain necessary sequences of the viral RdRp and that the analysis of revertants may help to identify essential viral functions.

RNA-dependent RNA polymerase (RdRp) is the key enzyme for the replication of all positive-strand RNA viruses. RdRps of several plant and animal viruses were grouped by Koonin & Dolja (1993) upon comparative analysis of their amino acid sequences. The ‘palm domain’ containing motifs A, B, C and D is found in many polymerases whereas the E motif is unique to RdRps and reverse transcriptases (Poch et al., 1989). Motif C comprises the highly conserved GDD motif of RdRps (Kamer & Argos, 1984; Koonin, 1991).

Tomato bushy stunt virus (TBSV), the prototypical tombusvirus, encodes the two overlapping replicase proteins, p33 and p92 (Russo et al., 1994), which are essential for TBSV replication (Oster et al., 1995; Calnan et al., 1995). Recently, Rajendra & Nagy (2003) characterized RNA-binding domains, one of them being an arginine/proline-rich motif termed RPR, which has the sequence RPRRRP. This motif is highly conserved among tombusviruses and related carmoviruses and lies in the overlapping domain of p33 and p92. It is similar to the arginine-rich motif present in the Tat transactivator protein of human immunodeficiency virus type 1 (Bayer et al., 1995; Calnan et al., 1991).

Wang & Gillam (2001) investigated the functional role of the GDD motif of Rubella virus (RUBV). RUBV is a small positive-strand RNA virus belonging to the genus Rubivirus of the family Togaviridae (Weaver et al., 2000). BHK cells were infected with RUBV RNA carrying mutations in the GDD motif and, by characterizing revertants isolated from infected BHK cells, Wang & Gillam (2001) could determine the essential amino acid residues of the GDD motif. To establish a similar test system for a plant virus, we first generated a TBSV RdRp GDD mutant. The glycine residue in the GDD motif of the TBSV RdRp in position 620 was substituted by alanine (G620A) by using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and primers 5′-GCAAATGTGAGATGACTG-3′ and 5′-CACAGTCATCTGACAGTTTCG-3′ (nucleotide position 2035–2075, mutated codons are underlined). The resulting viral mutant was tested for infectivity on mechanically inoculated Nicotiana benthamiana plants. In contrast to N. benthamiana plants that were inoculated with infectious transcripts of non-mutated TBSV, these plants did not show any symptoms and no systemic infection 5 days post-inoculation (p.i.). However, about half of these plants established systemic infection 14 days p.i., suggesting rever- sion of the mutation. Viral RNA was extracted from these plants and the mutated region was amplified by RT-PCR (RT-PCR kit; Gibco-BRL). PCR products were cloned into a T-tailed pUC19 vector and three individual clones were sequenced (Big dye reaction mix; MWG). In all three clones the substituted alanine residue had reverted to the original glycine (G620). The G620A exchange was a double base change (GGG→GCA), in which only one base (C) reverted back (G), resulting in GGA. The non-reverted base (A) functions as a ‘marker’ in our system, which helped us to exclude the possibility that our preparation of infectious transcripts or of viral RNA from infected plants was contaminated with (low amounts of) wild-type (wt) TBSV.
Table 1. Analysis of revertants

