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Molecular biotechnology of marine algae in China

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Abstract

Molecular biotechnology of marine algae is referred to as the biotechnology on the identification, modification, production and utilization of marine algal molecules. It involves not only the manipulation of macromolecules such as DNA, RNA and proteins, but also deals with low molecular weight compounds such as secondary metabolites.

In the last decade, molecular systematic researches to investigate the relationship and to examine the evolutionary divergence among Chinese marine algae have been carried out by Chinese scientists. For example, RAPD has been widely used in several laboratories to elucidate genetic variations of the reds, such as *Porphyra*, *Gracilaria*, *Grateloupia* and the greens such as *Ulva* and *Enteromorpha*. Some important data have been obtained. The study on molecular genetic markers for strain improvement is now in progress.

In 1990s, genetic engineering of economic seaweeds such as *Laminaria*, *Undaria*, *Porphyra, Gracilaria* and *Grateloupia* has been studied in China. For *Laminaria japonica,* the successfully cultivated kelp in China, a model transformation system has been set up based on the application of plant genetic techniques and knowledge of the algal life history. Progress has been made recently in incorporating a vaccine gene into kelp genome. Evidence has been provided showing the expression of gene products as detectable vaccines.

In the present paper, the progress of molecular biotechnological studies of marine algae in China, especially researches on elucidating and manipulating nucleic acids of marine algae, are reviewed.

Introduction

Algae form a broad and special group of living organisms. People from both the east and the west have long been interested in algae because of their morphological diversity, great biomass, abundance of metabolites and foreseeable economic potentiality (Critchley & Ohno, 1998). At present, the algal industry is worth several billion US dollars world-wide and the challenges facing the industry include stable supply of high quality raw materials, new algal product development and continuous search for new algal species with novel properties (Critchley, 2003). Large-scale cultivation of economically important algal species (Ask, 2003) and genetic engineering of desirable algal strains (Minocha, 2003) are approaches to ensure steady supply of quality raw materials to the algal industry.

China has been very successful in the cultivation of several commercially important algal species, such as *Laminaria*, *Undaria*, *Porphyra* and some agarophytes such as *Gracilaria*. It is now the world largest producer of kelps. The great success in Chinese kelp farming was partly due to the application of biotechnological approaches, such as seed selection and breeding techniques at the cytological level, in the process of cultivation. During the past ten years, more extensive biotechnological approaches at the molecular level, e.g. molecular genetic labeling techniques, have been adopted in marine algal research. The research goals were determination of taxonomic status of some species in dispute, establishment of evolutionary relationship and investigation of characters to be used to improve the quality of cultivated species. Genetic engineering of seaweeds was aimed at strain improvement and the utilization of cultivated seaweeds as bioreactors. This paper reviews and summarizes

some of the most recent results of algal molecular biotechnological researches carried out in China.

Molecular genetic markers

Analyses of phenotype mutation, chromosome polymorphism and protein polymorphism have been used to select and determine genetic markers. DNA polymorphism analysis is nowadays a more and more frequently used method. Many new genetic labeling techniques such as AFLP, RFLP, DNA fingerprinting, minisatellite DNA, microsatellite DNA, mitochondrion DNA, RAPD and sequence analysis have been set up (e.g. van Oppen et al., 1994, 1996; Ho et al., 1995; Minocha, 1998; Kusumo & Druehl, 2000; Wright et al., 2000). In the last decade, Chinese researchers have tried to employ some of these methods in marine algal research.

Sequence analysis

Partial sequence of the subunit gene of phycoerythrin (PE) in a red alga *Gracilaria lemaneiformis* (Bory) Dawson was analyzed by Sui & Zhang (1999), and high conservation level was seen by comparison with sequences from other three red algae *Rhodella violacea* (Kornmann) Wehrmeyer, *Polysiphonia boldii* Wynne *et* Edwards, and *Aglaothamnion neglectum* Feldmann–Mazoyer. Both α and β subunit genes have been highly expressed in *E. coli* (Sui & Zhang, 2001).

