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Abstract

Chloroplast transformation technology has emerged as an alternative platform offering many advantages over nuclear transformation. SAG1 is the main surface antigen of the intracellular parasite *Toxoplasma gondii* and a promising candidate to produce an anti-*T. gondii* vaccine. The aim of this study is to investigate the expression of SAG1 using chloroplast transformation technology in tobacco plants. In order to improve its expression in transplastomic plants, we also expressed the 90-kDa heat shock protein of *Leishmania infantum* (LiHsp83) as a carrier for SAG1 antigen. SAG1 protein accumulation in transplastomic plants was approximately 0.1-0.2 µg per gram of fresh weight (FW). Fusion of SAG1 to LiHsp83 significantly increased the level of SAG1 accumulation in tobacco chloroplasts (by up to 500-fold). We also evaluated the functionality of the chLiHsp83-SAG1. Three human seropositive samples reacted with SAG1 expressed in transplastomic chLiHsp83-SAG1 plants. Oral immunization with chLiHsp83-SAG1 elicited a significant reduction of the cyst burden that correlated with an increase of SAG1-specific antibodies. We propose the fusion of foreign proteins to LiHsp83 as a novel strategy to increase the expression level of the recombinant proteins using chloroplast transformation technology, thus addressing one of the current challenges for this approach in antigen protein production.

Keywords: *Toxoplasma gondii*, *Leishmania infantum*, SAG1, Hsp83, chloroplast transformation

1 Introduction

Plants have demonstrated the ability to produce functionally active proteins from mammals or other eukaryotic organisms with therapeutic activity, such as human serum and growth regulatory proteins, antibodies, antigens, hormones and cytokines [1, 2]. Despite providing many beneficial properties for protein production such as scalability, low production costs and in the case of vaccine production absence of pathogenic contaminants, one of the major challenges for the use of plants as an alternative expression system is the relatively low expression yields of recombinant proteins [3].

The stability of plant-derived recombinant proteins is considered the predominant factor that limits protein yields achieved by molecular farming [4]. In the past few years, chloroplast transformation technology has emerged as an alternative platform that offers many advantages over nuclear transformation such as high levels of transgene expression due to high copy number, transgene containment via maternal inheritance of plastids and multi-gene expression in a single transformation event [5, 6]. However, previous studies have also shown that, for certain eukaryotic proteins, chloroplast transformation has only provided a limited expression yield [7-11]. In this sense, new strategies should be explored in order to improve the production yield of foreign proteins expressed in plants and increase the commercial potential of plant-based production systems.

SAG1 is the main surface antigen of the intracellular parasite *Toxoplasma gondii*, the causal agent of toxoplasmosis [12, 13]. In human, toxoplasmosis is usually asymptomatic in healthy individuals. However, in pregnant women, congenital infection can cause severe neonatal malformations [14]. Although a vaccine against human infection with *T. gondii* is not yet available, an effective vaccine against toxoplasmosis may be valuable to prevent both fetal infection and reactivation. In this context, numerous studies

have established the potential of SAG1 as a candidate to produce an anti-*T. gondii* vaccine [15-21]. Previous attempts to express SAG1 using various classical heterologous expression systems have been challenged by low expression levels or poor antigenicity of the recombinant SAG1 protein [22-27]. SAG1 is thus both a promising antigen for vaccine development, and a good candidate to optimize an antigen production strategy using an alternative protein expression system such as plant-based protein production [26, 27].

The heat shock proteins belonging to the 90-kDa family (HSP90) are highly conserved among bacteria, yeast, plants, and animals [28]. HSP90s function as molecular chaperones to prevent inappropriate associations or aggregations of partially folded proteins [29]. In addition, HSP90s promote cell survival in response to many forms of cellular stress [30]. In general, HSP90s are ubiquitously expressed at high levels, with HSP90s making between 1 and 2% of total soluble proteins [31]. We hypothesize the high level of constitutive HSP90 expression combined with its chaperone properties may be utilized to enhance the expression of recombinant proteins in plants by using HSP90 as a carrier protein and by expressing recombinant proteins of interest as HSP90 fusion proteins. Previously Echeverria *et al.* [32] demonstrated that the 90-kDa heat shock protein of *Leishmania infantum* (LiHsp83) is a good candidate to carry *Toxoplasma* antigens produced as recombinant proteins in bacteria. Therefore, within this study we use LiHsp83 as a carrier for *T. gondii* SAG1 antigen in order to improve its expression in transplastomic plants. Our findings show that fusion of SAG1 to LiHsp83 significantly increases the level of SAG1 accumulation in tobacco chloroplasts.

