Available online at www.sciencedirect.com
ScienceDirect
Plant Science 173 (2007) 206–212
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Induction of sex pheromone secretion and observations of the secretory pathway in the *Closterium peracerosum-strigosum-littorale* complex

Machiko Imaizumi [a,b], Hiroyuki Sekimoto [c], Tetsuko Noguchi [d,*]

[a] *Department of Biology, Shiga University of Medical Science, Ohtsu 520-2192, Japan*
[b] *Graduate School of Humanities and Sciences, Nara Women's University, Nara 630-8506, Japan*
[c] *Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Bunkyo-ku, Tokyo 112-8681, Japan*
[d] *Department of Biological Sciences, Nara Women's University, Nara 630-8506, Japan*

Received 20 January 2007; received in revised form 28 April 2007; accepted 9 May 2007
Available online 17 May 2007

Abstract

Several sex pheromones function in the process of sexual reproduction in the *Closterium peracerosum-strigosum-littorale* complex. The secretory pathways of two sex pheromones, protoplast release-inducing protein (PR-IP) and PR-IP Inducer, were studied by means of Western blot analysis and immunoelectron microscopy. PR-IP Inducer is secreted from mating-type minus (mt^-) cells and induces the release of PR-IP from mating-type plus (mt^+) cells. PR-IP causes release of the protoplast from mt^- cells. Our studies were carried out using an experimental system that induces the secretion of sex pheromones in mt^+ or mt^- cells cultured separately, without mixing. Active secretion of PR-IP Inducer occurs 6–12 h after inoculation of mt^- cells in a nitrogen-depleted medium, and the secretion of PR-IP is substantial by 8 h after inoculation of mt^+ cells into medium containing PR-IP Inducer. In mt^+ and mt^- cells that actively secrete the pheromones, immunogold particles that label PR-IP Inducer and PR-IP were detected inside and along the surfaces of cell walls, vesicles 80–100 nm in diameter, Golgi cisternae, and in the region of the *trans*-Golgi networks. As gold particles were distributed entire the cell wall, exocytosis of pheromones may occur throughout the cell surface. This study is the first report to detect algal sex pheromones by means of electron microscopy.

Keywords: *Closterium peracerosum-strigosum-littorale*; Immunoelectron microscopy; Protoplast release-inducing protein; Sex pheromone; Sexual reproduction; Zygote formation

1. Introduction

Placoderm desmids *Closterium* is a genus of unicellular charophycean algae. These algae usually increase in number by means of binary fission, but they also engage in sexual reproduction (zygote formation) under special environmental conditions. Sexual reproduction can be readily induced in laboratories when the heterothallic strains of *Closterium* (mating-type plus, mt^+; mating-type minus, mt^-) are mixed in nitrogen-depleted mating medium (MI medium [1]). Because of the convenience of this induction system, sexual reproduction in *Closterium* has been more actively studied than in any of the other desmids.

Hogetsu and Yokoyama [2] suggested the necessity of cell–cell interactions between two partner cells during several processes of sexual reproduction in *Closterium ehrenbergii*: sexual cell division, cell aggregation, papilla formation, and the release of gametic protoplasts. Diffusible substances involved in these processes were proposed in the *Closterium peracerosum-strigosum-littorale* complex (*C. psl*c): chemotactic compounds involved in the formation of sexual pairs, and morphogenic compounds that induce sexual cell division, formation of papillae, and release of protoplasts [1,3,4]. Sekimoto et al. successfully isolated two glycoproteinaceous sex pheromones in *C. psl*c; a protoplast release-inducing protein (PR-IP) secreted by the mt^+ cells, which induces the release of protoplasts from the mt^- gametes [5], and PR-IP Inducer secreted by the mt^- cells, which is responsible for the production and secretion of PR-IP by the mt^+ cells [6,7].

* Corresponding author at: Department of Biological Sciences, Nara Women's University, Nara 630-8506, Japan. Tel.: +81 742 20 3416; fax: +81 742 20 3416.
E-mail address: noguchi@cc.nara-wu.ac.jp (T. Noguchi).

0168-9452/$ – see front matter
doi:10.1016/j.plantsci.2007.05.002

cDNAs encoding these pheromones have been cloned and characterized [8–11]. Subsequently, other kinds of pheromones involved in the sexual reproduction of *Closterium* spp. have been detected and purified [12–18] and one cDNA was also cloned and characterized [19,20]. Detailed information obtained from studying the genes that govern the sex pheromones has greatly increased our understanding of the regulatory system that governs their expression [12,21,22].

