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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

Abstract

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

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

Abbreviations :

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

1. INTRODUCTION

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

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

2. MATERIALS AND METHODS

2.1 Cloning of plasmid DNAs and recombinant viruses

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

2.2 Preparation and transfection of protoplasts.

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

2.3. Selection of zeocin-resistant Arabidopsis cells and generation of a stable suspension culture

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

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

2.4. Detection of GUS activity

β-glucuronidase (GUS) activity in protoplasts was measured as described previously [14], assays were performed in triplicate. GUS activity in leaves was assayed using a standard histochemical staining technique [14] 10 days after inoculation of 6-week-old Arabidopsis plants with 5 μg capped *in vitro* transcripts as described [15].

2.5. Analysis of protein extracts

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

2.6. RNA extraction and Northern blot hybridization

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

2.7. Replication complex isolation and activity assay

Fresh cells (~10 g) from zeocin-resistant or control wild-type (wt) Arabidopsis cell cultures were ground in a mortar and pestle using 2.5 ml extraction buffer (50 mM Tris-HCl pH 7.4, 15 mM $MgCl_2$, 1 mM EDTA, 8.7% glycerol, 0.1% β-mercaptoethanol) per gram of cells. After centrifugation (20,000 x *g*; 30 min; 4°C), TYMV replication complexes were solubilized using 0.5% Lubrol W and fractionated over a glycerol gradient as described previously [13,17]. Gradient fractions were assayed for *in vitro* RNA-dependent RNA polymerase (RdRp) activity with TYMV RNA, *Brome mosaic virus* (BMV) RNA, Q beta coliphage RNA or yeast tRNAs (2.5 μg each) as a template as previously described [13].

3. RESULTS AND DISCUSSION

3.1. Replication of TYMV replicons carrying reporter genes

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

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

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

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

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

3.2. Expression of reporter genes by TYMV replicons

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

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

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

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

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

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

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

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

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

3.4. Preparation of TYMV replication complexes from zeocin-resistant Arabidopsis cell lines

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

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

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

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5. REFERENCES

[1] Dreher, T.W. (2004). Turnip yellow mosaic virus: transfer RNA mimicry, chloroplasts and a C-rich genome. *Mol. Plant Pathol.* 5, 367-375.

[2] Ahlquist, P., Noueiry, A.O., Lee, W.M., Kushner, D.B., and Dye, B.T. (2003). Host factors in positive-strand RNA virus genome replication. *J. Virol.* 77, 8181-8186.

[3] Buck, K.W., 1996. Comparison of the replication of positive-stranded RNA viruses of plants and animals. *Adv. Virus. Res.* 47, 159-251.

[4] Martinez-Herrera, D., Romero, J., Martinez-Zapater, J.M., and Ponz, F. (1994). Suitability of Arabidopsis thaliana as a system for the study of plant-virus interactions. *Fitopatologia* 29, 132-136.

[5] Gatignol, A., Durand, H., and Tiraby, G. (1988). Bleomycin resistance conferred by a drug-binding protein. *FEBS Lett.* 230, 171-175.

[6] Perez, P., Tiraby, G., Kallerhoff, J., and Perret, J. (1989). Phleomycin resistance as a dominant selectable marker for plant cell transformation. *Plant Mol. Biol.* 13, 365-373.

[7] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987). Current Protocols in Molecular Biology. Wiley, New York.

[8] Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

[9] Drugeon, G., and Jupin, I. (2002). Stability in vitro of the 69K movement protein of Turnip yellow mosaic virus is regulated by the ubiquitin-mediated proteasome pathway. *J. Gen. Virol.* 83, 3187-3197.

[10] Prod'homme, D., Jakubiec, A., Tournier, V., Drugeon, G., and Jupin, I. (2003). Targeting of the turnip yellow mosaic virus 66K replication protein to the chloroplast envelope is mediated by the 140K protein. *J. Virol.* 77, 9124-9135.

[11] Jupin, I., Richards, K., Jonard, G., Guilley, H., and Pleij, C.W. (1990). Mapping sequences required for productive replication of beet necrotic yellow vein virus RNA 3. *Virology* 178, 273-280.

