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Efficient Stable Expression of Nuclear H5N1 Avian Influenza Virus HA2 Transgene in Chlamydomonus reinhardtii

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Abstract

Green biotechnology is the future of biopharmaceuticals production. The use of algae to produce biopharmaceuticals is of interest in vaccine-based control programs because of cost and environmental safety considerations. An attempt was made to express avian influenza virus (AIV) immunogens in algae because the virus is a serious economic, veterinary, and public health threat. A commercial system was modified to allow expression of H5N1 AIV hemagglutinin subunit 2 (HA2) in the microalga Chlamydomonas reinhardtii (C. reinhardtii). Codon-optimized AIV H5N1 HA2 (coHA2) sequence was synthesized and cloned into the transfer vector pChlamy_3/D-TOPO®. Cloned coHA2 sequence was then transformed in C. reinhardtii strain cc-125 by electroporation. Proper nuclear integration was confirmed in 16% of screened transformants selectively amplified in Hygromycin-containing TAP media. coHA2 mRNA transcription was confirmed using RT-PCR. AIV HA2 expression was confirmed using western blot analysis utilizing AIV polyclonal chicken antisera. Expressing transformants were maintained on Hygromycin-containing TAP agar for 26 weeks (15 subcultures). Expressing transformants maintained cell shape, motility and, growth characteristics similar to non-transformed C. reinhardtii cc-125. A coHA2-C terminus GFP was used to visualize HA2 expression in vivo using confocal microscopy. Background-normalized GFP-specific fluorescence of transformants was 15 % of the total cellular fluorescence. Fluorescence in GFP channels 508, 518, 528 and 538 nm was 3.9% of the total cellular fluorescence of non-transformed algae. Taken together, results indicate efficient stable expression of the AIV HA2 transgene and, warrant further investigation into immunogenic potential of the algae-expressed HA2.

Keywords: Avian influenza, Chlamydomonas reinhardtii, Nuclear, HA2, vaccine, subtypes, recombinant protein.

Introduction

Anumber of commercial heterologous protein expression platforms exist. Each platform offers distinct advantages in terms of protein yield, cost and versatility (Mayfield, Franklin and Lerner, 2003). Microalgae and transgenic plant expression platforms are considered green protein factories (Dove, 2002). Unicellular eukaryotic microalgae have considerable future in the biotechnology industry because of cost, and environmental safety considerations. Microalgae have been used for production of antibiotic resistance markers, reporter genes, valuable recombinant therapeutic proteins, industrial enzymes and, human and veterinary vaccines (Manuell et al., 2007; Surzycki et al., 2009; Gong et al., 2011; Soria-Guerra et al., 2014). In addition, they are used as a source for highly quality protein for the aquaculture (Chen et al., 2015), and have the potential to be used for oral delivery of biopharmaceuticals; most fall into the Generally Recognized as Safe (GRAS) category of the USA FDA (Mayfield et al., 2007; Mayfield and Miyake-stoner, 2010).

Chlamydomonas reinhardtii has been an excellent model freshwater microalga for many areas of research. As such, it has a well-developed molecular genetic makeup (Rasala et al., 2011) and has served as a genetic workhorse and an ideal organism for understanding the mechanisms of photosynthesis and nutrient regulated gene expression to the assembly and function of flagella (Hippler, Redding and Rochaix, 1998; Harris, 2001), C. reinhardtii has an

ability to grow phototrophically or heterotrophically (Jinkerson and Jonikas, 2015). In addition, C. reinhardtii has rapid growth and minimum nutrient requirements characteristics (Radakovits et al., 2010; Wang et al., 2012). Several genetic transformation techniques have been established for nuclear and chloroplast expression in C. reinhardtii (Kim et al., 2014). Compared to nuclear expression, transgene expression in chloroplasts has historically generated higher levels of transgene expression (Rasala et al., 2012). However, transgene expression from nuclear genome integration allows glycosylation (Mathieu-rivet et al., 2014) and other post-translational modifications (PTMs) (Mamedov and Yusibov, 2011), and transgene-targeting to sub-cellular locations (Rasala et al., 2014); which is not possible for proteins expressed in chloroplasts.

Glycoprotein expression in algae can facilitate oral vaccine development, and/or contribute positively to international efforts to control pathogens targeted by the one-health initiative (Bidaisee and Macpherson, 2014). A key example of the aforementioned pathogens is highly pathogenic (HP) avian influenza virus (AIV) H5N1. AIV is an influenza A virus (IAV) of family Orthomyxoviridae. It has a segmented negative-sense single-stranded RNA genome. The translation products of segments 1–3 assemble into the viral transcriptase complex (Fodor and Smith, 2004). The rapid evolutionary pattern of AIV is a result of its high rate of transcriptase errors, recombination, and reassortment (Boltz et al., 2010). The viral hemagglutinin (HA) is the main immunogen in AIV (Kaminski and Lee, 2011). Influenza A virus HA precursor (HA0) is a glycoprotein that is encoded on segment 4 of the AIV genome. HA is present as trimers on the envelope (Chen et al., 1998). Eighteen influenza A virus HA subtypes exist (Tong et al., 2013). During infection, influenza virus HA0 is cleaved by cellular proteases into two subunits, HA1 and HA2. The HA is responsible for virus attachment to host cell receptors, and for penetration by fusion after conformational changes in the endolysosomal compartment (Mair et al., 2014).

Recent evidence demonstrates that, unlike HA1, AIV HA2 is highly conserved (Steel, 2010). HA2 based antibodies provide cross-protection against several strains within subtypes in phylogenetic group protection (Ekiert et al., 2012) and subtypes cross group protection (Valkenburg et al., 2016). Additionally, conserved HA2 "stalk" epitopes of influenza H1N1 displayed on the globular head of the HA molecule have been shown to confer protection against heterologous influenza strains (Kim et al., 2016; Klausberger et al., 2016). Therefore, the goal of this study was to determine the feasibility of AIV HA2 glycoprotein expression from a nuclear transgene of C. reinhardtii, to examine its subcellular localization within algae and, to test its antigenicity in vitro as a prelude to its use as a vaccine antigen.

Materials and Methods:

2.1. Eukaryotic Algal Gene Expression System

Expression of HA2 gene of AIV in the eukaryotic microalgae C. reinhardtii was performed using GeneArt® Chlamydomonas TOPO® Engineering Kit; Cat. No. A14264 (Thermofisher, Waltham, MA). The kit is designed to facilitate directional TOPO® cloning and expression of recombinant proteins in C. reinhardtii. The kit contained a vector set (lot no. 1774297) comprises from pChlamy_3/D TOPO vector; a nuclear integrative algal expression vector (lot no. 1613461) and the control pChlamy_2 vector. It also includes frozen chemically competent E. coli TOP 10 and C. reinhardtii 137c cells, Gibco® TAP algal growth media, and the universal chlamy primers (Table 1).

2.2. C. reinhardtii Cells: Maintenance and Propagation

Ten vials of frozen C. reinhardtii 137c cells (240 µl each) were supplied as a component of Chlamydomonas TOPO® Engineering

Kit (Thermo-Fisher). These cells failed to fulfill the quality standards of the commercial products in the transformation experiments; no green color was observed during culture, and cell viability was low after 72 hours. Therefore, standard C. reinhardtii strains; including wild-type cell walled strain cc-125 mating type (+) mt+ and mutant cell walled strain cc-124 mating type (-) mt-, were purchased from Chlamydomonas research center (CRC), Minnesota, USA and were supplied in 2 ml yeast extract (YA) slants. C. reinhardtii is typically maintained onto 1.5% agar at room temperature, while cells propagated for transformation and protein expression are grown in Tris-acetate-phosphate (TAP) medium in shake flasks or bottles (Harris, 2001). Most strains grow well at temperatures ranging from 20 to 25°C, (temperature range 15-35°C). C. reinhardtii also requires continuous illumination with moderate light intensities of cool fluorescent white light (50 µE m–2 s–1) and constant agitation at 100-150 rpm on rotary shaker.

