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Heterologous expression of bacteriocin E-760 in *Chlamydomonas reinhardtii* and functional analysis

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Abstract. The use of antimicrobial peptides (AMPs) synthesized by bacteria (bacteriocins) is an alternative for combating multidrug resistant bacterial strains and their production by recombinant route is a viable option for their mass production. The bacteriocin E-760 isolated from the genus *Enterococcus* sp. has been shown to possess inhibitory activity against Gram-negative and Gram-positive bacteria. In this study, the expression of a chimeric protein coding for E-760 in the nucleus of *C. reinhardtii* was evaluated, as well as, its antibacterial activity. The synthetic gene E-760S was inserted into the genome of *C. reinhardtii* using *Agrobacterium tumefaciens*. A transgenic line was identified in TAP medium with hygromycin and also by PCR. The increment in the culture medium temperature of the transgenic strain at 35 °C for 10 minutes, increased the production level of the recombinant protein from 0.14 (Non-induced culture, NIC) to 0.36% (Induced culture, IC) of total soluble proteins (TSP); this was quantified by an ELISA assay. Recombinant E-760 possesses activity against Staphylococcus aureus in 0.34 U log, *Streptococcus agalactiae* in 0.48 U log, Enterococcus faecium in 0.36 U log, Pseudomonas aeruginosa in 2 U log and for *Klebsiella pneumoniae*, the activity was 0.07 U log. These results demonstrate that the nucleus transformation of *C. reinhardtii* can function as a stable expression platform for the production of the synthetic gene E-760 and it can potentially be used as an antibacterial agent.

Keywords: Heterologous expression; Antibacterial activity; Bacteriocin E-760; *Chlamydomonas reinhardtii*; Log inactivation; Nuclear transformation.

INTRODUCTION

The problem of bacterial resistance to antibiotics is a priority issue for global health agencies; such is the case of the World Health Organization (WHO), which in its global report on surveillance of antimicrobial resistance in 2014, mentioned that society is in a "post-antibiotic" era and that the crisis of antimicrobial resistance is increasingly serious (WHO, 2014). In response to this global problem, several strategies have been proposed to combat microbial resistance; one of these strategies is the synthesis and use of antimicrobial peptides (AMPs), whether natural or synthetic, which can be used as a model for the design and creation of new functional classes of antibiotics (Tillotson and Theriault 2013). Biologically, AMPs are indispensable components of the innate defense mechanisms of living beings; they are oligopeptides with a length of 10-50 aminoacid residues, which have a cationic-amphipathic or hydrophobic helical structure nature that facilitates the initial interaction with the negatively charged bacterial membrane, damaging it and causing cell death (Hassan et al., 2012). Furthermore, these peptides show a broad spectrum of activity against bacteria, fungi, viruses and eukaryotic parasites. AMPs synthesized by bacteria are called bacteriocins and are ribosomally synthesized peptides of small size, thermostable, active and highly specific against bacteria closely related to the producer organism (narrow spectrum) (Cotter et al., 2005). Unlike bacteriocins, eukaryotic AMPs are usually less specific because they have a broader action spectrum, encompassing a greater diversity of bacteria (Gram positive and Gram negative) (Hassan et al., 2012). The bacteria that produce AMPs are immune to their own bacteriocins, due to the

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synthesis of specific immunity proteins (Cotter et al., 2005).

The lactic acid bacteria (LAB) group is one of the main producers of bacteriocins and also one of the most studied, due to the fact that most of these bacteria are isolated from food sources, they are classified into the category GRAS (Generally Recognized As Safe). Among the most studied bacteriocins are nisin and pediocin PA-1, due to their potential of being used in the food industry (Woraprayote et al. 2016).

Line et al., 2008) isolated and purified a bacteriocin named enterocin E-760 from the genus *Enterococcus*, using biochemical and chemical methods. This peptide has 62 aminoacid residues, with a weight of 5,362 kDa. Also, it is thermostable, stable at a pH of 5.0 to 8.7, has a pI of 8.7, and has a broad inhibition spectrum against Gram positive and Gram negative bacteria. However, the synthesis and purification of peptides by biochemical and chemical methods has high production costs, even higher if the protein is to be massively produced. Therefore, several methods have been developed in order to avoid higher costs, which are based on the use of molecular techniques for the production of recombinant proteins that make possible the mass production of chimeric proteins with important pharmacological capacities (Parachin et al., 2012; Arbulu et al., 2015).

In the last decades, the microalga *Chlamydomonas reinhardtii* has been used as a platform for the production of recombinant proteins because it has a high reproduction rate; its growth requirements are minimal, since it is a photosynthetic organism that makes possible to obtain crops at commercial scales in a profitable way; it is classified as a GRAS organism; and its sequenced genome is available on the PlantGDB platform (http://www.plantgdb.org/CrGDB/), which facilitates the introduction of interest genes in any of its three genomes (nuclear, chloroplast and mitochondrial) by different techniques (Barrera and Mayfield 2013).

Although, the transformation methods of *C. reinhardtii* directed to the nucleus or plastids are relatively simple, the majority of the high-value recombinant proteins have been produced in the chloroplast of the microalgae through direct transformation by microprojectile bombardment, a method that requires the use of specialized equipment; whereas, the transformation directed to the nucleus mediated by *Agrobacterium tumefaciens* is a biological method that does not generate damage to the host cell, can transfer large fragments (150 kb) to the nuclear genome, integrates a low number of copies of the interest gene and does not require specialized equipment, making it a more cost-effective and accessible method (Mello-Farias and Chaves, 2008).

Therefore, the aim of this study was to design and produce a chimeric protein coding for the antibacterial peptide E-760, which was introduced into the nucleus of *C. reinhardtii* by *A. tumefaciens*, as well as, the evaluation of its bactericidal activity against some Gram positive and Gram negative bacteria.

