Chapter 4

The Study of Development Using Red Algae

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Control of development in algae including the effects of hormones and environmental factors such as light.

Introduction

Algae can be useful organisms for demonstrating developmental processes. The giant-celled marine red alga *Griffithsia pacifica* Kylin (Rhodophyta, Ceramiales) is particularly suited for experiments on developmental patterns and their control. Our unialgal isolate of this alga grows well at room temperature (20 to 25°C) on a 16 hr L:8 hr D photoregime. Its large shoot cells (up to 1.0 mm long and 0.2 mm wide) can be discerned with the naked eye and may easily be studied with a dissecting microscope. Development is rapid. Single, isolated shoot cells, readily excised from vegetative filaments, regenerate plants with 30–40 shoots cells in one week. The development from single cell to multicellular plant occurs in a regular and rhythmic manner. Cell division occurs at night; cell elongation takes place primarily during the day. Patterns of morphogenesis can be altered by changing environmental conditions. At low light intensities (40–50 ft-c) plants grow as deep red, sparsely-branched filaments; at higher intensities, filaments are pale red and highly branched. All these aspects of *Griffithsia pacifica* make it an excellent subject for classroom experiments in both elementary and advanced classes. We have used the experiments described below in classes in General Biology, General Botany, Cell Biology, Phycology and Plant Physiology. For beginning classes material is presented in demonstration form. In more advanced classes, students set up experiments themselves. The experiments fall into three general categories: I. Studies on development and its control at the cellular level, II. Environmental control of photosynthetic pigment content, and III. Studies of cell enlargement using fluoresence microscopy.

Instructors' Materials
I. Three Experiments on Cellular Development and Its Control

Introduction

Plants of *G. pacifica* have two types of cells: 1) upright, pear-shaped, deeply colored shoot cells, and 2) pale, elongate, prostrate rhizoidal cells. Individual shoot cells are easily excised from filaments; each shoot cell can regenerate a whole plant. Within 24–36 hrs after it is excised from a plant, an isolated cell regenerates a shoot cell at its apex and a rhizoid cell at its base. The shoot grows by apical division and subapical branching until at the end of one week a plant with 30–40 shoot cells and several multicellular rhizoids is produced. Since regeneration occurs so rapidly, students can readily follow the course of development from single cells to whole plants. The fate of individual cells can be followed as the multicellular plant grows. Below are described three experiments which can be used to illustrate development patterns and their control. Each of these exercises can be presented as a demonstration for large elementary courses or be done by students themselves in advanced courses.

Materials Required:

Glass slides

Fine forceps

Alcohol lamps and small jars of 95% alcohol for sterilizing forceps

Single-edged razor blades

6 × 1.5 cm disposable plastic petri dishes or sterile, glass petri dishes

Sterile enriched seawater

Stock cultures of *G. pacifica* (see Appendix A)

Dissecting microscopes with transmitted light bases

Cool white fluorescent lights with a timer to give 16 hr L:8 hr D

Experiment 1: Development of Plants From Single Cells

Procedure

For a demonstration, start individual plants from single cells each day for the week preceeding the laboratory. In the lab students can follow development by comparing cultures of different ages. For an advanced class, students can start their own plants. Appendix A describes techniques for growing stock cultures of *G. pacifica.*

1. To start plants from single cells, use fine forceps which have been dipped in 95% alcohol and flamed to transfer a small clump of *Griffithsia* from a stock culture to a small petri dish which contains enriched seawater. Sterile technique should be used to avoid contamination of cultures with other algae or fungi.

2. A few filaments from this dish should be placed on a clean microscope slide and viewed with a dissecting microscope. The fourth or fifth cell from the apex of a filament can be isolated by cutting through the cells on either side of it with a razor blade, which has been sterilized by being dipped in alcohol and flamed. The severed cells are thus killed and the desired cell can be isolated intact. (Figure 4.1)

3. Pick up the single cell by grasping the wall of one of the severed cells with fine forceps and transfer the cell to 10 ml of enriched seawater in a 6 × 1.5 cm disposable plastic petri dish.

4. Collect single shoot cells in the dish. Examine them after 15 min to see if they are alive. Dead cells will turn a bright fluorescent pink.

