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***A Turnip yellow mosaic virus infection system***  
**in Arabidopsis suspension cell culture**

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Running title: TYMV selectable replicon RNA

1 **Abstract**

2 *Turnip yellow mosaic virus* (TYMV) is a positive-strand RNA virus able to infect *Arabidopsis*  
3 *thaliana*. To establish a TYMV infection system in *Arabidopsis* cell culture, TYMV replicons  
4 with the capsid protein gene replaced by a reporter gene expressing the *Sh ble* protein  
5 conferring zeocin resistance were used to transfect *Arabidopsis* cells. Zeocin-resistant  
6 *Arabidopsis* calli were used to generate a suspension cell culture. Detection of viral proteins  
7 and RNAs after 18 months in culture demonstrated persistent replication of the replicon. The  
8 *Arabidopsis* cell culture yielded soluble, active replication complexes, providing a useful tool  
9 to study host factors involved in TYMV replication.

10

11 **Keywords** : *Turnip yellow mosaic virus*, selectable replicon, replication complex,  
12 *Arabidopsis* suspension culture, zeocin

13

14 **Abbreviations** :

15 TYMV, *Turnip yellow mosaic virus*; CP, coat protein; sg, subgenomic, PCR, polymerase  
16 chain reaction; GUS,  $\beta$ -glucuronidase; PAGE, polyacrylamide gel electrophoresis; RdRp,  
17 RNA-dependent RNA polymerase; wt, wild-type; hpt, hour post-transfection; PTGS, post-  
18 transcriptional gene silencing, BMV, *Brome mosaic virus*

19

# 1 1. INTRODUCTION

2

3 *Turnip yellow mosaic virus* (TYMV) - the type member of the tymovirus group - is a  
4 spherical plant virus with a positive-strand RNA genome that has proven useful in the study  
5 of fundamental aspects of viral multiplication (reviewed in [1]). The replication complexes of  
6 positive-strand RNA viruses comprise both virus and host components (reviewed in [2,3]).  
7 Such virus–host interactions are crucial in determining the success of viral infection, defining  
8 virus host range, and controlling pathogenicity. Identifying such interactions and the  
9 associated host factors is therefore a major focus of attempts to understand the viral  
10 multiplication cycle.

11 TYMV infects Crucifers, including the model plant *Arabidopsis thaliana* [4]. To  
12 extend the range of experimental tools to study TYMV replication and the composition of  
13 positive-strand RNA virus replication complexes, we sought to establish a TYMV-infected  
14 *Arabidopsis* suspension cell culture using TYMV-derived replicon RNAs in which the  
15 structural coat protein (CP) gene is replaced by a reporter gene that can be expressed via  
16 replication-mediated synthesis of subgenomic (sg) RNAs. As a reporter gene, we used the *Sh*  
17 *ble* gene from *Streptoalloteichus hindustanus* encoding a bleomycin-resistance gene that  
18 confers resistance to the drug zeocin™, which can be used as a dominant selectable marker in  
19 plants [5,6]. A zeocin-resistant *Arabidopsis* stable cell suspension culture containing  
20 autonomously replicating RNAs that recapitulate all steps of TYMV replication cycle was  
21 successfully generated. Soluble and active viral replication complexes were obtained from  
22 this TYMV-infected *Arabidopsis* cell suspension culture.

## 1 **2. MATERIALS AND METHODS**

### 2 **2.1 Cloning of plasmid DNAs and recombinant viruses**

3 All DNA manipulations used standard techniques [7,8]. The full-length TYMV cDNA clone  
4 E17, which produces infectious transcripts, and its derivative E17-stopCP, in which TYMV  
5 nucleotide positions 5707–6059 are replaced by a 58 bp polylinker were described previously  
6 [9,10]. TYMV-Rep was created via PCR site-directed mutagenesis on E17-stopCP using the  
7 primer CAATCAGCCCCAACtaGtAAATCGACAAAG to mutate the coat protein (CP)  
8 initiation codon and introduce a *SpeI* site (underlined) at nt 5895. The *uidA* gene from plasmid  
9 B6GΔES [11] was recloned in the unique *SnaBI* site of TYMV-Rep to create TYMV-Rep-  
10 GUS. Similarly, the *Sh ble* gene was PCR-amplified from pSP109, [12] and inserted into the  
11 *SnaBI* site of TYMV-Rep to create TYMV-Rep-Zeo. All clones were characterized by  
12 restriction-enzyme digestion and confirmed by sequencing. Cloning details are available on  
13 request.

14

### 15 **2.2 Preparation and transfection of protoplasts.**

16 Protoplasts ( $10^6$ ) of *Arabidopsis thaliana* ecotype Columbia prepared from a cell suspension  
17 culture as described previously [10,13] were transfected with 5  $\mu$ g capped *in vitro* transcripts  
18 generated from linearized DNA templates as described [9]. Transfected protoplasts were  
19 incubated at 24°C in the dark before harvesting.

