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Isolation and identification of gametogenesis-related genes in *Porphyra yezoensis* (Rhodophyta) using subtracted cDNA libraries

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Abstract

Gametogenesis of *Porphyra yezoensis* thalli is induced by ageing as well as by changing water temperature and photoperiod. Under laboratory conditions, thalli cultivated at 10 °C with a 10:14 h (light: dark) cycle develop vegetatively to adult form without gametogenesis. On the other hand, sexual reproduction, which involves differentiation of vegetative cells and subsequent gametogenesis, is induced by culturing at 15 °C with a 16: 8 h (light: dark) cycle. We have constructed subtracted cDNA libraries enriched for differentially expressed transcripts in vegetative and reproductive thalli, and randomly selected 1,152 cDNAs from each subtracted library. Results of the dot blot analyses used for identification of differentially expressed cDNAs indicated that mRNA levels of 176 and 138 cDNAs tended to increase in the vegetative and reproductive thalli, respectively. BLAST analysis of nucleotide and deduced amino acid sequences showed that the cDNAs represented 63 and 59 unique clones for the vegetative and reproductive cDNA enriched subtracted libraries, respectively. Interestingly, some of the cDNAs isolated from the reproductive subtracted library were homologous to genes encoding protein kinases, GTP-binding protein, and heat shock proteins involved in signal transduction and the molecular chaperon system.

Introduction

The genus *Porphyra* contains several species that include the edible laver, and is of considerable economic importance in many places of the world, especially in Asia (Zemke-White & Ohno, 1999). In Japan, approximately 60,000 tonnes (dry weight) were produced in cultivation farms each year (Zemke-White & Ohno, 1999). Thus, *Porphyra* is one of the most extensively cultivated seaweeds used as food in Japan. *Porphyra* displays a unique heteromorphic, digenetic life cycle that consists of a leafy gametophyte and a filamentous sporophyte. The difference between these two developmental phases is usually associated with different chromosome ploidy level. In addition, these two generations show many different structural features, such as chloroplast number and cell wall composition (Cole and Conway, 1975; Mukai et al., 1981). Therefore, in order to elucidate molecular mechanisms underlying these differences between the gametophytic and sporophytic generations, analyses of differentially expressed genes have been performed using subtracted cDNA libraries (Liu et al., 1994a) and expressed sequence tags (ESTs) (Nikaido et al., 2000; Asamizu et al., 2003) for both generations.

Some *Porphyra* species also have properties that make them a suitable system for the study of cellular differentiation, since the thallus is formed by a single cell layer. In the leafy gametophyte of *Porphyra*, vegetative cells differentiate into sexually mature male and female cells. In order to identify tissue-specific genetic markers of differentiation in *Porphyra*, RNA transcripts among morphologically distinct regions of the differentiated tissue have been compared using differential display, and a few genetic markers

specific to each tissue have been isolated (Hong et al., 1995). However, the molecular mechanisms underlying and controlling the differentiation of vegetative cells into reproductive cells are still poorly understood.

Among *Porphyra* species, *P. yezoensis* has recently been recognized as a useful model organism for fundamental and applied studies of marine algae (Waaland et al., 2004) since the life cycle can be completed within a few months in laboratory culture (Kuwano et al., 1996), the genome size is similar to other higher plant model organisms such as *Arabidopsis* and rice (Kapraun et al., 1991; Le Gall et al., 1993), and a public EST database exists (Nikaido et al., 2000; Asamizu et al., 2003). In addition, gamete formation (sexual differentiation) of vegetative cells within the leafy gametophyte can be easily induced by changing two extrinsic signals, photoperiod and water temperature, in laboratory culture (Iwasaki, 1979). Therefore, this alga is an ideal research tool to investigate the molecular mechanisms related to differentiation of vegetative cells, and to identify genes that regulate gametogenesis of *P. yezoensis* thalli that are induced or repressed in response to these changing conditions.

As a first step toward understanding the molecular mechanisms for reproductive cell differentiation induced by changing cultivation conditions, the present study was undertaken to construct subtracted cDNA libraries enriched for differentially expressed messages in vegetative and reproductive thalli and to investigate the levels of transcript accumulation for each subtracted cDNA by dot blot analysis. In addition, the cDNAs corresponding to putative differentially expressed transcripts in the vegetative and reproductive thalli were sequenced to identify candidate genes important for gametogenesis.

Materials and methods

Materials and cultivation conditions

Leafy gametophytes of *P. yezoensis* (strain FA-89) were grown in one-fifth strength Provasoli's enriched seawater (1/5 PES) medium (Provasoli, 1968). The culture medium was changed every three days throughout the experiments. The culture was aerated with filtrated air, irradiated with 40 μmol photons m^{-2} s^{-1} light on a 10:14 h (light: dark) cycle (10L/14D), and maintained at 10 °C. When the thalli reached an average length of 5 cm, the thalli were sampled as "vegetative thalli" after microscopic confirmation that only vegetative cells were present.

Gametogenesis of *Porphyra* thalli was induced by changing the photoperiod and water temperature. Thalli grown to an average length of 5 cm were inoculated into 1/5 PES medium and maintained on a 16 L/8D photoperiod at 15 °C. The morphology of the thallus cells was examined daily under a microscope. Thalli cultivated for three or seven days under the induction conditions were collected as "induced thalli" and "reproductive thalli", respectively. The cells in the induced thalli showed no differentiation, whereas in the reproductive thalli differentiation was limited to marginal files of cells in the upper one-third of the thallus, representing approximately 5% of the total surface area.

Construction of subtracted cDNA libraries

Poly(A)$^+$ mRNA was isolated directly from fresh thalli using a QuickPrep *Micro* mRNA Purification Kit (Amersham Biosciences). Two micrograms of mRNA from the vegetative and reproductive thalli were used for double-stranded cDNA synthesis and then for subtracted cDNA library construction using the PCR-Select^{TM} cDNA Subtraction Kit (Clontech), according to the manufacturer's instructions. In the case of preparation of the forward subtracted (FS) cDNA library that is enriched for differentially expressed transcripts in vegetative thalli, cDNAs from the vegetative and reproductive thalli were used as tester and driver cDNAs, respectively. The reverse subtracted (RS) cDNA library, enriched for differentially expressed transcripts in reproductive thalli, was prepared by using cDNAs from the reproductive and vegetative thalli as tester and driver cDNAs, respectively. Before subtractive hybridizations, sub-pools of each tester cDNA ligated to a different adaptor supplied in the kit were pooled as forward unsubtracted (FU) cDNA from the vegetative thalli and reverse unsubtracted (RU) cDNA from the reproductive thalli. The FS and RS cDNAs obtained were subcloned into a pT7Blue *T*-Vector (Novagen) and subsequently used to construct FS and RS cDNA libraries in *E. coli* JM109. After plating on LB-agar plates supplemented with ampicillin, X-gal, and ITPG, 1152 white colonies from each transformation were picked into twelve 96 well microtiter plates containing LB and ampicillin. The cultures were grown overnight with shaking and stored at −80 °C after the addition of an equal volume of 50% glycerol.