MATERIALS AND METHODS

Culture conditions of algae and bacteria strains. For the *C. reinhardtii* transformation, the cc137 mt (+) strain obtained from the *Chlamydomonas* Resource Center (www.chlamycollection.org) was used. The growth of the microalga was in a Tris-Acetate-Phosphate (TAP) medium in 2 L flasks at 25 °C with constant stirring (200 rpm), a photoperiod of 16:8 h (light: dark) and a light intensity of 5,000 lux. For the transformant strains, the same growing conditions were used and hygromycin was added as a selection agent (5 mg/L).

The *Agrobacterium tumefaciens* GV3101 strain was used as the transformation vector organism. The bacteria used for the inhibition tests were *Staphylococcus aureus*, *Staphylococcus agalactiae* and *Enterococcus faecium* (donated by the Facultad de Ciencias Químicas de la Universidad Autónoma de San Luis Potosí, San Luis Potosí, México), *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (provided by the Departamento de Química de la Universidad Autónoma de Aguascalientes, Aguascalientes, México). All bacteria were grown in Luria-Bertani (LB) medium at 37 °C under constant stirring (200 rpm) for 24 h before carrying out any protocol.

Design of the synthetic gene coding for the bacteriocin E-760 and plasmid construction. A coding chimera for Enterocin E-760 (Line et al., 2008) was designed; the synthesis and codon optimization for its expression in the nucleus of *C. reinhardtii* was carried out by GenScript Corp (Piscataway, NJ, USA). The chimeric gene was named as E-760S and contains 243 bp. Towards the 5' end, it has the *Xba*I restriction site followed by a Histidine tag (6 His), a thrombin cutting site and the mature sequence of the bacteriocin E-760; whereas towards the 3' end, it has the stop codon (TAA), the ribosome binding site (RBS) and the *Nde*I restriction site (Fig. 1).

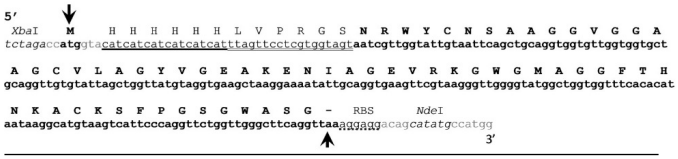

Fig. 1. Nucleotide and putative aminoacid sequence of E-760S. The sequence E-760S was subjected to codon optimization for its expression in the ***C. reinhardtii*** nucleus. The chimeric gene is flanked by the ***Xba*I** and ***Nde*I** restriction sites at the 5'and 3' ends, respectively. The start codon (M), the mature sequence of E-760 and the stop codon (-) are highlighted in bold letters; the Histidine tag (6 His) is underlined; the ribosome binding site (RBS) is shown as a discontinuous underline.

The synthesized E-760S gene was ligated into the pUC vector and was released with the *Xba*I and *Nde*I enzymes. On the other hand, the vector pChlamy_1 (Invitrogen, Waltham, MA, USA) was linearized with the same enzymes. Both, the E-760S fragment and the pChlamy_1 vector were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Fitchburg, WI). The fragments were ligated with

the T4 DNA ligase enzyme (Promega, Fitchburg, WI). With this construction (pChlamy1/E-760S) (Fig. 2), competent cells of *Escherichia coli* TOP10 were thermally transformed and selected in LB medium supplemented with 100 mg/L ampicillin. Plasmidic DNA was extracted by the Birboin-Dolly method (Sambrook et al., 1989) and a clone with the desired construction was identified by restriction analysis and subsequent sequencing.

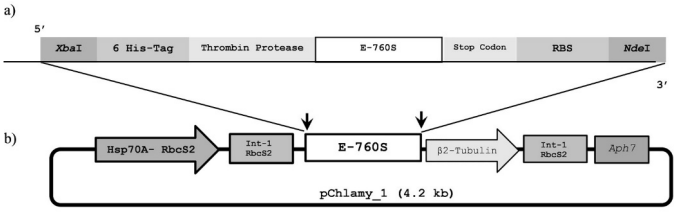

Fig. 2. Construction of pChlamy1/E-760S. a) E-760S scheme. b) The expression vector pChlamy_1 has the following elements: Hsp70A-Rbc S2 is a constitutive chimeric/hybrid promoter formed by the heat shock protein 70A and the RuBisCO small subunit; Int-1 Rbc S2 is the first intron of the RuBisCO small subunit (rbcs2) necessary to maintain the high expression of the interest gene; β2-Tubulin, strong native promoter of ***C. reinhardtii*** that drives the expression of the ***Aph***7 gene. ***Aph***7 is the aminoglycoside phosphotransferase gene from ***Streptomyces hygroscopicus***, which confers hygromycin resistance.

***In silico* analysis**. The E-760S sequence was bioinformatically analyzed in order to characterize the putative protein. The virtual translation was done on the ExPASY platform (http://web.expasy.org/translate/); the determination of the physico-chemical parameters such as, the isoelectric point (pI) and the Grand Average of Hydropathy (GRAVY) was performed in the ProtParam platform [http://web.expasy.org/protparam/] (Gasteiger et al., 2005). The aminoacid hydropathy charts were made with the ProtScale tool [http://web.expasy.org/protscale/] (Gasteiger et al., 2005), based on the Kyte and Doolittle scale. For the search of homologous aminoacid sequences, the BLASTX program of the Uniprot database was used (Uniprot, 2017). The conserved motifs were analyzed with the MEME platform [Motif-based sequence analysis tools; http://meme.nbcr.net/meme/] (Bailey et al., 2009). The multiple alignment of the homologous sequences was performed in the Clustal Omega program [http://www.ebi.ac.uk/Tools/msa/clustalo/] (Sievers et al., 2011). For the three-dimensional modeling of the E-760S structure, the PEP-FOLD 3.5 server was used [http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3] (Lamiable et al., 2016); while for the modeling edition, the USCF Chimera version 1.10.1 was used (Pettersen et al., 2004).