20

### 21 **2.3. Selection of zeocin-resistant Arabidopsis cells and generation of a stable suspension** 22 **culture**

23 Five days after transfection with TYMV-Rep-Zeo RNA, or water as a control, Arabidopsis  
24 protoplasts were collected and samples of Arabidopsis protoplast suspension (100  $\mu$ l  
25 containing ~ 80,000 cells) were plated on Gamborg's B5 medium (Sigma) supplemented with

1 different concentrations of zeocin™ (Invivogen; 50–500  $\mu\text{g/ml}$ ) to select for zeocin-resistant  
2 transformants. Plates were incubated at 21°C with a 16h/8h photoperiod and were evaluated  
3 weekly for formation of Arabidopsis microcalli. Five weeks after selection, individual calli  
4 were isolated from medium containing 500  $\mu\text{g/ml}$  zeocin and were propagated onto fresh  
5 drug-containing medium. These calli were subcultured on solid medium every 4 weeks until  
6 analysis. To establish a cell suspension culture, zeocin-resistant calli were cultured in 20 ml  
7 liquid medium supplemented with 10 or 50  $\mu\text{g/ml}$  zeocin in 100 ml flasks with constant  
8 shaking at 130 rpm. Cultures were propagated by dilution (1/5 every 12 days) and could be  
9 scaled up to 200 ml.

10

#### 11 **2.4. Detection of GUS activity**

12  $\beta$ -glucuronidase (GUS) activity in protoplasts was measured as described previously [14],  
13 assays were performed in triplicate. GUS activity in leaves was assayed using a standard  
14 histochemical staining technique [14] 10 days after inoculation of 6-week-old Arabidopsis  
15 plants with 5  $\mu\text{g}$  capped *in vitro* transcripts as described [15].

16

#### 17 **2.5. Analysis of protein extracts**

18 Proteins were extracted from Arabidopsis protoplasts as described [15], and from Arabidopsis  
19 calli or cell suspension culture using Tri-Reagent (MRC, Inc.) according to the supplier's  
20 instructions after grinding of the material in liquid nitrogen. SDS-PAGE and immunoblot  
21 analysis with polyclonal anti-66K or anti-140K antibodies were performed as previously  
22 described [10,13]. Antibodies raised against the *Sh ble* protein (Invivogen) were diluted 500-  
23 fold according to the supplier's instructions.

24

## 1 **2.6. RNA extraction and Northern blot hybridization**

2 Total RNA was extracted from transfected Arabidopsis protoplasts using Tri-Reagent (MRC,  
3 Inc.), and from calli or cell suspension culture using the Nucleospin® RNA plant method  
4 (Macherey-Nagel) according to the supplier's instructions. Equivalent amounts of total RNA  
5 were loaded onto 1% agarose-formaldehyde gels, using 2.5  $\mu$ g and 10  $\mu$ g for viral plus- and  
6 minus-strand detection, respectively. Blotting and hybridization using strand-specific probes  
7 were performed as described previously [16].

8

## 9 **2.7. Replication complex isolation and activity assay**

10 Fresh cells (~10 g) from zeocin-resistant or control wild-type (wt) Arabidopsis cell cultures  
11 were ground in a mortar and pestle using 2.5 ml extraction buffer (50 mM Tris-HCl pH 7.4,  
12 15 mM MgCl<sub>2</sub>, 1 mM EDTA, 8.7% glycerol, 0.1%  $\beta$ -mercaptoethanol) per gram of cells.  
13 After centrifugation (20,000 x g; 30 min; 4°C), TYMV replication complexes were  
14 solubilized using 0.5% Lubrol W and fractionated over a glycerol gradient as described  
15 previously [13,17]. Gradient fractions were assayed for *in vitro* RNA-dependent RNA  
16 polymerase (RdRp) activity with TYMV RNA, *Brome mosaic virus* (BMV) RNA, Q beta  
17 coliphage RNA or yeast tRNAs (2.5  $\mu$ g each) as a template as previously described [13].

### 1 3. RESULTS AND DISCUSSION

#### 2 3.1. Replication of TYMV replicons carrying reporter genes

3 To construct TYMV replicons in which the structural CP gene is replaced by a foreign gene,  
4 the full-length TYMV cDNA clone E17, which produces infectious transcripts [9], was  
5 modified to generate plasmid TYMV-Rep. The CP gene AUG initiation codon was  
6 inactivated to facilitate translational initiation from the endogenous start codon of the foreign  
7 gene, and a 58 nt multiple cloning site replaced nucleotides 5708 to 6058 of the viral genome  
8 (Fig. 1). The CP gene of TYMV is known to tolerate deletion and/or insertions in this region  
9 while maintaining its ability to replicate and synthesize sg RNA [18,19]. Introduction of the  
10 *Sh ble* gene or the *uidA* gene encoding  $\beta$ -glucuronidase (GUS) [14] into TYMV-Rep,  
11 generating TYMV-Rep-Zeo and TYMV-Rep-GUS, respectively (Fig. 1), resulted in a net  
12 increase in length of the viral genome of 129 nt or 1580 nt, respectively (Fig. 1).

13 Capped, *in vitro*-generated RNA transcripts of TYMV-Rep and the two reporter  
14 constructs were used to transfect Arabidopsis protoplasts. Accumulation of viral nonstructural  
15 proteins was assessed by Western blotting using specific antisera recognizing the TYMV  
16 replication proteins 140K and 66K [13,15], while synthesis of plus- and minus-strand TYMV  
17 RNA was analyzed by Northern blot. Viral proteins and RNAs were detected in all cases (Fig.  
18 2), indicating that all these TYMV-derived RNAs can replicate in plant cells. Note that the  
19 140K protein is processed *in vivo* into a shorter viral product of 115 kDa and into a non-  
20 specific degradation product of 85 kDa [13, Jakubiec *et al.*, unpublished data]), whose  
21 proportion is highly variable from sample to sample.

