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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.

Screening of subtracted cDNA libraries

In order to identify differentially expressed transcripts in the vegetative and reproductive thalli, dot blot analyses were performed on the FS and RS libraries. For each library, cDNA inserts were amplified directly from the 1152 liquid cultures in twelve 96 well PCR plates using the nested adaptor primers from the CLONTECH cDNA Subtraction Kit. The twelve plates from each library were divided into four sets of three plates each. PCR products from each set of three plates (288 independent clones) were arrayed in triplicate onto a Hybond^{TM}-N^{+} nylon membrane (Amersham Biosciences). Four replicate membranes were prepared from each set of three plates. In total, four sets of four replicate membranes were prepared for each library, and each replicate membrane within a set was hybridized separately with either the FS, RS, FU, or RU cDNA probe.

For preparation of cDNA probes, FS, RS, FU, and RU cDNAs were random DIG-labeled using DIG-High Prime (Roche Diagnostics). Membranes were pre-hybridized at 42 °C for 1 h in DIG Easy Hyb (Roche Diagnostics) and hybridized at 42 °C for 16 h in the same solution after adding 200 ng of the DIG-labeled probe. After hybridization, the membrane was washed twice with 2 X SSC containing 0.1% SDS at room temperature for 10 min and twice with 0.2 X SSC containing 0.1% SDS at 68 °C for 15 min. Signal generation of the DIG-labeled probe was performed using the ECF substrate (Amersham Biosciences) according to the manufacturer's instructions. The hybridized membranes were scanned using a STORM^{TM} 860 and quantified using ImageQuant V1.2 (Amersham Biosciences).

Sequence analysis

Purified plasmid DNA isolated using the QIAprep Spin Miniprep Kit (QIAGEN) was sequenced with the aid of a DYEnamic^{TM} ET Terminator Cycle Sequencing Kit (Amersham Biosciences) and an ABI PRISM Model 373A Sequencer (PE Biosystems). Database searches and similarity analyses of cDNA nucleotide sequences were carried out with the BLASTN and BLASTX programs (Altschul et al., 1990, 1997) against public nucleotide, EST, and protein databases.

Northern blot analysis

For the preparation of a cDNA probe, 10 pg of the cDNA fragment was DIG-labeled using the PCR DIG Probe Synthesis Kit (Roche Diagnostics). Ten micrograms of total RNA for each thallus type (vegetative, induced, and reproductive) were size-fractionated by electrophoresis on a 1.0% agarose-formaldehyde gel and capillary-transferred to a Hybond^{TM}-N^{+} nylon membrane. The RNA blot was pre-hybridized at 50 °C for 1 h in DIG Easy Hyb (Roche Diagnostics) and hybridized at 50 °C for 16 h in the same solution after adding the DIG-labeled cDNA probe. After hybridization, stringency washes and signal generation and detection of the DIG-labeled probe on the Northern blot were performed as for the dot blot analysis.

Results

Isolation of cDNAs for differentially expressed transcripts in the vegetative and reproductive thalli

Porphyra thalli grown to the average length of 5 cm in 1/5 PES medium under a 10L/14D photoperiod at 10 °C (vegetative thalli) contained only vegetative cells by microscopic examination. When these thalli were transferred to fresh medium and cultivated on a 16L/8D photoperiod at 15 °C for three days (induced thalli), differentiation of vegetative cells and subsequent gametogenesis was not observed. After four days, differentiated cells (sexually mature male cells) were clearly observed in the tips of thalli, and after seven days, the marginal cells in the thalli (reproductive thalli) had almost completely differentiated into reproductive cells.

Messenger RNA extracted from the vegetative and reproductive thalli was subsequently used for cDNA synthesis and construction of the FS and RS cDNA libraries enriched for differentially expressed transcripts in vegetative and reproductive thalli, respectively. After transformation of *E. coli* with the FS and RS cDNA libraries, 1152 recombinant (white) colonies obtained from each subtracted library were randomly selected and those inserted cDNAs were amplified by PCR. When 192 PCR products (8 per set of 96 colonies) were examined, the average size of cDNA inserts was approximately 400 bp.

