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Additional Information

Ribosomal protein L5 and transcription factor IIIA from *Arabidopsis thaliana* bind *in vitro* specifically *Potato spindle tuber viroid* RNA

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Abstract *Potato spindle tuber viroid* (PSTVd) contains an element of tertiary structure −loop E− also present in eukaryotic 5S rRNA. The ribosomal protein L5 and transcription factor IIIA (TFIIIA) from *Arabidopsis thaliana* bind 5S rRNA *in vitro* and *in vivo*, mediating different functions that include nucleocytoplasmic transport and transcription activation, respectively. We show that *A. thaliana* L5 and TFIIIA also bind PSTVd (+) RNA *in vitro* with the same affinity as they bind 5S rRNA, whereas the affinity for a chloroplastic viroid is significantly lower. These two proteins might participate in the synthesis and delivery of PSTVd RNA *in vivo*.

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Viroids are subviral plant pathogens with a genome composed by a small circular RNA not encoding any protein [1, 2]. Over 30 different viroid species that infect plants are classified in two families. Four viroid species whose RNAs self-cleave through hammerhead ribozymes during their replication in the chloroplast belong to the family *Avsunviroidae* [1]. The remaining species belong to the family *Pospiviroidae* and, like *Potato spindle tuber viroid* (PSTVd), contain in their most abundant (+) strand a central conserved region (CCR) and replicate in the nucleus through an asymmetric rolling-circle mechanism [3]. PSTVd (+) strands have been found in the nucleolus and the nucleoplasm, but (–) strands only in the nucleoplasm, suggesting that transcription of both polarity strands takes place in this latter compartment and the (+) strands are then transferred and processed in the nucleolus [4].

Viroid RNAs contain domains and motifs that, most probably through interaction with host proteins, mediate the different steps of the viroid infectious cycle. One of these motifs is loop E (Fig. 1), an element of local tertiary structure found within the CCR of PSTVd and related species forming genus *Pospiviroid*. The loop E motif, also present in some other RNAs like the eukaryotic 5S rRNA, was detected in PSTVd (+) RNA by UV cross-linking experiments, first *in vitro* [5] and later *in vivo* in infected tomato [6, 7]. The ribosomal protein L5 and transcription factor IIIA (TFIIIA) bind specifically the eukaryotic 5S rRNA *in vitro* and *in vivo*, serving different functions related to the biology of this RNA. L5 and TFIIIA binding sites on 5S rRNA cover loop E but also include other regions of the molecule: helices I, II, III and V, and loops A, B and C for L5, and helices I, II, IV and V, and loops A, B and D for TFIIIA [8, 9]. Here we show that these two proteins from *Arabidopsis thaliana* also bind *in vitro* PSTVd (+) RNA with affinities similar to those displayed for the physiological ligand 5S rRNA, while their affinities for the (+) RNA of the unrelated *Avocado sunblotch viroid* (ASBVd, family *Avsunviroida*e) are significantly lower.

To study the binding affinity *in vitro* of PSTVd (+) RNA by proteins L5 and TFIIIA from *A. thaliana*, recombinant versions of these two proteins (Genbank accession nos. AAM10263 and AAO73339, respectively) including a carboxy-terminal LEH_6 tag were expressed in *Escherichia coli* BL21(DE3)pLysS (Novagen) and purified in non-denaturing conditions using a nickel-nitrilotriacetic acid (Qiagen) resin [10]. Chromatography resulted in highly purified preparations of both proteins as revealed by SDS-PAGE in 12.5% gels stained with Coomassie blue (Fig. 2A). A western blot analysis of a parallel gel using an anti-His_6 antibody (Clontech) confirmed that the prominent bands in the purified preparations indeed corresponded to both full-length recombinant proteins (Fig. 2B).

