Possible involvement of eEF1A in *Tomato spotted wilt virus* RNA synthesis

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ABSTRACT

Tomato spotted wilt virus (TSWV) is a negative-strand RNA virus in the family *Bunyaviridae* and propagates in both insects and plants. Although TSWV can infect a wide range of plant species, host factors involved in viral RNA synthesis of TSWV in plants have not been characterized. In this report, we demonstrate that the cell-free extract derived from one of the host plants can activate mRNA transcriptional activity of TSWV. Based on activity-guided fractionation of the cell-free extract, we identified eukaryotic elongation factor (eEF) 1A as a possible host factor facilitating TSWV transcription and replication. The RNA synthesis-supporting activity decreased in the presence of an eEF1A inhibitor, suggesting that eEF1A plays an important role in RNA synthesis of TSWV.

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Introduction

Tomato spotted wilt virus (TSWV) is a type member of the genus *Tospovirus* within the family *Bunyaviridae* (King et al., 2012). TSWV was recently nominated as a top 10 plant virus based on scientific and economic importance (Scholthof et al., 2011). TSWV infects over 1000 plant species including many important crops, and is transmitted by insects in the order Thysanoptera (thrips) in a persistent and propagative manner (Parrella et al., 2003; Wijkamp et al., 1993). The genome of TSWV is composed of three viral strand RNAs (vRNAs) named L, M, and S. L vRNA is a negative-strand RNA, while M and S vRNAs are ambisense RNAs. In virions, these RNAs are associated with the nucleocapsid protein (N protein) and RNA-dependent RNA polymerase (L protein) to form viral ribonucleoprotein (vRNP) complexes (de Haan et al., 1991; German et al., 1992).

The vRNP complexes of TSWV perform two types of RNA synthesis: one to produce full-length, viral complementary strand RNA (cRNA) and progeny vRNA of each genome segment (replication; Fig. 1A) and the other to synthesize mRNA for each viral protein (transcription; Fig. 1A) (van Knippenberg et al., 2002).

Neither vRNAs nor cRNAs are 5′ capped during replication. In contrast, during transcription, viral mRNAs are capped by a cap-snatching mechanism, similar to other segmented negative-strand RNA viruses, including animal-infecting bunyaviruses and the influenza virus, in which the 5′ terminal 10–20 nucleotide (nt) RNA fragments of cellular mRNAs are cleaved and utilized as primers for transcription (Fig. 1A) (Bouloy et al., 1978; Duijsings et al., 2001; Geerts-Dimitriadou et al., 2011; Jin and Elliot, 1993; Kormelink et al., 1992a, 1992b; Mir et al., 2008; Plotch et al., 1979, 1981; Simons and Pettersson, 1991; van Knippenberg et al., 2005a; van Poelwijk et al., 1996). Transcription of mRNAs for the N protein and an RNA silencing suppressor protein NSs is terminated at a predicted stem–loop structure of the intergenic region of the S segment (Fig. 1A) (Clabbers et al., 2014; de Haan et al., 1990; van Knippenberg et al., 2005b). This stem–loop structure also plays a role as a translational enhancer of viral mRNAs (Geerts-Dimitriadou et al., 2012). However, the mechanism by which replication and transcription of TSWV are regulated remains unclear. Previous studies have shown that detergent-treated TSWV virions can perform genome replication in the absence of cellular factors (Adkins et al., 1995; van Knippenberg et al., 2002). Viral mRNA transcription was observed only in the presence of rabbit reticulocyte lysate (RRL), suggesting that RRL contains factors required for TSWV mRNA transcription (van Knippenberg et al., 2002).

Tobacco (*Nicotiana tabacum* L.) is a host plant of TSWV. We previously demonstrated that evacuolated tobacco BY-2 protoplast extract (BYL) supports the translation and subsequent genomic

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RNA replication of several positive-strand RNA viruses including *Tomato mosaic virus*, *Brome mosaic virus*, and *Turnip crinkle virus* (Komoda et al., 2004). Moreover, some studies have demonstrated that the genomic RNAs of other positive-strand RNA viruses (*Red clover necrotic mosaic virus* and *Tomato bushy stunt virus*) can be translated and replicated in this BYL system (Gursinsky et al., 2009; Iwakawa et al., 2007). Since the activities of vacuolar lytic enzymes such as proteases and ribonucleases are significantly decreased based on the evacuolation step during preparation (Komoda et al., 2004), BYL should be appropriate for not only studying the replication of positive-strand RNA viruses but also studying RNA synthesis of the negative-strand (and ambisense) RNA viruses, such as TSWV. In this report, we demonstrate that TSWV performed viral mRNA transcription when mixed with BYL and that the eukaryotic elongation factor (eEF) 1A is a possible host factor to activate TSWV RNA synthesis.

