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Identification of an *Arabidopsis thaliana* protein that binds to tomato mosaic virus genomic RNA and inhibits its multiplication

Koki Fujisaki, Masayuki Ishikawa *

Plant–Microbe Interactions Research Unit, National Institute of Agrobiological Sciences, 2-1-2 Kan-non-dai, Tsukuba 305-8602, Japan

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ABSTRACT

The genomic RNAs of positive-strand RNA viruses carry RNA elements that play positive, or in some cases, negative roles in virus multiplication by interacting with viral and cellular proteins. In this study, we purified *Arabidopsis thaliana* proteins that specifically bind to 5' or 3' terminal regions of tomato mosaic virus (ToMV) genomic RNA, which contain important regulatory elements for translation and RNA replication, and identified these proteins by mass spectrometry analyses. One of these host proteins, named BTR1, harbored three heterogeneous nuclear ribonucleoprotein K-homology RNA-binding domains and preferentially bound to RNA fragments that contained a sequence around the initiation codon of the 130K and 180K replication protein genes. The knockout and overexpression of *BTR1* specifically enhanced and inhibited, respectively, ToMV multiplication in inoculated *A. thaliana* leaves, while such effect was hardly detectable in protoplasts. These results suggest that BTR1 negatively regulates the local spread of ToMV.

Introduction

After entry into the host cell, the genomic RNA of a positive-strand RNA virus is released from virions into the cytoplasm, where the genomic RNA is translated to produce the replication proteins and other viral gene products. Then, the genomic RNA is specifically recognized, recruited to the cytoplasmic surfaces of organellar membranes to form the replication complex, and utilized as the template of negative-strand RNA synthesis (Ahlquist, 2006). The replicated genomic RNA assembles with the structural proteins into virions. For translation, replication, and encapsidation of the genomic RNA, specific RNA sequences or structures in the genomic RNA play important roles by interacting with each other (Miller and White, 2006) and/or with viral and/or cellular proteins (Martinez-Salas et al., 2008; White and Nagy, 2004). Such RNA elements often reside in the 5' and 3' untranslated regions (UTRs) (Dreher and Miller, 2006; Sullivan and Ahlquist, 1997), but some exist in the coding regions (McKnight and Lemon, 1998; Zimmern, 1977). Some specific interactions between viral genomic RNA and cellular proteins have negative effects on the viral life cycle (Paranjape and Harris, 2007; Zhu et al., 2007).

Tomato mosaic virus (ToMV) is a positive-strand RNA virus that belongs to the genus *Tobamovirus*. The genome of ToMV is a 5' m^7Gppp(G)-capped monopartite RNA of 6384 nucleotides (nts), and encodes at least four proteins: a 130-kDa (130K) protein and its readthrough product of 180 kDa (180K protein), a 30-kDa protein, and a coat protein (CP). The 130K and 180K proteins are synthesized from the genomic RNA and involved in viral RNA replication, suppression of RNA silencing, and cell-to-cell movement. The 30-kDa protein is required for viral cell-to-cell movement. The 30-kDa protein and CP are synthesized by translation of the respective subgenomic RNAs (Ishikawa and Okada, 2004; Kubota et al., 2003).

ToMV genomic RNA harbors 5' and 3' UTRs of 71 nts and 202 nts, respectively. Because some modifications in the 5' or 3' UTRs deleteriously affect infectivity (Takamatsu et al., 1990; 1991), these UTRs must play crucial roles in ToMV multiplication. The 5' UTR shows activity to enhance translational efficiency. The region contains a (CAA) repeat (nts 20–44), to which a host heat-shock protein HSP101 binds, and this binding contributes to the translational enhancement *in vitro* and in yeast (Tanguay and Gallie, 1996; Wells et al., 1998). The 3' UTR of ToMV RNA harbors three consecutive pseudoknots immediately downstream of the termination codon of the CP open reading frame (ORF), followed by a 3'-terminal transfer RNA (tRNA)-like structure. The pseudoknot structure enhances translation in *cis*, and interacts with HSP101 (Tanguay and Gallie, 1996; Wells et al., 1998) and eukaryotic elongation factor 1A (eEF1A) (Zeenko et al., 2002). The tRNA-like structures of tobamoviruses and many other plant viruses interact with various tRNA-modifying enzymes, including aminoacyl-tRNA synthetase and tRNA nucleotidyltransferase (Fechter et al., 2001), but the involvement of these host factors in tobamovirus multiplication is poorly understood.

