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EFFECTS OF CHLOROPHYLLIN ON ENCYSTMENT SUPPRESSION AND EXCYSTMENT INDUCTION IN *COLPODA CUCULLUS* NAG-1: AN IMPLICATION OF CHLOROPHYLLIN RECEPTOR

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Abstract–Among the molecules suppressing encystment and inducing excystment of *Colpoda cucullus* Nag-1, sodium copper chlorophyllin is the only molecule whose molecular structure is known. The present study showed that sodium iron chlorophyllin also had marked effects. When the encysting cells (2-day-aged immature cysts) of *C. cucullus* Nag-1 were treated with trypsin (1 mg/mL), excystment was suppressed. In this case, most of the cysts that failed to excyst were alive, because the selective permeability of the plasma membrane of these cysts functioned normally. These results suggest that presumed chlorophyllin receptors which are involved in the induction of excystment may occur on the plasma membranes of the resting cysts. Two-day-aged cysts (immature cysts) are surrounded by thick cyst walls. We assessed whether chlorophyllin and trypsin (23 kDa) penetrate across the cyst wall. When the cysts were immersed in the fluorescent molecule phycocyanin (40 kDa), a vivid phycocyanin fluorescence was observed inside or on the cyst wall, indicating that phycocyanin penetrates across the cyst wall. Judging from these results, chlorophyllin molecules and trypsin flowed in across the cyst wall to act on the receptors located on the plasma membrane.

INTRODUCTION

The resting cyst formation (encystment) is the most important adaptive strategy of terrestrial unicellular eukaryotes such as *Colpoda.* They transform into resting cysts surrounded by cyst walls that are resistant to hazardous environments such as desiccation (Corliss and Esser, 1974), ultraviolet rays (Matsuoka *et al*., 2017), freezing (Taylor and Strickland, 1936; Maeda *et al*., 2005), etc. The encystment of *Colpoda cucullus* Nag-1 can be effectively induced by suspending vegetative cells cultured for a couple of days (the growing phase just ended) at a high cell density in the presence of Ca^{2+} in the surrounding medium (encystment induction by Ca^{2+}/overpopulation) (Sogame *et al*., 2013). Encystment of *C. cucullus* Nag-1 is prevented by components released from bacteria suspended in the surrounding medium (Yamasaki *et al*., 2004), components contained in plant leaves, chlorophyllin (Tsutsumi *et al*., 2004), and the formation of food vacuoles and/or endosomes (Yamasaki *et al*., 2004; Kida *et al.* 2009). On the other hand, excystment of *C. cucullus* Nag-1 can be effectively induced by certain components of plant leaves (Tsutsumi *et al*., 2004), peptides (Akematsu and Matsuoka, 2007), and sodium copper chlorophyllin (Cu-Chl; Tsutsumi *et al*., 2004). Among these encystment-suppressing or excystment-inducing components, Cu-Chl is the only molecule for which the molecular structure is known.

The mature cysts and encysting immature cysts (aged more than 12 h) of *C. cucullus* Nag-1 are surrounded by the cyst wall (made up of a mucus/lepidosome layer, a rigid ectocyst layer, and endocyst layers, from the outside). The cyst wall seems to isolate the internal environment from the external environment. It remains to be clarified how

the resting cyst detects chlorophyllin molecules dissolved in the external medium. The present study discusses the location of the presumed chlorophyllin receptor that mediates excystment.

MATERIALS AND METHODS

Cell Culture and Induction of Encystment or Excystment

The *C. cucullus* Nag-1 strain (Funadani *et al*., 2016) (18S ribosomal RNA gene: GenBank accession no. AB918716) was cultured in a 0.05% (w/v) infusion of dried wheat leaves. Vegetative *Colpoda* cells were collected by centrifugation (1,500 g for 2 minutes) and suspended in an encystment-inducing medium [1 mM Tris-HCl (pH 7.2), 0.1 mM $CaCl_2$]. Thereafter, 500 mlvolumes of the cell suspension were dispensed in watch glasses and kept for 2 days under humid conditions.

Chemicals

Cu-Chl, trypsin, and phycocyanin were purchased from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan. Sodium iron chlorophyllin (Fe-Chl) was purchased from Nacalai Tesque Inc. Kyoto, Japan.

