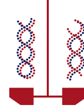

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Efficient transgene expression by alleviation of translational repression in plant cells

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Global translational repression under abiotic stress influences translation of both endogenous and transgene mRNAs. Even in plant cell culture, hypoxia and nutrient deficient stress arise during the growth process. In this study, we first demonstrated the existence of global translational repression in *Arabidopsis* T87 cultured cells over a time course following inoculation. Next, we performed genome-wide analysis, which revealed that the translational states of endogenous mRNAs differed significantly between growth and stationary phase cells. This analysis showed that translation from most mRNAs was repressed upon stationary phase. Otherwise, a part of mRNA including *alcohol dehydrogenase* (*ADH*) gene was recalcitrant to the repression. Furthermore, by polysome analysis and followed quantitative reverse transcription PCR analysis of transformants having 5′untranslated regions (UTRs) of *ADH* or translationally repressed At3g47610 mRNA fused to reporter gene, we demonstrated that polysomal associations of reporter mRNAs were in accordance with those the mRNAs from which their 5′UTR derived, suggesting that the 5′UTR is an important determinant of the translational state of mRNAs in stationary phase cells. Finally, we demonstrated the effectiveness of 5′UTR of *ADH* mRNA in transformants derived from the BY-2 tobacco cell line. These results suggested that 5′UTR of *ADH* mRNA would be a useful element for efficient transgene expression upon stationary phase.

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[**Key words:** Translation; Abiotic stress; 5′UTR; Recombinant proteins; Transgenic; Plant cell cultures; Translational repression]

Plant cell suspension cultures offer a number of advantages over traditional microbial and mammalian host systems for production of useful materials (e.g., glucocerebrosidase and interferon): intrinsic safety, cost-effective bioprocessing, and the ability to post-translationally modify exogenously expressed proteins (1,2). Many studies have attempted to achieve higher yields of expressed proteins in plant cells (1–8). Representative report described the development of an expression cassette that combined the efficient *Cauliflower mosaic virus* (CaMV) 35S promoter, a translational enhancer (4–6), and a heat shock protein gene terminator (7) to enable higher transcription, translation, and termination efficiency during transgene expression.

Translational status of mRNAs responds dramatically to abiotic stresses such as drought, elevated temperature, and high salinity (9,10). The translation state is a parameter reflecting the degree to which individual transcripts are translated into the corresponding proteins. Typically, the translation state is evaluated by comparing mRNA levels in polysomal (multiribosomal) and non-polysomal (free and/or monoribosomal) complexes in cell extracts fractionated through sucrose density gradients or by examining the correlation between total cellular and polysome-bound mRNA levels. Recent translatome analyses have shown that most mRNAs are translationally repressed under stress conditions. This phenomenon, referred to as global translational repression (11–13), is predicted to have a negative effect on transgene expression. Although synthesis of normal proteins is inhibited under stress conditions, a subset of transcripts is refractory to global translational repression (11–13). mRNA translation is primarily regulated by sequences in the 5′untranslated region (5′UTR) (9). Therefore, it is likely that the 5′UTR determines an mRNA's translation state under abiotic stresses. Indeed, we previously demonstrated that the 5′UTR plays an important role in abiotic stress–responsive translational control in stable transformed *Arabidopsis thaliana* cells; in that study, a chimeric reporter mRNA fused to a 5′UTR derived from an mRNA refractory to global translational repression was actively translated under heat stress (HS) conditions (14).

Severe culture stresses, such as hypoxia and nutrient deficiency, arise in plant cell suspension cultures, especially in stationary phase under normal culture conditions (3). It is likely that these stresses also negatively influence transgene expression at the translational level. In this study, we developed an efficient transgene expression cassette that allows transgenes to escape from global translational repression under culture stresses in plant suspension culture.

MATERIALS AND METHODS

Plant materials and conditions for transformation and culture *A. thaliana* T87 cultured cells were cultured in modified Murashige–Skoog medium and

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genetically transformed using *Agrobacterium tumefaciens* EHA105, as described previously (15). Culture of BY-2 tobacco cells and transformation were performed as described previously (16).

Measurement of fresh weight of cells A 500 μl of the suspension culture was transferred into CellTrics (20 μl: Partec, Gorlitz, Germany). The medium was removed by centrifugation at 100 ×*g* for 3 min at 25°C, and the fresh weight of cells were measured.

