Supporting Information

Target of rapamycin and ribosomal protein S6 transmit light signals to enhance protein translation in de-etiolating Arabidopsis seedlings

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Running title:
TOR and RPS6 in light-enhanced translation

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SI material and method

Materials and plant growth conditions

Arabidopsis seeds are surface sterilized by 10% bleach for 10 min, and then rinsed with distilled water for 4 times. The sterilized seeds were germinated on half-strength Murashige and Skoog (0.5x MS) medium (pH 5.7) with 0.8% (w/v) phytoagar and stratified in the dark at 4 °C for 1d followed by light treatment for 3 h and another 2 d in the dark at 4 °C. Mutants and their corresponding wild types used in this study include *phyA211* and *cop1-6* in Columbia-0 (Col-0) and *hy5 hyh* mutant in Wassiljevskaja (WS) background. The estradiol-inducible *tor* mutant was described previously (1). *rps6a* (SALK_048825) and *rps6b* (SALK_012147) were obtained from Arabidopsis Biological Resource Center (ABRC). *rps6a* mutant was confirmed by genotyping with primers rps6a-RP (5'-AAATGGAATCTGTACCTCGCC-3') and LBb1.3 whereas the wild-type *RPS6A* genomic fragment was amplified by rps6a-LP (5'-GTATTCATTCACCATCACCCG-3') and rps6a-RP. For genotyping *rps6b* mutant, we used primers rps6b-RP (5'-TGAATCCAAACCTTTGAATCG-3') and LBb1.3. *RPS6B* genomic fragment was amplified by rps6b-LP (5'-CTGCATCAGAGTTAGCCTTGG-3') and rps6b-RP.

For the light treatment, etiolated seedlings were illuminated with far-red light (1.2 W m^{-2} sec^{-1}), blue light (35 μmol m^{-2} sec^{-1}), red light (35 or 140 μmol m^{-2} sec^{-1}) or white light (70-80 μmol m^{-2} sec^{-1}) at 22^{o}C for the indicated times. 3-(3,4-Dichloropheneyl)-1-1-dimethylurea (DCMU), β-estradiol and glucose were purchased from Sigma. Torin2 was purchased from LC laboratories (cat no. T-8448). 5–(4–chlorophenyl)-4H-1,2,4–triazole-3–thiol (yucasin) was purchased from WAKO (cat no. 352-12001).

Isolation of total, non-polysomal and polysomal RNAs

Total RNAs were extracted from 4-d-old etiolated or light-treated seedlings by pine tree method (2). For the separation of non-polysomal and polysomal RNAs, 750 μl of frozen seedling powder were extracted by 375 μl polysome extraction buffer containing 200 mM Tris-HCl, pH8.5, 50 mM KCl, 25 mM $MgCl_2$, 50 μg/ml cycloheximide (Sigma), 100 μg/ml Heparin (Sigma), 400 U/ml RNasin (Promega), 2% polyoxyethylene 10 tridecyl ether and 1% deoxycholic acid. After incubation for

5 min on ice, the cell debris was cleared by 10,000 xg centrifugation for 5 min. Five hundred μl of supernatant was further separated by 11 ml sucrose gradient (15% to 50%) by ultracentrifugation at 210,000 xg for 3 hr 30 min at 4^{o}C. Non-polysomal (NP) and polysomal (PL) fractions were collected with the use of density gradient fractionator (ISCO). Total RNA from NP or PL fractions were extracted by acid phenol chloroform, pH4.5 (Ambion), precipitated by LiCl and dissolved in DEPC H_2O. PL% is calculated as polysome bound (polysomal) RNA relative to the sum of NP and PL RNAs as described (3).

Protein analyses

For total protein extraction, roughly 200 Arabidopsis seedlings for each treatment were grounded in the presence of liquid nitrogen to fine powder and extracted with 200 μl of 2X SDS sample buffer (100 mM Tris-Cl, pH6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol and freshly added 10% β-mecaptoethanol). Total protein was separated by 12% SDS-PAGE and transferred to PVDF membrane. For the detection of phosphorylated RPS6, total protein was separated by 12% SDS-PAGE with 35 μM Phos-tag (AAL-107, WAKO pure chemical industries, Ltd) and transferred to PVDF membrane. For immunoblot analyses, antisera specific to TOR (1), RPS6, α-tubulin (T5168, Sigma) and phospho-p70 S6 kinase (T389) (Cell Signaling, cat no. 9205) were used. RPS6-specific polyclonal antiserum was obtained by immunizing rabbits with RPS6A recombinant protein.

