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Impact of SV40 T antigen on two multiple fission microalgae species Scenedesmus quadricauda and Chlorella vulgaris

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Abstract

The combination of Simian Virus40 (SV40)'s large T antigen with its replication origin is commonly used in molecular studies to enhance the expression of heterogeneous genes through multiplying the plasmid copy number. There are no reports related to the impact of the SV40 T antigen on plant, multiple fissional, cell-type. This study explores the response of two multiple-fission microalgal cells, Scenedesmus quadricauda and Chlorella vulgaris, to the expression of the T-antigen, with aim of applying SV40 T-antigen to increase the expression efficiency of foreign genes in the two species. Different levels of low-expression have been constructed to control the expression of SV40 T antigen using three heterogenous promoters (NOS, CaMV35S, and CMV). Chlorella cultures showed slowdown in the growth rate for samples harboring the T antigen under the control of CaMV35S and CMV promoters, unlike Scenedesmus cultures which showed no significant difference between samples and could have silenced the expression.

Keywords: *Scenedesmus quadricauda, Chlorella vulgaris, multiple fission, SV40 T antigen, Retinoblastoma (RB)-family*

1. Introduction

SV40 T antigen is a viral initiator protein necessary for DNA replication of Simian virus 40 (SV40) which is a member of Polyomaviridae Virus family [1]. The genomic sequence of SV40 T-antigen contains two subunits, the large- and the small T-antigen (T-antigen & t-antigen). The Large T-antigen is a sequence-specific DNA-binding protein that also possesses intrinsic DNA helicase and ATPase activities that are required for its ability to influence viral DNA replication [1].

It has been reported that four functions of the SV40 T antigen, three in large T and one in small t, with obvious connection to mammalian and yeast cell transformation [2-4]. The SV40-transforming function correlates with the ability of one of the T antigens to bind to a cellular protein through the large T antigen that binds to the heat shock chaperone, hsc70, retinoblastoma family (Rb-family), and the tumor suppressor p53, while the small T antigen binds to the cellular phosphatase pp2A. Almost all previous reports about the SV40 T antigen were conducted on binary fissional cell types [1].

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The interaction of the SV40 large T antigen with Rb-family proteins is remarkable [1], causing mitotic regression through holding the cell for a longer time in the S-phase [1,5,6].

Although SV40 T antigen's mechanism of action has only been studied in animal cells, similarities between programmed cell death in plants and apoptosis of animals were observed, especially in pathways like (RB)-E2F, that is critical in regulation of the initiation of the DNA replication [7-9]. Retinoblastoma (Rb) homology has been found in all sequenced animal genomes as well as in some plants, and the first identification of Rb protein from plants has been found in maize, which was reported to be targeted by the RepA protein of the plant gemini virus for efficient viral DNA replication [7,10,11]. This interaction is similar to the interaction between animal cell Rb and animal viruses, which prove that plant and animal's Rb proteins at least share some similarities in functions.

Later, several studies showed that there is a functional similarity also between some microalgal proteins and Rb-family proteins. These proteins started by MAT3, which work in the regulation of the cell cycle in *Chlmydomonas reinhardtii,* followed by others in *Ostreococcus tauri*, *Volvox carteri*, and *Micromonas pusilla* [7,12-14].

However, no studies yet showed whether the SV40 T-antigen causes mitotic regression by interacting with any of plant's cellular proteins. Therefore, the initial trails of the current study were done using the two multiple-fissional microalgal species *Scenedesmus sp*. and *Chlorella sp*. with the T antigen as a complete gene and just the coding area of the large T antigen which is the most responsible part of cell transformation [1,15,16]. *Scenedesmus sp*. and *Chlorella* sp. are two different multiple-fissional algal models, their cell-cycle model has many patterns under different environmental conditions [17-19]. *Scenedemus* sp. is comprised of colony forming cells called "coenobia", but *Chlorella* stay as a single-cell after cytokinesis [17,18]. The current study has been conducted to explore the impact of the SV40 T-antigen on microalgae cells, which could lead to develop a stable gene transformation system for microalgae able to inherit from parent- to daughter cells without using selective pressure.

2. Experiments

2.1 Isolation, purification and cultivation of *Scenedesmus quadricauda* and *Chlorella vulgaris*

Freshwater algal samples were collected from a small pool next to Deokgok-Je reservoir, Josan-ri, Soramyeon, Yeosu, Korea. The collected samples were cultivated in slightly modified Beijerinck medium [20]. Isolation, purification, and identification were done based on cell morphology and 18S rDNA sequence. Purified species *Scenedesmus quadricauda* and *Chlorella vulgaris* were cultivated in 30 ml *Euglena gracilis* medium: Jaworski's medium (EG:JM medium) (Table 1). The cultures were submitted to continuous illumination for two weeks. The temperature of the culture was maintained at 24°C, with a light intensity of 8,000 Lm.

Table 1. EG:JM medium composition

Euglena Gracilis Medium	Medium	Per liter
1	Sodium acetate trihydrate	1 g
2	LabLemco powder	1 g
3	Tryptone	2 g
4	Yeast extract	2 g
5	CaCl2	0.01 g
Jaworski's Medium	Stocks	Per 200ml

1	$Ca(NO_3)_2 \cdot 4H_2O$	4.0 g
2	KH_2PO_4	2.48 g
3	$MgSO_4 \cdot 7H_2O$	10.0 g
4	$NaHCO_3$	3.18 g
5	EDTAFeNa	0.45 g
	EDTANa2	0.45 g
6	H_3BO_3	0.496 g
	$MnCl_2 \cdot 4H_2O$	0.278 g
	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.20 g
7	Cyanocobalamin	0.008 g
	Thiamine HCl	0.008 g
	Biotin	0.008 g
8	$NaNO_3$	16.0 g
9	$Na_2HPO_4 \cdot 12H_2O$	7.2 g
	Medium	Per liter
	Stock solutions 1-9	1 ml each
EG: JM medium	Medium	Per liter
	EG and JM medium	Mix 1:1

2.2 Cassette construction

SV40 genomic DNA (ATCC 45019D) and PRI201 plasmid vector (Takara Bio) were used as the template source for isolating the T antigen gene, GUS reporter gene, CaMV35S and NOS promoters, respectively. All primer designs were done using Primer3Plus online software [21,22] and listed in Table 2.