Fluorescence Microscopy

The encysting cells (2-day-aged cysts) which had been treated with 20 mg/mL phycocyanin (dissolved in water) for 20 minutes were then washed with water briefly and observed under a fluorescence microscope (OLYMPUS BX-50) equipped with a red fluorescence filter set (U-MWIG). Images were recorded with a Nikon Coolpix 4500.

RESULTS AND DISCUSSION

As shown in Figs. 1A and B, the Ca^{2+}/overpopulation-induced encystment of *C. cucullus* Nag-1 was significantly suppressed by the addition of Cu-Chl or Fe-Chl ($p < 0.01$, Mann-Whitney test). The cyst formation is known to be suppressed by the formation of food vacuoles and/or endosomes (Yamasaki *et al.*, 2004; Kida *et al*., 2009). However, neither food vacuoles nor endosomes were observed when the vegetative cells of *Colpoda* were incubated for 30 minutes in the encystment-inducing medium containing 0.5 mM Cu-Chl (Fig. 1C). This result suggests that the suppression of encystment

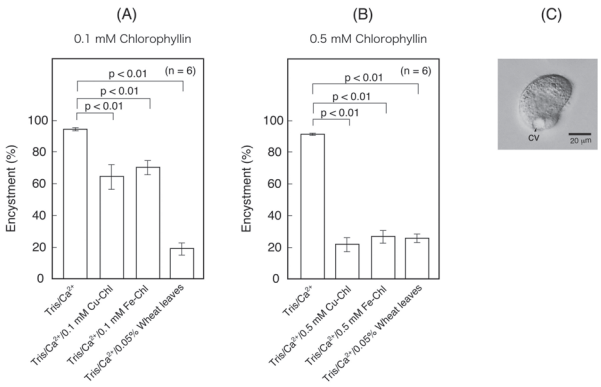

Fig. 1. Suppression effects of Ca^{2+}/over population-induced encystment of *C. cucullus* Nag-1 by the addition of 0.1 mM (A) or 0.5 mM (B) sodium copper chlorophyllin (Cu-Chl) or sodium iron chlorophyllin (Fe-Chl) in the surrounding encystment-inducing medium. (C) A photomicrograph of *Colpoda* vegetative cell which had been incubated for 30 minutes in the encystment-inducing medium containing 0.5 mM Cu-Chl. cv, contractile vacuole. All media contained at least 1 mM Tris-HCl (pH 7.2) and 0.1 mM Ca^{2+}. The columns and attached bars correspond to the means of six identical measurements (100 cells per measurement) and standard errors.

induced by chlorophyllin may not be attributed to food vacuoles and/or endosomes that are formed by the incorporation of chlorophyllins, but rather to the interaction of chlorophyllin with its presumed receptor located on the plasma membrane to suppress the signaling pathways of Ca^{2+}/overpopulation-induced encystment. It is not likely that relatively large water-soluble chlorophyllin molecules flow in across the plasma membrane to prevent signaling pathways leading to encystment induction.

The excystment of two-day-aged cysts was significantly induced by the addition of Cu-Chl or Fe-Chl into the surrounding encystment-inducing medium ($p < 0.01$, Mann-Whitney test) (Fig. 2). If the presumed chlorophyllin receptors are located on the plasma membranes of vegetative cells, excystment induction is expected to be prevented by trypsin digestion of the chlorophyllin receptors. When the vegetative cells of *Colpoda* were treated with 1 mg/mL trypsin for 30 minutes, they were killed. Presumably, the membrane function may be disordered by the digestion of membrane proteins.

When the 2-day-aged encysting cells of *Colpoda* were treated with Tris-HCl (pH 7.2) containing 1

