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RESEARCH REPORT

EF-1α silencing by feeding RNAi suppresses resting cyst formation in *Colpoda cucullus* Nag-1 strain

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Abstract

It is reported that the expression level of EF-1α in *Colpoda cucullus* Nag-1 is markedly enhanced within several hours after the onset of encystment induction. In this study, the *Colpoda* strain (stock EQ-1) known to promptly encyst also showed early EF-1α expression while the strain (stock ES-1) known to show prolonged encystment also showed delayed EF-1α expression. In cells in which EF-1α is silenced by feeding RNAi, the cyst formation was prolonged, but normal cyst walls were formed. These results suggest that *Colpoda* EF-1α is involved in the early morphogenetic events of the resting cyst formation by accelerating protein translation or cytosleletal dynamics such as microtubule disintegration.

Key Words: EF-1α; *Colpoda*; encystment; feeding RNAi

Introduction

Soil ciliates such as *Colpoda* quickly transform into resting cysts resistant to desiccation, high temperature, freezing, and acid (Taylor and Strickland, 1936; Maeda *et al*., 2005; Müller *et al*., 2010; Sogame *et al*., 2011) before the temporary puddles in which they dwell dry up. The resting cyst formation of *Colpoda cucullus* Nag-1 is promptly induced by suspension in a Ca^{2+}-containing food-free medium at a high cell density (encystment induction by Ca^{2+}/ overpopulation) (Yamaoka *et al*., 2004; Maeda *et al.,* 2005), while excystment is induced by components contained in wheat leaves or by sodium copper chlorophyllin (chlorophyllin-Cu) (Tsutsumi *et al*., 2004). When the vegetative cells of *C. cucullus* Nag-1 are stimulated to encyst, diffusion of external Ca^{2+} into the cell interior is accelerated by cell-to-cell mechanical stimulation (Matsuoka *et al*., 2009; Asami *et al*., 2010; Sogame and Matsuoka, 2013), followed by cAMP-dependent protein phosphorylation (Sogame *et al.,* 2012a, 2014a) and alteration of expression levels of proteins (Sogame *et al.,* 2014b) such as elongation factor 1α (EF-1α) (Sogame *et al*., 2012b). The fact that the expression of *Colpoda* EF-1α is prominently enhanced in the early phase of encystment (several hours after the onset of encystment induction), while its expression level is regained (reduced) within 1 h after onset of excystment induction (Sogame *et al*., 2013) implies that EF-1α may play an important role in the disintegration of the vegetative cell structure of *Colpoda* and its reconstruction into resting cysts. The present study showed that EF-1α plays a key role in certain processes in the encystment events of *C. cucullus* Nag-1.

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Materials and Methods

Cell culture and encystment induction

Colpoda cucullus Nag-1 strain, which was collected as a resting cyst from the soil suface in Kochi Prefecture in Japan, was cultured in a 0.05 % (w/v) infusion of dried wheat leaves inoculated with a non-pathogenic strain of bacteria (*Klebsiella pneumoniae*). *Klebsiella pneumoniae* were cultured on agar plates containing 1.5 % agar, 0.5 % polypepton, 1 % meat extract and 0.5 % NaCl. The vegetative cells of *C. cucullus* Nag-1 cultured for 1 - 2 days were rinsed 2 - 3 times with 1 mM Tris-HCl (pH 7.2), and subjected to sedimentation (1,500×*g* for 2 min) and resuspension; the cells were then induced to encyst by being suspended in encystment-inducing medium (1 mM Tris–HCl [pH 7.2], 0.1 mM $CaCl_2$) at a high cell density (50,000 cells/ml) (encystment induction by Ca^{2+}/overpopulation). As a control, the vegetative

