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## Editorial

# Recent Developments in Nuclear Reverse-Genetic Manipulation that Advance Seaweed Biology in the Genomic Era

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Seaweeds are multicellular aquatic plants that live in intertidal and subtidal zones; notably the former has harsh changes in environmental conditions. Thus, a high level of stress tolerance is indispensable for many seaweeds to carry out normal development and growth. Seaweeds generally have a haploid-diploid sexual life-cycle in which the haploid gametophyte and diploid sporophyte alternate [1-3]. Red and brown seaweeds have a gametophyte- and sporophyte-dominant life cycle, respectively, with morphological differences between the two generations, whereas green seaweeds have morphologically indistinguishable gametophytes and sporophytes. However, little is known about the physiological and molecular systems regulating multicellularity, diphasic life-cycle and acquisition of stress tolerance in seaweeds.

Since elucidating the regulatory mechanisms of life cycle and stress tolerance is fundamental to understanding the biological strategies enabling growth and survival of seaweeds in a stressful hydrosphere, master genes regulating morphogenesis, life-cycle progression and acquisition of stress tolerance should be identified in a variety of seaweed species. To this end, it is clear that a gene-targeting manipulation is a promising technique for direct analysis of gene function [4]. However, such a method has not been established in seaweeds, despite much effort by phycologists to develop reverse-genetic techniques such as gene-targeting disruption (knock-out) and insertion (knock-in) by homologous recombination via stable transformation. To contribute toward establishment of genetic manipulation systems, I summarize here the current progress in genome analysis and development of reverse-genetic methods in seaweeds.

The presence of master genes for morphogenesis and life cycle has been confirmed by genetic studies using mutants showing aberrant morphology and developmental processes. For instance, in the model brown seaweed species *Ectocarpus siliculosus*,

the *étiole* (*etl*) mutant showed enhanced branching with arrest of apical growth in sporophytes [5]; and two life-cycle mutants were reported, the *immediate upright* (*imm*) and the *ourobros* (*oro*) mutants with partial and complete conversion, respectively, of the sporophyte to the gametophyte [6,7]. In addition, *slender* and *lumpy* mutants showed a high growth rate with a lack of cell differentiation and with cell aggregation due to alteration of polarized cell division, respectively, in the green macroalga *Ulva mutabilis* [8]. However, despite the isolation of numerous mutants in brown, green and red seaweeds [8], identifying the genes responsible for their phenotypes is very difficult, because the mutants were obtained by spontaneous and chemical mutagenesis. Thus, genome sequencing was required as an indirect approach. The first sequencing analysis of the nuclear genome in seaweed was for *E. siliculosus* [9], which enabled identification of a gene encoding IMM by positional cloning [10]. This represents a strong contribution of genome information toward understanding molecular systems regulating development and life cycle. The same approach is also expected to result in identification of genes responsible for the *etl* and *oro* mutant phenotypes.

Following the *E. siliculosus* genome analysis, the nuclear genome was sequenced for several red and brown seaweeds, especially for economic species such as Nori (Laver), Kombu, Mozuku and agarophytes (Table 1). In relation to this progress, genome-wide gene expression profiling (also known as transcriptome or RNA-seq analysis) was also performed extensively for simultaneous identification of genes induced and repressed under various kinds of environmental stress conditions or in different life-cycle generations (for example, [19-27]). Therefore, much information on genes and their expression profiles has been gained as a result of genome and transcriptome analyses.

As mentioned previously, we cannot directly analyze the functions of genes of interest at present because of the lack of genome

Species	Genome size (Mb)	Gene no	GC content (%)	Ref
<b>Brown seaweed</b>				
<i>Ectocarpus siliculosus</i>	214	16,256	53.6	[9]
<i>Saccharina japonica</i> (Kombu)	545	18,733	49.1	[11]
<i>Cladosiphon okamuranus</i> (Mozuku)	140	13,640	54	[12]
<b>Red seaweed</b>				
Bangiophyceae				
<i>Pyropia yezoensis</i> (Nori, Laver)	43	10,327	63.6	[13]
<i>Porphyra umbilicalis</i> (Nori, Laver)	87	13,360	65.8	[14]
Floridophyceae				
<i>Chondrus crispus</i>	105	9,606	52	[15]
<i>Gracilariopsis chorda</i>	92	10,805	49.3	[16]
<i>Gracilaria changii</i>	36	10,912	50.6	[17]
<b>Green seaweed</b>				
Mating type locus				
<i>Ulva partita</i> mt-	1.0	46		[18]
<i>Ulva partita</i> mt+	1.5	67		[18]