5. Now transfer living cells to individual petri dishes. Each student should start at least 3 dishes.

6. Put the petri dishes at 300 ft-c, 20 to 25°C and long days (16 hr L:8 hr D).
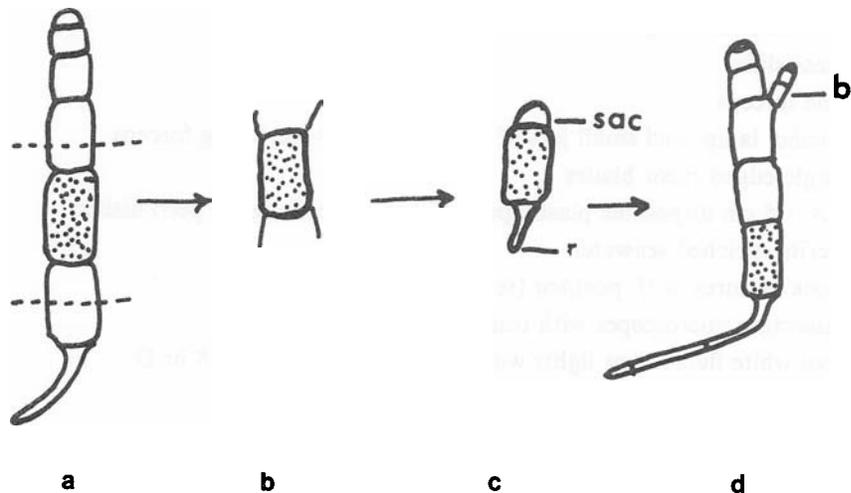
Figure 4.1. Isolation of single cells and regeneration of plants. a. A single shoot cell is excised by slicing through the cells on either side of it. b. day 0: isolated cell. c. day 1: cell has regenerated a new shoot apical cell from its apex and a rhizoid cell from its base. d. day 4: a small plant has been produced. (r= rhizoid cell, sac= shoot apical cell, b= branch)

7. Use a dissecting microscope to observe plants at 12–24 hr intervals. Make a drawing of each plant at each observation time. If possible measure the length of the first and second shoot cell produced. Follow development for at least 1 week.
8. Which cells divide? When does cell division occur? Which cells elongate? When does cell elongation occur? How are branches formed?

Expected results

The first divisions of isolated shoot cells should take place within 24 hrs after isolation. (Figure 4.1). Duffield et al. (1972) and Waaland and Cleland (1972) provide good descriptions of the process of regeneration. In *Griffithsia* only the apical cell of a filament or branch divides to increase the length of the filament. Branches are formed by budding on the sides of intercalary cells near a filament apex. Each branch is indeterminate. Division of the apical cells occurs with a diurnal rhythm just after the beginning of the dark cycle. The time of division can be changed by changing when day and night occur. Students can grow plants on a reversed cycle (dark from noon to 8 PM) to observe cell division; the rhythm will continue for at least a week under continuous light.

While only apical cells divide, intercalary cells do increase in length for 5–6 days after they are formed. Cell elongation is most rapid in the early part of the day and slows at night. Cells bearing branches grow for a shorter time than do cells without branches.

Experiment 2: Environmental Control of Development

The pattern of regeneration of plants from single cells is strongly influenced by light intensity. Comparison of the morphology of plants grown at low and high light intensities demonstrates how environmental conditions may drastically influence the appearance of a plant.

Procedure

1. Isolate 6–8 individual shoot cells as described above (Experiment 1).
2. Put each in a 6 × 1.5 cm petri dish with 10 ml enriched seawater.
3. Grow half the plants at 50 ft-c and half at 300 ft-c; both should be grown at 20–25°C and 16 hr L:8 hr D.
4. Plants can be observed every 24 hrs to see how different morphologies develop. As in Experiment 1, drawings should be made and cell lengths measured at each observation time.
5. What makes plants grown at low intensities look so different from those grown at high intensities? Does the rate of cell division change? Does the rate of cell elongation change?

Expected Results

At the end of one week, the difference in the morphology of the plants will be quite evident (Table 4.1). At low intensities (40–50 ft-c), plants grow as unbranched or sparsely branched filaments. At higher intensities (300 ft-c), plants are highly branched. The rate of cell division does not change, but the number of dividing apical cells is much higher at the higher intensity (Waaland and Cleland 1972).

