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Recombinant expression of *rt-PA* gene (encoding Reteplase) in gametophytes of the seaweed *Laminaria japonica* (Laminariales, Phaeophyta)

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The life cycle of seaweed *Laminaria japonica* involves a generation alternation between diploid sporophyte and haploid gametophte. The expression of foreign genes in sporophte has been proved. In this research, the recombinant expression in gametophyte was investigated by particle bombardment with the *rt-PA* gene encoding the recombinant human tissue-type plasminogen activator (Reteplase), which is a thrombolytic agent for acute myocardial infarction (AMI). Transgenic gametophytes were selected by their resistance to herbicide phosphiothricin (PPT), and proliferated in an established bubble column photo-bioreactor. According to the results from quantitative ELISA, Southern blotting, and fibrin agarose plate assay (FAPA) for bioactivity, it was showed that the *rt-PA* gene had been integrated into the genome of gametophytes of *L. japonica*, and the expression product showed the expected bioactivity, implying the proper post-transcript modification in haploid gametophyte.

Laminaria japonica, gametophyte, genetic engineering, Reteplase, rt-PA, bioreactor

Proteins can be used as diagnostic reagents, vaccines and drugs, and this creates a strong demand for producing recombinant proteins on a large-scale[1]. Compared with microbial fermentation and mammalian cell lines, plants provide an inexpensive and convenient system for the large-scale production of valuable recombinant proteins[2-5]. Another important reason is that plants contain no known human pathogens that could contaminate the final product[6]. Over the past ten years, many plant-based expression systems have been established in a range of different species, e.g. leaf crops (tobacco), cereals (Maize), legumes (soybean), fruit and vegetables (tomato)[4]. Besides these land plants, the marine plants, such as the kelp (brown algae) *Laminaria japonica,* are also expected to be a promising marine bioreactor for the biopharmaceutical end[7,8].

The life cycle of *L. japonica* involves an alternation of generations between microscopic gametophyte (haploid) and macroscopic sporophyte (diploid). Gametophytes-targeted genetic transformation has been developed to obtain foreign gene expression in sporophytes generated by either fertilization[9] or parthenogenesis[7]. In this way, the *HBsAg* gene (encoding human hepatitis B surface antigen) was the first function gene expressed in sporophytes of *L. japonica*, with products possessing natural epitope[7]. Alternatively, to put the potential gene-flow under better control, it is necessary to develop the gene expression at gametophyte stage of kelp, to

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gether with strategies of culture in closed photo-bioreactor[8].

Up to now, the expression of *lacZ* reporter gene has been detected in *Undaria pinnatifida*, a species closely relate to *L. japonica*[10], and the integration of *bar* gene, whose product contributes resistance to herbicide BASTA®, in which phosphiothricin (PPT) is the effective ingredient, has been proved in *L. japonica*[11]. However, no function gene expression has been observed at the gametophyte stage.

In this study, the recombinant expression in kelp gametophyte was investigated by transferring *rt-PA* gene encoding Reteplase, which is the third-generation of thrombolytic agents for acute myocardial infarction (AMI). Structurally, rt-PA is a single-chain nonglycosylated deletion mutant of wild-type tissue plasminogen activator, consisting of just the kringle 2 and protease domains[12–16]. Although rt-PA is the most effective for clinical thrombolysis, thousands of dollars per therapy dose made it still expensive, especially in developing countries. rt-PA was produced mainly by *Escherichia coli* so far[17,18]. To explore new alternative expression host, *Pichia pastoris*[19] and CHO mammalian cell line[13,20] were still being tested, and there was no report yet on producing rt-PA in plant.

Accounting for better safety control, an illuminated bubble column bioreactor has been set up to culture transgenic gametophytes in a closed water body[21].

1 Materials and methods

1.1 Kelp strain and proliferation

Clonal stock lines of *Laminaria japonica* were maintained in our laboratory. Male gametophyte strain M039 was employed for this research. To get enough amounts of gametophytes after transformation and herbicide selection, a controllable bubble column photo-bioreactor was established. Every batch culture lasted for 20 day using modified culture conditions[21], by which the gametophytes biomass could proliferate about 10 times more than the initial inoculation.

1.2 Vector construction and transformation

The expression vector pSVrPA/CaMV-bar was constructed as indicated in the ref. [22]. The herbicide PPT resistance gene *bar* was driven by CaMV35S promoter. The expression of the *rt-PA* gene was regulated by the SV40 promoter and enhancer (Figure 1). The vector was introduced to the M039 strain according to the methods described in ref. [9].