22 Plant cells infected with TYMV-Rep transcripts accumulated much lower amounts  
23 (>10-fold) of plus-strand RNA as compared to wt transcripts (Fig. 2B, lanes 1-3), whereas  
24 there was no major effect on accumulation of minus-strand RNA or replication proteins,  
25 indicating that the lack of CP specifically affects accumulation of plus-strand RNAs but does

1 not affect replication *per se*. This observation most likely reflects the protective effect of  
2 packaging on viral RNA stability, as suggested also in previous reports [18,19].

3 The insertion of reporter gene sequences in TYMV-Rep caused a further decrease in  
4 the efficiency of viral RNA replication, affecting the accumulation of plus- and minus-strand  
5 RNAs as well as replication proteins (Fig. 2, lanes 4 and 5). The reduction was more  
6 pronounced with TYMV-Rep-GUS than with TYMV-Rep-Zeo transcripts. Whether this  
7 effect – a common problem reported in the engineering of viral vectors [20] – relates to the  
8 total length of the viral genome is presently unknown.

9

### 10 **3.2. Expression of reporter genes by TYMV replicons**

11 Arabidopsis protoplasts transfected with capped *in vitro* transcripts corresponding to each  
12 TYMV-derived RNA replicon were monitored for reporter gene expression. Two days after  
13 transfection of TYMV-Rep-Zeo, the *Sh ble* protein could be detected by Western blotting  
14 (Fig. 3A). Similarly, GUS protein was expressed from TYMV-Rep-GUS (Fig. 3B).  
15 Expression of both proteins increased over time, consistent with the expression kinetics of CP  
16 during the infectious cycle [15]. Mechanical inoculation of Arabidopsis leaves with TYMV-  
17 Rep-GUS capped *in vitro* transcripts resulted in readily detectable levels of GUS expression,  
18 visualised as blue precipitates within local lesions following standard histochemical GUS  
19 assays [14], (Fig. 3C, right).

20 These results constitute the first report that TYMV-derived replicons can tolerate the  
21 insertion of foreign sequences and promote the transient expression of reporter genes in plant  
22 cells. The lack of CP – which is required for long distance movement of TYMV through the  
23 plant [18] – precludes the use of these replicons for expression of foreign genes in whole  
24 plants. However, because the CP is dispensable for local cell-to-cell movement [18], these  
25 vectors may produce foreign proteins effectively in inoculated plant leaves (see Fig. 3C). The

1 TYMV-Rep vector may thus be useful for expression of foreign proteins in discrete regions of  
2 Arabidopsis leaves, or for high-throughput screening of genes as reported recently for TMV-  
3 derived vectors in *Nicotiana benthamiana* [21]. Since no viral particles are formed, no spread  
4 to other tissues or vector transmission can occur, providing the necessary containment for this  
5 vector to meet stringent safety requirements.

6

### 7 **3.3. Generation of a zeocin-resistant Arabidopsis cell line recapitulating features of** 8 **TYMV-infected cells**

9 Arabidopsis protoplasts transfected with TYMV-Rep-Zeo capped *in vitro* transcripts were  
10 transferred to solid medium containing various concentrations of zeocin. In the presence of  
11 500 $\mu$ g/ml zeocin, cells transfected with the TYMV-Rep-Zeo replicon were able to actively  
12 divide and form microcalli, while cells from control transfections died (Fig. 3D). The plating  
13 efficiency (calculated as the percentage of microcolonies growing from the total number of  
14 protoplasts plated) was in the range of 0.1%, which given the routine transfection level of  
15 10% [10], indicates that  $\sim$  1% of the infected cells gave rise to a zeocin-resistant callus.  
16 Microcalli were recovered individually and propagated on solid zeocin-containing culture  
17 medium, where they generated calli. Upon transfer to liquid medium containing zeocin, a  
18 stable zeocin-resistant Arabidopsis cell suspension culture was established.

19 To detect the presence of TYMV-derived replicons in the zeocin-resistant Arabidopsis  
20 suspension cell culture, total protein extracts were probed by Western blotting using anti-  
21 140K and anti-66K antisera; both replication proteins were detected (Fig. 4A). Northern blot  
22 analysis using strand-specific probes confirmed the presence of both positive- and negative-  
23 sense RNAs of the expected sizes (Fig. 4B), indicative of a complete viral replication cycle.  
24 Detection of viral proteins and RNAs in cell cultures passaged >40 times in zeocin-containing  
25 medium over 18 months indicates that the zeocin-resistant Arabidopsis cell cultures

1 continuously produced TYMV RNA and proteins throughout this period. No obvious  
2 cytotoxicity was observed, but the level of viral RNAs were reduced and there appeared to be  
3 relatively more sgRNA synthesized compared to a wt TYMV infection (Fig. 4B, lanes 2, 3).  
4 In addition to individual variation between independent cell lines, it is possible that adaptive  
5 mutations accumulating in the viral genome also contribute to such differences. Whether the  
6 TYMV replicon retains its original sequence in the zeocin-resistant suspension cell culture  
7 remains to be elucidated.