Experiment 3: Phototropism

Plants of *G. pacifica* respond rapidly to unilateral illumination. This experiment provides a good demonstration of phototropism in lower plants.

Table 4.1 The effect of light intensity on apical cell division and branch formation

Light intensity	Plants After One Week		
	# of cells	# of branches	# divisions per apical cell per day
50 ft-c	7	0.8	1
300 ft-c	32	8.2	1

Procedure

1. Start at least 4 plants from single cells as described in Experiment 1.
2. Grow for 4–5 days at 50 ft-c, 20–25°C, at least 16 hr light per day.
3. Put in unilateral light.
4. Observe under a dissecting microscope after 3 hrs to see rhizoid phototropism. Observe again at 24 hrs to see shoot phototropism.

Expected results

Rhizoids are negatively phototropic and begin to grow away from unilateral light in 2–3 hours. Curvature can be seen with a dissecting microscope at 2.5 to 3 hrs, and with the unaided eye after 6 to 8 hrs (Waaland et al. 1977). Shoots are positively phototropic and grow toward unilateral light within 18 to 24 hrs. Shoot phototropism can be observed with the unaided eye by 18 to 24 hrs.

Students can do a rough action spectrum to see which colors of light stimulate phototropism. In *Griffithsia,* as in higher plants, blue light is the most effective color of light for phototropism.

II. Environmental Control of Photosynthetic Pigment Content

Introduction

Griffithsia is a good organism with which to demonstrate the photosynthetic pigments found in red algae. Because its cells are large and thin-walled, they are easy to disrupt. Thus it is relatively easy to extract the phycobiliproteins and chlorophyll a. In *Griffithsia* R-phycoerythrin is by far the most abundant phycobiliprotein pigment present. The light intensity under which plants are grown regulates the relative concentrations of phycobiliproteins and chlorophyll a. The effect of varying light intensity on pigment content can be demonstrated both qualitatively, by observing whole plants, and quantitatively, by extracting the pigments and measuring their concentrations.

Materials required

Two stock cultures of *G. pacifica:* grown for 2 weeks to a month at 50 ft-c and 300 ft-c

Mortar and pestle

Distilled water

80% acetone

50 ml centrifuge tubes (glass)

Clinical centrifuge

Spectrophotometer

Procedure

1. Plants grown at 50 ft-c should be deep red in color while those grown at 300 ft-c should be brownish red.
2. Remove a portion of each stock culture. Blot gently on a towel; weigh. Keep the two samples of plant separate and follow the procedure outlined below for each sample.
3. Put the plant material in a test tube and cover with ice-cold distilled water. Let stand for 5 to 15 min on ice; during this time, many of the cells will explode.
4. Pour the water and plant material into a chilled mortar. Add 20 ml ice-cold distilled water. Grind without sand for 5 min until all cells are broken. Pour liquid and cellular debris into a 50 ml glass centrifuge tube. Rinse mortar and add rinse water to solution in test tube.
5. Centrifuge at full speed in a clinical centrifuge for 15 min to pellet chlorophyll-containing membranes and cellular debris.
6. Decant and save the supernatant which contains the water-soluble phycoerythrin. If the pellet still contains red color, reextract it with distilled water and repellet membranes.
7. Extract green, chlorophyll-containing pellet with 80% acetone; if necessary, centrifuge this solution to clarify it. The supernatant will contain the chlorophyll a.
8. To determine the amount of phycoerythrin present in each sample, measure the volume of the water extract. Then use a spectrophotometer to measure its absorption at 566 nm. To calculate the mg of phycoerythrin in the extract, use the following formula.

$$\frac{\text{O.D. at 566 nm}}{8.20} \times \text{Volume (in ml) of extract} = \text{Total mg Phycoerythrin in extract}$$

9. To determine the amount of chlorophyll a present in each sample, measure the volume of the acetone extract. Then measure its absorption at 663 nm. To calculate the mg of chlorophyll a in the extract, use the following formula:

$$\frac{\text{O.D. at 663 nm}}{8.20} \times \text{Volume (in ml) of extract} = \text{Total mg chlorophyll a in extract}$$

10. Now calculate the ratio of chlorophyll a to phycoerythrin for each sample.
11. How does the ratio of phycoerythrin to chlorophyll a differ between the plants grown at the two light intensities?

TABLE 4.2 The effect of light intensity on photosynthetic pigment content.

