Algal Research 17 (2016) 293–299

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Chloroplast virus causes green-spot disease in cultivated *Pyropia* of Korea

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ARTICLE INFO

Article history:
Received 5 February 2016
Received in revised form 14 May 2016
Accepted 21 May 2016
Available online xxxx

Keywords:
Anaaki disease
Green-spot disease
Pyropia
Red algae
Virus

ABSTRACT

Viruses are potential regulators of primary production in marine algal populations, which also manipulate the life history and evolution of their hosts. Recent development of an intensive algal aquaculture has enabled emerging viral diseases to spread faster than ever. The pathogen of green-spot disease which causes serious economic loss to *Pyropia* sea farms in Korea is confirmed for the first time. Ultrastructural study and infection experiment showed that the pathogen is a novel virus, PyroV1 (*Pyropia*-infecting virus 1), that causes cellular lysis in the blade of the three most commonly cultivated *Pyropia* species in Korea; *Pyropia yezoensis*, *Pyropia tenera*, and *Pyropia dentata*. Susceptibility test showed that PyroV1 could not infect the conchocelis filaments of the above species nor 16 strains of other red algae, including *Pyropia plicata* from New Zealand and an Australian strain of *Porphyra lucasii*. The shape of PyroV1 was isometric, apparently spherical and up to 100 nm in diameter. Chloroplast of the infected cells showed loosely arranged thylakoids and enlarged pyrenoid. Most viral particles were observed around the pyrenoid of the chloroplast and none of them were found in the cytoplasm. In the infected cells, floridean starch granules disappeared and the cytoplasm was filled with swollen vesicles. The burst size was roughly 400–500 infectious units/cell and the latent period was <36 h. To the best of our knowledge, this is the first report of an isolation of a virus, which infects and causes disease in marine red algae.

1. Introduction

Viruses are the most abundant biological entities in the oceans and are known to infect representatives of every major phylum, causing cellular lysis upon completion of their life cycle [1]. Viruses may affect the evolution of their hosts through gene swapping between compatible hosts [2]. Viruses from one environment can successfully infect and replicate in marine organisms from unrelated environments, contributing to the movement of genes between different ecosystems [[3].] The recent development of intensive marine aquaculture may radically change viral abundance and increase viral diseases in marine ecosystems. Aquaculture has been one of the fastest growing global industries with total annual production exceeding 50 million tons and estimated value of almost US$ 100 billion, which has overtaken world beef production recently [4]. Sea farmers are now challenged with many new emerging viral diseases, some of which are already established in cultivation beds and have begun to affect surrounding environments [5–6].

In marine eukaryotic algae, over 50 different viruses or virus-like particles (VLPs) have been isolated and characterized during the last three decades [7–8]. Algal viruses play an important ecological role in regulating the population dynamics of their host algae [9]. Recently, algal viruses have attracted public attention because some algal viruses were shown to infect human and cause a reduced cognitive functioning [10]. Viral diseases have never been reported in red algae although many extensively cultivated commercial algae are included in this phylum. Virus-like particles (VLPs) were reported in the cytoplasm of some red algae [11]. Two RNA viruses have been isolated recently from the surface microbiome of *Delisea pulchra* [8]. However, no red algal virus has been isolated or shown to infect its host.

Green-spot disease (GSD), similar to Anaaki disease that was reported in Japanese *Pyropia* farms, is one of the most serious diseases in Korean *Pyropia* sea farms, causing economic losses of millions of US dollar every year [6]. The symptoms of GSD include the development of numerous holes on the infected blades [12]. Aerobic Gram-negative bacteria, such as *Pseudomonas* and *Vibrio*, were first claimed to be the causative agents of this disease in *P. yezoensis* [12]. A *Flavobacterium* LAD-1 was isolated from the blades of *P. yezoensis*, which were severely infected with the disease and was proposed as the pathogen for Anaaki disease [13]. Although the above bacteria could macerate *Pyropia* blades in certain laboratory conditions and form pin holes on the blades, the progress of the disease and morphological symptoms were different from those observed in GSD [6]. The infection process of GSD on *Pyropia* does not follow typical maceration process as seen in the above experiments, but shows successive cellular lysis forming a row of dead cells.

