

# Expression of an HBcAg-based antigen carrying angiotensin II in *Chlamydomonas reinhardtii* as a candidate hypertension vaccine

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**Abstract** In contrast to traditional pharmacological treatments for hypertension, immunotherapies serve as promising alternatives as they are low-cost and afford better patient compliance. In this study, a chimeric protein targeting Angiotensin II via genetic fusion to a nucleocapsid antigen from Hepatitis B virus (HBcAg), serving as a carrier, is designed. This candidate immunogen designated as HBcAgII has been expressed in the alga specie *Chlamydomonas reinhardtii*, serving as an attractive vaccine expression system and delivery host. This alga can be grown on minimal media under controlled environmental conditions, and can serve as a safe oral delivery vehicle. Transgenic *C. reinhardtii* lines have been developed, and the expected recombinant protein has been detected by Western blot and ELISA analyses. Levels of expression of this recombinant protein in some transgenic lines have reached 0.05 % of total soluble protein. The immunogenic properties of the HBcAgII algae-derived antigen will be assessed.

**Keywords** Chronic disease · Green biofactory · Hypertension · Molecular farming · Vaccination

## Introduction

One of the major risk factors contributing to the development of cardiovascular disease is high blood pressure. This contributes to almost 7.5 million deaths annually, representing almost 12.8 % of reported global annual deaths in the human population (WHO 2009). Among the primary drawbacks of current pharmacological treatments against hypertension are adverse symptoms (nausea, fatigue, and dizziness), metabolic effects, including changes in serum concentrations of uric acid, glucose, and total cholesterol, as well as low patient compliance due to necessity for multiple and continuous dosage administration, thus resulting in high treatment costs (Bramlage and Hasford 2009).

Recently, there has been a renewed interest in vaccination, first explored in 1941, as a treatment against hypertension (reviewed by Maurer and Bachmann 2010). In pursuing vaccines against hypertension, components of the renin angiotensin aldosterone system have been proposed as vaccine targets. These components would elicit antibodies that in turn would lower blood pressure by partially blocking activity of this physiological system. Thus far, the most advanced candidate vaccine, designated as CYT006-AngQb, targets angiotensin II, and comprises of chimeric Virus Like Particles (VLPs) as these structures are highly immunogenic and capable of evoking strong immune responses even to unrelated antigens fused to these structures (Roldão et al. 2010). The CYT006-AngQb vaccine has been tested in clinical phase II trials, and has demonstrated capability in lowering blood pressure values along

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with acceptable safety standards (Ambühl et al. 2007; Tissot et al. 2008).

Vaccine production costs under conventional platforms, such as bacterial and mammalian, are often associated with low vaccination coverage and safety concerns also exists when parenteral administration is followed (WHO 2011). In this context, photosynthetic organisms have been proposed as efficient and low cost platforms for the production of recombinant proteins. Plants in several modalities have been used for the successful production of antigens, antibodies and enzymes (Hahne et al. 2011; Soria-Guerra et al. 2011). Outstandingly, some biopharmaceuticals produced in these systems are in advance degree of development and close to be marketed (Yusibov et al. 2011; Wirz et al. 2012). Recently, a number of studies have focused on the use of *Chlamydomonas reinhardtii* for the production of recombinant antigens in algae-based vaccines. *C. reinhardtii* offers several advantages as a vaccine production platform. This microorganism serves as a safe oral delivery vector, easily propagated in photo bioreactors under full containment, easily genetically modified, and high capable of assembling complex proteins. To date several *Chlamydomonas*-based vaccine candidates have proved to be functional in test animals and functional antibodies have also been produced in this host (Specht et al. 2010; Rosales Mendoza 2013). Thus far, several vaccination models have been reported using *Chlamydomonas* as a source of recombinant vaccines (Rosales Mendoza et al. 2012).

In an effort to develop an effective immunization scheme against hypertension, this study aimed at producing VLPs carrying angiotensin II in *C. reinhardtii* as a low-cost production platform and a delivery vector for a hypertension vaccine.

