

Full Length Research Paper

Cloning and characterization of a female gametophyte-specific gene in *Gracilaria lemaneiformis* (Gracilariales, Rhodophyte)

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In this study, forward and reverse suppression subtractive hybridization (SSH) libraries were constructed between female and male gametophyte of *Gracilaria lemaneiformis* to isolate genes differentially expressed between gametophytes. Dot-blots were performed to screen 384 colonies randomly selected from each subtracted libraries. Partial gene (designated as GMF-01) which is female gametophyte-specific was selected to isolate its cDNA full length sequences and to be characterized. Then we got its cDNA full length sequences using SMART-RACE technic. Sequence analysis showed that the open reading frame (ORF) of GMF-01 is 1002 bp long with a GC content of 47.7%, encoding 333 amino acids. GMF-01 does not have significant match in the databases when examined on NCBI website. Amino acid sequence analysis showed that the theoretical pI of this translated protein is 7.92, while the instability index is computed to be 43.61, which has classified the protein as unstable. Results of prediction showed 45.05% of its secondary structure has random coil and sub-cellular location prediction indicated that it is probably a extracellular protein.

Key words: *Gracilaria lemaneiformis*, suppression subtractive hybridization (SSH), sex determination, gametophyte.

INTRODUCTION

Gracilaria lemaneiformis is a commercially important agarophyte that can be used to produce agar, a major ingredient of dairy products, surgical jellies, ointments, cosmetics and healthcare products (Tseng, 2001). *Gracilaria*, along with *Porphyra*, *Laminaria* and *Undaria* has a bulk production by farming in China. It is not only an economically important algal species, but also a good material for genetical studies (Chen et al., 2009). Thus, it is important to pursue basic studies on *G. lemaneiformis*.

Gracilaria has a life history involving three distinct stages: gametophytes, carposporophytes and

tetrasporophytes. Even though the phases and sexes of *Gracilaria* look identical before sexual maturation, there are physical differences between them (Kain and Destombe, 1995), such as growth rate in phases of *G. lemaneiformis* (Zhang and van der Meer, 1988), levels of polyamines in sexes and phases of *G. cornea* (Guzman-Uristegui et al., 2002), and lipid composition among different developmental stages of *Gracilaria verrucosa* (Khotimchenko, 2006). Owing to their particular life history, differentiation of phase and sex in red algae has already attracted researchers' attention. Researchers have been engaged in the study of the mechanisms of phase formation since 1976 (Ren and Zhang, 2008). However, no satisfactory results were obtained due to the limitation of applicable methods in the past. With the development of molecular biology, great progress has been made recently, such as that made by Ye et al. (2006). 6 ISSR primers, which had proved previously to

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be able to yield clear bands in *Gracilaria*, were used to distinguish the phases and sexes of *G. lemaneiformis* (Sun et al., 2003).

Until now, several phase-specific and sex-specific genes have been identified. Eight unique cDNAs for the sporophyte and seven specific for the gametophyte, including elongation factor alpha and lipoxygenase encoding genes have been isolated from *Porphyra purpurea* (Liu et al., 1996). A heat-shock protein encoding gene, which might be involved in the differentiation of female gametophyte, has been identified from *Griffithsia japonica* (Lee et al., 1998). An ubiquitin gene of *G. lemaneiformis* during phase formation is identified and characterized (Ren et al., 2009). GlRab11, the first functional Rab-like protein identified in *G. lemaneiformis* was isolated and the cDNA full-length of GlRab11 was obtained (Ren et al., 2008). cDNA subtracted hybridization was employed to study *Porphyra purpurea* phase-specific genes (Liu et al., 1994) and suppression subtractive hybridization (SSH) was developed by Diatchenko et al. (1996), which turned out to be a successful tool for rapid screening of differentially expressed genes (Shim and Dunkle, 2002; De la Vega et al., 2007). SSH had been applied to study differential expression of genes in developmental stages (Brun et al., 2003; Zhu et al., 2003; Singh et al., 2007) and under stress conditions (Bahn et al., 2001; Caturla et al., 2002). Sun et al. (2002) reported an analysis of 180 ESTs of the *G. lemaneiformis* tetrasporophyte cDNA library. Suppression subtractive hybridization (SSH) was employed between RNA extracted from female gametophyte and tetrasporophyte. Fourteen cDNAs were identified, among which *SSH466* was a putative tetrasporophyte-specific gene (Ren et al., 2006).

In this study, we constructed SSH libraries between female and male gametophyte of *G. lemaneiformis* and isolated the cDNA full-length of *GMF-01*, which is a female gametophyte-specific Gene.

