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Viral factor TAV recruits TOR/S6K1 signalling to activate reinitiation after long ORF translation

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The protein kinase TOR (target-of-rapamycin) upregulates translation initiation in eukaryotes, but initiation restart after long ORF translation is restricted by largely unknown pathways. The plant viral reinitiation factor transactivator–viroplasmin (TAV) exceptionally promotes reinitiation through a mechanism involving retention on 80S and reuse of eIF3 and the host factor reinitiation-supporting protein (RISP) to regenerate reinitiation-competent ribosomal complexes. Here, we show that TAV function in reinitiation depends on physical association with TOR, with TAV–TOR binding being critical for both translation reinitiation and viral fitness. Consistently, TOR-deficient plants are resistant to viral infection. TAV triggers TOR hyperactivation and S6K1 phosphorylation *in planta*. When activated, TOR binds polyribosomes concomitantly with polysomal accumulation of eIF3 and RISP—a novel and specific target of TOR/S6K1—in a TAV-dependent manner, with RISP being phosphorylated. TAV mutants defective in TOR binding fail to recruit TOR, thereby abolishing RISP phosphorylation in polysomes and reinitiation. Thus, activation of reinitiation after long ORF translation is more complex than previously appreciated, with TOR/S6K1 upregulation being the key event in the formation of reinitiation-competent ribosomal complexes.

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Introduction

During cap-dependent translation initiation, a preassembled 43S preinitiation complex (43S PIC) containing eIFs 1, 1A and 3, and a ternary complex (TC; eIF2/GTP/Met-tRNAi^{Met}) is recruited to the RNA cap-structure—a process facilitated by the heterotrimeric eIF4F complex together with eIF4B (Pestova *et al*, 2007). The PIC scans along the mRNA in search of the start codon to form the 48S PIC at this AUG codon (Kozak, 1999). Translation begins after joining of a 60S ribosomal subunit (60S).

The terminating ribosome normally dissociates from the mRNA, except if the first translated ORF is short (sORF, less than ~30 codons), in which case a downstream ORF can be translated via reinitiation (Morris and Geballe, 2000). In plants, eIF3 subunit h is critically required to overcome the inhibitory effect of multiple sORFs (Kim *et al*, 2004) and a role for the 60S ribosomal protein L24 in reinitiation was suggested (Nishimura *et al*, 2005). In mammals, several canonical eIFs (eIF3, eIF4F and eIF4A) have been shown to promote reinitiation after sORF translation (Kim *et al*, 2004; Pöyry *et al*, 2004). eIF3 was found to promote reinitiation after a long ORF translation in some exceptional cases of its activation in eukaryotes (Park *et al*, 2001; Pöyry *et al*, 2007). Why reinitiation after translation of a long ORF is limited is still an open question.

An unusual case of a reinitiation mechanism that allows translation of polycistronic mRNA in plants is found in *Cauliflower mosaic virus* (CaMV; Bonneville *et al*, 1989; Ryabova *et al*, 2006). A CaMV reinitiation factor—transactivator/viroplasmin (TAV)—promotes translation of both the viral polycistronic 35S pregenomic RNA and the artificial bicistronic RNAs. To control reinitiation after a long ORF translation, TAV interacts with the host translation machinery via eIF3 (subunit g), 60S ribosomal protein L24 (L24 N-terminus; Park *et al*, 2001) and a novel reinitiation-supporting protein (RISP), where RISP itself serves as a scaffold protein between eIF3 (binds to subunits c/a) and 60S (binds to C-terminus of L24; Thiébeauld *et al*, 2009). In addition, RISP was identified as binding to the PIC (Thiébeauld *et al*, 2009). We previously proposed that TAV enters the host translational machinery around the 60S-joining step via interaction with 40S-bound eIF3, and prevents dissociation of eIF3/RISP from the translating ribosomes during the long elongation event, positioning eIF3/RISP for reinitiation at the downstream ORF. It was also hypothesized that RISP/TAV/eIF3 promotes either 60S recruitment or 80S scanning (Thiébeauld *et al*, 2009). These interactions between TAV and eIF3/RISP are essential, but not sufficient, to accomplish polycistronic translation in plants.

Several steps of translation initiation are positively regulated by the serine/threonine protein kinase TOR (target-of-rapamycin), which is the key component of a nutrient- and hormone-dependent signalling pathway controlling cell growth. Mammalian TORC1 (mTORC1; Kim *et al*, 2002)

signals to its downstream targets in translation initiation, with major targets being the 4E-BPs that regulate cap-dependent translation initiation (Gingras *et al*, 1999) and S6Ks (40S ribosomal protein S6 kinases; Ma and Blenis, 2009). Activated S6K1 has the potential to phosphorylate multiple downstream targets, including its major target the 40S ribosomal protein S6 (Ma and Blenis, 2009) and eIF4B (Raught *et al*, 2004; Holz *et al*, 2005). Both mTOR and S6K1 contribute to the assembly of PIC, where eIF3 serves as a scaffold for either S6K1 or mTOR binding (Holz *et al*, 2005). Upon activation, mTORC1 binds eIF3 and phosphorylates eIF3-bound S6K1 at Thr389, triggering its dissociation and further activation by phosphoinositide-dependent kinase 1 (PDK1; Alessi *et al*, 1998; Frodin *et al*, 2002).

Like mammals, plants possess a single *TOR* gene encoding a protein that regulates the activity of S6K1 (Menand *et al*, 2002; Mahfouz *et al*, 2006) and the S6K1 downstream target S6 (Turck *et al*, 2004), suggesting the existence of a functional TOR kinase pathway in plants. *Arabidopsis* genes *At*S6K1 and S6K2 (Zhang *et al*, 1994) encode proteins highly homologous to mammalian S6K1 and S6K2. The two key phosphorylation sites of mammalian S6K1—T229 and T389—are conserved between plants and mammals, and the activation loop of *At*S6K1 could be phosphorylated by *At*PDK1 (Mahfouz *et al*, 2006). Plants lack orthologues of genes phosphorylated by TOR in other organisms such as those coding for eIF4E-BPs.

Here, we uncover the role of the TOR signalling pathway in TAV-activated reinitiation after long ORF translation. We demonstrate that TAV can interact with TOR, triggers TOR activation as manifested by phosphorylation of S6K1 and stimulates phosphorylation of RISP at S267—a novel downstream target of the TOR/S6K1 pathway. Upon TAV overexpression, TOR can associate with polysomes and this correlates with a high phosphorylation level of polysome-associated RISP. Our data suggest that activation of polycistronic translation in plants is a complex process depending on a dynamic sequence of events leading to the formation of reinitiation-competent post-termination ribosomal complexes, in which TOR/S6K1 activation has a crucial role.

Results

TOR binds the TAV minimal transactivation domain via HEAT repeats

Stringent screening of a yeast genomic two-hybrid library with TAV as bait yielded an N-terminal fragment of yeast TOR1 with five HEAT repeat domains (aa 290–866). Thus, we subcloned the gene encoding *Arabidopsis thaliana* TOR (TOR; 250 kDa), and its 5′- and 3′-terminal fragments—one encoding the entire HEAT repeat domain (NTOR, aa 1–1449) and the other including the sequence coding for kinase domain (CTOR, aa 1449–2481; Figure 1A, upper panel) to determine the ability of *Arabidopsis* TOR to interact with TAV.

To test whether TAV interacts physically with TOR, GST-TOR, GST-NTOR, GST-CTOR and GST were each subjected to pull-down assays with full-length recombinant TAV. TAV directly bound both full-length TOR and NTOR, but not CTOR or GST alone (Figure 1A, bottom panel). Next, we studied colocalization of these proteins *in planta* (Supplementary Figure S1). NTOR and CTOR fusions with enhanced green fluorescent protein (EGFP–NTOR and EGFP–CTOR), and TAV with red fluorescent protein (RFP–TAV) under the control of the CaMV 35S promoter were expressed in *Nicotiana benthamiana* plants via Agrobacterium-mediated transformation. Analysis of leaf epidermal cells by confocal microscopy revealed RFP–TAV aggregates (Supplementary Figure S1A), consistent with its role as a major component of viroplasm, whereas expression of either EGFP–NTOR or EGFP–CTOR alone induced diffuse fluorescent signals. However, coexpression of RFP–TAV and EGFP–NTOR fusion proteins produced structures that exactly superimposed with RFP–TAV aggregates (Supplementary Figure S1B), indicating their interaction, with a high proportion of TAV particles colocalizing with a subpopulation of NTOR as confirmed by high values of Pearson's correlation coefficient, $Rr = 0.776$ (see Supplementary data). The intracellular behaviour of the EGFP–CTOR fusion protein was not modified in the presence of RFP–TAV.

We identified TOR and TAV interaction domains using the yeast two-hybrid assay (Figure 1C). NTOR bound TAV via the minimal transactivation domain (MAV; De Tapia *et al*, 1993) (Figure 1C). Accordingly, TAV lacking MAV was unable to interact with AD-NTOR, confirming that MAV is essential for TOR binding.

