Translation of the shallot virus X TGB3 gene depends on non-AUG initiation and leaky scanning

Alexander A. Lezzhov,1 Vladimir A. Gushchin,2,3 Ekaterina A. Lazareva,2 Valery K. Vishnichenko,4 Sergey Y. Morozov2,5 and Andrey G. Solovyev4,5

1Department of Genetics, Biotechnology, Plant Breeding and Seed Science, Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Moscow 127550, Russia
2Department of Virology, Biological Faculty, Moscow State University, Moscow 119992, Russia
3Genetic Department, Russian Center of Forest Health, Pushkino 141207, Russia
4Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences, Moscow 127550, Russia
5Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119992, Russia

Triple gene block (TGB), a conserved gene module found in the genomes of many filamentous and rod-shaped plant viruses, encodes three proteins, TGB1, TGB2 and TGB3, required for viral cell-to-cell movement through plasmodesmata and systemic transport via the phloem. The genome of Shallot virus X, the type species of the genus Allexivirus, includes TGB1 and TGB2 genes, but contains no canonical ORF for TGB3 protein. However, a TGB3-like protein-encoding sequence lacking an AUG initiator codon has been found in the shallot virus X (ShVX) genome in a position typical for TGB3 genes. This putative TGB3 gene is conserved in all allexiviruses. Here, we carried out sequence analysis to predict possible non-AUG initiator codons in the ShVX TGB3-encoding sequence. We further used an agroinfiltration assay in Nicotiana benthamiana to confirm this prediction. Site-directed mutagenesis was used to demonstrate that the ShVX TGB3 could be translated on a bicistronic mRNA template via a leaky scanning mechanism.
other viruses (Morozov & Solovyev, 2003) (Fig. 1a). Conservation of a TGB3-like protein-encoding sequence in allexivirus genomes suggested that it could be a functional gene expressed by use of a non-canonical translation initiation mechanism.

In this study, we carried out sequence analysis to predict a possible non-AUG initiator codon in the ShVX TGB3-encoding sequence. We further used an agroinfiltration assay in Nicotiana benthamiana to confirm this prediction and to provide evidence showing that the ShVX TGB3 can be translated on a bicistronic mRNA template via a leaky scanning mechanism.

The multiple amino acid sequence alignment generated for potentially translated allexivirus TGB3-related polypeptides
revealed that these proteins were closely related to each other (percentage identity 38–60) and exhibited more distant relation to the TGB3 proteins encoded by viruses of other genera (percentage identity 13–31) (Fig. 1b). In particular, these potentially expressed polypeptides possessed the C-terminal regions containing the conserved sequence signature and a single highly hydrophobic transmembrane sequence segment, both known to be typical for the TGB3 proteins of viruses with potex-like TGB (Morozov & Solovyev, 2003). If the allexivirus TGB3 translation could involve a non-canonical non-AUG initiation codon, it should be conserved in all allexviruses in terms of position and sequence context. Examination of aligned sequences of allexivirus TGB3-like polypeptides revealed the presence of an invariant Leu residue (Fig. 1b) encoded in all cases by the CUG triplet, which has been previously shown to be the most efficient non-AUG initiator in different systems and, in particular, in plant viruses (Firth & Brierley, 2012; Gordon et al., 1992). Importantly, in all allexivirus sequences this CUG was found to have an adenine residue in the (−3) position and a guanine residue in the (+1) position (Fig. 1c), which perfectly matched the optimal context for the translation initiation (Kozak, 1983, 1989) known to considerably enhance initiation from non-AUG codons (Firth & Brierley, 2012).

In other plant viruses, partially overlapping TGB2 and TGB3 genes are expressed from a single subgenomic RNA (sgRNA), and the translation of the 5′-distal TGB3 gene requires leaky ribosome scanning (Jackson et al., 2009; Morozov & Solovyev, 2003; Verchot-Lubicz et al., 2010). The canonical scanning model of translation initiation implies that 40S ribosomal subunits bind at the 5′-end of capped mRNA and then scan the template until they reach the first AUG codon, at which translation is initiated. Leaky scanning occurs when a proportion of ribosomal subunits can pass by the first AUG triplet and continue further scanning until they reach a downstream initiation codon (Kozak, 2002). Analysis of allexivirus TGB sequences revealed that in all viruses of this genus the TGB2 initiator AUG codon was found in a weak context (Kozak, 1983) characterized by either a cytidine or uracil residue in the (−3) position (Fig. 1c) that would reduce the initiation frequency on this codon and thus increase the probability of leaky scanning. Furthermore, in agreement with the observations previously made for other TGB-containing viruses (Morozov & Solovyev, 2003), no AUG triplets were found in allexivirus TGB2 genes between the TGB2 initiator AUG and the proposed TGB3 CUG initiator codon (data not shown). Therefore ribosomal subunits, which failed to initiate translation at the TGB2 initiator AUG codon, would continuously scan the template up to the predicted TGB3 CUG initiator codon. Taken together, these observations suggested that translation of allexivirus TGB3 could be initiated at the CUG triplet and involve the ribosome leaky scanning of a functionally bicistronic sgRNA.