Overlapping PCR was used with the whole T antigen gene to construct the following DNA cassettes: CaMV35S+T antigen, NOS+T antigen, and CaMV35S+GUS. By excising intron (small T antigen) from large T antigen, we constructed CaMV35S+large T antigen and NOS + large T antigen.

The primers on the flanks regions of all cassettes contained the two restriction sites SacI and ApaI for double digestion purpose. The overlapping PCR products were examined on 1.5%agarose gels, and the chosen band was extracted using the Qiagen gel extraction kit (Tumberry Lane, Valencia, CA, USA). All PCR reactions were carried out using the high-fidelity DNA polymerase (TLA polymerase) (Bioneer Inc., Daejeon, Korea).

Table 2. Primers used in the current study

Primer No.	Primer name	Sequence (5'-3')	Purpose
1	LT-Antigen-OF	TGCAACTGAGATTCCAACCTATGGAACTGATG	Primers 1 & 2 are used in combination
2	LT-Antigen-OR	AGGTTGGAATCTCAGTTGCATCCCAGAAGC	with Primers 3 & 4 to splice the intron from the Large T-antigen coding region
3	SV40-TAD-F	CAGCGAGCTCTTGGAGGCCTAGGCTTTTG	Primers 3&4 are the forward and reverse
4	SV40-TAD-R	GCACGGGCCCGTTAACAACAACAATTGCATTCAT	of the SV40 T-antigen total gene
5	cDNA-TA-F	GGAATCTTTGCAGCTAATGGA	Primers 5&6 used with RT-PCR to Check
6	cDNA-TA-R	GGAGGTGTGGGAGGTTTTT	the transcription of the T-antigen 250bp
7	O-F1H-1	CGTTATCCCCTTACCGCCATGCATTAGTTATT	The Set of primers (7 to 15), are used to
8	O-RSL-2	GCGGGACTATTTCCACAGCTGGTTCTTTCC	disintegrate the pAcGFP1-Hyg-N1
9	O-F1SL-3	AGCTGTGGAAATAGTCCCGCCCCTAACTCC	plasmid vector into four fragments and
10	O-R2B-3	TATGATCCTCACAGACGTCGCGGTGAGTT	reconstruct it again to remove some
11	O-F1B-4	CGACGTCTGTGAGGATCATAATCAGCCATACCA	unnecessary genes like GFP, Hygromycin
12	O-R1H-4	ATGGCGGTAAGGGGATAACGCAGGAAAGA	resistance gene, and SV40 promoter
13	R1SC-1	CAGCGAGCTCTCGAGATCTGAGTCCGGTAG	

14	F2A-2	CAGCGGGCCCACGGCATGGATGAGCTGT	
15	R1SC-4	CAGCGAGCTCGGGGATAACGCAGGAAAGA	
16	Gus-F	TACGTCCTGTAGAAACCCCAAC	Primers 16&17 designed to amplify the GUS gene from PRI201-AN-GUS plasmid vector
17	Gus-R	TCGAGGTCGATCATTGTTTG	
18	NOS-DF1	CAGCGAGCTCTTCCAGTCGGGAAACCTGT	Primers 18, 19, and 20 designed to amplify the NOS promoter from PRI201-AN-GUS vector and connect it to the T-antigen via Overlapping-PCR
19	NOS-OR1	AGGCCTCCAAAATCATGCGAAACGATCCAG	
20	SV-TA-NOS-F1	TCGCATGATTTTGGAGGCCTAGGCTTTTG	
21	SV-TA-pri+ F1	AGAACACGGGTTGGAGGCCTAGGCTTTTG	Primers 21, 22, and 23 designed to amplify the CaMV35S promoter from PRI201-AN-GUS vector and connect it to the T-antigen via Overlapping-PCR
22	CaM35S-R1	AGGCCTCCAACCCGTGTTCTCTCCAAATGA	
23	CaMV35S-F	CTGCAGGAGATTAGCCTTTTCA	
24	CaMV35S pro-F	CTGCAGGAGATTAGCCTTTTCA	Primers 24&25 designed to check the presence of CaMV35S promoter
25	CaMV35S pro-R	CCCGTGTTCTCTCCAAATGA	
26	CMV pro-F	CGGGGTCATTAGTTCATAGCC	Primers 26&27 designed to check the presence of CMV promoter
27	CMV pro-R	GGCGGAGTTGTTACGACATT	
28	NOS Pro-F	TTCCAGTCGGGAAACCTGT	primers 28&29 designed to check the presence of NOS promoter

2.3 Vector construction

Linearization and modification of pACGFP1-Hyg-N1 plasmid vector (Clontech, CA, USA) using TLA polymerase were done on four levels. First, primers were designed to open the vector from the MCS area including the CMV promoter. Second, the CMV promoter was excluded from the level one vector. Third, the level-two vector was modified via removing the GFP and Hygromycin resistance gene. Finally, the fourth level was achieved by excluding the SV40 promoter from the level-three vector (supplementary data, E).

The primers used for linearizing the vector contained the restriction sites SacI and ApaI for double digestion Table 2.

Seven reconstructed vectors were prepared through digestion ligation reactions for vectors and DNA cassettes. Cassettes 1 to 5 were in complete pACGFP-Hyg-N1 vector. Cassettes 6 and 7 were in modified vectors (supplementary data, D, C) and listed in Table 3. Transformations were done using HIT competent cells, followed by plasmid preparation using Qiagen miniprep (Tumberry Lane, Valencia, CA, USA). Sequencing was carried out to check the success of cassettes construction including the modified parts of the last two vectors.