2 Material and Methods

2.1 Vector construction

The vectors pAF-SAG1 and pAF-LiHsp83:SAG1 were constructed as previously described [11, 34, 35] with slight modifications. The *SAG1* sequence was amplified by polymerase chain reaction (PCR) from the pRSET-SAG1₇₇₋₃₂₂ vector [33] using the primers 5'-cccgggCACTTCACTCTCAAGTGCCC-3' (upstream, lowercase indicates *Sma*I restriction site) and 5'-ctcgagAGCAAAGATGGAAACATGAG-3' (downstream, lowercase indicates *Xho*I restriction site). The *SAG1* fragment was cloned into the vector pAF-TDHIS to generate the plasmid pAF-SAG1 as was previously described by [11]. The *LiHsp83* sequence was amplified by PCR from the pQ-LiHsp83 vector [36] using the primers 5'-cccgggACGGAGACGTTTCGCGTTCCAG-3' (upstream, lowercase indicates *Sma*I restriction site) and 5'-gttaacGTCCACCTGCTCCATGCTGG-3' (downstream, lowercase indicates *Hpa*I restriction site). The *LiHsp83* fragment was digested with *Sma*I and *Hpa*I restriction enzymes and cloned into the pAF-SAG1 plasmid digested by *Sma*I, to give plasmid pAF-LiHsp83:SAG1.

2.2 Transformation and regeneration of transformed chloroplast plants

In vitro-grown tobacco (*Nicotiana tabacum* cv. Petite Havana) leaves were transformed by bombarding pAF-SAG1 or pAF-LiHsp83:SAG1 plasmids-coated gold particles using the PDS1000/He (Bio Rad, Hercules, CA) biolistic device, as previously described [11, 37]. Leaves were then cut into small pieces and placed on regeneration RMOP medium containing 500 mg/L spectinomycin [11, 37]. After 2 cycles of shoot regeneration, the regenerated plants were moved to a greenhouse for homoplasmy confirmation and seed production.

2.3 Southern blot analysis

Ten µg of plant DNA was digested with *Bgl*III, separated on a 0.8% (w/v) agarose gel and transferred on to a nylon membrane. The digestion by *Bgl*III and *Bam*HI of the chloroplast vector generated a 0.8-kb probe (P1) homologous to the flanking sequences (Figure 1A). Probe labelling, hybridization and detection were performed as previously described [11]. Homoplastic plants were grown in a greenhouse for further analysis.

2.4 Protein Extraction

Leaves from transplastomic plants and wild-type tobacco plants were ground in liquid nitrogen. For total protein extraction, 100 mg of grounded leaves were homogenized in 300 µl Laemmli buffer (0.5 M Tris–HCl pH 6.5, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.1% bromophenol blue). The total protein content was measured using the RC-DC protein assay (Bio Rad) with BSA as a standard according to the manufacturer's instructions.

To separate soluble and insoluble protein fractions, powdered leaves (100 mg) were homogenized in 500 µL of protein extraction buffer (20 mM NaPO₄, 250 mM NaCl, 0.1% Triton X-100) [11, 38] and sonicated for 15 s. After centrifugation at 13,000 rpm, Laemmli buffer was added to soluble fraction (supernatant) and insoluble fraction (pellet).

2.4 SDS-PAGE and Western blot analysis

Leaf protein extracts were heated at 100°C for 5 min and separated by 10% (chLiHsp83-SAG1) or 15% (chSAG1) SDS-polyacrylamide gels which were either stained with coomassie brilliant blue or transferred onto PVDF membrane (GE Healthcare) using

an Electro-transfer unit (Bio Rad). The membranes were incubated with rabbit anti-SAG1 polyclonal antibody (1:500) or rabbit anti-LiHsp83 polyclonal antibody (1:500) followed by anti-rabbit IgG alkaline phosphatase conjugate as secondary antibody (1:5000; Sigma). Phosphatase activity was detected using NBT/BCIP substrate (Promega, Fitchburg, WI). The accumulation of recombinant proteins (chSAG1 and chLiHsp83-SAG1) in tobacco leaves was estimated by comparing plant extract samples with a serial dilution of known concentration of *E. coli*-purified SAG1 (EcSAG1) [33], or *E. coli*-purified LiHsp83 (EcLiHsp83) [39] electrophoresed on the same polyacrylamide gel. The band intensity of purified EcSAG1 and EcLiHsp83 detected by Western blot was estimated with the “Gel-Pro analyzer” software (Media Cybernetics, Rockville, MD) and used as a standard to build a calibration curve as previously described [11, 27]. The leaf-expressed chSAG1 band intensity detected by Western blot was estimated using the “Gel Pro analyzer” software and compared with a calibration curve obtained by the band intensity of EcSAG1, while the leaf-expressed chLiHsp83-SAG1 band intensity was compared with two calibration curves obtained from the band intensity of EcSAG1 or from the band intensity of EcLiHsp83. Exclusively the intact band detected by Western blot was taken into account to estimate the chSAG1 and chLiHsp83-SAG accumulation. This analysis allowed us to have an estimation of the concentration of chSAG1 and chLiHsp83-SAG1 expressed in chloroplast transformed plants.

2.5 Reactivity of human serum samples

Human serum samples were obtained during routine serologic screening of pregnant women at Parasitology Department, ANLIS “Dr. Carlos G. Malbrán”, Argentina, and analyzed by IgG-Indirect immunofluorescence assay (IFA) and in-house IgM-ISAGA test.

Two of the three positive sera used in this study were grouped as IgG+, IgM- and the other was grouped as IgG+, IgM+. Three seronegative samples (IgG-, IgM-) were used as negative control. The use of these serum samples was approved by the Independent Ethics Committee for the Medical Federation of Buenos Aires Province, Argentina (FE.ME.BA., note 592, September 23rd, 2014).