Binding *in vitro* of PSTVd (+) RNA by L5 and TFIIIA proteins from *A. thaliana* was examined by electrophoretic mobility shift and UV cross-linking label transfer assays, using as a probe a full-length linear ^{32}P-labeled PSTVd (+) RNA of 359 nt obtained by transcription in the presence of 5 μCi/μl of [α-^{32}P]UTP (400 Ci/mmol) and quantified with a scintillation counter. This RNA (Genbank accession no. M16826, positions 95 to 94) contained vector tails at the 5' (GGGAU) and 3' (AUCU) ends. In the electrophoretic mobility shift assay, the ^{32}P-labeled RNA probe was mixed with the proteins at different ratios in 10 μl of binding buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol), incubated on ice for 15 min and separated by non-denaturing PAGE in 5% gels in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2) at 4 ºC. The gels were fixed in 10% acetic acid and 20% ethanol, vacuum dried and exposed for autoradiography or phosphorimage analysis. L5 started retarding the probe at a protein concentration of 0.5 μM and completely retarded it at 2.5 μM (Fig. 2C, lanes 4 and 5), whereas TFIIIA retarded the probe completely at 0.1 μM (Fig. 2C, lanes 8 to 10), indicating a higher affinity of this second protein for PSTVd (+) RNA. No probe retardation was observed at lower protein concentrations or in a control without protein (Fig. 2C). The presence of bands corresponding

to the free probe and the fully retarded complex, but not to partially retarded complexes, suggested a cooperative binding, particularly for TFIIIA.

These results were confirmed by the UV cross-linking label transfer assay. For this purpose, the ^{32}P-labeled RNA probe was mixed with the proteins at different ratios in 10 µl of binding buffer, spotted on a piece of Parafilm layered on ice, and irradiated with 0.5 J/cm^2 of UV light using a UV cross-linker (Hoefer). After adding 1 µg of RNase A and incubating at 37 ºC for 30 min, reactions were stopped and analyzed by SDS-PAGE in 12.5% gels. PSTVd (+) RNA-protein binding was observed starting at 0.5 µM for L5 (Fig. 2D, lanes 4 and 5) and at 0.1 µM for TFIIIA (Fig. 2D, lanes 8 to 10). No label transfer was observed at lower protein concentration or in a control without protein (Fig. 2D). In this assay, prominent bands arising from label transfer were only obtained for full-length L5 and TFIIIA (Fig. 2D, lanes 5 and 10), indicating that no *E. coli* contaminant proteins or L5 and TFIIIA fragments bound appreciably the probe.

To further dissect the binding affinity of *A. thaliana* L5 and TFIIIA proteins for PSTVd (+) RNA, a series of competition experiments were performed by the electrophoretic mobility shift assay using as a probe *A. thaliana* 5S rRNA. The mixtures included the full-length *A. thaliana* 5S rRNA (120 nt, Genebank accession no. M65137, position 220 to 339) flanked by two short vector tails (identical to those described above flanking the PSTVd RNA), and proteins L5 and TFIIIA at 1.25 µM and 0.25 µM respectively. These protein concentrations resulted in the complete retardation of the RNA probe in the absence of competitors (Fig. 3A and B, lanes 10). Incorporation of the unlabeled competitors (quantified spectrophotometrically and premixed with the probe before adding the protein in a final volume of 20 µl) *A. thaliana* 5S rRNA (identical to the probe), exactly monomeric-length linear PSTVd (+) RNA (359 nt, positions 88 to 87), and exactly monomeric-length linear ASBVd (+) RNA (247 nt, Genebank accession no. X52041, positions 56 to 55), showed a similar behavior for PSTVd and 5S rRNAs (Fig. 3C and D): both RNAs were good competitors, with a minor fraction of the probe remaining bound by L5 (Fig. 3A, lanes 3 and 6) and TFIIIA (Fig. 3B, lanes 3 and 6) at a 500 molar excess. However, the ASBVd (+) RNA, with a rod-like secondary structure similar to that of PSTVd (+) RNA, was a poorer competitor (Fig. 3C and D) because at the same molar excess significant amounts of the probe were still bound by L5 (Fig. 3A, lane 9) and, particularly, by TFIIIA (Fig. 3B, lane 9). The molar ratio competitor/probe inhibiting probe binding by 50% (I_{50}) was calculated. For L5, whereas the I_{50} for 5S rRNA and PSTVd (+) RNA were comparable (185±15 and 131±41, respectively), the I_{50} for ASBVd (+) RNA was larger (538±248). Similarly, for TFIIIA, the I_{50} for 5S rRNA and PSTVd (+) RNA were again comparable (223±2 and 250±25, respectively) and the I_{50} for ASBVd (+) RNA larger (1148±262).

Finally, to test the relevance of the loop E motif in PSTVd (+) RNA binding by *A. thaliana* L5 and TFIIIA proteins, mutant versions of this RNA and of the *A. thaliana* 5S RNA with extensive deletions in their respective loop E motifs (Fig. 1) were assayed as competitors in the electrophoretic mobility shift assays. The two mutant RNAs competed with the probe similarly to the wild-type RNAs, suggesting a complex interaction of both RNAs with the proteins not relying exclusively on binding to the loop E motif.