RPS6 dephosphorylation assay was performed by treating 15 μg of soluble protein isolated from de-etiolating Arabidopsis seedlings with extraction buffer containing 1x NEBuffer (50 mM HEPES, 100 mM NaCl, 2 mM DTT and 0.01% Brij 35), 1 mM $MnCl_2$, 0.01% Triton X-100 (1%), 1x proteinase inhibitor (EDTA-free) and 600 units of lambda phosphatase (NEB) for 45 min. The reaction was stopped by adding SDS sample buffer and boiling for 10 min before subjecting to Phos-tag PAGE and SDS-PAGE analyses.

Cotyledon opening kinetic assay

After 3 d of cold stratification, the surface sterilized seeds were grown in darkness for 4 d. These etiolated seedlings were shifted to white light (15 μmol m^{-2} sec^{-1}) and the angles between the 2 cotyledons were measured at 24, 48, 72 and 168 h

to represent the degree of cotyledon opening with Image J software (http://rsp.info.nih.gov/ij/). For yucasin treatment, seedlings were grown with or without yucasin (100 μM) in darkness for 4 d and shifted to white light (15 μmol m^{-2} sec^{-1}) to measure cotyledon angles at 48, 72 and 168 h.

Analyses of *de novo* protein synthesis

Seedlings were incubated with 100 μM L-azidohomoalaine (AHA, a methionine analog, Invitrogen) for 4-5 h before total proteins were extracted. Forty μg protein isolated with extraction buffer (1% SDS, 50 mM Tris-HCl, pH 8.0) were subjected to separation by 4-12 % NuPAGE Bis-Tris gel (Invitrogen). The *de novo* synthesized proteins were detected with the use of Click-IT™ Tetramethylrhodamine (TAMRA) Protein Analysis Detection Kit (Invitrogen). The fluorescence was detected and quantified with the use of Typhoon 9200 with excitation at 545 nm and emission at 589 nm. Fluorescence signal was first normalized by corresponding coomassie blue stained PAGE and the relative fold changes were then shown by normalizing to values of dark-grown wild type seedlings.

Generating of *cop1 XVE:TOR-RNAi* lines

XVE:TOR-RNAi line (1) were crossed to *cop1-6* mutant to generate F1 seeds. F1 seeds are screened in half-strength MS plate containing kanamycin to obtain F1 plants carrying the construct of *XVE:TOR-RNAi* and the homozygous plants were screened from F2 population by genotyping for *cop1-6* and kanamycin resistance for *XVE:TOR-RNAi*. Two primers, cop1-6-gt-sp 5' GCCGATTGGACTTCAGAGAGA 3' and cop1-6-Rv-wt 5' ACGTATAACCCCTGAATGTTAG 3') were designed for amplifying *COP1* genomic fragment. The mutation site in *cop1-6* was sequence confirmed.

Literature cited

1. Xiong Y & Sheen J (2012) Rapamycin and glucose-target of rapamycin (TOR) protein signaling in plants. *J Biol Chem* 287(4):2836-2842.
2. Chang S, Puryear J, & Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol Rep* 11(2):113-116.
3. Liu MJ, Wu SH, Chen HM, & Wu SH (2012) Widespread translational control contributes to the regulation of Arabidopsis photomorphogenesis. *Mol Syst Biol* 8(1):566.