Leaf extract samples were separated by 10% SDS-PAGE under reducing conditions and transferred onto PVDF membrane (GE Healthcare). Human serum samples were assayed at 1:100 dilution. Phosphatase alkaline conjugated anti-human IgG was used as secondary antibody (1:2500; Sigma). The reaction was developed by the addition of NBT/BCIP substrate (Promega). Pre-stained proteins (Fermentas, Waltham, MA) was included in Western blots as molecular weight marker.

2.6 Oral immunization of mice

Female C57BL/6 (H-2b) mice were obtained from the Biotechnology Research Institute (IIB), National University of General San Martin (UNSAM), Buenos Aires, Argentina. The mice were bred and housed according to institutional guidelines approved by the Independent Ethics Committee for the Care and Use of Experimental Animals of the National University of General San Martin (C.I.C.U.A.E., IIB-UNSAM, 09/2011).

Lyophilized plant leaf stocks were used for oral administration to 8- to 10-wk-old mice as previously performed [11]. The leaf extracts was homogenized in PBS 1X buffer. The chLiHsp83-SAG1 group received five doses of transplastomic leaf extracts (4-6 µg of chLiHsp83-SAG1), at one-week intervals, while the control group received five doses of

wild-type leaf extracts. A PBS group was also included. This group was orally immunized with five doses of PBS buffer.

2.7 Measurement of antibody responses

Antigen-specific antibodies were analyzed by enzyme linked immunosorbent assay (ELISA) as previously described [11, 26, 27]. Two weeks after the last immunization, blood was collected from the tail vein and sera were stored at -20°C until the antibody response analysis. Briefly, microtiter plates (Immuno Plate Maxiorp; Nunc, Roskilde, Denmark) were coated with rSAG1 (5 µg/ml, EcSAG1). Rat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate (1:3000) was used as a secondary antibody (Sigma). Immune complexes were revealed with TMB (Invitrogen, Waltham, MA) and optical density was read at 650 nm with an automatic ELISA reader (Synergy H1, Bio-Tek, Winooski, VT). Serum samples were used at a 1:100 dilution. Pre-immune serum samples were used as negative controls.

2.8 Challenge infection

Two weeks after the last immunization, mice (eight per experimental group) were orally infected with 20 Me49 tissue cysts (sublethal dose) as previously achieved [11, 27]. One month after the challenge, animals were sacrificed and their brains removed for tissue cyst quantification. The mean number of cysts per brain was determined under an optical microscope, by counting four samples of each homogenized brain.

2.9 Statistical analysis

Statistical analysis was carried out with the Prism 5.0 Software (GraphPad, La Jolla, CA) using one-way analysis of variance (ANOVA). A Bonferroni's test was used to compare means. $P < 0.05$ was considered significant.

3 Results

3.1 Cloning of SAG1 and LiHsp83-SAG1 fusion constructs

The SAG1 and LiHsp83-SAG1 sequences were fused to the promoter and 5'-untranslated region of the *psbA* gene (PpsbA) with a downstream box composed by the first 15 codons of the green fluorescence protein (GFP15) [34, 40] and to the tetramerization domain (TD) from the human transcription factor p53, inclusion of which has previously been demonstrated to favor the accumulation of antigenic peptides in tobacco chloroplasts [34]. Finally, a 6xHis-tag was included at the C-terminal region of the recombinant proteins (Figure 1A). The expression cassettes were inserted between the *trnI* and *trnA* genes into the chloroplast genome (Figure 1A).

3.2 Confirmation of transgene integration in transplastomic plants

The pAF-SAG1 and pAF-LiHsp83:SAG1 constructs were introduced into *Nicotiana tabacum* cv. Petite Havana chloroplasts by biolistic transformation [37]. Stable integration of transgenes into the chloroplast genome and homoplasmy of the transformed plants were confirmed by Southern blot analysis (Figure 1B). A 4.5-kb DNA fragment was detected in wild-type chloroplasts, whereas transformed chloroplasts released two DNA fragments either of 5.2 and 1.8-kb (for SAG1 plants) or 5.2 and 3.7-kb (for LiHsp83-SAG1 plants) (Figure 1A, B). The absence of 4.5-kb bands in the transformed lines indicates homoplasmy.

3.3 Morphology of transplastomic plants

After rooting on Murashige and Skoog medium, transgenic plants were transferred to the greenhouse for seed production and morphological studies. SAG1 transplastomic

lines exhibited normal growth and morphology. In contrast, LiHsp83-SAG1 transplastomic lines showed stunted growth during both *in vitro* and greenhouse conditions. Moreover, leaves from all transplastomic LiHsp83-SAG1 plant lines were chlorotic and pale in color as compared to wild-type tobacco plants, and this phenotype persisted in the successive generations (Figure 2). Despite the stunted growth and chlorotic leaf phenotype of the LiHsp83-SAG1 transplastomic plants, they reached maturity, produced fertile seeds and yielded the same amount of seeds that are usually produced in wild-type plants.