8         The zeocin-resistant Arabidopsis cell culture constitutes a stable cell line that  
9 recapitulates the features of TYMV-infected cells. The concept of using replicon-based stably  
10 infected cell lines has been applied to several animal- or human-infecting positive-strand  
11 RNA viruses, e.g. alphaviruses, flaviviruses and rubiviruses [22-24]. The system described  
12 here indicates that a similar approach is applicable to plant RNA viruses.

13         The replicon system reported here provides an alternative to the use of amplicons, i.e.  
14 transgenic expression of viral genomes [25-30], with a presumably lower risk of induction of  
15 post-transcriptional gene silencing (PTGS), as constitutive transgene expression was reported  
16 to be a strong inducer of PTGS [28-30].

17

#### 18 **3.4. Preparation of TYMV replication complexes from zeocin-resistant Arabidopsis cell** 19 **lines**

20         Membrane fractions of zeocin-resistant Arabidopsis cell suspension cultures were  
21 prepared, and TYMV replication complexes were solubilized using Lubrol W and  
22 fractionated through a glycerol gradient as previously described [13,17]. A peak of RdRp  
23 activity was detected, with >50% of the total RdRp activity being present in fractions 10–14  
24 (Fig. 5A). An identical procedure performed on wild-type Arabidopsis cell suspension culture  
25 revealed no significant RdRp activity. The template specificity of the peak fraction was

1 verified using genomic RNAs of *Brome mosaic virus* (BMV), Q beta coliphage or a mixture  
2 of yeast tRNAs (Fig. 5A inset). Western blots using anti-140K and anti-66K antisera revealed  
3 viral replication proteins within RdRp-containing fractions (Fig. 5B). These data indicate that  
4 soluble and active TYMV replication complexes can be purified from the *Arabidopsis* zeocin-  
5 resistant cell line.

6 Suspension-cultured plant cells have the advantages of uniformity, greater  
7 reproducibility and ease of mass production compared with whole plants or transfected  
8 protoplasts [31], hence the virus infection system established here provides a convenient tool  
9 to study virus–cell interactions and should facilitate the identification of host factors involved  
10 in the life cycle of TYMV.

11

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19

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1 **6. FIGURE LEGENDS**

2

3 **Figure 1 : TYMV genome and replicons used in this study**

4 Schematic representation of the TYMV genome (top) showing the major viral ORFs (*open*  
5 *bars*). The encoded 206K protein is proteolytically processed at a peptide bond (*filled square*)  
6 to generate 140K and 66K proteins. The 3' region of the wild-type genome and replicons used  
7 in this study are expanded below. *Bent arrow* Initiation site of sgRNA; *dashed lines* deleted  
8 sequences, *grey boxes* reporter genes (not to scale). Useful restriction sites are indicated.  
9 Predicted sizes of genomic and sgRNAs are listed on the right.

10

11 **Figure 2 : Infectivity of TYMV replicons in Arabidopsis protoplasts**

12 Arabidopsis protoplasts transfected with *in vitro* transcripts as indicated were harvested 24  
13 hours post-transfection (hpt) for Western blot analysis and 48 hpt for Northern blot analysis.  
14 (A) Equivalent amounts of proteins were analyzed by Western blot using anti-140K (top) and  
15 anti-66K (middle) antibodies, based on Ponceau Red staining as a loading control (bottom).  
16 (B) Equivalent amounts of total RNA (or dilutions as indicated) were analyzed and plus-  
17 strand genomic (g) and subgenomic (sg) TYMV RNAs (top) and minus-strand genomic RNA  
18 (bottom) were detected by strand-specific Northern blot.

19

20 **Figure 3 : TYMV replicons express reporter genes in Arabidopsis cells and plants**

21 (A), (B) Arabidopsis protoplasts transfected with *in vitro* transcripts as indicated were  
22 harvested 24 and 48 hpt. (A) Equivalent amounts of proteins were analyzed by Western blot  
23 using anti-*Sh ble* antibodies. (B) GUS activity in transfected protoplasts was quantified  
24 fluorometrically and normalized to the protein content in cell extracts. The data represent the  
25 mean  $\pm$  standard deviation of three concomitant triplicate samples. (C) Histochemical staining  
26 for GUS activity in Arabidopsis leaves 10 days post-inoculation with *in vitro* transcripts. (D)

1 Arabidopsis protoplasts transfected with TYMV-Rep-Zeo *in vitro* transcripts or water as a  
2 control were plated on solid medium containing 500  $\mu\text{g/ml}$  zeocin. Protoplast-derived  
3 microcalli were observed 5 weeks post-transfection.

