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# Extracellular $Ca^{2+}$ Influx is involved in Tip Growth of the Marine Red Alga *Pyropia yezoensis*

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## Abstract

The conchocelis, the filamentous sporophyte generation of Bangiales, proliferates through tip growth in which only the tips of branch initial cells elongate and subsequently divide **perpendicularly to the apical–basal axis to** form filamentous structures. As was found in terrestrial plants, the tip growth of conchocelis in the marine red alga *Pyropia yezoensis* is regulated by auxin, actin polymerization, and phosphatidylinositol signaling. Although extracellular $Ca^{2+}$ influx is critical for tip growth in terrestrial plants, little is known about its involvement in the tip growth of algae. We therefore investigated whether extracellular $Ca^{2+}$ influx is required for tip growth in *P. yezoensis* conchocelis. Treatment of isolated single-celled conchocelis with a $Ca^{2+}$ chelator, ethylene glycol tetraacetic acid, inhibited both the formation of branch initials and the tip growth of branches in a dose-dependent manner. These findings indicate that extracellular $Ca^{2+}$ influx is indeed involved in the tip growth of conchocelis, suggesting that the basic regulatory mechanisms governing tip growth may be conserved between marine red algae and terrestrial plants. Further confirmation of this possibility will require the characterization of the spatiotemporal patterns of $Ca^{2+}$ oscillations and F-actin accumulation, as well as the subcellular localization of phosphoinositides and their catabolic enzymes in the *P. yezoensis* conchocelis.

***Keywords:*** *Branch initial, $Ca^{2+}$, Conchocelis, Extracellular influx, Pyropia yezoensis, Red alga, Tip growth*

## Introduction

Patterns of anisotropic cell growth give rise to the body shapes of terrestrial plants and multicellular algae [1,2]. Diffuse growth, which results in the expansion of cell surfaces [3,4], produces planar or foliose shape, whereas tip growth [5-7], characterized by directional and highly localized expansion at the apex, produces a filamentous body architecture. Algal cells grow anisotropically through both diffuse and tip growth. We previously demonstrated that tip growth produces the filamentous structures of the conchocelis (sporophyte) and conchosporangium (conchosporophyte) of the red alga *Pyropia yezoensis* [8-12], although regulatory mechanisms of tip growth in *P. yezoensis* has not yet been fully elucidated. Moreover, how diffuse growth produces a foliose shape in the thallus (gametophyte) of *P. yezoensis* remains largely unknown. Therefore, characterizing the contrasting mechanisms that regulate patterns of diffuse and tip growth is necessary to understand the differences in growth–morphology relationships between the two life-cycle generations of Bangiales.

The mechanisms that regulate diffuse and tip growth systems have been studied extensively in terrestrial plants. Cortical microtubules (MTs), actin filament (F-actin), Rho GTPases, the histone variant H2A.Z, gibberellin, and brassinosteroids have all been implicated in the regulation of diffuse growth [1,3,13] likewise, MTs, F-actin, Rho GTPases, $Ca^{2+}$ influx, reactive oxygen species, phosphatidylinositol signaling, auxin, and jasmonate have been shown to regulate tip growth [7,13-21]. Although research on the regulation of diffuse growth in macroalgae has shown little progress, recent studies have provided basic information about the mechanisms regulating tip growth in red and brown macroalgae [2,11,12,22]. Indeed, we demonstrated that auxin, phosphoinositide turnover, and actin polymerization are involved in the regulation of tip growth in *P. yezoensis* [11,12], consistent with findings in terrestrial plants [13,17,18,21]. Although extracellular $Ca^{2+}$ influx is required for tip growth in terrestrial plants [17-19], little is known about its role in the tip growth of macroalgae. We therefore sought to determine whether extracellular $Ca^{2+}$ influx is required for tip growth in *P. yezoensis* conchocelis.

## Materials and Methods

Single-celled conchocelis of *P. yezoensis* strain U-51 were used for these experiments, enabling us to visualize and quantitatively analyze the initiation and progression of tip growth in detail [11]. Conchocelis were maintained in artificial seawater at 15°C under 60 μE/m²/s of light with a short-day cycle (10-h light/14-h dark) [23]. Single-celled conchocelis were prepared as described in [11]. In brief, aggregates of multicellular conchocelis were chopped with a razor blade, and small fragments of conchocelis were separated from larger pieces by filtration through a 10-μm nylon mesh. The separated conchocelis

were then incubated in 30 mL of artificial seawater [23] at 15°C for 10 min. Unbranched single-celled conchocelis (Figure 1) were identified by observation under an Olympus IX73 light microscope equipped with an Olympus DP22 camera (Olympus Corporation, Tokyo, Japan), drawn into a micropipette, and transferred to 96-well plates (one cell per well containing 200 µL of artificial seawater). Ethylene glycol tetraacetic acid (EGTA; Dojindo Laboratories, Japan), an effective $Ca^{2+}$ chelator, was dissolved in artificial seawater to create a 0.5 M stock solution (adjusted to pH 8.0 with NaOH) and stored at −30°C before use. Single-celled conchocelis were treated with 0, 500, or 750 µM EGTA for 3 days, and the growth and morphology of the side branches were observed using the microscope described above.