More sequence analysis researches have been performed on red tide-microalgae. The sequences of the 5.8S rDNA and the flanking ITS (internal transcribed spacers) 1 and 2 from twelve strains (five species) of *Alexandrium*, a dinophyceous genus with many toxic species, were analyzed by Chen & Qu (1999). The calculated genetic distance among these species suggested that ITS regions are practical molecular genetic markers for *Alexandrium* (Chen et al., 1999a).

Similar work was performed on another red tide-related species *Ceratium furca* Ehrenberg (Claparède *et* Lachmann) collected from Liaoning Province in northern China (Zhuang et al., 2001). Sequence alignments on over 1700 nucleotides of 18S rDNA of *C. furca* with 15 other representative species of dinoflagellates from Genebank have been analyzed in order to investigate the phylogenetic relationships within this highly divergent and taxonomically controversial group. A coherent and convincing evolutionary tree was obtained, using *Tetrahymena corlissi* Corliss as the outgroup. The results also showed that the ITS region of rDNA had a high level of sequence divergence, which could be a suitable target sequence for developing genus or species-specific oligonucleotide probes. Such probes would also be available in genus *Microcystis* based on the sequence analysis on its ISR region between 16S and 23S rDNA (Chen et al., 1999c). These results provide new perspectives on instant diagnosis of red tide marine algae and cloning of functional gene with known conservative sequences.

Restriction fragment length polymorphism (RFLP)

The small subunit ribosomal RNA genes (Ss-rDNA) of eight strains of the red tide toxic alga *Alexandrium tamarense* (Labour) Balech were amplified for a RFLP assay. PCR-RFLP analysis of the Ss-rDNA of samples from the South China Sea revealed that they only had gene A but lack gene B. This result suggested that gene B could be used as a molecular biogeographic marker for this species (Chen et al., 1999b). Chen et al. (1999a) conducted RFLP analysis in ITS region of two morphologically alike species *Alexandrium catenella* (Whedon *et* Kofoid) Balech and *A. tamarense* and found that all *A. catanella* samples (different collection sites) shared the same polymorphism pattern.

Random amplified polymorphic DNA (RAPD)

RAPD is regarded as a rapid, convenient and economic method (van Oppen et al., 1996). This method is now being used by Chinese phycologists and is widely applied in algal systematic research.

Under the optimized reaction condition for polymerase chain reaction (PCR) by using a single arbitrary primer, a high degree of reproducibility of the amplified bands could be obtained by gel electrophoresis. Such genetic markers are derived from priming sites randomly distributed throughout a genome, whose polymorphism makes the analysis of a complex genome without any prior knowledge of the DNA sequence possible. Application of RAPD to investigate phylogenetic relationship, to evaluate genetic variation and to clarify taxonomic ambiguities within several groups of seaweeds has been documented in China.

New ideas challenging traditional taxonomy were proposed when RAPD was carried out on *Ulva* and *Enteromorpha* (Chlorophyta) (Yang et al., 2000).

Based on the dendrogram of UPGMA and N-J analysis, convincing results showed that RAPD was effective in discriminating these two genera. The divergence between *Enteromorpha linza* (L.) J. Ag. and *Ulva* is shown to be even closer than that between *E. linza* and other *Enteromorpha* species. Similar results were also found in *Grateloupia* (Q. Wang et al., 2000). The deduced genetic distance between *G. filicina* f. *lomentaria* Howe and other *Grateloupia* species suggested that the former should be listed as a new genus, namely *Sinoyubimorpha*.

Genetic diversity of *Gracilaria* was also studied by using RAPD (Li et al., 1998). Different PCR patterns were derived in three mutants of *G. lemaneiformis*, and the relationship between different strains from different habitats was elucidated. In the same report, the phase and sex related markers were also discussed.

Most RAPD analysis was done to the important cultivated red alga *Porphyra*. Phylogenetic divergence was generated between geographic populations (Jia et al., 2000; Kuang et al., 1998a; Song et al., 1998; Y. Wang et al., 2000), between wild and cultivated populations (Xu et al., 2001), or among different cultivated populations (Mei et al., 2000; Shi et al., 2000). Some species trait related markers have been proposed which may be used further in strain improvement (Song et al., 1998).