Due to their lack of protein-coding capacity, viroids RNAs depend for replication and movement on host factors. Experiments with inhibitors and with a monoclonal antibody against a conserved domain of the major subunit of the DNA-dependent RNA polymerase II indicate that elongation of both strands of PSTVd, and most likely of the other nuclear viroids (family *Pospiviroidae*), is catalyzed by this enzyme redirected to transcribe RNA templates [11, 12]. A viroid RNA-binding protein (Virp1) from tomato with a nuclear localization signal was shown to be required for viroid infection [13] and has been implicated in viroid transfer to the nucleus [14]. Also in the context of the family *Pospiviroidae*, the phloem

protein 2 from cucumber interacts *in vitro* and *in vivo* with *Hop stunt viroid* RNA, possibly assisting its long-distance movement [15]. Within the family *Avsunviroidae,* a UV cross-linking screening for host factors interacting *in vivo* with ASBVd RNA retrieved two closely-related chloroplast RNA-binding proteins from avocado (PARBP33 and PARBP35), the first of which behaves as an RNA chaperone and facilitates *in vitro* the hammerhead-mediated self-cleavage of dimeric ASBVd RNA [10]. On the other hand, northwestern hybridization analyses have identified six peach proteins interacting *in vitro* with *Peach latent mosaic viroid* that include the elongation factor 1-alpha [16]. However, the complex infectious cycle of viroids predicts the involvement of more host proteins that remain to be identified.

In a search for additional host proteins interacting with PSTVd (+) RNA, we have focused on loop E because this motif, conserved in PSTVd and members of its genus [1], has been involved in replication [3, 17], host specificity [18] and pathogenesis [19]. Using two independent *in vitro* approaches we have found that two proteins from *A. thaliana*, ribosomal L5 and TFIIIA, which bind *in vivo* the eukaryotic 5S rRNA also bind PSTVd (+) RNA with the same affinity, while the affinity for ASBVd (+) RNA, a chloroplastic viroid, was significantly lower. Despite *A. thaliana* being a non-host for PSTVd, this model plant has the enzymatic machinery for replicating representative viroid species of the family *Pospiviroidae*, being therefore, an appropriate system for the study of viroid-host interactions [3, 20].

L5 and TFIIIA might serve different functions in the PSTVd infectious cycle, as previously proposed [21]. Because *A. thaliana* L5 binds 5S rRNA and accumulates in the nucleolus, although it is also present in the cytoplasm [22], by binding to PSTVd (+) strands this protein could mediate their selective trafficking into the nucleolus [4]. Moreover, L5 is also involved in the nucleocytoplasmic transport of 5S rRNA [23], and could likewise facilitate nuclear export of newly synthesized PSTVd (+) RNA. On the other hand, TFIIIA is a transcription factor of RNA polymerase III specifically required for transcribing 5S rRNA genes that is also involved in a network of interactions coupling 5S rRNA synthesis to accumulation of ribosomal proteins [24]. TFIIIA also binds 5S rRNA, as well as the DNA gene coding for it, and concentrates at several nuclear foci including the nucleolus in *A. thaliana* [22]. This dual binding capacity makes TFIIIA a candidate for being involved in PSTVd replication, because viroid RNAs most likely require proteins with these properties to be accepted as templates by RNA polymerases that, under normal conditions, transcribe DNA. TFIIIA could thus act as a bridge between the viroid RNA template and the RNA polymerase, even if the available evidence indicates that this polymerase is RNA polymerase II. Further studies are needed to confirm that the specific interactions between L5 and TFIIIA with PSTVd (+) RNA showed here *in vitro* exist also *in vivo*.

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References

1. Flores R, Hernández C, Martínez de Alba AE, Daròs JA, Di Serio F (2005) Viroids and viroid-host interactions. Annu Rev Phytopathol 43: 117-139
2. Ding B (2009) The biology of viroid-host interactions. Annu Rev Phytopathol 47: 105-131