## Materials and methods

### Plasmid constructs and bacterial strains

A synthetic gene encoding a recombinant protein, HBcAgII, was synthesized by GeneScript Corp. (Piscataway, NJ, USA). This HBcAgII antigenic protein comprises the coding region of the Hepatitis B core protein along with the angiotensin II sequence flanked by Gly-rich linkers, inserted into the central *c/e1* epitope of the truncated assembly-competent HBcAg protein (aa 1–149). A histidine tag was also included to facilitate detection of the recombinant protein. The restriction sites *Xba*I and *Sac*I were included at the 5' and 3' ends, respectively, to facilitate subcloning into the binary vector pBI-121. This expression vector harbors the *nptII* gene for kanamycin resistance, and the gene of interest is driven by the CaMV 35S promoter. *Escherichia coli* TOP10 clones carrying the

desired expression vector, designated as pBI-HB, were selected by restriction analysis and sequencing. *Agrobacterium tumefaciens* strain GV3101 was used for nuclear transformation following the mobilization of the pBI-HB vector by electroporation.

### Chlamydomonas maintenance and transformation

A wild-type *C. reinhardtii* strain CC-137 (mt+) was donated by Dr. Manfredo Seufferheld (University of Illinois) and used for transformation. Cells were grown in Tris–acetate–phosphate (TAP) medium. Liquid cultures were kept in an incubation shaker under cool fluorescent light at  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 16 h light/8 h dark cycle and 25 °C.

*C. reinhardtii* cells were co-cultivated with *A. tumefaciens* cells according to Kumar et al. (2004), but with some modifications. Briefly,  $\sim 1 \times 10^8$  *Chlamydomonas* cells were plated onto a solid Tris–acetate–phosphate medium supplemented with 100  $\mu\text{M}$  acetosyringone (AS), and incubated under light conditions for a period of 2 days. An overnight grown *Agrobacterium* culture grown on Luria–Bertani (LB) broth was pelleted and subsequently resuspended in liquid TAP medium. A total of 300  $\mu\text{l}$  of *Agrobacterium* cell suspension culture ( $OD_{600} = 0.5$ ) was plated over a thin layer of *Chlamydomonas* cells growing on agar plates. Following co-cultivation for 48 h in the dark, cells were harvested by centrifugation at 8,000 rpm for 3 min and washed three times with liquid TAP medium containing 500  $\text{mg l}^{-1}$  cefotaxime. Cells were resuspended in 200  $\mu\text{l}$  of TAP medium and plated onto solid TAP plates containing 100  $\text{mg l}^{-1}$  of kanamycin and 500  $\text{mg l}^{-1}$  of cefotaxime. Kanamycin resistant colonies were grown in liquid TAP medium, and used for molecular analysis.

### PCR analysis

Genomic DNA from putative transformed colonies was isolated following the protocol of the Chlamydomonas Resource Center (<http://www.chlamy.org/methods.html>). PCR analysis was performed using specific primers for the *HBcAgII* and *nptII* genes to amplify 580 and 610 bp amplicons, respectively. Two hundred ng of genomic DNA was used as template in PCR mixtures containing the following: 2.5 mM  $\text{MgCl}_2$  (for *HBcAgII* gene) or 2.0 mM  $\text{MgCl}_2$  (for *nptII* gene), 0.2 mM dNTPs, 0.5  $\mu\text{M}$  of each primer, and 0.5 U *Taq* DNA polymerase. After a 7 min initial denaturation at 95 °C, samples were subjected to 35 PCR cycles under the following conditions: 95 °C for 50 s, 55 °C for 30 s, and 72 °C for 60 s. The final extension was carried out for 10 min at 72 °C. PCR products were electrophoresed on 1 % agarose gels.

Candidate colonies were also evaluated for presence of persistent *A. tumefaciens* in PCR reactions using *virG* gene specific primers (5'ATCATTTGTAGCGACT-3' and 5'-AGCTCAAACCTGCTTC-3') as reported by Hass et al. (1995).

#### Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed in a 48-well optical grade plate using an Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems, Mulgrave, VIC, Australia). DNA amplification products were monitored using SYBR Green. Each reaction contained 10 µl of SYBR Green Master Mix reagent (Applied Biosystems, Mulgrave, VIC, Australia), 100 ng genomic DNA, and 0.5 pmol of gene specific forward and reverse primers in a final volume of 20 µl. The following standard PCR reaction conditions were used: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Melting curves were generated by a cycle consisting of 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C.