Individual clones of the subtracted cDNA libraries were screened for differential expression by hybridizing with either FS, RS, FU, or RU cDNA probes. The distribution of signal intensity ratios (FS/RS and RS/FS for the screening of the FS and RS cDNAs, respectively) in dot blot analyses for the 1152 randomly selected cDNAs from each subtracted

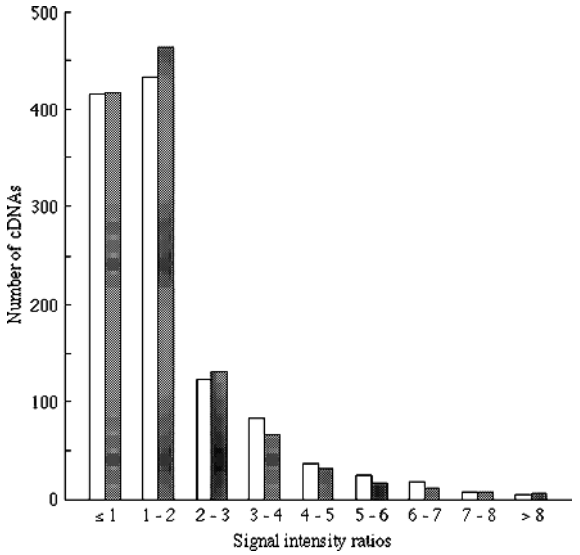

Figure 1. Distribution of signal intensity ratios in dot blot analyses for 1,152 randomly selected cDNAs from each subtracted library. Open and gray bars represent the number of cDNAs from the forward subtracted (FS) and reverse subtracted (RS) cDNAs, respectively. The signal intensity ratios for the FS and RS cDNAs were calculated from the signal intensity of FS to RS cDNA probes and of RS to FS cDNA probes, respectively. cDNA clones which showed a difference in signal intensity ratio of more than 3-fold in dot blot analyses using FS and RS cDNA probes were selected for further studies.

library is shown in Figure 1. Quantitative analysis of signal intensity for the dot blots showed that approximately three-quarters of the recombinant colonies in the FS and RS cDNA libraries had less than a two-fold difference in signal intensity. On the other hand, 176 of the clones screened from the FS library and 138 of the clones screened from the RS library, had greater than a three-fold difference in signal intensity. Therefore, these clones in the FS and RS cDNA libraries were selected for further investigation.

Identification of differentially expressed transcripts in the vegetative and reproductive thalli

Positive clones selected by dot blot analyses were sequenced to identify the corresponding genes. The 176 forward subtracted and 138 reverse subtracted clones were found to represent 63 and 59 unique sequences, respectively. Approximately 84% (53/63) of the forward subtracted clones showed similarity (E <0.005, score >90) to genes and *Porphyra* ESTs registered in the public databases, while 73% (43/59) of the reverse subtracted clones also had significant matches in the public databases (Table 1). Therefore, a significant number of the clones remain to be categorized.

Table 1. BLAST results of similarity searches against the public nucleotide and protein databases.

	Number of unique cDNAs	
Similarity	Forward subtracted	Reverse subtracted
Genes of known or putative function[a]	33 (12)	26 (15)
Porphyra ESTs of unknown function[b]	20	17
No similarity[c]	10	16
Total	63	59

[a]Similarity threshold set at E < 0.005 and score >90 for genes of known or putative function. Number of cDNAs in parentheses also showed significant similarity (E < 1e-60 and score >120) to *Porphyra* ESTs.
[b]Similarity threshold set at E < 1e-40 and score >90.
[c]Number of cDNAs that showed no similarity ($E \geq 0.005$ and score ≤90) to genes or ESTs of known or putative function.

Thirty-three of the forward subtracted clones and 26 of the reverse subtracted clones that showed sequence similarity to genes of known or putative functions were classified according to their putative biological roles and biochemical functions (Table 2). The largest category (12/33) was forward subtracted clones with similarities to genes that function in protein synthesis, mainly corresponding to genes encoding various ribosomal proteins (E < 1e-23, score >270). Interestingly, several homologues of genes associated with signal transduction (e.g. a small GTP-binding protein (E = 2e-24, score = 281), a MAP kinase (E = 1e-22, score

Table 2. Functional classification of the subtracted cDNA clones based on similarity to known protein genes.

	Number of protein genes	
Functional categories	Forward subtracted	Reverse subtracted
Energy metabolism	5	5
Protein fate	5	6
Protein synthesis	12	1
Transport and binding proteins	2	1
Transcription and regulation	1	4
Signal transduction	0	3
Structure and membrane proteins	0	2
Fatty acid metabolism	3	0
Intermediary metabolism	5	4
Total	33	26

= 265), and a SNF1/AMP-activated protein kinase (E = 5e-12, score = 174)) and protein fate (e.g. HSP90 (E = 4e-18, score = 151), HSP70 (E < 1e-46, score >470), ubiquitin (E = 3e-30, score = 331), and some proteasome subunits (E < 1e-16, score >210)) were also identified.