A putative RNA-binding motif (dsR, aa 136–182; Evans and Bycroft, 1999) within MAV contains two α-helices and three β-sheets (Figure 1B). Two stretches of three amino acids—^{145}AsnGlyPro (ΔNGP) and ^{171}LysLysPhe (ΔKKF)—highly conserved in CaMV strains were deleted separately to test the specificity of TAV binding to TOR. Both deletions abolished TAV–TOR binding (Figure 1C), while their binding to the other TAV-interacting partner eIF3g was unaffected (Figure 1D). Indeed, eIF3g binds to the multiple protein-binding domain (MBD; Park *et al*, 2001)—a site adjacent to MAV (Figure 1B).

Next, we tested whether these deletion mutants affect CaMV replication and the reinitiation capacity of TAV *in planta* (Figure 1E). Replication was assayed in protoplasts, where TAV is required to allow polycistronic translation of the 35S pregenomic RNA to produce proteins essential for CaMV replication (capsid protein precursor (GAG) and polyprotein with protease, reverse transcriptase and RNase H activity (POL)) as described previously (Kobayashi and Hohn, 2003). We found that the TAVΔKKF and TAVΔNGP mutants, which are expressed as efficiently as wild-type TAV (Figure 1F), did not support CaMV replication in plant protoplasts (Figure 1E). DNA replication was restored only when the viral proteins GAG and POL were provided *in trans*, strongly suggesting that deletion of either of these sets of three amino acids specifically affects TAV transactivation function.

Thus, deletion of either KKF or NGP motifs specifically abolishes TAV transactivation function in plant protoplasts as well as TAV binding to TOR.

TAV association with TOR is required to upregulate phosphorylation of the hydrophobic motif residue T449 of S6K1 in a rapamycin-sensitive manner

To identify a link between TOR/TAV complex formation and TAV-mediated transactivation, we examined whether this interaction activates the TOR/S6K1 signalling pathway. Activation of S6K1 in mammals involves phosphorylation of S6K1 Thr389 (T389), located in the hydrophobic motif by mTORC1 (Pearson *et al*, 1995). Comparing mammalian S6K1 ($p70^{S6K}$) with its *Arabidopsis* orthologues S6K1 and S6K2

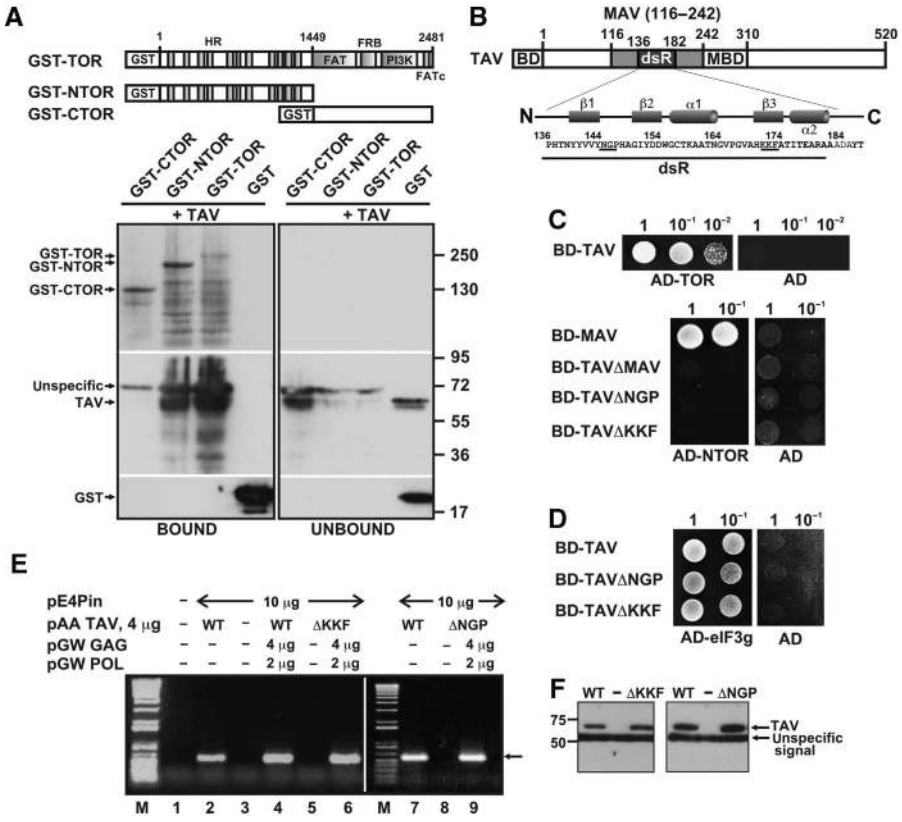

Figure 1 Mapping of TAV and TOR interaction domains. (**A**) TAV interacts with the N-terminal domain of TOR. Schematic representation of *Arabidopsis* TOR, N- and C-terminal (NTOR/CTOR) domains fused to GST. GST-fusion proteins bound to glutathione beads were incubated with purified TAV. The beads were washed, and the unbound and bound fractions were analysed by western blot using antibodies against GST and TAV. (**B**) Schematic representation of TAV. The sequence and predicted secondary structure, and two conserved motifs (NGP and KKF) in the putative dsRNA-binding domain (dsR) are shown underneath. (**C**) (Upper panel) Yeast two-hybrid interactions between BD-TAV and AD-TOR. (Lower panel) Yeast two-hybrid interactions between AD-NTOR and MAV or MAV deletion mutants fused to BD. (**D**) Yeast two-hybrid interactions between AD-eIF3g and TAV and TAV deletion mutants fused to BD. Equal OD_{600} units and 1/10 and 1/100 dilutions were spotted from left to right and incubated for 2 days. (**E**) Semiquantitative reporter-targeted PCR analysis of total low molecular weight DNA from transfected protoplasts. (Left panel) (Lane 1) Mock-transfected protoplasts; transfections with pE4Pin alone (lane 3), or with pAA TAV (wild type (lane 2)); or pAATAV, pGWGAG and pGWPOL (lane 4); or with pAATAVΔKKF (mutant) either alone (lane 5), or with pGWGAG and pGWPOL (lane 6). (Right panel) Transfections with pE4Pin and either pAATAV (lane 7), or pAATAVΔNGP (mutant) without or with pGWGAG and pGWPOL (lane 8). (**F**) Immunoblot analysis of TAV, or TAVΔKKF and TAVΔNGP mutant, accumulation levels in plant protoplasts during CaMV amplification, see (**E**).

suggests that plant S6K1 T449 is the functional equivalent of mammalian $p70^{S6K}$-T389 (S6K1-T389; Figure 2A). TOR does not respond to rapamycin in *Arabidopsis* due to the inability of the *Arabidopsis* homologue of the peptidyl-prolyl isomerase FKBP12 to present rapamycin to *At*TOR (Mahfouz *et al*, 2006; Sormani *et al*, 2007), but phosphorylation of S6K1 can be blocked by rapamycin in maize plants (Reyes de la Cruz *et al*, 2004). However, phosphorylation of recombinant S6K1 by rice extracts detected after 5 min of incubation was abolished by rapamycin treatment (Figure 2B), confirming that phospho-specific anti-S6K1-T389-P antibodies are able to recognize *Arabidopsis* phosphorylated S6K1 in a rapamycin-sensitive manner (for band density quantification of Figure 2B–E, see Supplementary data S2).

Next, we compared the capacities of plant extracts prepared from either TAV *Arabidopsis* transgenic line AT7 or wild-type plants to phosphorylate the hydrophobic motif of S6K1 (Figure 2C). The results revealed that the levels of phosphorylated S6K1 (S6K1-T449-P) were elevated about eight-fold after a 15-min incubation of recombinant S6K1 with AT7 extracts as compared with wild-type extracts; the endogenous S6K1 phosphorylation level in AT7 extracts (no incubation) was also strongly increased. The TOR-specific phosphorylation site mutation (T449A) completely abolished S6K1 recognition by anti-S6K1-T389-P antibodies. The ability of S6K1-T389-P antiserum to distinguish between wild-type S6K1 and the S6K1 T449A mutant suggests that the antiserum is specific for *Arabidopsis* S6K1 phosphorylated at T449.

