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Heat treatment alleviates the growth and photosynthetic impairment of transplastomic plants expressing *Leishmania infantum* Hsp 83-Toxoplasma gondii SAG1 fusion protein

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Abstract

Previously, we showed that transplastomic tobacco plants expressing the LiHsp83-SAG1 fusion protein displayed a chlorotic phenotype and growth retardation, while plants expressing the SAG1 and GRA4 antigens alone did not. We conducted a comprehensive examination of the metabolic and photosynthetic parameters that could be affecting the normal growth of LiHsp83-SAG1 plants in order to understand the origin of these pleiotropic effects. These plants presented all photosynthetic pigments and parameters related to PSII efficiency significantly diminished. However, the expression of *CHLI*, *RSSU* and *LHCa/b* genes did not show significant differences between LiHsp83-SAG1 and control plants. Total protein, starch, and soluble sugar contents were also greatly reduced in LiHsp83-SAG1 plants. Since Hsp90s are constitutively expressed at much higher concentrations at high temperatures, we tested if the fitness of LiHsp83-SAG1 over-expressing LiHsp83 would improve after heat treatment. LiHsp83-SAG1 plants showed an important alleviation of their phenotype and an evident recovery of the PSII function. As far as we know, this is the first report where it is demonstrated that a transplastomic line performs much better at higher temperatures. Finally, we detected that LiHsp83-SAG1 protein could be binding to key photosynthesis-related proteins at 37 °C. Our results suggest that the excess of this molecular chaperone could benefit the plant in a possible heat shock and prevent the expected denaturation of proteins. However, the LiHsp83-SAG1 protein content was weakly decreased in heat-treated plants. Therefore, we cannot rule out that the alleviation observed at 37 °C may be partially due to a reduction of the levels of the recombinant protein.

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Keywords

Pleiotropic Effects; Transplastomic Plants; heat shock protein; Tobacco; *Toxoplasma gondii*

1. Introduction

In recent years, great efforts have been conducted to improve the yields of recombinant proteins produced in plants [1–3]. In this sense, chloroplast transformation has emerged as an alternative platform to raise the low yields obtained in transgenic plants [4,5]. The plastid transformation has allowed the increase of protein expression levels with respect to nuclear transformation since it is possible to find a large number of chloroplasts within a single leaf cell and, therefore, multiple copies of the transgene [5,6]. In addition, in contrast to nuclear transformation, in chloroplast transformation there are no positioning effect on the transgene because the transgene insertion into plastid DNA occurs via homologous recombination. Therefore, it is not necessary to evaluate several transformation events due to the genetic background in all transplastomic lines produced are identical [7]. Up to now, different kind of interesting proteins was expressed in transplastomic plants. Some of them are related to agronomic resources, phytoremediation and biofuels, while others correspond to pharmaceutical molecules and vaccine antigens [4]. It has been reported that some transplastomic lines present pleiotropic effects related to plant physiology, like plant growth retardation, chlorosis, and male sterility [8,9]. Some authors attributed these side effects to the transgene overexpression [10]. However, there are some examples where the pleiotropic effects are due to the presence of the recombinant protein since they disturb the chloroplast metabolism affecting the photosynthetic mechanism and, in consequence, the correct plant development [10].

Previously, the correct insertion and homoplasmy of *Toxoplasma gondii* GRA4, SAG1, and LiHsp83-SAG1 antigens were demonstrated [11,12]. In particular, SAG1 yields were significantly increased when this antigen was fused to the carrier/adjuvant *Leishmania infantum* Hsp83 (up to 100 µg/g of fresh leaves). Noticeably, all the leaves from the LiHsp83-SAG1 transplastomic line showed a chlorotic phenotype and growth retardation [12]. Nevertheless, the expression levels reached for the LiHsp83-SAG1 line were not superior to 3% of the total soluble protein and it remained to be determined if the overexpression of LiHsp83-SAG1 would explain by itself the pleiotropic effects observed. Therefore, we conducted a comprehensive examination of the metabolic and photosynthetic parameters that could be affecting the normal growth of LiHsp83-SAG1 plants in order to understand the origin of these pleiotropic effects and whether there is a way to overcome them. The implications of such findings are discussed.