3.4 Expression and quantification of the SAG1 protein

The expression of SAG1 in transformed plastome (chSAG1) was detected by Western blot using an anti-SAG1 polyclonal antibody. A faint 34-kDa band was observed in total protein extracts obtained from mature leaves of transplastomic plants grown for 90 days after transplantation (Figure 3A). In addition to the 34-kDa band, other nonspecific signals were present in both SAG1 transplastomic plants and wild-type tobacco plants. As expected, the chSAG1 migrated at a slower rate on the gel compared to the *E. coli*-derived SAG1 (EcSAG1) due to the N- and C-terminal fusions mentioned above (Figure 3).

To determine the levels of chSAG1 expression, plant tissue was lyophilized with the final weight reduced to 10-fold relative to the initial fresh weight (10X). The chSAG1 expression levels were then estimated by Western blot with an anti-SAG1 polyclonal antibody using a dilution series of EcSAG1 as reference (Figure 3B). The accumulation of chSAG1 protein in transplastomic plants was approximately 1-2 μg per gram of lyophilized weight (LW) that represents 0.1-0.2 μg per gram of FW.

3.5 Expression and quantification of LiHsp83-SAG1 fusion protein

We also investigated whether utilization of LiHsp83 as a carrier of SAG1 can improve the protein expression in transplastomic plants. When 25 µg of total protein from leaf extract was resolved by SDS-PAGE, a band of corresponding size to the chLiHsp83-SAG1 fusion product could be observed upon staining with coomassie blue. This band of approximately 130-kDa was absent in wild-type tobacco plants and it was clearly visualized in samples obtained at 50, 75 and 100 days after transplantation (Figure 4A). Samples of LiHsp83-SAG1 transplastomic plants showed a decrease in the intensity of the band corresponding to the large subunit of pro-homeostatic RuBisCo enzyme with respect to the wild-type tobacco plants at each 50, 75 and 100 days after transplantation.

The 130-kDa band was excised from the gel and analyzed by MALDI-TOF/TOF, which revealed several peptides (22% sequence coverage) that matched with chLiHsp83-SAG1 fusion sequence (Figure S1). In addition, Western blot analysis with an anti-LiHsp83 or an anti-SAG1 polyclonal antibody revealed the 130-kDa band expected for chLiHsp83-SAG1 fusion protein (Figure 4A). A second specific band of approximately 100-kDa was also revealed by both antibodies. This secondary band is probably a degradation product of chLiHsp83-SAG1 fusion protein. Finally, multiple bands with lower molecular weight were only detected by anti-LiHsp83 antibody suggesting that these bands are N-terminal degradation products due to these bands were not detected by anti-SAG1 antibody (Figure 4A).

In order to evaluate the solubility of the fusion protein, total soluble and insoluble proteins were extracted from developed mature leaves. The specific 130-kDa band was observed mainly in the soluble fraction in 50 and 100-day-old plants by coomassie blue staining (Figure 4B).

3.6 Effect of leaf age on LiHsp83-SAG1 expression

To determine whether the leaf age affects the expression level of the recombinant fusion protein, transplastomic plants were grown in the greenhouse for 100 days and analyzed. The total protein content is normally higher in young and mature leaves than in old leaves because of higher proteolytic activity in senescent leaves [41]. Usually, the profiles of recombinant proteins matched those of the total protein content [41]. Contrary to expectation, the expression level of chLiHsp83-SAG1 in young leaves was barely detectable by coomassie blue staining (Figure 5A). However, we observed that the fusion protein mainly accumulated in mature and old leaves (Figure 5A).

ChLiHsp83-SAG1 accumulation in mature and old leaves was estimated by Western blot analysis using EcSAG1 and EcLiHsp83 for reference as described in Material and Methods (Figure 5B). The relative amount of chLiHsp83-SAG1 fusion protein accumulated in transplastomic plants was up to approximately 100 µg per gram of FW (~2.5% of total cellular proteins) (Figure 5B). We estimated the yield of chLiHsp83-SAG1 expressed in tobacco chloroplast according to the number and weight of mature and old leaves per plant (2.8 g of FW/leaf). A total of 3.6 mg of chLiHsp83-SAG1 could be produced per plant, the great majority of this coming from mature and old leaves.

3.7 Antigenicity and immunogenicity analysis of the chLiHsp83-SAG1

In order to evaluate the antigenic functionality of the chLiHsp83-SAG1, the reactivity of human serum samples against SAG1 was analyzed by Western blot under reducing conditions. A total of six human serum samples (3 positive and 3 negative sera) were tested. A band around 130-kDa (chLiHsp83-SAG1 protein) was detected by the positive serum samples. In addition, these positive sera reacted with other band around 100-

kDa. This lower band was also detected by the rabbit anti-SAG1 antibody that was used as positive control (Figure 6A). Immunoblot analysis using samples extracted from wild-type plants and EcLiHsp83 protein did not show reactivity with any of the positive serum samples (Figure 6B and C).

In order to study the immunogenicity of the chloroplast-made LiHsp83-SAG1, we included an oral immunization protocol using C57BL/6 (H-2b) mice. Lyophilized material was used for oral administration to mice (Figure S2). The amount of chHsp83-SAG1 protein was estimated to be 200 µg/g of lyophilized weight (Figure S2).