2.3. Amplification of HA2 Gene of AIV Using RT-PCR

Two Egyptian strains of AIV H5N1 (A/chicken/Egypt/1575S/2015 and A/chicken/Egypt/12186F-12/2012) were kindly provided by Virology Dept. Vet. Med. Cairo University, Egypt. Viral RNA was isolated using QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized using SuperScript™ III reverse transcription system (Thermo-Fisher, USA) at 50°C for 30 min. PCR amplification of the complete HA2 gene was carried out using high fidelity Platinum® Pfx DNA Polymerase, which generates blunt end products for directional TOPO cloning, and the oligonucleotide primers Forward HA2 TOPO Bay and Reverse HA2 Bay primers (Table1) in Gene-Amp 9700 (Applied Biosystems, Foster City, CA). Specific PCR products were separated by electrophoresis in 1.2 % agarose gel stained with ethidium bromide, visualized by Gel Doc XR gel documentation system (BIORAD laboratories, Milan, Italy), and purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). All protocols were performed according to the instructions of the manufacturers.

2.4. Cloning of HA2 Gene In pChlamy_3/D-TOPO® Vector

TOPO® cloning of the purified HA2 gene in pChlamy_3/D-TOPO® vector was performed with a molar ratio of 1:1 as recommended by the manufacturer. The cloning mixture was incubated at room temperature for 20 minutes with salt solution (final concentration of 0.2 M NaCl and 0.01 M MgCl2). Two microliters of the cloning reaction were used to transform competent E. coli TOP10 cells by heat shock for 45 second at 42°C. After incubation at 37°C for 1 hour with shaking (200 rpm), the transformed cells were spread onto LB agar plates containing ampicillin (100µg/ml) and were incubated at 37°C overnight till appearance of colonies. Bacterial transformants were identified using two colony PCR reactions. The first utilized the gene specific primers; Forward HA2 TOPO Bay and Reverse HA2 Bay primers, and the second utilized the universal Chlamy primers supplied by GeneArt® Chlamydomonas TOPO® Engineering kit (Table 1). Appropriate controls including E. coli transformed with control vector and non-transformed bacteria were considered in all cloning experiments.

2.5. Verification of Algal Vector Contamination and Isolation Of The Purified pChlamy_3/D-TOPO® Vector

During TOPO cloning experiments, contamination of the vector was investigated either with the algal transformation control vector pChamy2, or with other algal expression vectors produced by Thermo-Fisher (e.g. pChlamy1 and pChlamy4). To verify such possibility, the restriction digestion profile of the four vectors; pChlamy1-4, was in silico simulated using SnapGene® software (GSL Biotech, Chicago, IL). Accordingly, five series of digestion reactions were proposed

for differentiation between the vectors including: 1) digestion with KpnI, 2) double digestion with KpnI and BamHI, 3) double digestion with ScaI and EcoRI, 4) double digestion with EcoRI and BamHI, and 5) digestion with NotI (Table 2). The vector stock was propagated onto LB agar plates containing 100µg/ml ampicillin and a few number of separate well-spaced colonies were picked up and purified for analysis. The different proposed digestion reaction sets were performed on the vector stock and the purified bacterial colonies using the proper enzyme buffers and the reaction conditions described by the manufacturer (Clontech, Takara, Japan). The products of digestion reactions were separated in 0.8% agarose gel stained with ethidium bromide and DNA bands were visualized by Gel Doc XR gel documentation system (BIO-RAD). Purified pChlamy_3/D-TOPO® vector was confirmed by colony PCR (as described above in section 3.3) and propagated for use in subsequent cloning experiments.

2.6. Preparation and Cloning of Synthetic HA2 Gene Cassette

The complete sequence of HA2 gene of AIV H5N1 strain A/chicken/Egypt/ 1575S/2015 was chemically synthesized (Biomatik, Canada) as an alternative approach to promote robust gene expression in C. reinhardtii. The green fluorescent protein (GFP) gene (GenBank accession: KX431221) was added downstream of HA2 gene sequence. Codon optimization of the gene cassette was performed using the codon optimization tool at integrated DNA technologies (https://eu.idtdna.com/ CodonOpt). Different tags were included between HA2 and GFP sequences including TEV protease recognition sequence, 6x His tag, V5 epitope, and FMD 2A sequence that encodes post translational self-cleaving sequence, respectively (Rasala et al., 2012). The synthetic HA2-GFP gene cassette was cloned via KpnI and NotI into pChlamy_3/D TOPO vector. Verification of bacterial transformants that carry synthetic HA2-GFP gene in correct orientation was achieved by: 1) colony PCR using universal Chlamy primers (Table 1) that generates a specific amplification product of 2267 bp, 2) double digestion with KpnI and NotI that generates two digestion products of 1588 and 4491 bp, 3) DNA Sequencing using universal chlamy primers, forward Bay HA2 and reverse Bay GFP primers (Table 1).

2.7. Transformation of C. reinhardtii By Electroporation

C. reinhartdii cc-125 was propagated in TAP medium till reach a density of 1-2 x 10^6 cells/ml. Cells were harvested by centrifugation at 2500 rpm for 5 min and resuspended in MAX Efficiency transformation reagent (Thermo-fisher) to a final density of 2-3 x 10^8 cells/ml. One microgram of the purified recombinant pChlamy3 vector (i.e. carrying HA2-GFP gene cassette) was added to each 250 µl of cell suspension and was incubated at 4°C for 5 min. A separate tube was used to prepare a control transformation reaction using pChlamy2 vector. Transformation of algal cells was obtained by electroporation at 400 V and 25 µF capacity with no resistance (BTX630, Amika, Holliston, MA). After recovery for 15 minutes at room temperature, cells were mixed with 10 ml of TAP-40mM sucrose medium and were incubated at 23˚C under continuous illumination of 5000 Lux on a rotary shaker (150 rpm) for 14-16 hours. Cells were harvested again and plated onto TAP-agar containing 8 µg/ml hygromycin B. The plates were further incubated for 5-7 days for the development of colonies (Shimogawara et al., 1998).

2.8. PCR Screening of The Transformed C. reinhartdii Cells for Nuclear Integration With HA2-GFP

Recombinant C. reinhartdii colonies were recovered from TAP agar plates and were propagated in TAP medium supplemented with 3 µg/ml hygromycin B till reach a density of 3 x 10^6 cells/ml. Algal DNA extraction was performed using plant extraction kit (Tiangen, Beijing, China) according to the manufacturer's

instructions. The cultures were screened by PCR for: 1) stable nuclear integration of HA2-GFP gene cassette using forward Bay HA2 and reverse Bay GFP primers (Table 1) that generates an amplification product of 1559 bp, 2) integration of HA2 gene in correct orientation using forward universal Chlamy primer and reverse Bay GFP primers (Table 1) that generates a PCR product of 1835 bp, 3) confirm successful extraction of C. reinhartdii DNA utilizing Chlamydomonus specific primers (Raheem Haddad, Esmat Alemzadeh, Ali-Reza Ahmadi, Ramin Hosseini, 2014).

2.9. Verification of HA2 Gene Expression By The Transformed C. reinhartdii Cells

2.9.1. Detection of HA2 mRNA Using RT-PCR

Total RNA extraction from each 10^6 recombinant C. reinhartdii cells was performed using RNeasy Mini kit (Qiagen). Detection of the HA2 mRNA was conducted using both primer sets, in separate one-step RT-PCR reactions (Qiagen). All steps of RNA extraction and RT-PCR were performed according to manufacturer's guidelines.