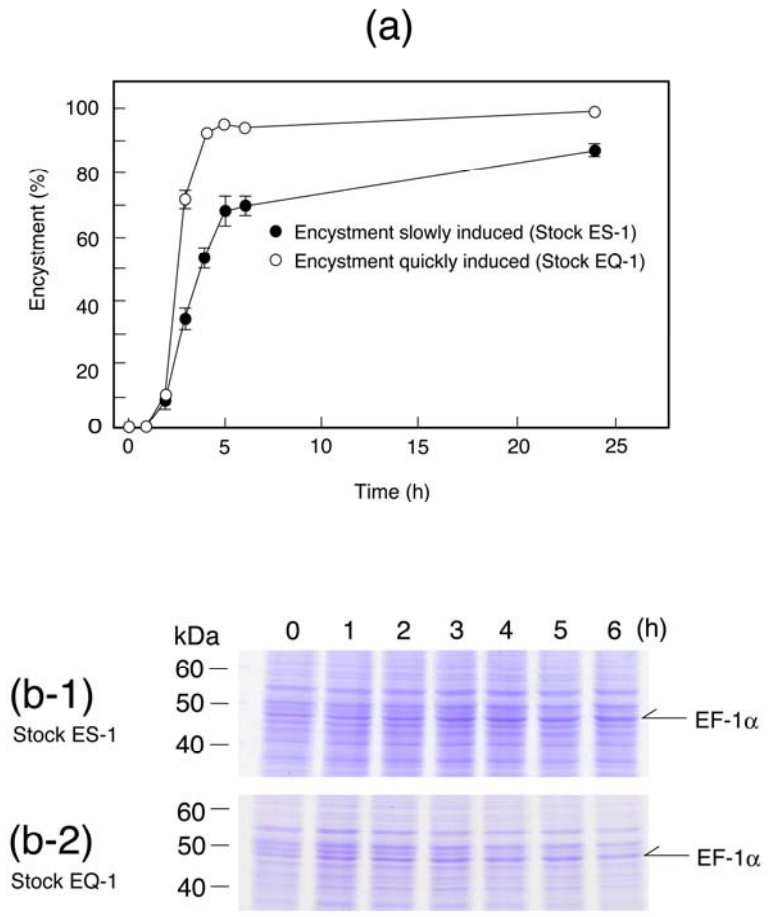

Fig. 1 Comparison of the time course of encystment and EF-1α expression between two stock cultures of *C. cucullus* Nag-1. (a) Time course of encystment rate. Closed circles, Stock ES-1, with slowly induced encystment; open circles, Stock EQ-1, with quickly induced encystment. In this measurement, rounded cells with an ectocyst layer were counted as encysting cells. (b) SDS-PAGE of total proteins contained in the cells of encysting *Colpoda,* showing EF-1α expression level (arrows). The solubilized samples of Stock ES-1 and EQ-1 were obtained at 0 to 6 h (labeled on the top of each lane) after the onset of encystment induction.

cells were suspended in 1 mM Tris-HCl (pH 7.2) at a low cell density (2,000 cells/ml) so that the resting cyst formation could be inhibited as much as possible. Excystment induction was carried out by replacing the surrounding medium (encystment-inducing medium) of 10-day-old resting cysts by a fresh 0.05 % (w/v) wheat-leaf infusion.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli's method (Laemmli, 1970) with a slight modification. The vegetative cells and encysting cells of *C. cucullus* Nag-1 were solubilized in SDS-PAGE sample buffer containing 1% SDS, 30 mM Tris-HCl (pH 6.8), 5 % 2-mercaptoethanol and 10 % glycerol, followed by boiling for 3 min. A sample containing 5,000 cells was applied in each lane, and electrophoresed on a 10 % gel at 150 V. The gels were stained with a solution containing 0.2 % Coomassie brilliant blue (CBB) R250, 45 % (v/v) methanol and 10 % glacial acetic acid, and then destained in a 27 % (v/v) methanol, 9 % glacial acetic acid solution.

Determination of partial nucleotide sequence of the EF-1α of C. cucullus *Nag-1*

PCR amplification of *C. cucullus* Nag-1 EF-1α (using TAKARA Prime STAR HS) was performed using genomic DNA, and the nucleotide sequence was determined (GenBank accession No. AB918921.1). Amplification primers (sense: 5'-AAGAACATGATTACCGGT; antisense: 5'-GAACCAGGTAAGGTTGGG) were designed based on the sequence of *C. inflate* (GenBank, accession No. AF056098.1).

