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Artificial Reestablishment of the Kelp and Red Algal Symbiosis

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A type of symbiosis was previously described from nature in which the gametophytes of Laminariales were endophytic in filamentous red algae. Here we reconstruct this symbiosis for the first time in laboratory culture using zoospores of the kelp, *Undaria pinnatifida*, and the red alga, *Aglaothamnion oosumiense*. Zoospores of *U. pinnatifida* readily attached to *A. oosumiense*. In 48 h these spores germinated and the initial germ tube penetrated into the host cell wall leaving only an empty zoospore wall outside the host. Within ten days, four to five-celled endophytic gametophytes were present. Zoospores of *Laminaria religiosa* which were also inoculated into cultures of *A. oosumiense* rarely attached to the red alga and never became endophytic. Within ten days the free-living gametophytes of *L. religiosa* on cover slips became fertile and produced young sporophytes. These observations demonstrate the ability of *U. pinnatifida* to become endophytic, and show differences in host specificity among kelp species.

The life history of Laminariales has been well established in culture since the early part of this century (Sauvageau, 1915; 1918). Accordingly, the life history consists of a macroscopic diploid, spore-producing sporophytic generation that alternates with a microscopic, haploid, gamete producing gametophytic generation (Bold and Wynne, 1985). This life history has been confirmed in all species that have been investigated by means of culture studies (e.g. Hollenberg, 1939; Cole, 1968; Lüning and Neushul, 1978; Lüning, 1980).

The ecology of the haploid phase of Laminariales has remained poorly understood primarily because of the difficulty in studying microscopic phases *in situ* when their presumed substratum were rock surfaces. Thus, only a few studies record observations of gametophytes on natural substrata (e.g. Funano, 1969; Kaneko, 1973), and these are mostly from cultivated kelp beds or artificial substrata (Garbary et al., 1999b). These difficulties were partly overcome by using settlement plates (mostly glass) that could subsequently be examined in the laboratory (e.g. Reed et al., 1988), or by settling spores onto substrata in the laboratory and outplanting the substrata into nature (e.g. Hsiao and Druehl, 1973). More recently, Garbary et al. (1999a, b) described a type of symbiosis in which the gametophytes of kelp were found as endosymbionts in the cell walls of primarily filamentous and polysiphonous red algae. This work raised the possibility that the cell walls of red algae were a primary substratum for haploid kelp. Among the limitations of this work were that: 1) it was based exclusively on field observations that did not permit the identification of the kelp species involved, and 2) the degree of host specificity could not be evaluated. Here we present the artificial reestablishment of the symbiosis in culture and provide the first evidence of host specificity among kelp gametophytes.

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Materials and Methods

Reproductive portions of fronds of *Undaria pinnatifida* (Harvey) Suringar were collected from a wild population at Namae-Do on the southern coast of Korea. Reproductive fronds of *Laminaria religiosa* Miyabe were collected from a cultivated population at Haenam on the southwestern coast of Korea. After collecting samples, sori and associated stipe or blade tissue were wrapped in moist paper towel and refrigerated. For release of zoospores, sori were cut from the plants and immersed in sterile, filtered seawater. A unialgal culture of female plants of *Aglaothamnion oosumiense* Itono that was originally isolated from Eochundo, the western coast of Korea (Cha and Kim, 1998), was used

in the infection experiments. Plants were placed in 250 ml of IMP medium and 20 ml of seawater containing a dense solution of recently released zoospores of *U. pinnatifida* and *L. religiosa* was added to each culture vessel. Coverslips were placed at the bottom of the dish to serve as a control for germination. The zoospore/red algal culture was placed at 18°C under continuous light of 20-25 µmol photons cm^{-2} sec^{-1} provided by an 80 watt, white fluorescent bulb. Plants were initially examined after two days, and then at day 5, 10, and 15. Culture medium was changed after 3 days and at day 10. The experiment was terminated at 15 days because of the growth of contaminating diatoms.

For observation, small clumps of the host were removed from the culture dish and mounted on a slide. All photography was carried out using a Zeiss Axioskop microscope equipped with differential interference optics and with a digital camera and image analysis system by Seung Won (Seoul, model SW-961), equipped with the software package Image-Pro Plus v. 2.0 (©Media Cybernetics). Digital images were saved as TIF files and subsequently manipulated with Photoshop v. 4.0.1 prior to printing with a high resolution Digital Image printer (Pictography 3000).