In the present study, we isolated and characterized a novel chloroplast virus which causes GSD on the blades of three important commercial algae, *P. yezoensis*, *P. tenera*, and *P. dentata*. The morphological symptoms, susceptibility and progress of the disease were described using time-lapse videography and transmission electron microscopy.

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http://dx.doi.org/10.1016/j.algal.2016.05.023
2211-9264/

2. Materials and methods

2.1. Sample collection and culture

P. yezoensis blades infected with green-spot disease were collected from commercial farms in Seocheon (36°12′ N 126°50′ E) on 21 March, 2012 and Jindo (34°46′ N 126°36′ E) in March–April 2013. Host plants were kept in MGM medium [14] with constant aeration at 10 °C and 30 μmol m^{-2} s^{-1} irradiance (16 L, 8D).

Infected blades of *Pyropia* were stored at −78 °C with 20% dimethyl sulfoxide (DMSO) as a cryoprotectant. Cultures were also maintained by transferring a piece of infected blade to MGM medium containing healthy *P. tenera* blades every 2 days.

2.2. Infection experiment using ultra-filtrated lysate

Freshly cultured *P. tenera* blades were incubated with an infected blade in 1.8 L of MGM medium for 3 days until cellular lysis occurred on the thalli. The thalli were removed and the resultant lysate was sequentially passed through 4.3 μm, 1.2 μm, 0.45 μm, 0.22 μm and 0.1 μm filters to remove cell debris and bacteria. Finally, the lysate was concentrated to 50 mL using 100 kDa molecular membrane filter and was stored in the deep freezer at −78 °C. To check if any bacteria have passed through the filter, a drop of the concentrated solution was smeared on the marine agar plate and incubated in 37 °C chamber for 48 h. When no bacterial growth was observed from the assay, the concentrated lysate solution was stored at 4 °C and used for infection experiment.

To determine the latent period of the virus, freshly cultured healthy *P. tenera* thalli were cut into pieces with razor, incubated in a concentrated lysate of the virus for 24 h. Two thalli were taken from the solution every 6 h and transferred to a fresh MGM medium. The total required time for the successful infection of the newly introduced *P. tenera* blade was used as the latent period of the virus.

2.3. Host susceptibility test

Sixteen strains of marine macroalgae including blades and conchocelis filaments of four *Pyropia* species were incubated with the concentrated lysate for 5 days (Table 1). Each strain was observed every 2 days to check if cellular lysis occurred. As *P. tenera* thalli showed GSD symptom in 2 days of incubation, the tested strains were incubated with the concentrated lysate about three times longer than *P. tenera*. When infection was observed the thalli were transferred to fresh MGM medium and checked to see if the typical pinkish lesion area surrounding green spot in the center developed.

Table 1
Infection specificity of PyroV1 against marine macroalgal strains.
All unialgal strains were cultured at 15 °C, 30 μmol m^{-2} s^{-1} irradiance (12 L, 12D).

Species	Result of infection by PyroV1	Country of strain origin
Pyropia dentata (blade)	+	Korea
Pyropia tenera (blade)	+	Korea
Pyropia yezoensis (blade)	+	Korea
Pyropia plicata (blade)	−	New Zealand
Porphyra lucasii (blade)	−	Australia
P. dentata (conchocelis)	−	Korea
P. tenera (conchocelis)	−	Korea
P. yezoensis (conchocelis)	−	Korea
P. lucasii (conchocelis)	−	Australia
Bostrychia tenuissima	−	Australia
Bostrychia moritziana	−	Madagascar
Dasysiphonia chejuensis	−	Korea
Griffithsia monilis	−	Australia
Heterosiphonia japonica	−	Korea
Bryopsis pennatula	−	Korea, *Chlorophyta
Bryopsis plumosa	−	Korea, *Chlorophyta

2.4. Microscopy

Light micrographs were taken with Olympus DP73 camera affixed to an Olympus BX50 microscope (Olympus, Tokyo, Japan). For time-lapse video-microscopy, the infected algal thalli were placed on a glass slide and a coverslip was lowered and sealed with VALAP (1:1:1; vaseline/lanolin/paraffin) melted on a hot plate at 70 °C. The slide preparations were examined and recorded using a digital imaging time-lapse recorder (AllBT, Daejon, Korea) mounted on the microscope.