Polysome fractionation assays Polysomes were fractionated by sucrose density gradient centrifugation according to previously described methods (12). Approximately 0.2 ml of packed cells (about 200 mg fresh weight) were harvested by suction filtration and pulverized using a mortar and pestle. The resulting frozen powder was homogenized in 1.5 ml of buffer U (200 mM Tris–HCl [pH 8.5], 50 mM KCl, 25 mM $MgCl_2$, 2 mM EGTA, 100 μg ml^{-1} heparin, 2% polyoxyethylene 10-tridecyl ether, and 1% sodium deoxycholate) and centrifuged at 15,000 ×*g* for 10 min at 4°C. Aliquots of supernatant were layered on 4.6 ml of a 15–60% sucrose density gradient in buffer B (50 mM Tris–HCl [pH 8.5], 25 mM KCl, and 10 mM $MgCl_2$) and centrifuged at 55,000 rpm. for 50 min at 4°C in an SW55 rotor (Beckman Coulter, Brea, CA, USA). The gradient was collected from the bottom using a peristaltic pump, with simultaneous recording of the absorbance profile at 254 nm using a UV monitor.

RNA isolation from sucrose gradients For quantitative reverse transcription PCR (qRT-PCR) analysis, cell lysates were separated through a 15–60% sucrose density gradient, which was then collected in eight fractions of approximately 650 μl each. Guanidine hydrochloride at a final concentration of 5.5 M, together with 5 ng of *in vitro* synthesized, capped, and polyadenylated *Renilla luciferase* (*r-luc*) mRNA (17), was added to each collection tube. The *r-luc* mRNA was used as an external control to correct for varying efficiencies of subsequent RNA isolation and qRT-PCR. RNA was precipitated from each fraction by the addition of an equal volume of ethanol, overnight incubation at −20°C, and centrifugation at 10,000 rpm. for 45 min in a JA-20 rotor (Beckman Coulter). The resulting precipitate was washed with 80% ethanol. RNA was purified using an RNeasy kit (Qiagen Inc., Hilden, Germany) with on-column DNase I treatment. RNA was eluted with 30 μl of RNase-free water, and equal volumes of RNA solution from each fraction were subjected to qRT-PCR analysis (Fig. 1).

For microarray analysis, RNA was isolated from sucrose gradients as described above for qRT-PCR analysis, with some modifications. First, polysomal RNA (fractions 1–3 in the polysome profile shown in Fig. 2) and total RNA (fractions 1–8 in the polysome profile shown in Fig. 2) were individually collected and pooled in tubes containing guanidine hydrochloride (final concentration, 5.5 M); the direction of sedimentation was from fraction 8 to fraction 1. As external controls, 5 μl of Spike A and Spike B Mix (Two-Color RNA Spike-In Kit; Agilent Technologies, Santa Clara, CA, USA) were added to the polysomal and total RNA, respectively, concomitant with collection of the sucrose gradient. Each spike-in mixture contained 10 *in vitro* synthesized, polyadenylated transcripts at various pre-determined ratios. These transcripts are derived from adenovirus *E1A* and are represented by corresponding spots on the Agilent oligonucleotide microarrays used in this study. Eluted RNA solution from RNeasy Mini spin columns (Qiagen) was further subjected to LiCl precipitation by addition of LiCl at a final concentration of 1.5 M, followed by centrifugation. After ethanol precipitation, RNA integrity was examined using a Bioanalyzer 2100 (Agilent Technologies).

qRT-PCR analysis qRT-PCR analysis was performed as described previously (17). Briefly, cDNAs were synthesized at 20 μl reaction scale with equal volumes of RNA solution (typically 1–2 μl) from each gradient fraction, or with 1 μg of total RNA, using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) with anchored oligo$(dT)_{18}$ primers. PCR was performed using 1 μl of a 1:10 dilution of first-strand cDNA, LightCycler 480 SYBR Green I Master (Roche Applied Science), and gene-specific primer sets (Table S1) in a LightCycler 480 (Roche Applied Science). Melting-curve analysis was carried out for each primer set to verify the presence of a single melting peak after amplification. 'No cDNA' samples (water) and 'no RT' samples were included as negative controls.

DNA oligonucleotide microarray hybridization and scanning Fluorescent cRNA was generated from polysomal and total RNAs (RNAs from all fractions) from day 1 and day 8 cells after inoculation using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). A 500 ng aliquot of RNA was subjected to reverse transcription using MMLV reverse transcriptase and an oligo(dT) primer containing the T7 promoter, and subsequently *in vitro* transcribed using T7 RNA polymerase, ultimately yielding Cy3-labeled and Cy5-labeled cRNAs. The cRNA was purified using RNeasy Mini spin columns. Mixtures of 0.85 μg each of Cy3-labeled and Cy5-labeled cRNAs were co-hybridized at 65°C for 17 h on an Agilent Technologies 4 × 44K *Arabidopsis* 4 60-mer oligo microarray. The slide was washed, and then scanned using a G2505 DNA Microarray Scanner (Agilent Technologies), at a pixel size of 5 μm.