Fig. S1

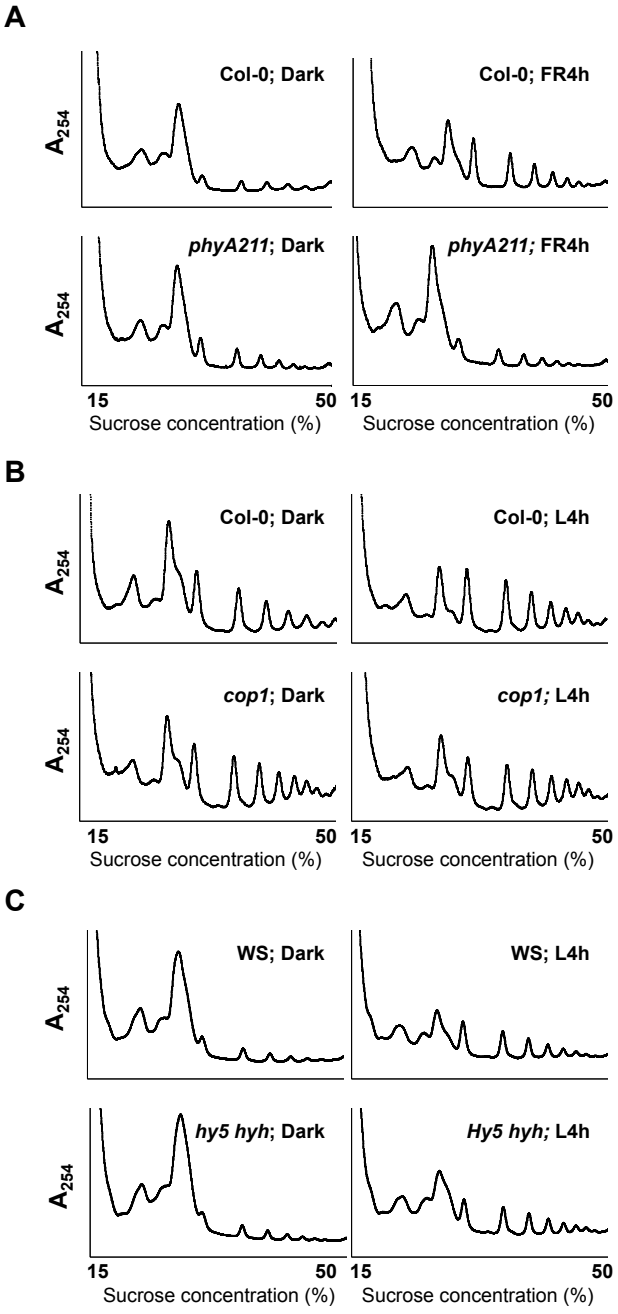

Fig. S1. Representative polysome profiles of wild type (Col-0 or WS), *phyA211* (A)*, cop1* (B) and *hy5 hyh* mutants (C). Plant materials and growth conditions were identical to those in Fig. 1.

Fig. S2

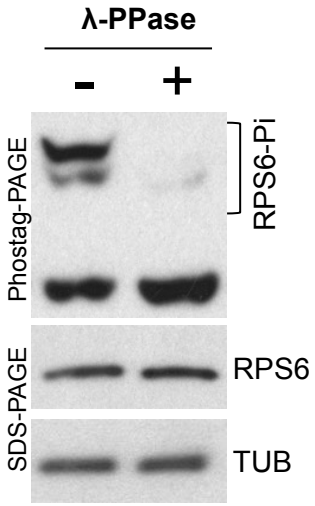

Fig. S2. RPS6 signals with mobility shifts on Phostag-PAGE represents phosphorylated RPS6.
(A) Soluble proteins isolated from L4h seedlings were treated with (+) or without (-) phosphatase and incubate at 30 °C for 45 min before SDS-PAGE and Phostag-PAGE analyses. RPS6 and TUB were detected by RPS6- and α-Tubulin-specific antiserum, respectively. TUB level was an internal control.

Fig. S3

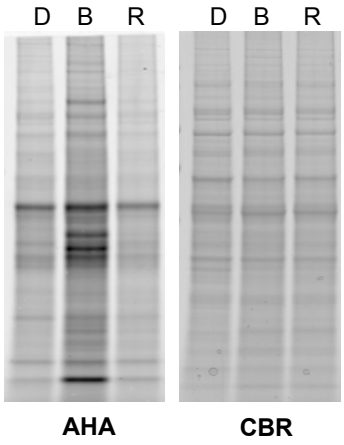

Fig. S3. Measurement of *de novo* protein synthesis of Col-0 under different light conditions. Representative images of newly synthesized proteins labeled by L-azidohomoalaine (AHA) for Col-0 under dark (D), treated with blue (B) or red (R) light. The same blot was stained with Coomassie blue (CBR) to show equal loading of proteins.