Light intensity	Phycoerythrin/chlorophyll a (mg/mg)
50 ft-c	1.5
300 ft-c	0.8

Expected results

The ratio of (mg Phycoerythrin): (mg chlorophyll a) will be much higher in plants grown at 50 ft-c than in those grown at 300 ft-c (Table 4.2). (Waaland et al. 1974).

If a scanning spectrophotometer is available absorption spectra for the phycobiliproteins and chlorophyll can be measured and compared. NOTE: The calculations of phycoerythrin and chlorophyll a concentrations described above assume that the optical pathlength through the samples is 1 cm.

III. Cell Elongation Patterns: Study Using Fluorescent Label

Introduction

This exercise illustrates a technique for studying patterns of cell elongation in unicellular and filamentous organisms and structures. The site of addition of new cell wall material to an elongating cell can be detected if a label can be used which will label either the new wall added after a particular point in the experiment, or the old wall, present at the start of the experiment. The former type of labelling is usually done by using radioactive wall precursors whose presence can be detected by autoradiography. The latter type of labelling may be done by applying particulate matter (charcoal grains, resin beads, or starch grains) to the wall or by using a vital stain for cell walls; in this way the old wall is labelled and can be distinguished from the newly synthesized wall which is unlabelled. In this exercise we will use a fluorescent label, Calcofluor White, which stains cellulose and chitin, to study cell elongation patterns in cells of *Griffithsia pacifica*. These techniques may also be tried on other red algae such as *Callithamnion* and *Antithamnion,* and on fungal hyphae, pollen tubes, root hairs, other algae and other filamentous structures.

Calcofluor White can be detected by the use of fluorescence microscopy. Fluorescence microscopy makes use of the fact that many chemical substances will absorb light of one wavelength and reemit light (fluoresce) at a longer wavelength. In biological materials the *exciting radiation* is typically ultraviolet light. It may be short wavelength UV (200–300 nm) or long wavelength UV (300–400 nm). If shorter wavelength UV is to be used, special optics

made of quartz or mirrors must be used because normal glass absorbs these wavelengths strongly.

In the fluorescence microscope, one wants to illuminate the specimen with UV light and then block out this light with filters so that only the light which is fluoresced by the specimen reaches the observer's eye. The exciting UV light is passed through a filter which removes all visible wavelengths of light. This filter, called the *exciter filter,* is chosen so that it will transmit wavelengths of light which will be absorbed by the specimen. The fluorescence of the specimen then enters the microscope objective, but so does some of the exciting light. To get rid of this UV light, a *barrier filter* (or filters) is placed betweeen the specimen and the observer's eye. The barrier filter combination is chosen such that all the UV light is absorbed, but the wavelengths of light fluoresced by the specimen are transmitted to the eye. *Because UV light is harmful to human eyes, one should always be sure that the barrier filters are in position before looking through the microscope.*

Materials

Week-old plants of *G. pacifica* started from single cells

Enriched seawater

0.01% w/v Calcofluor White ST powder (available from Polysciences, Warrington, PA 18976) in enriched seawater—prepared 24 hrs in advance to allow powder to dissolve

Monocular microscope

UV absorbing goggles

Exciting Radiation: 360 nm, long Wave UV (See Appendix B from UV light sources)

Barrier Filter: Use a Zeiss 41 filter or a yellow K-2 photographic filter to act as a barrier between the specimen and your eyes

Procedure

1. Set up the fluorescence microscope as follows. First, put on UV-absorbing goggles to protect your eyes from stray UV light. Put the barrier filter over the ocular of the microscope. (This filter absorbs UV light and transmits the wavelengths of light which are fluoresced by Calcofluor). Now direct UV light up through the condensor and the specimen. If you are using a low-intensity light source, the microscope should be set up in a totally dark room, and you will need to dark-adapt your eyes.
2. Filaments with growing cells should be stained by placing them in 0.01% w/v Calcofluor White in enriched seawater in a 6 X 1.5 cm petri dish for 20–30 min. Swirl the dish several times during the labelling period. Plants

should then be washed in three 5-min washes of enriched seawater without dye. Cells should be examined immediately in the fluorescence microscope to see if the cell walls have stained and to see what the pattern of staining is before growth occurs. Measure and record cell lengths.