Since TAV overexpression correlates with increased S6K1 phosphorylation at T449 *in vitro*, it seemed possible that the level of phosphorylated TOR and S6K1 would be constitutively high in TAV-transgenic plants. To examine this hypothesis, we compared phosphorylation levels of S6K1 and TOR in extracts prepared from AT7, wild-type plants, the T-DNA insertion line G548 expressing high levels of TOR, and the TOR-deficient RNAi silenced line 35–7 (Supplementary Figure S3A; Deprost *et al*, 2007). While G548 plants displayed enhanced plant growth compared with wild-type plants, TOR-deficient plants were smaller, often having a particular phenotype characterized by the presence of serrated and pointed leaves (Supplementary Figure S3B). Extracts prepared from these plants were subjected to SDS–PAGE and

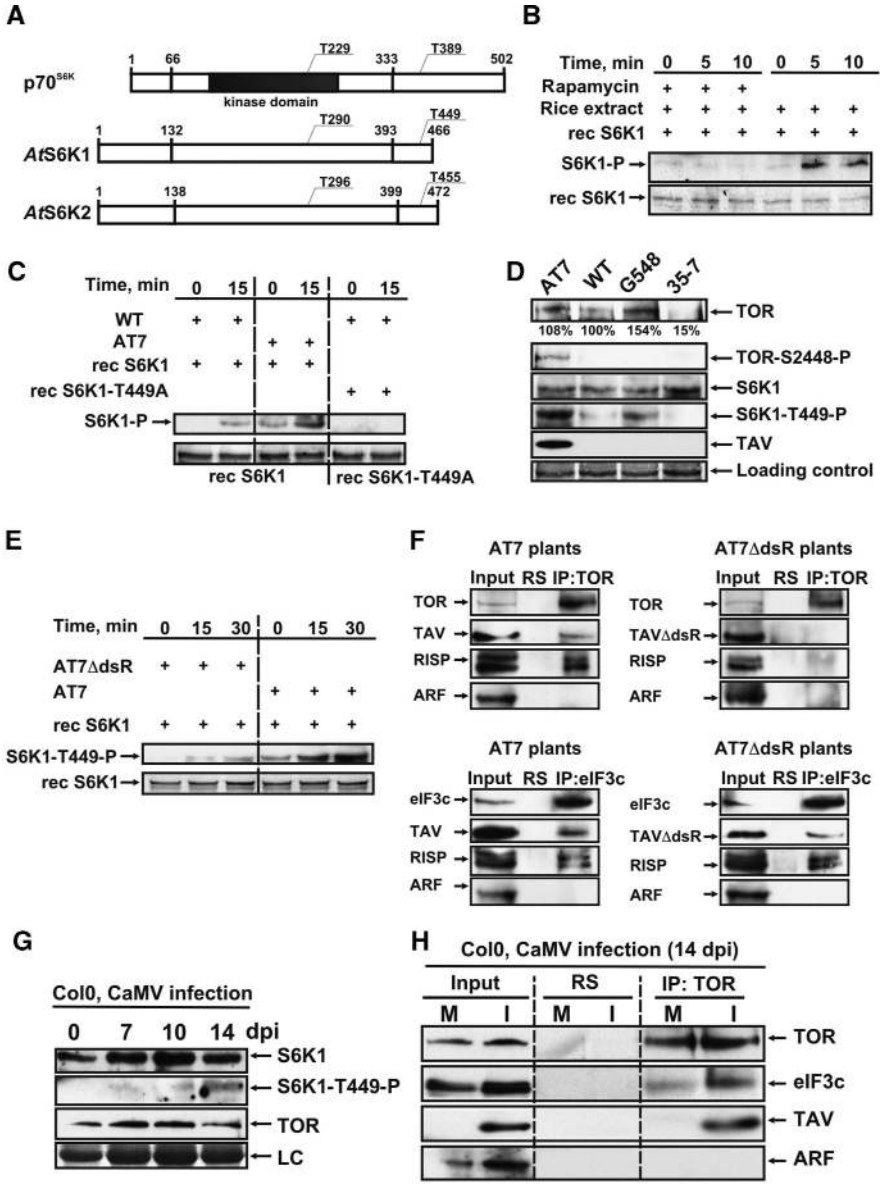

Figure 2 Phosphorylation of S6K1 on TOR-specific residue T449 is specifically upregulated in plants overexpressing TAV, and correlates with TOR and TAV complex formation *in vivo*. (**A**) Schematic representations of *Arabidopsis* S6K1 and S6K2 with mammalian p70^{S6K} as a reference. Two key phosphorylated threonines (T) are indicated. (**B**) The *in vitro* phosphorylation assay using rice extract. Recombinant S6K1 (rec S6K1) was incubated with rice extracts with or without rapamycin followed by western blot analysis using anti-S6K1-T389-P or anti-S6K1 antibodies. S6K1 was visualized by Coomassie blue staining. (**C**) Phosphorylation of S6K1 at T449 is upregulated by TAV. Rec S6K1 and its mutant S6K1-T449A were incubated with extracts prepared from wild type or AT7 plants followed by western blot analysis. (**D**) Immunoblot analysis of either TOR phosphorylation (using anti-TOR-S2448-P antibodies), or S6K1 phosphorylation levels at T449, as well as their accumulation levels in the TAV-transgenic line AT7; the control line (wild type); the TOR-overexpressing line G548, and the TOR-deficient RNAi silenced line 35–7. (**E**) *In vitro* phosphorylation of Rec S6K1 in extracts prepared from either AT7- or AT7ΔdsR-expressing plants; panels show western blot analysis using anti-S6K1-T389-P antibodies. (**F**) AT7 and AT7ΔdsR plants were used for coimmunoprecipitation with either anti-TOR or anti-eIF3c antibodies. The panels show immunoblotting of either TOR or eIF3c with TAV, RISP or control small GTPase ADP-ribosylation factor (ARF) present in Input, normal rabbit serum (RS) and the entire immunoprecipitate (IP) using appropriate rabbit polyclonal antibodies. (**G**) Time course of S6K1, T449-phosphorylated S6K1 and TOR protein levels in extracts of CaMV-infected *Arabidopsis* plants. (**H**) Lysates prepared from flowers of mock-inoculated (M) and CaMV-infected (I) *Arabidopsis* plants were used for immunoblotting of eIF3c, TOR, TAV or ARF present in Input, normal rabbit serum (RS) and the entire immunoprecipitate (IP).

probed with phosphospecific-S2448 mTOR antibody (p70^{S6K} was found as a major effector of mTOR phosphorylation at S2448; Chiang and Abraham, 2005) or S6K1-T389-P antibodies (Figure 2D). TOR was detected in AT7 and wild-type plants; a somewhat stronger TOR band was detected in TOR-overexpressing plants, while TOR-deficient 35–7 plants contained TOR levels at the limit of detection (Figure 2D). Phosphorylated TOR was detected easily only in AT7 plants, with the signal again being below the detection limit for G548 and WT plants, strongly indicating an influence of TAV on TOR phosphorylation (Figure 2D). As for phosphorylation of the TOR-dependent substrate S6K1, phosphothreonine 449 was also higher in AT7 compared with G548 and WT plants. No significant amount of phosphothreonine 449 was detected

in 35–7 plants with our antibodies, further suggesting that TOR or TOR-dependent kinase are major effectors of S6K1 phosphorylation in *Arabidopsis*. To strengthen our conclusion that TAV is specifically activating TOR phosphorylation of S6K1, we attempted to inhibit TOR kinase activity in AT7 plants using a specific TOR inhibitor, Torin-1 (Supplementary Figure S2A). Torin-1 interacts with the TOR kinase domain within the ATP-binding pocket and blocks TOR phosphorylation with an ATP-competitive mode of action to inhibit cap-dependent mRNA translation (Guertin and Sabatini, 2009; Thoreen *et al*, 2009). Growth of *Arabidopsis* seedlings on medium with Torin-1 eliminated the increased levels of S6K1-T449-P and thus TOR-S2448-P, suggesting inactivation of the TOR pathway despite the presence of high levels of TAV (Supplementary Figure S2A).

To study the effect of TAV/TOR binding on phosphorylation of endogenous TOR and S6K1 in *Arabidopsis* plants, we employed two transgenic lines expressing either TAV or its ΔdsR mutant lacking the TOR-binding site (Δaa 136–182). Interestingly, AT7ΔdsR plants expressing the TAV mutant allele displayed no phenotypic defects, despite accumulation of comparable levels of mutant TAV (Figure 6C), while plants constitutively expressing TAV display strong stunting and a chlorotic dwarf phenotype resembling CaMV-elicited symptoms (Supplementary Figure S3A). Deletion of the TOR-binding site of TAV decreased the ability of AT7ΔdsR extracts to phosphorylate recombinant S6K1 *in vitro* by about 10-fold compared with that of AT7 extracts (Figure 2E), strongly suggesting that TAV dsR is critical for TOR signalling activation.

To determine if the dsR domain is critical for TAV/TOR complex formation *in vivo*, we immunoprecipitated TOR from plants expressing TAV or its ΔdsR mutant and assayed TOR association with TAV and RISP. As expected, TOR immunoprecipitates from AT7 plants contained both TAV and RISP (Figure 2F), but those from AT7ΔdsR plants were devoid of both proteins. In contrast, complex formation between TAV, eIF3 and RISP is not dependent on TOR/TAV interaction and proceeds efficiently with either TAV or TAVΔdsR. Thus, we can dissect MAV into domains that are responsible largely for interaction with TOR (the dsR domain) or RISP (the MAV C-terminus).

To verify whether S6K1 phosphorylation is also elevated during the course of CaMV infection, we mechanically inoculated *Arabidopsis* plants with CaMV and monitored S6K1 induction kinetics within pools of these plants (viral symptoms were seen 10 days post-inoculation, dpi). Strikingly, levels of S6K1-T449-P (normalized to a loading control) increased significantly in CaMV-infected plants up to 14 dpi, while TOR and S6K1 levels were unaffected (Figure 2G). These results confirm that overexpression of TAV either alone or via virus propagation correlates with TOR activation.

Furthermore, as shown in Figure 2H, eIF3 coimmunoprecipitated with TOR in both wild-type and CaMV-infected plants. The high phosphorylation level of S6K1 in extracts prepared from CaMV-infected plants at 14 dpi (Figure 2G) correlates with increased amounts of eIF3 pelleted by TOR in flower extracts prepared from CaMV-infected plants compared with uninfected plants (14 dpi; Figure 2H), indicating that eIF3 can serve as a platform for activated TOR binding *in planta*, as suggested in mammals (Holz *et al*, 2005).