Mice were orally immunized with lyophilized LiHsp83-SAG1 leaves (LiHsp83-SAG1 group) or lyophilized wild-type leaves (control group). Mice orally immunized with PBS buffer were also included as an additional control (PBS group). Two weeks after last immunization, mice were challenged with cysts from *T. gondii* M49 strain [11, 27]. A month later, the number of *T. gondii* brain cysts was analyzed. The LiHsp83-SAG1 group showed a significant reduction in the cysts (57%) compared to both control and PBS groups (Figure 7A). In addition, humoral response was determined by ELISA using rSAG1. Two weeks after the immunization protocol was finished, serum samples were taken from immunized mice. LiHsp83-SAG1 group showed a significant increase of SAG1-specific IgG antibodies compared to control and PBS groups (Figure 7B).

4 Discussion

4.1 LiHsp83 protein used as a carrier improve the expression of *T. gondii* SAG1 antigen in the chloroplast

SAG1 is well conserved at the immunological and amino acid sequence levels making it an attractive antigen for diagnostics and immunoprophylaxis of Toxoplasmosis [42]. For this reason, this protein has been produced using a variety of expression systems [22-27]. The SAG1 expression using eukaryotic cell systems, such as *Pichia pastoris* [43, 44] or CHO cells [45], resulted in an hyper-glycosylation of the protein (N- and O-glycosylation) that affected its immunogenicity [44]. Since *Toxoplasma*, like other Apicomplexans does not have N-glycosylation, the chloroplast expression would offer the unique chance to express properly folded non-glycosylated SAG1 protein featuring the native amino acid sequence, which is not possible in the other eukaryotic expression systems like *P. pastoris* or CHO cells. In the present study, we investigated chloroplast transformation technology as a method to improve the SAG1 expression. In addition, SAG1 was fused to *L. infantum* Hsp83 as a strategy to enhance recombinant protein stability.

SAG1 transplastomic lines exhibited normal growth and morphology. In contrast, LiHsp83-SAG1 transplastomic lines showed chlorosis and growth retardation. Several publications have previously reported changes in the phenotype of transplastomic plants [9, 46]. Some of these reports suggest that the potential causes of the phenotypes observed include recombinant protein interference in different metabolic processes or/and the effect of transgene over-expression by itself [46]. In our case, other possible explanation for the phenotypic changes observed in LiHsp83-SAG1 transplastomic plants could include potential disruption of chloroplast Hsp90 function. Chloroplast Hsp90 (Hsp90C) plays an

important role in chloroplast biogenesis [47, 48]. *hsp90C* mutant in *Arabidopsis*, alters responses to red light and delays chloroplast development [47]. More recently, Feng *et al.* [48] showed that the co-suppression of Hsp90C cause albino phenotypes in seedlings of *Arabidopsis* down-regulating the expression of genes involved in photosynthesis. According to these antecedents, it remains to be determined whether chLiHsp83-SAG1 fusion protein is competing with Hsp90C for the binding to co-chaperones or client proteins affecting the normal phenotype of plants.

In previous reports, we determined that it was feasible to produce the *T. gondii* SAG1 protein in plants of *Nicotiana* spp. by means of transient expression via recombinant *A. tumefaciens* [26, 27]. We demonstrated that codon sequence optimization showed a negative impact on protein yield, while endoplasmic reticulum (ER) targeting improved the native SAG1 protein expression reaching yields of approximately 1.3 µg per gram of fresh weight (FW) [27]. Here, the relative amount of SAG1 protein accumulated in transplastomic plants was approximately 0.1-0.2 µg per gram of fresh weight (FW). These expression levels of chSAG1 were lower to those obtained by previous studies that utilized vacuum agroinfiltration method for SAG1 expression in tobacco leaves [26, 27]. Laguía Becher *et al.* [27] reported that ER localization allow the accumulation of SAG1 (~1.3 µg per gram of FW) to levels higher than that achieved by chloroplast transformation. The higher levels of expression yield achieved in these studies may be attributable to ER that provides a relatively protective environment with molecular chaperones and stabilizing agents. Similar to studies examining exogenous expression of SAG1 in plants, Bellucci *et al.* [49] demonstrated that the plant storage protein zeolin also exhibits reduced accumulation when expressed from the chloroplast genome compared to the targeting of the recombinant protein to the ER. Bellucci *et al.* [49] suggested that higher levels of

proteolytic activity present within the chloroplast may account for the reduced zeolin stability compared to the ER accumulation. According to this report we hypothesize that, for some specific proteins, targeting to ER provides a better protective and stabilizing environment than expression in the chloroplast, resulting in increased SAG1 stability and an enhanced level of SAG1 accumulation.