MATERIALS AND METHODS

Algae materials and cultivation

G. lemaneiformis used in this study were collected from Zhanshan Bay (Qingdao, China). The healthy and mature fronds were used. Tetrasporophytes with released tetraspores were separated from female and male gametophytes under the microscope. Then one female gametophytes and one male gametophytes developed from tetraspores were picked out. The separated algae materials were brushed and rinsed in sterilized seawater until they were completely divorced from epiphytes. The materials were cultivated in Provasoli medium (Provasoli, 1966) under a light intensity of 50 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ with a 12:12 (L: D) cycle at $15\pm 1^\circ\text{C}$. The thallus were used for RNA extraction.

SSH library construction

To isolate sex-relative genes, suppression subtractive hybridization (SSH) was performed between RNA isolated from male and female

gametophytes of *G. lemaneiformis*. Total RNA was extracted from each sample with RNeasy Plant Mini Kit (Qiagen, China), reverse-transcribed and amplified using a SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech, USA). Both forward (female gametophyte as tester and male gametophyte as driver) and backward (male gametophyte as tester and female gametophyte as driver) SSH were performed using a PCR-select cDNA subtraction kit (BD Biosciences Clontech) according to the manufacturer's instructions. In order to confirm differential expressions of the clones, cDNA dot-blots were performed. Based on the results of cDNA dot-blots, clones that expressed differentially between female and male gametophyte were sequenced and aligned with the BLAST algorithms at the National Center for Biotechnology Information (NCBI). The sequences were also analysed with ContigExpress (Vector NTI Suite 6.0) to find contigs.

Cloning of the cDNA full-length and sequence analysis of GMF-01

According to the partial sequence previously obtained and to get the full length of *GMF-01*, 5' and 3' cDNA ends were amplified with a BD SMARTTM RACE cDNA amplification kit (BD Biosciences Clontech). Primers B5 (5'-GCGCTACCGTTGCTCCATAATCCAC-3') and B3 (5'-CATCCAGTTCTACTCGCTTCTTATACC-3') were designed and respectively used in the 5' and 3' RACE.

Sequence searches were performed by the BLAST algorithm on NCBI databases. Theoretical isoelectric point (pI) and molecular weight were predicted using ExPasy ProtParam tools (<http://us.expasy.org/tools/protparam.html>). Protein analysis was performed with ProtComp (www.softberry.com), TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) and PSORT (<http://psort.nibb.ac.jp/form2.html>).

RESULTS

Screening of the SSH cDNA libraries

A total of 768 clones were randomly selected from the SSH libraries (384 clones from each of the two SSH libraries) and screened by macro-array dot-blots. Both forward and backward subtracted radioactively labeled cDNA populations were then used as individual probes for identical blots (Figure 1). 411 clones were found to be positive clones. When analysing the sequencing results with ContigExpress (Vector NTI Suite 6.0), 136 contigs were found and one female gametophyte-specific sequence (480 bp) showed significantly differential expression between the two sexes. It was designated as *GMF-01* and was chosen to clone its full-length cDNA sequence. We are still analyzing the rest partial sequences and they will be published later. The partial sequence of *GMF-01* is as follows:

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GCAGACTTCTACTATTTCAGTATAGATGGGTTGAAGAA
TATAGGGCCACGTTTACCACCAAAGTGGAGATCGGA
GAAATTATTTCGGACGACGAGACATCATCAACTCGCCT
GACTTTAACATGGGACAATCCGTTTCATTTCGATGGAG
TCGAGTGGAGTCCCTCCAGTCAGCGACCGGAAGCCG
CCGAACATTGGGGTAGCATACAAGGTTGACACGAAC
GCTCTGCATCCAGTTCTACTCGCTTCTTATACCATGA
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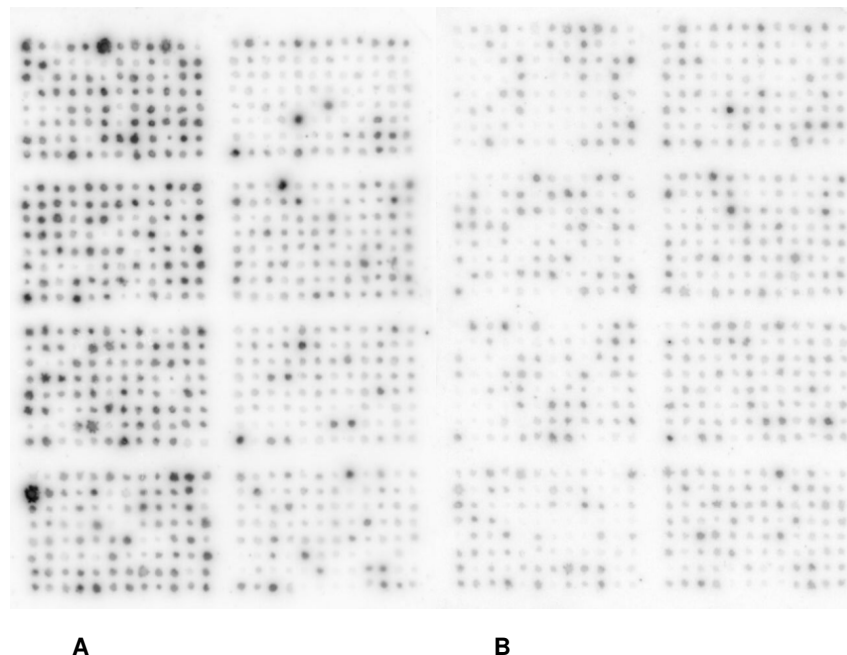