TOR overexpression in plant protoplasts stimulates TAV-dependent reinitiation after long ORF translation

As TAV stimulates TOR signalling, we next asked whether TOR phosphorylation activity is required to promote the TAV reinitiation function. For transient expression experiments in mesophyll protoplasts prepared from *Arabidopsis* leaves, we used two reporter plasmids: pmonoGFP, containing a single GFP ORF; and pbiGUS, containing two consecutive ORFs: CaMV ORF VII and β-glucuronidase (GUS; Bonneville *et al*, 1989), where GUS serves as a marker of transactivation, and GFP as a control for transformation/cap-dependent translation initiation efficiency (Figure 3A). In addition, plants were cotransformed with reporter plasmids encoding TAV and either TOR or its functionally inactive version TOR-D2247A (corresponds to functionally inactive mutant of mTOR, mTOR-D2338A; Brunn *et al*, 1996).

Upon transfection of protoplasts, the biGUS construct did not give rise to any significant GUS activity (Figure 3B). In contrast, the monoGFP vector was fully active. Thus, upstream ORF VII blocks downstream GUS gene expression, and no GUS activity appeared as a result of reinitiation or any putative *biGUS* mRNA cleavage in *Arabidopsis* mesophyll protoplasts. As expected, activation of translation of the second ORF (GUS) via reinitiation depended exclusively on the presence of TAV (Figure 3B). TOR, when overexpressed above its endogenous level in the presence of TAV, enhanced GUS ORF transactivation two-fold. Overexpression of both TAV and TOR correlates with a strongly increased endogenous level of S6K1 phosphothreonine 449 (Figure 3C, lane 3). In contrast, expression of TOR-D2247A lacking phosphorylation capacity (Figure 3C, lane 4) did not increase the level of TAV-mediated transactivation (Figure 3B). Monitoring of RNA transcript levels using RT–qPCR and semiquantitative RT–PCR in extracts did not reveal any significant differences in *biGUS* mRNA length or level (Figure 3B, bottom panels) with or without TAV and TOR. The levels of transiently expressed TAV, and either TOR or TOR-D2247A in protoplasts did not differ significantly under the different experimental conditions after 24 h of incubation (Figure 3C).

Thus, the kinase activity of TOR is required to stimulate TAV function in reinitiation after translation of a long ORF.

TOR-deficient plants fail to support TAV-mediated transactivation of reinitiation and are resistant to CaMV infection

TAV mutants lacking the capacity to interact with TOR are unable to promote polycistronic translation and abolish viral replication, suggesting that TOR knockdown would increase plant resistance to CaMV (Figure 1). To test whether CaMV indeed recruits TOR to overcome cell resistance, we studied susceptibility to viral infection of TOR-deficient RNAi 35–7 plants that display the serrated and pointed leaf phenotype (see Supplementary Figure S3B).

Appearance of symptoms (Figure 4A) and virus replication kinetics (Figure 4B) in wild-type and 35–7 plants mechanically inoculated with CaMV were compared in two independent experiments. In all 13 wild-type plants tested, TAV and viral coat protein (CP) accumulation was first observed at 10 dpi, while we failed to detect viral proteins in 10 tested 35–7 plants up to 21 dpi (Figure 4B) and 28 dpi (data not shown). Although symptom appearance at 14 dpi was strong for wild-type plants (typical vein-clearing symptoms indicative

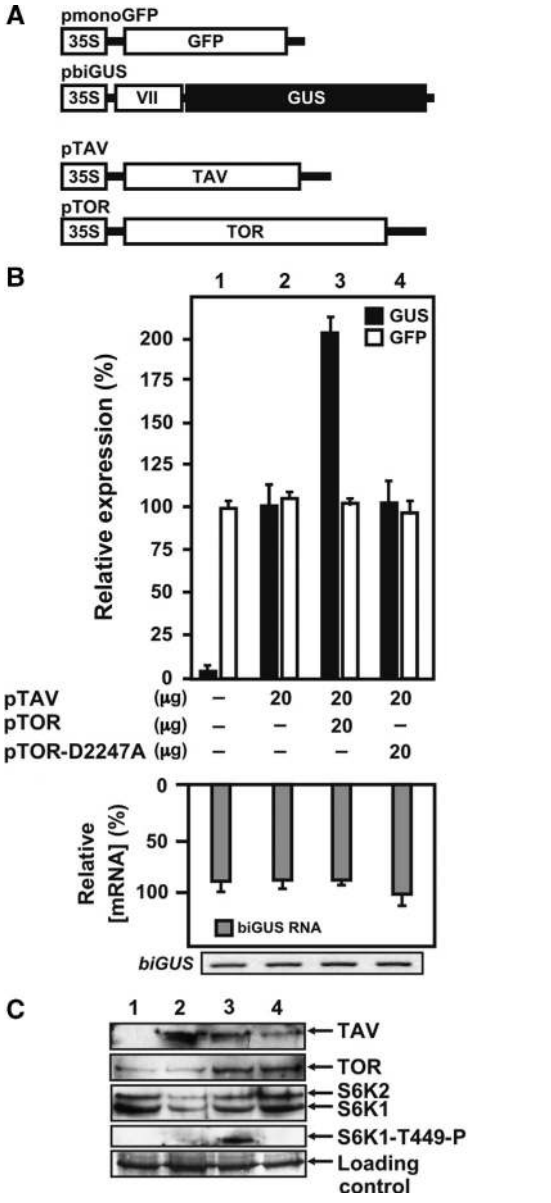

Figure 3 Inactive TOR kinase mutant fails to stimulate TAV-mediated transactivation. (**A**) Schematic diagram of the monocistronic and dicistronic reporter constructs. (**B**) *Arabidopsis* mesophyll protoplasts were cotransfected with the two reporter plasmids pmonoGFP and pbiGUS, as well as effector plasmids expressing TAV and either TOR or its inactive derivative (D2247A) as indicated. Activity of GUS synthesized in protoplasts transfected with pTAV only was set as 100% (150 000 r.f.u.), and quantified GFP expression in protoplasts transfected with only reporter constructs—as 100%. *biGUS* mRNA levels were analysed by qPCR (grey bars; RNA value in the presence of pTOR(D2247A) was set at 100%), and semi-quantitative RT–PCR 24 h after protoplast transformation. The data shown are the means of three independent assays: error bars indicate s.d. (**C**) TAV, TOR, S6K1 and S6K1-T449-P accumulation was analysed by western blot using appropriate antibodies.

of systemic infection), 35–7 plants displayed no symptoms at either 14 dpi (Figure 4A) or 28 dpi (data not shown). Comparable RT–qPCR and western blot analyses of these WT and 35–7 plants revealed a drastic decrease in *TOR* RNA, and TOR protein levels were practically nondetectable in our conditions (Figure 4C). We concluded that this resistance to CaMV is due to low TOR availability limiting viral replication in *Arabidopsis* plants.

We next determined whether these TOR knockdown plants fail to support TAV-mediated transactivation. Protoplasts prepared from 35–7 and wild-type plants were transformed with the pmonoGFP and pbiGUS plasmids used above. Again, upon transfection of protoplasts, the biGUS construct did not give rise to any significant GUS activity without TAV (Figure 4D), indicating that ORFVII is intact and suppresses reinitiation at the GUS ORF. Transient expression of GUS was activated only upon cotransfection with the pTAV vector (Figure 4D). As expected, protoplasts prepared from TOR-deficient plants had no capacity to mediate reinitiation of the downstream GUS ORF with or without TAV (see Figure 4D and Supplementary Figure S4A), while GFP synthesis was largely unaffected. *biGUS* RNA transcript length and levels were monitored by qPCR and semiquantitative RT–PCR (Figure 4D, bottom panels). In addition, TAV-mediated transactivation in 35–7 protoplasts was rescued upon transient expression of TOR (Supplementary Figure S4B). Therefore, we conclude that TOR is an essential partner of TAV in activation of reinitiation after long ORF translation, and that substantial TOR protein levels are required for TAV transactivation function.

TOR associates with polyribosomes in TAV-overexpressing plants

Previous studies have suggested that TAV functions in transactivation by promoting retention of eIF3 and RISP on translating ribosomes; in sucrose gradient sedimentation experiments, TAV, eIF3 and RISP comigrated with polyribosomal fractions in CaMV-infected plants (Park *et al*, 2001; Thiébeauld *et al*, 2009). In order to understand the mechanism whereby TAV-dependent activation of TOR promotes reinitiation, it would be useful to know whether TOR is recruited to polysomes in TAV-transgenic plants.

This was tested using cytoplasmic extracts prepared from young *Arabidopsis* wild-type and TOR-deficient seedlings fractionated on sucrose gradients (Figure 5A and B, respectively). Evaluation of eIF3, RISP and TOR in sucrose gradient fractions demonstrated their comigration largely with 40S ribosomal subunit fractions of the gradients (Figure 5, cf. A and B). WT extract polysomes treated with EDTA dissociated into 60S- and 40S-containing complexes, where eIF3, RISP and TOR shifted to 40S and top gradient fractions (Figure 5C).