Nuclear transformation of *C. reinhardtii*. With the construction of pChlamy1 / E-760S, *A. tumefaciens* GV3101 was transformed by electroporation (BioRad, Hercules, CA, United States) under the following conditions: 2400 V, 200 Ω, 25μF. Transformed cells were inoculated on LB solid medium supplemented with ampicillin (100 μg/mL) and incubated at 37 °C for 19 hours. For the genetic transformation of *C. reinhardtii* cc137 mt (+) the protocol described by Kumar et al. (2004) was followed. First, the *A. tumefaciens* transformant was grown in LB medium with ampicillin (100 mg/L) until reaching an OD osf 0.6 and acetosyringone was added to a final concentration of 100 μM. A pre-inoculum of the *C. reinhardtii* strain was grown in liquid TAP medium for 5-7 days, the biomass was concentrated by centrifugation at 2400 rpm and solid TAP medium was inoculated; finally, the *A. tumefaciens* culture with acetosyringone was added. The co-culture was incubated for 2 days at 25 °C in total darkness. On the third day, several washes with liquid TAP medium and cefotaxime (500 mg/L) were performed in order to eliminate *A. tumefaciens*. Subsequently, the biomass was obtained and plated on solid TAP medium with hygromycin at 5 mg/L.

Selection of recombinant strains by PCR. The putative transformants of *C. reinhardtii* were separated after several selection rounds with hygromycin (3 rounds at a concentration of 5 mg/L, a selection at 10 mg/L and a last round of hygromycin at 20 mg/L). In order to confirm the presence of the transgene by PCR analysis, genomic DNA extractions were performed according to the protocol previously described by Newman et al. (1990). We used specific oligonucleotides for the coding region E760-F: 5′-ggtgcagcaggttgtgtttt-3′ and E760-R: 5′-cccaaccactaccagggaat-3′. The amplification conditions were: an initial denaturation for 5 min at 94 °C, followed by 35 cycles of 94 °C for 30 seconds, 62 °C for 30 seconds and 72 °C for 30 seconds; finally, a final extension stage at 72 °C for 3 minutes. The amplified products were evaluated in 2% agarose gels, stained with EtBr (0.5 μg/mL) and visualized with UV light at 260 nm.

Induction assay of the recombinant protein E-760S production. The transgenic strain of *C. reinhardtii* was inoculated in two flasks of liquid TAP medium at 25 °C under constant stirring (200 rpm) until logarithmic growth; then, an induction was performed at 35 °C for 10 minutes and another at 40 °C for 30 minutes. After induction, the culture was recovered at room temperature for 2 h and the extraction of Total Soluble Proteins (TSP) was performed.

Extraction and quantification of the Total Soluble Protein (TSP). Liquid cultures of the transgenic strain of *C. reinhardtii* and the wild type strain (WT) were grown until a late logarithmic phase; the biomass was concentrated by centrifugation at 10,000 rpm per 5 minutes and re suspended in extraction buffer (50 mM Tris pH8, 40mM NaCl and 0.1% Tween 20). Subsequently, cell lysis was performed by sonication (Sonics Vibra-Cell™ Processors VCX 130 PB) at an 80% amplitude during 20 cycles with 10s of lysis pulses and 5s of rest; the whole process was performed in cold. The lysates were clarified by centrifugation at 13,000 rpm per 10 min. Crude extracts were used to quantify the TSP content of each culture by the Bradford assay (Bradford, 1976) and subsequently evaluated by 12% SDS-PAGE (w/v).

Purification of the recombinant protein E-760S. Purification of the recombinant protein was performed by Immobilized Metal Affinity Chromatography (IMAC), using Ni-NTA agarose (Invitrogen, Waltham, MA, USA) following the manufacturer's recommendations. The transgenic strain

of *C. reinhardtii* was grown to a late logarithmic phase in a 1 L flask; the cell biomass was concentrated as mentioned above and re suspended in loading buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0 adjusted with NaOH). Lysis was performed by sonication and cell debris was removed by centrifugation at 15,000 g for 20 minutes at 4 °C. The final crude extract was added to a Ni^{2+}-NTA resin suspension pre-equilibrated with loading buffer; affinity was carried out for 12 hours at 4 °C under gentle stirring. Then, the resin was packed in a column and 10 washings were performed with loading buffer in order to remove unwanted proteins; the level of eluted protein was monitored by spectrophotometry at 280 nm. The elution of the recombinant protein was carried out by washing 10 times the resin with elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, 250 mM Imidazole, pH 8.0), monitoring the eluted protein by spectrophotometry.

Quantification of the chimeric protein E-760S by ELISA assay. For quantification by ELISA assay, a microtiter plate was coated with TSP (90 µg/well) of both the transgenic and the wild type strains and incubated 12 hours at 4 °C, together with a standard curve made of known amounts of the E-760 protein purified from *C. reinhardtii*. The wells were washed with Phosphate Buffered Saline + Tween (PBST, 8mM Na_2HPO_4, 150 mM NaCl, 2 mM KH_2PO_4, 3 mM KCl, 0.05% Tween® 20, pH 7.4) and blocked for 1 hours at room temperature with 5% milk dissolved in PBS. The plate was washed with PBST and incubated for 1 hours at 37 °C with a primary antibody directed to the His tag. After washing with PBST, the plates were incubated 1 hour at room temperature with a secondary antibody conjugated with horseradish peroxidase (HRP). The activity reaction of the peroxidase was developed with ABTS Substrate (Thermo Scientific Pierce, Rockford, IL, USA). The content of E-760S was quantified by measuring the optical density at a wavelength of 450 nm in a microplate reader (GloMax®-Multi+ Detection System. Promega). The amount of the E-760 protein was expressed as % of TSP. The statistical significance of the differences (P<0.05) between the non-induced culture (NIC), induced culture (IC) and wild type of *C. reinhardtii* was determined by a one-way analysis of variance (ANOVA) using the Tukey test. Each reading was done in triplicate.