Results

Spores of *Laminaria religiosa* successfully germinated on the cover slips. Spores occasionally attached to the cell wall surface of the host, but gametophytes did not become endophytic. Spores of *L. religiosa* readily attached to the bottom of the dish and germinated. Germ tubes tended to be very short (Fig. 1A and D) and all of the cytoplasm from the spore moved into the initial cell. Within ten days spores had germinated, formed both antheridia and oogonia, and fertilization had occurred to produce young zygotes that were up to seven cells (Fig. 1). Female gametophytes were very small and tended to produce oogonia directly upon spore germination. Male gametophytes were considerably larger and formed two to ten cells prior to forming antheridia (Fig. 1). Following initiation of sporophytes, further development did not occur.

Germination and growth of *Undaria pinnatifida* was excellent on the control cover slips and floating debris in the medium (Fig. 2). A dense settlement of spores occurred, and within two days there was extensive spore germination. Spores tended to form a long germination tube and cells had discoid chloroplasts without chloroplasts typical of Laminariales (Fig. 2A). Within five days, 2-5 celled gametophytes were present. Maturation of gametophytes was not observed, and cultures were abandoned after two weeks because of heavy growth of diatoms.

Numerous zoospores of *U. pinnatifida* attached to *A. oosumiense* and germination occurred within 48 h (Fig. 3A). Spores formed a germ tube into which all of the cytoplasm moved, leaving the empty spore wall behind. The germ tube typically penetrated into the cell wall of the host leaving only the empty zoospore wall on the surface (Fig. 3B-E). Typically, the germ tubes penetrated directly into the host cell wall, and there was

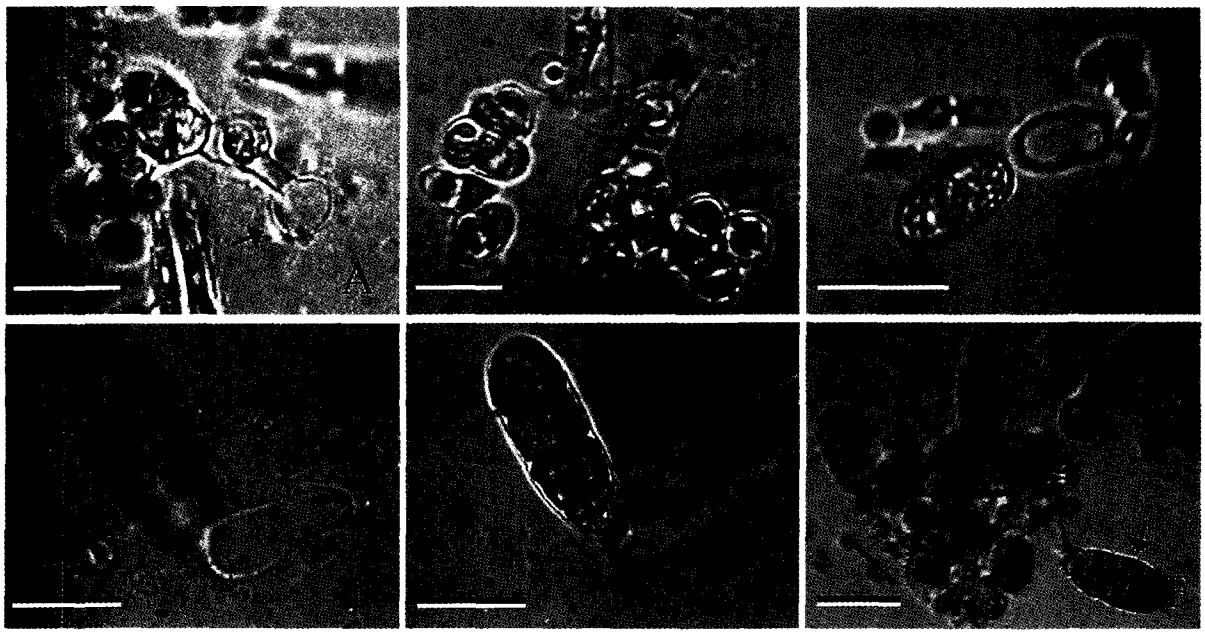

Fig. 1. Gametophyte development in *Laminaria religiosa*. A, B, Mature gametophytes with many antheridia and empty spore (arrows). C, Female gametophyte with egg and empty oogonial wall (o). D, Empty oogonial wall (o) with attached young sporophyte (out of focal plane). Note original spore (arrow) and germ tube of thallus. E, Different focal plane of plant in D with young sporophyte showing transverse divisions. F, Cluster of gametophytes with young sporophytes (arrows). Scale bars=5 µm (A, B), 10 µm (D, E), and 15 µm (C, F).