In TAV-transgenic plants, TAV, eIF3 and RISP comigrate with polysomes (Figure 5D). Strikingly, TOR was also found associated with polysomes in the presence of TAV. We next asked whether TOR, eIF3 and RISP polysomal distribution is affected by removing the TOR-binding site of TAV. To answer this question, polysomes prepared from seedlings expressing TAVΔdsR were analysed as above; TOR binding to polysomes was completely abolished (Figure 5E). Although comigration of eIF3 and RISP with polysomes was not significantly affected by dsR removal, and TAVΔdsR was detected in polysomes, its abundance in polysomes was somewhat reduced. We conclude that TOR associates with polysomes in TAV-transgenic plants in the presence of TAV, but seems not to be required for TAV, eIF3 and RISP recruitment to polysomes.

While EDTA treatment, which dissociates polysomes, shifted western blot signals to the top of gradients (Figure 5F), some TAV, eIF3g and RISP seems to cosediment not only in 40S, but also 60S fractions (Figure 5F). To analyse this unusual comigration with 60S in detail, we

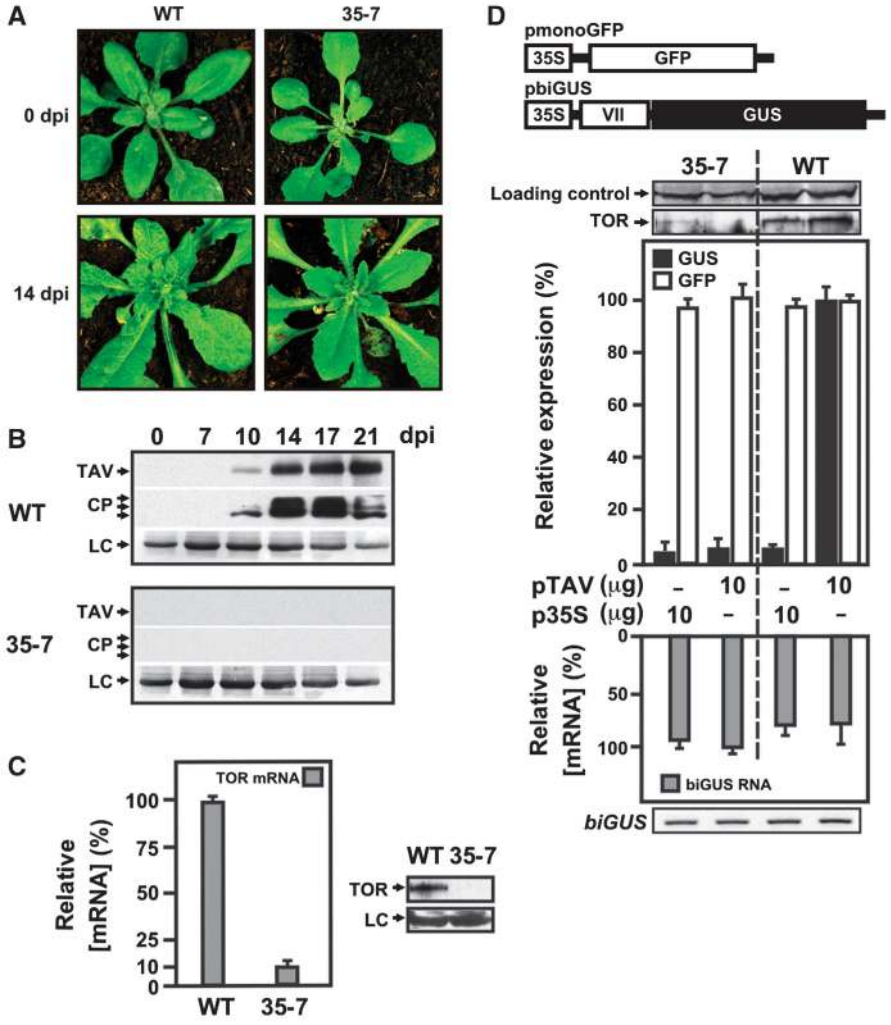

Figure 4 TOR-deficient plants fail to promote TAV-mediated transactivation of reinitiation after long ORF translation, and are resistant to CaMV. (**A**) Analysis of healthy plants (top panels) and disease symptoms (bottom panels) in wild type and the TOR-deficient RNAi silencing line 35–7. (**B**) TAV and CP proteins accumulate in CaMV-infected wild type, but not in 35–7 plants. (**C**) TOR mRNA levels in wild-type and mutant plants were analysed by qPCR (grey bars; RNA value in wild-type plants was set at 100%). TOR expression levels were analysed by immunoblot. (**D**) TAV-mediated transactivation is efficient in mesophyll protoplasts prepared from WT and abolished in TOR-deficient mutant plants. Activity of GUS synthesized in protoplasts transfected with pTAV was set as 100% (110 000 r.f.u.). GUS and GFP activities are shown as black and open bars, respectively. TOR expression levels were analysed by immunoblot. *biGUS* mRNA levels were analysed by qPCR (grey bars; RNA value in the presence of pTAV in 35–7 line was set at 100%) and semiquantitative RT–PCR. The data shown are the means of three independent assays: error bars indicate s.d.

adjusted sedimentation conditions to resolve 60S- and 40S-containing complexes after EDTA treatment of polysomes (Figure 5G and H). The data obtained show that TAV, eIF3g and RISP are shifted into 60S-containing fractions of the gradient, indicating their association with 60S also within polysomes (for band density quantification see Supplementary data S5). Interestingly, TOR cosediments with 60S-containing complexes in AT7 plants.

RISP is phosphorylated at S267 in a rapamycin-sensitive manner

The data presented above provide evidence that the activation state of TOR as mediated by TAV governs its binding to polysomes. We hypothesized that either activated TOR or its downstream target kinases phosphorylate protein(s) associated with polysomes in a TAV-dependent manner. This hypothesis was supported by the fact that RISP harbours the pattern RGRLES found in many Akt or S6K1 substrates (phospho-Ser/Thr preceded by Lys/Arg at positions −5 and −3 (R/KxR/KxxS/T)) within the central domain of RISP, which was implicated earlier in TAV binding (Figure 6A).

First, we investigated whether TOR promotes RISP phosphorylation. Initial experiments examining TAV or RISP phosphorylation capacities in the presence of an *Arabidopsis* extract and ^{32}P orthophosphate/ATP suggested that, like recombinant S6K1, recombinant RISP could indeed serve as a substrate for phosphorylation (Supplementary Figure S6). In both cases, RISP and S6K1 phosphorylation was elevated in extracts prepared from AT7 plants. We further examined the phosphorylation capacity of RISP by using the rapamycin-sensitive rice extract and phospho-specific anti-(RxRxxpS/T) antibodies (these antibodies have been used successfully to identify putative protein substrates of the TOR/S6K1 pathway (Ma *et al*, 2008)). The Ser residue 267 (S267) is a predicted