4

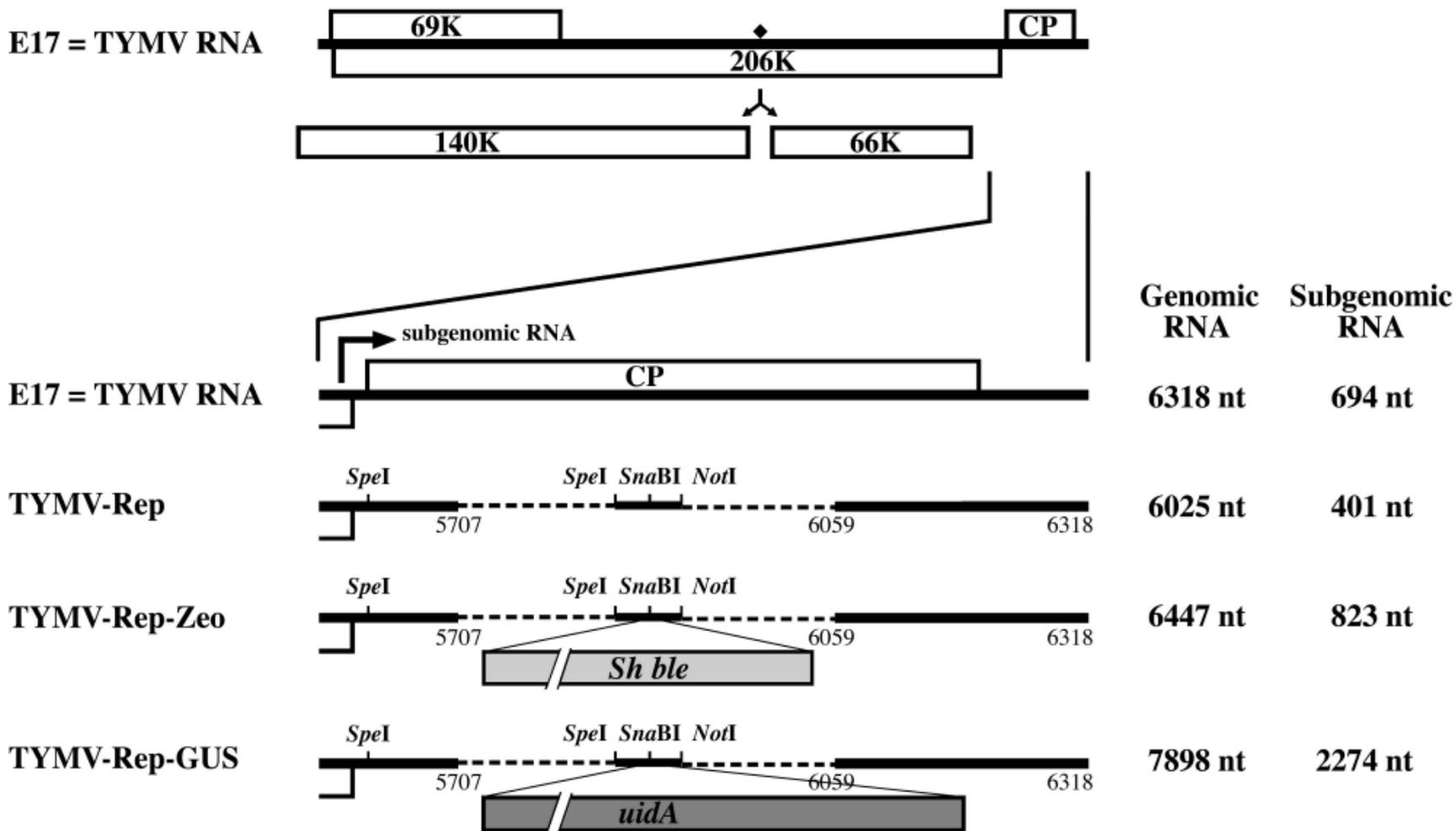
5 **Figure 4 : Accumulation of viral products in the zeocin-resistant Arabidopsis suspension**  
6 **cell culture**

