

Construction of an expression vector for production of tissue plasminogen activator (t-PA) in a transgenic microalgae bioreactor

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Abstract. Microalgae systems have opened up new platforms for molecular farming in closed conditions. Recently, *Dunaliella salina* has been considered to be a suitable expression host for the production of recombinant proteins due to its significant advantages. It is a unicellular eukaryotic organism lacking cell wall and having post-translational processing for proteins which can grow in high salinity environments. To construct an expression vector, different fragments including Ubiquitin promoter, the gene of interest (t-PA; K2 and S domains), Omega leader sequences, KDEL, 6-Histidine tag, SYQ and Nos terminator (as the stop signal for gene transcription) were considered in upstream strategy. pCAMBIA 3301 and 1304 plasmids which contained *bar* gene (phosphinothricin resistance gene as a plant selectable marker) were used as the framework for construction of the expression vector and driving the expression of gene of interest. Further studies are undergoing to transform the constructed vector into *D.salina*.

Keywords: *Dunaliella salina*, heterogeneous expression, molecular farming, recombinant protein, photobioreactor, transgenic microalgae.

1. Introduction

The use of *Dunaliella salina* as the host cell, as in the present research, is considered to have significant advantages due to the following reasons: *D.salina* is probably the most halotolerant unicellular eukaryote known. The cell of *D.salina* has no cell wall, which permits rapid volume changes in adaptation to extracellular changes in osmotic pressure (1, 4). As a result, its adaptability to the environments is extremely strong, and can live in a variety of salt concentrations from as low as 0.2% up to salt saturation concentration (35%). *D.salina* has a strong capability of photosynthesis and can synthesize many organic molecules like proteins from water, carbon dioxide and inorganic salts under sunlight. For this reason, mass cultivation of *D.salina* can be considered as easy and cheap.

It is believed that, as compared with other transgenic bioreactors, the transgenic *D.salina* bioreactor of the present research possesses many advantages:

- *D.salina* is a unicellular organism and its protein content is relatively high. The alga propagates very fast and grows without seasonal restriction, and it can grow to dense populations in a shorter time, which make the screening of the transformed *D.salina* easier.
- *D.salina* is a lower eukaryotic green alga lacking of cell wall, therefore genetic manipulation thereof is easier. In addition, there is single, large, cup-shaped chloroplast in the cell, which is useful for the chloroplast transformation of foreign genes. The chloroplast transformation is harmless to the environments and does not induce gene silencing.

- The cultivation of *D.salina* is based on autotrophic growth in media containing inorganic salts, e.g. nitrate as the nitrogen source and carbon dioxide as the exclusive carbon source. Hence, the cultivation cost can be considered low. *D.salina* can grow in the high salinity media in which other organisms are not able to withstand. The cost of production can be decreased greatly because two models of cultivation, intensive and extensive, have been used in the large-scale cultivation of *Dunaliella salina*.
- *D.salina* has the eukaryotic post-translational processing for proteins, such as the formation of disulfide bond and glycosylation, which simplifies processing of the downstream of gene engineering and assures bioactivity and quality of the protein products.
- *D.salina* is, because of being nontoxic and abundant in natural vitamins and polyunsaturated fatty acids, valuable edible alga. Oral vaccines or drugs produced with *D.salina* can be taken directly even without purification, so that the cost of production may be reduced markedly.
- The bioreactor system according to the present research may be used to produce drugs or vaccines for humans and/or animals, phytohormones and other bioactive materials. So, it may be reasonable to study its application in production of recombinant pharmaceutical proteins (1, 4, 5, 6, 10).

According to a new WHO report, heart diseases and stroke have been reported as the chief causes of death globally, and they will remain at the top until 2030 (9). It appears that the t-PA has the highest affinity for fibrin compared to the uPA group of activators. Tissue plasminogen activator (t-PA) is a naturally occurring protein that cleaves the inactive zymogen plasminogen into the active enzyme plasmin. Plasmin then cleaves fibrin clots. This process is important in removing clots after the repair of a wound has been completed. Clots often form in response to disease conditions such as atherosclerosis, stroke, cancer and some complications during pregnancy. Coadministration of heparin (as an auxiliary anticoagulant) and t-PA clears up to 75% of obstructed coronary arteries in 90 min and decreases mortality 25%. Therefore, t-PA has been known as the best available effective medication and the only drug approved by the U.S. Food and Drug Administration in this case. Doctors now use recombinant t-PA as a powerful drug to help dissolve these clots and prevent much of the damage associated with loss of blood flow to vital organs (3).

The production of t-PA by recombinant techniques should provide sufficient quantities to examine its clinical usefulness in the treatment of pulmonary embolism, deep vein thrombosis, heart attacks and strokes. Hence, the development of a more effective and cheaper t-PA by genetic engineering techniques is significant for the treatment of these diseases world-wide.

Expression of recombinant proteins is an important method to make proteins. Different systems including bacteria, yeasts, fungi, mammalian cells, insect cells, transgenic animals, and transgenic plants have been used as necessary. None of these numerous systems is general. All systems use independent organisms.

Major object of the present study is providing methods for production of biologically active t-PA in *D.salina*. Hence, it is an objective of the present research to provide a new and effective transgenic *D.salina* bioreactor, which can be used to produce t-PA and possibly other valuable pharmaceutical proteins for humans and/or animals.