2. Materials and methods

2.1. Plant material and heat treatment

Transplastomic tobacco genotypes expressing SAG1, GRA4, and LiHsp83-SAG1 were generated as described in Del Yácono et al. [11] and in Albarracín et al. [12] and compared to wild-type non-transformed (WT) tobacco control (*Nicotiana tabacum* L. cv Petite

havana). Since the insertion of transgenes into the plastid DNA occurs via homologous recombination at the specific *trnI-trnA* insertion site, all transplastomic lines are identical [7]. Therefore, the SAG1 line 1, the GRA4 line 1 and the LiHsp83-SAG1 line 1 were used in further experiments. Sterile seeds from transplastomic lines or WT plants were germinated in sterile Murashige Skoog medium (MS, Sigma) supplemented with sucrose 3% and agar 8 g/L in a growth chamber with a 16 h day/8 h night photoperiod (photosynthetic flux photon density (PPFD) of 350 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) provided by cool-white fluorescent lamps, 24 \pm 2 °C temperature. After 21 days, ten plants per genotype were grown in sterile sand: soil: perlite (1:1:1) mixture and watered with Hoagland nutrient mix [13] every 2 days. Physiological, biochemical and gene expression analyses were performed between 20 and 40 days after transplantation (dat). The heat treatment was administered at 37 °C for 3 weeks to 40-days-old WT and LiHsp83-SAG1 plants. Control plants were maintained at 25 °C the same period. At the end of the treatment, photosynthetic parameters were measured, and plants were immediately harvested and stored at -80 °C until their use for RNA isolation.

2.2. Foliar area

The youngest fully completely developed leaf was photographed, the images were analyzed with *Gel-Pro analyzer* software (Media Cybernetics) and the foliar area was calculated.

2.3. Estimation of chlorophyll content

Total chlorophylls of the youngest fully completely developed leaf were determined used an optical leaf-clip chlorophyllmeter (Cavadevices <http://www.cavadevices.com>, Argentina). Chlorophyll *a*, chlorophyll *b* and carotenoids were measured according to Lichtenthaler [14]. Briefly, 40 mg of plant material of every line and WT plants were ground in liquid nitrogen and incubated in 80% acetone with agitation in darkness overnight at 4 °C. The extracts were centrifuged at 5000 \times g at 4 °C and the supernatant was saved. The absorbance was read at 470 nm, 663 nm and 647 nm in a spectrophotometer (Perkin Elmer Lambda 25 UV/VIS spectrometer), and pigments concentration was calculated according to Lichtenthaler [14], using the following formulas:

$$\text{Chlorophyll "a"} = 11,25 \times A_{\lambda 663} - 2,79 \times A_{\lambda 647}$$

$$\text{Chlorophyll "b"} = 21,5 \times A_{\lambda 647} - 5,1 \times A_{\lambda 663}$$

$$\text{Carotenoids} = (1000 \times A_{\lambda 470} - (1,82 \times [\text{Chl "a"}] - 85,02 \times [\text{Chl "b"}])) / 198 \times 13,33$$

2.4. Chlorophyll fluorescence fast-transient analysis

The non-invasive OJIP analysis [15] was performed in the youngest fully developed leaf with a portable chlorophyll fluorometer (Pocket PEA v.1.1, Hansatech Instruments Ltd.). Leaves were pre-darkened 20 min before analysis. After that, they were exposed during 3 s to 3500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (637 nm peak wavelength) and Chlorophyll *a* fluorescence

was recorded. The data were analyzed with PEA Plus software (Hansatech Instruments Ltd.). The maximum quantum yield of primary photochemistry (F_v/F_m) was determined. In addition, the contribution to photosynthesis regulation by the three functional steps namely ABS (absorption of light energy), TR (trapping of excitation energy) and ET (conversion of excitation energy to electron transport) was analyzed. The definition of each parameter is provided in Supplementary Table 1.