3. Gas ME, Hernández C, Flores R, Daròs JA (2007) Processing of nuclear viroids in vivo: an interplay between RNA conformations. PLoS Pathog 3: 1813-1826
4. Qi Y, Ding B (2003) Differential subnuclear localization of RNA strands of opposite polarity derived from an autonomously replicating viroid. Plant Cell 15: 2566-2577
5. Branch AD, Benenfeld BJ, Robertson HD (1985) Ultraviolet light-induced crosslinking reveals a unique region of local tertiary structure in potato spindle tuber viroid and HeLa 5S RNA. Proc Natl Acad Sci USA 82: 6590-6594
6. Wang Y, Zhong X, Itaya A, Ding B (2007) Evidence for the existence of the loop E motif of *Potato spindle tuber viroid* in vivo. J Virol 81: 2074-2077
7. Eiras M, Kitajima EW, Flores R, Daròs JA (2007) Existence in vivo of the loop E motif in potato spindle tuber viroid RNA. Arch Virol 152: 1389-1393
8. DiNitto JP, Huber PW (2003) Mutual induced fit binding of Xenopus ribosomal protein L5 to 5S rRNA. J Mol Biol 330: 979-992
9. Theunissen O, Rudt F, Guddat U, Mentzel H, Pieler T (1992) RNA and DNA binding zinc fingers in Xenopus TFIIIA. Cell 71: 679-690
10. Daròs JA, Flores R (2002) A chloroplast protein binds a viroid RNA *in vivo* and facilitates its hammerhead-mediated self-cleavage. EMBO J 21: 749-759
11. Mühlbach HP, Sänger HL (1979) Viroid replication is inhibited by α-amanitin. Nature 278: 185-188
12. Warrilow D, Symons RH (1999) Citrus exocortis viroid RNA is associated with the largest subunit of RNA polymerase II in tomato *in vivo*. Arch Virol 144: 2367-2375
13. Kalantidis K, Denti MA, Tzortzakaki S, Marinou E, Tabler M, Tsagris M (2007) Virp1 is a host protein with a major role in *Potato spindle tuber viroid* infection in *Nicotiana* plants. J Virol 81: 12872-12880
14. Martínez de Alba AE, Sägesser R, Tabler M, Tsagris M (2003) A bromodomain-containing protein from tomato specifically binds potato spindle tuber viroid RNA in vitro and in vivo. J Virol 77: 9685-9694
15. Gómez G, Pallás V (2004) A long-distance translocatable phloem protein from cucumber forms a ribonucleoprotein complex in vivo with *Hop stunt viroid* RNA. J Virol 78: 10104-10110
16. Dubé A, Bisaillon M, Perreault JP (2009) Identification of proteins from *Prunus persica* that interact with peach latent mosaic viroid. J Virol 83: 12057-12067
17. Baumstark T, Schröder ARW, Riesner D (1997) Viroid processing: switch from cleavage to ligation is driven by a change from a tetraloop to a loop E conformation. EMBO J 16: 599-610
18. Wassenegger M, Spieker RL, Thalmeir S, Gast FU, Riedel L, Sänger HL (1996) A single nucleotide substitution converts potato spindle tuber viroid (PSTVd) from a noninfectious to an infectious RNA for *Nicotiana tabacum*. Virology 226: 191-197
19. Qi YJ, Ding B (2003) Inhibition of cell growth and shoot development by a specific nucleotide sequence in a noncoding viroid RNA. Plant Cell 15: 1360-1374
20. Daròs JA, Flores R (2004) *Arabidopsis thaliana* has the enzymatic machinery for replicating representative viroid species of the family *Pospiviroidae*. Proc Natl Acad Sci USA 101: 6792-6797
21. Flores R, Di Serio F, Hernández C (1997) Viroids: the noncoding genomes. Seminars in Virology 8: 65-73
22. Mathieu O, Yukawa Y, Prieto JL, Vaillant I, Sugiura M, Tourmente S (2003) Identification and characterization of transcription factor IIIA and ribosomal protein L5 from *Arabidopsis thaliana*. Nucleic Acids Res 31: 2424-2433
23. Guddat U, Bakken AH, Pieler T (1990) Protein-mediated nuclear export of RNA: 5S rRNA containing small RNPs in xenopus oocytes. Cell 60: 619-628

24. Pittman RH, Andrews MT, Setzer DR (1999) A feedback loop coupling 5 S rRNA synthesis to accumulation of a ribosomal protein. J Biol Chem 274: 33198-33201

Figures

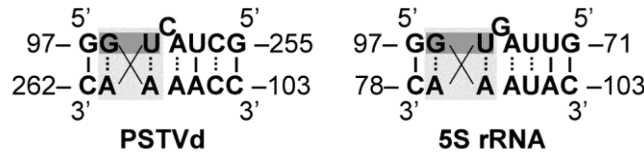

Fig. 1 Scheme of loop E in PSTVd (+) RNA and *A. thaliana* 5S rRNA. UV cross-linked residues are on a dark gray background and canonical and non-canonical base pairs are indicated with continuous and broken lines, respectively. Crossed lines depict the continuity of the phosphodiester backbone. Residues of loop E deleted in the mutant versions of PSTVd (+) RNA and 5S rRNA from *A. thaliana* are boxed (light gray striped background).