After total RNA isolation, two sets of primers were utilized: in the first one, RT-PCR mix contains forward primer designed to bind the promoter sequence (forward universal chlamy) and the reverse primer bind with end of the HA2-GFP sequence (Table 1; Primers 3, 6), if mRNA transcribed, it will not include promoter sequence inside (as promoters does not transcribed by cellular polymerases) and in the same time control for DNA contamination after total RNA extraction. In the Second aliquot of the extracted RNA, RT-PCR mix contains forward primer designed to bind the first of the HA2-GFP sequence and the reverse the last of the HA2-GFP sequence (Table 1; Primers 5, 6), if mRNA transcribed, it generated the expected PCR-product fragment size 1559 bp confirmed with absence of amplification of the previous aliquot. The two types controls were properly added in all PCR and mRNA screening experiments; negative electroporated not transformed and electroporated with the control vector pChlamy2.

2.9.2. Detection of The Reporter Green Fluorescent Protein (GFP) Using Confocal Microscopy

The mRNA-positive transformants and negative control cells (non-transformed cells and cells transformed with the algal control vector pChlamy2) were cultured in TAP medium supplemented with hygromycin B in shaking flasks. When cells reached the late log phase with a density of 4-5 $x10^6$ cells/ml, three drops of each PCR positive and negative samples were placed on a clean microscopic slide. Cells were let to adhere on the slide for 5 min before placing of cover slips and sealing with adhesive wax. Slides were examined by the confocal microscope and all wave length emissions (no=23), plus the photomultiplier channel (PMT), were captured. The images were analyzed using ZEN LE image-processing program (http://www.zesiss.co.jp/micro) in single or overlapped channels (black edition 2012). Image analysis for qualitative estimation of specific protein expression was performed using different parameters including:

Intensity Means Values Significance:

ZEN LE image-processing program (Blue edition 2012) enable semi-quantitative tool for reporter transgenic protein expression (GFP) that can be used in case of absence of data about absolute quantification of total soluble protein (TSP). Software tool can be used for calculation of intensity means values per each cell, which give numerical values to each wave length (n=23) plus the value of photomultiplier (PMT), these values was statistically analyzed which for their significance difference in positive transformants and negative control cells in chlorophyll specific windows (the last 7 channels plus PMT), and significance difference in positive and negative cells in GFP specific windows (the first 4 channels) compared to total cell fluorescence.

The Relative Quantification of GFP Transgenic Protein:

The relative quantification of the positive transformants transgenic protein was determined by devising the sum of intensity mean value of specific GFP channels (2-5), by the sum of intensity mean value of all channels (n= 24, 23+ PMT) after background normalization.

Transgenic Protein Localization:

Transgenic protein localization was determined by following the emission fluorescence from the GFP specific windows channels (2-5) with development of emission per each channel with and without overlapping.

2.9.3. Characterization of The Expressed Protein Using Immunoblotting Assay

Detection of HA2-GFP transgenic protein in the algal extracts was performed according to (Franklin et al., 2002). Proteins were harvested from C. reinhardtii cells using lysis buffer (750mM Tris-HCl; pH 8.0, 15% sucrose (w/v), 100mM β-mercaptoethanol (BME), 2 mM AEBSF, 1 mM Phosphoramidon, 130 mM Bestatin, 14 mM E-64, 1 mM Leupeptin, 0.2 mM Aprotinin, 10 mM Pepstatin A). The total soluble proteins (TSP) and cell pellets were denatured using Laemmli buffer (containing 0.05% BME) at 95˚C for 8 min. Proteins were separated in 12% polyacrylamide gels at 100 volts for 90 min and were transferred to nitrocellulose membrane at 230 mAmp for 20 min using semidry blotting (Bio-Rad). The membranes were blocked using 5% skimmed milk before being reacted with chicken anti-AIV H5N2 polyclonal serum (diluted 1/100) (Harbin, China). After 3 times wash with PBS-Tween, the membrane was incubated with horseradish peroxidase anti-chicken antibodies (Clontech). Specific bands were developed by incubation with 4-Chloro-1-naphthol/H_2O_2 solution.

Results and Discussion

3.1. TOPO Cloning Of RT-PCR Amplified AIV H5 HA2 Into Supplied Expression Vector Was Unsuccessful

AIV H5 HA2 cloning into the pChlamy_3/D-TOPO® algal expression vector was originally attempted using RT-PCR-amplified products (Section 3.3). The primers used allowed the amplification of fragments that are ready for directional TOPO cloning (Table1). Multiple well-controlled TOPO cloning experiments (n=6) were performed using the supplied pChlamy_3/D-TOPO® algal expression vector. Out of 57 colonies picked randomly from a selection plate containing 240 TOP10 E. coli transformants, none contained HA2 sequences. Control insert-specific primers generated the expected PCR-product fragment size when AIV viral RNA was used. The expected PCR product size that would indicate a successful transgene integration were not observed as well (Figure S2) when the same colonies were testing utilizing the universal chlamy primers (Table 1; Primers 3, 4). Moreover, the amplification of variable sizes of PCR products (250 bp, 400 bp and, 500 bp) from these colonies indicated that multiple species of vectors may present (since colonies acquired antibiotic resistance) and, indicated that problems exist with insert integration into the vector preparation provided.

3.2. Restriction Digestion Analysis Of The Surviving Colonies Revealed The Presence Of A Non-Homologues Population Of Plasmids In The Supplied Vector Vial

To investigate whether the commercial expression vector vial contained a non-homologous population of plasmids, competent TOP 10 bacteria were transformed using insert-free expression vector vial contents. Twenty colonies were selectively amplified following transformation indicating that circular plasmids carrying the antibiotic resistance gene were successfully transformed. This result may be interpreted in two different ways. The first interpretation is that topoisomerase activity resulted in vector circularization prior to transformation; the cloning strategy depends on directional TOPO cloning. The second interpretation is that the vector vial provided with the kit contained circular plasmids. Nevertheless,

none of the interpretations above explain why cloning of RT-PCR products failed. Restriction digestion analysis was performed on plasmid extracts from 13 randomly selected bacterial clones of the 20 selectively amplified colonies mentioned above. This analysis was done to investigate whether the circularized plasmids transformed were identical, and if not, whether their restriction patterns resemble any of the vectors produced by the manufacturer. The predicted digestion patterns of four vectors of the commercial vector series of the manufacturer (pChlamy_1, pChlamy2, pChlamy_3/D-TOPO and, pChlamy4) were used for comparison purposes (Table 2).

Restriction digestion of extracted plasmids using the five different enzyme combinations (Table 2) confirmed the presence of a non-homologous population of plasmids (Figure 1). A single colony of transformants carried a plasmid with restriction pattern similar to pChlamy_3/D-TOPO; a single 4525 bp fragment generated by NotI or KpnI digestion, and two fragments (2775 bp and 1750 bp) generated by KpnI and BamHI double digestion. Double digestion with ScaI/EcoRI and EcoRI/BamHI also generated the expected fragments' lengths (Table 2; Figure 1), however, it was easier to differentiate between plasmids using the KpnI/BamHI combination. Other colonies contained plasmids with restriction patterns similar to pChlamy 2 (Figure 1; Table 2). A second transformation experiment was conducted to investigate whether pChlamy_3/D-TOPO can be recovered more effectively. The vector vial content was incubated for 15 minutes rather than 5 min to allow for more topoisomerase activity; up to 30 minutes' incubation is allowed by the manufacturer. Longer periods of incubation that result in higher transformants yield would indicate that linear topoisomerase-bound vectors do exist. Indeed, transformation followed by selective amplification resulted in recovery of 110 colonies. The KpnI digestion pattern of 32 randomly-selected colonies of surviving colonies was not similar to that of pChlamy_3/D-TOPO vector. Taking in consideration the results of the first restriction analysis described above, it can be concluded that the plasmids extracted from the 32 colonies were probably pChlamy2. Further restriction analysis was not conducted due to financial considerations.