Results

TSWV mRNA transcription was supported by plant cell extracts

We first examined the RNA synthesis activity of TSWV in the absence and presence of RRL to reproduce the results of Adkins et al. (1995) and van Knippenberg et al. (2002). TSWV was incubated with replication buffer or RRL reaction mixture (GE Healthcare, Milwaukee, WI) as described previously. After incubation at 30 °C for 90 min in the presence of $[\alpha\text{-}^{32}P]CTP$, ^{32}P

incorporation into RNA products was analyzed by denaturing urea polyacrylamide gel electrophoresis (Urea PAGE). In the presence of replication buffer, two ^{32}P-labeled RNA bands of about 3.0 kb and larger products (marked by an asterisk) were detected (Fig. 1B, top panel, lane 3). In the presence of RRL, the signal intensity of these bands increased, and two additional bands of approximately 1.2 kb and 1.7 kb were detected (Fig. 1B, top panel, lane 6). The 1.2-, 1.7-, and 3.0-kb RNA bands were observed in previous reports and described as N mRNA, NSs mRNA, and S RNA segment, respectively (van Knippenberg et al., 2002, 2004; de Medeiros et al., 2005). To identify these RNA bands, we performed Northern hybridization analysis. Note that not only vRNA but also cRNA of the S segment was already present in virions, as previously reported (Tsuda et al., 1992; Fig. 1B, middle and bottom panels, lanes 2 and 5). The band intensity of vRNA and cRNA of the S RNA was not increased during the RNA synthesis reaction (Fig. 1B, middle and bottom panels, lanes 2, 3, 5, and 6), suggesting that the accumulated levels of newly synthesized S RNA were much lower than that of input viral S RNA. We confirmed that the 1.2-kb N mRNA was synthesized in the presence of RRL (Fig. 1B, bottom panel, lane 6). Unfortunately, we could not detect the 1.7-kb NSs mRNA band since it may have been masked by the presence of excess vRNA of S RNA (Fig. 1B, middle panel, lane 6). Although this 1.7-kb band is likely the NSs mRNA according to the previous reports, it is deemed as an uncharacterized RNA (marked by double asterisks) in this study. Next, we explored whether membrane-depleted BYL (mdBYL) (Komoda et al., 2004, 2007) facilitated TSWV RNA synthesis as RRL did. When TSWV was incubated with the mdBYL reaction mixture, the ^{32}P-labeled 1.2-kb N mRNA and 1.7-kb products synthesized in the presence of RRL were clearly detected (Fig. 1B, top panel, lane 9), although the 3.0-kb S RNA band was weak in the mdBYL sample (Fig. 1B, top panel, lanes 6 and 9). The
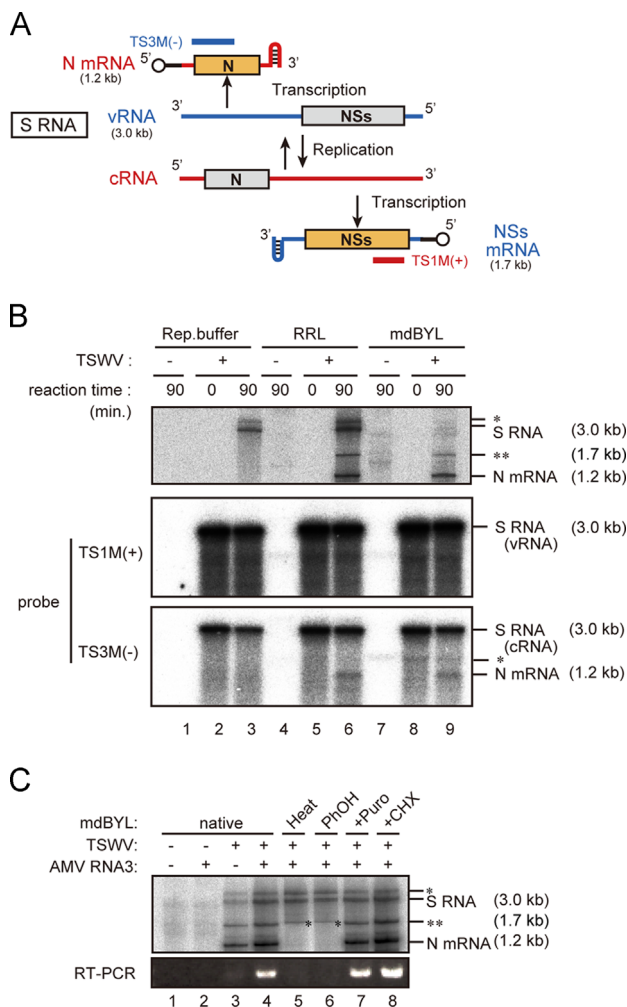
Fig. 1. The effect of addition of cell extracts on TSWV RNA synthesis. (A) Gene expression strategy of the TSWV S RNA segment. In the replication reaction, viral strand RNA (vRNA: long blue line) is used as template to synthesize full-length viral complementary strand RNA (cRNA: long red line). This cRNA strand is used as template to synthesize full-length vRNA strands. In the transcription reaction, the mRNA for NSs is transcribed from cRNA of the S RNA, whereas the mRNA for N is transcribed from vRNA of the S RNA. Both NSs and N mRNA possess the 5′ cap structures (depicted as open circles) and several nucleotides of cellular mRNA 5′-terminal sequences (black line) are introduced via a cap snatching mechanism. The positions of probe TS1M(+), which anneals to NSs mRNA and vRNA, and probe TS3M(−), which anneals to N mRNA and cRNA, are shown. The RNA strands, which possess the same polarity as vRNA, are represented by blue lines, whereas those that possess the same polarity as cRNA are represented by red lines. (B) Detection of synthesized TSWV-related RNAs. TSWV virions purified from the infected leaves (lanes 2, 3, 5, 6, 8, and 9) or the equivalent fraction prepared from mock-inoculated leaves (lanes 1, 4, and 7) are incubated with the replication buffer (lanes 1, 2, and 3), with rabbit reticulocyte lysate (RRL; lanes 4, 5, and 6) or with membrane-depleted BY-2 evacuolated protoplast extract (mdBYL; lanes 7, 8, and 9) at 30 °C for 90 min in the presence (top panel) or the absence (middle and bottom panels) of $[\alpha\text{-}^{32}P]CTP$. Total RNAs are obtained from samples and analyzed by 8 M urea–2.4% PAGE (top panel) or Northern analysis (middle and bottom panels). The ^{32}P-labeled RNA bands are detected by autoradiography (top panel). For Northern analysis, the ^{32}P-labeled TS1M(+) probe is used to detect vRNA of S RNA and NSs mRNA (middle panel), and the TS3M(−) probe is used to detect cRNA of S RNA and N mRNA (bottom panel). Positions of S RNA and N mRNA are indicated on the right of each panels. Positions of uncharacterized RNAs (*) and 1.7-kb RNA (**) are indicated on the right of each panels. (C) TSWV RNA synthesis and assays in the presence of mdBYL under various conditions. Capped AMV RNA3 leader fragment is added (lanes 2, 4–8) or not added (lanes 1 and 3) to the mdBYL reaction mixture before incubation. TSWV virions purified from infected leaves (lanes 3–8) or the equivalent fraction from mock-inoculated leaves (lanes 1 and 2) are mixed with native mdBYL (lanes 1–4), heat-treated (65 °C for 20 min) mdBYL (lane 5), a phenol-extracted and ethanol-precipitated fraction from mdBYL (lane 6), native mdBYL with puromycin (lane 7), or native mdBYL with cycloheximide (lane 8). ^{32}P-labeled RNA products are detected using autoradiography (top panel). Incorporation of capped AMV RNA3 to N mRNA (cap snatching) is examined by reverse transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis (bottom panel). Positions of S RNA, N mRNA, uncharacterized RNA (*) and 1.7-kb RNA (**) are indicated on the right of panel. An asterisk at lanes 5 and 6 also indicates an uncharacterized RNA band. Puro, puromycin; CHX, cycloheximide.