Gene silencing by feeding RNAi method

The region (336 bp) of the open reading frame of *C. cucullus* Nag-1 EF-1α (GenBank accession No. AB918921.1) was amplified by PCR, followed by cloning into the Litmus 28i vector (New England BioLabs) between the two T7 promoters. For PCR, a set of primers connected with *EcoRI*-HF (New England BioLabs) or *HindIII*-HF (New England BioLabs) recognition sequences (underlined) and 4 extra nucleotides (5'-CCGC) (sense: 5'-CCGCGAATTCAAGAACATGATTACCGGT/antisense: 5'- CCGCAAGCTTGAACCAGGTAAGGTTGGG)

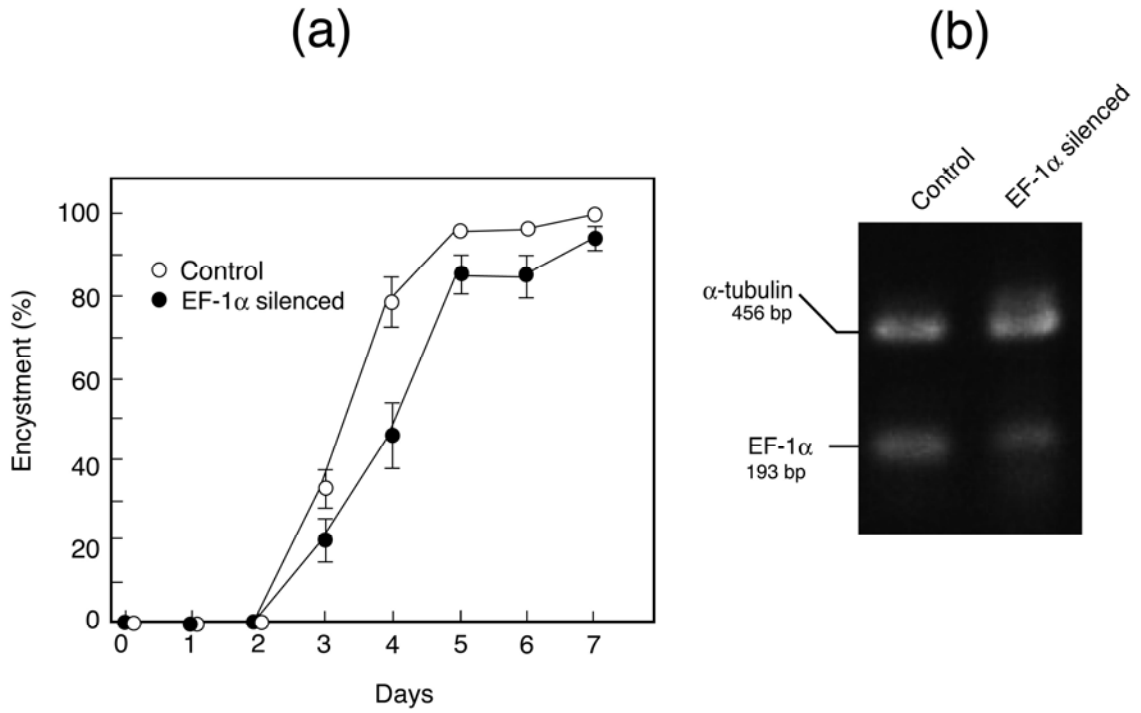

Fig. 2 Suppression of encystment and semi-quantitative RT-PCR in *C. cucullus* Nag-1 cells whose EF-1α had been silenced by RNAi. (a) Time course of encystment rates of EF-1α-silenced cells (closed circles) and nonsilenced cells (open circles) of Stock EQ-1. In this measurement, rounded cells with an ectocyst layer were counted as encysting cells. (b) Semi-quantitative RT-PCR of EF-1α, showing electrophoresis images of EF-1α and α-tubulin expression in nonsilenced cells (Control), left lane, and EF-1α-silenced cells (3 days after induction of gene silencing), right lane.

was used. *Escherichia coli* strain HT115 was transformed by the introduction of the obtained constructs. In order to confirm that the *E. coli* strain HT115 might be transformed by the introduction of an EF-1α-gene-cloned Litmus 28i vector, the vector was isolated from the *E. coli* HT115 strain, and the nucleotide sequence was determined. In this case, a set of primer sequences (M13 primer M3: 5'-GTAAAACGACGGCCAGT/M13 primer RV: 5'-CAGGAAACAGCTATGAC) was used.