3.3. Synthetic Codon-Optimized HA2-GFP Gene Cassette Was Successfully Cloned Into A Rescued pChlamy_3/D TOPO Vector.

In the absence of topoisomerase-ready pChlamy_3/D-TOPO vector, an alternative cloning strategy had to be devised. The TOP 10 E. coli colony containing the plasmid that had a pChlamy_3/D-TOPO-like restriction pattern was expanded in selective broth, and individual colonies were isolated on selective plates. Plasmids from the purified colonies were PCR-tested using universal chlamy primers (Table 1; Primers 3, 4). A single 708 bp PCR product was amplified indicating that pChlamy_3/D-TOPO sequences were present. The purified plasmid preparation was then used in cloning experiments.

For cloning, the RT-PCR amplified AIV H5 HA2 was replaced with a synthetic codon-optimized HA2 sequence in which KpnI recognition sequences were replaced without affecting the translated a.a. identity. The HA2 sequences were linked using a self-cleaving sequence with a reporter codon-optimized green fluorescent protein (GFP) sequence. This strategy was designed to allow temporal and spatial in vivo monitoring of transgene expression. Reports have demonstrated that codon optimization enhanced expression of target transgenes (Heitzer and Zschoernig, 2007), and thus it was deemed critical for the nuclear HA2 transgene.

Double digestion with KpnI and NotI generated the expected 4491 bp and 1588 bp fragments and confirmed successful gene cloning. Additional confirmation of successful cloning was obtained when vector specific primers (Table 1; Primers 3, 4) amplified a 2267 bp product; equivalent

to the sum of the 1559 bp insert plus a 708 vector-specific flanking sequences. DNA sequencing using vector and gene specific primers (Table 1; Primers 3-6) verified the correct cloning and orientation of the synthetic transgene. The promoter and 3'-UTR sequences of the construct were identical to those of the expression vector pChlamy_3/D-TOPO®.

3.4. The Synthetic Codon-Optimized HA2-GFP Sequence Was Successfully Cloned into The Nuclear Genome Of C. reinhardtii cc-125

3.4.1. Transformation with pChlamy_3/D-TOPO®-HA2-GFP Transferred Hygromycin B Resistance to C. reinhardtii cc-125

A controlled algal transformation experiment resulted in the recovery of 158 hygromycin B-resistant colonies of C. reinhardtii cc-125, 1 week after transformation with the recombinant pChlamy_3/D-TOPO®-HA2-GFP.

pChlamy2 transformants (transformation control) generated 250 colonies. No colonies were observed in TAP selection plates that were seeded with an identical number of non-transformed C. reinhardtii cc-125; plates contained 8ug/ml of hygromycin B (the MIC was calculated at 6ug/ml). It is important here to mention that the electroporation parameter of the manufacturer did not result in the generation of transformed colonies. A reduction in electroporation voltage to 400 V, and capacitance 25 μF (with no shunt resistance) resulted in increased recovery of transformants and reduced cell loss.

3.4.2. The Nuclear DNA Of Transformed Hygromycin B Resistant C. reinhardtii cc-125 Cells Contained Integrated HA2-GFP Sequences

Out of 51 algal colonies picked randomly from a selection TAP agar plate containing 158 hygromycin B resistant C. reinhardtii cc-125 transformants, 8 contained HA2-GFP sequences preceded by vector upstream promoter sequence. All colonies generated the expected PCR-product using the forward universal chlamy with reverse Bay GFP primers (Table 1; Primers 3, 6) with a fragment size of 1835 bp; equivalent to the sum of the 1559 bp insert plus a 267 upstream vector-specific flanking sequences (Figure 3a), rather than using gene specific primers.

Survival of algae on selective TAP agar indicates hygromycin B-resistance gene must have been integrated within the correct context within the nuclear genome of all 51 colonies. However, since only about 16 % of colonies had the HA2-GFP sequences, it can be concluded that integration of different fragments of the vector is independent of other fragments. The mechanism of integration and the relative efficiency of integration of each section of the vector was not investigated in this study. It is important to mention that isolation of algal nuclear DNA was successful using the three technologies tested.

3.5. The Synthetic Codon-Optimized HA2-GFP Sequence Was Efficiently and Stably Expressed Into C. reinhardtii cc-125

3.5.1. HA2-GFP mRNA Was Transcribed In A Fraction Of Hygromycin B Resistant C. reinhardtii cc-125 Transformants

Properly controlled experiments demonstrated that only 3 of the 8 algal colonies containing the nuclear transgene produced mRNA transcripts; generated the expected 1559 bp RT-PCR amplicon (Figure 3b). This indicated that these colonies may allow protein expression. It also indicates that the context in which the transgene is integrated differs between different colonies. mRNA level and survival time inside cells was not measured.

3.5.2. Robust GFP Expression Was Demonstrated in Hygromycin B Resistant C. Reinhardtii Cc-125 Transformants Expressing HA2-GFP mRNA

GFP-specific fluorescence at 528 nm [28] was detected in the three algal colonies expressing HA2-GFP mRNA transcripts (Figure 4). Clones expressing the recombinant GFP also emitted chlorophyll-specific fluorescence at 658 nm (Neupert,

Karcher and Bock, 2009); indicating that expressing cells retained viability. Viability was also confirmed by color and retention of motility using conventional microscopy. GFP-specific fluorescence was mostly diffuse with some granular structures evident within regions of the cytoplasm, and co-localized with fluorescence emitted from chlorophyll and not within the cup harboring the nucleus.

It was important to detect the robustness of transgene expression in the three transgene-expressing clones described above. Expression of nuclear transgenes is often plagued by gene silencing. The contributing molecular mechanism(s) behind poor transgenes expression from the nuclear genome are not fully understood. Possible reasons implicate poor promoters, nuclear integration position effects, and transcriptional and translational levels of gene silencing (Cerutti et al., 1997; Casas-Mollano et al., 2007). Strategies adopted to overcome expression level issues include using UV mutant strains that enhance transgene expression (Neupert, Karcher and Bock, 2009) and, fusion of the transgene with and essential resistance marker in a manner that would allow downstream purification of target proteins (Rasala et al., 2014). None of the aforementioned techniques were used. Moreover, tools were not available to detect HA2 expression level. Therefore, estimation of transgene expression robustness was done based on relative fluorescence data generated from confocal LASER-scanning microscopy images.

Many biological problems are addressed by using fluorescence microscopy-based approaches and the confocal fluorescence microscope has become a centerpiece in the cell biology laboratories. Fluorescence intensity measurements within the specific channels offers the possibility to study quantitatively molecular interactions in live cells with high degree of accuracy and precision (Waters, 2009). However, a simple analysis approach was adopted using Blue edition ZEN LE software (graphics quantitative analysis tool) since reference fluorescence material was not available for device calibration and precision quantification of fluorescence molecules in cells.