2.5. Gas Exchange determination

The net photosynthesis (P_n), stomatal conductance (g_s) and CO_2 concentration of sub-stomatal cavity (C_i) were measured in the youngest completely developed leaf at light saturation ($1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ illumination led light) using an infrared gas analyzer TPS-2 Portable Photosynthesis System (PP Systems In.).

2.6. Protein extraction and quantitation

For total protein extraction, the youngest completely developed leaf from transplastomic lines and WT tobacco plants were ground in liquid nitrogen and homogenized in Laemmli buffer (0.5 M Tris-HCl pH 6.5, 4% SDS, 20% glycerol, 10% β -mercaptoethanol and 0.1% bromophenol blue) in a 1:3 ratio. The total protein content was determined following the instructions of the RC-DC protein assay (BioRad) and using a standard solution of bovine serum albumin protein (BSA).

2.7. Sugar and starch determination

Sugar and starch were measured by anthrone assay with modification [16]. Leaves of every line and WT plants were homogenized with liquid nitrogen, 100 mg of powder were separated and 1 ml of EtOH 80% v/v was added to each sample and the samples were incubated at 80°C for 30 min. After heating, the samples were centrifuged 10 min at 10000 rpm and the supernatants were saved. These last steps were conducted three times. For starch extraction, the pellet was dried and then 2 ml of 9.2 N $HClO_4$ were added to analyze the starch content in this extract.

2.8. LiHsp83-SAG1 accumulation in transplastomic lines

The analysis of the accumulation of LiHsp83-SAG1 expressed in transplastomic plant was performed as Albarracín et al. [12] with minor modifications. Protein extracts from WT and LiHsp83-SAG1 plants maintained at 25°C or at 37°C were separated in a 12% SDS-PAGE, transferred onto a PVDF membrane (GE Healthcare) and immunoblotted with an anti-SAG1 polyclonal antibody. A serial dilution (100, 50 and $25 \text{ ng}/\mu\text{L}$) of known concentration of an *Escherichia coli*-purified SAG1 protein (Ec-SAG1) was used as the reference. LiHsp83-SAG1 amount was estimated with the Gel-Pro Analyzer software (Media Cybernetics) as described by Albarracín et al. [12]. Page Ruler™ Prestained Protein Ladder (Fermentas) was used as a molecular marker.

2.9. RNA isolation and gene expression profiling

Total RNA was isolated from WT and LiHsp83-SAG1 leaves using Trizol reagent (Invitrogen), following the manufacturer's instructions. The RNA concentration and its

integrity were analyzed as previously [17]. cDNA was synthesized using oligo dT₂₀ (Invitrogen) and M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. This cDNA was used as a template for Real-Time quantitative PCR (qRT-PCR). The steady-state mRNA levels were analyzed by qRT-PCR as indicated previously [18]. Primer sequences for all the experiments are listed in Supplementary Table S1. Relative quantification was performed by the comparative cycle threshold method. The elongation factor alpha from *Nicotiana tabacum* gene (NtEF α) was used as endogenous control. Reactions were carried out in MicroAmp™ Fast Optical 96-Well Reaction Plate (Thermo Scientific) using the StepOnePlus Real-Time PCR System and the Mx3005P qPCR Software 4.0 (Stratagene). For comparative purposes, relative gene expression was defined with the value of $-\text{Log}_2$ in each control plants.

2.10. Co-Immunoprecipitation assay

The Co-Immunoprecipitation assay was conducted as Scotti et al. [19] and Inoue et al. [20] with modifications. Briefly, 1 g of LiHsp83-SAG1 leaves exposed at 25 °C or 37 °C during 3 weeks were ground in liquid nitrogen and 5 ml of lysis buffer was added (Sucrose 250 mM; Hepes 50 mM, pH 7.2; EDTA 50 mM; DTT 100mM; L-Cistein 6,3 mg; MgCl₂ 1 mM; PVP 0,06 g) and incubated for 1 h at 4 °C. Separately, 20 μ L of ProteinA A/G PLUS-Agarose beads (Thermo Fisher) were pre-incubated with 20 μ L of anti-LiHsp83 polyclonal sera for 1 h on ice and washed twice with lysis buffer. The extracts were centrifugated at 12000 g and 100 μ L of each supernatant was incubated with LiHsp83-beads for 4 h at 4 °C. Then, the samples were washed twice with lysis buffer and the beads were separated onto a 12 % 1D-SDS-PAGE. Gels were fixed for 90 minutes (40% Methanol; 10% glacial acetic acid), stained overnight (10% p/v (NH₄)₂SO₄; 2% Orthophosphoric acid; 15 g/L Coomassie brilliant Blue G250; 5% Methanol) and washed with water.