**Table 1:** Current status of nuclear genome sequencing in seaweeds (*Gracilariopsis chorda* and *Gracilaria changii* of Floridophyceae are agarophytes. Analysis of *Ulva partita* has been performed for only mating type loci of mt- and mt+ strains, whose GC contents were not mentioned in the reference).

manipulation techniques in seaweeds [28,29]. Nonetheless, two significant achievements toward development of reverse-genetic experimental systems were published in 2013 and 2015. One was the establishment of a functional RNA interference method using embryos of the brown alga *Fucus serratus* [30], in which microinjection of double-strand RNA (dsRNA) corresponding to the  $\alpha$ -tubulin gene transcript reduced intracellular microtubules and arrested growth of injected embryos. This was the first report of gene-targeting down-regulation in seaweeds due to the functional significance of dsRNA and is expected to contribute to analysis of gene function in developmental regulation of Fucales. However, applying this method to other seaweeds is likely to be very difficult, because vegetative cells and spores in most other seaweeds are much smaller than in *Fucus* embryos and so are likely to be killed by injection using a needle. Thus, microinjection is currently restricted to *Fucus* embryos.

The second method is confirmation of integration of a foreign plasmid into the genome of the green seaweed *U. mutabilis* [31]. The plasmid containing the bleomycin resistance (*ble*) or *ble-gfp* fusion coding region sandwiched by the 5'- and 3'-regions of the nuclear *rbcS* gene was successfully introduced into genomes of gametes, spores and protoplasts by polyethylene glycol (PEG) method, in which the plasmid DNA was randomly integrated into the genome of antibiotic-resistant cells. In addition, since some transgenic lines showed aberrant phenotypes, random integration seems to have potential to disrupt genes involved in regulation of morphogenesis. Thus, so-called tagging lines, a collection of mutants showing abnormal morphology or devel-

opment, might be generated by this approach, by which genes responsible for mutant phenotypes may be isolated more easily than for the positional cloning performed in *E. siliculosus*. However, in turn, random integration of a plasmid indicates an inability for gene-targeted modification using this method. It is therefore necessary to develop a powerful reverse-genetic system, such as a homologous recombination-based gene modification, to enable analysis of the functions of genes of interest. Alternatively, genome editing is expected to develop based on the PEG method because only transfer of a foreign gene to the nucleus, and not integration into the genome, is required for transient expression of genes in plasmids for genome editing.

Despite above significant progresses, gene-targeting homologous recombination and genome editing have not been established in seaweeds at present. Thus, functional analysis of genes involved in morphogenesis, development, life cycle and stress tolerance have not yet reported, except for the *E. siliculosus* *DISTAG/TBC-Cd1* gene controlling production of rhizoids, which was recently isolated by positional cloning [32]. It is however clear that seaweed genomes can accept foreign genes by integration [31]. Thus, homologous recombination is expected to be successful, if efficient selection of transformed cells or tissues is available, because seaweeds usually tolerate high concentrations of antibiotics [33]. Identification of an excellent selection marker for individual seaweed species could accelerate establishment of transformation systems based on gene-targeting modifications. In addition, to establish genome editing based on the CRISPR/Cas9 system, a promoter of the U6 or U3 small nuclear RNA

(snRNA) gene should be isolated to express a guide RNA [4]; however, it is currently difficult to identify these genes in genome and transcriptome data. In contrast, since microinjection of proteins and RNAs is possible, it is plausible that genome editing might be established at least in *Fucus* embryos by injection of both Cas9 protein and guide RNA into the nucleus. Accordingly, future studies toward establishing reverse-genetic manipulations in seaweeds other than *Fucus* embryos should focus on identifying selection markers and cloning promoter regions of snRNA genes. Progress in these approaches should provide essential techniques contributing to rapid development of seaweed biology in the genomic era.

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