Sequences of RT-PCR products derived from TBSV KB1 transcripts and from infectious revertants (RNA was extracted from systemically infected leaves of N. benthamiana plants 7 days p.i.). Bold letters indicate the RPR motif. Mutated codons are underlined. The reversion is indicated in italics. Asterisks indicate the mutated nucleotides.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Amino acid sequence</th>
<th>Nucleotide sequence</th>
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<tbody>
<tr>
<td>TBSV wt</td>
<td>STGR$<em>{213}$P$</em>{214}$R$<em>{215}$R$</em>{216}$R$_{217}$PYA</td>
<td>TCAAACAGGGACGCCCTCAGCAGAAGGCCCTAGCGG</td>
</tr>
<tr>
<td>TBSV KB1</td>
<td>STG$<em>{213}$G$</em>{214}$P$<em>{215}$S$</em>{216}$S$_{217}$PYA</td>
<td>TCAAACAGGGACCTAGAAGAGCTCCCTAGCGG</td>
</tr>
<tr>
<td>Revertant</td>
<td>STG$<em>{213}$G$</em>{214}$R$<em>{215}$R$</em>{216}$R$_{217}$PYA</td>
<td>TCAAACAGGGACCTAGAAGAGCTCCCTAGCGG</td>
</tr>
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Our data are in accordance with previous studies on viral RdRps suggesting that the glycine residue of the GDD motif is somewhat flexible and that replacement of this residue does not completely abolish the functionality of RdRps (Hong & Hunt, 1996; Jablonski et al., 1991; Lohmann et al., 1997). Similarly, the G$_{620}$ of the TBSV RdRp appeared to not completely abolish virus replication, allowing the mutation to revert. Until now, for tombusviruses, only the strict requirement of the first aspartate residue of the GDD motif has been demonstrated (Molinari et al., 1998), confirming that any changes at this position are not tolerated for in vivo virus replication and/or in vitro RNA synthesis (Lohmann et al., 1997; Longstaff et al., 1993; Inokuchi & Hirashima, 1987). Moreover, we were able to demonstrate that N. benthamiana is a suitable plant system to detect revertants of tombusviruses.

In a second experiment we generated a TBSV clone carrying mutations within the RPR motif (primers 5'-TCAAGGGAGCTCTGAGGGAAGTCCCTACGCGG-3' and 5'-CCGGCTAGAGCTAGACATGCTGTGTA-3', nucleotide position 795–829, mutated codons are underlined). In this clone the RNA-binding domain was mutated by changing R$_{213}$G, P$_{214}$S, R$_{215}$G and R$_{217}$S (Table 1). After verification of the sequence transcripts of the mutated (TBSV KB1) and non-mutated TBSV clones, the clones were inoculated on N. benthamiana and Chenopodium quinoa (local lesion host of TBSV) plants, respectively. In contrast to the TBSV-infected plants that developed typical symptoms 4 days p.i., plants infected with transcripts of TBSV KB1 did not display symptoms during the whole period of the experiment (4 weeks) (Fig. 1). RT-PCR with TBSV-specific primers using total RNA extracted from upper leaves of these plants also failed to amplify any product, demonstrating that TBSV KB1 was not able to establish (systemic) infection. In summary, these data clearly indicate that the RPR motif is essential for TBSV RdRp function and does not tolerate the above sequence alteration.

It was reported that the RdRp of tombusviruses is able to function in trans (Rubino & Russo, 1995). In order to investigate whether a functional TBSV RdRp would complement the TBSV KB1, we produced transgenic N. benthamiana plants carrying the open reading frame (ORF) of the TBSV RdRp by applying the leaf-disc transformation procedure (Horsch et al., 1985). The transgene construct comprised the 5' untranslated region and the ORF of the TBSV RdRp being introduced into a cauliflower mosaic virus 35S promoter-driven expression cassette. Moreover, upstream of the p92 gene we incorporated the leader sequence of the Tobacco etch virus, an efficient translational enhancer (Carrington & Freed, 1990). Regenerated transformants were self-pollinated, and in the transgenic progeny the presence of RdRp mRNA was confirmed by Northern blot analysis. Ten plants of one of the RdRp-expressing lines were infected with 2 µg TBSV KB1 transcripts. Four of these plants showed local symptoms 5 days p.i. and systemic symptoms 7 days p.i. N. benthamiana plants were subinoculated with plant sap from these four plants and all of them showed local and systemic virus symptoms 4 and 6 days p.i., respectively (data not shown). This result indicated that the ability of the TBSV KB1 to replicate was restored by expression of the viral RdRp in trans.

Several revertants were isolated, and the genetic changes in their RdRps were analysed by RT-PCR amplification and

![Fig. 1. Symptom expression in non-transgenic N. benthamiana plants 7 days p.i. after inoculation with a non-mutated infectious clone of TBSV (a) and with TBSV KB2 (b). N. benthamiana plants infected with TBSV KB1 did not develop an infection and never showed symptoms (c).](image-url)
sequencing of the corresponding products. In all three clones tested only the \( G_{216} \) had reverted to the original arginine residue while all other substitutions remained unchanged (Table 1). To confirm that the reversion of the \( G_{216} \) to arginine is sufficient to restore infectivity, we created a second mutated clone (TBSV KB2), displaying the same sequence as the infectious revertant of TBSV KB1. Transcripts of TBSV KB2 were inoculated on non-transgenic \( N. \ benthamiana \) plants. By 4 days p.i. all inoculated plants developed typical symptoms as plants inoculated with the \( \text{wt} \) virus (Fig. 1). This clearly demonstrates that this single reversion was sufficient to restore infectivity.