mdBYL reaction samples showed almost similar pattern of Northern hybridization to that obtained with RRL (Fig. 1B, bottom panel, lanes 4–9).

To determine whether cap snatching occurred during the reaction with mdBYL, we added *in vitro*-synthesized capped RNAs of the leader sequence of *Alfalfa mosaic virus* (AMV) RNA3. AMV RNAs are known to be an efficient cap donor *in vivo* and *in vitro* (Duijsings et al., 1999, 2001; van Knippenberg et al., 2005a). The addition of cap-donor RNAs did not significantly change the pattern of ^{32}P-labeled RNA bands, but slightly enhanced their intensity including the S RNA band (Fig. 1C, lanes 3 and 4 of the upper panel). AMV RNA-primed N mRNA synthesis (i.e. cap snatching) in the presence of mdBYL was confirmed using reverse transcription-polymerase chain reaction (RT-PCR) analysis with a primer corresponding to the leader sequence of AMV RNA3 and another primer complementary to the TSWV N protein-coding sequence (Fig. 1C, lanes 1–4 of the lower panel).

To characterize host factors required for TSWV transcription, we examined the effect of heat treatment and deproteinization of mdBYL on TSWV RNA synthesis *in vitro*. After these treatments, mdBYL no longer facilitated N mRNA production (Fig. 1C, lanes 5 and 6). Based on this result, a proteinaceous factor is required for N mRNA transcription. The addition of translational inhibitors (puromycin and cycloheximide) did not affect N mRNA transcription in the presence of mdBYL (Fig. 1C, lanes 7 and 8), as previously reported in experiments using RRL (van Knippenberg et al., 2004). This result is in contrast to observations in animal bunyaviruses, in which ongoing translation is required for viral mRNA transcription (Abraham and Pattnaik, 1983; Barr, 2007; Bellocq and Kolakofsky, 1987; Bellocq et al., 1987; Ikegami et al., 2005, Patterson and Kolakofsky, 1984; Pattnaik and Abraham, 1983, Raju and Kolakofsky, 1987; Vialat and Bouloy, 1992). This unique characteristic of TSWV should make it feasible to identify host factors required for viral mRNA transcription based on biochemical fractionation of mdBYL.