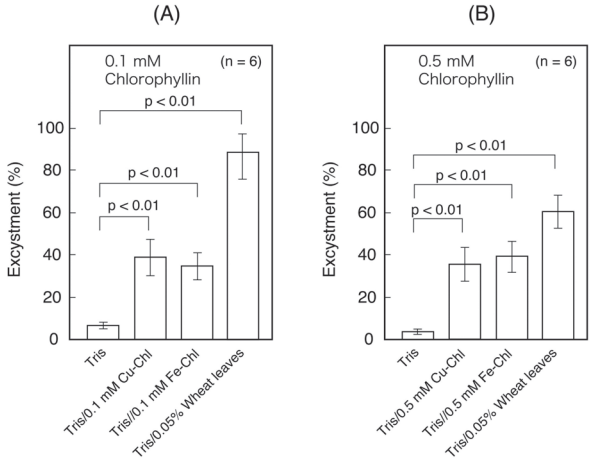

Fig. 2. Excystment induction of *C. cucullus* Nag-1 by the addition of 0.1 mM (A) or 0.5 mM (B) sodium copper chlorophyllin (Cu-Chl) or sodium iron chlorophyllin (Fe-Chl) in the surrounding medium. All media contained at least 1 mM Tris-HCl (pH 7.2). The columns and attached bars correspond to the means of six identical measurements (100 cells per measurement) and standard errors.

mg/mL trypsin for 30 minutes, and the surrounding medium was then replaced by excystment-inducing medium containing 0.5 mM Cu-Chl, the rate of excystment was extensively reduced ($p < 0.01$, Mann-Whitney test) (Fig. 3). The trypsin treatment can lead to death of the cysts because the vegetative

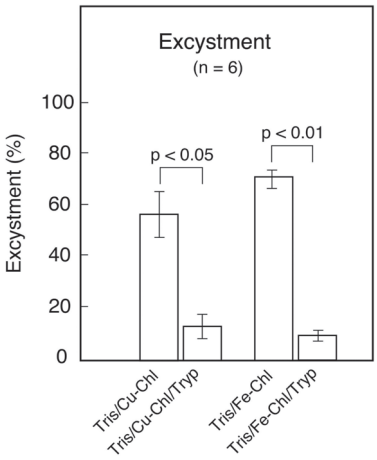

Fig. 3. Suppression of chlorophyllin-induced excystment of *C. cucullus* Nag-1 by the addition of 1 mg/mL trypsin. Left two columns, excystment induction by Cu-Chl. Right two columns, excystment induction by Fe-Chl. All media contained 1 mM Tris-HCl (pH 7.2) and 0.5 mM chlorophyllin. The columns and attached bars correspond to the means of six identical measurements (100 cells per measurement) and standard errors.

cells of *Colpoda* were quickly killed by the treatment. In order to determine whether or not the trypsin-treated cysts are alive, the membrane selective permeability of the cysts was examined. When the trypsin-treated and non-excysted cysts were kept for 10 minutes in hypertonic solution (0.25 M sucrose solution), most cells (83%, n=30 cells) shrank to 90% or less. This result indicates that selective permeability normally functions in most cysts that have not excysted. These results suggest that trypsin may destroy the presumed chlorophyllin receptors on the plasma membrane of the cysts rather than killing the cells.

It is not clear whether chlorophyllin receptors are exposed outside of the cyst wall as shown in Fig. 4A. If the chlorophyllin receptors are located inside the cyst wall, trypsin (23 kDa) is required to penetrate the cyst wall as shown in Fig. 4B. In order to know whether large molecules such as trypsin (23 kDa) can penetrate the cyst wall, a fluorescent protein phycocyanine (about 40 kDa) that is larger than trypsin was chased in 4-day-aged cysts which had been incubated for 20 minutes in an aqueous solution of phycocyanine (Fig. 5). As shown in Fig. 5B-2, a vivid red fluorescence emission was observed around the cyst wall (on the endocyst layers, in the space between the endocyst layers, or in the space between the endocyst layers and the plasma membrane), although in the cyst that did not receive the phycocyanin treatment, only a faint autofluorescence was observed (Fig. 5A-2). These results indicate that even large molecules can penetrate the cyst wall and act on the plasma membrane.

When the vegetative cells of *Colpoda* were treated with 1 mg/mL trypsin, the cells were quickly killed. On the other hand, the 2-day-aged cysts were not killed despite the fact that the plasma membrane of the resting cysts may have been exposed to 1 mg/mL trypsin. It is possible that the plasma membrane of the cysts may be somewhat resistant to trypsin.