In order to verify that the inability of the RPR mutant to infect plants systemically was caused by a defect in replication and not by e.g. a block in long-distance movement, we did a protoplast assay. Protoplasts were prepared from \( N. \ benthamiana \) leaves as described by Panaviene et al. (2003). Purified \textit{in vitro} RNA transcripts of \( \text{wt} \) TBSV, TBSV KB1 and KB2 (1 \( \mu \)g) were electroporated into \( 5 \times 10^5 \) protoplasts. After electroporation, the samples were incubated in 2 ml protoplast culture medium in the dark for 48 h at 22 °C. Total RNA was extracted from protoplasts (Nagy et al., 2001) and analysed.

Aliquots of the total RNA were electrophoresed through a 1% formaldehyde denaturing agarose gel, and viral RNAs were detected by transfer to a Nylon membrane (Amersham) followed by a Northern blot analysis using a digoxigenin probe complementary to the 3’ end of genomic TBSV RNA. The result shows that the infectious TBSV clone and TBSV KB2 replicated in the protoplasts, whereas in the protoplasts infected with TBSV KB1 viral RNA could no longer be detected (Fig. 2).

Our result confirms the finding of Panaviene et al. (2003). To test which amino acids in the RPR motif play an essential role in RNA binding they generated 19 different cucumber necrosis virus (CNV) mutants containing one or more altered amino acid residues in the RPR motif. In an \textit{in vitro} RNA-binding test they showed that the third arginine within the RPR motif is important for RNA binding and even the positively charged lysine was detrimental in this position. In our transgenic-\textit{trans}-complementation system this was exactly the arginine that reverted. Moreover, Panaviene et al. (2003) could show that replacing separately the second and third arginine residue by lysine residues decreased CNV replication by 95 to 98%. In contrast to Panaviene et al. (2003), who did not replace more than one arginine residue at the same time (except the strictly replication-deficient mutant 19 in which the three central arginine residues were replaced by alanine residues), we replaced in our mutant two of the central arginine residues and the first arginine residue.

Panaviene et al. (2003) demonstrated that mutations within the RNA-binding domains of the CNV replicate proteins affected the frequency of recombination by delaying the formation of recombinants. Because the RNA-binding domain was also affected in the TBSV KB1, we assume that the recombination frequency was also hampered in the complementation system. Thus, to restore a functional RPR motif, reversion was more likely to occur than recombination. Alternatively or in addition, low expression of the RdRp construct, as was observed in all of our transgenic \( N. \ benthamiana \) plants, may explain the failure to detect any recombination of the TBSV KB1 with the transgene mRNA. However, it cannot be excluded that recombinants can also be isolated when the replica function is provided \textit{in trans}. Only three of the infectious TBSV isolates were analysed in detail. Characterization of a large number of infectious clones may result in detection of recombinants. However, in this study we would like to focus on revertants. In contrast to recombinants, revertants highlight single amino acid residues that are essential for the functionality of the motifs to be analysed.

Analysis of revertants was also successfully used to elucidate viral RNA and/or gene function of MS2 and \( Q \Phi \) phages (Klovins & van Duin, 1999; Licis et al., 2000; Olsthoorn et al., 1994). In contrast to our work, however, viable phages were mainly restored by insertions or deletions and not by single base changes as we showed with our system.

Reversion of the TBSV KB1 mutation as reported here may illustrate the power of selection as a means of eliminating genetic drifts in viral genomes. A practical aspect of this observation concerns the identification of essential amino acids or amino acid motifs in viral proteins. Analysis of a population of viruses carrying random mutations in one or more essential viral genes and characterization of evolving revertants may help to identify essential virus functions.

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References