3. After about 24 to 48 hrs of growth, remeasure the lengths of cells to be sure that they have grown. Now the plants should be examined in the fluorescence microscope to see where new material has been added to growing cells walls. The new material will appear dark (unlabelled) in UV light while the old material will fluoresce.

4. Diagram cells showing where pre-existing wall material was and which part of the wall is new.

5. If one wants to make quantitative measurements plants should be photographed at the beginning of the experiment and again at the end of the experiment in both visible and UV light. The photographs made with visible light can be used to measure how much cell elongation has occurred; the photographs made with UV light will show where new cell wall material has been added to the wall during elongation. If low intensity light sources are used photographs should be taken using either TRI-X film which should then be developed with Acufine to give an ASA of approximately 1200, or Polaroid High Speed Black and White film (ASA 3000). Make trial exposures to determine proper exposure times. Depending on the intensity of the UV source exposures may need to be as long as 4 min.

Expected results

In *Griffithsia pacifica* new material is added to the cell wall in bands which are located near the top and bottom of each growing intercalary call (Waaland et al. 1972; Waaland and Waaland 1975). This is also true for a number of other filamentous red algae. If this technique is used with cells which elongate along their total length, then the dye fluorescence will become dimmer as cells elongate, but there will be no distinct bands of new wall material. Because chloroplasts absorb some of the light fluoresced by Calcofluor, varying concentrations of chloroplasts along a cell may cause spurious banding patterns. To check for this, isolate cell walls and examine their staining patterns.

References

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APPENDIX A
General Culture Conditions

1. Organism:

The organism used in these experiments is *Griffithsia pacifica,* a marine red alga. This species of *Griffithsia* grows intertidally and subtidally along the West Coast of North America. Other species of the genus, such as *G. globulifera* can be collected on the East Coast of the U.S.A.

II. Culture Media:

This alga will grow in a variety of enriched seawater media, including Provasoi's enriched seawater (PES) and Guillard's f/2 (see McLachlan 1973) or in artificial sea salt media such as "Instant Ocean" to which the vitamin and mineral enrichment of PES or f/2 has been added. All media should be sterilized before use.

III. Culture Conditions:

Although cultures of *Griffithsia* are not axenic, sterile technique should be used in handling them to avoid introduction of other algae, fungi or foreign bacteria. Fine forceps can be used to transfer plants from dish to dish. These can be sterilized by dipping them in 95% ethanol and flaming them.

Stock cultures are grown in deep culture dishes or in 400 to 800 ml beakers covered with plastic wrap or in 500 ml Erlenmeyer flasks at 20 to 25°C under 50–300 ft-c illumination from cool white fluorescent lamps on a 16 hr L: 8 hr D cycle. Fastest growth occurs at 300 ft-c. Light intensities higher than 500 ft-c may cause cell bleaching and death. The culture medium should be changed at fortnightly or monthly intervals. For best growth the volume of plant material should not exceed ¼ to ⅓ the volume of the medium.

APPENDIX B
Ultraviolet Light Sources

A number of UV light sources can be used for fluorescence microscopy. The brightness of the specimen fluorescence depends in part on the intensity of the exciting light. With low intensity sources especially, microscopes will need to be set up in a totally dark room so that the fluorescence can be seen.

1. The least expensive UV light sources are black light fluorescent tubes. These are available for use in standard fluorescent light fixtures or as "light sticks" or wands (G.E. "Bright Stik"). The fluorescent tubes are placed under the microscope conden-

sor; two student microscopes can share one tube. Cover the tube between microscopes with aluminum foil to eliminate stray UV radiation.

2. Another relatively inexpensive light source is a 4 W, longwave UV (365 nm) mineral light. This is available from Ultraviolet Products, San Gabriel, CA as Model UVL-21.

3. A more powerful light source is a 100 W low pressure UV lamp (G.E. Mercury Reflector Spot). A metal tube should be constructed to funnel light from this type of lamp to the substage mirror of the microscope. A UG-1 exciter filter must be used if a broad-band UV source is used.

4. Finally there is a variety of high-pressure mercury or xenon arc lamps available from various microscope manufacturers. These range up to a brightness of 200 W and are very expensive. *CAUTION:* Protect eyes and skin from stray UV light; protect eyes, skin and optics from excessive heat produced by high intensity lamps.