Numerical intensity means values generated from each of the 23 channels were calculated for five cells of positive transformants and five cells of negative non-transformed controls. The analysis revealed that channels 508, 518, 528 and 538 showed significant differences in fluorescence intensities when expressing and control algal cells were compared. The remaining channels were not suitable for detection of GFP fluorescence, but were rather detected chlorophyll autofluorescence. The background-corrected mean GFP-specific fluorescence intensity (measured from the 4 channels described) of 5 cells was 15% of the total cellular fluorescence (sum of intensities from all 23 channels) of the same cells. Mean background-corrected fluorescence from the same 4 channels was only 3.9% for negative transformants. Image background was usually low (around 600 intensity mean value unit) and was subtracted prior to calculations. Therefore, the fluorescence detected in the 4 GFP-specific channels may have been due to bleed through from other channels or fluorescence energy transfer (Kim et al., 2012; Bajar et al., 2016) . Cells showing specific GFP fluorescence also retained the average cell size (10 um). Taken together, these finding indicated that the expressed protein(s) were not toxic to the algae.

3.5.3. AIV H5 Polyclonal Chicken Antisera Recognize the HA2 Expressed In C. reinhardtii cc-125 Transformants

GFP expression analysis does not guarantee that HA2 is expressed correctly, or that it is retained within expressing algal clones; both are essential characteristics for commercial downstream applications. It was essential to detect HA2 protein expression using Western blot analysis.

Antigens are viewed as linear or conformational epitopes by the host's immune system and successful clearance of infections is linked to the ability of the immune system to recognize and mount an effective response to critical viral epitopes (van den Berg et al., 2008). Therefore, AIV H5 polyclonal chicken antisera were used to detect HA2 transgene expression in algal transformants. Positive signals were developed in the three algal colonies expressing HA2-GFP mRNA transcripts at the predicted molecular size (58 kDa). No signals were detected in wild-type C. reinhardtii and in cells transformed with pChlamy2.

3.6. Transformed Colonies Retained mRNA Transcription and Protein Expression for Prolonged Culture Times in Selective Media Containing 10X The MIC Of Hygromycin B.

The effect of incubating the transformed cells in selective marker hygromycin B for prolonged time was tested to evaluate the stability of transgene expression. Increasing the hygromycin B concentration up to 10X of the calculated MIC (15, 30, 60 ug/ml) did not affect gene nuclear integration or mRNA transcription in subcultures of the transformants. Epigenetics may be one of the causes which display high resistance levels either due to insertion of hygromycin gene into a highly euchromatic or high-transcription region of the C. reinhardtii genome or an inadvertent integration event next to a strong endogenous C. reinhardtii promoter (Haring and Beck., 1997; Berthold, Schmitt and Mages, 2002). Multiple subcultures (n=15) still have HA2-GFP and mRNA nuclear stable transformants even after preservation in liquid nitrogen (Scarbrough and Wirschell, 2016) more than 6 months.

References:

Bajar, B. T., Wang, E. S., Zhang, S., Lin, M. Z. and Chu, J. (2016) 'A guide to fluorescent protein FRET pairs', Sensors (Switzerland), 16(9), pp. 1–24. doi: 10.3390/s16091488.

van den Berg, T., Lambrecht, B., Marché, S., Steensels, M., Van Borm, S. and Bublot, M. (2008) 'Influenza vaccines and vaccination strategies in birds', Comparative Immunology, Microbiology and Infectious Diseases, 31(2–3), pp. 121–165. doi: 10.1016/j.cimid.2007.07.004.

Berthold, P., Schmitt, R. and Mages, W. (2002) 'Gene Mediates Dominant Resistance against Hygromycin B in Chlamydomonas reinhardtii', Protist, 153(December), pp. 401–412.

Bidaisee, S. and Macpherson, C. N. L. (2014) 'Zoonoses and one health: A review of the literature', Journal of Parasitology Research. Hindawi Publishing Corporation, 2014. doi: 10.1155/2014/874345.

Boltz, D. A., Douangngeun, B., Phommachanh, P., Sinthasak, S., Mondry, R., Obert, C., Seiler, P., Keating, R., Suzuki, Y., Hiramatsu, H., Govorkova, E. A. and Webster, R. G. (2010) 'Emergence of H5N1 avian influenza viruses with reduced sensitivity to neuraminidase inhibitors and novel reassortants in Lao People's Democratic Republic', Journal of General Virology, 91(4), pp. 949–959. doi: 10.1099/vir.0.017459-0.

Casas-Mollano, J. A., van Dijk, K., Eisenhart, J. and Cerutti, H. (2007) 'SET3p monomethylates histone H3 on lysine 9 and is required for the silencing of tandemly repeated transgenes in Chlamydomonas', Nucleic Acids Research, 35(3), pp. 939–950. doi: 10.1093/nar/gkl1149.

Cerutti, H., Johnson, A. M., Gillham, N. W. and Boyntonl, J. E. (1997) 'Epigenetic Silencing of a Foreign Gene in Nuclear Transformants of Chlamydomonas', the plant cell, 9(June), pp. 925–945.

Chen, C., Lee, P., Tan, C. H., Lo, Y., Huang, C. and Show, P. L. (2015) 'Improving protein production of indigenous microalga Chlorella vulgaris FSP-E by photobioreactor design and cultivation strategies', Biotechnology Journal, pp. 905–914. doi: 10.1002/biot.201400594.

Chen, J., Lee, K. H., Steinhauer, D. A.,

Stevens, D. J., Skehel, J. J. and Wiley, D. C. (1998) 'Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation', Cell, 95(3), pp. 409–417. doi: 10.1016/S0092-8674(00)81771-7.

Dove, A. (2002) 'Uncorking the biomanufacturing bottleneck As biomanufacturing capacity becomes strained , several new methods for producing biologics are being investigated by biotechnology companies .', Nature Reviews, M, 20, pp. 777–779.

Ekiert, D. C., Friesen, R. H. E., Bhabha, G., Kwaks, T., Yu, W., Ophorst, C., Cox, F., Korse, H. J. W. M., Vogels, R., Brakenhoff, J. P. J., Kompier, R., Martin, H., Cornelissen, L. A. H. M., Poon, L. L. M., Peiris, M. and Koudstaal, W. (2012) 'A Highly Conserved Neutralizing Epitope on Group 2 Influenza A Viruses', Science, 333(6044), pp. 843–850. doi: 10.1126/science.1204839.A.

Fodor, E. and Smith, M. (2004) 'The PA Subunit Is Required for Efficient Nuclear Accumulation of the PB1 Subunit of the Influenza A Virus RNA Polymerase Complex', Journal of virology, 78(17), pp. 9144–9153. doi: 10.1128/JVI.78.17.9144.

Franklin, S., Ngo, B., Efuet, E. and May, S. P. (2002) 'Development of a GFP reporter gene for Chlamydomonas reinhardtii chloroplast', the plant journal, 30(30(6)), pp. 733–744.

Gong, Y., Hu, H., Gao, Y., Xu, X. and Gao, H. (2011) 'Microalgae as platforms for production of recombinant proteins and valuable compounds : progress and prospects', J Ind Microbiol Biotechnol, 38, pp. 1879–1890. doi: 10.1007/s10295-011-1032-6.

Haring, M. and Beck., C. (1997) 'A promoter trap for chlamydomonus reinhardtii: development of a gene cloning method using 5'RACE-based probes', The Plant Journal, 11(6), pp. 1341–1348.

Harris, E. H. (2001) 'CHLAMYDOMONAS AS AMODELORGANISM', Annu. Rev. Plant Physiol. Plant Mol. Biol, 52, pp. 363–406.

Heitzer, M. and Zschoernig, B. (2007) 'Construction of modular tandem expression vectors for the green alga Chlamydomonas reinhardtii using the Cre/lox-system', BioTechniques, 43(3), pp. 324–332. doi: 10.2144/000112556.

Hippler, M., Redding, K. and Rochaix, J. D. (1998) 'Chlamydomonas genetics, a tool for the study of bioenergetic pathways', Biochimica et Biophysica Acta - Bioenergetics, 1367(1–3), pp. 1–62. doi: 10.1016/S0005-2728(98)00136-4.