Purification of host factors supporting TSWV RNA synthesis

We partially purified mdBYL using the ammonium sulfate precipitation method (Fig. 2A), and each fraction was incubated with TSWV to analyze RNA synthesis activity. Before the assay, each fraction was buffer-exchanged, and AMV RNA3 leader RNA was added as a cap donor. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining, we demonstrated that the majority of proteins was precipitated with 50% saturated ammonium sulfate (the 0–50% P fraction; lane 2 of Fig. 2B), but the transcription-supporting activity was mainly observed in the fraction precipitated between 50% and 80% saturated ammonium sulfate (50–80% P fraction; lane 3 of Fig. 2B and lane 4 of Fig. 2C). Notably, the intensity of the S RNA band increased in the presence of the 50–80% P fraction compared to that of unfractionated mdBYL (Fig. 2C, lanes 2 and 4; see *Discussion*).

We next fractionated the 50–80% P fraction using a HiTrap SP FF cation exchange column (GE Healthcare; Fig. 3A and B). The transcriptional activity of TSWV N mRNA was mainly observed in fraction #8, although most proteins did not bind to the column and were found in the flow-through fraction (Fig. 3B). The HiTrap SP #8 fraction was subsequently subjected to size-exclusion chromatography (SEC) using Superdex 200 10/300GL (GE Healthcare; Fig. 3A and C). Using SDS-PAGE and silver staining, an approximately 50-kDa polypeptide was found to be a major component of SEC fractions that had N mRNA transcriptional activity (Fig. 3C, top and middle panels). Based on the elution volume in SEC, the 50-kDa protein was estimated to be a monomer. Because our procedure to purify this 50-kDa protein was similar to the method reported for maize eEF1A (54 kDa) (Sun et al., 1997), we hypothesized that the 50-kDa protein
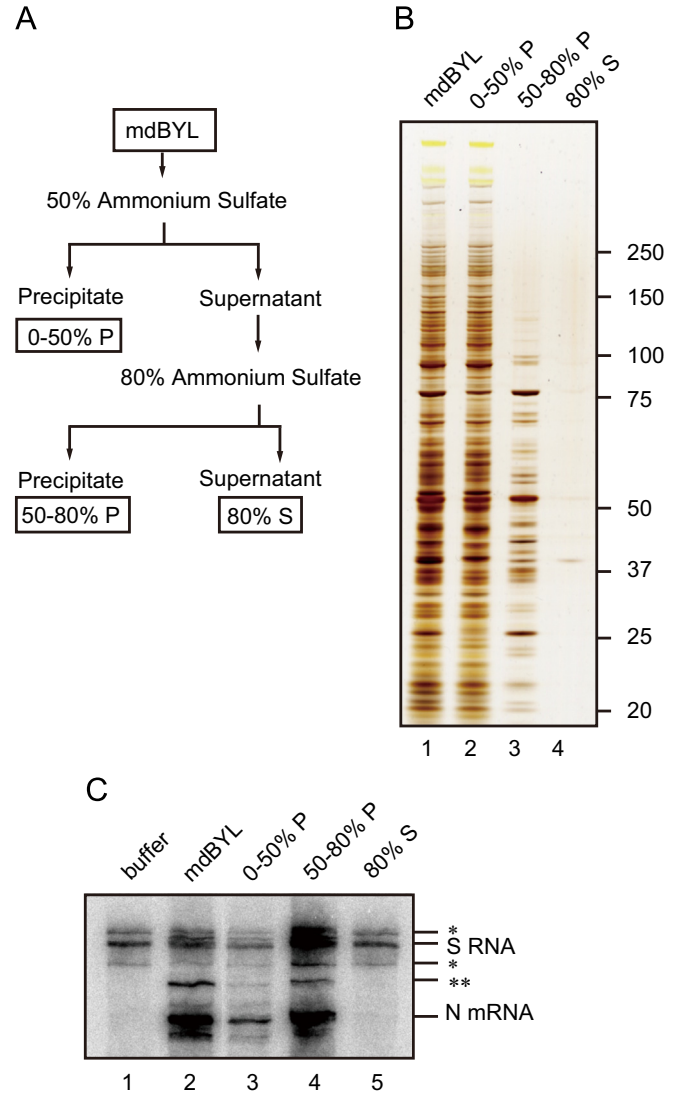

Fig. 2. Ammonium sulfate purification of the host factor that supports TSWV mRNA transcription. (A) Diagram of mdBYL fractionation by ammonium sulfate precipitation. (B) The silver-stained SDS-PAGE gel of each fraction. The positions of protein markers are shown with their sizes in kDa. (C) TSWV RNA synthesis assay with the mdBYL ammonium sulfate precipitation fraction. After fractionation, the ammonium sulfate of each fraction (500 μL) is removed through Vivaspin 500 (MWCO: 10,000). TSWV RNA synthesis assay with the buffer-exchanged fractions is performed as shown in Fig. 1. Positions of each RNAs are indicated on the right as in Fig. 1B.

may be eEF1A. To confirm this hypothesis, we performed Western blotting analysis using anti-eEF1A antibody. As expected, an immunopositive band was detected at approximately 50 kDa (Fig. 3B and C, bottom panel), suggesting that eEF1A is a host factor endowing TSWV with transcriptional activity. We also found that the intensity of the S RNA band increased in the presence of the eEF1A-containing fractions (Fig. 3B and C, top panel). A similar result was observed by incubating TSWV with the 50–80% P fraction (Fig. 2B). These observations suggested that the replication activity of TSWV is enhanced by eEF1A-containing fractions.