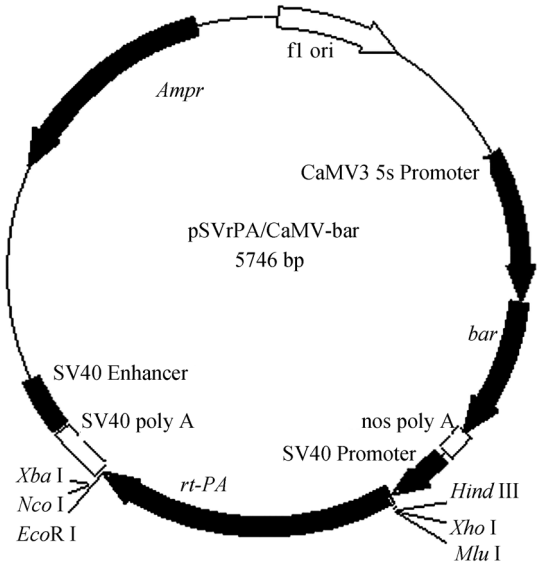

Figure 1 Illustration of transformation vector pSVrPA/CaMV-bar used to transform *L. japonica*. A *Hin*d III-*Nco* I fragment containing the intact *rt-PA* gene was labeled as blotting probe.

1.3 Herbicide selection and PCR detection

After one week of recovery culture following the bombardment, PPT in concentration of 40 mg/L was added to the media to screen the transgenic gametophytes, and it was removed three weeks later by renewing the normal media. Forty days later, the surviving colonies were picked out for primary biomass accumulation. Following the PCR detection with primers (Shanghai Sagon) for *bar*[11] and *rt-PA* gene (FW: 5′-GCTATTCCAGAAGTAGTGAGG-3′, RV: 5′-TTTGATGCGAAACTGAGG-3′) respectively, the positive colonies were introduced into a 300 mL bubble column bioreactor for vegetative proliferation.

1.4 ELISA quantitative determination for rt-PA

Gametophyte colonies were ground in an ice bath, and suspended in PBS (pH 7.0). Total soluble proteins were extracted and determined by the Bradford method[23]. The dissolved rt-PA was determined using an ELISA kit (Shanghai Sun).

1.5 Southern blotting

Total genomic DNA of gametophytes was prepared by Mini Plant Genome Extraction Kit (TaKaRa) according to the instruction. A *Hin*d III-*Nco* I fragment from the vector containing intact *rt-PA* gene was labeled as a

blotting probe. All genomic DNA sapmles and vector DNA were digested with *Hin*d III and *Nco* I. About 3 μg of each DNA sample was separated on 1% agarose gel, and subjected to the Southern blotting using the procedure from ref. [9].

1.6 Bioactivity assay

The fibrin agarose plate assay (FAPA) provides a quick, sensitive method to examine the fibrinolysis activity[24,25]. rt-PA converts plasminogen into plasmin, which then degrades fibrin to produce cleared zones. All reagents were obtained from the NICPBP (National Institute for the Control of Pharmaceutical and Biological Products). Followed the method from ref. [26] and Chinese Pharmacopoeia (appendix XI), the fibrin agarose plate was prepared with 0.1 mg coagulable protein/mL. The gametophyte in about 100 mg of wet weight was washed three times with sterilized PBS and then thoroughly ground in an ice bath. After centrifuge at 4℃ by 10000×*g* for 10 min, 30 μL supernatant was transferred into the wells fixed on the fibrin agarose plate, followed by incubation for 16 h at 37℃.

2 Results

2.1 PPT selection and PCR detection

Totally 33 gametophytes colonies were obtained after PPT selection and 19 colonies (about 58%) were proved positive in PCR detection for both *bar* and *rt-PA* gene. No PCR signal was detected in negative control which was transformed with naked golden particle. The specificity of amplified fragment was verified by PCR-Southern bloting (Figure 2).

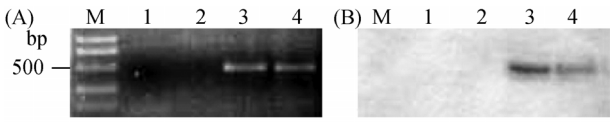

Figure 2 Results of PCR detection for *rt-PA* gene (A) and PCR- Southern blotting analysis (B). M: DL2000 marker (TaKaRa); 1: blank control (PCR without DNA template); 2: negative control (bombarded with naked golden particle); 3: positive control (vector pSVrPA/CaMV-bar as PCR template); 4: survival sample after PPT selection.

2.2 ELISA quantitative determination

Among ten PCR-positive transgenic samples detected by quantitative ELISA, seven samples showed positive results. The concentration of rt-PA in total soluble protein ranged from 0.023 to 0.470 μg/mg soluble proteins, averaging 0.159 μg/mg soluble proteins, and no background was detected in ten control samples (Table 1).