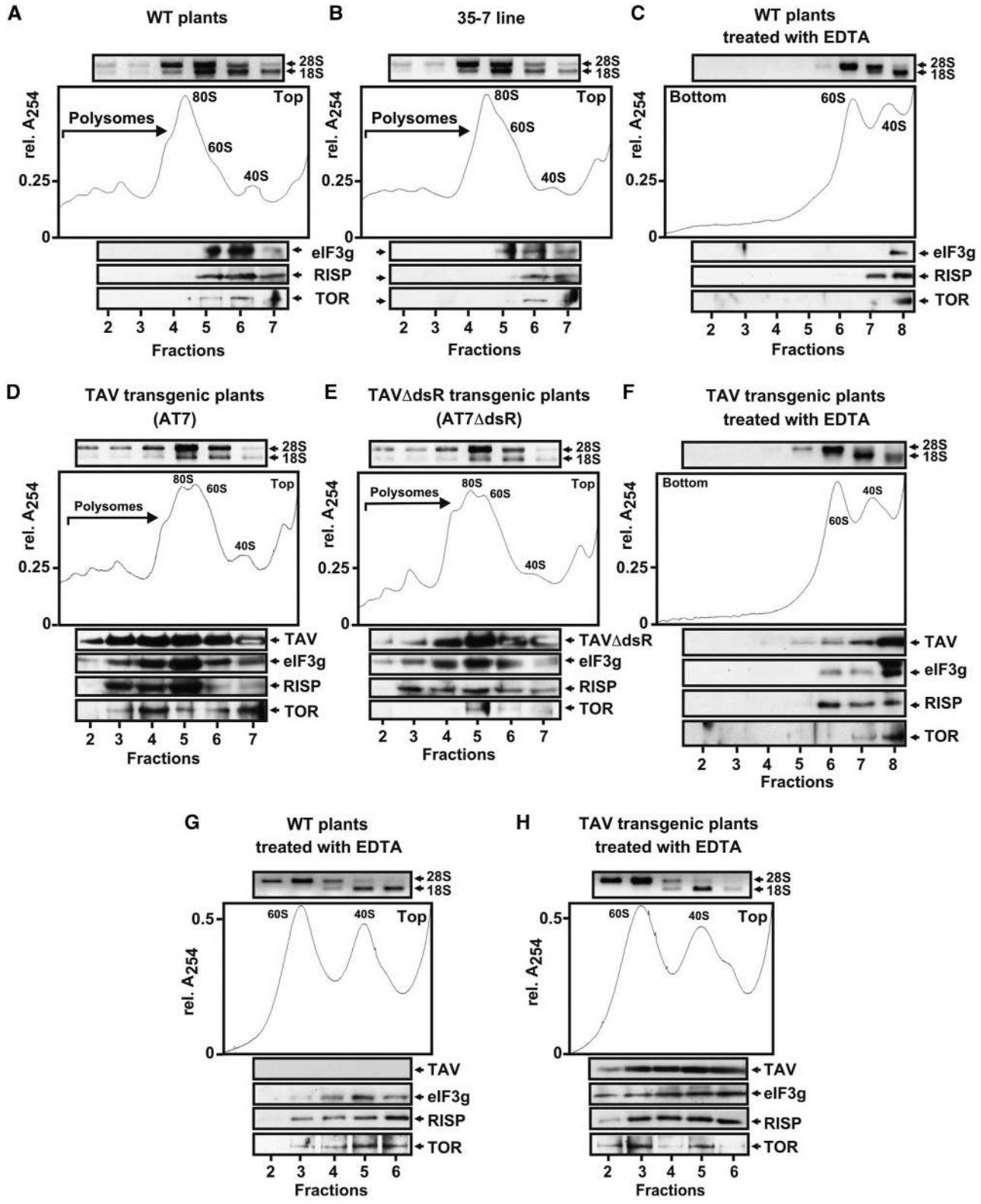

Figure 5 TOR-binding site of TAV is required for TOR loading on polysomes. (**A–C**) Ribosomal profiles of polyribosomes (polysomes) and ribosomal species from wild type (WT, **A**), TOR-deficient line 35–7 (**B**) and wild-type extracts treated with 50 mM EDTA (**C**). (**D–F**) Ribosomal profiles from plant extracts containing TAV (AT7, **D**) or its mutant TAVΔdsR (AT7ΔdsR, **E**) and TAV-containing plant extracts treated with 50 mM EDTA (AT7, **F**). (**G–H**) Ribosomal profiles of polysomes from WT (**G**) or TAV-expressing (AT7, **H**) plants treated with 50 mM EDTA. (**A–H**) In all, 1 ml aliquot fractions were precipitated with 10% TCA, and analysed by western blot using corresponding antibodies (lower panels); or analysed by agarose gel electrophoresis (upper panels). Positions of 18S and 28S rRNAs are indicated. A minimum of four replicate experiments were performed for all sucrose gradients presented; all repeats gave reproducible results.

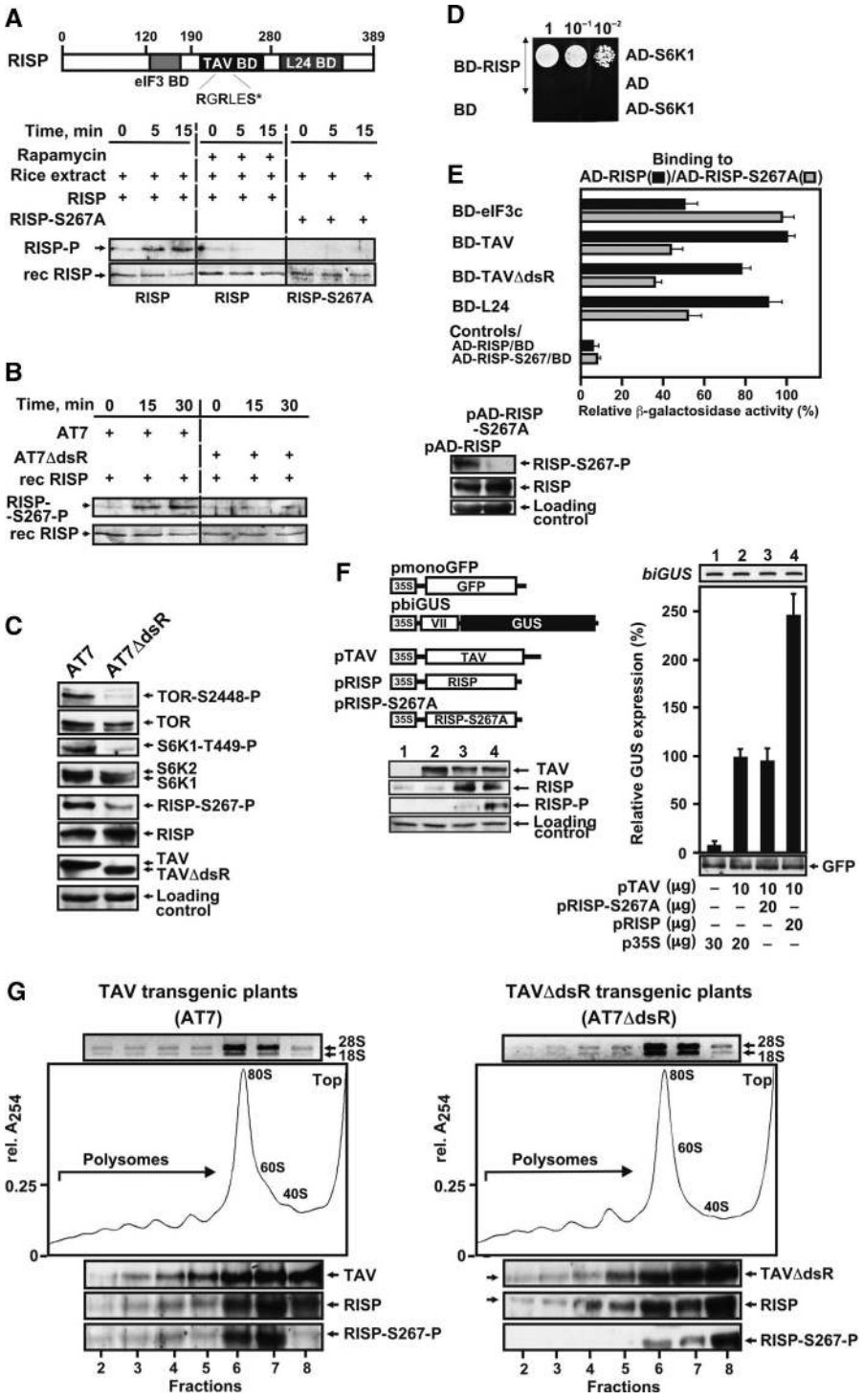

Figure 6 Identification of RISP as a novel substrate of the TOR/S6K1 pathway. (**A**) Schematic representation of RISP. A conserved consensus phosphorylation site (R/KxR/KxxpS/T) for Akt/S6K1 is present in the central region. BD denotes binding sites. Rec RISP or RISP-S267A was incubated with rice extracts with or without rapamycin followed by immunoblotting with anti-R/KxR/KxxpS/T (anti-RISP-P) antibodies. RISP was visualized by Coomassie staining. (**B**) Rec RISP was incubated with extracts prepared from either AT7- or AT7ΔdsR-expressing plants followed by immunoblot analysis with anti-RISP-P antibodies. (**C**) Immunoblot analysis of phosphorylation levels of either TOR at S2448, or S6K1 at T449 or RISP at S267, as well as TOR, S6K1, RISP and TAV accumulation levels in TAV- and TAVΔdsR-transgenic lines. (**D**) Yeast two-hybrid interactions between BD-RISP and AD-S6K1. Equal OD_{600} units and 1/10 and 1/100 dilutions were used for one experiment. (**E**) Quantification of interactions between either RISP or RISP-S267A fused to Gal4 AD, and BD-eIF3c, -TAV, -TAVΔdsR and L24. Immunoblot analysis of AD-RISP accumulation levels or phosphorylation in yeast transformed with either pAD-RISP or pAD-RISP-S267A. Results represent the mean values from triplicates ± s.d. (**F**) Schematic diagram of reporter constructs used for protoplast transformations are shown. *Arabidopsis* suspension culture protoplasts were cotransfected with pmonoGFP and pbiGUS, as well as plasmids expressing TAV and either RISP or its mutant (RISP-S267A) as indicated. Activity of GUS synthesized in protoplasts transfected with pTAV was set as 100% (52 000 r.f.u.). GFP, TAV and RISP expression levels as well as RISP phosphorylation levels were analysed by immunoblot with appropriate antibodies. Results shown represent the means obtained in three independent experiments. *biGUS* mRNA levels were monitored by semiquantitative RT–PCR. (**G**) Ribosomal profiles from TAV- and TAVΔdsR-overexpressing plant extracts. In all, 1 ml aliquot fractions were precipitated with 10% TCA, and analysed by western blot using anti-TAV, anti-RISP and R/KxR/KxxpS/T antibodies; or analysed by agarose gel electrophoresis (rRNA profiles).

phosphorylation site within the consensus pattern of RISP (Figure 6A, top panel). Accordingly, we found specific phosphorylation of RISP in this rice extract, whereas rapamycin abrogated RISP phosphorylation (Figure 6A). The S267A mutation completely abolished RISP recognition by anti-(RxRxxpS/T) antibodies, strongly indicating that RISP is phosphorylated at S267 (for band density quantification of Figure 6A–C, see Supplementary Figure S7).