RNAi gene silencing was carried out according to the method described previously (Galvani and Sperling, 2002; Kutomi *et al*., 2012) with modifications. Resting cysts of *Colpoda cucullus* Nag-1 were stimulated to encyst in 0.05 % wheat leaves infusion (culture medium), and cultured for about 12 h in this medium. Thus, cultured cells were suspended using a thin pipette at a cell density of 10 cells/ml in culture medium containing 100 μg/mL ampicillin, 100 μg/ml tetracycline and 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). Gene silencing was initiated by the addition of *E. coli* strain HT115 into the culture medium at the cell density of OD_{600} of 0.25, which was transformed to produce double-stranded RNA of EF-1α. For the control (nonsilenced *Colpoda*), *E. coli* strain HT115 transformed by the introduction of a Litmus 28i vector from which the EF-1α gene had been excluded was added into the culture medium at the cell density of OD_{600} of 0.25.

Semi-quantitative RT-PCR

Total RNA was extracted from *C. cucullus* Nag-1 vegetative cells by an acid guanidinium thiocyanate-phenol-chloroform technique using ISOGEN-II (NIPPON GENE Co., Ltd, Tokyo, Japan) according to the attached protocol. The total RNA (4 μg) was reverse transcribed using GoScript Reverse Transcription System (Promega) according to the attached protocol. The 100 μl of PCR mixture for competitive PCR amplification (30 cycles using Go Taq Green Master Mix [Promega]) contained 5 μl cDNA solution (containing 1 ng cDNA) as a template. PCR amplification of EF-1α gene of *C. cucullus* Nag-1 was performed using EF-1α primers 5'-TAAGTCCACCTCCACTGG (sense) and 5'-TGGCGGTTTCGAACTTCC (antisense), which had been designed based on the sequence of *C. inflate* (GenBank, accession No. AF056098.1). As an internal control for cDNA quantity, the α-tubulin gene of *C. cucullus* Nag-1 was amplified using the primers 5'-CTGAAACTGGTGCTGG (sense) and 5'-CAGTGTGTTCAAGAAGGG (antisense), designed based on the sequence of *Colpoda* sp. (GenBank, accession No. X94348.1).

Amplified PCR products were electrophoresed in 2.0% agarose gels, followed by visualization with ethidium bromide staining. In order to confirm that each band was a PCR product of the EF-1α (194 bp) or α-tubulin (456 bp) gene, DNA was extracted from each gel band using phenol, and their sequences

were determined (EF-1α: GenBank accession No. AB976559.1; α-tubulin; GenBank accession No. LC004697.1).

The rate of encystment and excystment is expressed as a percentage of the total number of tested cells (142-161 cells). Points (columns) and attached bars correspond to the means of six identical measurements (140 - 163 cells per measurement) and standard errors.

Results and Discussion

We compared the time course of Ca^{2+}/overpopulation-induced encystment initiation between two stock cultures of *C. cucullus* Nag-1 cells, those in which encystment was slowly induced (Stock ES-1) and those in which encystment was quickly induced (Stock EQ-1) (Fig. 1a). As shown in Figure 1a, compared to Stock EQ-1 (open circles), encystment initiation of 'Stock ES-1' (closed circles) was significantly prolonged ($p < 0.01$ in 3, 4, 5, 6 h after the onset of encystment induction, Mann-Whitney test). SDS-PAGE of total proteins of the cells obtained from these two stocks showed that the expression of a protein around 48 - 49 kDa which had been identified EF-1α (Sogame *et al*., 2012b) was enhanced 1 hour after onset of encystment induction in Stock EQ-1 cells (Fig. 1b-2), but several hours after onset of encystment induction in Stock ES-1 cells (Fig. 1b-1).

Encystment occurred spontaneously in the culture medium. We silenced EF-1α expression in Stock EQ-1 cells by feeding *E. coli* containing knockdown plasmid, and examined the effect of EF-1α silencing on the spontaneously induced encystment during culturing (Fig. 2a). Compared to nonsilenced cells (Fig. 2a, open circles), the encystment initiation of EF-1α-silenced cells was significantly suppressed ($p < 0.01$ at 4 days after initiation of culturing, Mann-Whitney test). We used competitive PCR to examine whether the EF-1α mRNA expression in *Colpoda* fed *E. coli* containing the knockdown plasmid was actually silenced. Semi-quantitative RT-PCR showed that the amount of EF-1α mRNA contained in the silenced *Colpoda* was decreased (Fig. 2b) while the amount of α-tubulin mRNA used as an internal control was almost identical between the silenced and nonsilenced cells (Fig. 2b).