For transmission electron microscopy (TEM), *P. yezoensis* blades infected with GSD in the laboratory were fixed in 2.5% glutaraldehyde in PBS buffer with an adjusted sodium chloride concentration at 4 °C for 2 h. Samples were processed for TEM on the same day or 1 day after they were collected. Samples were then rinsed with the same buffer and post-fixed with 1% osmium tetroxide at 4 °C for 2 h. Thereafter, the tissues were rinsed out with PBS buffer and were dehydrated in a graded ethanol series with 10% increments (each step was 20 min), embedded in Spurr's epoxy resin and polymerized overnight at 72 °C oven (Polysciences Inc., USA). Resin-embedded samples were sectioned with a diamond knife, and thin sections stained with TI Blue (Nisshin EM) for 30 min and 2% (w/v) lead citrate for 10 min were viewed and photographed on a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan).

3. Results

Green-spot disease in *Pyropia* was recognizable by the numerous holes on the blades which coalesced to make bigger holes and eventually lead to lysis of the whole blade (Fig. 1a). Cultured *P. yezoensis* blades were incubated with a solution containing 0.1 μm filtered lysate from infected blades to observe the infection process. In the initial stages of infection, chains of green cells radiated from the center forming a green spot (Fig. 1b). A chain of pinkish cells formed a distinct circular border around the green cells (Fig. 1c). The lesions occurred anywhere on the blade, but usually the infection was much faster in wounded regions. As lesions grew (Fig. 1d), numerous bacteria forming a slimy film, developed at the center of the lesion. Thereafter, holes coalesced on the blades and whole blades broke down in a day or two (Fig. 1e). Numerous vesicles developed in the cells before cellular lysis occurred (Fig. 1f). Successive cellular lysis of neighboring cells resulted in the growth of a pinkish chain of dead cells (Fig. 1g, Suppl. video 1). As estimated from the time-lapse video-microscopy, it took about 5–15 min for the infected cell to undergo large vesicle formation and collapse (Fig. 1f). Approximately 10–20 neighboring cells underwent cellular lysis and formed a pinkish chain of dead cells. When observed in slow motion, under time-lapse video-microscopy, it appeared as though infected cells melted and fused with each other. Dead cells leaked cytoplasm, therefore bacterial contamination followed. Later, the pinkish lesions turned green and many bacteria were observed in the region.

When the concentrated lysate was stored at 4 °C in the dark the infectivity was maintained over 6 months and it could be maintained for even longer periods at −78 °C. Successive infection of blades in culture was maintained for over 30 generations. Before the infection of sixteen experimental strains the algal thalli were cut with a razor blade to aid infection. Although the virus was isolated initially from field-collected *P. yezoensis*, it infected thalli of *P. yezoensis* and *P. tenera* as well as *P. dentata* in 1–3 days. *P. tenera* showed the symptoms of infection earlier (1–2 days) than the other two species. It is noteworthy that no infection was observed in any conchocelis (the diploid stage) cells of the above species. The blades of *Pyporia plicata* from New Zealand and *Porphyra lucasii* from Australia showed no signs of infection. No other tested macroalgae showed any sign of infection (Table 1).

Ultrastructure of the infected cell was quite different from normal cells (Fig. 2). Cytoplasm of healthy uninfected cells of *Pyropia* contained abundant floridean starch granules, observed as electron-dense black granules under TEM (Fig. 2a). Also, healthy cells had numerous irregular