To gain further evidence that TOR/S6K1-mediated phosphorylation of RISP is under TAV control, we determined if an extract expressing TAV is more efficient in RISP phosphorylation than one expressing TAVΔdsR. Strikingly, in TAV-containing *Arabidopsis* extracts, the RISP phosphorylation level was more than three-fold higher than in extracts with mutant TAV (Figure 6B). Taken together, these results strongly suggest that RISP is a substrate of the TOR pathway, and that phosphorylation of S6K1 and RISP is increased by the TAV dsR domain. Indeed, western blot analyses of extracts prepared from AT7 or AT7ΔdsR plants confirmed hyperactivated phosphorylation levels of TOR, S6K1 and RISP in TAV-expressing plants (Figure 6C). However, in TAVΔdsR-overexpressing plants, a significant level of phosphorylated RISP is maintained by TOR/S6K1 activated independently of TAV (Figure 6C).

RISP phosphorylation in polysomes depends on activation and binding of TOR to polysomes, and stimulates TAV-mediated reinitiation after long ORF translation

Our previously published results showed that endogenous RISP coimmunoprecipitates with endogenous eIF3 and eIF2 in plants, and that the RISP–eIF3 complex interacts with the 40S ribosomal subunit *in vitro*, suggesting RISP participation in 40S-containing PIC (Thiébeauld *et al*, 2009). After identifying RISP S267 as a putative phosphorylation site of the TOR/S6K1 signalling pathway, we set out to analyse whether RISP can potentially interact with S6K1. Indeed, RISP fused to the Gal4-binding domain interacts strongly with S6K1 fused to the activation domain of Gal4 under our yeast two-hybrid system conditions (Figure 6D).

Phosphorylation of S6K1-T389 regulates the interaction between S6K1 and eIF3-PIC in mammalian cells, and therefore the S6K1-T389A mutant is characterized by increased affinity to eIF3 (Holz *et al*, 2005). Thus, we tested if the S267A mutation of RISP would alter complex formation between RISP and its interacting partners in the yeast two-hybrid system. First, we confirmed that RISP, but not its S267A mutant, is phosphorylated in yeast extracts (Figure 6E, bottom panel). As shown in Figure 6E, the phosphorylation inactive mutant of RISP has a reproducibly stronger interaction with eIF3c than wild-type RISP. These results suggest that phosphorylation of RISP in wild-type plants can occur within eIF3-containing complexes that seem to preferentially bind unphosphorylated RISP. In contrast, the S267A mutation had the opposite effect on RISP binding to TAV, decreasing the interaction by 50%. Binding of the RISP mutant to other RISP partner, L24 was decreased by about 40%, as was TAV (Figure 6E).

Thus, RISP phosphorylation seems to strengthen its binding to both TAV and 60S L24, and both complexes are implicated in RISP loading on polysomes in AT7 and CaMV-infected plants. To address the question of whether phosphorylation of RISP stimulates reinitiation, we used protoplasts prepared from *Arabidopsis* suspension culture to study the effect of the S267A mutation on the capacity of transiently expressed RISP to stimulate TAV-mediated transactivation (Figure 6F). Little to no increase in TAV-mediated GUS activity was detected with a construct encoding RISP-S267A (Figure 6F, right panel), which was characterized by a negligible phosphorylation level (left panel, lane 3), while highly phosphorylated transiently expressed wild-type RISP (left panel, lane 4) increases TAV-mediated transactivation by more than 2.5-fold (right panel, lane 4). Together, these results indicate that RISP phosphorylated at S267 is largely active in reinitiation of translation.

In sucrose sedimentation gradients, TOR is seen associated with ribosomal subunits with or without TAV, and monosomes/polysomes in TAV-overexpressing plants (Figure 5D). We tested whether TAV-mediated association of TOR with polysomes is required for phosphorylation of RISP. We thus investigated segregation of RISP and its phosphorylated form between polysomes, monosomes and ribosomal subunits in the presence of overexpressed TAV or dsR mutant TAV (Figure 6G). Polysomes were analysed as described above using anti-TAV, anti-RISP and anti-(RxRxxpS/T) antibodies. In preparations from AT7 extracts, a significant amount of phosphorylated RISP (RISP-S267-P) sedimented preferentially with polysomes, monosomes and 60S ribosomal subunits (Figure 6G, left panel). This pattern roughly matched the distribution of total RISP, except that 40S fractions contain less RISP-S267-P. As shown already in Figure 5E, removal of the dsR domain did not abolish association of total RISP with polysomes: TAVΔdsR binds polysomes and mediates recruitment of RISP. In contrast, while RISP-S267-P was still associated with fractions of monosomes, 60S and preferentially 40S ribosomal subunits in TAVΔdsR-expressing extracts, polysomes were occupied by RISP in the nonphosphorylated state (Figure 6G, right panel). RISP-P distribution between polysomes, monosomes and ribosomal subunits likely reflects the distribution of TOR in TAV or TAVΔdsR plants. Thus, there was no phosphorylated RISP in polysomes without associated TOR (cf. Figures 5E and 6G).

Discussion

In eukaryotes, production of two or more proteins from the same mRNA via a translation reinitiation mechanism is strongly disallowed except for reinitiation after sORF translation. It was proposed that reinitiation after sORF translation depends on the participation of certain eIFs supporting reinitiation that are recruited during the initiation event at sORF and then not released during the short time required for sORF translation (Kozak, 2001). These eIFs associated with post-termination ribosomes can then regenerate reinitiation-competent 40S complexes capable of scanning to the next ORF, rebinding the TC along the way. We study an exceptional case of translation restart controlled by the CaMV reinitiation factor TAV, which can promote translation of consecutive multiple long ORFs on the same RNA via reinitiation in eukaryotes. How does TAV activate such polycistronic translation in eukaryotes? Our previous studies suggested that activation of reinitiation proceeds via a mechanism that includes retention of reinitiation-supporting factors on translating ribosomes during the long primary

translation event, and reuse of these factors for reinitiation, and we identified such factors as eIF3 (Park *et al*, 2001) and a novel plant protein RISP (Thiébeauld *et al*, 2009).

The present report identifies the essential role of the protein kinase TOR in reinitiation after long ORF translation, and demonstrates that this process depends strictly on TOR signalling activation. We demonstrated that TAV functions as a trigger of TOR activation as manifested by phosphorylation of *Arabidopsis* S6K1 at TOR-specific T449 in TAV-transgenic and CaMV-infected plants. Only functionally active TOR was able to stimulate TAV-mediated reinitiation efficiency, and TOR knockdown abolishes TAV function in plant protoplasts and CaMV infection *in planta*. Accordingly, transient overexpression of TAV and TOR increases both phosphorylation of S6K1 and the level of reinitiation in mesophyll protoplasts. Thus, TOR is identified as a host protein that is essential at least for TAV transactivation function during the CaMV life cycle. Furthermore, we identified a putative target of the TOR signalling pathway as a factor known to support reinitiation: RISP is a novel putative substrate of S6K1 in *Arabidopsis*, and RISP phosphorylation at S267 is mandatory for its function in reinitiation.

Both TAV central domains essential for reinitiation, MAV and MBD, act largely via interaction with the cell translation machinery: MBD functions in transactivation via eIF3 subunit g and the 60S ribosomal protein L24 (Park *et al*, 2001), while MAV, in addition to TOR binding (via dsR), interacts with RISP (via the C-terminal MAV domain adjacent to dsR; Thiébeauld *et al*, 2009). The dsR domain may also contact two other 60S ribosomal proteins, L13 and L18 (Leh *et al*, 2000; Bureau *et al*, 2004). TOR binds TAV via its HEAT repeat domain, which is responsible for Raptor binding (Hara *et al*, 2002), but whether TAV and Raptor cooperate or compete for TOR binding remains to be determined. Although the potential contribution of Raptor and LST8 to TAV-mediated activation of TOR is not known, recent data suggest that raptor is dispensable for mTORC1 to phosphorylate S6K1 in a rapamycin-sensitive manner *in vitro* (Yip *et al*, 2010). Binding of TAV to TOR seems to be highly specific, since a deletion of three amino acids within the dsR domain abolished both binding to TOR and CaMV amplification in plant protoplasts. How TAV contributes to TOR activation is unclear. Presumably, TAV relies on direct interaction with TOR and possibly also on contacts with other TAV- or TOR-interacting partners. TOR activation and phosphorylation of S6K1 at T449 and RISP at S267 upon TAV overexpression are abolished by removal of the TOR-binding site within MAV (Figure 6C), or by the TOR-specific inhibitor Torin-1 (Supplementary Figure S2A).

What could be the function of TOR in reinitiation of translation? Based on biochemical data (Kozak, 2001; Unbehaun *et al*, 2004), removal of eIFs (except that of eIF2-GDP, which occurs after the eIF5-dependent GTP hydrolysis) seems likely to occur upon 60S binding to the 48S PIC, or a few elongation cycles later for some eIFs including eIF3 (Pöyry *et al*, 2004). The affinity of TAV for eIF3 and RISP in addition to its capacity to interact with the 60S ribosomal subunit can explain the unusual accumulation of both proteins in polysomes prepared from CaMV-infected plants (Park *et al*, 2001; Thiébeauld *et al*, 2009) or TAV-transgenic plants (this study). According to our data, TAV functions in recruitment of eIF3 and RISP independently of TOR, and TAV lacking dsR is still active.