Suppression of BYL-mediated enhancement of TSWV RNA synthesis by an eEF1A inhibitor

To explore the role of eEF1A in TSWV RNA synthesis, we utilized an inhibitor of eEF1A, namely, didemnin B (DB) (Li et al., 2010; Vera and Joullié, 2002). The intensity of ^{32}P-labeled RNA signals observed
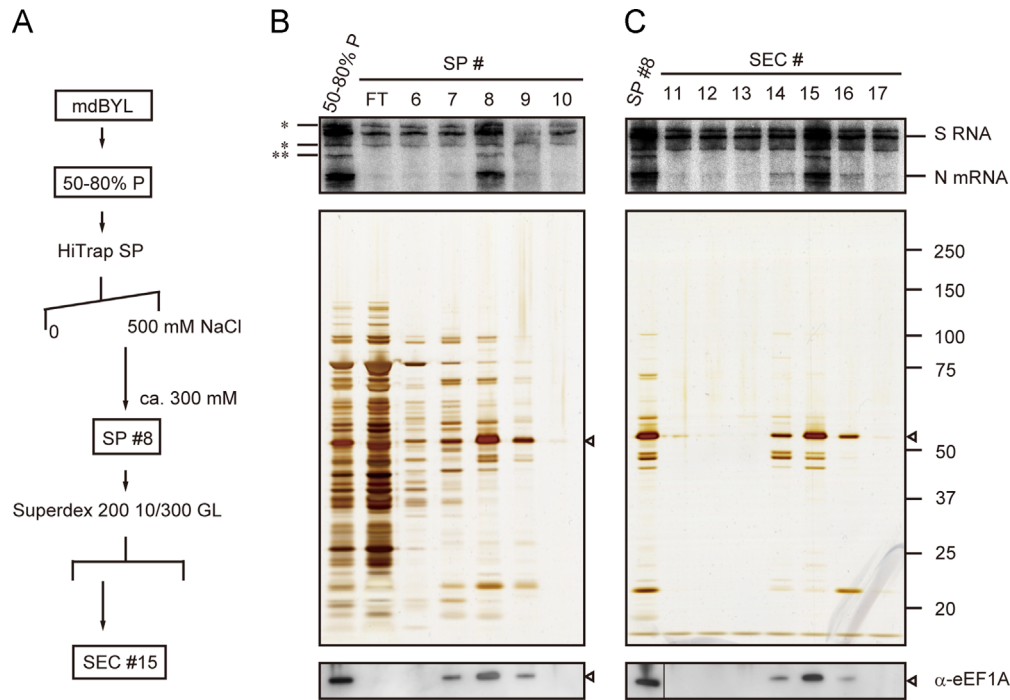
Fig. 3. Further purification of the 50–80% P fraction by cation exchange and size-exclusion chromatography. (A) Purification diagram of the host factor involved in TSWV RNA synthesis using column chromatography. The 50–80% P fraction of ammonium sulfate precipitation is loaded onto a HiTrap SP FF column and eluted with a linear gradient of 0–500 mM NaCl. The HiTrap SP #8 fraction is then loaded onto a Superdex 200 10/300GL column. (B) Fractionation of the 50–80% P fraction by HiTrap SP. TSWV RNA synthesis assay in the presence of mdBYL fractions (top). The silver staining of the SDS-PAGE gel of each fraction (middle). Western blotting using anti-eEF1A antibody (bottom). FT, flow-through fraction. (C) Fractionation of the HiTrap SP #8 fraction by Superdex 200 10/300GL. TSWV RNA synthesis assay in the presence of mdBYL fractions (top). The silver staining of the SDS-PAGE gel of each fraction (middle). Western blotting using anti-eEF1A antibody (bottom). Positions of S RNA and N mRNA are indicated on the right of C, top panel. The positions of uncharacterized RNAs are indicated on the left of B, top panel as Fig. 1B. The positions of protein markers are shown on the right of C with their sizes in kDa (middle). The position of the 50-kDa protein are indicated by open triangles on the right of B and C (middle and bottom).
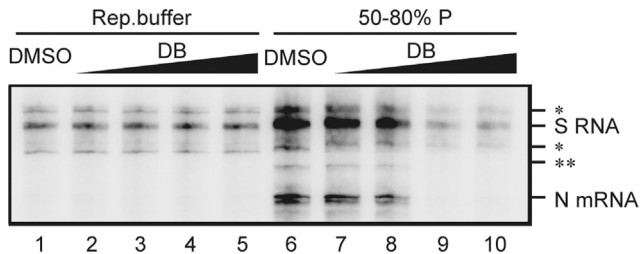