Data were analyzed using the Applied Biosystems StepOne™ Real-Time PCR System Software (Applied Biosystems). Baseline data were collected from cycles 1 to 40. Amplification plots ( $\Delta R_n$  vs. cycle) were analyzed using an  $R_n$  threshold of 0.04 to obtain cycle threshold ( $C_t$ ) values. Gene copy number index for each sample was obtained using the following formula:  $GCI = \text{Eff}^{\text{Ref}C_t} / \text{Eff}^{C_t}$  (Casu et al. 2012; Shepherd et al. 2009), wherein GCI is the gene copy number index,  $\text{Eff}^{\text{Ref}C_t}$  is the PCR efficiency achieved using reference gene primers (*ubiquitin ligase* gene) to the power of the  $C_t$  value generated from the reference gene for each sample, and  $\text{Eff}^{C_t} = \text{PCR efficiency achieved using test gene primers (HBcAgII) to the power of the } C_t \text{ value generated from the test gene for each sample.}$

#### Protein analysis

Total soluble protein (TSP) samples from both untransformed and transgenic lines were extracted as reported by Campos-Quevedo et al. (2012), and protein concentrations were measured using the Bradford assay (Bradford 1976). For Western blot analysis, protein extracts were denatured by the addition of SDS-PAGE loading buffer (Laemmli), and followed by incubation at 95 °C for 5 min. Proteins were separated onto a 12 % SDS-PAGE gel at 120 volts and transferred onto nitrocellulose membranes at 350 mA for 30 min in a Trans-Blot SD (BioRad, Hercules, CA, USA). After blocking with 6 % fat-free milk, the membrane was probed with an anti-HBc monoclonal antibody (1:3,000 dilution; Abcam ab8638; Cambridge, MA, USA), and detected using an anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:10,000 dilution; Sigma

Missouri, USA). An ECL substrate (Thermo Scientific, USA) was then added, and the signal was detected on an X-ray film using standard protocols (Sambrook et al. 1989).

A quantitative ELISA assay for the HBcAgII protein was conducted as described by Campos-Quevedo et al. (2012). A microtiter plate was coated with 10 µg of TSP from the wild type or the transformed *C. reinhardtii* lines. A standard curve for the HBcAg protein (ranging from 0.1 to 4 ng/µl) was established using a pure Hepatitis B virus core antigen protein standard of known concentration (Abcam ab49014; Cambridge, MA, USA). This was used for quantification of the recombinant protein HBcAgII expressed in *C. reinhardtii* lines in the form of TSP. All antibody dilutions were the same as described above for Western blot analysis.

## Results

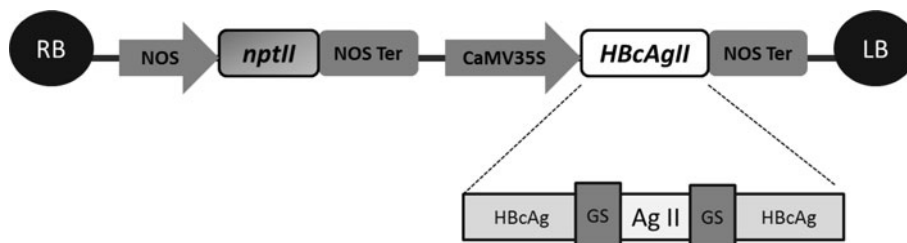
The HBcAgII gene is successfully transferred to *C. reinhardtii*

The presence of the cloned insert *HBcAgII* into the binary vector pBI-121 was successfully confirmed by restriction analysis and verified by sequencing (Fig. 1). Following the *Agrobacterium*-mediated transformation of *C. reinhardtii*, several transformants were rescued on a kanamycin containing selection medium 2 weeks after transformation. Following several rounds of selection, six kanamycin-resistant lines were rescued for further analysis.

DNA transfer events were assessed by PCR analysis to detect the *HBcAgII* and *nptII* genes in Kan<sup>R</sup> colonies. The expected 0.58 kb amplicons of the *HBcAgII* transgene were obtained in all lines and the positive control (pBI-HB); whereas, no signal was detected in wild-type colonies (Fig. 2a). The *nptII* transgene was also detected by PCR, and 0.15 kb amplicons were observed in these lines, but these were completely absent in the wild type (Fig. 2b). These PCR analyses indicated presence of the transgene of interest in candidate algal clones.