Our earlier results suggested that TAV can enter the host translation machinery upon removal of 40S- and eIF3-bound eIF4B (Cheng and Gallie, 2006), which is able to out-compete TAV for eIF3g binding somewhere during the 60S-joining step of translation initiation (Park *et al*, 2004). Our previous observations that RISP immunoprecipitates with eIF3 and eIF2 in *Arabidopsis* plants and, together with eIF3, binds 40S *in vitro* led us to propose that RISP may enter the cell translation machinery together with eIF3 at the stage of 43S PIC formation (Thiébeauld *et al*, 2009). Thus, phosphorylation of RISP may occur within 40S initiation complexes with or without TOR activation by TAV (Figure 6G). Although, in mammals, eIF3-bound inactive S6K1 dissociates when phosphorylated by TOR (Holz *et al*, 2005), RISP phosphorylation results in reduced affinity to eIF3 but higher binding capacity to TAV (Figure 6E), which indicates that TAV preferentially binds phosphorylated RISP to stabilize it on polysomes after 60S-joining (see model in Figure 7). Accordingly, RISP stimulates TAV-mediated reinitiation only in its phosphorylated state (Figure 6F).

Several lines of evidence suggest that the essential function of TOR in TAV-mediated reinitiation would be to keep RISP in its phosphorylated state in polysomal complexes. Indeed, upon overexpression of dsR deleted TAV, when TOR is not present in polysomes, RISP was found in polysomes in a dephosphorylation state, despite the presence of phosphorylated RISP in fractions of 40S ribosomal subunits (Figure 6G). Given that the presence of phosphorylated RISP correlates with the presence of TOR in polysomal fractions, our data support the contention that the phosphorylated state of RISP in polysomes is maintained only upon TOR binding to polysomes. Although a protein phosphatase 2A can form a complex with $p70^{S6K}$ and counteracts its phosphorylation (Westphal *et al*, 1999), the presence of active TOR in polysomes will trigger S6K1 phosphorylation. Thus far, we are unable to say whether activated TOR has the capacity to interact with polysomes, or whether TOR association with polysomes is mediated by TAV. Whatever the mechanism of TOR recruitment, the role of TOR/S6K1 in TAV-mediated transactivation most likely involves phosphorylation of RISP in polysomes (Figure 7D).

Based on structural data (Srivastava *et al*, 1992; Spahn *et al*, 2001), it appears that eIF3 binds primarily to the solvent-exposed side of the 40S subunit during translation initiation, and that eIF3 may stay associated with the 40S of elongating ribosomes for a few cycles as previously proposed (Kozak, 2001; Pöyry *et al*, 2004). If translation proceeds for long enough (e.g. at ORFVII, Figure 7), TAV-bound eIF3 and RISP may disengage from 40S and translocate to the 60S subunit of the translating ribosome via TAV binding to L18 (Park *et al*, 2001), where the complex will not interfere with 40S dynamics and substrate binding during elongation of translation. This hypothesis is consistent with earlier observation showing that TAV strongly binds 60S, but not 40S (Park *et al*, 2001). In support to our hypothesis of eIF3/RISP/TAV complex shuttling between ribosomal subunits (Figure 7), eIF3 and RISP remain partially bound to 60S after dissociation of 80S, as shown by the sucrose gradient analysis of EDTA-treated polysomes prepared from TAV-transgenic plants (Figure 5H). Notably, recycling and phosphorylation of eIF2 on 60S subunits of polysomes have been observed (Ramaiah *et al*, 1992).

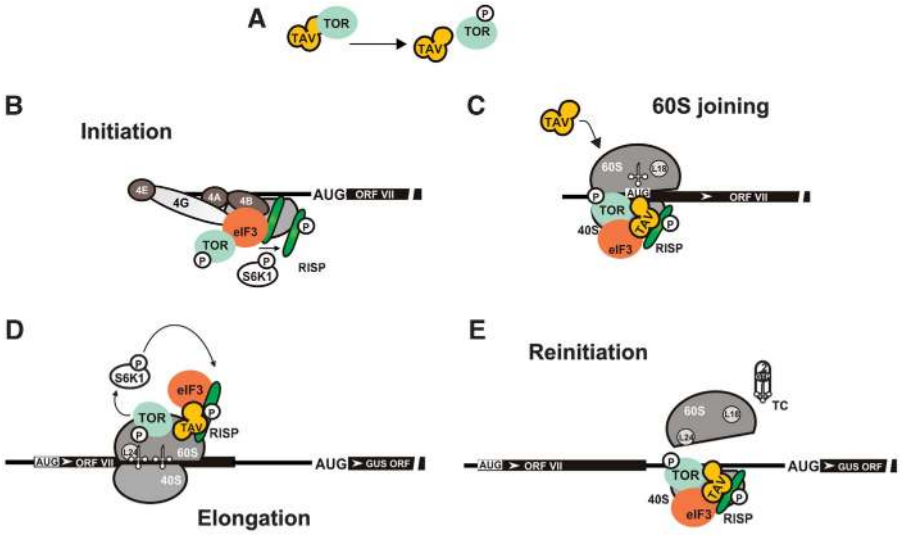

Figure 7 Provisional model of TAV/TOR function in reinitiation after translation of a long ORF. (**A**) TAV maintains TOR in a constitutively activated state. (**B**) Activated TOR binds eIF3-PIC to trigger S6K1 and RISP phosphorylation. (**C**) TAV joins 40S-associated eIF3 during the 60S-joining step via interactions with eIF3 and RISP-P. (**D**) TAV retains eIF3/RISP on the elongating ribosome, likely by transferring to the rear side of 60S. Activated TOR with or without TAV binds polysomes and maintains the phosphorylation state of RISP. (**E**) TAV/eIF3/RISP-P is relocated back to 40S via eIF3/40S interaction to form a reinitiation-competent 40S capable of resuming scanning and 60S/TC recruitment.

Together, our results suggest a model of how TOR signalling may contribute to TAV-mediated transactivation (Figure 7). Upon overexpression, TAV binds to and activates TOR (Figure 7A). As in the mammalian scenario, TOR may phosphorylate S6K1 using eIF3-containing PIC as a platform for phosphorylation. Upon S6K1 activation, RISP is phosphorylated within eIF3-PIC (Figure 7B). TAV joins 48S-PIC at the 60S-joining step via binding eIF3 and RISP-P. TAV binding prevents eIF3 and RISP-P dissociation by stabilizing these proteins on 80S (Figure 7C). During elongation, the TAV-containing complex can be relocated to the 60S subunit via interaction of TAV with L18 located on the rear side of 60S, where the complex will not interfere with translation elongation. The phosphorylation (or rephosphorylation) state of RISP in polysomes is maintained upon binding of activated TOR (or TOR-P/TAV) to polysomes, which in turn leads to activation of S6K1 (Figure 7D). During termination of the first ORF, the reinitiation-competent eIF3/TAV/RISP-P complex, with or without TOR, is relocated back to the 40S subunit via eIF3 affinity to 40S and begins to scan to the second GUS ORF, recruiting the TC and 60S on the way (Figure 7E).

This report identifies TOR as an essential host factor critical for CaMV infection. TOR-deficient plants are immune to CaMV due to a serious defect in transactivation of translation of the 35S pregenomic RNA, and may fail to support other steps of viral replication that rely on TOR as a major effector from the host cell. In mammals, another special case of reinitiation after long ORF translation, termed 'termination-reinitiation', has been shown to rely on recruitment of eIF3/40S complexes by an RNA *cis*-acting element located upstream of the first ORF stop codon on the bicistronic subgenomic RNA of feline calicivirus, in part mimicking TAV function (Meyers, 2007; Pöyry *et al*, 2007).

Control of mRNA translation pathways has a fundamental role in many aspects of gene expression, cell growth and proliferation. A growing number of examples speak in favour of a widespread influence of TOR signalling on different steps of protein synthesis. Our data suggest that unregulated TOR signalling activation may not only perturb the capacity of ribosomes to upregulate initiation of translation but also under certain conditions, activate normally restricted reinitiation events.

Materials and methods

Expression constructs, antibodies

Detailed description of plasmid construction, oligos and antibodies is found in the Supplementary data.

Pull-down experiments

Glutathione-*S*-transferase pull-down assays were carried out as described in Park *et al* (2001). Coimmunoprecipitation experiments were performed as described (Thiébeauld *et al*, 2009; Supplementary data).

Yeast two-hybrid assay

GAL4-based yeast two-hybrid protein interaction assays were performed according to Thiébeauld *et al* (2009).

Arabidopsis *protoplasts*

Arabidopsis protoplasts were prepared from either suspension culture or from 3- to 4-week-old plantlets (mesophyll protoplasts) transfected with plasmid DNA by PEG method as described in Supplementary data.

In vitro kinase assay

The *in vitro* kinase assay was carried out in buffer (25 mM HEPES pH 7.6, 10 mM $MgCl_2$, 1 mM ATP), with small aliquots of extracts prepared from *Arabidopsis* Col0, AT7, AT7ΔdsR plants, or rice homogenized suspension culture supernatants with or without 0.1 mM. Rapamycin in the presence of 10 mg of recombinant *Arabidopsis* S6K1 at 30°C. The aliquots taken as indicated were analysed by immunoblot with corresponding antibodies.

Polyribosome isolation

Polyribosomes were isolated from 7-day-old *Arabidopsis* samples and analysed by density sucrose centrifugation (Supplementary data).

Real-time and semiquantitative PCR analyses

Quantitative PCR analysis was performed according to the SYBR Green qPCR kit protocol (Supplementary data).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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