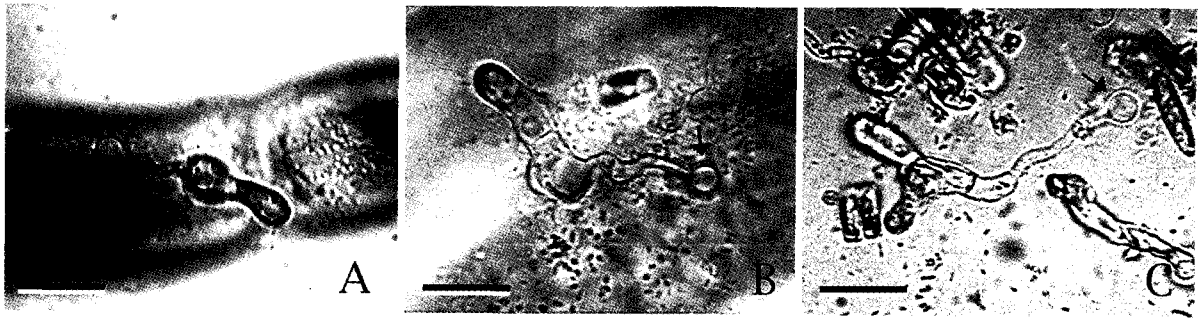

Fig. 2. Free-living gametophytic plants (A-C) of *Undaria pinnatifida* showing empty spore germination (arrows), long germination tubes and filamentous construction. Note discoid chloroplasts (C) without pyrenoids in cell of kelp gametophyte. Scale bars=10 μm.

little tendency for the germ tube to grow along the host surface. After penetrating the host, only the empty spore wall was left on the host surface. At five days the gametophytes were entirely within the host cell wall and were usually multicellular (Fig. 3D). At ten days gametophytes were three to five celled and growing parallel the surface of the host cell wall (Fig. 3E) and the empty spore wall was often present on the host surface. By 15 days, vegetative growth had become more extensive, but there was no evidence of reproduction (Fig. 3F). The original spore walls had disappeared. In all endophytic gametophytes the filaments grew only in the host cell wall. Penetration of the gametophyte into the cytoplasm of the host, or even the inner portion of the cell wall parallel to the protoplast was not observed. At 15 days, cultures were discarded as a result of diatom growth.

Discussion

Previous reports of Laminariales endophytic in red algae were based exclusively on field observations from a limited geographic area in the San Juan Islands of Washington State (U.S.A.) (Garbary et al., 1999a, b). In addition to the limited geographic extent of the symbiosis, those studies did not identify any of the kelp species involved. Although seventeen species of red algae were hosts (Garbary et al., 1999b), the kelp species involved in the symbiosis could not be identified. Thus it is possible that all the observations were from a single kelp species, and that the endophytism was associated with a particular species of kelp rather