Evaluation of the antibacterial activity of E-760S. The antibacterial activity of E-760S was analyzed against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus aureus*, *Staphylococcus agalactiae* and *Enterococcus faecium*, all belonging to clinical isolates. From the axenic cultures of each strain, a colony was taken and inoculated in 5 mL of liquid LB medium for 24 hours at 37 °C under constant stirring (200 rpm). A volume of 1.5 mL of the culture was centrifuged at 12,000 rpm per 5 minutes, the supernatant was removed and the pellet was resuspended in 1 mL of sterile potassium phosphate buffer (0.05 M pH 7). The microbial suspension was adjusted to obtain a titre of 10^6 CFU/mL (Colony Forming Units per milliliter). Once the bacterial load was obtained, the necessary amount of TSP was added until reaching 560 µg/mL and 1,120 µg/mL concentrations and it was brought to a final volume of 1 mL by adding phosphate buffer. As controls, bacterial suspensions eluted in TSP of unprocessed *C. reinhardtii* were used in phosphate buffer and ampicillin (0.3 mg/mL) under the same growing conditions. The inhibition of microorganisms was evaluated at 0, 30, 60, 120, 240 minutes and 480 minutes. After each exposure period, aliquots were taken from the suspension and inoculated in solid LB medium for 19 h at 37 °C and the CFU/mL number was counted. The log inactivation was calculated as $\log_{10}$ (N/N_0), where N_0 represents the initial CFU/mL and N the CFU/mL treatment survivors (Garcidueñas-Piña et al., 2016). The experiments were performed for triplicate and maintained under sterile conditions.

RESULTS

***In silico* analysis of the synthetic gene E-760S**

The virtual translation of the E-760S chimera shows that each element that conform the synthetic gene is in the correct Open Reading Frame, since the protein synthesis begins with the aminoacid Methionine (M) followed by the Histidine tag (6 His), a cutting site for Thrombin and the sequence of the bacteriocin E-760 (Fig. 1). The analysis of physical and chemical parameters showed that the peptide is composed by 40.8% of hydrophilic residues (GSTCYNQ), 36.7% of hydrophobic residues (AVLIPMFW), 18.4% of basic residues (KRH) and 3.9% of acid residues (DE). Additionally, it has a hypothetical weight of 7.8 kDa, an isoelectric point (pI) of 9.3, a net charge of 4.5 at pH 7 and a -0.308 GRAVY, which indicates that the protein is slightly hydrophilic (Fig. 3).

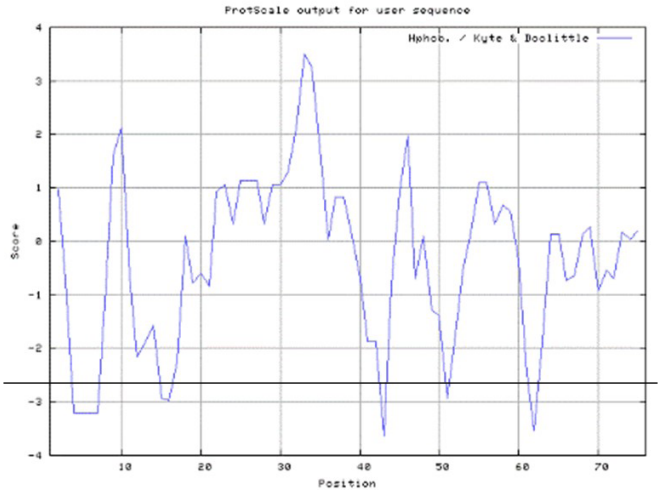

Fig. 3. Aminoacid hydropathicity of E-760S. Plotted data were obtained based on the scale of Kyte and Doolittle, where: the lower the value, the more hydrophilic the aminoacid is.

In the search for homologous genes of the E-760S chimera, high similarity was found with the sequence of the bacteriocin Amylovorin-L471 (P80696) produced by

Lactobacillus amylovorus and with an uncharacterized protein (UP) (A0A0R1GLB9) produced by *Lactobacillus crispatus* with antibacterial activity.

The alignment of E-760, Amylovorin-L471 and UP showed the presence of two conserved motifs, the NRW motif which in the three sequences is located towards the *N*-terminal end and the GGVGGAAVCGLAGYV motif, which is towards the *N*-terminal end of E-760; for Amylovorin-L471 and the UP, it was identified towards the *C*-terminal end. E-760 lacks the intermediate aminoacids between both motifs unlike the other analyzed sequences (Fig. 4). The putative three-dimensional model of E-760 is illustrated in Fig. 5.

Fig. 4. Alignment of E-760, Am and UP. The conserved motifs are framed in black boxes. The GxxxG and SxxxS motifs present in E-760 are shaded in black. Dotted line boxes show the missing sequence in E-760. E-760= Enterocin E-760 (***Enterococcus sp.***); amyL= Amylovorin-L471 (***Lactobacillus amylovorus***); UP= Uncharacterized protein (*Lactobacillus crispatus*).

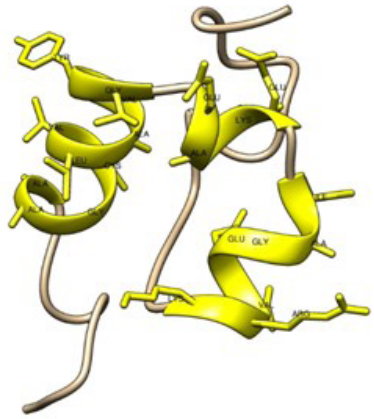

Fig. 5. Hypothetical structure of the bacteriocin E-760. According to the three-dimensional model elaborated by the PEP-FOLD 3.5 server, it contains 3 α-helix.