Jinkerson, R. E. and Jonikas, M. C. (2015) 'Molecular techniques to interrogate and edit the Chlamydomonas nuclear genome', the plant journal, 82, pp. 393–412. doi: 10.1111/tpj.12801.

Kaminski, D. A. and Lee, F. E. H. (2011) 'Antibodies against conserved antigens provide opportunities for reform in influenza vaccine design', Frontiers in Immunology, 2(DEC), pp. 1–14. doi: 10.3389/fimmu.2011.00076.

Kim, J., Li, X., Kang, M. S., Im, K. Bin, Genovesio, A. and Grailhe, R. (2012) 'Quantification of protein interaction in living cells by two-photon spectral imaging with fluorescent protein fluorescence resonance energy transfer pair devoid of acceptor bleed-through', Cytometry Part A, 81 A(2), pp. 112–119. doi: 10.1002/cyto.a.21150.

Kim, J. Y., Sim, S., Kim, J. Y., Seong, B. L., Nguyen, H. H. and Chang, J. (2016) 'Baculovirus Displaying Hemagglutinin Elicits Broad Cross-Protection against Influenza in Mice Baculovirus Displaying Hemagglutinin Elicits Broad Cross-Protection against Influenza in Mice', PLoS ONE, 11(March), pp. 1–17. doi: 10.1371/journal.pone.0152485.

Kim, S. S., Lee, Y. C., Cho, D. H., Lee, H. U., Huh, Y. S., Kim, G. J., Kim, S. S. and Kim, H. S. (2014) 'A simple and non-invasive method for nuclear transformation of intact-walled Chlamydomonas reinhardtii', PLoS ONE, 9(7), pp. 1–9. doi: 10.1371/journal.pone.0101018.

Klausberger, M., Tscheliessnig, R., Neff, S.,

Nachbagauer, R., Wohlbold, T. J., Wilde, M., Palmberger, D., Krammer, F., Jungbauer, A. and Grabherr, R. (2016) 'Globular Head-Displayed Conserved Influenza H1 Hemagglutinin Stalk Epitopes Confer Protection against Heterologous H1N1 Virus', PLoS ONE, 11(April), pp. 1–21. doi: 10.1371/journal.pone.0153579.

Mair, C. M., Ludwig, K., Herrmann, A. and Sieben, C. (2014) 'Receptor binding and pH stability - How influenza A virus hemagglutinin affects host-specific virus infection', Biochimica et Biophysica Acta - Biomembranes. Elsevier B.V., 1838(4), pp. 1153–1168. doi: 10.1016/j.bbamem.2013.10.004.

Mamedov, T. and Yusibov, V. (2011) 'Green algae Chlamydomonas reinhardtii possess endogenous sialylated N-glycans', FEBS Open Bio. Federation of European Biochemical Societies, 1, pp. 15–22. doi: 10.1016/j.fob.2011.10.003.

Manuell, A. L., Beligni, M. V., Elder, J. H., Siefker, D. T., Tran, M., Weber, A., McDonald, T. L. and Mayfield, S. P. (2007) 'Robust expression of a bioactive mammalian protein in Chlamydomonas chloroplast', Plant Biotechnology Journal, 5(3), pp. 402–412. doi: 10.1111/j.1467-7652.2007.00249.x.

Mathieu-rivet, E., Vanier, G., Ovide, C., Kiefer-Meyer, M.-C., Vanier, G., Ovide, C., Burel, C., Lerouge, P. and Bardor, M. (2014) 'Protein N-glycosylation in eukaryotic microalgae and its impact on the production of nuclear expressed biopharmaceuticals', Frontiers in Plant Science, 5(July), pp. 1–13. doi: 10.3389/fpls.2014.00359.

Mayfield, S. and Miyake-stoner, E. S. S. (2010) 'Micro-algae come of age as a platform for recombinant protein production', Biotechnol Lett, 32, pp. 1373–1383. doi: 10.1007/s10529-010-0326-5.

Mayfield, S. P., Franklin, S. E. and Lerner, R. a (2003) 'Expression and assembly of a fully active antibody in algae.', Proceedings of the National Academy of Sciences of the United States of America, 100(2), pp. 438–42. doi: 10.1073/pnas.0237108100.

Mayfield, S. P., Manuell, A. L., Chen, S., Wu, J., Tran, M., Siefker, D., Muto, M. and Marin-navarro, J. (2007) 'Chlamydomonas reinhardtii chloroplasts as protein factories', Current Opinion in Biotechnology, 18, pp. 1–8. doi: 10.1016/j.copbio.2007.02.001.

Neupert, J., Karcher, D. and Bock, R. (2009) 'Generation of Chlamydomonas strains that efficiently express nuclear transgenes', Plant Journal, 57(6), pp. 1140–1150. doi: 10.1111/j.1365-313X.2008.03746.x.

Radakovits, R., Jinkerson, R. E., Darzins, A. and Posewitz, M. C. (2010) 'Genetic Engineering of Algae for Enhanced Biofuel Production □ ', EUKARYOTIC CELl, 9(4), pp. 486–501. doi: 10.1128/EC.00364-09.

Raheem Haddad, Esmat Alemzadeh, Ali-Reza Ahmadi, Ramin Hosseini, M. M. (2014) 'Identification of Chlorophyceae based on 18S rDNA sequences from Persian Gulf', Iranian Journal of m icrobiology, 6(6), pp. 437–442.

Rasala, B. A., Chao, S. S., Pier, M., Barrera, D. J. and Mayfield, S. P. (2014) 'Enhanced genetic tools for engineering multigene traits into green algae', PLoS ONE, 9(4). doi: 10.1371/journal.pone.0094028.

Rasala, B. A., Lee, P. A., Shen, Z., Briggs, S. P., Mendez, M. and Mayfield, S. P. (2012) 'Robust expression and secretion of xylanase1 in Chlamydomonas reinhardtii by fusion to a selection gene and processing with the FMDV 2A peptide', PLoS ONE, 7(8). doi: 10.1371/journal.pone.0043349.

Rasala, B. A., Muto, M., Lee, P. A., Jager, M., Cardoso, R. M. F., Behnke, A., Kirk, P., Hokanson, C. A., Crea, R., Mendez, M. and Mayfield, S. P. (2011) 'Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of Chlamydomonas reinhardtii', Plant Biotechnol J., 8(6), pp. 719–733. doi: 10.1111/j.1467-7652.2010.00503.x.Production.

Scarbrough, C. and Wirschell, M. (2016) 'Cryobiology Comparative analysis of cryopreservation methods in Chlamydomonas reinhardtii', Cryobiology.

Elsevier Ltd, pp. 1–5. doi: 10.1016/j.cryobiol.2016.07.011.

Shimogawara, K., Fujiwara, S., Grossman, A. and Usuda, H. (1998) 'High-efficiency transformation of Chlamydomonas reinhardtii by electroporation', Genetics, 148(4), pp. 1821–1828. doi: 10.1126/science.2897716.

Soria-Guerra, R. E., Ramírez-Alonso, J. I., Ibáñez-Salazar, A., Govea-Alonso, D. O., Paz-Maldonado, L. M. T., Bañuelos-Hernández, B., Korban, S. S. and Rosales-Mendoza, S. (2014) 'Expression of an HBcAg-based antigen carrying angiotensin II in Chlamydomonas reinhardtii as a candidate hypertension vaccine', Plant Cell, Tissue and Organ Culture, 116(2), pp. 133–139. doi: 10.1007/s11240-013-0388-x.