The excystment induction and encystment suppression of *C. cucullus* Nag-1 have been induced by the addition of an infusion of dried wheat leaves (Tsutsumi *et al*., 2004). It is known that certain water-soluble components produced by the degradation of purified chlorophyll molecules prominently induced excystment and suppressed encystment (Maeda *et al*., 2005). It is known that magnesium chlorophyllin can be artificially produced by treatment with methanol and KOH (Fig. 6, upper) (Krüger *et al*., 2019), and can possibly be produced

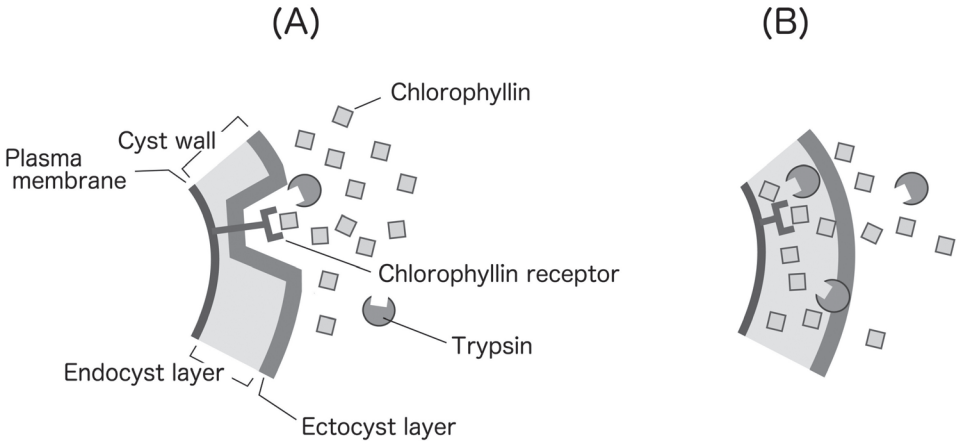

Fig. 4. Schematic diagrams showing the location of presumed chlorophyllin receptors. (A) Receptors exposed to the outside surface of the cyst wall. (B) Receptors located inside the cyst wall.

by the natural degradation of chlorophyll (Fig. 6, lower). In this case, the production of chlorophyllide from chlorophyll was proven (Kräutler, 2016), but the production of magnesium chlorophyllin has not been reported. One of the excystment-inducing and encystment-suppressing molecules contained in the infusion of wheat leaves may be magnesium chlorophyllin.

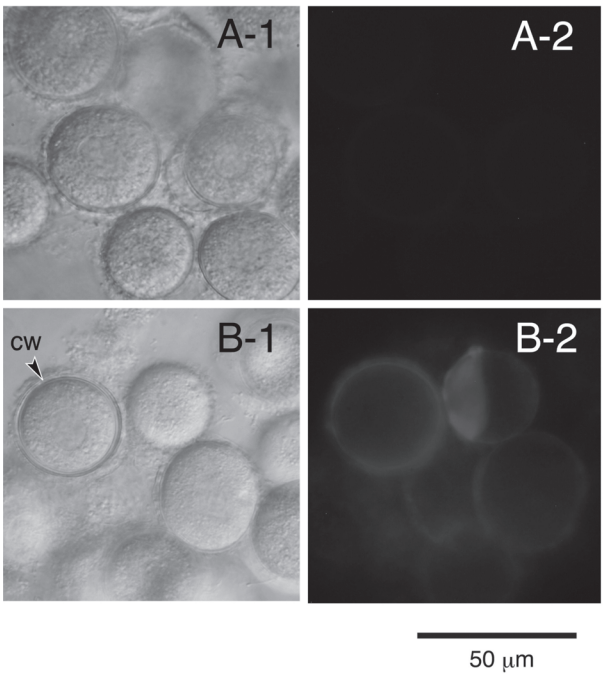

Fig. 5. Red fluorescence of phycocyanin which flowed in across the cyst wall of *C. cucullus* Nag-1. Each set of photographs shows Nomarski (left) and its fluorescence images (right). (A-1), (A-2) A 4-day-aged cyst without phycocyanin treatment.(B-1), (B-2)4-day-aged cyst treated with 20 mg/mL phycocyanin for 20 minutes. cw, cyst wall.

CONCLUSION

Sodium iron chlorophyllin and sodium copper chlorophyllin had an encystment-suppressing effect and excystment-inducing effect of unicellular eukaryote *Colpoda cucullus* Nag-1. Judging from the fact that excystment of *Colpoda* was suppressed by trypsin treatment (1 mg/mL) and that large molecules such as trypsin (23 kDa) penetrate across the cyst wall, it is likely that presumed chlorophyllin receptors for the induction of excystment may occur on the plasma membranes of the resting cysts.