Fig. S4

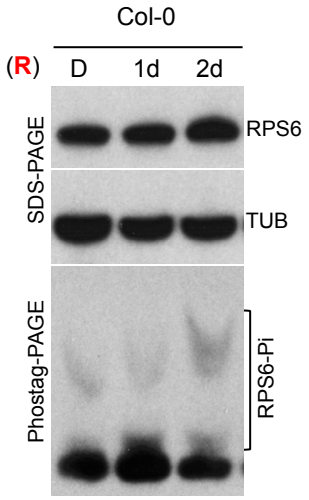

Fig. S4. RPS6 phosphorylation under high irradiance red light.
Four-d-old etiolated seedlings were treated with 140 μE red light (R) for 0 d (Dark; D) 1 d and 2 d. Total protein from each treatment was extracted and subjected to SDS-PAGE and Phostag-PAGE analyses. RPS6 and TUB were detected by RPS6- and α-Tubulin-specific antiserum, respectively. Tubulin (TUB) level was an internal control.

Fig. S5

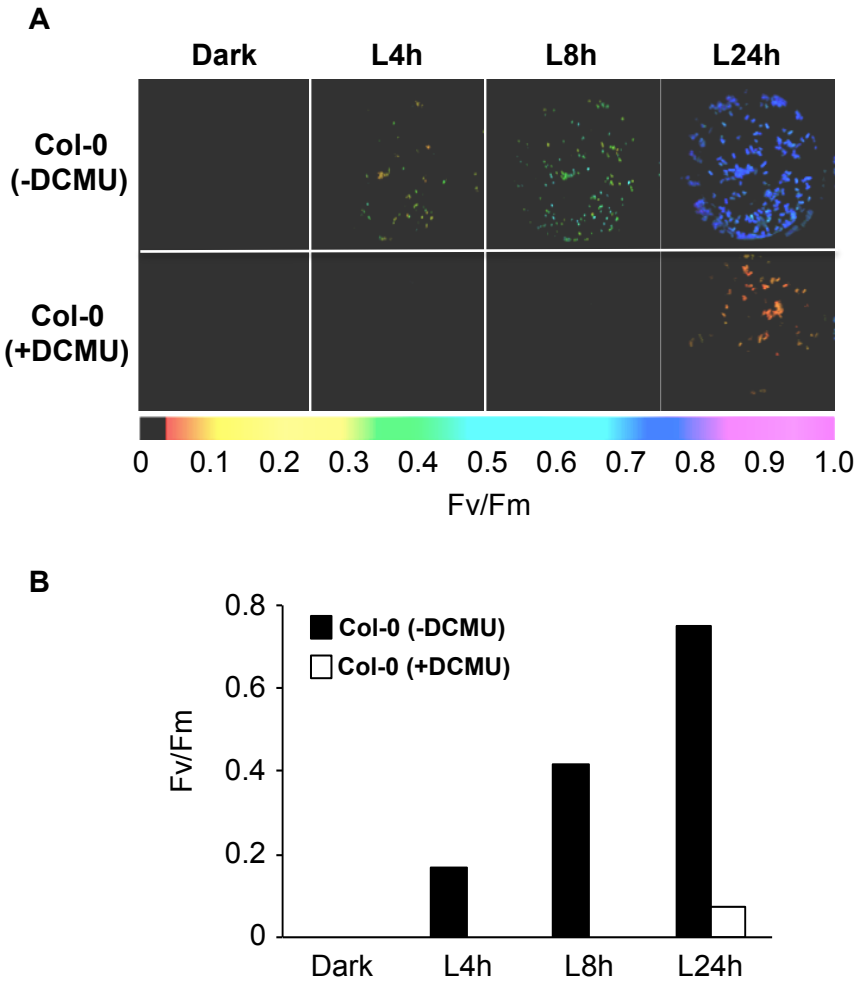

Fig. S5. DCMU treatment effectively inhibited the PSII activity.
After strafication at 4°C for 3 d, surface-sterilized seeds of Col-0 were germinated in half-strength MS medium with or without 20 μM DCMU for 4 d in darkness, then treated with white light for the indicated times (L4h, L8h or L24h). The PSII activity is represented by Fv/Fm measured by a chlorophyll fluorometer (MAXI IMAGING PAM). Representative images are shown in (A). Quantification of Fv/Fm is shown in (B).