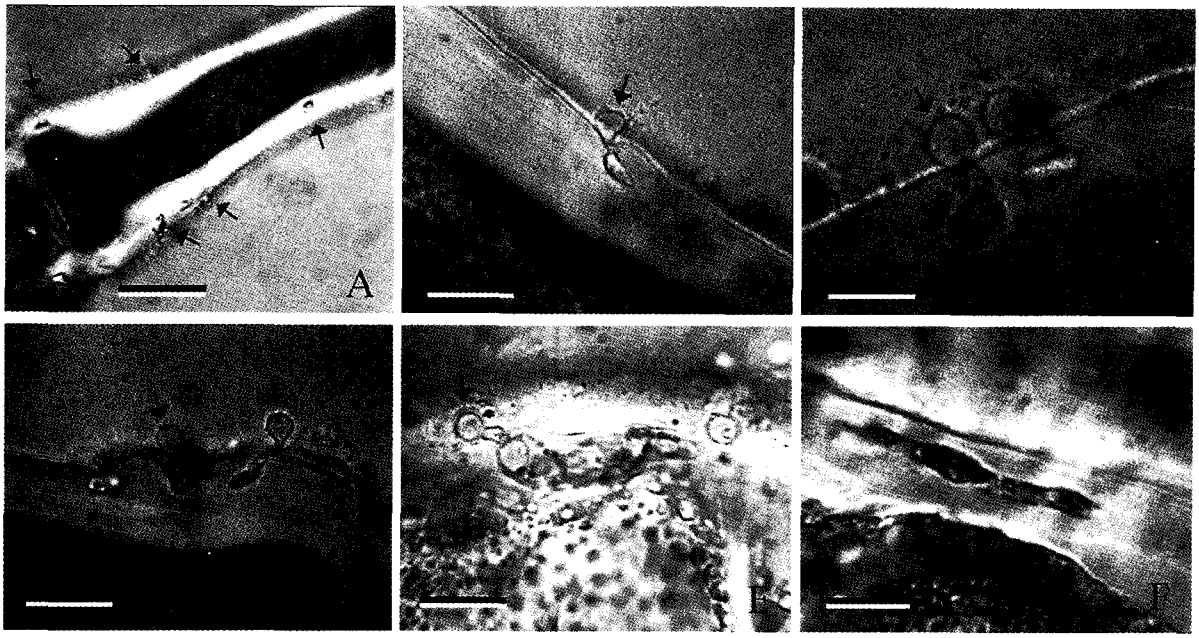

Fig. 3. *Undaria pinnatifida* symbiotic with *Aglaothamnion oosumiense*. A, Portion of single host cell with settled zoospores (arrows) attached to host. B, Penetration of host cell wall by kelp gametophyte leaving empty spore wall (arrow) on outer surface of host. C, Two adjacent kelp gametophytes following empty spore germination (arrows) and penetration of host cell wall. D, Growth of gametophytes after 5 days in host with intact spore walls on surface (arrows), E, Three-celled endophytic gametophyte after ten days in host with intact spore wall on surface. F, Portion of five celled gametophyte at 15 days growing parallel to host surface. Scale bars=10 μm (B, C, F), 15 μm (E), 20 μm (D), and 50 μm (A).

than being a general phenomenon of the order. Because *Undaria pinnatifida* does not occur in the eastern Pacific (Scagel et al., 1993), at least two kelp species from opposite sides of the Pacific Ocean must be capable of developing endophytic gametophytes.

As in other algal groups with endophytic species (e.g. Acrochaetiaceae and Ulvellaceae, Garbary et al., 1982, Nielsen and McLachlan, 1986), not all endophytes are able to establish symbiosis with their hosts. Endophytes may be restricted to a single host species or even to one life history stage of that host (Correa and McLachlan, 1991). The inability of spores of *Laminaria religiosa* to become endophytic in *Aglaothamnion oosumiense* suggests either that *L. religiosa* is unable to form symbiosis with red algae, or that there is some host specificity involved. In either case, there are important implications for the biology of species of Laminariales and the ecology of kelp beds. Given the huge number of spores that are produced by each kelp plant (Chapman, 1984), it is also possible that in a given species some spores may become symbiotic and others may become free-living.

Here we selected a kelp species based on the occurrence of reproductive fronds at our field site and a random species of potential host that was available in culture, for example, *A. oosumiense*. The fact that the symbiosis was so readily established between these taxa is consistent with our previous field observations from the San Juan Islands (Garbary et al., 1999a, b). This further raises the possibility that endophytism is a generalized phenomenon in the Laminariales. Since all of the observations to date involve only limited sites and species in the North Pacific Ocean, it would be of interest to know if this symbiosis occurs elsewhere.

We have not yet demonstrated an association between *U. pinnatifida* and red algae in nature, nor have we demonstrated that this mechanism is important in kelp recruitment. Because of the economic importance of *Undaria* spp. (e.g. Saito, 1975) it would be useful to resolve the actual relationship between host and symbiont, and the importance of this symbiosis in the ecology of natural kelp beds.

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