2.11 LC-MS/MS

Each lane of the gel was cut into 3 individual pieces. Each band was then cut into 1 mm³ cube and washed with 50 mM NH₄HCO₃ in 50% CH₃CN. Each group of gel cubes was then dehydrated in CH₃CN for 10 min and dried in a Speed Vac. Protein samples were reduced by dithiothreitol (DTT) and alkylated by iodoacetamide [21]. A solution of 10 ng/ μ L trypsin in 50 mM NH₄HCO₃ was used to re-swell the gel pieces completely at 4°C for 30 min, followed by a 37°C digestion overnight. A small amount of 10% formic acid was then added to stop the digestion. The sample was then centrifuged at 2,800 \times g, and the supernatant was collected for LC-MS/MS.

Two microliters of the tryptic digests were analyzed on the Thermo Q-Exactive plus mass spectrometer coupled to an EASY-nLC 1200 system (Thermo Scientific). Peptides were separated on a fused silica capillary (12 cm \times 100 μ m I.D) packed with Halo C18 (2.7 μ m particle size, 90 nm pore size, Michrom Bioresources) at a flow rate of 300 nl/min. Peptides were introduced into the mass spectrometer via a nanospray ionization source at a spray voltage of 2.2 kV. Mass spectrometry data were acquired in a data-dependent top-10 mode, and the lock mass function was activated (m/z, 371.1012; use lock masses, best; lock mass injection, full MS). Full scans were acquired from m/z 350 to 1,600 at 70,000 resolution (automatic gain control [AGC] target, 1⁶; maximum ion time [max IT], 100 ms; profile

mode). Resolution for dd-MS2 spectra was set to 17,500 (AGC target: 1^5) with a maximum ion injection time of 50 ms. The normalized collision energy was 27 eV. A gradient of 0 to 40% acetonitrile (0.1% FA) over 55 min was applied. The spectra were searched against the *Nicotiana tabacum* protein database (UP000084051) by Proteome Discoverer (PD) 1.4. The search parameters allowed a 10ppm precursor MS tolerance and a 0.02 Da MS/MS tolerance. Carboxymethylation of cysteines was set up as fixed modifications and Oxidation of methionine (M). Up to two missed tryptic cleavages of peptides were considered with the false-discovery rate set to 1% at the peptide level.

2.12. Statistical analysis

Statistical analysis was carried out with the Prism 5.0 Software (GraphPad) using parametric or non-parametric one-way analysis of variance (ANOVA). Besides, RT-qPCR results were analyzed with fgStatistics (<https://sites.google.com/site/fgstatistics/>) and permutation test.

3. Results

3.1. Reduced growth and leaf area of LiHsp83-SAG1 plants

In a previous report, we assessed the expression and functionality of *T. gondii* GRA4 and SAG1 antigens and the LiHsp83-SAG1 fusion protein expressed in transplastomic plants [11,12]. In addition, the homoplasmy and correct insertion was demonstrated previously [11,12]. Noticeably, growth retardation was only observed in the LiHsp83-SAG1 line from 20 days after germinating and became more evident when plants were potted in soil (Fig. 1-A, B; Fig S1). Since this phenotype was observed in 4 independent transplastomic lines (Fig. S1), a single line was used in the next experiments. Given this phenotype, the leaf area was measured as a growth parameter. Leaves were photographed, and the images were analyzed to establish the foliar area. The analysis showed that the leaf area of LiHsp83-SAG1 line was between 5- and 6-fold lower than the other lines and non-transplastomic (WT) plants (Fig. 1-C).