To test these predictions experimentally, a region of ShVX genome comprising overlapping TGB2 and TGB3 genes (positions 6005–6473 in the ShVX genome sequence according to Arkhipov et al. (2013)) was cloned in the binary vector pLH (* Solovyev et al., 2013) under the control of the cauliflower mosaic virus 35S promoter, with the ShVX TGB3 gene being translationally fused to the GFP gene. An Agrobacterium tumefaciens strain carrying the resulting construct, pLH-ShVX-TGB2/TGB3-GFP (Fig. 2a), was used for infiltration of *N. benthamiana* leaves. Western blot analysis of infiltrated leaf regions with GFP-specific monoclonal antibodies was carried out 3 days post infiltration (p.i.). A specific band of approximately 39 kDa, revealed by Western blotting (Fig. 2c), corresponded in size to the TGB3–GFP fusion. This observation demonstrated that the ShVX TGB2 could be expressed in plants from bicistronic templates.

To verify the TGB3 alternative initiator codon prediction and analyse the mechanism of TGB3 expression, site-directed mutations were introduced into pLH-ShVX-TGB2/TGB3-GFP. The predicted TGB3 initiator CUG codon was replaced with either CAG, which has never been reported as an alternative initiator codon, or AUG (Fig. 2b). The TGB2 initiator codon was either replaced with CAG, or left intact but placed in the context optimal for translation initiation (Fig. 2b). *N. benthamiana* leaves were infiltrated with agrobacterial cultures carrying the mutant constructs and analysed by Western blotting with GFP-specific antibodies. The experiment was repeated three times, and a representative blot is shown in Fig. 2(d).

Point mutation of the predicted TGB3 initiator CUG codon to CAG completely blocked translation of the TGB3–GFP fusion protein (Fig. 2d), confirming therefore that this triplet could serve as the initiator codon for ShVX TGB3 synthesis in plants. When the TGB3 CUG initiator codon was replaced with AUG, the expression level of TGB3 increased considerably (Fig. 2d). Previous estimations based on translation of reporter constructs in protoplasts demonstrated that the efficiency of translation initiation at a CUG codon was approximately 30 % of the initiation at an AUG codon (Gordon et al., 1992). Although Western blotting cannot be considered as a quantitative method, our data indicate clearly that mutation of the wild-type CUG codon to AUG greatly increased expression of the TGB3–GFP fusion protein (Fig. 2d).

In the mutant construct with the TGB2 initiator AUG context changed to optimal, translation of the TGB3–GFP fusion was completely blocked, whereas for the mutant with the TGB2 AUG codon replaced with CAG, TGB3–GFP translation was considerably enhanced (Fig. 2d). These data demonstrate that the efficiency of translation initiation at the TGB3 initiator CUG codon depends on the initiation at the TGB2 AUG codon. Since efficient initiation at the TGB2 AUG suppressed TGB3 synthesis, while blocked initiation at the TGB2 AUG enhanced the translation of TGB3, we conclude that TGB3 synthesis is initiated via leaky scanning rather than an internal ribosome entry.
It should be noted that Western blot analysis of translation products with GFP-specific antibodies revealed an additional minor band of approximately 36 kDa, which was smaller than the TGB3–GFP fusion protein (Fig. 2d) but bigger than a non-fused GFP (Fig. 2e). We presume that this translation product can arise due to translation initiation at a cryptic initiator codon in the TGB3 gene. As the TGB3 gene sequence has no in-frame AUG...
codons, a non-AUG cryptic initiator could be considered. Taking into account that the minor translation product is approximately 3 kDa less in size compared to the full-length TGB3–GFP (Fig. 2d), we presume that its translation can be initiated at a GUG codon in positions 6306–6309 of the ShVX genome (Arkhipov et al., 2013), located in a suboptimal context GCCGUGU. Since the amount of this minor translation product is increased for the mutant lacking the TGB2 AUG codon and not detected for the mutant with the TGB2 AUG codon placed in the optimal context, one can speculate that the translation of this protein is initiated by leaky scanning, when ribosomal subunits pass by both TGB2 AUG and TGB3 CUG initiator codons. Indeed, more than two proteins are known to be translated by leaky scanning on single RNA genomes in several plant RNA viruses (Castano et al., 2009; Ling et al., 2013; Smirnova et al., 2015; Turina et al., 1998). However, it remains unknown why mutations affecting the TGB3 initiator CUG codon have no effect on minor product translation.