Fig. 4. Effect of the eEF1A inhibitor didemnin B (DB) on TSWV RNA synthesis. DB is added to TSWV virions with replication buffer (lanes 1–5) or the 50–80% P ammonium sulfate fraction (lanes 6–10) at a final concentration of 0.1 μM (lanes 2 and 7), 1 μM (lanes 3 and 8), 10 μM (lanes 4 and 9), or 100 μM (lanes 5 and 10). Positions of S RNA and N mRNA are indicated on the right. The positions of uncharacterized RNAs are indicated on the right as shown in Fig. 1B.

in the absence of the mdBYL fraction was not affected by DB (Fig. 4, lanes 1–5). In contrast, the N mRNA transcriptional activity in the 50–80% P fraction was completely inhibited by DB at concentrations above 10 μM (Fig. 4, lanes 9 and 10). Moreover, the intensity of the S RNA band that increased upon addition of the 50–80% P fraction reverted to basal or lower levels in the presence of DB (Fig. 4, lanes 6–10). These results suggest that eEF1A is required for transcription and facilitates the replication of TSWV RNA.

Discussion

BYL has been used as an in vitro system to study RNA synthesis of various positive-strand RNA viruses (Gursinsky et al., 2009; Iwakawa et al., 2007; Komoda et al., 2004). In this study, we developed the BYL system to investigate the transcription of

TSWV, a negative-strand RNA virus, by optimizing the BYL mixture (see *Materials and methods*). BYL must contain all host factors involved in TSWV RNA synthesis since tobacco is a host plant of TSWV. Previously, it was reported that three insect-encoded proteins are involved in TSWV multiplication: 50 kDa and 96 kDa thrips proteins associated with the TSWV glycoproteins and thought to be receptors (Bandla et al., 1998; Kikkert et al., 1998; Medeiros et al., 2000), and the other is a putative transcription factor (FoTF) required for the replication of TSWV M and L RNA segments, but not for mRNA transcription (de Medeiros et al., 2005). In this study, we identified eEF1A as a plant host factor that supports the transcription of TSWV using a BYL in vitro system.

eEF1A is one of the abundant proteins and highly conserved across kingdoms (Mateyak and Kinzy, 2010). In addition to its canonical role as a translation factor, eEF1A has a variety of noncanonical functions (Li et al., 2013; Mateyak and Kinzy, 2010). For example, many positive strand RNA viruses (*Tombusviridae*, *Flaviviridae*, *Tymoviridae*, and *Virgaviridae*) are known to employ eEF1A for RNA synthesis (Davis et al., 2007; Li et al., 2010; Matsuda et al., 2004; Yamaji et al., 2010). However, a few reports have described the involvement of eEF1A in RNA synthesis of other virus groups. In retroviruses, HIV-1 was recently reported to recruit eEF1A and eEF1G as components of the reverse transcription complex (Warren et al., 2012). Moreover, *Vesicular stomatitis virus* (VSV), one of the 'non-segmented' negative-strand RNA viruses (*Mononegavirales*), is the only virus whose transcription reaction (but not replication reaction) occurs with the assistance of eEF1A (Qanungo et al., 2004). The transcriptional mechanism of *Mononegavirales* differs from that of 'segmented' negative-strand RNA viruses such as TSWV. For example, VSV transcription occurs in a sequential start–stop process, and VSV does not employ cap-snatching (Barr et al., 2002). Our study

provides the first evidence that eEF1A is engaged in the transcription of 'segmented' negative-strand RNA viruses. Unlike in VSV, eEF1A activates the replication reaction in addition to the transcription reaction in TSWV because the S RNA band intensity increased in the eEF1A-containing fraction but decreased in the presence of DB, as shown in Fig. 4. However, the increased amount of S RNA band may reflect the erroneous transcription termination read-through. This possibility is yet to be analyzed.

In the family *Bunyaviridae*, active protein synthesis (or ribosome scanning) was reportedly required for mRNA transcription of viruses in the genus *Phlebovirus* (*Rift Valley Fever virus*) (Ikegami et al., 2005) and *Orthobunyavirus* (*Bunyamwera virus*, *La Crosse virus*, *Germiston virus*, and *Akabane virus*) (Abraham and Pattnaik, 1983; Barr, 2007; Bellocq and Kolakofsky, 1987; Bellocq et al., 1987; Patterson and Kolakofsky, 1984; Pattnaik and Abraham, 1983; Raju and Kolakofsky, 1987; Vialat and Bouloy, 1992). The molecular mechanism of the coupled translation and transcription in *Orthobunyavirus* was proposed as nascent RNA unfolding by ribosome scanning to inhibit premature termination of transcription (Bellocq and Kolakofsky, 1987; Barr, 2007). Despite the close phylogenetic relationship between *Tospovirus* and *Orthobunyavirus* (Marklewitz et al., 2013), TSWV does not require a translation reaction for mRNA synthesis (van Knippenberg et al., 2004). We propose two possible explanations for this discrepancy. The first explanation is that eEF1A might bind and activate TSWV RNA polymerase directly. Previously, it was reported that bacteriophage Qβ replicase consists of virus-encoded RNA-dependent RNA polymerase (β-subunit) and host-donated EF-Tu (a bacterial counterpart of eEF1A), EF-Ts (a bacterial counterpart of eEF1B), and ribosomal protein S1 (Blumenthal et al., 1972; Takeshita and Tomita, 2010). In this case, eEF1A is thought to play chaperone-like roles in the assembly and maintenance of the structure of the active Qβ replicase (Takeshita and Tomita, 2010). Furthermore, in the case of VSV, eEF1A binds to VSV RNA-dependent RNA polymerase and activates viral transcription (Qanungo et al., 2004). The second possibility is that eEF1A binds to TSWV genomic RNA, especially the predicted stem–loop structure localized between N and NSs ORFs (Clabbers et al., 2014; de Haan et al., 1990; van Knippenberg et al., 2005b). This binding might induce correct transcription termination. Since eEF1A delivers aminoacyl-tRNAs to the ribosome during the translation reaction, RNA binding should be a canonical feature of eEF1A. Future studies should elucidate the role of eEF1A in TSWV RNA synthesis.