The yields of many proteins that have shown to be difficult to produce in chloroplasts have also been shown to accumulate at higher concentrations when fused to carrier proteins [8, 35, 41, 50]. These findings suggest that the carrier protein would act to stabilize the recombinant protein expressed in chloroplasts and may also protect against proteolysis. Here, we expressed LiHsp83 to function as a carrier for SAG1 antigen in order to improve its expression in transplastomic plants. Fusion of SAG1 to LiHsp83 significantly increased the level of SAG1 accumulation in tobacco chloroplasts (by up to 500-fold). In addition, chLiHsp83-SAG1 protein accumulation does not decrease significantly as the plant ages, although the peak yield would appear to be at 75 days after transplantation, and also highlights a greater stability than the non-fusion SAG1. Also samples of LiHsp83-SAG1 transplastomic plants showed a decrease in the intensity of the band corresponding to the large subunit of pro-homeostatic RuBisCo enzyme with respect to the wild-type tobacco plants at each 50, 75 and 100 days after transplantation. This is consistent with findings from Bally *et al.* [51, 52], who demonstrated that the over-expression of recombinant proteins in chloroplasts commonly resulted in reduced expression of endogenous proteins including RuBisCo. However, in our case it remains to be elucidated whether the reduction of RuBisCo observed in LiHsp83-SAG1 transplastomic plants is due to direct interference of the recombinant protein, as it was already mentioned above.

The relative amount of chLiHsp83-SAG1 fusion protein accumulated in transplastomic plants was up to approximately 100 µg per gram of FW (2.5 % of total protein), the majority of which was observed in the soluble protein fraction. These results show that fusion of SAG1 to LiHsp83 significantly increased the level of chSAG1 accumulation in tobacco chloroplasts. Since chSAG1 and chLiHsp83-SAG1 are synthesized from the same expression cassette, we infer that the low levels of chSAG1 expression are not due to inefficient transgene transcription or translation. In addition, accumulation of the chLiHsp83-SAG1 recombinant fusion protein was found to be higher in plant tissues (mature and old leaves) with higher levels of proteolytic activity. Given that HSP90s are highly stable in different stress conditions, in addition to the functions of HSP90s in promoting correct protein conformation [53, 54], our results suggest that the fusion of SAG1 to the molecular chaperone LiHsp83 promotes SAG1 stabilization and/or protects it from degradation. Similarly, using tobacco plant chloroplasts, Sanz-Barrio *et al.* [55, 56] found improved expression and solubility of recombinant human serum albumin (HSA) when fused or co-expressed with thioredoxin f, a plastidic oxidoreductase with chaperone-like properties. Together, these findings support the use of molecular chaperones as carriers to improve the expression yield and solubility of recombinant proteins in chloroplasts.

4.2 LiHsp83-SAG1 fusion protein retain the immunogenic and antigenic properties of *T. gondii* SAG1 antigen

In order to determine if an antigenic SAG1 version is expressed in LiHsp83-SAG1 transplastomic plants, the reactivity of human serum samples against SAG1 was analyzed by Western blot assays. Our results showed that chLiHsp83-SAG1 was recognized by IgG

antibodies in human serum samples from *Toxoplasma*-infected individuals, indicating that SAG1 fused to LiHsp83 can be recognized by antibodies under reducing condition. Although conformational epitopes were found in SAG1 protein [24, 57], the appreciable immunoreactivity of chLiHsp83-SAG1 observed in Western blots against positive sera, suggests that electrophoresis of proteins under reducing conditions makes the antigen react even if chLiHsp83-SAG1 is denatured. Recently, Velmurugan *et al.* [58] showed that recombinant non-folded SAG1 protein reacted with positive sera from goat, rabbit and humans by Western blot analysis. More recently, Cardona *et al.* [59] showed that, in humans, anti-SAG1 antibodies recognize lineal epitopes and they are directed mostly against peptides from the SAG1 carboxy-terminus. Additionally, Siachoque *et al.* [60] observed that the humoral response is specifically directed against these carboxy-terminus peptides in a murine model. In agree with these authors our results suggest that lineal epitopes of SAG1 would be recognized by IgG antibodies from *Toxoplasma*-infected humans.

Finally, we evaluated the immunogenicity of LiHsp83-SAG1 expressed in chloroplasts. We previously showed that oral immunization with SAG1-expressing leaves did not elicit evident humoral immune responses and protection [27]. A heterologous protocol based on oral immunization with plant-derived SAG1 protein prior to rSAG1 intradermal boost was necessary to elicit significant protection [27]. Here, the oral immunization with chLiHsp83-SAG1 elicited an important reduction of the cyst burden (~57% when compared to control) that correlated with an increased humoral response. Our results suggest that chLiHsp83-SAG1 retain the structural integrity to elicit immunological responses in mice. Although it remains to prove the efficacy of LiHsp83 as oral adjuvant in the antigen presentation, a potential for the use of LiHsp83 as an adjuvant for vaccine

antigens has been largely previously based on its immunomodulatory properties [32, 36]. In addition to this feature of LiHsp83, we demonstrate in the current study that LiHsp83 also enhances the solubility and expression of the SAG1 protein during antigen production.

4.3 Conclusion

We thus identify further applications of LiHsp83 properties that together with its immunomodulatory attributes, suggest LiHsp83 to be a promising candidate to function as a carrier protein for the expression of vaccine antigens in plants.

We conclude that the fusion of recombinant proteins of interest to LiHsp83 is a novel strategy that presents advantages over other carrier proteins used in heterologous plant expression systems promoting the use of chloroplast transformation technology in molecular farming.