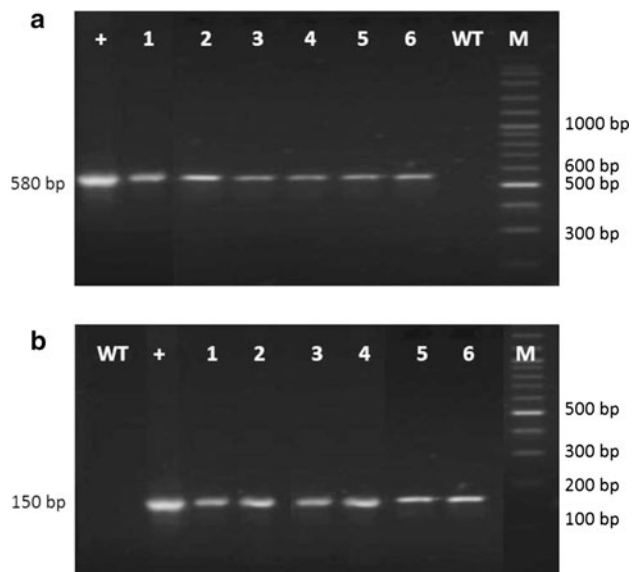
#### Screening for persistent *Agrobacterium*

Transformant colonies were subsequently evaluated for absence of any bacterial cells. PCR analysis was used to evaluate these colonies for *Agrobacterium* contamination. Using specific primers to amplify the *virG* gene of *Agrobacterium*, no amplicons were observed in all transformed and wild-type colonies, while amplification was observed in the positive control (*A. tumefaciens* strain GV3101) (data not shown). These findings confirmed that these transformed *C. reinhardtii* were free of bacterial contamination.



**Fig. 1** Schematic diagram of transformation vector pBI-HB, which contains the *HBcAgII* gene cassette, which comprises the promoter and terminator of the cauliflower mosaic virus 35S (CaMV 35S), the *HBcAgII* gene encoding the recombinant protein, including the

angiotensin II (Ag II) flanked by the amino acids GS, which are useful to form disulfide bridges, to facilitate the display of Angiotensin II, and the *nptII* gene which confers kanamycin resistance to transformants clones



**Fig. 2** PCR analysis of *C. reinhardtii* transformants for detection of the *HBcAgII* (a) and *nptII* (b) transgenes. **a** Lane+: positive control (pBI-HB); Lanes 1–6: putative transgenic lines (C.HB1 to C.HB6). Lane WT Untransformed *C. reinhardtii*; M: DNA ladder. **b** Lane WT Untransformed *C. reinhardtii*; Lane+ positive control (pBI-HB); Lanes 1–6: putative transgenic lines (C.HB1–C.HB6); Lane M Molecular weight 100 bp

#### Gene copy number

Genomic DNA was extracted from each of the six transgenic lines carrying the *HBcAgII* transgene, and used as a template for qRT-PCR using *HBcAgII*-specific primers and *ubiquitin ligase*-specific primers (used as a reference gene). Each reaction was run in triplicate. All amplification reactions showed a single peak in the dissociation curve analysis (data not shown). PCR efficiencies were higher than 90 % for both *HBcAgII* and *ubiquitin ligase* genes. The gene copy number index for *HBcAgII* is directly related to the transgene copy number for a given clone and similar to that of the copy reference gene. Given this assumption, an estimated transgene copy number was generated for each transgenic line (Table 1). A single copy

of the transgene was estimated for four analyzed lines, including C.HB1, C.HB2, C.HB3, and C.HB5, and two copies of the transgene were detected in clones C.HB4 and C.HB6.

The *HBcAgII* protein is produced in transgenic *C. reinhardtii* lines

Western blot analysis performed for the detection of *HBcAgII* revealed the presence of a band corresponding to a molecular weight of 20.7 kDa, which is in agreement with the predicted size for the expected protein. No signal was observed for the WT sample (Fig. 3). This assay indicates that the expressed antigen retains the antigenic determinants of the carrier *HBcAg*.

The amount of the *HBcAgII* protein, in a sample of known concentration of total soluble protein (TSP), was determined by ELISA using a standard curve generated with pure *HBcAg*. Overall, significantly higher absorbance readings were recorded for transformed clones in comparison to those obtained for the wild-type sample, thus confirming presence of an immunoreactive protein (Fig. 4). Levels of the alga-derived *HBcAgII* protein ranged between 0.02 and 0.05 % of TSP in different transgenic lines.