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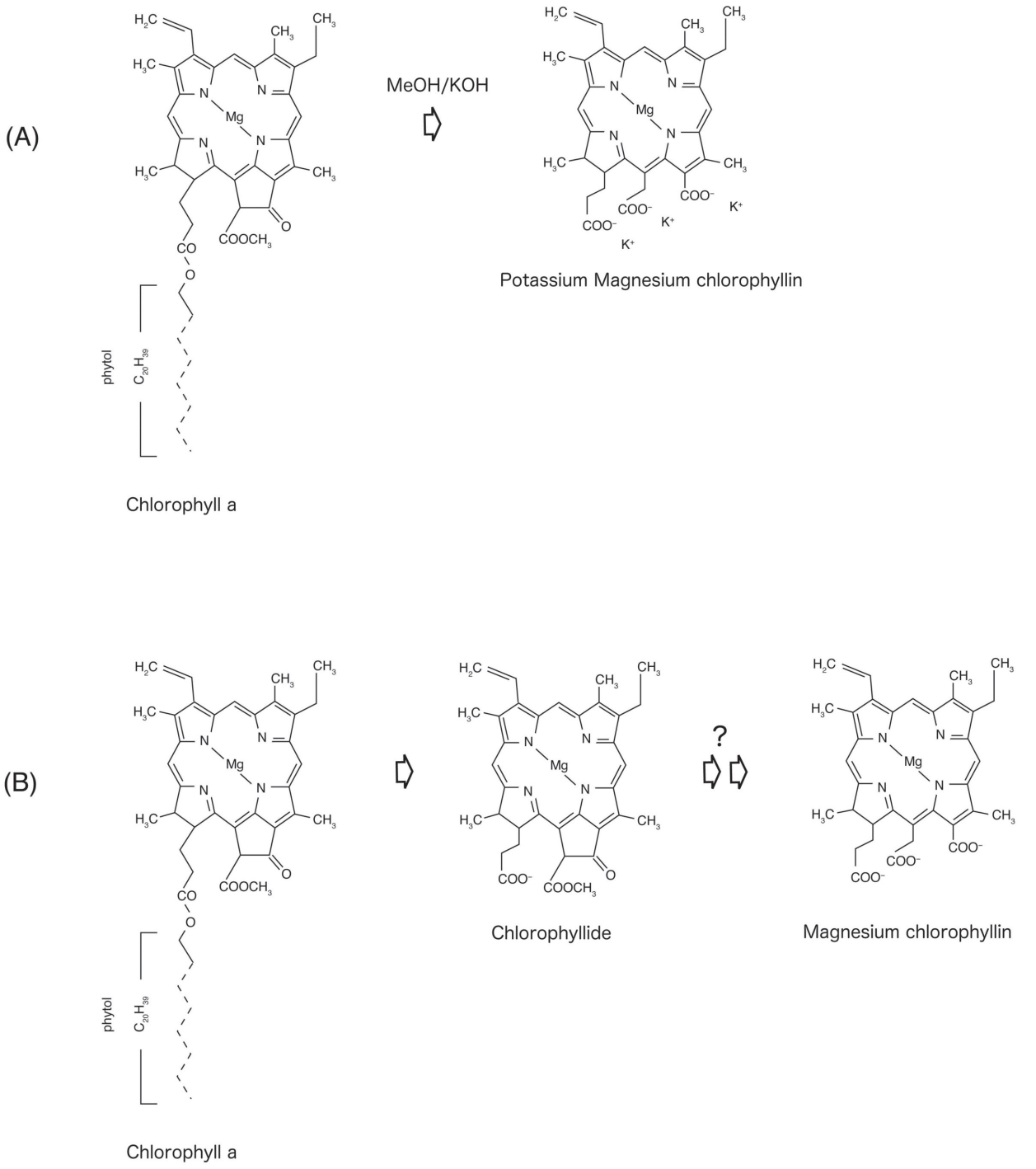

Fig. 6. Artificial production of magnesium chlorophyllin (Mg-Chl) from chlorophyll (A) and a natural degradation of chlorophyll showing the possible production of Chl-Mg (B).

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