Microarray data analysis The fluorescence intensity of individual features (spots) on scanned images was quantitated and corrected for background using the Feature Extraction software (Agilent Technologies). In order to ensure that we analyzed only microarray data of high quality, we evaluated the reproducibility of the microarray analyses according to a publication provided by the manufacturer (18); only features that passed three criteria described therein were used for further analysis. Features flagged in Feature Extraction software as saturated ('IsSaturated' field) or as low-signal ('IsWellAboveBG' field) were omitted. Polysomal RNA profiling data were normalized via a Linear and LOWESS method followed by spike-in normalization using the Two-Color RNA Spike-In Kit. As described above, because 10 *in vitro* synthesized *E1A* transcripts were added to each fraction in various pre-determined ratios concomitant with the collection of the sucrose gradient, the ratios of these transcripts between fractions can be used to monitor variations in preparation steps such as RNA isolation, reverse transcription, sample amplification, dye labeling, or hybridization. High correlations between observed and expected log ratios for these spike-in RNAs were observed for all microarray analyses (data not shown). Out of the ten spike-in RNAs, E1A_r60_a20 RNA, whose expected ratios are 1:1, was used for spike-in normalization. The ratio of the intensity value in the polysomal RNA sample (Cy3) to that in the total RNA sample (Cy5) was calculated, and defined as the translation state or polysome ratio (PR).

Vector construction A DNA fragment containing the firefly (*Photinus pyralis*) luciferase (*F-luc*) was PCR amplified using primers Fluc-F (5′-aggtaccatggaagacgtcaaaaacataaa-3′), Fluc-R (5′-agagctcactagtttacacggcgatctttccgc-3′) and pT3-FL-pA (17) as the template. In the corresponding F-LUC protein, Ala4 (gcc) was changed to Val (gtc) in order to introduce an AatII site. A DNA fragment containing CaMV 35S promoter, *ADH* 5′-UTR, and the 5′-terminal part of *F-luc* containing the AatII site was PCR-amplified using primers 35S XbaI-F (5′-aaatctagattagccttttcaatttcag-3′), ADH-R (5′-tttgacgtcttccattatcaacagtgaagaacttgcttttg-3′), and At1g77120_5′UTR:GUS (14) as the template. These two fragments were ligated via their AatII sites, and inserted into the XbaI–SacI gaps of At1g77120_5′UTR:GUS to generate ADH_5′UTR:*Fluc*. A plasmid At3g47610_5′UTR:*Fluc* was constructed in a same way as ADH_5′UTR:*Fluc* by using primer At3g47610-R (5′-tttgacgtcttccatcttcttctccgattctctccgtc-3′) and template At3g47610_5′UTR:GUS (14). A DNA fragment containing CaMV 35S promoter, *ADH* 5′UTR or At3g47610_5′UTR, and the 5′-terminal part of horseradish peroxidase C1a (*HRP C1a*) containing the NsiI site was PCR-amplified using primers 35S XbaI-F, ADH-C1a-R (5′-ttatgcattatcaacagtgaagaacttgcttttg-3′) or At3g47610-C1a-R (5′-ttatgcatcttcttctccgattctctccgtc-3′), and At1g77120_5′UTR:GUS or At3g47610_5′UTR:GUS (14) as the template. These XbaI-NsiI fragments were inserted into the XbaI-NsiI gaps of AtAGP21mod 5′UTR HRP C1a (6) to generate ADH_5′UTR:*HRP C1a* and At3g47610_5′UTR: *HRP C1a*, respectively.

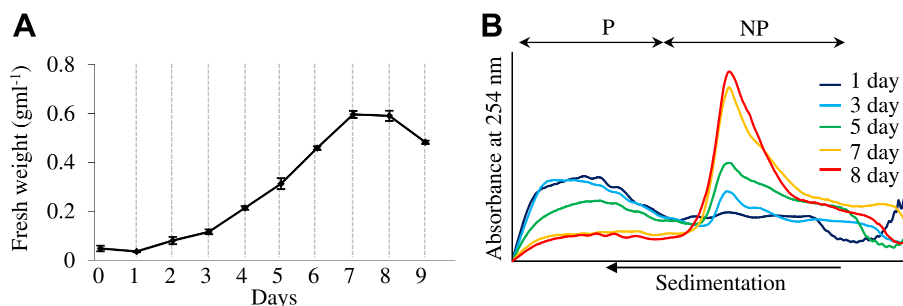

FIG. 1. Representative growth curves for *A. thaliana* T87 suspension cells, and polysome fractionation assays. (A) The fresh weight of cells in 1 ml of broth was measured at 24-h intervals after inoculation. Plotted values are means of experiments conducted three times, and the standard deviation for each point is indicated by bars. (B) Detergent-treated cell extracts were prepared from cultured cells, and then fractionated over a 15–60% sucrose density gradient. Absorbance was measured at 254 nm; the corresponding polysome and non-polysome fractions were shown. P, polysome fraction; NP, non-polysome fraction. The direction of sedimentation is from right to left. Each experiment was independently repeated three times with similar results, and representative profiles were shown.

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