Expression of a small GTP-binding protein in different growth phases

We chose a 368 bp cDNA putatively encoding a small GTP-binding protein to confirm that the isolated cDNA clones truly represent mRNAs differentially expressed in vegetative and reproductive thalli. The clone was hybridized to mRNA from the three phases (vegetative, induced, and reproductive) in Northern hybridization experiments (Figure 2). The apparent larger size of the band in the reproductive thalli total RNA lane is most likely due to polysaccharides inhibiting the rate of RNA migration in the gel, since the ethidium bromide-stained gel also showed the same shift of rRNA in this lane relative to the other two (data not shown). The relative mRNA level of the small GTP-binding protein gene was almost the same in the vegetative and induced thalli, but the level was 2.6 times higher in the reproductive thalli than in the vegetative thalli.

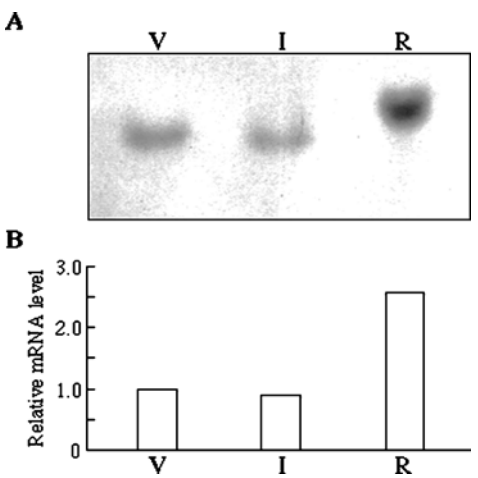

Figure 2. Northern blot analysis (A) and relative expression levels (B) of mRNA encoding a small GTP-binding protein in *P. yezoensis* thalli. In panel A, total RNA (10 μg per lane) from vegetative (V), induced (I), and reproductive thalli (R) were electrophoretically separated on a 1.0% agarose-formaldehyde gel, blotted onto a nylon membrane, and hybridized with DIG-labeled cDNA probe from the insert of clone pT7B-RS-H3-8. In panel B, expression levels in the induced (I) and reproductive thalli (R) represent transcript abundance relative to the vegetative thalli (V). The rRNA band stained with ethidium bromide was used to adjust values for equal loading (data not shown).

Discussion

Many studies have been performed that focused on morphological and physiological differences between the leafy gametophyte and the filamentous sporophyte in the life cycle of *Porphyra* species. Gametophyte- and sporophyte-specific cDNAs that encode proteins such as elongation factors, serine protease-like proteins, polysaccharide-binding proteins, and lipoxygenases have been isolated by differential screening and subtraction of phase-specific cDNA libraries (Liu et al., 1994a,b, 1996a,b,c). Recently, EST analysis has been performed to identify candidate genes related to the morphological and physiological differences between the gametophytic and sporophytic generations, and large numbers of cDNAs have been identified (Nikaido et al., 2000; Asamizu et al., 2003). Thus, potential genetic markers specific to two generations are well known. However, because these are generation-specific cDNAs rather than cell differentiation-specific genes, the genes regulating the maturation process of *Porphyra* thalli remain poorly understood. In this paper, we describe initial results examining the molecular mechanisms regulating the reproductive maturation process in *Porphyra* thalli.

We constructed two subtracted cDNA libraries, enriched for differentially expressed transcripts in vegetative thalli and reproductive thalli that were artificially induced to undergo differentiation in laboratory culture by changing the photoperiod and water temperature. In order to remove false positives, 1152 recombinant clones from each subtracted cDNA library were screened by dot blot hybridization with four DIG-labeled cDNA probes corresponding to forward and reverse, subtracted and unsubtracted cDNAs. cDNAs that showed greater than a three-fold difference in signal intensity between the forward and reverse subtractions were sequenced, and the sequence data subjected to clustering by BLAST analysis. These cDNAs were found to represent 63 and 59 unique clones from the forward and reverse subtracted libraries, respectively (Table 1), with average size of approximately 370 bp. This insert size is shorter than those reported previously (Diatchenko et al., 1996), which may reflect an increased frequency of *Rsa*I restriction sites in the *P. yezoensis* genome compared to the human genome.

The size also is shorter than the average insert and EST sequences in the *Porphyra* normalized library (approximately 970 and 470 bp, respectively) (Nikaido et al., 2000). Therefore, in order to generate more sequence information for each clone, one possibility is to use a six-base recognition enzyme rather than the four-base recognition enzyme *Rsa*I used in the CLONTECH PCR-Select^{TM} cDNA Subtraction Kit.