Steel, J. (2010) 'Influenza Virus Vaccine Based on the Conserved Hemagglutinin Stalk Domain Influenza Virus', mBio, 1(1). doi: 10.1128/mBio.00018-10.

Surzycki, R., Greenham, K., Kitayama, K., Dibal, F., Wagner, R., Rochaix, J., Ajam, T. and Surzycki, S. (2009) 'Factors effecting expression of vaccines in microalgae', Biologicals. Elsevier Ltd, 37(3), pp. 133–138. doi: 10.1016/j.biologicals.2009.02.005.

Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., Yang, H., Chen, X., Recuenco, S., Gomez, J., Chen, L., Johnson, A., Tao, Y., Dreyfus, C., Yu, W., Mcbride, R., Carney, P. J., Gilbert, A. T., Chang, J., Guo, Z., Davis, C. T., Paulson, J. C., Stevens, J., Rupprecht, C. E., Holmes, E. C., Wilson, I. A. and Donis, R. O. (2013) 'New World Bats Harbor Diverse Influenza A Viruses', PLoS pathogen, 9(10). doi: 10.1371/journal.ppat.1003657.

Valkenburg, S. A., Mallajosyula, V. V. A., Li, O. T. W., Chin, A. W. H., Carnell, G., Temperton, N., Varadarajan, R. and Poon, L. L. M. (2016) 'Stalking influenza by vaccination with pre-fusion headless HA mini- stem', Nature. Nature Publishing Group, pp. 1–11. doi: 10.1038/srep22666.

Wang, B., Wang, J., Zhang, W., Meldrum, D. R. and Peebles, C. A. M. (2012) 'Application of synthetic biology in cyanobacteria and algae AND ALGAE', frontiers in microbiology, 3(September), pp. 1–15. doi: 10.3389/fmicb.2012.00344.

Waters, J. C. (2009) 'Accuracy and precision in quantitative fluorescence microscopy', Journal of Cell Biology, 185(7), pp. 1135–1148. doi: 10.1083/jcb.200903097.

Table 1: Oligonucleotide primers utilized in the study.

	Primer name	**Primer sequence**	**Company**
1	Forward HA2 TOPO Bay	5'- CAC CGG ACT ATT TGG AGC TAT AGC -3'	Invitrogen, USA
2	Reverse HA2 Bay	5'- TTA AAT GCA AAT TCT GCA CTG TAG -3'	
3	Universal forward chlamy	5'- GCA AGC AGT TCG CAT GCA G -3'	Life technologies, USA
4	Universal reverse chlamy	5'- GCT CGC CCT GGA GCG GCA TCG G-3'	
5	Forward Bay HA2	5'- CAC CAG AGG GCT CTT TGG -3'	Metabion, Germany
6	Reverse Bay GFP	5'- GAT ATC TTA CTT GTA CAG CTC ATC CA -3'	
7	Forward Chlamydomonus	5'-AAC CTG GTT GAT CCT GCC AG-3'	Sigma-Aldrich, USA
8	Reverse Chlamydomonus	5'-CAC CAG ACT TGC CCT CCA-3'	

Table 2: Restriction digestion profile of the different pChlamy vectors

Restriction enzymes Vector	KpnI	KpnI and BamHI	ScaI and EcoRI	EcoRI and BamHI	NotI
pChlamy1	4291 bp	2775 bp 1516 bp	2304 bp 1987 bp	3219 bp 1072 bp	4291 bp
pChlamy2	--------	3692 bp	2086 bp 1606 bp	2620 bp 1072 bp	----------
pChlamy_3/D-TOPO	4525 bp	2775 bp 1750 bp	2538 bp 1987 bp	3453 bp 1072 bp	4525 bp
pChlamy4	3640 bp	3637 bp 3 bp	2455 bp 1185 bp	3541 bp 99 bp	----------

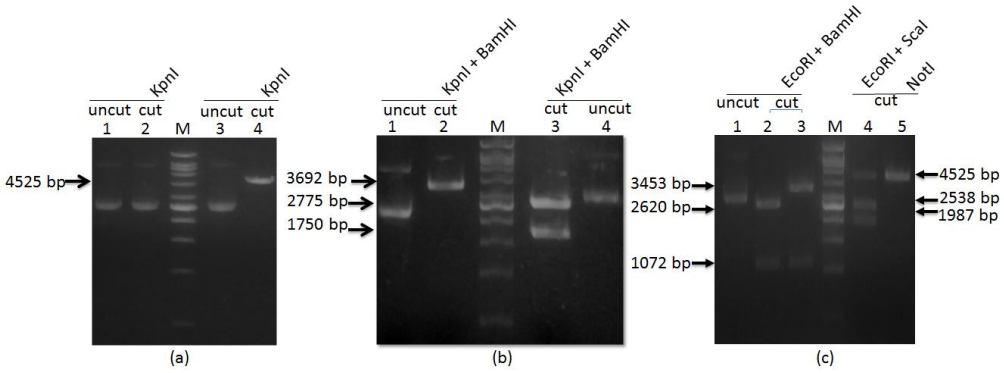

Figure1 (a): Restriction digestion products of the purified pChlamy_3/D TOPO® vector in comparison to the algal control transformation vector pChlamy2 using KpnI enzyme (a), KpnI and BamHI enzymes (b). Lanes 1 and 2 show the digestion products of untreated and treated pChlamy2 vector, respectively. Lanes 3 and 4 show the digestion products of untreated and treated pChlamy_3/D TOPO® vector, respectively. Lane M shows bands of 1 Kb DNA ladder (Vivantis). (c): Restriction digestion product of the purified pChlamy_3/D TOPO® vector using EcoRI and BamHI enzymes in comparison to the algal control transformation vector pChlamy2. Lanes 1 and 2 show the digestion products of untreated and treated, respectively showing 2 bands of 2620 and 1072 bp pChlamy2 vector. Lanes 3 shows the digestion products of treated pChlamy_3/D TOPO® vector showing 2 bands of 3453 and 1072 bp. Restriction digestion products of the purified pChlamy_3/D TOPO® using EcoRI and ScaI enzymes (Lane 4) and NotI (Lane 5). Lane 4 shows 2 digestion products of 2538 and 1987 bp beside a band that represents remnants of the undigested plasmid. Lane 5 shows a single band of the linearized vector at 4525 bp, Lane M show bands of 1 Kb DNA ladder (Vivantis).

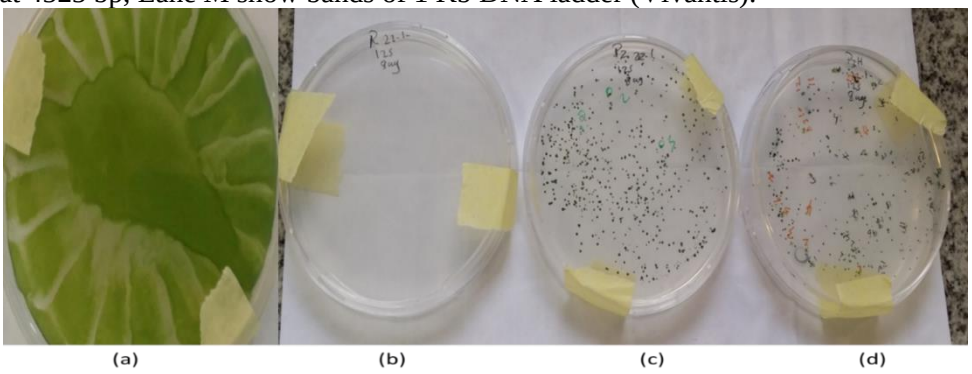

Figure2: Chlamydomonus reinhardtii cc-125 transformation (a) Viability control plate showing extensive growth of C. reinhardtii cells (b) Resistance control plate showing no colony formation (c) Algal transformation control plate (d) Algal expression plate.