Table 3. Strains and plasmids used in the current study

Strains/plasmids	Relevant characteristic	Reference or source
Strains		
Chlorella vulgaris	Wild type	Deokgok-Je reservoir, Josan-ri, Soramyeon, Yeosu, Korea
Scenedesmus quadricauda	Wild type	Deokgok-Je reservoir, Josan-ri, Soramyeon, Yeosu, Korea
Escherichia coli-NOS1	*E. coli* harboring plasmid pAcGFP1-Hyg-N1-Cassette1	This study

Escherichia coli-CaMV2	*E. coli* harboring plasmid pAcGFP1-Hyg-N1-Cassette2	This study
Escherichia coli-CMV3	*E. coli* harboring plasmid pAcGFP1-Hyg-N1-Cassette3	This study
Escherichia coli-NOS4	*E. coli* harboring plasmid pAcGFP1-Hyg-N1-Cassette4	This study
Escherichia coli-CaMV5	*E. coli* harboring plasmid pAcGFP1-Hyg-N1-Cassette5	This study
Escherichia coli-NOS6	*E. coli* harboring plasmid Modified pAcGFP1-Hyg-N1-Cassette6	This study
Escherichia coli-NOS7	*E. coli* harboring plasmid Modified pAcGFP1-Hyg-N1-Cassette7	This study
Plasmids		
pAcGFP1-Hyg-N1	CMV promoter;AcGFP, Hyg^r; *ori*SV40	Clontech, CA, USA
PRI201-AN-GUS	CaMV 35S Promoter; GUS; NOS promoter;	Takaka Bio. Inc., Shiga, Japan
pAcGFP1-Hyg-N1-Cassette1	NOS promoter; Complete T-antigen; AcGFP, SV40pro-Hyg^r; *ori*SV40	This study
pAcGFP1-Hyg-N1-Cassette2	CaMV35S promoter; Complete T-antigen; AcGFP, SV40pro-Hyg^r; *ori*SV40	This study
pAcGFP1-Hyg-N1-Cassette3	CMV promoter; Complete T-antigen; AcGFP, SV40pro-Hyg^r; *ori*SV40	This study
pAcGFP1-Hyg-N1-Cassette4	NOS promoter; Large T-antigen Coding region; AcGFP, SV40pro-Hyg^r; *ori*SV40	This study
pAcGFP1-Hyg-N1-Cassette5	CaMV 35S promoter; Large T-antigen Coding region; AcGFP, SV40pro-Hyg^r; *ori*SV40	This study
MD-pAcGFP1-Hyg-N1-Cassette6	NOS promoter; Large T-antigen Coding region; Hyg^r; *ori*SV40	This study
MD-pAcGFP1-Hyg-N1-Cassette7	NOS promoter; Large T-antigen Coding region; Hyg^r; *ori*SV40	This study
pAcGFP1-Hyg-N1-GUS	CaMV 35S promoter; GUS gene; AcGFP, SV40pro-Hyg^r; *ori*SV40	This study

2.4 CaMV35S promoter activity

CaMV35S promoter was connected to the GUS reporter gene taken from PRI201-AN-GUS vector. The short cassette introduced into the pACGFP-hyg-N plasmid. The CMV promoter of the original vector has been replaced with the new cassette.

2.5 Electroporation and Cultivation

A 30 ml volume of EG: JM medium in a 100 ml conical flask was inoculated with 1ml of algae grown in EG: JM medium. Algae were grown with orbital agitation (80-100 rpm) at 25-27°C under continuous illumination for 3 days. At the end of the period, cell density was 10^5 cells/ml. Cells were collected by low speed centrifugation at 4000rpm for 9 minutes. They were washed with 20 ml of 7 mM HEPES and 252 mM glucose at pH 7.0. After centrifugation, washing was repeated. The cells were then suspended in 2ml of sterile distilled water and were kept on ice for 2 h. Eighty micro liter of cell suspension was mixed with a maximum volume of 5µl of plasmid DNA. All DNA concentrations were assayed by measuring optical density at 260 nm and fixed as 33.6 ng by dilution using DW for all samples. Plasmids were used as super coiled and open circular DNA. Electroporation was carried out in the following conditions: electric field 1800 V/cm and shunt 200 Ω. Pre-tests were carried out to select the proper DNA concentration for electroporation (Supplementary data, A). Colonies number and applied pulse were considered. After

electroporation, the algal cells were kept on ice for 5 min and suspended in EG:JM medium (5 ml) containing mannitol (1 M) and sorbitol (1 M). The cells were placed in the dark for 12-24 h without agitation. After shaking, 1 ml was taken from each tube into a 100ml conical flask containing new 30ml EG: JM sterilized medium. All flasks were cultivated under continuous illumination with agitation. Culture regeneration was done using 1ml from the old culture to a new 30 ml EG: JM medium. The whole experiment has been repeated three times sequentially on the two species. Three selected cultures of *Scenedesmus sp*. (Empty vector, CMV+ T antigen, CaMV + T antigen) were regenerated until G3.

2.6 Modified protocol for screening the mitotic-division

In the current experiment there are two parameters involved in the vector design. These two parameters are the plasmid vector stability and the impact of the T-antigen. Thus, calculating the probabilities which come out from combining the two parameters will result in three possibilities. Comparing to the control sample which only have the empty vector in algal cell, so the first possibility is the vector would be stable with normal mitotic division; secondly the vector also would stable but with mitotic regression. Otherwise, the third possibility would be that the vector is unstable with normal or mitotic regression.

To go through the previous calculated probabilities there was a need to screen the differences in the mitotic divisional rate with the previous-mentioned two parameters and without applying any selective pressure. No protocols adopted previously for such case, so a simple method applied to compare between the wild type and the transformed ones. Considering the impact of the T-antigen which slowing down the mitotic division of the host cell. So, after transformation; there was no separation between transformed and non-transformed cells of the same species under the same conditions (Fig. 1). The wild-type and the non-affected recombinant cells (Fig. 1, “Culture A”) were divide normally, resulting in a fixed ratio of cell-number in each generation. Therefore, the foreign DNA concentrations will be almost equal within samples of the same generation. On the other side a mixture of wild-type and mitotic-affected recombinant cells in one culture (Fig. 1, “Culture B”), the cell-number of the mitotic affected cells will decrease compared to the number of wild-type cells. So, considering the three expression levels, the result should appear with some variation in the DNA concentrations and band patterns through generations.