Plant vacuoles contain high activities of proteases and nucleases that can hamper the biochemical analyses of RNA- and protein-related functions. Indeed, we found that cell extracts prepared from evacuolated plant protoplasts have high translation activity, while those prepared by direct disruption of the original (vacuole-containing) protoplasts do not (Komoda et al., 2004). In this study, we

* Corresponding author. Fax: +81 29 838 7009.
E-mail address: ishika32@affrc.go.jp (M. Ishikawa).

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searched for proteins that interact with 5' or 3' terminal regions of ToMV genomic RNA from the extracts of evacuolated *Arabidopsis thaliana* protoplasts, which were expected to have low nuclease and protease activities.

Results

Purification of A. thaliana proteins that bind to ToMV RNA

The genomic RNA of ToMV is 6384 nts in length, and harbors 5' and 3' UTRs of 71 nts and 202 nts, respectively (Fig. 1A). To isolate plant proteins that specifically bind to the 5' or 3' terminal regions of ToMV genomic RNA, StreptoTag, an RNA aptamer that binds to streptomycin (Fig. 1B) (Wallace and Schroeder, 1998; Bachler et al., 1999), was utilized. We prepared probe RNAs that carry the StreptoTag and a 5'-terminal sequence (nts 1–277 for st-L5 RNA) or a 3'-terminal sequence (nts 6166–6384 for st-L3 RNA) of ToMV genomic RNA. As a control, st-LR RNA that carries the StreptoTag and an internal region of ToMV RNA (nts 764–1004 within the coding region for the 130K and 180K proteins) was also prepared (Figs. 1A, B).

st-L5, st-L3, and st-LR RNAs were separately incubated with the 30,000 ×*g* supernatant (the S30 fraction) of evacuolated *A. thaliana* protoplast extracts, followed by affinity purification using streptomycin-conjugated Sepharose beads. Analysis of the purified fractions by SDS-PAGE and silver staining revealed that the fractions contained st-L5, st-L3, or st-LR RNAs and their degradation products, as well as putative proteins that were removed by phenol–chloroform extraction (Fig. 1C). The band patterns of putative proteins in the st-L3 and st-L5 RNA-purified fractions were different from each other, and most of these bands were absent in the st-LR RNA-purified fraction (Fig. 1C, asterisks), suggesting that some *A. thaliana* proteins were specifically co-purified with st-L5 or st-L3 RNAs.

Previously, Zeenko et al. demonstrated that eEF1A binds to the 3' terminal region of TMV genomic RNA (Zeenko et al., 2002). The band of approximately 50 kDa in the st-L3-purified fraction (marked by 'X' in Fig. 1C) was identified as eEF1A by immunoblot analysis (Fig. 1D) and by mass spectrometry analysis (MALDI-TOF-MS) of its tryptic digest peptides. This result confirms the results of Zeenko et al. and demonstrates the usefulness of the StreptoTag affinity purification method to isolate ToMV sequence-specific RNA-binding proteins.

Identification of a protein that binds to the 5' terminal region of ToMV genomic RNA

Proteins specifically co-purified with st-L5 or st-L3 RNA were excised from the SDS-PAGE gel, digested with trypsin, and analyzed by mass spectrometry (LC–MS/MS). A MASCOT analysis of the LC–MS/MS data suggested that many of the protein bands represented known or putative RNA-binding proteins. Among these proteins, we focused on a protein found in band 'Y,' which was co-purified with st-L5 RNA (Fig. 1C). The protein showed an approximate molecular mass of 37 kDa on SDS-PAGE, and its 11 tryptic digest peptides showed MS/MS spectra similar to those predicted for an *A. thaliana* protein that contained three KH (heterogenous nuclear ribonucleoprotein K-