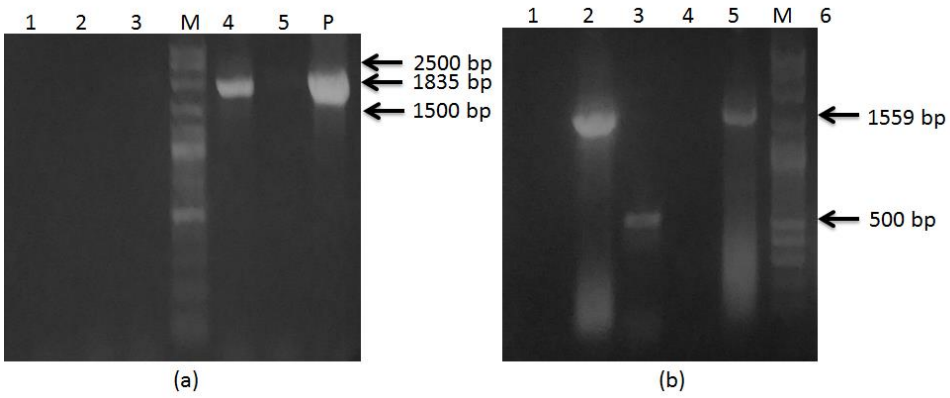

Figure 3: (a) Verification of nuclear integration of the synthetic HA2-GFP gene cassette using the universal forward chlamy and the reverse Bay GFP primers. Lanes 1-3 show no amplification of the no template control, MOCK transformed cells, and pChlamy2 vector transformed cells, respectively. Lane M shows bands of 100 bp plus DNA ladder (Vivantis), Lanes 4 and 5 illustrate the amplification products of positive and negative transformed algal colonies, respectively, Lane P show specific amplification of the complete synthetic HA2 gene including the upstream promoter of the positive control recombinant pChlamy_3/D TOPO vector. (b) mRNA detection in two recombinant algal colonies using RT-PCR. Lanes 1 and 4 shows no specific amplification of 1835 bp fragment using forward universal chlamy and reverse synthetic HA2 primers (no DNA contamination) for both colonies. Lanes 2 and 5 show specific amplification of 1559 bp fragment using synthetic forward and reverse HA2 primers. Lane 3 shows the amplification product using Chlamydomonas gene 18sDNA primers control. Lane M, Vivantis 1 kb DNA ladder.

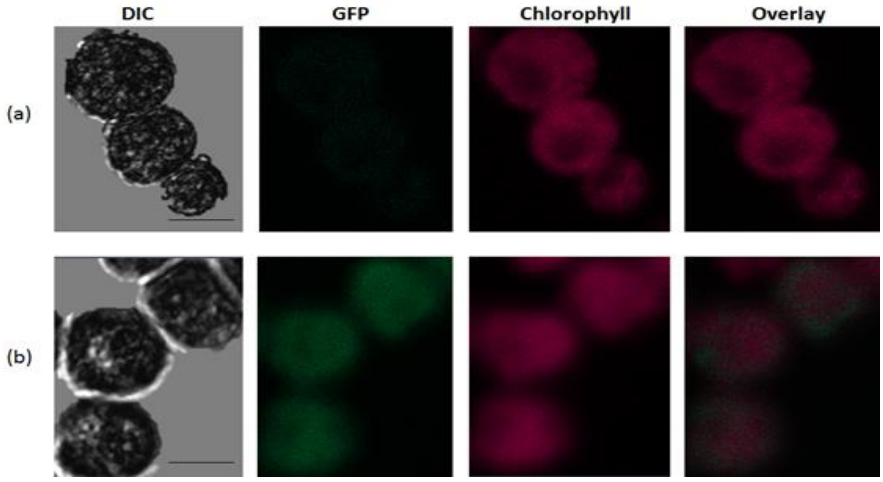

Figure 4: Confocal microscopy for GFP signals detection using live algal cells, scale bar 5 um. (a) Negative control cell pool (b) mRNA Positive transformants cell pool at 528 nm to GFP, 658 nm for chlorophyll, Differential interference contrast (DIC) images are also shown.

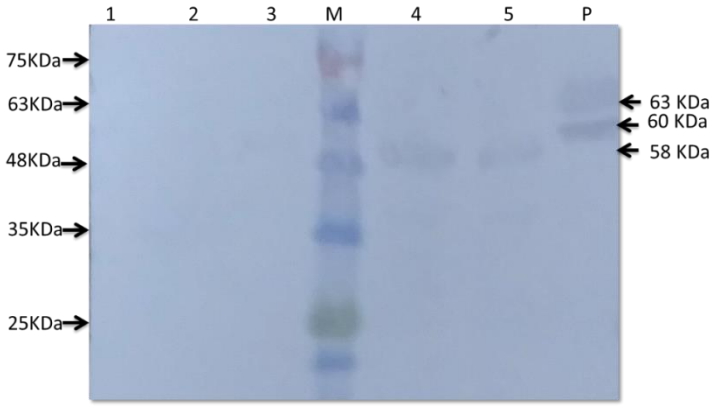

Figure 5: Analysis of HA2 gene expression in C. reinhardtii transformants by Western immunoblotting. Lanes 1 and 2 indicate negative results of wild-type and pChamy2 transformed cells, respectively, Lane 3 shows a very weak band at the predicated molecular weight of HA2 gene (58 kDa) in cell pellet of a representative algal transformant, Lane M shows the bands of BLUelf prestained protein ladder, Lanes 4 and 5 show distinct bands of HA2 gene in TSP extracts of two representative colonies, Lane P specifies two bands of the positive control AIV H5N1 at the molecular weight sizes 60, 63kDa of NA, HA Proteins of AIV.

المستخلص العربي

تعتبر البيوتكنولوجيا الخضراء هي المستقبل لانتاج المستحضرات الحيوية البيولوجية، ويعتبر استخدام الطحالب لانتاج المستحضرات الحيوية البيولوجية ذو أهمية قصوى لبرامج انتاج اللقاحات. وقد تم عمل محاولة للتعبير عن انتجينات لفيروس انفلونزا الطيور داخل الطحالب لما له من أهمية اقتصاية وأخرى لخطورة صحية عامة. فقد تم التعديل على النظام التعبيرى التجارى حقيقى النواة لكى يسمح بالتعبير عن بروتين التلزن الدموى 2 لفيروس انفلونزا الطيور اتش5ان1 داخل خلايا الطحالب (كلاميدوموناس رنهارتى). فقد تم تخليق الجين المطلوب بحيث يسمح بالتعبير داخل الطحالب على ناقل فرعى pUC-57 وتم اخذ الجين المطلوب وكلونته داخل الناقل التعبيرى الطحلبى pChlamy_3/D- TOPO® ومنه الى داخل خلايا الطحالب. وقد تم تأكيد دخول الجين السليم الى جينوم الطحالب النووي في 16% فى الخلايا التي تم البحث داخلها.وقد تم التأكد من نسخ الرنا الرسول فى هذه الخلايا للجين المطلوب باستخدام تفاعل البلمرة المتسلسل باستخدام انزيم النسخ العكسى، وتم التأكد من انتاج البروتين المطلوب باستخدام ميكروسكوب متحد البؤر وتم التأكد بعمل اختبار اللطعة الغربية باستخدام اجسام مضادة خاصة للبروتين. ومن هذه النتائج نامل في اجراء تقصيات أخرى حول النواحى المناعية للبروتين المنتج داخل خلايا الطحالب.

الكلمات الدالة : انفلونزا الطيور، كلاميدوموناس رنهارتى، بروتين التلزن الدموى2، بروتين مخلق