Figure 1. Dot blots of cDNA in subtractive. Two identical membranes blotted with PCR amplified cDNA sequences from subtracted libraries were probed with the forward subtracted probes (A) and the backward subtracted probes (B).

ACGGGCAAAGAAATGGAGGCCGTTTTACTACGGCG
 TTTCTGTAATTTCTGGAAACACTTTGGTAGGCTTCCC
 CGTGATTAAGCTTCAGATCCAACCTCGGTACTGCTATC
 AAGGATAGCGCATTCGTAGGAAGTATAATCGGCAAC
 AGTCTCGAAGTGGATTATGGAGCAACGGTAGCGCAA
 ACAGTGTGCGTGACTCCCGTGAACGTGAGACCACG
 GTGAGGCT

Cloning of the full-length cDNA and sequence analysis of GMF-01

Based on the partial sequence obtained from the SSH library, two primers B5 and B3 were designed to amplify the 5' and 3' cDNA ends of *GMF-01*. 1019 bp and 548 bp were amplified in the 5' and 3' SMART RACE reactions respectively. The full length of *GMF-01* cDNA had 1357 nucleotides. Sequence analysis showed that the open reading frame (ORF) of *GMF-01* is 1002 bp long with a GC content of 47.7%, encoding 333 amino acids. Searches from the public sequence databases using NCBI BLASTx showed that there was no significant match with *GMF-01*. The predicted protein had a calculated molecular weight of 36.7 kDa and a theoretical pI of 7.92.

The instability index was computed to be 43.61 which classified the protein as unstable. The GRAVY (Grand average of hydropathicity) of this protein was -0.131, which indicated that the protein was hydrophilic. Results of prediction showed 45.05% of its secondary structure is

random coil (Figure 2). Sub-cellular location prediction results with ProtComp, TMHMM (Figure 3) and PSORT all indicated that it's probably an extracellular protein. The cDNA and amino acid sequences are indicated in Figures 2 and 3.

DISCUSSION

As an agarophyte, components of *G. lemaneiformis* cells are extremely complicated. In the construction of SSH library, RNA isolated from *G. lemaneiformis* was too difficult to purify enough due to the polysaccharides. The SMART approach is a PCR-based amplification system that allows the creation of cDNA from a very small amount of total RNA (Cramer and Lawrence, 2004; Shary and Guha-Mukherjee, 2004; Pavan, 2011).

Thus, SMART strategy was taken before SSH library construction was carried out. That is the key point of the successfully construction of SSH library. We identified 3 practical sequences that are differentially expressed between female gametophytes and male gametophytes. One putative female gametophyte-specific gene *GMF-01* was selected to isolate the full-length of cDNA for further analysis.

In this study, we identified a female gametophyte-specific gene of *G. lemaneiformis*. The protein encoded by *GMF-01* may be an extracellular protein. It was not known yet whether GMF-01 was red-algae specific.

In the SSH library, we found some special gene

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      10      20      30      40      50      60      70
      |      |      |      |      |      |      |
MTFFALLLNFLCLFFLASSSTSEGRPIQVPERLKPLGYPPETVGYLQKPVSLSENSCKCESTSSNPTCHLA
hhhhhhhhhhheeeeecccccccccccccccccttcttceeeccccccccccccccccccccceec
SDSCQKTHEAICSTSGECSCICQPAFANIGFEPEKYSFFAAYRQARAPSLVLNIIVDRSAARNINAQRL
ccccccchhhheccccccccccccchhhhttccttthhhhhhhhtccccceeeeeechhhhhhhhhhhh
NLGVSKGFASSGDQSFNTVWMFEPYTSLOTSTIQYRWVEEYRATFTTKVEIGEIIIRTQDIINSPDFNMGQ
eeeecccccttccccceeecccccccccheehhhhhhhhhheeeecttceeecccccccccccccc
SVSFDGVESPPVSDRKPPNIGVAYKVDINALHPVLLASYTMNGQKKWRPFYYGVSVISGNTLVGFPVIK
eeeetteccccccccccccceeeectttcchheehhecttccccceeeeeettccccceeee
LQIQLGTAIKDSAFVGSIIIGNSLEVYDYGATVAQIVCVYSRERETTVRLKSGRC
eeccccccccchhhhhheetcceeecttcehhheeeeeccccceeeetccc