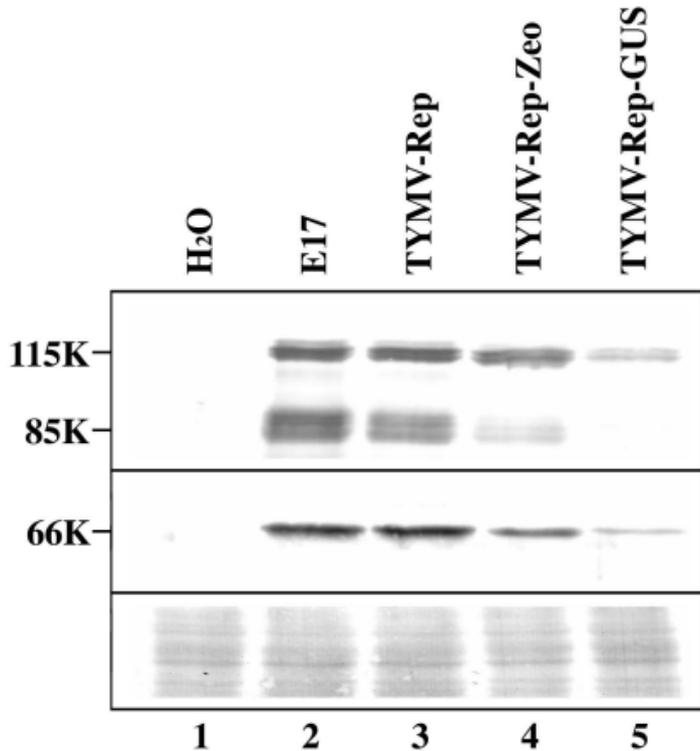
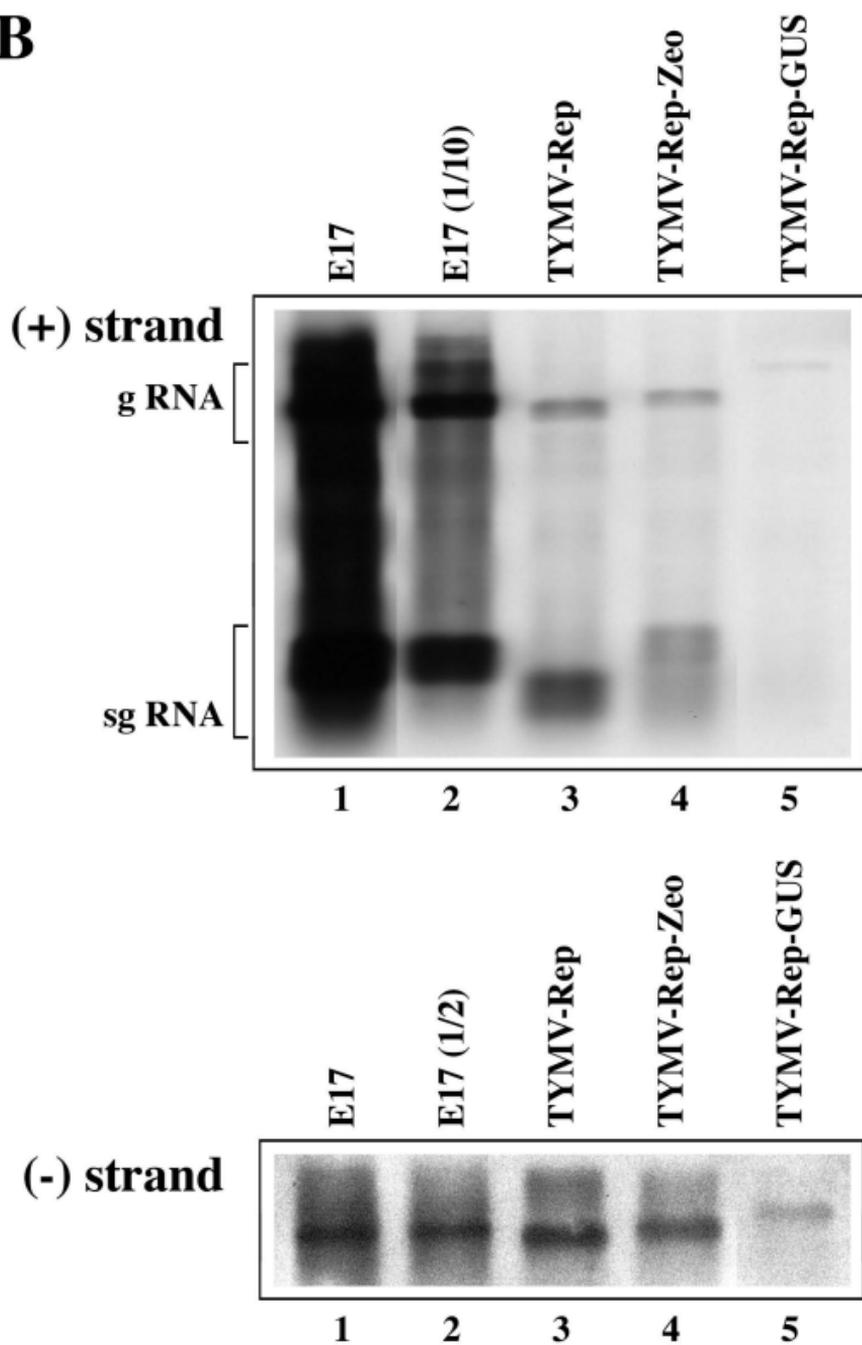
7 Protein and RNA were extracted from the zeocin-resistant (ZeoR) Arabidopsis suspension  
8 cell culture maintained for 18 months or a non transformed (wt) Arabidopsis suspension  
9 culture. **(A)** Equivalent amounts of proteins were analyzed by Western blot using anti-140K  
10 (top) and anti-66K (bottom) antibodies. **(B)** Equivalent amounts of total RNA (or dilutions as  
11 indicated) were analyzed and plus-strand genomic (g) and subgenomic (sg) TYMV RNAs  
12 (left) and minus-strand genomic RNA (right) were detected by strand-specific Northern blot.  
13 RNA extracted from protoplasts transfected with viral RNA (E17) was used as a control (lane  
14 3).