**Figure 1:** Representative image of an isolated single-celled conchocelis. Nonbranched single-celled conchocelis were used for EGTA treatment experiments. Scale bar, 25 µm.

## Results and Discussion

To evaluate the effects of EGTA on the initiation and elongation of side branches, we measured the branching rate (the percentage of observed cells that produced side branches) and branch length, respectively. Side branches were initiated and underwent elongation when treated with 0 (control) or 500 µM EGTA (Figure 2A and 2B), but 750 µM EGTA completely prevented both the initiation and growth of side branches (Figure 2C). The branching rate and branch length were affected by the presence of EGTA in a dose-dependent manner, being strongly inhibited at 750 µM (Figure 2D and 2E). These results demonstrate that extracellular $Ca^{2+}$ influx is important for

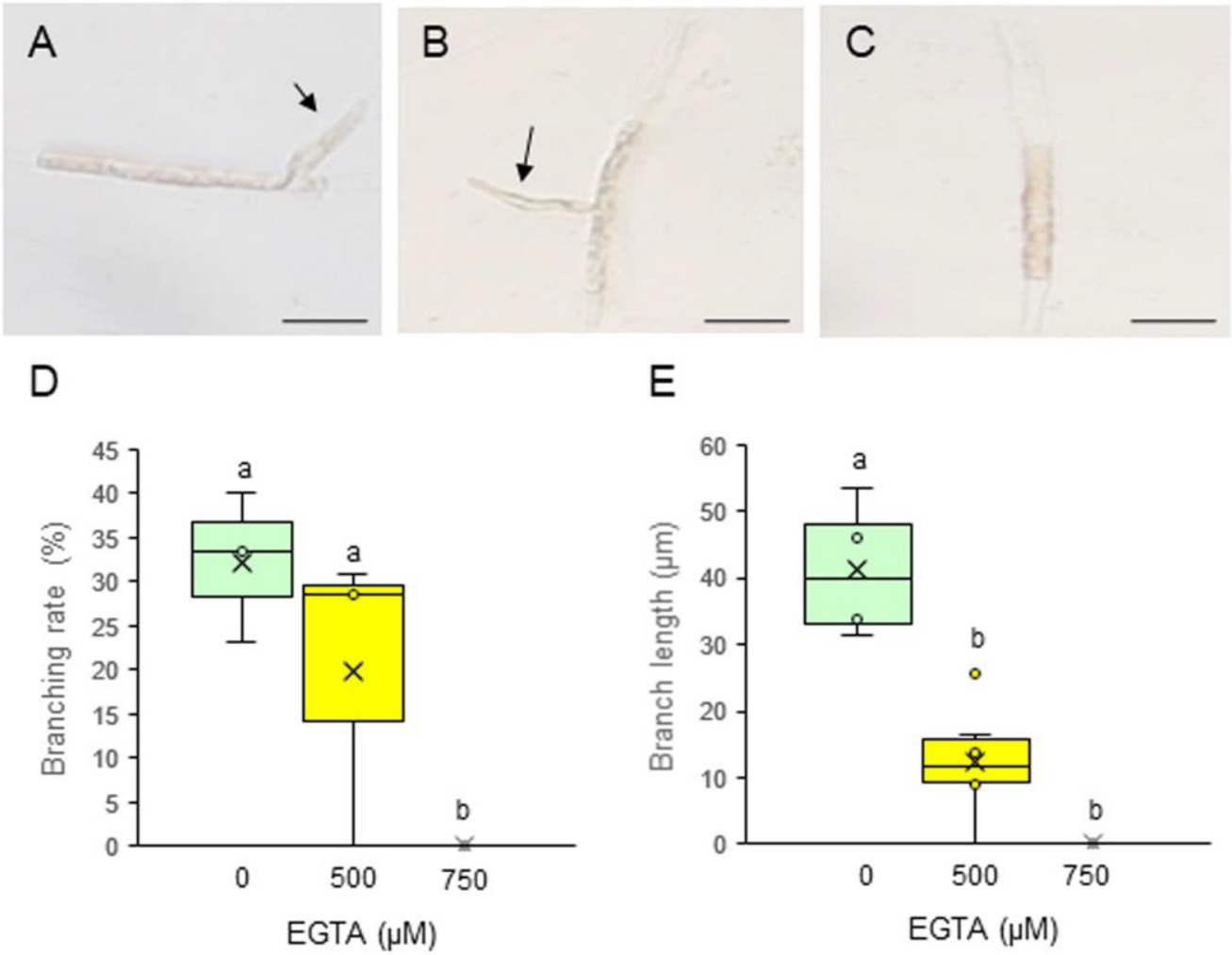


**Figure 2:** Effects of EGTA treatment on the tip growth of side branches from single-celled conchocelis. (A–C) Photographs of single-celled conchocelis treated with 0 (A), 500 (B), or 750 µM EGTA (C) for 3 days, for which each treatment was employed total 16 isolated cells for observation. Arrows indicate tip-growing branches initiated from single-celled conchocelis. Scale bars, 25 µm. (D, E) Branching rate (D) and branch length (E) after treatment of single-celled conchocelis with 0, 500, or 750 µM EGTA for 3 days. Center line, median; cross, mean; box limits, interquartile range with upper and lower quartiles; points, individual data points; whiskers, range with maximum and minimum values. Lowercase letters in (D) and (E) denote significant differences between treatments based on three independent experiments (n = 3) as determined by the Tukey–Kramer test ($p < 0.05$).

the production and tip growth of branch initials from differentiated conchocelis cells. It reminds to be elucidated whether washing out of EGTA and subsequent addition of $CaCl_2$ in the medium recover branch initiation and branch growth and whether inhibition of channel-mediated $Ca^{2+}$ influx by treatment with $LaCl_3$ prevents tip growth.

There are two important steps in the tip growth of conchocelis: the formation of branch initials in non-dividing differentiated cells (initiation of tip growth) and the polar directional growth of these initials to form elongated branches [12]. Because the treatment of single-celled conchocelis with EGTA impaired both branch initiation and branch growth, we concluded that $Ca^{2+}$ is required for both critical steps in tip growth. To confirm this proposal, measurements of the time-course of both production of branch initial and its growth in single-celled conchocelis are necessary for elucidation of a question which step requires extracellular $Ca^{2+}$ influx. In addition, we previously demonstrated that these two steps are regulated by auxin, phosphoinositide