[12] Lumbreras, V., Stevens, D.R., and Purton, S. (1998). Efficient foreign gene expression in Chlamydomonas reinhardtii mediated by an endogenous intron. *Plant J.* 14, 441-447.

[13] Jakubiec, A., Notaise, J., Tournier, V., Héricourt, F., Block, M.A., Drugeon, G., van Aelst, L., and Jupin, I. (2004). Assembly of turnip yellow mosaic virus replication complexes: interaction between the proteinase and polymerase domains of the replication proteins. *J. Virol.* 15, 7945-7957.

[14] Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: betaglucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901–3907.

[15] Prod'homme, D., Le Panse, S., Drugeon, G., and Jupin, I. (2001). Detection and subcellular localization of the turnip yellow mosaic virus 66K replication protein in infected cells. *Virology* 281, 88-101.

[16] Jakubiec, A., Tournier, V., Drugeon, G., Pflieger, S., Camborde, L., Vinh, J., Héricourt, F., Redeker, V., and Jupin, I. (2006). Phosphorylation of viral RNA-dependent RNA polymerase and its role in replication of a plus-strand RNA virus. *J. Biol. Chem.* 281, 21236-21249.

[17] Deiman, B.A., Séron, K., Jaspars, E.M., and Pleij, C.W.A. (1997). Efficient transcription of the tRNA-like structure of turnip yellow mosaic virus by a template-dependent and specific viral RNA polymerase obtained by a new procedure. *J. Virol. Methods* 64, 181-195.

[18] Bransom, K.L., Weiland, J.J., Tsai, C.H., and Dreher, T.W. (1995). Coding density of the turnip yellow mosaic virus genome: roles of the overlapping coat protein and p206-readthrough coding regions. *Virology* 206, 403-412.

[19] Schirawski, J., Voyatzakis, A., Zaccomer, B., Bernardi, F., and Haenni, A.L. (2000). Identification and functional analysis of the Turnip yellow mosaic tymovirus subgenomic promoter. *J. Virol.* 74, 11073-11080.

[20] Scholthof, H.B., Scholthof, K.B.G., and Jackson, A.O. (1996). Plant virus gene vectors for transient expression of foreign proteins in plants. *Annu. Rev. Phytopathol.* 34, 299–323.

[21] Escobar, N.M., Haupt, S., Thow, G., Boevink, P., Chapman, S., and Oparka, K. (2003). High-throughput viral expression of cDNA–green fluorescent protein fusions reveals novel subcellular addresses and identifies unique proteins that interact with plasmodesmata. *Plant Cell* 15, 1507-1523.

[22] Frolov, I., Hoffman, T.A., Pragai, B.M., Dryga, S.A., Huang, H.V., Schlesinger, S., and Rice, C.M. (1996). Alphavirus-based expression vectors: strategies and applications. *Proc. Natl. Acad. Sci. U S A* 93, 11371-11377.

[23] Bartenschlager, R. (2002). Hepatitis C virus replicons: potential role for drug development. *Nat. Rev. Drug. Discov.* 1, 911-916.

[24] Chen, M.H., Frolov, I., Icenogle, J., and Frey, T.K. (2004). Analysis of the 3' cis-acting elements of rubella virus by using replicons expressing a puromycin resistance gene. *J. Virol.* 78, 2553-2561.

[25] Mori, M., Kaido, M., Okuno, T., and Furusawa, I. (1993). mRNA amplification system by viral replicase in transgenic plants. *FEBS Lett.* 336, 171–174.

[26] Dohi, K., Nishikiori, M., Tamai, A., Ishikawa, M., Meshi, T., and Mori, M. (2006). Inducible virus-mediated expression of a foreign protein in suspension-cultured plant cells. *Arch. Virol.* 151, 1075-1084.

[27] Chen, J., Li, W.X., Xie, D., Peng, J.R., and Ding, S.W. (2004). Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microrna in host gene expression. *Plant Cell* 16, 1302-1313.

[28] Angell, S.M., and Baulcombe, D. C. (1997). Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *EMBO. J.* 16, 3675–3684.