Materials and methods

Virus preparation

The TSWV infected leaves of *Pericallis* x *hybrida* (MAFF number: 260050) were obtained from the National Institute of Agrobiological Sciences (NIAS) Genebank (Tsukuba, Ibaraki, Japan). For viral propagation, we ground the leaves in a mortar and pestle with cold resuspension buffer consisting of 0.1 M phosphate (pH 7.0) and 10 mM sodium sulfite, and the aqueous layer was mechanically inoculated into 2-week old *Nicotiana rustica* leaves. The virus was purified using the method described by Gonsalves and Trujillo (1986) with minor modifications. All experiments during purification were performed on ice. Batches of 50 g of TSWV infected *N. rustica* leaves were homogenized in a Waring blender at 10 times high speed with 150 mL of 0.1 M sodium phosphate pH 7.0 and 10 mM sodium sulfite. After filtering through a twofold aseptic gauze, the extract was centrifuged at 10,000g for 15 min, and the resulting pellets were thoroughly dispersed with a DOUNCE glass homogenizer (Wheaton, capacity: 15 mL) in 10 mM sodium sulfite solution. After clarification (8000g for 15 min), the supernatant

was centrifuged at 100,000g for 30 min. The precipitate was then suspended using a DOUNCE glass homogenizer (Wheaton, capacity: 1 mL). Aliquots (50 μL) were stored at −80 °C until use.

Preparation and fractionation of BYL

BYL (the extract of evacuolated BY-2 protoplasts) was prepared from tobacco BY-2 (*N. tabacum* cv. Bright Yellow 2) cells, as described previously (Komoda et al., 2004). BYL (200 μL) was centrifuged at 30,000g for 15 min, and the supernatant was stored as mdBYL (Komoda et al., 2007). Then, mdBYL was fractionated using ammonium sulfate precipitation methods. For the preparation of 50% ammonium sulfate-saturated mdBYL, 94 mg of ammonium sulfate was dissolved with 300 μL of mdBYL and incubated on ice for 10 min. This mixture was then centrifuged at 6500g for 15 min at 4 °C. The precipitate fraction was referred to as "0–50% P", and 300 μL of the supernatant fraction was added to 64 mg of ammonium sulfate to make 80% ammonium sulfate-saturated mdBYL. The mixture was then centrifuged at 6500g for 15 min at 4 °C; the precipitate fraction was referred to as "50–80% P", and the supernatant fraction was called "80% S". The 50–80% P fraction was desalted with Vivaspin 500 (MWCO:10,000; Sartorius, Hannover, Germany) using buffer A [30 mM HEPES–KOH (pH 7.4), 10 mM potassium acetate, 1.8 mM magnesium acetate, 0.5 mM DTT, and 5% (v/v) glycerol]. This fraction showed high TSWV transcription-supporting activity. The desalted fraction was injected into a HiTrap SP FF cation exchange column (column volume 1 mL; GE Healthcare) equilibrated with buffer A. The fractionation (2 mL each) was started after injection. The column was washed with 9 mL of buffer A, and bound proteins were eluted with 8 mL of buffer A containing 0–500 mM NaCl in a linear gradient. The eighth fraction, which was eluted at approximately 300 mM NaCl, showed high TSWV transcription-supporting activity. This fraction was subjected to gel filtration using a Superdex 200 10/300GL column (GE Healthcare) with buffer A containing 150 mM NaCl. The eluted fraction was collected at 1.0 mL volume each. For TSWV RNA synthesis assay, each fraction was buffer-exchanged with buffer B [30 mM HEPES-KOH (pH 7.4), 80 mM potassium acetate, 1.8 mM magnesium acetate, 2 mM DTT] by Vivaspin 500 (MWCO: 10,000).