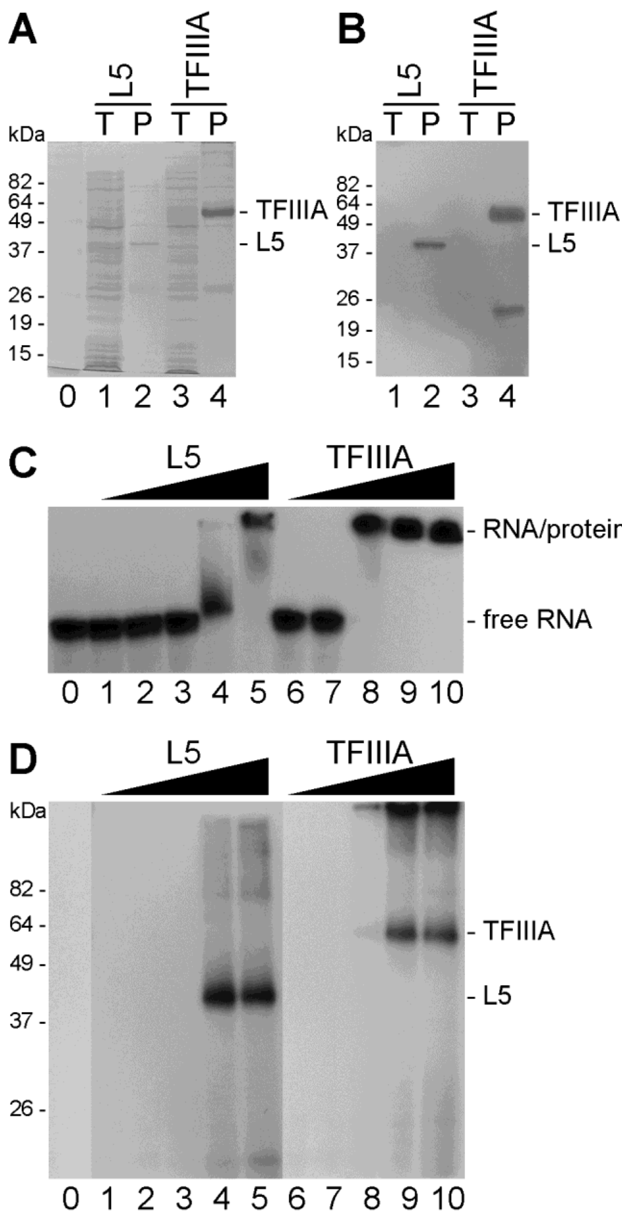

Fig. 2 Purification of recombinant L5 and TFIIIA proteins from *A. thaliana* and binding *in vitro* to PSTVd (+) RNA. (A and B) Proteins were separated by SDS-PAGE followed by (A) blue Coomassie staining or (B) western blot analysis with an anti-His_6 antibody. Lanes 0, protein markers with their molecular masses indicated at the left. Lanes 1 and 3, total proteins (T) from *E. coli* cultures induced to express L5 and TFIIIA, respectively. Lanes 2 and 4, purified preparations (P) of L5 and TFIIIA, respectively. Positions of recombinant proteins L5 and TFIIIA are indicated at the right of panels A and B. (C) Electrophoretic mobility shift assay. The labeled PSTVd (+) RNA probe (0.2 nM) was mixed with increasing amounts of L5 or TFIIIA and separated by non-denaturing PAGE. (D) UV cross-linking label transfer assay. The same PSTVd (+) RNA probe was mixed with increasing amounts of L5 and TFIIIA, and then subjected to UV cross-linking, RNase digestion and separation by SDS-PAGE. Lanes 0, no protein. Lanes 1 to 5 and 6 to 10, addition of 0.004, 0.02, 0.1, 0.5 and 2.5 µM of L5 and TFIIIA, respectively. Positions of the RNA-protein complex and of the free RNA probe are indicated at the right of panel C. Positions and molecular masses (in kDa) of the markers, and of the labeled L5 and TFIIIA, are indicated at the left and right of panel D, respectively.