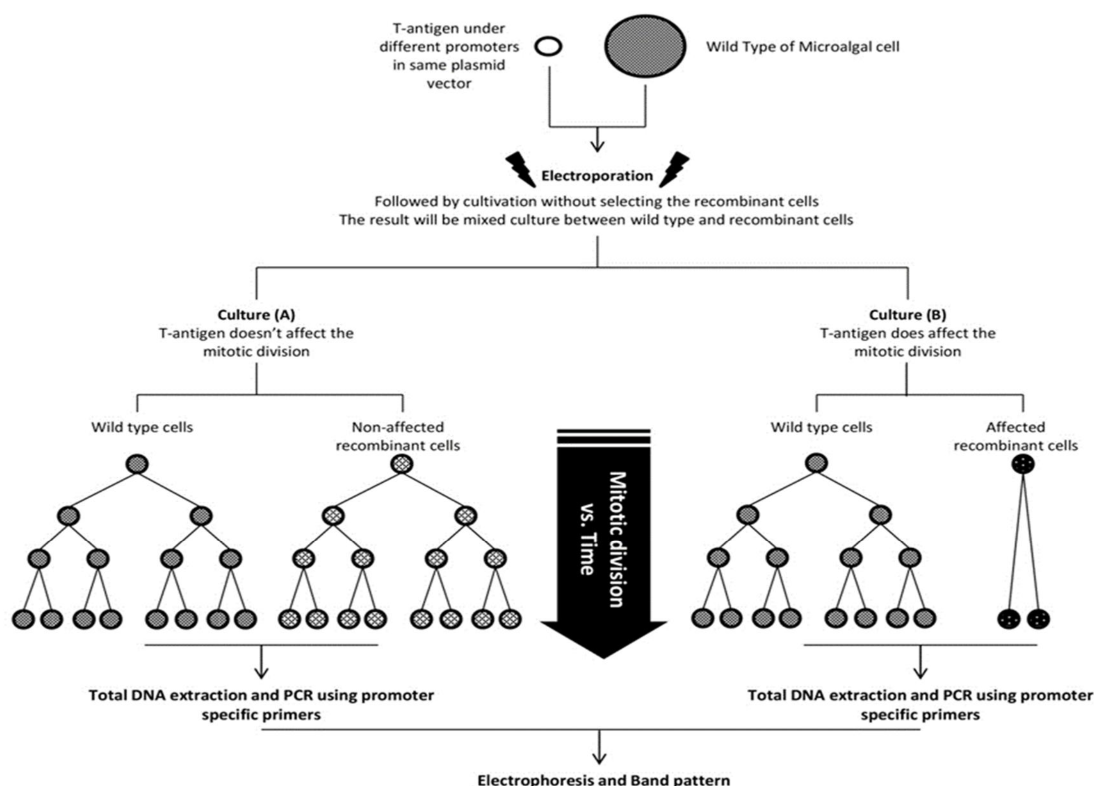

Figure 1. Description of the principle of the simple rapid screening method used in this study. Culture A, is a mixture of wild type cells with non-affected recombinant cells. Culture B, a mixture of wild type cells with mitotic division affected recombinant cells

2.7 DNA extraction, Quantitative and normal PCR

The isolation method of genomic DNA was modified from previous study [23]. Five milliliters of algal culture were centrifuged, and the pellet homogenized using mortar and pestle in 1 ml of CTAB buffer (54 mM CTAB, 0.25 mM Tris [pH 8.0], 1.4 M NaCl, 10 mM EDTA and 2% beta-mercaptoethanol). The mixture was incubated at 65°C for 2 h and shaken every 15 min. After incubation, an equal volume of phenol: chloroform was added and mixed by inversion. The aqueous phase was recovered after centrifugation at 13,000g for 20 min. The phenol: chloroform step was repeated 2 times until the aqueous layer was no longer cloudy. Genomic DNA was precipitated with 0.8 volume of isopropanol and 0.1 volume of 3M sodium acetate, centrifuged at 13,000 rpm for 30 min, washed with 70% ethanol, centrifuged (13,000 rpm for 5 min), dried and suspended in 30 µl of sterilized distilled water. Elimination of RNA was carried out by adding 0.5 µl of RNase (10mg/ml) and incubating the mixture at room temperature for 10min. All DNA samples were measured, and the concentrations were adjusted to be 20 to 30 ng per µl. A volume of 4µl of genomic DNA was used for Q-PCR and normal PCR reactions. Q-PCR performed only once at the end of generation 0 in 20µl reaction volume using SYBR® Premix Ex Taq ™ II (Tli RNaseH Plus) (Takara Bio. Inc., Shiga, Japan). Normal PCR performed at different stages in a 50µl reaction volume using Ex-taq (Takara Bio. Inc., Shiga, Japan), and specific primers for each promoter, Table 2. The PCR program followed the polymerase type of certain reaction with fixed annealing temperature 60°C. The normal PCR products were analyzed on a 1.5% agarose gel by loading 10µl from each tube.

2.8 Immunofluorescence and Flow cytometry

Immunofluroescenece was done using SV40 T antigen-specific primary-antibody SV40 T Ag mouse monoclonal IgG2a with goat anti-mouse IgG-FITC as secondary antibody (Santa Cruz Bio., Inc., USA). Harvesting 5ml from five *Scenedesmus* cultures with cassettes contain the T-antigen plus one culture contains the negative control Empty vector. The pellets were washed three times using sterilized distilled water then re-suspended in 5ml of 100% acetone at -80 °C to fix and permeabilize the membrane for 1hr. Then, the cells were harvested by centrifugation and washed two times using 1X PBS buffer. The pellet was re-suspended again, now in 1ml of 1:100 diluted primary antibody and then then it was shaked at room temperature for 2hr. Pelleting and washing were done using PBS buffer two times. The pellet was re-suspended in 1ml of 1:100 diluted secondary antibody, then was shaken at room temperature for 2hr. The pellet was washed and re-suspended in 3ml PBS buffer. The flow cytometry settings were as follows: 10000 events, Detectors/Amp FL1 "505" and FL2 "412", Threshold FL1-H "13", Compensation FL1-FL2 "73", FL2-FL1 "21.2". The results were analyzed via dot blot and histogram between FL1 vs. FL2, and Count vs. FL1, respectively. Each sample was acquired 16 times to represent the whole sample, and to detect cells containing the FITC-labeled secondary antibody.