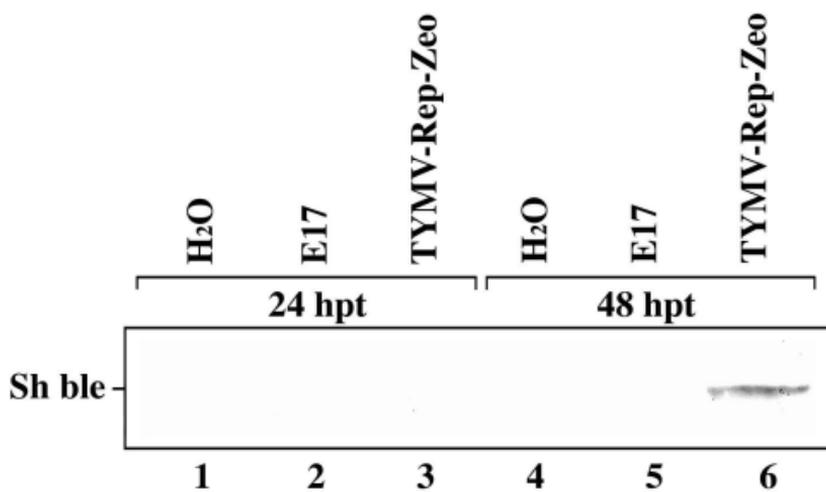
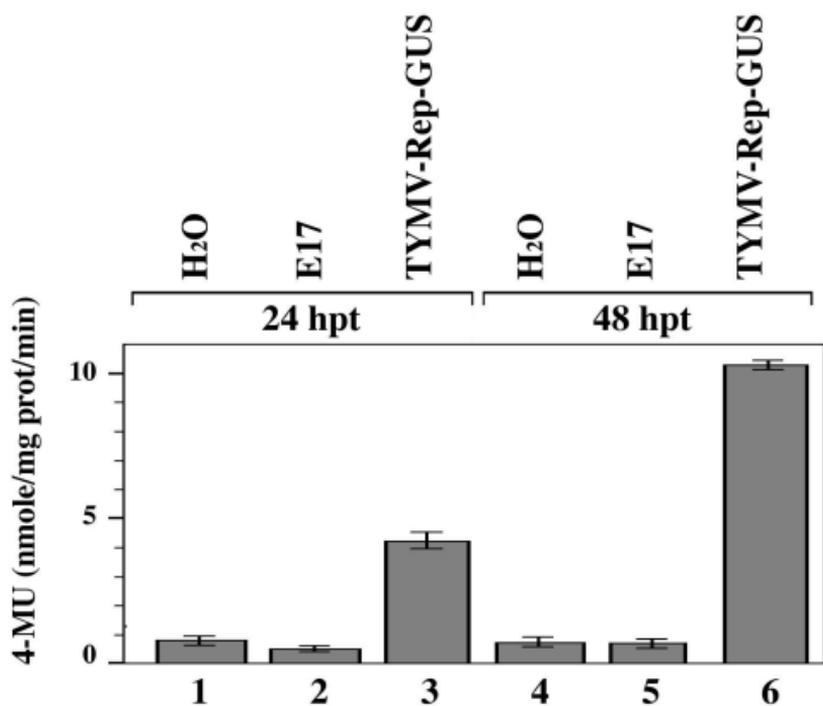
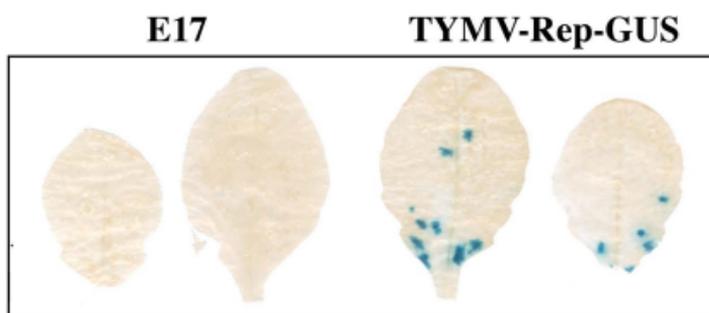
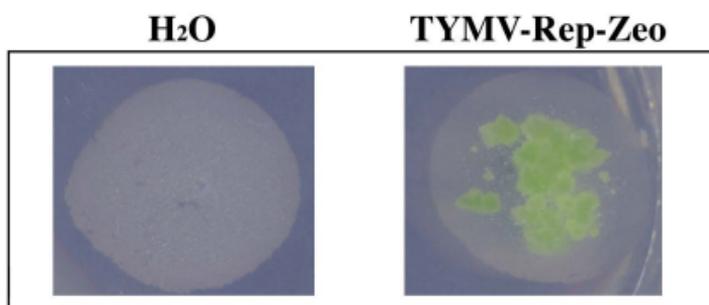
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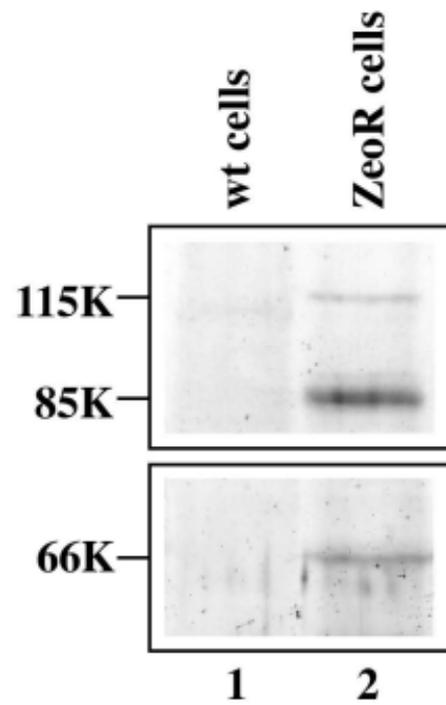
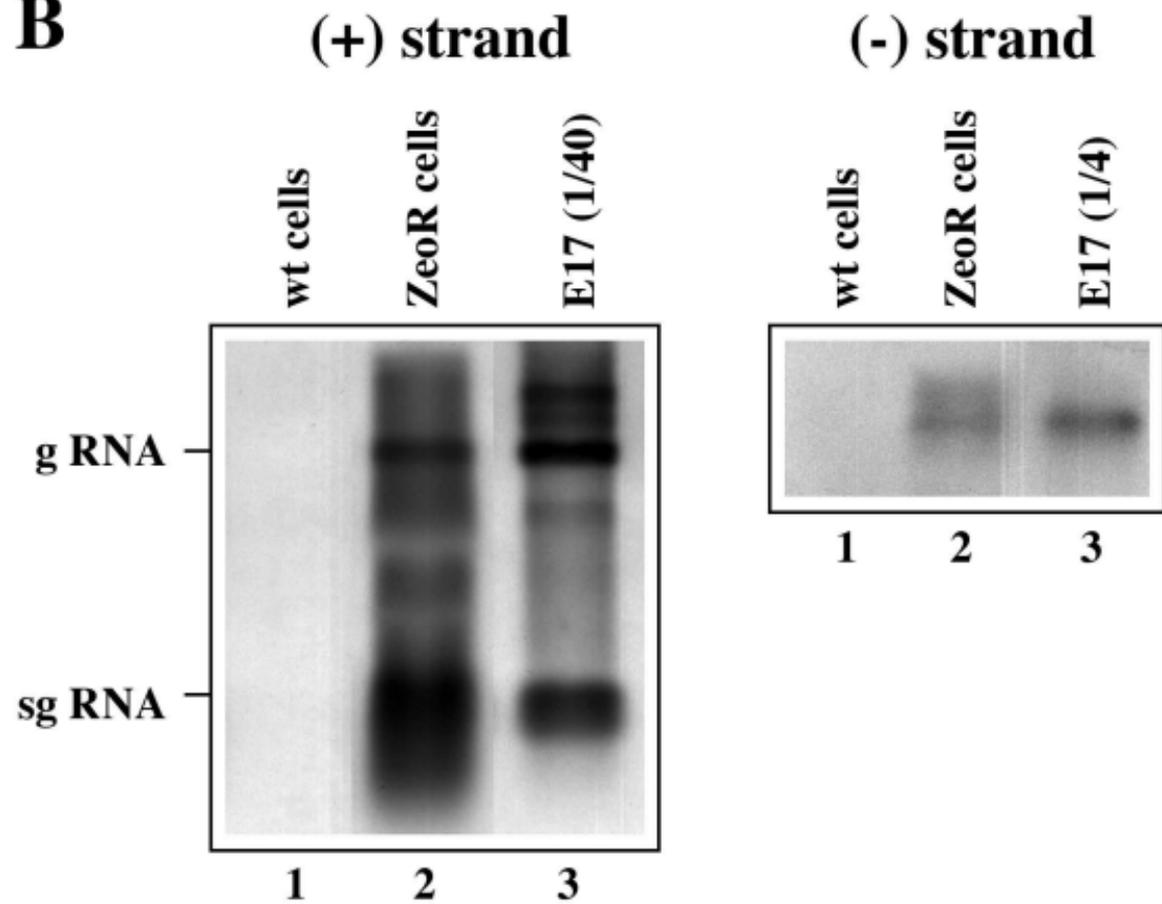
16 **Figure 5 : Preparation of TYMV replication complexes from zeocin-resistant**  
17 **Arabidopsis suspension cell culture**