Selection of *C. reinhardtii* nucleus transformants and PCR analysis

Transformation by *A. tumefaciens* was successful in obtaining 20 presumptive transformants; however, due to contamination problems, only one clone was used for further analysis. DNA was extracted from the presumed transformant clone in order to detect the transgene presence by PCR. The size fragment of both, transformant and positive control, was 139 bp, while for the negative control (*C. reinhardtii* untransformed) there was no amplification (Fig. 6). The amplification of this fragment indicated the presence of the transgene in the clone and was confirmed by its growth after five rounds in the selection medium.

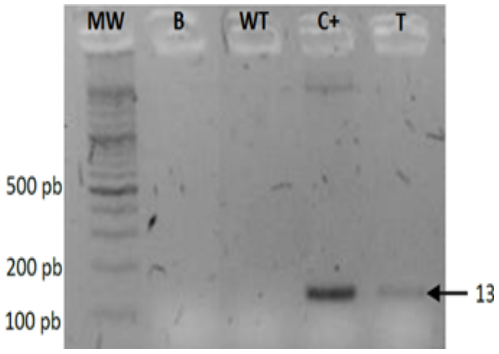

Fig. 6. PCR analysis of ***C. reinhardtii*** transformants using specific primers for the E-760S gene. MW: 100 bp molecular weight marker; B: Negative control of the reaction (Without DNA); WT: wild type strain of ***C. reinhardtii***; C+: Positive control, pUC18 / E-760S; T: Transformant of ***C. reinhardtii*** with the pChlamy1 / E-760S construction.

Quantification of the expressed protein by ELISA

The expression level of the E-760S gene in the *C. reinhardtii* transformant was quantified by an ELISA assay and compared with the untransformed strain using as standard the purified E-760 protein from *C. reinhardtii*. The amount of protein E-760 was expressed as TSP percentage, which was 0.36% for the *C. reinhardtii* induced culture (IC) at 35 °C; while for the non-induced culture (NIC), the percentage was 0.14% (Fig. 7). It should be mentioned that the E-760 value for *C. reinhardtii* induced at 40 °C was practically null (results not shown), which could be due to cell death; so, this treatment was not evaluated in the antibacterial activity tests.

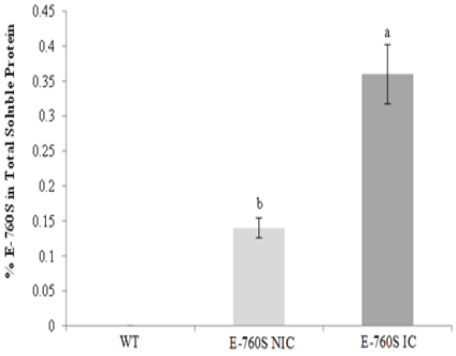

Fig. 7. Accumulation levels of the recombinant protein E-760 in the evaluated transgenic line. Quantification of the recombinant protein by ELISA assay was determined at a known concentration of transformed TSP of ***C. reinhardtii*** (90 µg/ well) and standardized against a linear curve of a known quantity of the purified E-760 protein standard of ***C. reinhardtii***. The amount of protein was expressed as TSP percentage. The TSP absorbance of the wild type strain (WT) used as control was almost equal to the one of the bottom of the plate wells. The assay was performed with an antibody directed to the Histidine tag. Values are the averages of two replicates with standard deviations. WT, Untransformed ***C. reinhardtii***; E-760S NIC, Transformed

culture of *C. reinhardtii* with non-induced E-760; E-760S IC, Transformed culture of *C. reinhardtii* with E-760S Induced at 35 °C/10 minutes. Different letters indicate statistically significant differences between treatments ($P < 0.05$).

Evaluation of the antibacterial activity of TSP extracts of transgenic and wild type *C. reinhardtii*

The TSP extracts from the E-760 transformed strain induced at 35 °C showed greater inactivation than the TSP extracts of not induced E-760 against *Staphylococcus aureus*, *Streptococcus agalactiae* and *Enterococcus faecium*; whereas for *Klebsiella pneumoniae*, the inactivation results between both extracts were very similar. For *Pseudomonas aeruginosa*, the non-induced E-760 extract showed the greatest inhibitory effect.