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6 Conflict of interest

The authors do not have a conflict of interest to declare

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Figure legends

Figure 1: Chloroplast genome transformation with SAG1 and LiHsp83-SAG1 sequences and morphological analysis of transplastomic plants. A: Schematic representation of chloroplast genome transformation. Map of the wild-type, SAG1 and LiHsp83-SAG1-transformed chloroplast genomes, the transgenes are positioned to the intergenic region between *trnI* and *trnA*. The SAG1 and LiHsp83-SAG1 expression cassette consists of the *psbA* promoter and 5-untranslated region (*PpsbA*) and the *psbA* terminator (*TpsbA*) from tobacco (*Nicotiana tabacum* cv. Petite Havana). The selectable marker gene *aadA* is driven by the ribosomal RNA operon promoter (*Prrn*). The expected sizes of DNA fragments in Southern blot analysis with the restriction enzyme *Bgl*III are indicated. The 0.8-kb fragment (P1) of the targeting region for homologous recombination was used as probe for Southern blot analysis. B: Homoplasmy verification in transplastomic plants. Southern blot analysis of two independent transplastomic lines for pAF-SAG1 and pAF-LiHsp83-SAG1 constructions. The blots were probed with P1. WT ptDNA, wild-type chloroplast genome; Transplastomic ptDNA, SAG1 and LiHsp83-SAG1 transformed chloroplast genomes; DB, downstream box with the first 15 amino acids of the green fluorescent protein; TD, tetramerisation domain from the human transcription factor p53 with a C-terminal 7xHis-tag. C: Morphological analysis of SAG1 and LiHsp83-SAG1 transplastomic plants (chSAG1 and chLiHsp83-SAG1, respectively). Growth retardation and chlorotic phenotype was observed within LiHsp83-SAG1 transplastomic plants. No overt morphological difference was observed in SAG1 transplastomic plants compared to wild-type (WT) tobacco plants.

Figure 2: Expression of chSAG1 protein in transplastomic tobacco plants. A: Western blot analysis of total protein extracts from mature leaves harvested 90 days after transplantation. The equivalent to 5 mg leaf fresh weight (25 µg of total proteins) per well were loaded in a 15% SDS-PAGE, transferred to PVDF membrane and immunoblotted with an anti-SAG1 polyclonal antibody. Coomassie blue stained SDS-PAGE gel was used as a loading control. B: Quantification of chSAG1 protein by Western blot. Total protein was extracted from chSAG1 lyophilized leaves harvested 90 days after transplantation. The equivalent to 5 mg of lyophilized tissue weight (250 µg of total proteins) per well were resolved by 15% SDS-PAGE, transferred to PVDF membrane and immunoblotted with an anti-SAG1 polyclonal antibody. A dilution series of purified *E. coli*-derived SAG1 (2, 4, 6, 8 and 10 ng) was used as a reference for protein quantification. The asterisk indicates a proteolytic product. chSAG1: transplastomic plant expressing SAG1 protein; EcS: 100 ng of *E. coli*-derived and purified SAG1 protein; WT: wild-type *Nicotiana tabacum* cv. Petite Havana, M: molecular weight marker (Fermentas).

Figure 3: Analysis of chLiHsp83-SAG1 fusion protein accumulation in transplastomic plants. A: Total protein extracts from mature leaves harvested 50, 75 and 100 days after transplantation (dat) were analyzed by coomassie blue-stained SDS-PAGE gel (upper panel) and by Western blot analysis of the same samples immunoblotted with an anti-SAG1 polyclonal antibody (middle panel) or an anti-LiHsp83 polyclonal antibody (lower panel). The equivalent to 5 mg leaf fresh weight (25 µg of total protein per well) is shown with the chLiHsp83-SAG1 fusion protein resolving to form a 130-kDa band as indicated. B: chLiHsp83-SAG1 fusion protein expression in soluble (SF) and insoluble (InF) fractions.

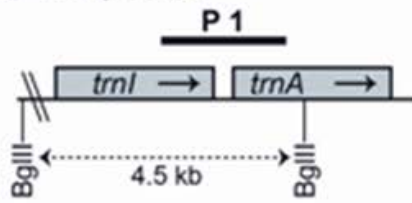
Total soluble and insoluble proteins were extracted from mature leaves harvested 50 and 100 days after transplantation (50 and 100, respectively) and resolved by SDS-PAGE and stained with coomassie blue. chLiHsp83-SAG1: transplastomic plant expressing LiHsp83-SAG1 protein; WT: wild-type tobacco plant; RbcL: Ribulose biphosphate carboxylase large subunit; M: molecular weight marker (Fermentas).