[29] Kaido, M., Mori, M., Mise, K., Okuno, T., and Furusawa, I. (1995). Inhibition of brome mosaic virus (BMV) amplification in protoplasts from transgenic tobacco plants expressing replicable BMV RNAs. *J. Gen. Virol.* 76, 2827-2833.

[30] Mori, M., Fujihara, N., Mise, K., and Furusawa, I. (2001) Inducible high-level mRNA amplification system by viral replicase in transgenic plants. *Plant J*. 27, 79-86.

[31] Fischer, R., Emans, N., Schuster, F., Hellwig, S., and Drossard, J. (1999). Towards molecular farming in the future: using plant-cell-suspension cultures as bioreactors. *Biotechnol. Appl. Biochem*. 30, 109–112.

6. FIGURE LEGENDS

Figure 1 : TYMV genome and replicons used in this study

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

Figure 2 : Infectivity of TYMV replicons in Arabidopsis protoplasts

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

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

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

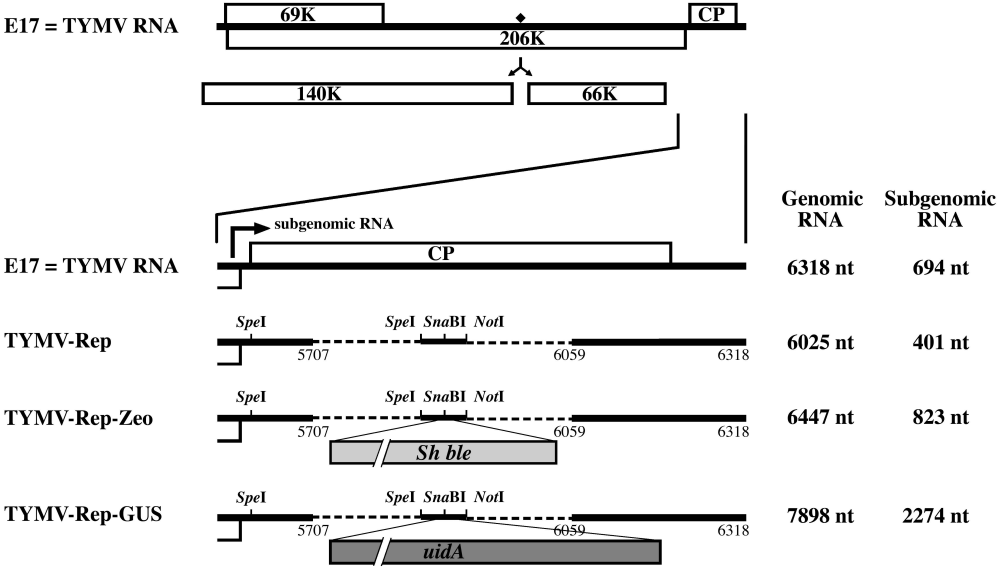
Arabidopsis protoplasts transfected with TYMV-Rep-Zeo *in vitro* transcripts or water as a control were plated on solid medium containing 500 μg/ml zeocin. Protoplast-derived microcalli were observed 5 weeks post-transfection.