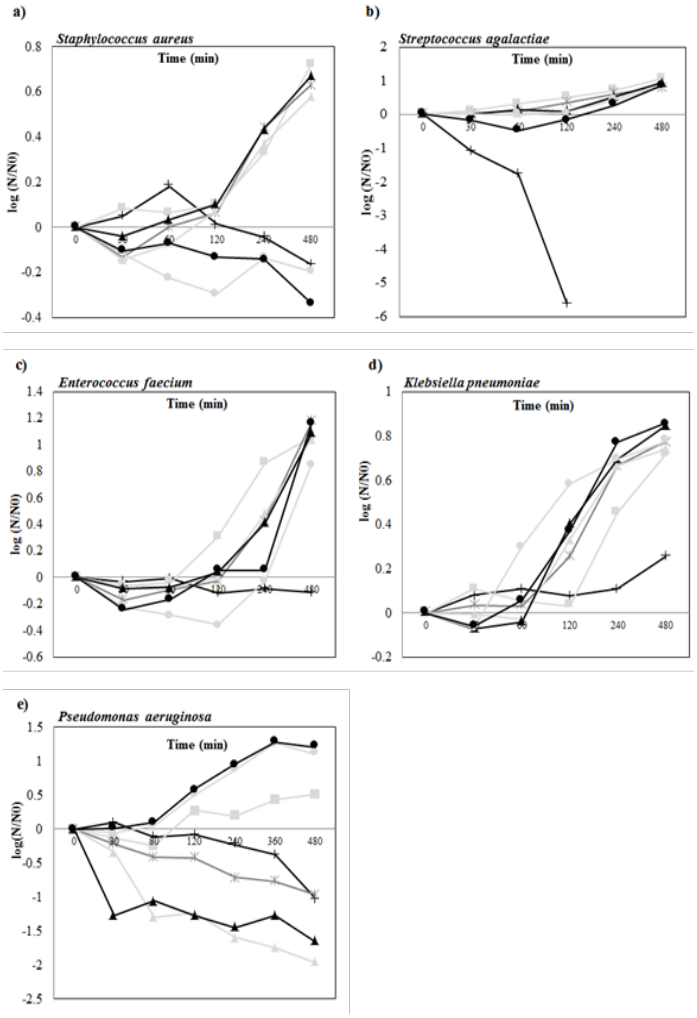

Fig. 8. Bacterial inactivation by TSP extracts of WT, E-760S and E-760S Induced.

Specifically for *S. aureus*, the TSP extract of the induced E-760S in both concentrations registered a gradual increase in the inhibition of bacterial growth in each evaluated exposure time, in addition to having a higher bactericidal effect than the one obtained for ampicillin. The maximum inactivation value for the 560 µg concentration was 0.29 U log at 120 minutes of exposure, while for the 1,120 µg concentration was 0.34 U log at the maximum evaluated time. The greatest bactericidal effect of the ampicillin treatment was achieved at 480 minutes with an inactivation of 0.16 U log. For both TSP extracts of the non-induced E-760S, minimal inhibition values were obtained, almost comparable with the extracts of the wild type strain (Fig. 8a).

For *S. agalactiae*, the TSP extract of induced E-760S at 1,120 µg was the one that showed the best inhibitory effect achieving a reduction of 0.5 U log at 60 minutes of exposure; however, bacterial growth increased after 120 minutes. It is important to mention that ampicillin exerted a strong inhibitory effect at 0.3 mg/mL, achieving total inactivation of the cells at 120 minutes of exposure. For the rest of the treatments TSP of the recombinant, wild strain as well as the phosphate buffer, very similar growth kinetics was recorded during all exposure times of the bacterial culture (Fig. 8b).

For *E. faecium*, the treatment with the best inhibitory effect was the TSP extract of E-760S induced at 560 µg with 0.36 U log at 120 minutes of exposure; however, the growth kinetics increased after 240 minutes. On the other hand, ampicillin showed a lower inhibitory effect (0.1 U log) compared to the aforementioned extract, however, the bacterial growth was maintained throughout, simulating a bacteriostatic effect. After 30 minutes of exposure, all the analyzed treatments showed a slight inactivation, however, after 60 minutes the growth kinetics of bacteria increased considerably (Fig. 8c).

Specifically for *K. pneumoniae*, the TSP extracts E-760S induced and E-760S non-induced in both concentrations exerted a slight inactivation at 30 minutes exposure (0.07 U log), however after the incubation time the growth kinetics increased considerably, even surpassing the CFU number of the positive control (Phosphate Buffer) in the longest exposure time (480 minutes). On the other hand, ampicillin did not show any inhibitory effect throughout the experiment, since after 30 minutes the bacterial cells began to increase; presumably this strain had a certain level of resistance for this antibiotic (Fig. 8d).

For *P. aeruginosa*, the TSP extracts of non-induced E-760 in both concentrations were more efficient. The maximum inactivation value was recorded at the longest exposure time, being 560 µg of TSP, the concentration with the highest inhibition (1.9 U log); while for the of 1,120 µg concentration, it was 1.6 U log. The TSP of WT also showed inactivation (0.9 U log) against *P. aeruginosa*, registered at the maximum exposure time. It should be noted that the aforementioned TSP extracts were more efficient in bacterial inhibition than ampicillin (0.3 mg/mL), since their maximum inactivation value was 1 U log at 480 minutes (Fig. 8e).

Ampicillin 0.3 mg/mL; PB, potassium phosphate buffer 0.05 M pH 7; WT, *C. reinhardtii* wild type strain 560 mg/mL TSP; E-760S NIC *C. reinhardtii* transgenic strain non-induced 560 mg/mL TSP; E-760S NIC *C. reinhardtii* transgenic strain non-induced 1,120 mg/mL TSP; E-760S

CI *C. reinhardtii* transgenic strain Induced at 35 °C/10 min, 560 mg/mL TSP; —●— E-760S CI *C. reinhardtii* transgenic strain Induced at 35 °C/10 minutes, 1,120 mg/mL TSP.

DISCUSSION

In this study, the expression ability of the antibacterial peptide E-760 by recombinant route in the nucleus of the microalga *Chlamydomonas reinhardtii* was analyzed, in order to be used as a stable platform for the mass production of peptides of pharmacological interest. *C. reinhardtii* has been reported as a robust platform for the production of recombinant proteins specifically in chloroplasts (Rasala et al., 2010; Campos-Quevedo et al., 2013). However, there are few reports regarding the production of recombinant proteins in the microalga nucleus through the transformation performed by *Agrobacterium tumefaciens*.

Antibacterial peptides are a promising alternative for the inhibition of antibiotic resistant pathogens. In order to achieve a better characterization of the interesting peptide (e.g., to determine action mechanisms, structure and function, or its potential use as a medicine) it is necessary to obtain a reasonable amount of this peptide; however, there are some issues that prevent it from being obtained in a massive way, such as the low yield of the interest peptide when it is purified directly from the natural host, the production of potential virulence factors of some bacterial producers and the high production costs by chemical synthesis (Parachin et al., 2012; Arbulu et al., 2015). Due to the aforementioned, the production of antimicrobial peptides by recombinant pathways using heterologous expression systems such as bacteria (Mesa-Pereira et al., 2017), yeast (Arbulu et al., 2015), microalgae (Mu et al., 2012) and plants (Zakharchenko et al., 2013) has become a rapidly expanding research area.

