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Identification and characterization of a green-type mutant of *Porphyra tenera* Kjellman var. *tamatsuensis* Miura (Bangiales, Rhodophyta)

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Abstract

We identified two green-colored conchocelis strains of cultivated *Porphyra*, 'Oba-green' and HGT-6 (F_1 strain of 'Oba-green'), based on PCR-RFLP and sequence analyses of the nuclear ITS region and the plastid RuBisCO spacer region. These molecular analyses confirmed that the two strains were green-type mutants of the endangered species *Porphyra tenera*, although their blade color was not green. Under the same culture conditions, blade length increase was significantly higher in HGT-6 than in a wild strain HGT-1 of *P. tenera*, and the blade shape of HGT-6 was extremely elongate compared with that of HGT-1. From the growth characteristics and ITS-1 sequence data, HGT-6 was confirmed to be a green-type mutant of *P. tenera* var. *tamatsuensis*, a vigorously growing cultivar. Although photosynthetic pigment contents were lower in HGT-6 than in HGT-1, total content of four major free amino acids was higher and the blade thickness was almost the same in HGT-6 and HGT-1. These results suggest that the green-type mutant HGT-6 has potential as breeding material for further development of *Porphyra* breeding.

Keywords: Breeding; Free amino acid; Internal transcribed spacer (ITS); PCR-RFLP; *Porphyra tenera* var. *tamatsuensis*; RuBisCO spacer

1. Introduction

The marine red algal genus *Porphyra*, commercially known as nori, exhibits a dimorphic life cycle with a conspicuous gametophytic blade and a microscopic, filamentous, shell-boring sporophyte called the conchocelis phase. According to Yoshida et al. (1997), over 130 species of *Porphyra* have been described worldwide. Since several *Porphyra* species are extensively cultivated in Japan, Korea and China, this genus is one of the most economically important mariculture crops.

In Japan, *P. yezoensis* Ueda and *P. tenera* Kjellman are known as the representative species used for mariculture (Miura, 1984; Miura and Aruga, 1987). Before artificial seeding of cultivated *Porphyra* became popular in Japan, *P. tenera* was the most widely used species in nori farms (Ueda, 1932; Miura, 1988). However, with the spread of artificial seeding, it is considered that the main species of cultivated *Porphyra* has changed to *P. yezoensis*, because gametophytic blades of *P. yezoensis* are darker in color under low-nutrient conditions and more tolerant of strong waves and windy conditions than those of *P. tenera* (Miura, 1998). Furthermore, *P. yezoensis* f. *narawaensis* Miura and *P. tenera* var. *tamatsuensis* Miura have been bred as vigorously growing cultivars (Miura, 1984), and until now many strains of cultivated *Porphyra* have been established by selective breeding and used in Japanese nori farms. However, because *P. tenera* and *P. yezoensis* are extremely similar to each other in their morphological features, it is difficult to discriminate between the two based on only morphological observations. Therefore, most of the strains have not been identified even to the species level with certainty.

To identify these cultivated strains at the species level and to reveal their genetic relationships, we have performed combined studies of morphological observations and molecular analyses (Niwa and Aruga, 2003, 2006; Niwa et al., 2004). In our studies, PCR-RFLP analyses of the nuclear internal transcribed spacer (ITS) region and the plastid ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) spacer region were also developed for reliable and rapid species identification of cultivated *Porphyra* (Niwa et al., 2005a,b). The results of these studies

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indicated that many strains currently cultivated in nori farms are *P. yezoensis* f. *narawaensis*, and suggested that the recent intensive selective breeding has led to a reduction of genetic diversity in cultivated *Porphyra* even within the species of *P. yezoensis*. For further development of *Porphyra* breeding, it is necessary to improve the diversity of strains by collecting genetically different strains as breeding materials (Niwa et al., 2004, 2005a, in press).

On the other hand, *P. tenera*, formerly the main species used in nori farms, is currently listed as an endangered species in Japan (Miura, 1998; Yoshida et al., 1999; Yoshida, 2000). Because the blade of wild *P. tenera* is thinner, making the finished product softer than that of *P. yezoensis* f. *narawaensis*, it is suggested that *P. tenera* has a potential to be used as breeding material for development of high quality nori (Niwa et al., 2005a). A variety of this species, *P. tenera* var. *tamatsuensis*, was reported to be a more vigorous cultivar compared with the wild species (Miura, 1984). However, it is currently unclear whether strains with growth characteristics like *P. tenera* var. *tamatsuensis* still exist or not as cultivars.

Although conchocelis color of cultivated *Porphyra* is usually dark brown, we recently obtained a green-colored conchocelis strain of cultivated *Porphyra*, which was labeled as *P. tenera* var. *tamatsuensis* but without strict species identification. In the present study, we refer to the strain as a green-type mutant based on the conchocelis color. Pigmentation mutants of *Porphyra* are useful for genetic and breeding studies of cultivated *Porphyra* (Miura, 1985; Ohme et al., 1986; Ohme and Miura, 1988; Miura and Shin, 1989; Niwa et al., 1993, 2002, 2003; Yan and Aruga, 2000; Yan et al., 2000). Our objectives in the present study are to identify whether the green-type mutant is really a strain of *P. tenera* var. *tamatsuensis*, and to evaluate the green-type mutant as a breeding material for cultivated *Porphyra*. For these objectives, first, we tried to identify the green-type mutant by PCR-RFLP analyses of the nuclear ITS and plastid RuBisCO spacer regions, and by sequence analysis of the RuBisCO spacer and ITS-1 regions. Second, we investigated the growth characteristics, photosynthetic pigment contents and free amino acid contents of the mutant blades in comparison with those of wild *P. tenera* HGT-1.