3.2. LiHsp83-SAG1 plants displayed a chlorotic phenotype and very low pigment content

The chlorotic phenotype is the second most evident pleiotropic effect in the LiHsp83-SAG1 line (Fig. 1-B), which could be indicating degradation of photosynthetic pigments, specially chlorophylls [22]. Therefore, chlorophyll (Chl) and accessory pigments content was determined (Fig. 2). SAG1 and GRA4 lines showed no significant differences compared to WT plants in total Chl, Chl *a*, Chl *b* and carotenoids content, while LiHsp83-SAG1 line presented all these pigments significantly diminished compared to WT plants and the other transplastomic lines. Total Chl content of LiHsp83-SAG1 line was 1.6-fold lower than WT plants (Fig. 2-A), while Chl *a* content was 8-fold (Fig. 2-B) and Chl *b* and carotenoids content were 5-fold lower than WT plants (Fig. 2-C, D).

3.3. Very low net photosynthesis and impaired photosystem II in LiHsp83-SAG1 plants

To determine whether the reduction of the foliar area and the decrease of the pigment content observed in LiHsp83-SAG1 plants are correlated with possible damage of the photosynthetic capacity, we evaluated the net photosynthesis (Pn) as a parameter of the normal plant physiology. Not only WT plants but also SAG1 and GRA4 lines showed

similar Pn, while LiHsp83-SAG1 plants showed a 22-fold drop compared to WT (Fig. 3). Since the photosynthetic deficiencies could be also related to damage in photosystem II (PSII), the physiological parameters associated to the normal function of PSII were analyzed through the OJIP test (Table 1, Fig. S2). Parameters related to the PSII efficiency (Fv/Fm and Phi(Eo)) and energy flux (ABS/RC, ETo/RC, and TRo/RC) were analyzed (Table 1; Fig. S2). The two parameters related to PSII efficiency were strongly diminished in the LiHsp83-SAG1 (Table 1). In particular, we evaluated the maximum quantum yield (Fv/Fm) to determine the physiological state of LiHsp83-SAG1 line. The Fv/Fm values for WT, SAG1, and GRA4 plants were normal, while it was remarkably low for LiHsp83-SAG1 plants (Table 1). On the other hand, the analysis of the PSII energy flux parameters in LiHsp83-SAG1 line showed that the apparent antenna size per reaction center (ABS/RC) and trapped energy flux per RC (TRo/RC) are highly increased (Table 1), meanwhile the electron transport flux per RC (ETo/RC), suggesting that functional RC are not frequent in this transplastomic line (Table 1, Fig. S2). Finally, the PI_{ABS} index was measured as a parameter of the global state of the PSII and the lowest values were observed in LiHsp83-SAG1 plants (Table 1).

Once confirmed that the photosynthetic processes are altered in LiHsp83-SAG1 plants, we wondered whether it could be occurring due to a limitation on the CO₂ entrance into the plant cells. To confirm this, the concentration of CO₂ in the substomatal cavity (C_i) and stomatal conductance (g_s) in the three transplastomic lines and WT plants were measured (Fig. 4). The results showed that the CO₂ levels in the stomatal cavity in LiHsp83-SAG1 plants are significantly increased (Fig. 4-A), while no differences in g_s among the three lines and WT plants were observed (Fig. 4-B). According to these results and given the important role of the RuBisCO enzyme in the carbon fixation, relative quantification of RuBisCO in the three transplastomic lines and WT plants was performed (Fig. S3). The relative quantification of the major subunit of RuBisCO (RbcL) showed that LiHsp83-SAG1 plants presented the lowest amount of this subunit (26% of total proteins), while SAG1 and GRA4 lines showed similar RbcL levels contrasted to WT plants (59%, 61% and 65% of total proteins, respectively) (Table 2).

3.4. Reduced levels of total protein, sugars, and starch in LiHsp83-SAG1 plants

Since photosynthetic damage could affect the carbon availability for the proper functioning of other metabolic processes in plant cells, we evaluated the total protein levels, the soluble sugar content and the starch accumulation. SAG1 and GRA4 lines did not show significant differences in comparison to WT plants. On the other hand, the total protein levels were 2.5-fold lower in LiHsp83-SAG1 plants. Although sugars were not able to be detected by this method, the starch content was around 25-fold lower in this transplastomic line, as regards the others (Table 2).