2.3 Integration detection by Southern blotting

The samples with both positive signal in PCR and higher value in ELISA were analyzed by Southern blotting. An expected 1.1-kb band was detected in tested sample and no signal found in negative control (Figure 3). The result showed that the *rt-PA* gene has been stably integrated into the genome of *L. japonica* gametophyte.

2.4 Fibrinolysis activity assay

According to the results of FAPA analysis, the transparent circle produced by a transgenic sample showed the effective bioactivity of the rt-PA expressed in kelp gametophyte, and no such activity was shown in negative controls (Figure 4).

3 Discussion

The results showed that *rt-PA* gene has been successfully integrated into the genome of gametophyte of *L. japonica*, and the recombinant expression product showed expected bioactivity, which indicating that the recombinant rt-PA underwent a proper post-translational modification and structural folding in haploid kelp gametophytes. To our knowledge, this was the first report of recombinant expression of rt-PA in plant.

The structural stability and biological activity of rt-PA depends on the correct formation of nine disulfide bonds[17]. Usually, rt-PA is produced as inactive inclusion bodies in *E. coli* and requires *in vitro* folding to become active[16,17]. However, the renaturation ratio is still low[27], which contributes to the high cost for this drug. This research showed that the *in vitro* renaturation procedure could be saved when using gametophyte of *L. japonica*, a eukaryotic expression host, to produce rt-PA.

Table 1 ELISA quantitative determination for rt-PA in transgenic samples (μg/mg soluble proteins)

Sample No.	S1	S2	S3	S4	S5	S6	S7
rt-PA content	0.277 ± 0.031	0.054 ± 0.012	0.470 ± 0.020	0.023 ± 0.004	0.089 ± 0.016	0.112 ± 0.024	0.091 ± 0.007

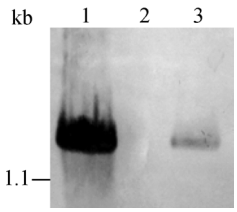

Figure 3 Southern blotting analysis. 1: Positive control (digested vector pSVrPA/CaMV-bar); 2: negative control (bombarded with naked golden particle); 3: ELISA-positive sample S3.

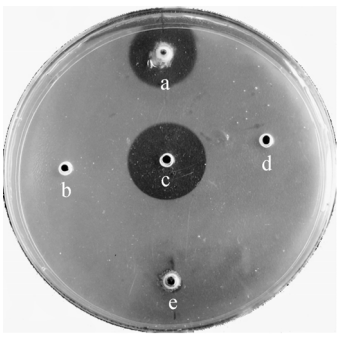

Figure 4 Result of FAPA analysis. a: Positive sample; b: blank control 1 (30 μL PBS added); c: positive control (rt-PA added); d: blank control 2 (no sample added); e: negative control (bombarded with naked golden particle).

In this study, the average concentration (0.159 μg/mg soluble proteins) of rt-PA expressed in gametophyte is lower than that (0.529 μg/mg soluble proteins) of HBsAg in the transgenic sporophyte of kelp, using the same SV40 promoter[7]. This may partially result from the difference in the recognization efficiency to the heterologous promoter between gametophyte and sporophyte. To increase the expression efficiency in gametophyte, further work to isolate the endogenous constitutive promoter at gametophyte stage of kelp is necessary. Only seven of ten PCR-positive samples showed ELISA signals, and significant difference of rt-PA content was detected among those positive samples, implying an un-uniform expression pattern in gametophyte of kelp, produced by the random integration mechanism for introduced gene. More efforts should be made to set up directional integration techniques in kelp.

It has been reported that both gametophyte and sporophyte of kelp were more sensitive to PPT than to antibiotics[11,28]. This study proved that PPT may select the transformants effectively at the gametophyte stage with low dosage.

Up to now, the integration and expression of several genes have been detected in kelp sporophyte, generated from either fertilization or parthenogenesis after gametophyte-targeted transformation[8]. However, to be an expression host for recombinant protein in a large scale, the macroscopic sporophytes still need to overcome such challenge on bio-safety issue as potential gene-flow since they need to be cultivated in a open water body. Alternatively, to develop the microscopic gametophyte as a new expression host functioned in closed water body would be a better choice. As shown in this research, all procedures including transformation, selection and proliferation could be accomplished at gametophyte stage. Especially, the establishment of a photo-bioreactor had been proved to be a feasible way to rapidly accumulate the biomass of transgenic gametophyte. This system could avoid the accidental release of transgenic gametophyte effectively, which is a potential risk to water environment. Moreover, it can also be considered as an effective "seed"-supplying system to generate transgenic sporophyte when under entire control.

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