2.9 Total RNA extraction, RT-PCR, and Transcription analysis by PCR

Harvesting 1.5ml from six *Scenedesmus* sp. cultures with cassettes NOS-T antigen, CaMV 35S-T antigen, NOS-large T antigen, CaMV 35S-large T antigen, CaMV35S+GUS, and negative control sample (Empty pACGFP1 vector). These samples were from generations, early G0, late G0, and G1.

Total RNA extraction was performed using ISO-RNA reagent (Takara Bio. Inc., Shiga, Japan), and 6 µl from the total RNA of each sample was used in Reverse Transcriptase reaction.

PCR reaction was performed for the T antigen-positive samples using primers specific for the first 250bp of T antigen Table 2, and primers specific for GUS gene regards to the GUS sample.The confirmation of the absence of DNA contamination in the extracted RNA was done using primers specific to the promoters in each sample, namely NOS, CaMV 35S, and CMV promoters.

3. Results and discussion

3.1 Sequencing result

After the sequencing was done for the first 300bp of the T antigen and the SV40 replication origin of each reconstructed vector, the result demonstrates absolute homology regarding the sequenced parts. Seven successful reconstructed vectors have been confirmed as shown in Fig. 2 (Supplemntary data, C, D).

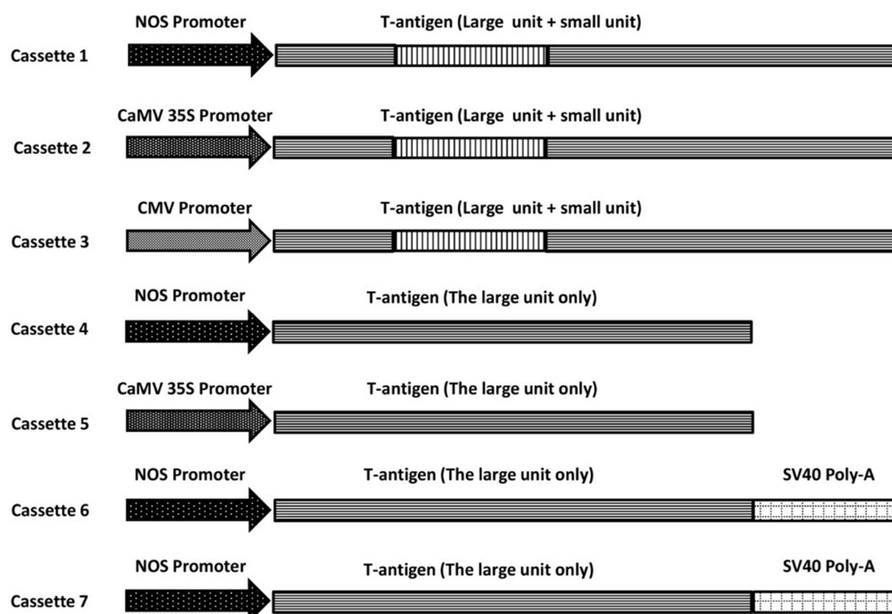

Figure 2. The designs of the seven DNA cassettes used in the current study which been introduced in the reconstructed vectors. The constructed-DNA used in the current experiment includes three promoters, two constructions of T antigen, and three designs of the vector backbone (Supplementary data, C).

The initial design of the current experiment was to develop marker-less transformation-system for microalgae to avoid using the costly selective pressures. The strategy which had been followed, was to increase the DNA vector stability via increasing the plasmid copy number inside microalgae cell, specifically microalgal nucleus. The steps which been followed were to combine the SV40 large T antigen, which contains Nuclear Localization Sites (NLS), with its replication origin on the same plasmid, which is commonly used in several other mammalian and yeast studies to efficiently enhance the transient expression by increasing the number of vectors inside the cell [24,25].

Additionally, it has been reported [1,26-28,6], that SV40 genomic DNA was able to integrate easily with host genomic DNA by nonhomologous recombination through NLS. Also, previous studies on plant cells used the SV40 T antigen as a NLS showed promising results [29,30].

Being *Scenedesmus sp*. and *Chlorella* sp. two different multiple-fissional algae, which make the nucleus undergoes several mitotic divisions to produce a number of nuclei followed by cell division of more than two cells, that in general have some differences between the binary and multiple fission cell cycles, especially with regard to the length of each stage [31,17,18].

3.2 Polymerase chain reaction and Quantitative real time PCR

The best conditions between the DNA concentrations, cells number, and the growth rate of the algal cells have been optimized as showed in Supplementary data (A, B). Regarding to the cultures of *Scenedesmus* sp., the presence of the promoter was checked for each vector within all transformed samples including the empty vector sample. The results showed that all vectors used were stable during G0 and G1 with almost same concentration of the empty vector sample (Fig. 3), also that two highly expressed vectors (CaMV35S+Tantigen and CMV + T antigen) were regenerated with the empty vector sample till G3, and

that low concentration regards to the three samples in G3 (Fig. 4, A). After the comparison of the concentrations of the empty vector through generations in *Scenedesmus* sp. G0, early G1, late G1, and G3, were checked that the concentration of the empty vector drop-down during the sequential generations (Fig. 4, B, C). Regards to the *Chlorella* sp. The observation was a variation in the DNA concentrations and band patterns (Fig. 5), when samples were checked for the presence of the vectors that were all checked in early and late G0 (Fig. 5). The products related to the vectors carrying the cassettes under the control of the CaMV35S and CMV promoters were in very low concentrations compared to the NOS controlling vectors. The NOS vectors were almost in equal concentration with the empty vector (Fig. 5). In G1, PCR products and band patterns disappeared for all samples including the empty vector sample, except some nonspecific bands and primer-dimers.