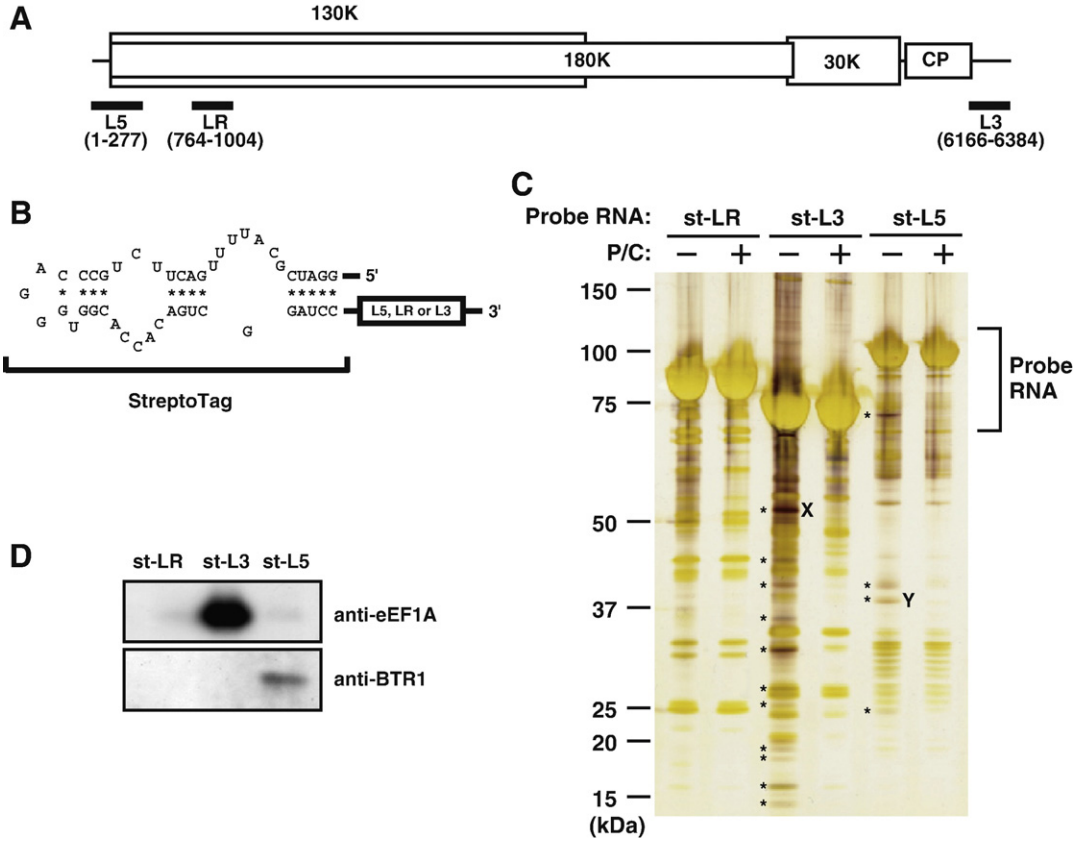

Fig. 1. Purification of *A. thaliana* proteins that bind to ToMV RNA. (A) ToMV genome organization. Thick bars show the regions carried by the probes that were used for protein purification. (B) Structure of the probes. (C) SDS-PAGE patterns of the StreptoTag-purified samples. StreptoTag-purified fractions (for P/C − lanes) were subjected to SDS-PAGE followed by silver staining. The fractions were also extracted with phenol–chloroform, and the aqueous phase was recovered and analyzed in parallel (for P/C + lanes). The positions and molecular masses (kDa) of protein markers are shown on the left side of the gel. Probe RNAs (st-LR, st-L3, and st-L5) used for purification are indicated above the gel. Putative protein bands are denoted by asterisks. The protein bands corresponding to eEF1A and BTR1 are indicated by X and Y, respectively. The positions of the probe RNA bands are shown on the right side of the gel. (D) Specific purification of eEF1A and BTR1 with st-L3 and st-L5 probes, respectively. StreptoTag-purified fractions (P/C-untreated samples in panel C) were subjected to immunoblot analysis using anti-eEF1A or anti-BTR1 antisera.