Fig. S6

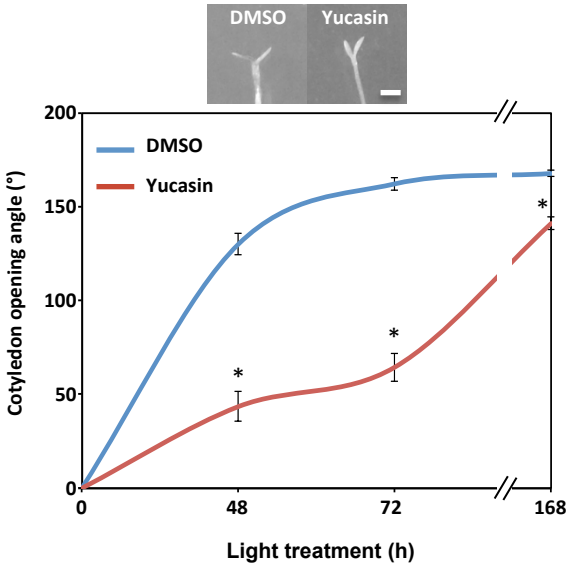

Fig. S6. Auxin is required for an optimal light-induced cotyledon opening in de-etiolating Arabidopsis seedlings.
Four-d-old etiolated Col-0 grown with or without 100 nM of yucasin was treated with 15 μE white light to observe cotyledon opening for 7 d. Cotyledon opening angles are shown as mean ± SE. *P<0.001 vs DMSO (n=18-28). Representative image shows cotyledons from seedlings treated with white light for 48 h. Scale bar: 1 mm.

Fig. S7

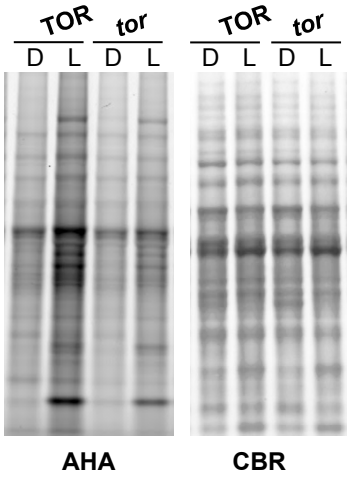

Fig. S7. TOR is required for light-enhanced *de novo* protein synthesis. Measurement of *de novo* protein synthesis in *XVE:TOR-RNAi* lines treated with (*tor*) or without (TOR) estradiol treatment in dark (D) or light (L) conditions. The same PAGE was stained with Coomassie blue (CBR) to show equal loading of proteins.

Fig. S8

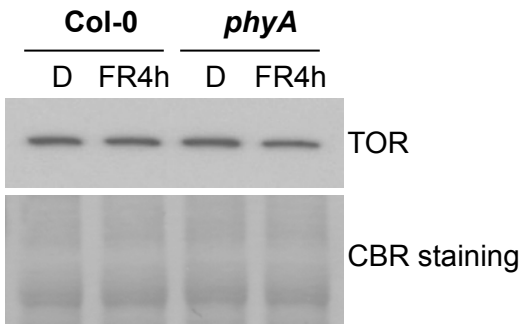

Fig. S8. TOR protein abundance is not regulated by far-red light.
Four-d-old etiolated (D) seedlings of Col-0 and *phyA* were treated with far-red light for 4 h (FR4h). Total protein was extracted for SDS-PAGE and immunoblot analysis with TOR-specific polyclonal antiserum. The same blot was stained with Coomassie blue (CBR) to show equal loading of proteins.