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

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

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

TYMV replication complexes prepared from the ZeoR Arabidopsis suspension cell culture were solubilized from membranes and centrifuged in a glycerol gradient. (**A**) The *in vitro* RdRp activity of gradient fractions determined using TYMV RNA as a template [expressed as cpm of [^{32}P]-UMP incorporated (x 10^{-3})]. Fractions obtained from a non transformed (wt) Arabidopsis suspension culture were analyzed in parallel. *Inset* Peak Fraction 12 was used in an RdRp assay with TYMV RNA, BMV RNA, Q beta RNA or yeast tRNAs as templates. Relative template activities (%) is the mean of two independent experiments. (**B**) Samples of proteins from each fraction were analyzed by Western blotting using anti-140K (top) and anti-66K (bottom) antibodies.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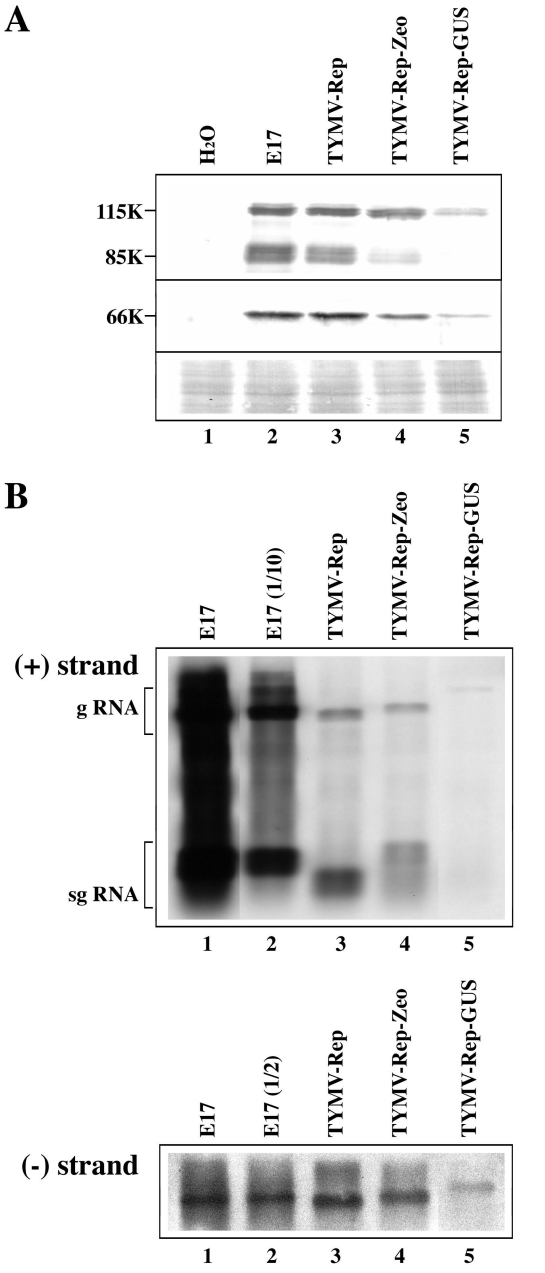
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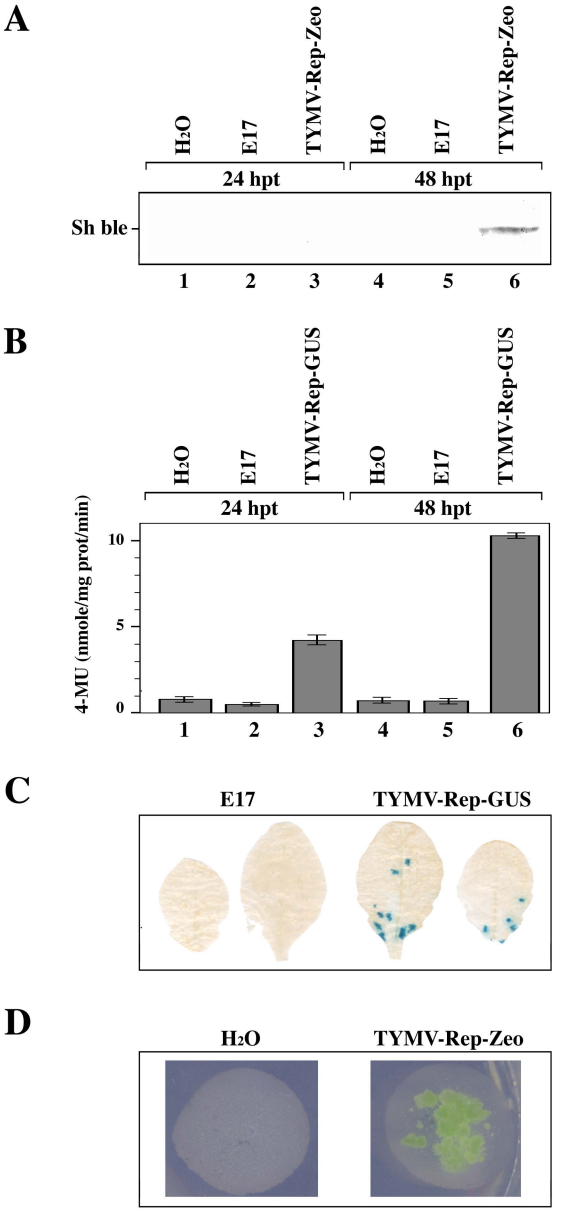