AMPs are generally cationic, with charges ranging from +2 to +9. This positive charge is important for the initial attraction and interaction with the anionic cell membranes of bacteria and other pathogenic microorganisms; so, relatively less anionic membranes will not attract electrostatically the AMPs (Ebenhan et al., 2014). According to its physico-chemical characteristics, the bacteriocin E-760 can be defined as a cationic peptide according to the *in silico* results, since it contains 40.8% of hydrophilic aminoacids and 36.7% of hydrophobic residues, coinciding with Line et al. (2008), but it differs in the isoelectric point (i.e., they report it as 8.7, whereas we reported as 9.3). In most AMPs, the hydrophobicity is around 50% and is essential for the functioning of the peptide, since it allows the peptide to interact and penetrate the phospholipid bilayer (Ebenhan et al., 2014); in our case, the peptide E-760 was slightly hydrophobic according to the scale of Kyte and Doolittle (Fig. 3). The influence of the hydrophobicity degree in an AMP on the antibacterial specificity can be modified by using analogs of variable hydrophobicity. For example, by increasing the hydrophobicity in an analogue of the Magainin 2 Amide (M2a) peptide, the activity against *E. coli* was increased; on the contrary, the selectivity against *P. aeruginosa* decreased (Wieprecht et al., 1997), which demonstrates the low correlation between the hydrophobicity of the peptides and their ability to permeabilize biomembranes.

Antibacterial peptides synthesized by lactic acid bacteria (LAB) are divided into two classes. Class I consists of bacteriocins named lantibiotics, which contain one or more residues of modified aminoacids, while Class II consists of small (<10kDa) and thermostable bacteriocins that lack modified residues (Nissen-Meyer et al., 2010; Cotter et al., 2013). Class II bacteriocins are divided into four subclasses. Class IIa contains pediocin-type bacteriocins that have similar aminoacid sequences; Class IIb contains bacteriocins of two peptides; Class IIc consists of cyclic bacteriocins, whose *N*- and *C*- ends are covalently bound; and Class IId, contains non-cyclic bacteriocins that do not show any similarity in the sequence of pediocin-type bacteriocins (Nissen-Meyer et al., 2010; Cotter et al., 2013). Peptide E-760 is classified within Class II bacteriocins, since it is a thermostable, small peptide (5.3 kDa) which does not contain modified residues (Line et al., 2008). However, there is some discrepancy regarding its classification, because it does not fully comply with the distinctive characteristics of Class IIa or Class IIb bacteriocins. Some authors classify E-760 within Class IIa (Belguesmia et al., 2011), which is characterized by the presence of an YGNGVxC motif (where V can be replaced by L in some cases) in the *N*-terminal region of the peptide (Cotter et al., 2013); but E-760 lacks this motif (Fig. 4). On the other hand, Uniprot (UniProt, 2017) and BACTIBASE (available in http://bactibase.hammamilab.org/BAC174) (Hammami et al., 2007) platforms, indicates that E-760 belongs to the Class IIb, including the Amylovorin peptide. An interesting structural feature of all Class IIb bacteriocins is the presence of GxxxG motifs (where x represents any residue) and GxxxG type motifs [conformed by A (AxxxA) or S (SxxxS)] that facilitate helix-helix interactions and promote the oligomerization of transmembrane helical peptides or membrane protein domains (Nissen-Meyer et al., 2010; Kyriakou et al., 2016). Based on the aforementioned, the E-760 sequence presents two GxxxG motifs (GGVGG and GMAGG) and one SxxxS motif (SFPGS) (Fig. 4). Although E-760 shares this Class IIb uniqueness, it lacks the second characteristic peptide of this category; since in order to achieve an optimal antibacterial activity, the presence of both peptides is required in approximately equal amounts (Nissen-Meyer et al., 2010).

In the absence of structural models of the peptide E-760, 13 hypothetical models were obtained, which had α-helix structures. Figure 5 shows the model obtained from PEP-FOLD 3.5 (Lamiable et al., 2016). It is important to mention that there are few studies related to bacteriocin E-760 (Line et al., 2008; Arbulu et al., 2015); so this peptide has not been fully characterized yet at the biochemical and molecular levels.

Due to the fact that transgenes with a strong deviation of codons or with low GC content are poorly expressed in *C. reinhardtii* (Barahimipour et al., 2016) because the nuclear genome of this microalga presents a high content of GC (64%) (Merchant et al., 2007), a codon optimization was carried out for an efficient expression of the E-760S transgene in the nucleus of *C. reinhardtii*. PCR analysis with specific primers

demonstrated the integration of the E-760S gene into the nuclear genome of the microalga (Fig. 6). Derived from the event of nuclear transformation mediated by *A. tumefaciens*, 20 possible transformed clones were obtained; however, only one clone was analyzed due to contamination problems. There are few studies of stable genetic transformation of the nuclear genome of *C. reinhardtii* mediated by *A. tumefaciens*; however it is reported that the transformation frequency of this method is 50 times higher than the transformation with glass beads (Kumar et al., 2004). Different methods for the nuclear transformation of microalgae have been developed, including biobalistics (Koop et al., 2007), electroporation (Kang et al., 2015), stirring with glass beads (Neupert et al., 2009) or with silicon carbide fibers (Dunahay, 1993); so, the research related to the effective use of nuclear transformation mediated by *A. tumefaciens* in microalgae is still minimal (Kumar et al., 2004; Pratheesh et al., 2014; Salas-Montantes et al., 2018).