turnover, and actin polymerization [11,12]. These findings are consistent with our understanding of tip-growth regulation in the pollen tubes and root hairs of terrestrial plants [13,14,16-18,21]. It is therefore possible that the basic regulatory mechanisms governing tip growth are conserved between aquatic red algae and terrestrial plants. Factors such as $Ca^{2+}$, enzymes related to phosphoinositide turnover, and F-actin act at the cell apex to enable the polar growth of filamentous tissues such as root hairs and pollen tubes [14,16,18]; however, little is known about the spatial distribution of these factors during the initiation and elongation of branches in *P. yezoensis* conchocelis. Therefore, determining the specific regions where auxin and $Ca^{2+}$ exert their effects and characterizing the subcellular distributions of F-actin and enzymes involved in phosphoinositide turnover will be critical for fully understanding tip-growth regulation in conchocelis branches. A live-imaging technique for conchocelis tip growth has recently been reported [10]. Improvements to this system are expected to enable the visualization of $Ca^{2+}$ influx, F-actin distribution, and the localization of phosphoinositides and related enzymes, providing spatiotemporal details of polarity establishment and tip growth in *P. yezoensis* conchocelis.

Despite progress in understanding tip growth in the conchocelis, much less is known about the regulation of diffuse growth that gives rise to the foliose thallus of the gametophyte. It is remarkable that foliose and filamentous generations survive independently in *P. yezoensis* and other Bangiales [9], suggesting differences in the regulatory mechanisms governing these two growth patterns. However, the shared involvement of MTs, F-actin, and Rho GTPases in both diffuse and tip growth of terrestrial plants [1] raises the question of how common factors can regulate different types of growth. Characterizing the contrasting regulatory mechanisms that govern diffuse and tip growth will be essential for understanding the physiological and molecular bases of the distinct multicellular body plans of heteromorphic generations in *P. yezoensis*.

## Author Contributions

RI: Methodology, Investigation, Data curation, Formal analysis, Validation, Visualization.

KM: Conceptualization, Methodology, Formal analysis, Validation, Visualization, Supervision, Writing—original draft preparation and reviewing and editing, Funding acquisition.

## Conflict of Interest

The authors declare no conflicts of interest. No aspects of the study required informed consent, and the work did not involve human or animal subjects. All authors have read and agreed to authorship and to the submission of the manuscript for peer review.

## Data Availability Statement

Data are contained within the article.

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