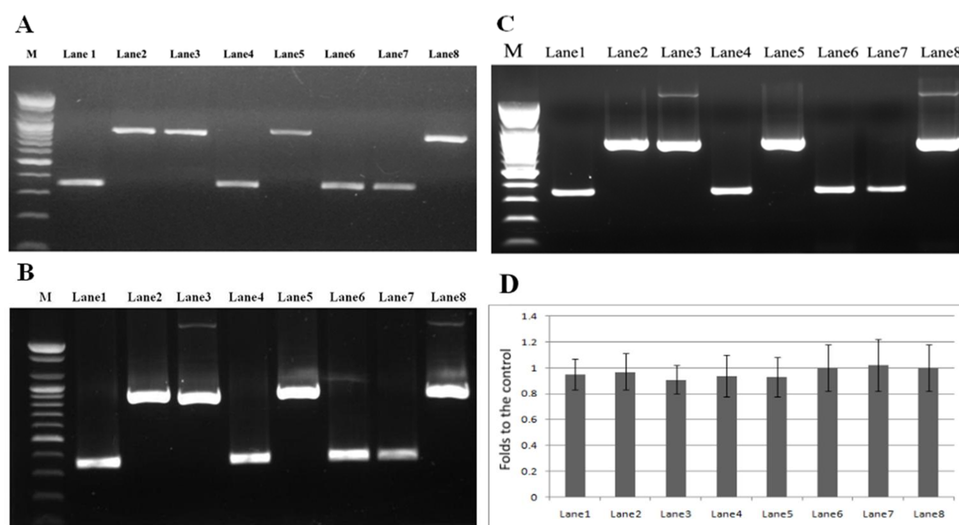

Figure 3. Band patterns of *Scenedesmus* sp. cultures during generations. A, Band patterns of Generation 0 (G0). B, Band patterns in early generation 1 (G1). C, Band patterns in late generation 1 (G1). M, a 100bp DNA ladder (Bioneer). Lane1, NOS promoter of cassette No.1. Lane2, CaMV35S promoter of cassette No.2. Lane3, CMV promoter of cassette No.3. Lane4, NOS promoter of cassette No.4. Lane5, CaMV35S promoter of cassette No.5. Lane6, NOS promoter of cassette No.6. Lane7, NOS promoter of cassette No.7. Lane8, CMV promoter of the original vector. D, Quantitative real time-PCR for late generation 0 of *Scenedesmus* sp. cultures mentioned by folds for each sample in regard to the control empty vector (Lane8).

Horstmann *et al.* (2004), published that the expressions of CMV and NOS promoters are 10% and 3%, respectively comparing to the CaMV 35S promoter, although choosing three different promoters were on the basis of the expression percentage. Reports about the usage of these promoters in microalgal transformation research are rare even having the previous studies showed that the three used promoters have low expression rate in micro and macroalgal cells, the advantage of the low expression rate of the later promoters, also observed in this study, has been taken to express the T antigen in low quantity in the two microalgae species, so that can avoid cell-toxicity which could come from over expression of foreign proteins.

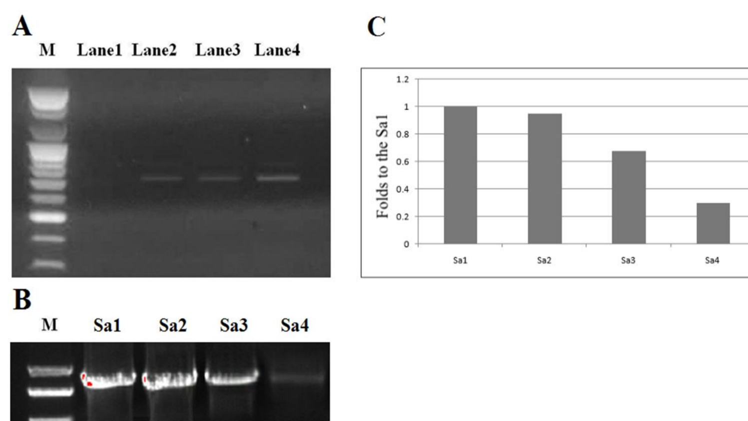

Figure 4. Band patterns of *Scenedesmus* sp. cultures during generations. A, Band patterns of three different vectors in generation 3 (G3) of *Scenedesmus* sp. cultures. M, a 100bp DNA ladder (Bioneer). Lane1, negative control empty cells amplified using SV40 promoter's primers. Lane2, SV40 promoter of the empty vector. Lane3, SV40 promoter of reconstructed vector No.3. Lane4, SV40 promoter of reconstructed vector No.2. B, Band patterns of the control empty vector during generations G0, early G1, late G1, and G3 of *Scenedesmus* sp. cultures. M, a 100bp DNA ladder (Bioneer), Sa1, SV40 promoter of the empty vector in G0. Sa2, SV40 promoter of the empty vector in early G1. Sa3, SV40 promoter of the empty vector in late G1. Sa4, SV40 promoter of the empty vector in G3. C, Quantitative real time-PCR for the SV40 promoter from the empty vector in *Scenedesmus* sp. during generations G0, early G1, late G1, and G3 mentioned by folds for each sample in regards to the G0's value (Sa1).