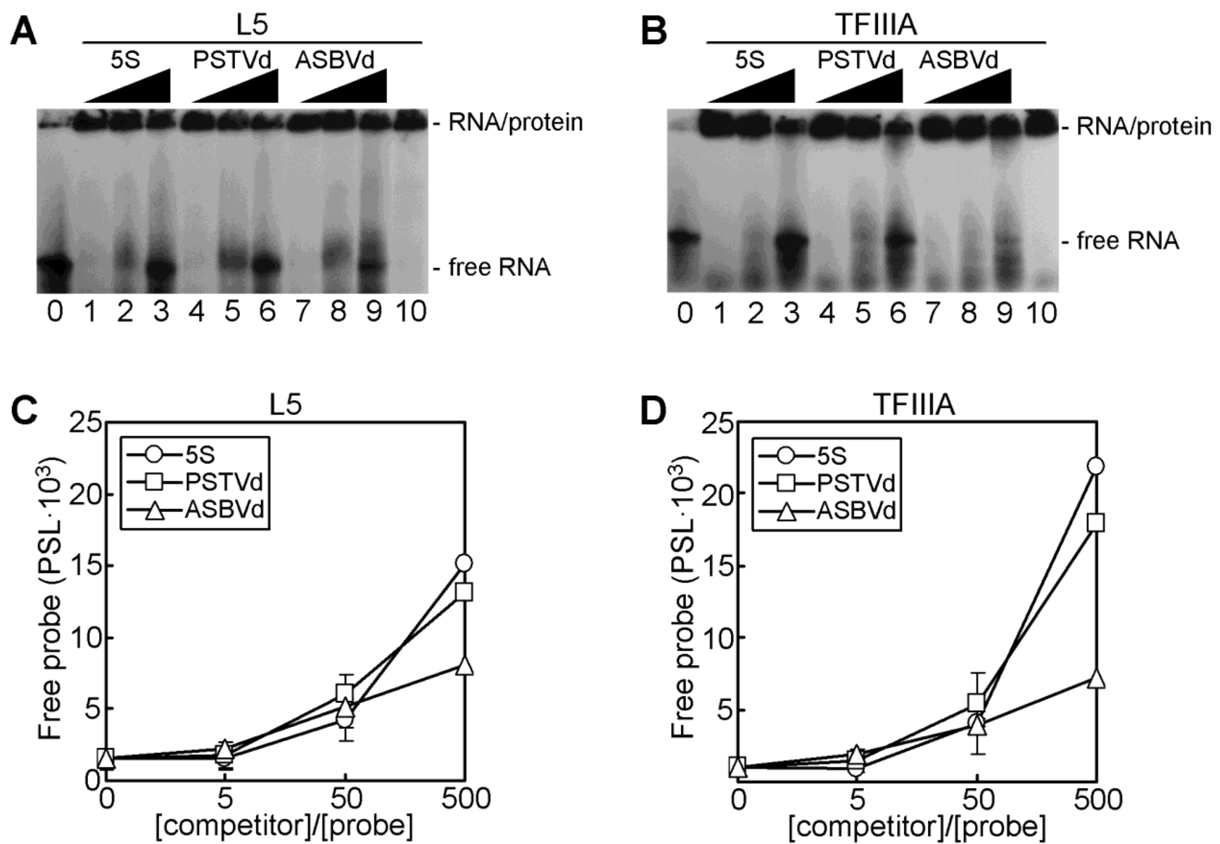

Fig. 3. Effects of three competitors on the binding *in vitro* of 5S rRNA by recombinant proteins L5 and TFIIIA from *A. thaliana* determined by electrophoretic mobility shift assays. (A and B) Representative autoradiograms of the binding of L5 (1.25 µM) and TFIIIA (0.25 µM) to a 5S rRNA radiolabeled probe (0.1 nM), respectively. Lanes 0, no protein. Lanes 1 to 9, assays in the presence of 5, 50 and 500 molar excesses of unlabeled 5S rRNA from *A. thaliana* (1 to 3), PSTVd (+) RNA (4 to 6) and ASBVd (+) RNA (7 to 9). Lanes 10, no competitor. (C and D) Graphical representation of free probe (as photostimulated luminescent −PSL− units) versus competitor/probe molar ratio for L5 and TFIIIA, respectively. Points represent the average of three independent experiments with the standard deviation given as errors bars.