Genetic engineering

Difficulties in genetic engineering of seaweeds were recognized in early 1990s (Saga, 1991). There was very poor knowledge on how to introduce foreign DNA into seaweed cells as well as on how to regenerate and select transformed plants. Moreover, nobody at that time knew where were the vectors for expressing foreign genes in seaweeds. From then on, efforts have been made in the world towards establishing a so-called transformation model for seaweeds.

In China, scientists have studied genetic transformation of economic seaweeds such as *Laminaria, Undaria*, *Porphyra, Gracilaria* and *Grateloupia* since 1991, and visible progress has been made in model research, especially in *Laminaria*.

The work in red algae

Wang et al. (1994) reported transient expression of the exogenous GUS gene in the protoplasts of *Porphyra haitanensis* Chang *et* Zheng by using electroporation. Kuang et al. (1998b) used five species of economically important red algae, i.e., *Porphyra yezoensis* Ueda, *Gracilaria asiatica* Zhang *et* Xia, *G. lemaneiformis*, *Grateloupia filicina* (Wulf.) C. Ag. and *Ceramium tenuissimum* J. Ag. as materials for transformation studies. The gene donors were plasmids containing GUS gene and CaMV35S promoter. Four DNA introduction methods, i.e., electroporation, PEG treatment, PEG + electroporation and Biolistic bombardment (Minocha & Wallace, 2000) were employed on *P. yezoensis*. Meantime, Biolistic bombardment was employed on explants of other four reds. The results suggested that PEG is the best method for transformation of protoplasts, and that Biolistic bombardment is effective in the transformation of protoplasts, thallus tissues and free-living conchocelis. In the transformation of *Gracilaria asiatica* by using Biolistic bombardment, a positive PCR result was obtained. The workers on reds are now trying to find applicable selectable markers to get true transformants at a certain scale. The development of a transformation model for the red algae is still incomplete.

The work in brown algae

Research has been done on the kelps *Laminaria japonica* Aresch. and *Undaria pinnatifida* (Harv.) Sur. in China. People want to use these algae as a cheap source of high value products by gene transfer. Genetic engineering is being expected to make kelp a useful bioreactor to produce drugs such as edible vaccines and to absorb excess N, P and other eutrophication elements from the marine environment. So far a model transformation system for kelp has been set up. The model includes a series of methods of gene introduction, vector construction, transformants' regeneration and screening (Qin et al., 1998a–c). By using this model, progress has been made in strain improvement with genes that serve particular purposes.

Application of Biolistic bombardment

Due to incomplete understanding of genomes of seaweed associated bacteria and viruses, direct physical methods have to be tried first in the transformation of seaweeds. Equally or even more difficult, protoplasts from either the sporophytes or the gametophytes of *L. japonica* failed to regenerate. Therefore, the search for method which can get the target DNA through the cell wall directly, e.g. ultrasonic treatment or Biolistic bombardment, was given the first priority.

Ultrasonic treatment was tried since it was less costly and more time saving, but the result did not suggest that it was a promising method. It could break up the filamentous female gametophytes into shorter fragments and partially break down cell walls, but at the same time it killed the cells and decreased their parthenogenesis efficiency (Wang et al., 1998a).

It has been proved that Biolistic bombardment could effectively introduce foreign DNA through cell walls into intact kelp cells, with either haploid or diploid thallus as recipient. By using this method, activities of CAT gene (*cat*) and LacZ gene (*lacZ*) have been detected in mature sporophytes regenerated by parthenogenesis. This suggested that random integration of foreign genes could occur in this way (Qin et al., 1998a–c).

Promoter selection

Promoter availability and selection is a critical factor in genetic transformation. Promoters from seaweed or seaweed-infective viruses have seldom been isolated (Henry & Meints, 1994). It was therefore necessary to examine some effective promoters from other organisms including higher plants and unicellular algae.