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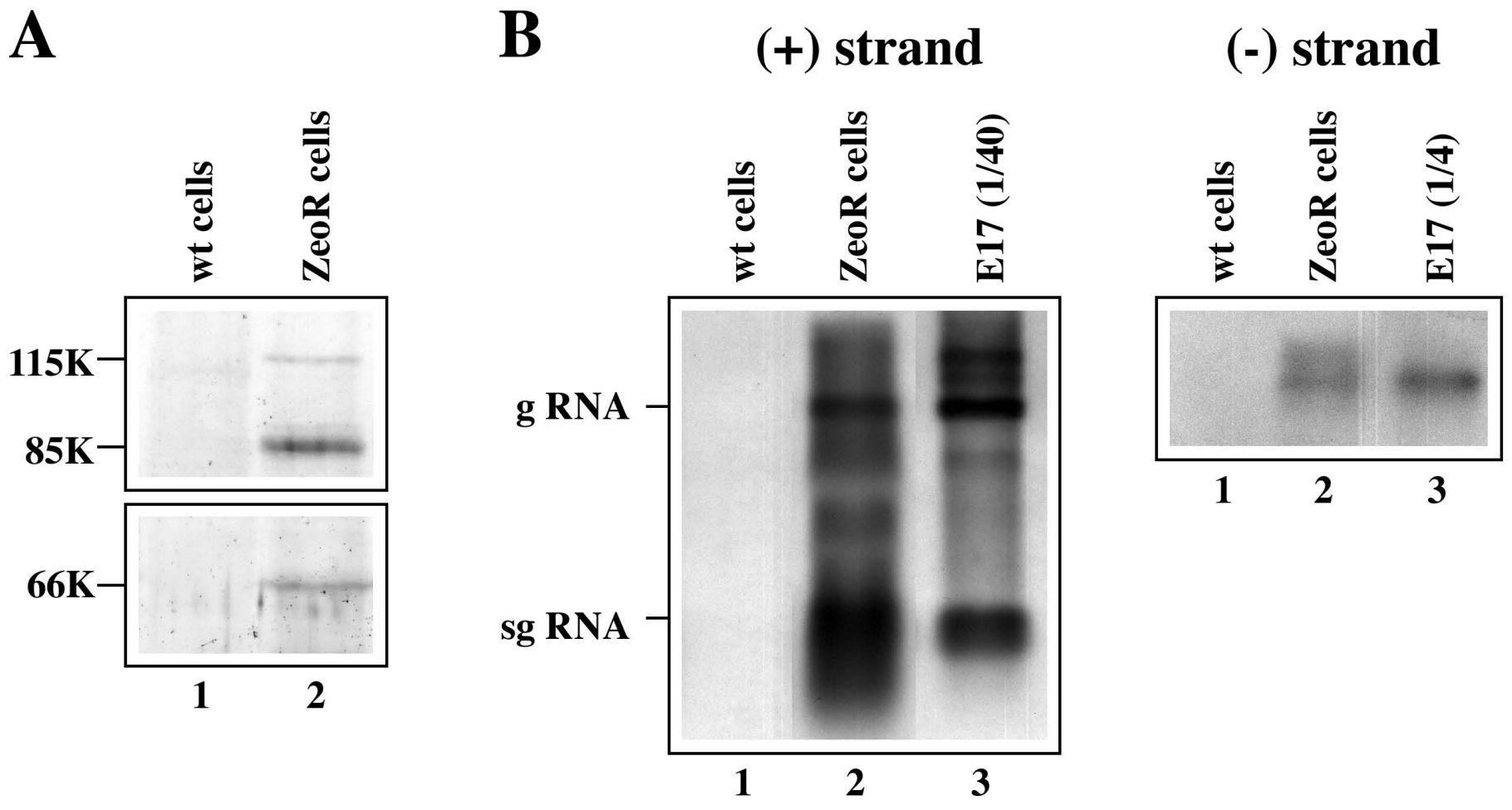
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1
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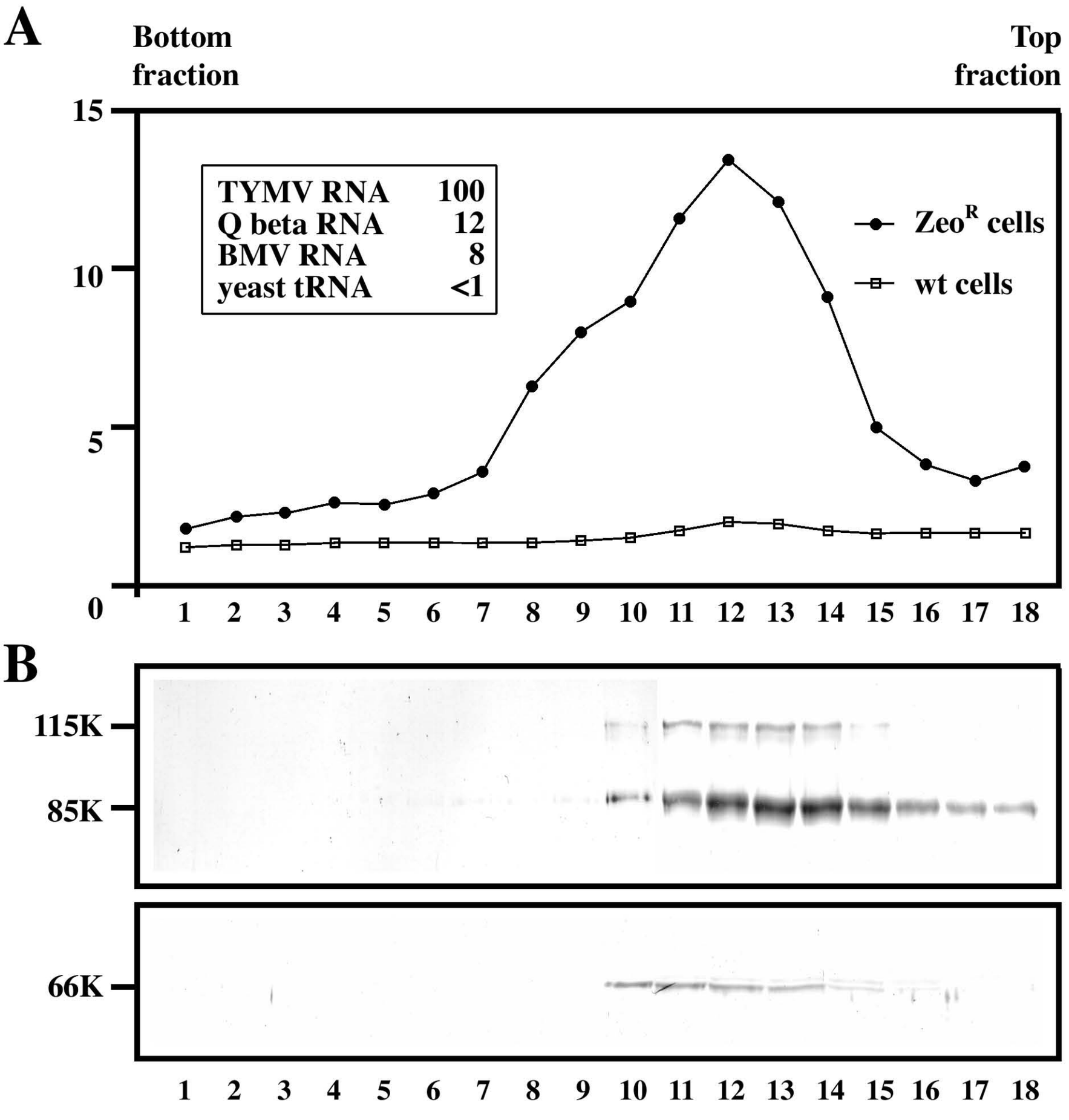
3
(-) strand
wt cells
ZeoR cells
E17 (1/4)
1
2
3


A
Bottom fraction
Top fraction
15
10
5
0
TYMV RNA 100
Q beta RNA 12
BMV RNA 8
yeast tRNA <1
Zeo^R cells
wt cells
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
115K
85K
66K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18