In contrast, there have been no reports of morphological detection of sex pheromones in *Closterium* spp. or other algal cells. This may be because of the difficulty of detecting sex pheromones that are present at concentrations as small as 4.1 nM [5]. In the present study, we successfully detected two sex pheromones in *C. psl*c, PR-IP and PR-IP Inducer, by means of immunoelectron microscopy. Our clear results were made possible by using the experimental system established by Sekimoto et al. [6], which induces the secretion of sex pheromones in mt^+ or mt^- cells cultured separately. Without this experimental system, it would have been impossible to distinguish between mt^+ and mt^- cells by means of electron microscopy, especially in low-contrast images from sections prepared for immunoelectron microscopy. This study is the first report to detect algal sex pheromones by means of electron microscopy.

2. Materials and methods

2.1. Strains and culture conditions

The strains of heterothallic *C. psl*c used in this study were NIES-67 (mt^+) and NIES-68 (mt^-), obtained from the National Institute for Environmental Studies (Ibaraki, Japan). They were cultured in C medium (http://www.nies.go.jp/biology/mcc/home.htm) at 24 °C with a 16-h illumination under fluorescent light at a photon flux density of 50 μmol m^{-2} s^{-1}, with 8 h of darkness per day.

2.2. Induction of secretion of PR-IP Inducer or PR-IP

Vegetatively growing mt^+ and mt^- cells at the late logarithmic phase were harvested and separately washed three times with MI medium. To induce secretion of PR-IP Inducer, 1.25×10^5 mt^- cells were inoculated into a 100-mL Erlenmeyer flask that contained 25 mL of MI medium, and were cultured at 24 °C under continuous illumination at a photon flux density of 75 μmol m^{-2} s^{-1}. After 24 h of culture, part of the medium was used as "PR-IP Inducer-containing medium" after filtration through a membrane filter with 0.45-μm pores (Durapore, Millipore Co.). To induce PR-IP secretion, 2.5×10^5 mt^+ cells were inoculated into a 100-mL Erlenmeyer flask along with 10 mL of PR-IP Inducer-containing medium and were cultured under the same conditions used to induce secretion of PR-IP Inducer. Cells were counted using a hemacytometer and the counting was repeated three times in each case.

After 0, 6, 12, 18, or 24 h of induction for PR-IP Inducer, and after 0 or 8 h of induction for PR-IP according to the previous study [10], the entire medium in each flask was separated from the cells by filtration through a Durapore membrane filter with 0.45-μm pores and the filtrate was lyophilized.

2.3. Western blotting

The lyophilized residues of the culture medium were dissolved in distilled water. Residues corresponding to 7.5×10^4 cells were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gel with a gradient of 5–20% (SDS-PAGE; PAJEL NPG-R520L, Atto) and separated. The residues were then transferred onto Immuno-Blot PVDF membranes (Bio-Rad Laboratories). The membranes were blocked with 5% bovine serum albumin (BSA) in 0.01 M Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 (TBST) at room temperature for 3 h, and were incubated with rabbit antiserum against the 18.7-kDa of PR-IP Inducer or 19-kDa of PR-IP produced by Sekimoto [23] in 2% BSA-TBST at 4 °C overnight. The working dilution for these antisera was 1:1000. After washing the membranes three times for 10 min with TBST, they were treated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Medical and Biological Laboratory, working dilution; 1:10,000) in 2% BSA-TBST at room temperature for 1 h. The immunoreactive bands were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotechnology).

2.4. Immunoelectron microscopy

Cells after 12 h of induction for PR-IP Inducer, and after 8 h of induction for PR-IP were fixed with 2% glutaraldehyde in 0.07 M phosphate buffer (pH 7.4) that contained 0.2% (v/v) picric acid, at 4 °C for 1 h. The cells were then washed with the same buffer for 2 h, dehydrated with an ethanol series and embedded in LR White resin (The London Resin Co., London, UK).

Thin sections (100 nm thickness) were attached to nickel grids coated with Formvar film. They were blocked with 3% BSA in 0.02 M phosphate-buffered saline (PBS, pH 7.4) at room temperature for 30 min, and treated with rabbit antiserum directed against the 18.7-kDa PR-IP Inducer, the 19-kDa subunit of PR-IP, or pre-immune serum after dilution to 2% of the original concentration in PBS, at 4 °C overnight, followed by washing with PBS for 30 min. Then, the sections were treated with a 2% (v/v) solution of protein A conjugated with 15-nm gold particles (EY Laboratories, Inc.) in PBS at 30 °C for 1 h, followed by washing with PBS followed by distilled water. They were then stained with 2% aqueous uranyl acetate and Reynold's lead citrate solution, and then viewed with a Hitachi H-7500 transmission electron microscope.

2.5. Observation of cell ultrastructure by electron microscopy

Cells were fixed with 1% glutaraldehyde by mixing the culture with an equal volume of 2% glutaraldehyde solution buffered with phosphate at pH 7.4, at 4 °C for 45 min. After