2. Materials and Methods

2.1. *Dunaliella salina* strains and media

D.salina obtained from Prof. M. Shariati (Department of Biology, Isfahan University) was used in this study. Liquid modified Johnson medium (11) was used to cultivate cells. The cultures were incubated at 25°C under 50 $\mu\text{mol photon/m}^2/\text{s}$ illumination with a 16:8 h light:dark cycle.

2.2. Vector construction

To construct an expression vector, different fragments including Ubiquitin promoter, Omega (12) and TEV (Tobacco Etch Virus) as enhancers, the gene of interest (t-PA; K2 and S domains), KDEL, MARs

(Matrix Attachment Regions) sequence, 6-Histidine tag, SYQ and Nos terminator (as the stop signal for gene transcription) were considered in the upstream strategy.

Ubiquitin promoter was isolated from a vector (pGEM4z) incorporating Ubi. The Omega leader sequence designed in the Ubi primer set to be amplified along with Ubi. Regarding to the gene of interest, all of the required sequences as mentioned above were considered in the primer pairs so that the GOI amplified through 3 continuous PCR from a plasmid containing K2S domains of t-PA. All of the PCRs were carried out using High Fidelity Polymerases (Roche and Fermentas).

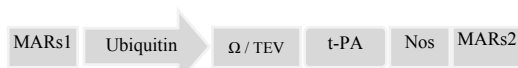


Fig. 1: The specific construct designed to be made for *Dunaliella salina* transformation.

pCAMBIA 3301 and 1304 plasmids which contained *bar* gene (phosphinothricin resistance gene as a plant selectable marker) were used as the framework for construction of the expression vector and driving the t-PA expression (4).

pCAMBIA 3301 and the plasmid containing t-PA were cut with the same restriction enzymes simultaneously. The cuts of pCAMBIA 3301 and t-PA fragments were purified from the gel and ligated. The ligated plasmid was transformed to *E. coli* (BL21) through electroporation.

Verification of the ligations was carried out through colony PCR, double digestion and sequencing (Fig. 2).

3. Results & Discussion

The gene of interest obtained via 3 continuous PCR to add different fragments of interest, so that each PCR product was the template for the next one. Final PCR product included all of the required sequences KDEL (amino acid; a sequence in the amino acid structure of a protein which keeps it from secreting from the ER; The KDEL sequence is Lys-Asp-Glu-Leu. This sequence is responsible for retrieval of ER luminal proteins from the Golgi), 6-Histidine tag (an amino acid motif in proteins that consists of at least six histidine residues, often at the N- or C-terminus of the protein. It is also known as hexa histidine-tag, 6xHis-tag, and by the trademarked name His-tag), Xa factor and SYQ.

The final PCR product of t-PA was cloned through TA cloning and the successful ligation and transformation was confirmed by Colony PCR. The positive clones were cultured for plasmid extraction.

Digestion of pCAMBIA3301 vector and the plasmid containing t-PA was carried out successfully so that *gus* and t-PA fragments were cut off. The digested pCAMBIA3301 and t-PA sequence containing the required elements were ligated and transformed into *E. coli*. The proper ligation was verified through PCR, double digestion and sequencing. Sequencing results confirmed the proper sequence of inserted gene.

In the next steps the construct will be transformed into microalgae cells and the constructed vector will be further analyzed regarding to expression level, stability of transformants, as well as the bioactivity of the protein.

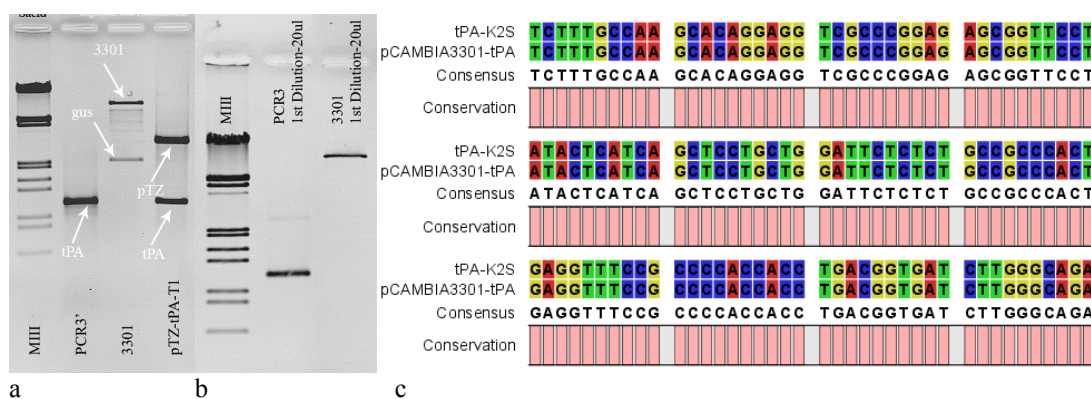


Fig. 2: Verification of the inserted sequence. a: Digestion of pCAMBIA3301 vector and the plasmid containing tPA using NcoI and BstEII, b: Digested fragments purified from the gel, c: Alignment of the ligated sequence with the original one.

4. Acknowledgment

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