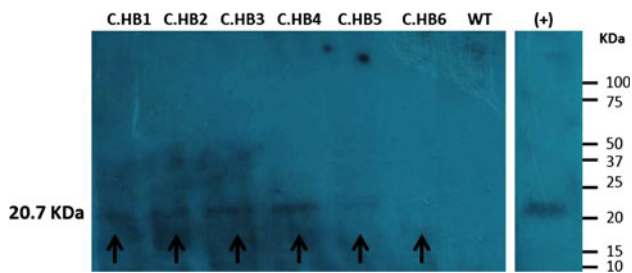
#### Discussion

As current treatments for hypertension have substantial limitations, including side effects and low patient compliance, new therapeutic approaches are needed to combat the epidemiologic impact of cardiovascular diseases. In this study, a new candidate for the development of a low-cost vaccine is reported. *Agrobacterium*-mediated transformation allowed the development of *Chlamydomonas* transgenic lines producing VLPs based on a chimeric protein that comprises *HBcAg* and *AngII*. Accumulation levels of this protein in transgenic lines were in the range of 0.02–0.05 % of TSP, as determined by ELISA assays.

**Table 1** Estimated *HBcAgII* copy number for each line based on gene copy number index (GCI) generated using the *ubiquitin ligase* gene used as a reference

Transgenic lines	C.HB1	C.HB2	C.HB3	C.HB4	C.HB5	C.HB6
PCR efficiency for the <i>ubiquitin ligase</i> gene	94.2 %	94.2 %	94.2 %	94.2 %	94.2 %	94.2 %
PCR efficiency for the <i>HBcAgII</i> gene	92.5 %	92.5 %	92.5 %	92.5 %	92.5 %	92.5 %
Ct for <i>ubiquitin ligase</i> gene	21.55	20.6	20.71	19.98	21.83	19.73
Ct for <i>HBcAgII</i> gene	21.39	20.58	20.68	19.40	21.72	19.14
$GCI = \text{Eff}^{\text{RefCt}}/\text{Eff}^{\text{Ct}}$	1.19	1.04	1.00	1.8	1.1	1.8
Estimated copy number	1	1	1	2	1	2

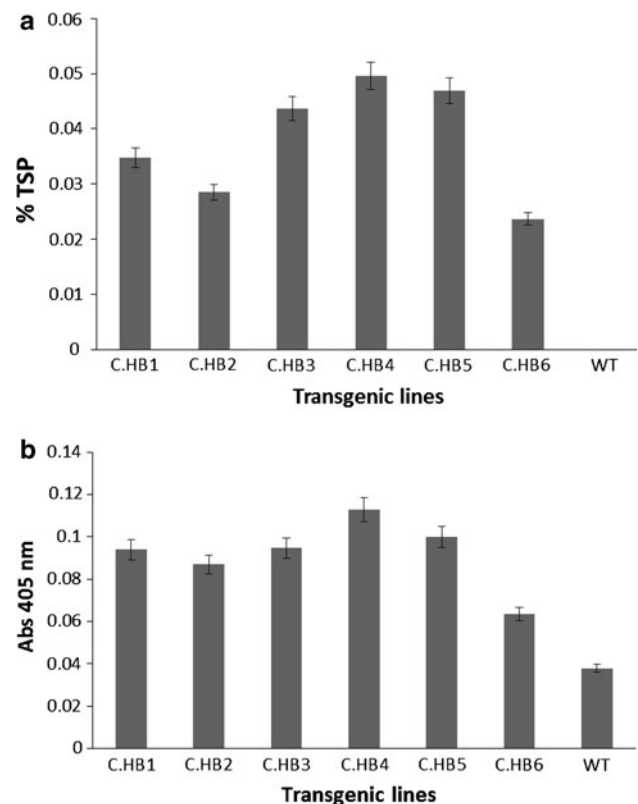
The GCI was obtained using the formula:  $GCI = \text{Eff}^{\text{RefCt}}/\text{Eff}^{\text{Ct}}$  as described in “Materials and methods” section  
Ct cycle threshold



**Fig. 3** Western-blot assay of HBcAgII protein in *C. reinhardtii* transformants. Equal amounts of total soluble protein were loaded in each lane (20  $\mu\text{g}$ ). The HBcAgII protein was detected by anti- HBcAg antibody, using a secondary antibody conjugated to horseradish peroxidase (HRP). Lane C.HB1–C.HB6: Transformed lines. Lane WT: Untransformed *C. reinhardtii*; Lane (+): 20 ng of HBcAgII pure protein

Although a single copy of the transgene is detected in four lines (C.HB1, 2, 3, and 5) and two copies in lines C.HB4 and C.HB6, the observed variation in protein accumulation may also be attributed to other factors as well such as site positional effects of integration of the transgene, which is a well-known phenomenon reported in nuclear transformation approaches (Leon and Fernandez 2007).