3.5. Transcription of nuclear-encoded photosynthesis-related genes was not altered in LiHsp83-SAG1 plants

To rule out if the pleiotropic effects related to the chlorotic phenotype observed in LiHsp83-SAG1 line are also accompanied by the down-regulation of photosynthesis-related genes, we examined the expression levels of magnesium protoporphyrin chelatase subunit I (*CHL1*),

ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RSSU*) and light harvesting chlorophyll a/b binding protein (*LHCa/b*). Interestingly, the expression of *CHLI*, *RSSU*, and *LHCa/b* genes did not show significant differences between LiHsp83-SAG1 and WT plants (Fig. 5). We also tested the chloroplast heat shock protein (*Hsp90C*) expression levels. Similarly, to the other photosynthesis-related genes, no differences between LiHsp83-SAG1 and WT plants were observed (Fig. 5).

3.6. Heat treatment partially reverts the pleiotropic effects observed in LiHsp83-SAG1 transplastomic line

Since LiHsp83-SAG1 line over-expresses LiHsp83, a member of the family of molecular chaperones, we tested if a heat treatment at 37 °C would improve its physiological state. After the heat treatment, LiHsp83-SAG1 plants showed an important alleviation of their chlorotic phenotype (Fig. 6-A). Also, leaves of heat-treated LiHsp83-SAG1 plants were bigger than LiHsp83-SAG1 plants raised at 25 °C (Fig. 6-A), their mass (Fresh Weight, FW) was significantly augmented and presented an increment of 8-fold compared to 25 °C (Fig. 6-B). The Pn was also measured and it was greatly improved. At 25 °C, Pn was 22-fold lower than WT (Fig. 3), while at 37 °C it was only 2-fold lower than control (Fig. 6-C), indicating that LiHsp83-SAG1 physiology is almost recovered. To verify whether the greening phenotype observed in the LiHsp83-SAG1 line exposed at 37 °C correlates with a better function of photosynthetic processes, we evaluated the PSII parameters. Interestingly, heat-treated LiHsp83-SAG1 plants showed an evident recovery of the PSII function. Almost all parameters assessed in LiHsp83-SAG1 line at 37 °C were closer to reference values (Fig. 6-D). The maximum quantum yield of primary PSII photochemistry (Fv/Fm) raised up to 0.73 ± 0.01 values, showing a 90% recovery according to non-treated WT plants (Fig. 6-D). Similarly, the apparent antenna size (ABS/RC) in heat-treated LiHsp83-SAG1 plants also showed a significant reduction (3.08 ± 0.54 at 37 °C vs. 12.34 ± 3.06 at 25 °C), suggesting an increment in the number of functional reaction centers. Finally, the PI_{ABS} index was measured and the heat-treated plants belonging to the LiHsp83-SAG1 line showed a strong recovery of this value (0.72 ± 0.09 at 37 °C vs. 0.01 ± 0.007 at 25 °C); although it did not reach the levels of the non-heated WT plants (3.50 ± 1.13). It should be mentioned that the heat treatment produced a delay in the growth of WT plants (Fig. S4), however it did not alter any of the parameters measured in the WT plants at 37 °C compared to WT at 25 °C (Fig. 6-D). To determine whether the recovery of the PSII parameters in heat-treated LiHsp83-SAG1 plants is due to changes in the expression of photosynthesis-related genes, the levels of *Hsp90C*, *LHCa/b*, *CHLI* and *RSSU* genes were measured (Fig. 7). In particular, *Hsp90C* and *LHCa/b* expression did not show differences at 37 °C along the time. After 1 week of heat treatment, *Hsp90C* expression tends to be upregulated in WT plants compared to LiHsp83-SAG1 plants, but no significant differences were observed (Fig. 7-A). Besides, after 3 weeks of heat treatment, *LHCa/b* expression showed a tendency to be down-regulated, but there were no differences with WT plants (Fig. 7-B). On the other hand, the *CHLI* and *RSSU* expression were down-regulated in heat-treated LiHsp83-SAG1 line and WT plants, but it was only significantly different in LiHsp83-SAG1 line after the 3 weeks-treatment (Fig. 7-C, D). Regarding the total protein content, there was an increase in heat-treated LiHsp83-SAG1 plants compared to those non-treated (Fig. 8-A). More importantly, a significant increase in the relative RbcL content in heat-treated LiHsp83-SAG1 plants