Figure 4: Analysis of chLiHsp83-SAG1 accumulation in young, mature and old leaves of transplastomic plants. A: Coomassie blue-stained SDS-PAGE of young, mature and old leaves (YL, ML and OL, respectively). Total protein extracts from chLiHsp83-SAG1 young, mature and old leaves harvested 100 days after transplanting. Twenty micrograms of plant total protein per well were resolved by 10% SDS-PAGE. B: Quantification of LiHsp83-SAG1 fusion protein by Western blot. Total protein extracts were extracted from a pool of chLiHsp83-SAG1 mature and old leaves harvested 100 days after transplantation. Twenty micrograms of total plant protein was resolved by 10% SDS-PAGE, transferred to PVDF membrane and immunoblotted with an anti-SAG1 or anti-LiHsp83 polyclonal antibody. A dilution series of purified *E. coli*-derived SAG1 (2, 4, 6, 8, 16 and 24 ng) and *E. coli*-derived LiHsp83 (10, 20, 40, 80 and 100 ng) were used as reference for protein quantification. chLiHsp83-SAG1 protein migrates as a 130-kDa band. The asterisks indicate proteolytic degradation products. chLiHsp83-SAG1: transplastomic plant expressing LiHsp83-SAG1 protein; WT: wild-type tobacco plant; RbcL: Ribulose biphosphate carboxylase large subunit; MW: molecular weight marker (Fermentas).

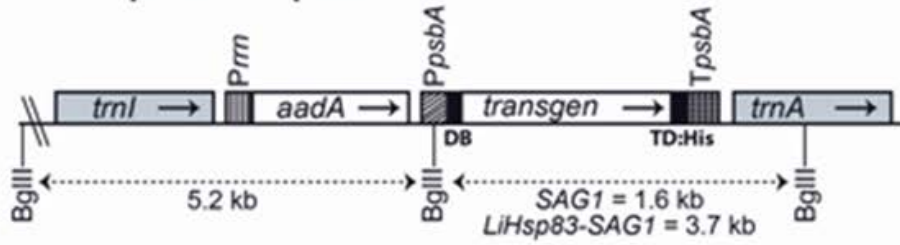
Figure 5: Antigenic and immunogenic properties of SAG1 expressed in LiHsp83-SAG1 transplastomic plants. A: The reactivity of the recombinant chLiHsp83-SAG1

protein by IgG immunoblot assays. Leaf extract samples were separated by 10% SDS-PAGE under reducing conditions. A: Immunoblot profiles of chLiHsp83-SAG1 leaf extracts were probed with human negative sera (1:100) (lanes 1–3), human *Toxoplasma*-positive sera (1:100) (lanes 4–6), and rabbit anti-SAG1 polyclonal antibody (1:1000) (C+). B: Immunoblot profiles of wild-type leaf extracts probed with human *Toxoplasma*-positive sera (1:100) (lanes 4–6) and chLiHsp93-SAG1 leaf extracts probed with rabbit anti-SAG1 polyclonal antibody (1:1000) (C+). The asterisks indicate the two bands that reacted with *Toxoplasma*-positive sera and rabbit anti-SAG1 polyclonal antibody. C: Immunoblot profiles of recombinant LiHsp83 (EcLiHsp83) probed with human *Toxoplasma*-positive sera (1:100) (lanes 4-6) and rabbit anti-LiHsp83 polyclonal antibody (1:1000) (C+). D: Protection assay after a challenge with *T. gondii* cysts in orally immunized C57BL/6(H-2d) mice. The results are showed as the cyst number per brain. E: Humoral response in orally immunized C57BL/6(H-2d) mice. Specific total anti-SAG1 IgGs (IgGt) in sera from vaccinated mice were determined by ELISA. Control: mice vaccinated with wild type leaf extracts, chLiHsp83-SAG1: mice vaccinated with transplastomic leaf extracts, PBS: mice vaccinated with PBS buffer. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's Multiple Comparison Test. Different letters indicate significant differences among groups. $a = p < 0.05$. Results are expressed as the means value \pm S.E.M. Values for each serum sample were determined in duplicate.

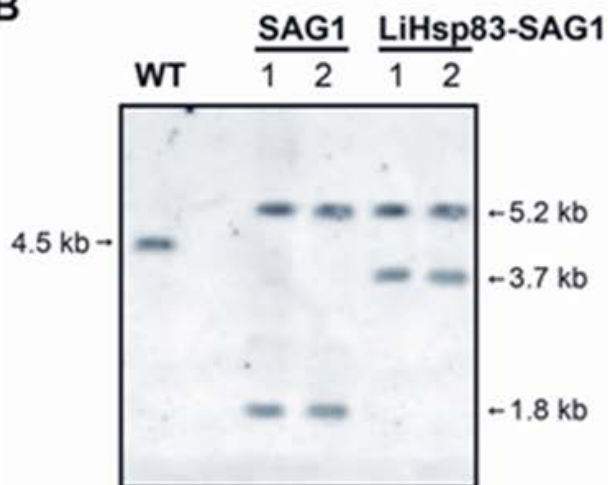
A WT ptDNA



Transplastomic ptDNA

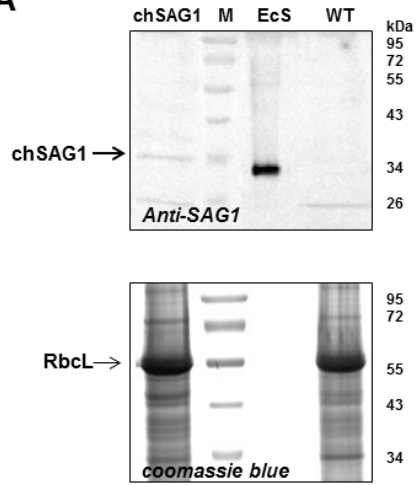


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