Results of similarity searches for each unique cDNA using the BLASTX program showed that 33 forward subtracted and 26 reverse subtracted cDNA clones were putative homologues of known functional genes registered in public databases (Table 1). The largest functional category, genes involved in protein synthesis, appeared to be down regulated in reproductive thalli compared to vegetative thalli (Table 2). This apparent down regulation does not result in a decreased growth rate in reproductive thalli (I. Kaneko & M. Kakinuma, unpublished data). However, at approximately seven days of induction (i.e., the time point at which "reproductive" mRNA was sampled), the pace of differentiation picks up rapidly, suggesting that the protein synthesis machinery already present may be sufficient to carry the thallus through the rest of the developmental program with reduced expression of protein synthesis genes. As is the case with many of the genes identified in this screen, further confirmation of differential expression is required.

Since, in this study, thallus maturation was artificially induced by changing photoperiod and water temperature, we are largely interested in the cDNAs involved in signal transduction (e.g. small GTP-binding protein and protein kinases) and protein fate (e.g. HSP90). Small GTP-binding proteins have been shown to participate in signal transduction, cell proliferation and differentiation, and membrane vesicle transport (Balch, 1990; Bourne et al., 1990; Hall, 1990). In higher plants, it has been reported that light regulates the changes in steady-state levels of several small GTP-binding protein mRNAs, and that phytochrome mediates the changes in a negative manner (Yoshida et al., 1993; Inaba et al., 1999). On the other hand, some small GTP-binding proteins in *Chlamydomonas* are used for household functions responsible for vesicle transport rather than for cell differentiation (Dietmaier et al., 1995). In the case of maturation process of *P. yezoensis*, expression of the small GTP-binding protein was dramatically increased in the reproductive thalli (Figure 2). This result suggests a possibility that the small GTP-binding protein plays an important, yet unknown role in cell differentiation in the thallus maturation process.

For other interesting genes such as protein kinases and HSP90, expression profiles during the maturation process have not yet been investigated by Northern blot analysis. However, it is well known that the MAP kinases mediate intracellular phosphorylation events linking receptor activation to the control of cell proliferation, chemotaxis, differentiation, and stress responses (Schaeffer & Weber, 1999). Also, the SNF1/AMP-activated protein kinase is commonly activated in response to cellular and environmental stress responses (Hardie et al., 1998). The HSP90 family in most eukaryotic cells binds to and regulates the activity of functionally important proteins such as steroid hormone receptors and protein kinases (Pratt et al., 2001). In addition, it has been reported that HSP90 function is linked to the development of pollen in higher plants and of the female gametophytes in algae (Marrs et al., 1993; Yabe et al., 1994; Lee et al., 1998). Cells within *P. yezoensis* thalli have differentiated specific signal transduction pathways for response to and integration of extracellular stimuli. Therefore, it is possible that protein kinases and HSP90 in *P. yezoensis* cells also link perception of extracellular stimuli and sexual differentiation.

One of the surprising results of the similarity search using isolated cDNAs from FS and RS libraries is that many of cDNAs isolated by subtractive hybridization have no known homologues, even among the available *Porphyra* ESTs (Nikaido et al., 2000; Asamizu et al., 2003). Because the *Porphyra* ESTs were derived from vegetative (non-induced or reproductive) gametophyte tissue, it is possible that these clones might correspond to rare genes related to the maturation processes of *Porphyra* thalli. Northern blot analyses using these clones probed against RNA from different phases of the maturation process are currently being carried out in our laboratory.

In this paper we have identified a number of interesting candidate genes that might play an important role in gametogenesis in *P. yezoensis*. However, it is possible that changes in expression of these genes may only be a response to the change in temperature and photoperiod and are unrelated to gametogenesis. We have observed that under our laboratory conditions gametogenesis is induced even in young thalli when either or both conditions are changed, suggesting a direct link between the increase in temperature and photoperiod and induction of gametogenesis. We are currently developing an *in situ* hybrizidation protocol to determine if changes in expression of the candidate genes are focused in the area of sexual differentiation. We are

also constructing subtracted libraries using induced and reproductive tissue. Theoretically, since both of these phases will have been grown under the increased temperature and photoperiod conditions, differences in expression of temperature and light response genes will be minimized. Ultimately, we would like to be able to knock out expression of these genes and look for the effect on gametogenesis, although this approach is not yet technically feasible.

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