In this study, low transformation efficiency and a relatively low expression levels were observed. One may speculate that these findings are associated to possible toxicity of the foreign protein in the studied host. Among recently reported strategies that may lead to enhanced levels of nuclear expression of transgene(s), Rasala et al. (2012) have used the 2A peptide, placed in between two open reading frames, one of them coding for the protein of interest. During translation, a self-cleavage reaction known as a skip translation mechanism is induced by the 2A peptide in this mRNA chimeric arrangement, resulting in synthesis of two independent polypeptides from a single mRNA (Ryan et al. 1991). Using this approach, higher levels of expression of the *ble* marker gene and xylanase enzyme was observed, contributing to a substantial improvement in yield, reaching up to 0.25 % TSP for xylanase, corresponding to a 100-fold increase in yield. As



**Fig. 4** ELISA assay of the recombinant protein HBcAgII expressed in *C. reinhardtii* transformants. **a** The quantitation of the HBcAgII protein was determined by ELISA comparing the absorbance values of the transgenic lines against a standard curve of a known amount of HBcAg protein, ranging from 200 to 6.25 ng. The amount of HBcAgII protein was expressed as a percentage of TSP. **b** Qualitative ELISA assay probed with anti-Histidine tag antibody. The graph shows absorbance of the total soluble protein (TSP) from transgenic lines and from the wild type (WT), used as a negative control. These values are averages of three experiments along with standard deviations

relatively high expression levels of the *ble* marker gene are required to confer resistance to zeocin, it is proposed that this likely promotes expression of other ORFs included in the chimeric mRNA arrangement (Rasala et al. 2012).

Chloroplast-based expression is deemed as a potential alternative in achieving higher levels of expression of this candidate recombinant antigen as a number of vaccine antigens, enzymes, antibodies, immunotoxins, and human therapeutics have been produced at high accumulation levels using transplastomic technologies (Rosales-Mendoza et al. 2012). However, it should be taken into consideration that VLPs are typically effective at low doses due to its high immunogenicity, thus all of these transgenic strains may also be proposed as candidates for further characterization (Pniewski et al. 2011; Scotti and Rybicki 2013). As revealed by Western blotting using anti-HBc antibodies, the algae-synthesized recombinant HBcAgII protein seems to retain the HBcAg antigenic determinants suggesting an immunogenic potential.

It is important to note that recent reports have addressed the development of vaccine candidates against hypertension. For example Ou et al. (2013) have developed a candidate vaccine based on four repetitions of AngII fused along the surface of the hepatitis virus, designated as pHAV-4AngII, and produced in sf9 insect cells. Successful assembly of VLPs was achieved as demonstrated by electron microscopy. Functional assays indicated that this candidate vaccine is capable of inducing high antibody titers of anti-AgII IgG and a decrease of 23 mmHg in the systolic blood pressure of spontaneously hypertensive rats (SHR).

Although the development of immunotherapies against hypertension is a topic of relevance, considering the possible costs of these formulations produced under conventional treatments, it is important to note that *C. reinhardtii* has been established in the last decade as an attractive platform for the production of biopharmaceuticals due to its efficient and rapid methods of growth and cultivation. Moreover, this organism is capable of correctly performing post-transcriptional and post-translational modifications, and shows high photosynthetic efficiency than higher plants (Shimizu 1996; Surzycki et al. 2009). As *C. reinhardtii* is Generally Recognized As Safe (GRAS) by the FDA, it serves as an attractive host that can be used for safe production of parenteral vaccines under good manufacturing practices. Another approach consists in formulating oral vaccines avoiding purification, which dramatically reduces costs (Rosales-Mendoza 2013).

The algae-derived HBcAgII protein described in this study offers a new approach that would contribute in the development of low cost vaccines against hypertension. Further studies will focus on determining the immunogenic potential and the anti-hypertensive effect of this candidate vaccine.

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