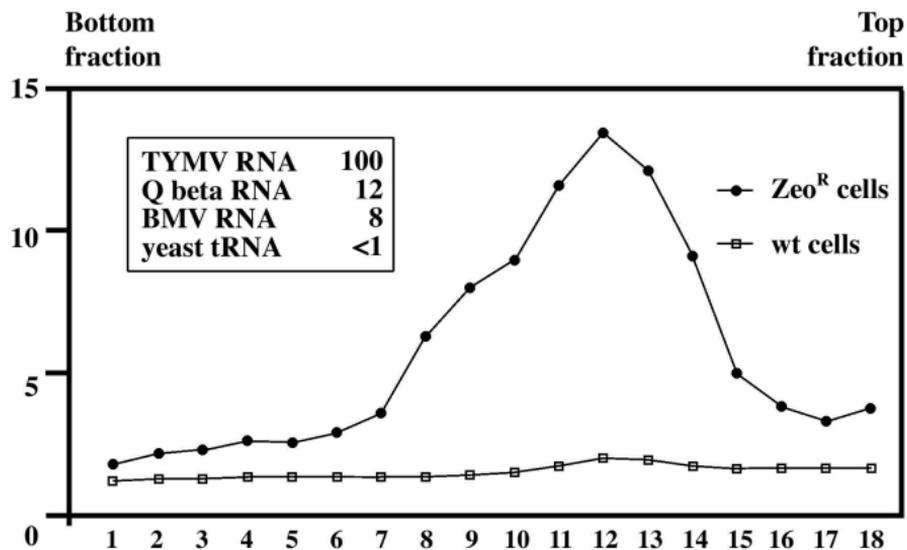
18 TYMV replication complexes prepared from the ZeoR Arabidopsis suspension cell culture  
19 were solubilized from membranes and centrifuged in a glycerol gradient. **(A)** The *in vitro*  
20 RdRp activity of gradient fractions determined using TYMV RNA as a template [expressed as  
21 cpm of [ $^{32}\text{P}$ ]-UMP incorporated ( $\times 10^{-3}$ )]. Fractions obtained from a non transformed (wt)  
22 Arabidopsis suspension culture were analyzed in parallel. *Inset* Peak Fraction 12 was used in  
23 an RdRp assay with TYMV RNA, BMV RNA, Q beta RNA or yeast tRNAs as templates.  
24 Relative template activities (%) is the mean of two independent experiments. **(B)** Samples of  
25 proteins from each fraction were analyzed by Western blotting using anti-140K (top) and anti-  
26 66K (bottom) antibodies.



**A****B**

**A****B****C****D**

**A****B**

**A****B**