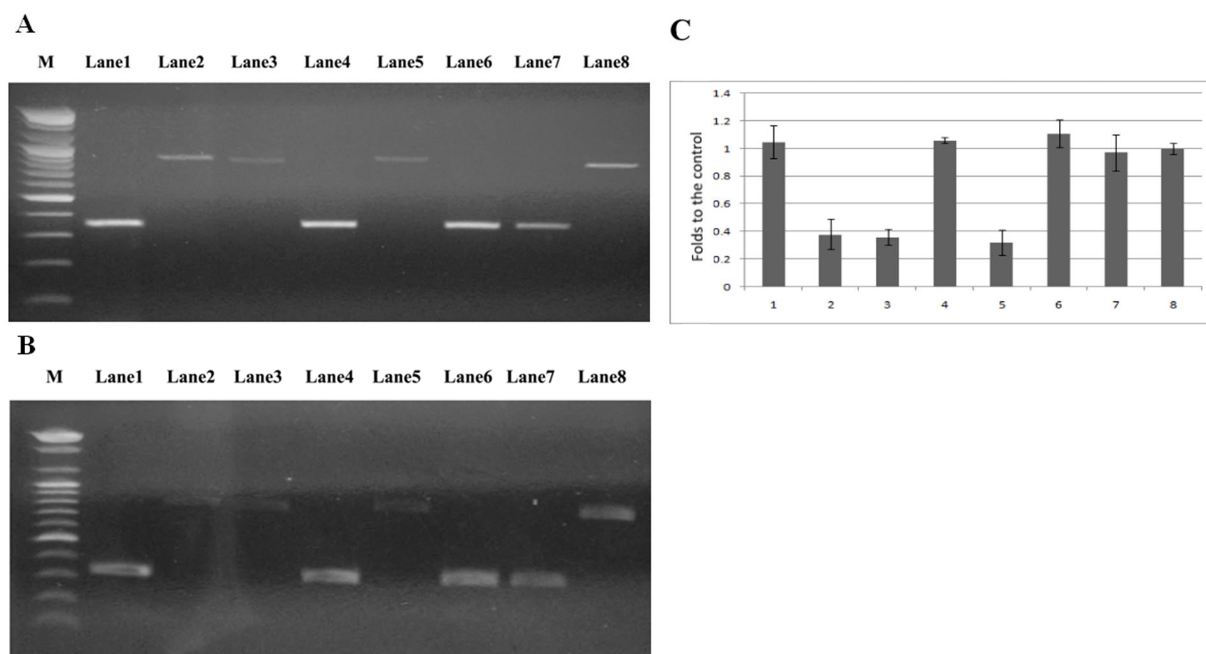

Figure 5. Band patterns during generations of *Chlorella* sp. cultures. A, Band patterns in early generation 0 (G0). B, Band pattern in late generation 0 (G0). M, a 100bp DNA ladder (Bioneer). Lane1, NOS promoter of cassette No.1. Lane2, CaMV35S promoter of cassette No.2. Lane3, CMV promoter of cassette No.3. Lane4, NOS promoter of cassette No.4. Lane5,

CaMV35S promoter of cassette No.5. Lane6, NOS promoter of cassette No.6. Lane7, NOS promoter of cassette No.7. Lane8, CMV promoter of the original vector. C, Quantitative real time-PCR data of late generation 0 mentioned by folds for each sample in regard to the control empty vector (Lane8).

3.3 Flow cytometry analysis for *Scenedesmus* cultures

The differences were non-significant between the acquired recombinant samples compared to the negative control. The FITC couldn't be compensated from the florescence in the background within the 5 samples, thus, all samples were analyzed with regard to the green emission and as mentioned, every sample was acquired 16 times and the most significant difference was selected between the acquired samples (Fig. 6, 7).

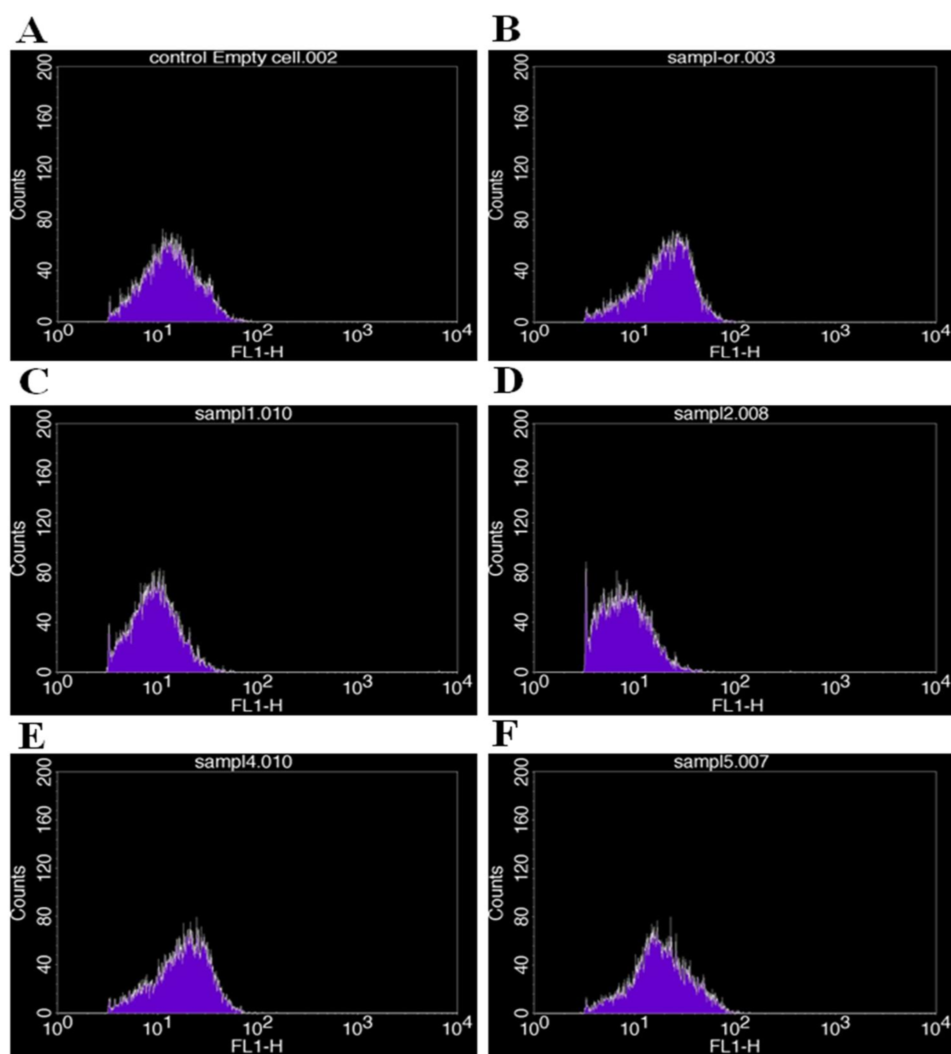

Figure 6. Histogram for the flow cytometry acquisition of *Scenedesmus* sp. samples. A, Negative control empty cells. B, Cells with original empty vector. C, NOS-T antigen cassette sample. D, CaMV 35S-T antigen cassette sample. E, NOS-large T antigen cassette sample. F, CaMV 35S-large T antigen cassette sample.