The transgenic line generated with the construction of pChlamy1 / E-760S showed that the nucleus of *C. reinhardtii* is a stable system for the expression of heterologous proteins. According to the ELISA quantification and the use of the anti-His antibody, it was determined that the evaluated transgenic line produced a recombinant protein between 0.14 (NIC) and 0.36% (IC) in TSP extracts (Fig. 7). Transgene expression yields have been reported in the nucleus of microalgae and plants, which are very similar or lower than the ones obtained in this investigation. For example, the reporter gene encoding for the green fluorescent protein GFP expressed in mutant strains of *C. reinhardtii* had a 0.2% yield, which is considered a relatively high value for nuclear expression in algae (Neupert et al., 2009); the P24 antigen of HIV obtained by recombinant route in *C. reinhardtii*, had an accumulation up to 0.25% of total cellular protein (Barahimipour et al., 2016). This comparison can be made also with plants such as tobacco, where the HIV P24 antigen reached an accumulation of 3.5 mg/g of soluble protein in leaf (Zhang et al., 2002); potato tubers, where human proinsulin was expressed with a 0.1% TSP yield (Arakawa et al., 1998) and in *Arabidopsis* (0.1% of total protein in seed) (Nykiforuk et al., 2006). These data and the fact that our transgenic clone accumulated the chimeric protein at levels above 0.1% of TSP, confirm that the microalga nucleus is capable of producing heterologous proteins under the chimeric promoter HSP70A / RBCS2.

The induction process by thermal shock at 35 °C per 10 minutes carried out in this study was effective in the expression of E-760S in 2.5 more times than the non-induced culture (Fig. 7). This increased expression phenomenon of recombinant proteins by induction with thermal shock in *C. reinhardtii* under the chimeric promoter HSP70A / RBCS2, has already been reported in the expression of the antibacterial peptide Cecropin B, where the induction condition was a thermal shock at 40 °C for 30 minutes; although, the expression increase of the recombinant protein is not mentioned (Mu et al., 2012). In this investigation, the induction was analyzed at 35 °C and 40 °C at 10 minutes and 30 mintes, respectively; however, the cell culture changed from an intense green color to a yellowish green one at a longer induction time. Although, the sample was processed and when it was quantified by ELISA, the values of the recombinant protein were practically null (results not shown), which could be due to cell death. *C. reinhardtii* is a mesophilic microalga, whose optimum growth temperature is between 20-32 °C; therefore, an abrupt transfer at a temperature above the ideal growth range triggers adaptive responses that may or may not allow the resumption of growth or survival to the new temperature. A prolonged exposure to incompatible temperatures results in a cessation of metabolism, chlorosis and finally cell death (Xie et al., 2013).

Regarding the functional analysis of the recombinant bacteriocin E-760 against the analyzed bacteria, it showed a moderate inhibitory effect but still greater than that produced by the TSP of the wild strain. E-760S resulted with inhibitory activity against *S. aureus*, *E. faecium*, *K. pneumoniae* and *P. aeruginosa* bacteria, achieving inhibition values of 0.33U log, 0.36U log, 0.07 U log and 2 U log, respectively; these values are higher than those shown by the TSP of the wild strain (0.13, 0.17, no inhibition and 0.97 U log, respectively) and by ampicillin (0.16, 0.12, no inhibition and 1 U log, respectively). Only for *S. agalactiae*, the control treatment of ampicillin (0.3 mg/mL) was efficient, since it exerted a strong bactericidal activity to achieve a complete inhibition at 120 minutes of exposure, so it could be hypothesized that most of the strains analyzed have a certain level of resistance to the antibiotic. Regarding the inhibitory activity shown by the TSP extracts of the WT strain, it has been reported that exist some secondary metabolites such as hydrocarbons, phenols, alcohols and esters produced by *C. reinhardtii* cultures which are attributed antimicrobial activity against bacteria such as *Bacillus subtilis*, *P. aeruginosa* and *K. pneumoniae*, in addition to the yeast *Candida albicans* (Renukadevi et al., 2011).

Other studies have reported, the production and purification of E-760 by chemical methods and has been characterized as an effective bacteriocin with a broad activity spectrum against Gram-positive (*S. aureus, S. epidermidis* and *L. monocytogenes*) and Gram-negative (sero-varieties of *Salmonella enterica, E. coli, Yersinia enterocolitica, Citrobacter freundii, Klebsiella pneumoniae, Shigella dysenteriae* and *Campylobacter jejuni*) bacteria at concentrations ranging from 0.1 to 3.2 μg/mL (Line et al., 2008); concentrations lower than the ones used in our research (0.8-3.8 μg/mL). However, it has been reported in chemically synthesized AMPs of animal origin, the use of concentrations higher than those analyzed in this research ranging from 4 to > 128 mg/L against multi-drug resistant bacteria, such as *Acinetobacter baumannii, E. coli, P. aeruginosa* and Methicilin Resistent *S. aureus* (MRSA) (Liu et al., 2015). The interest for producing the synthetic bacteriocin E-760 by recombinant routes is latent, since it was tried to be produced previously in the *Pichia pastoris* yeast; however, the expression of the heterologous protein failed because the sequencing analysis determined that none of

the obtained peptide fragments coincided with the expected aminoacid sequence, suggesting the existence of truncated bacteriocins, interaction of bacteriocins with unknown biological compounds or post-translational modification processes (Arbulu et al., 2015).

In this study we demonstrate the stable expression of a heterologous gene coding for the antibacterial peptide E-760 in the nucleus of the transformed microalgae *C. reinhardtii* via *A. tumefaciens*, as well as its antibacterial activity, which highlights the feasibility of using the nuclear genome of microalgae as a potential producer of pharmacologically important proteins.

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