2. Materials and methods

2.1. Algal materials

The green-type mutant 'Oba-green (No. 416)' of cultivated *Porphyra* and the F_1 strain HGT-6 (see below) were examined. The conchocelis of 'Oba-green (No. 416)' is a strain that was used in nori farms of Ariake Bay in Japan and has been maintained in laboratory culture at the Aichi Fisheries Research Institute. The strain HGT-1 of wild *P. tenera* (Niwa et al., 2005a) was used for comparisons with the two strains, 'Oba-green (No. 416)' and HGT-6.

The strain HGT-6 was established in laboratory culture as follows. A mature conchocelis colony of 'Oba-green (No. 416)' was cultured in a 300 mL flask with NPM medium (Niwa and Aruga, 2003) and agitated by aeration at 15 °C under 100 μmol photons m^{-2} s^{-1} (10L:14D). Vinylon monofilaments (approximately 6 cm long) were placed in the flask for attachment of conchospores. Upon confirmation of conchospore attachment on the monofilaments, the conchocelis colony was removed from the flask. The conchospores on the monofilaments were cultured until they developed into gametophytic blades. The culture medium was renewed twice a week. When the blades had grown to approximately 2 cm long, they were detached from the monofilaments, transferred into a 1 L flask and further cultured. Before the blades became mature, a single blade was separated and further cultured. After confirming complete maturation of the blade, carpospores produced by self-fertilization were collected and grown to conchocelis, i.e. the conchocelis strain HGT-6.

2.2. Molecular analyses

Total DNA was extracted from three conchocelis strains, 'Oba-green', HGT-6 and HGT-1. The extraction method was the same as described previously by Niwa and Aruga (2003). The nuclear internal transcribed spacer (ITS) and plastid RuBisCO spacer regions were amplified using the PCR technique as in Niwa and Aruga (2006). The ITS-1 region was amplified using the same PCR conditions for the ITS region, and the primers used were F-intron 2 and R-5.8S2 (Niwa et al., 2005a). PCR-RFLP analyses of the ITS and RuBisCO spacer regions for 'Oba-green' were carried out as in Niwa and Aruga (2006). The strain HGT-1 of wild *P. tenera* was also examined by the PCR-RFLP analyses to compare the DNA patterns of 'Oba-green'. The ITS-1 and RuBisCO spacer sequences of HGT-6 were determined using the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with a 377A DNA sequencer (Applied Biosystems, Foster City, CA, USA). Their sequence data have been submitted to GenBank (accession numbers: ITS-1 AB365190, RuBisCO spacer AB365191).

2.3. Comparisons of HGT-6 and HGT-1

The strains HGT-6 and HGT-1 were compared for their growth characteristics, photosynthetic pigment contents and free amino acid (FAA) contents. The gametophytic blades of HGT-6 and HGT-1 were cultured under the same conditions as described above. The length and the width of blades were measured each week over a 6 week period after conchospore attachment. The blade shape of the two strains was compared using the blade length-to-width ratio, and the thickness of sectioned blades was measured under a light microscope. Photosynthetic pigment (chlorophyll *a*, phycoerythrin and phycocyanin) contents and FAA contents of gametophytic blades were measured as described previously by Niwa et al. (1993) and Niwa et al. (2003), respectively. Comparisons of the data of growth characteristics, pigment contents and FAA contents between HGT-6 and

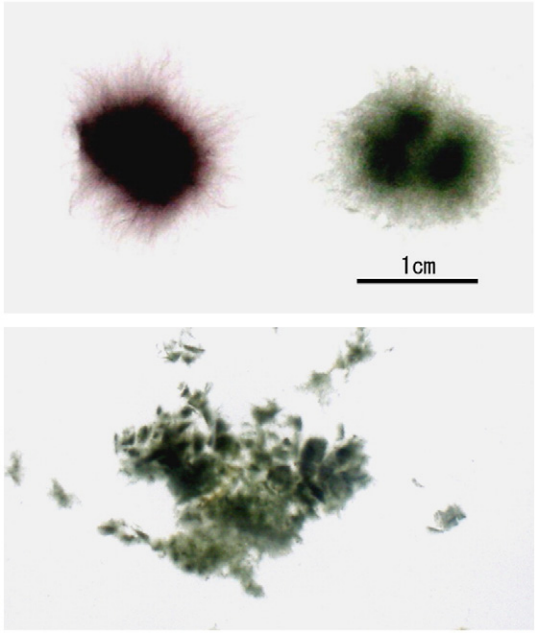

Fig. 1. Conchocelis of wild *Porphyra tenera* HGT-1 (upper left), cultivated *Porphyra* 'Oba-green (No. 416)' (upper right) and *Porphyra* HGT-6 (F_1 strain of 'Oba-green (No. 416)', lower).