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Sequence length : 333

SOPMA :

Alpha helix	(Hh) :	64 is	19.22%
3 ₁₀ helix	(Gg) :	0 is	0.00%
Pi helix	(Ii) :	0 is	0.00%
Beta bridge	(Bb) :	0 is	0.00%
Extended strand	(Ee) :	91 is	27.33%
Beta turn	(It) :	28 is	8.41%
Bend region	(Ss) :	0 is	0.00%
Random coil	(Cc) :	150 is	45.05%
Ambiguous states (?)	:	0 is	0.00%
Other states	:	0 is	0.00%

Figure 2. Prediction of secondary structure of GMF-01.

expression: (1) expression of rDNA and ribosomal protein, such as 16SrDNA, 23SrDNA, ribosomal protein S8, L23, L27. This may indicate that a number of genes are synthesized during sex differentiation, (2) expression of α -1,4-glucan lyase (AglI) which could degrade the floridean starch into glucose which can provide energy for sex differentiation, (3) expression of some protein genes related to cellular process, including aminotransferase, COP9 signalosome, Ran (one of GTP-binding proteins).

These proteins play important roles in cell division and signal transduction. However, their function in sex regulation needs additional research.

The results in this study strongly suggest that *GMF-01* is a functional gene that may play important roles during the sex determination of *G. lemaneiformis*. We are now committed to further studies of *GMF-01*, such as gene expression analysis and semi quantitative determination by RT-PCR, more work are required to elucidate the

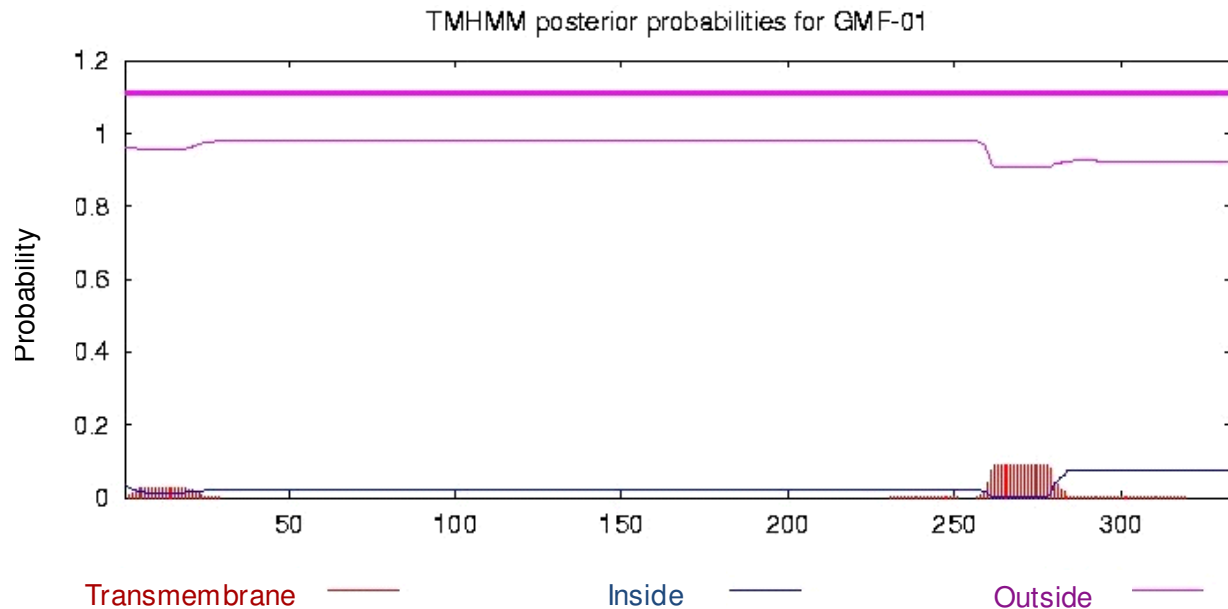


Figure 3. TMHMM posterior probabilities for GMF-01.

function of *GMF-01*. The construction of additional transgenic clones in which *GMF-01* is knocked out should allow a better assignment of its function. Based on the SSH libraries constructed in this study, more differentially expressed genes could be found. The differentially expressed genes obtained in this study are closely related with gametogenesis of *G. lemaneiformis*. Studies on these genes may play important roles in understanding sex determination mechanisms and will provide clues for red algal evolution pathways.

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