Photomicrographs shown in Figure 3a, b are motile cells surrounded by endocyst layers (en) just emerging from the mechanically ruptured ectocyst (ec) of a 10-day-old resting cyst, and they indicate that an ectocyst layer and endocyst layers are formed in EF-1α-silenced *Colpoda* cysts (Fig. 3b) identical to the case with the nonsilenced *Colpoda* cyst (Fig. 3a). In addition, there was no difference ($p > 0.05$, Mann-Whitney test) in the emergence rate (%) between nonsilenced (Fig. 3c, 'Control') and EF-1α-silenced cells (Fig. 3c, 'EF-1α silenced') when excystment was induced by replacing the surrounding encystment-inducing medium with fresh 0.05 % (w/v) wheat-leaf infusion. These resuts indicate that EF-1α-silenced cells may ultimately become mature resting cysts despite the cyst formation was prolonged.

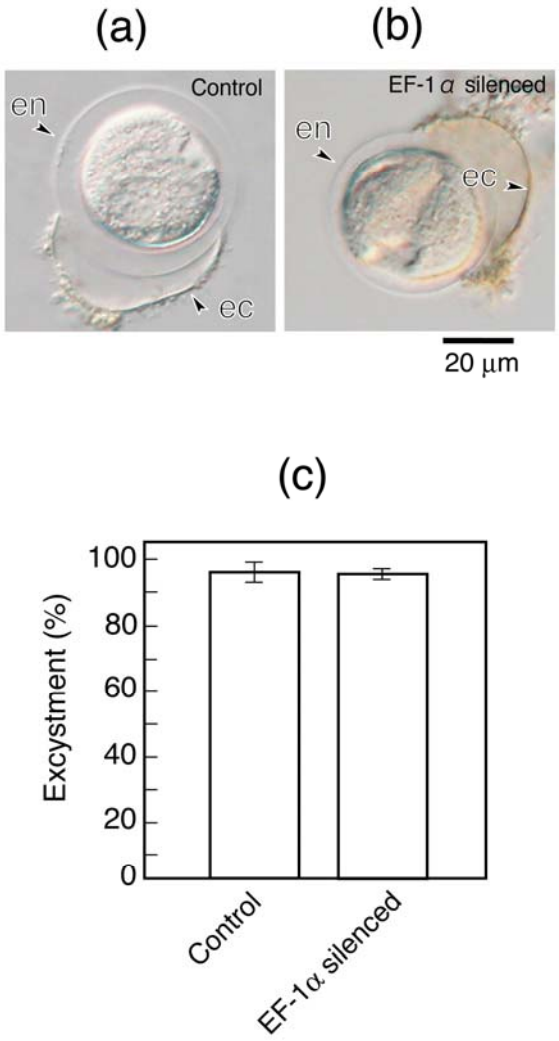

Fig. 3 Excystment of *C. cucullus* Nag-1 cells whose EF-1α had been silenced by RNAi. (a), (b) Photomicrographs of EF-1α-nonsilenced cells (a) and silenced cells (b), showing just-emerging cells surrounded by endocyst layers (en) through the rupture of the hard ectocyst layer (ec). (c) Effect of RNAi silencing of EF-1α on the emergence rate (%) in the excystment-induced cysts. Left and right columns correspond to the EF-1α-nonsilenced (Control) and -silenced cells. The photomicrographs (a, b) and the emergence rate (c) were obtained at 1 h after the onset of excystment induction in the resting cysts which had been spontaneously formed until the 10th day of culture of vegetative cells (Stock EQ-1) in normal culture medium or EF-1α-silencing medium.

The results obtained in the present study suggest that *Colpoda* EF-1α may play a role in early events in the encystment process. EF-1α is one of the subunits of translation elongation factor 1 composed of four different subunits (Ejiri, 2002), and has multiple functions such as a translation in ribosomes (Ejiri, 2002), bundling of actin filaments (Kurasawa *et al*., 1996), severing of microtubules

(Shiina *et al*., 1994) and regulation of the proteasome-dependent degradation of proteins (Gonen *et al*., 1994). Judging from the multiple functions of EF-1α, it is suggested that enhancement in the EF-1α expression level may be involved in the acceleration of morphogenetic events such as cyst wall formation by promoting protein translation in the regulation of protein disintegration, or in cytoskeletal dynamics such as microtubule disintegration.

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