CaMV35S promoter was first used in brown seaweeds (*Laminaria* and *Undaria*) and transient expression of GUS reporter gene was observed (Qin et al., 1994). Further study was performed to select better promoters, including two from land plant, i.e. CaMV35S promoter and Ubiquitin promoter (from maize), and two from unicellular algae, i.e. fcp promoter (from diatom fucoxanthin, chlorophyll a/c-binding protein gene,) and amt promoter (from adenine methyltransferase gene of *Chlorella* virus). With young parthenogenetic sporophytes used as gene recipients and Biolistic bombardment as method, the quantitative detection of GUS transient expression using fluorometric assay indicated that CaMV35S and fcp promoter were more efficient in kelp than in the other algae (Wu, 2001).

So far two promoters have been shown to have efficient power in driving stable expression of foreign genes in kelp. They are fcp promoter (Wu, 2001) and SV40 promoter (from simian virus) (Qin et al., 1998a; Jiang et al., 2003). Utilization of SV40 promoter even resulted in uniform expression of *lacZ* reporter gene in regenerated *Laminaria* sporophytes, suggesting its high transcription recognition efficiency without histospecificity (Jiang et al., 2003). This promoter also worked well in *Undaria* for both transient (Yu et al., 2002) and stable expression (Qin et al., 2003).

Introduction of foreign genes into gametophytes and generation of sporophytes

Protoplasts, single cells and tissues are routine hosts for gene introduction in land plants, while neither haploid and diploid protoplasts nor single cells of *L. japonica* can be regenerated into new plants. Tissue culture in Laminariales has been studied extensively, but as yet no efficient regeneration system has been obtained in *L. japonica* since the regeneration efficiency from calli to sporophytes should still be increased (Wang et al., 1998b).

Fang (1978) reported that female gametophytes could develop by parthenogenesis. Also, female gametophytes could grow vegetatively to form filamentous clones which could be maintained for a long time in the laboratory. Stimulated under certain conditions, these vegetative clones can be developed into parthenogenetic sporophytes. Transgenic kelp has been obtained by bombarding foreign genes into female gametophytes and inducing the development of parthenogenetic plants (Qin et al., 1998a, c). Recently, success has also been obtained by using male gametophytes of *L. japonica* as transformation targets to generate zygotic sporophytes after hybridization (Jiang et al., 2003). The reproducibility of this newly developed pathway has also been confirmed in *U. pinnatifida* (Qin et al., 2003).

Employment of antibiotics and herbicide to select positive transformant

Antibiotics and herbicides are widely used as screening agents in genetic engineering of higher plants. Sensitivities of *L. japonica* to nine antibiotics and one herbicide were tested, including lincomycin, ampicillin, streptomycin, kanamycin, neomycin, chloramphenical, hygromycin, zeocin, G-418 and basta (Qin et al., 1998c). Results showed that both *L. japonica* and *U. pinnatifida* were sensitive only to chloramphenical, hygromycin and basta. The LD_{50} of hygromycin to parthenogenetic sporophytes was much lower than that of chloramphenical, and was not correlated with thallus length of the kelp while that of chloramphenical was. So *cat*, *hpt* and *bar* are applicable selectable markers for screening kelp transformants (Li et al., 1999; Wu et al., 2000; Yu et al., 2003).

By using the transformation model described above, stable expression of reporter genes such as *cat* and *lacZ* have been detected in parthenogenetic sporophytes after cultivation in the sea (Qin et al., 1998a, c; 1999). Recently progress has been made by using hepatitis B surface antigen gene (*HBsAg*). Positive results of PCR and ELISA for *HBsAg* detection were obtained, which suggested the integration of *HBsAg* into the genome of *L. japonica* (Jiang et al., 2002).

Conclusion

Much remains to be done for successful application of molecular biotechnological techniques in marine algal research in China to obtain strain improvement, development of algae as bioreactors, and for clarification of phylogenetic and evolutionary relationships among algal species of interest. Some progress has been achieved and it is anticipated that more will be coming in the near future as more and more people and resources are being invested towards these efforts.

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