TSWV RNA synthesis assay

TSWV RNA synthesis assays were performed using 5 μL of purified TSWV in a final volume of 25 μL. These reactions were performed as described by van Knippenberg et al. (2002) with minor modifications. Briefly, the ^{32}P-incorporation assay without cell extracts was performed in replication buffer containing 30 mM HEPES (pH 7.4), 0.5 mM magnesium acetate, 5 mM manganese(II) chloride, 5 mM DTT, 1 mM of each ATP, GTP, and UTP, 25 μM of CTP, 0.1% NP-40, 0.8 U/μL RNasin (Promega, Madison, WI), and 5 μL of [α-^{32}P]CTP (800 Ci/mmol) at 30 °C for 1.5 h. The transcription assay was performed with 10 μL of RRL- (GE Healthcare), mdBYL-, or mdBYL-derived fractions, and incubated in a 25 μL of reaction mixture at 30 °C for 1.5 h. The RRL reaction mixture contained 4 mM magnesium acetate, 1 mM of ATP, GTP, and UTP, 25 μM of CTP, 0.1% NP-40, 0.8 U/μL RNasin, and 5 μL of [α-^{32}P]CTP (800 Ci/mmol). The reactions using mdBYL- or mdBYL-derived fractions were performed with buffer containing 30 mM HEPES–KOH (pH 7.4), 4 mM magnesium acetate, 1 mM of ATP, GTP, and UTP, 25 μM of CTP, 0.1% NP-40, 5 mM DTT, 20 mM EGTA, 25 mM creatine phosphate, 5 μg of creatine phosphokinase, 0.8 U/μL RNasin, 1 μg of AMV3-derived RNA (except for Fig. 1B), and 5 μL of [α-^{32}P]CTP (800 Ci/mmol). AMV3-derived RNA [m^{7}G (5′)ppp(5′)GUAUUAAUACCAUUUUCAAAAUAUUCCAAUUCAACUCA-AUUAACGCUUUUAGAAUU-3′] was synthesized by *in vitro*

transcription using AmpliCap-Max T7 High Yield Message Maker Kit (Cellscript, Madison, WI). RNA products were phenol–chloroform-extracted, ethanol-precipitated, and resuspended in sterile water. Radio-labeled RNA products were resolved by 8 M urea–2.4% PAGE. To detect RNAs in a sequence-specific manner, we also analyzed the reaction products by Northern blotting and RT-PCR. Both the in vitro replication and transcription reactions were performed using the same procedure as described above, except for the addition of 1 mM non-radiolabeled CTP instead of [α-^{32}P] CTP. RNAs of the reaction mixture were phenol–chloroform-extracted, ethanol-precipitated, and resuspended in sterile water. For Northern blotting, the extracted RNAs were separated on 1% agarose gels, transferred onto GeneScreen membranes (DuPont-NEN, Hamburg, Germany), and hybridized with ^{32}P-labeled riboprobes described below. To synthesize riboprobes, we constructed two types of plasmids containing partial sequences of TSWV S RNA. The plasmids pTS1M and pTS3M contain the 110–603-nt and 2316–2896-nt region of the TSWV genomic S RNA in the ClaI site of pSP72 (Promega), respectively. To detect the S cRNA and N mRNA, the ^{32}P-labeled TS3M(−) riboprobe was transcribed from EcoRI-digested pTS3M using T7 polymerase. For detecting the S vRNA and NSs mRNA, ^{32}P-labeled TS1M(+) riboprobe was transcribed from the EcoRV-digested pTS1M by SP6 polymerase. For RT-PCR analysis, the OneStep RT-PCR Kit (Qiagen, Hilden, Germany) was used with primers corresponding to the leader sequence of AMV RNA3 (5′-GTATTAATACCATTTTC-3′) and primers complementary to the TSWV N protein-coding sequence (5′-AAGCACAACACACAGAAAGC-3′). Amplification conditions were 30 min at 50 °C, 15 min at 95 °C, 30 cycles of 0.5 min at 94 °C, 1 min at 30 °C, and 2 min at 72 °C, with a final 5 min at 72 °C. Products were analyzed on a 1.0% agarose gel.

Protein analysis

Proteins were analyzed by SDS-PAGE and visualized by silver staining (Silver Stain 2 Kit; Wako Pure Chemical Industries, Osaka, Japan). The SEC fractions were subjected to Western blotting, and antigens on the blots were detected using rabbit antisera against eEF1A and the ECL Plus Western blotting detection system (GE Healthcare). Rabbit antisera against eEF1A purified from wheat germ extract were generous gifts from Karen S. Browning (The University of Texas at Austin). We confirmed that this antibody recognizes tobacco eEF1A polypeptides produced in *Escherichia coli* with the expected molecular mass of 50 kDa.

The effect of denaturing BYL and adding eEF1A inhibitor on TSWV N mRNA

Heat denaturation of mdBYL was performed at 65 °C for 20 min. The phenol-extracted and ethanol precipitated (protein-removed) mdBYL fraction was also used. Puromycin (final concentration: 5 mM) or cycloheximide (5 μg) was added to mdBYL before the transcription assay. We confirmed that the concentration of puromycin and cycloheximide was sufficient to inhibit the translation reaction in mdBYL. The eEF1A inhibitor (DB; NSC 325319) was provided by the Drug Synthesis and Chemistry Branch NCI (Bethesda, MD, USA). DB was added to the detergent-treated TSWV virion with the replication buffer or the 50–80% P ammonium sulfate fraction at a final concentration of 0.1, 1, 10, or 100 μM.

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