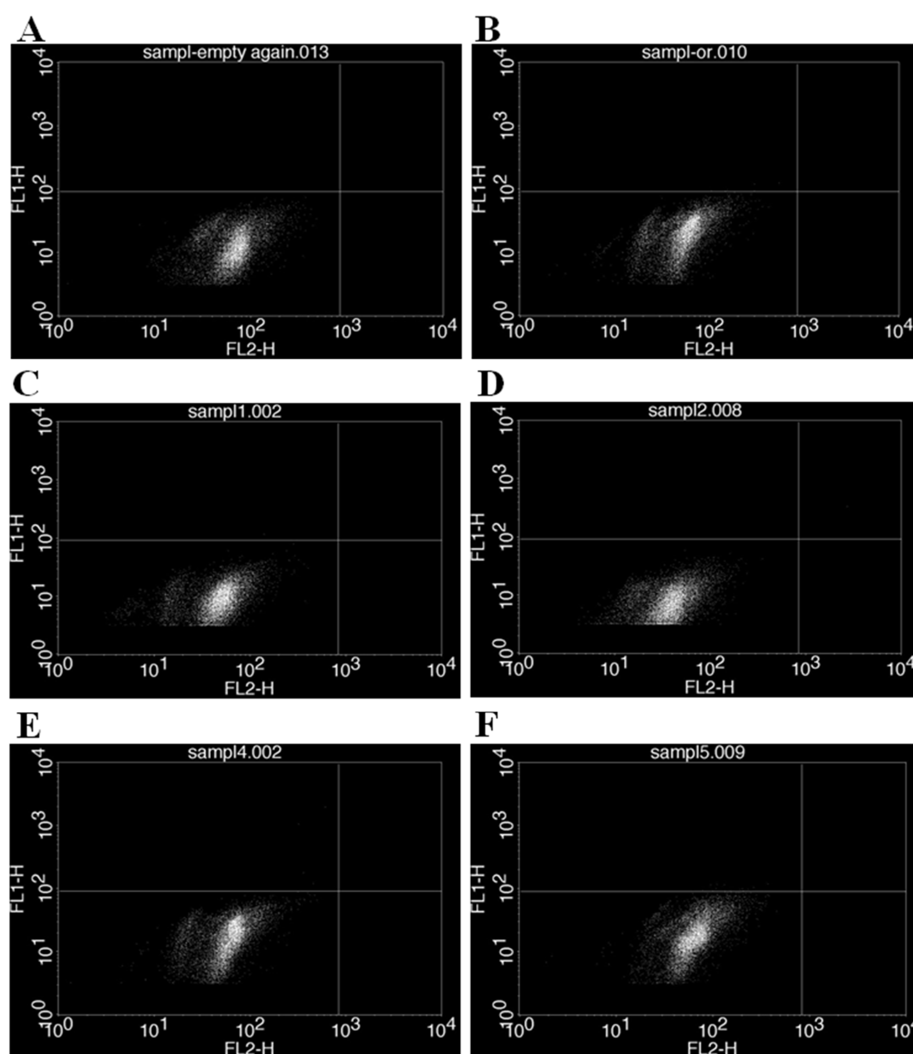

Figure 7. Dot plot for the flow cytometry acquisition of *Scenedesmus* sp. samples. A, Negative control empty cells. B, Cells with original empty vector. C, NOS-T antigen cassette sample. D, CaMV 35S-T antigen cassette sample. E, NOS-large T antigen cassette sample. F, CaMV 35S-large T antigen cassette sample.

3.4 RT-PCR and Transcription analysis

The expression pattern of the T antigen in the two microalgal species under the used promoters was done and confirmed via RT-PCR followed by normal PCR (Fig. 4, 5).

Instead of using Northern blot for detecting the targeted RNA, which predicted to be in a very low concentration, PCR was used to amplify the specific fragment as much as possible with the intention to increase the concentration.

The PCR results of the first strand cDNA indicate that there is no, or rather an undetectable transcription level for the T antigen in *Scenedesmus sp*.

The possibility of Genomic or plasmid DNA contamination in cDNA was eliminated by targeting promoter regions from the first strand cDNA of each sample. The results were all negative indicating that there was no- or undetectable DNA contamination.

3.5 Confirming the activity of the CaMV35S Promoter in *Scenedesmus* sp.

The activity of CaMV35S promoter in *Scenedesmus sp.* had been confirmed by checking the successful transcription of GUS gene in pACGFP plasmid vector. However, GUS reporter doesn't work properly in *Scenedemus sp*., as the histochemical assay has failed to check the GUS activity.

In *Scenedesmus quadricauda* cultures, which showed the same concentration in almost all bands, it was suspected that there is no T antigen expression. Then comparing the transcription of the reporter Gene GUS and the T-antigen under the 35S promoter, it was concluded that T antigen transcription was negative, and it could be silenced by the cell.

Relatively to *Chlorella* cultures, they clarified that the large T antigen owns the main responsibility of slowing the rate of cellular mitotic division, besides the fact that, they showed no stability within the genomic DNA.

Considering the Rb-like proteins in some microalgal species could give a key explanation to why *Chlorella* got affected by the T-antigen, and *Scenedesmus* acted against the expression [7,12-14], the results obtained from the current experiment suggested that it is necessary to remove some DNA-domains from the large T-antigen before re-applying the strategy in any further trails, according to previous reports [32-35], the large T antigen contains an LXCXE motif (residues 103–107) that is essential for its interaction with the Rb family of tumor suppressors, so that motif was altered, which showed defective for transformation in nearly all assays.

4. Conclusion

Although, the current study could be useful information for researchers interested in the algal cell-cycle models and molecular similarity between plant and animal cells. However, the main target was to enhance the stability of the transformed DNA in microalgae without using selective pressures. The result obtained from the present study suggested that the success of the current strategy depend mainly of the impact of the expressed T antigen on the algal cell cycle.

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