Thus, the data presented in this paper support the hypothesis that the TGB3 gene of ShVX and other allexiviruses is an expressed gene, which can be translated together with TGB2 from a single sgRNA.

The TGB3 proteins were shown to be expressed at very low levels in virus-infected tissues (Jackson et al., 2009; Shemyakina et al., 2011). Moreover, TGB3 proteins expressed at higher levels negatively affect subcellular transport of other TGB proteins to plasmodesmata (Lim et al., 2009) or viral movement (Lauber et al., 2005), and cause severe structural rearrangements of the endoplasmic reticulum in plant cells (Solovyev et al., 2012). As suggested earlier, the mechanism of TGB expression involving two sgRNA and, in particular, translation of TGB2 and TGB3 proteins from a single template via leaky scanning ensures the optimal ratio of the three movement proteins, calculated to be approximately 100 : 10 : 1 (Jackson et al., 2009). Taking into account that the alternative ShVX TGB3 CUG initiation codon is considerably less efficient than the AUG codon, the initiation of allexivirus TGB3 translation at the non-AUG codon could be required to further reduce the level of TGB3 synthesis. Since the biological significance of the low-level TGB3 expression is unclear, one can speculate that it can be of benefit for allexviruses in adaptation to their hosts. Interestingly, only in one of many potexivirus genomes sequenced thus far, namely in that of Lily virus X, was the TGB3 gene also found to lack the initiator codon (Memelink et al., 1990). This observation might support the hypothesis that this feature can be pertinent to specific virus–host adaptation.

Another hypothesis explaining the low-level expression of allexivirus TGB3 can be based on our recent analysis of new TGB types. The Hibiscus green spot virus (HGSV) genome was found to have TGB with unusual features of encoded proteins; in particular, the TGSV TGB3 is rather small and exhibits no sequence similarity to TGB3 proteins of other viruses (Morozov & Solovyev, 2012). Further computer analysis of plant transcriptome databases revealed HGSV-like TGBs in virus-like RNA assemblies (VLRA) corresponding to genomic RNAs of viruses infecting plants subjected to transcriptome analysis (Morozov & Solovyev, 2015). Amazingly, a Litchi chinensis VLRA was shown to encode a HGSV-like transport gene block lacking the TGB3 gene (Morozov & Solovyev, 2015). We hypothesized that early in TGB evolution, TGB1 and TGB2 proteins were sufficient for viral cell-to-cell movement, while the TGB3 gene was acquired later in TGB evolution, possibly as an accessory protein, initially unnecessary for viral transport but increasing its efficiency (Morozov & Solovyev, 2015). This hypothesis is supported by the finding that potato virus X (PVX; genus Potexvirus) can move from cell to cell even in the absence of TGB3, although the process is inefficient (Tamai & Meshi, 2001). Taking into account that PVX TGB2 is able to increase the plasmodesmata size exclusion limit, while PVX TGB3 targets TGB2 to cell peripheral sites and most probably plasmodesmata (Schepetilnikov et al., 2005; Tamai & Meshi, 2001), the conclusion about the auxiliary function of TGB3 was further reinforced by the observation that the PVX TGB3 deficiency can be compensated by increased amounts of TGB2 (Tamai & Meshi, 2001). One can speculate that the allexivirus TGB partially reverted to an evolutionary early mode of TGB function, which relies mostly on the activities provided by TGB1 and TGB2, while TGB3 retained accessory functions. This could occur due to the persistence of allexviruses in vegetatively propagated plants and/or their natural existence as a component of viral complexes, including also carlaviruses and potyviruses (Chen et al., 2001; Katis et al., 2012).

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (grant 13-04-00667-a). We thank Dr T. N. Erokhina for providing GFP-specific monoclonal antibodies.

References