reaching similar levels to those obtained in non-heated WT plants was observed (Fig. 8-B). On the other hand, no differences of RbcL accumulation between heat- and non-heat-treated WT plants were detected (Fig. S2, Fig. 8-B).

In an attempt to find the reason why the reversion phenotype of the LiHsp83-SAG1 line at 37 °C, we performed a co-immunoprecipitation (CO-IP) assay using anti-LiHsp83 polyclonal antisera (Table S3). Surprisingly, LiHsp83-SAG1 is interacting with key photosynthesis-related proteins at 37 °C, while at 25 °C is not. Among the critical interactors we found Ribulose biphosphate carboxylase large chain (Accession number in UniProt database: A0A140G1S3), Ribulose biphosphate carboxylase small chain (A0A1S3YB15, Q84QE5), Ribulose biphosphate carboxylase/oxygenase activase (A0A1S4AKW3), Chlorophyll a-b binding protein (A0A1S3YAU9, A0A1S4CEW8, A0A1S3YPE9), oxygen-dependent coproporphyrinogen-III oxidase (A0A1S3XMU0) and ATP synthase subunit alpha (A0A140G1P7).

3.7. Heat treatment improves the LiHsp83-SAG1 yield

Finally, the accumulation of LiHsp83-SAG1 protein from heat-treated transplastomic plants was evaluated by Western blot (Fig. 8-C). The yield of LiHsp83-SAG1 obtained in transplastomic plants raised at 25 °C was approximately 2.4 µg per gram of FW. In contrast, LiHsp83-SAG1 accumulation in heat-treated plants was around 1 µg per gram of FW (Fig. 8-C). However, as the heat treatment produced larger LiHsp83-SAG1 plants, a yield improvement equivalent to 5–7 mg of LiHsp83-SAG1 per plant (Fig. 8-D) was observed.

4. Discussion

LiHsp83-SAG1 overexpression in transplastomic plants led to pleiotropic effects, such as chlorosis, reduced RbcL content and growth retardation [12]; and this phenotype could limit the ability to produce the protein of interest. In this study, we performed an integrated analysis of the metabolism and the photosynthetic parameters of these plants, together with the assessment of the LiHsp83-SAG1 line response to a heat treatment.

4.1. Photosynthesis process is damaged in the LiHsp83-SAG1 line

Over-expression of transgenes in chloroplasts often leads to detrimental effects on chloroplast development and photosynthesis. The green color of chloroplasts is due to photosynthetic pigments which are associated with electron transfer proteins and all together form the antenna complex [23]. This pigment-protein complex is able to collect the light energy, transfer it to the reaction centers (RC) and use it for plant biomass production [24]. Therefore, growth retardation and chlorosis phenotype are linked to the chlorophylls and carotenoids disposal and the reduced photosynthesis capacity [10]. In this study, we analyzed three transplastomic lines, but we observed that only LiHsp83-SAG1 line present such phenotype and we determined that the photosynthetic processes are damaged. We first hypothesized that the expression levels reached for the LiHsp83-SAG1 line were toxic for plant metabolism. Although LiHsp83-SAG1 protein accumulation is not superior to 3% of the TSP, it has been reported that a wide range of heterologous production is able to lead deleterious effects. As it was reported by others, an exceptional production of exogenous

protein (up to 70 % of total soluble proteins (TSP)), intermediate production (18 % of TSP) or as little as 0.2 % of TSP have led to chlorosis and growth retardation, without affecting flowering and seed setting [19,25–27]. On the other hand, accumulation of 2 % of TSP, not only led to chlorosis and growth retardation but also produced male sterility in all transplastomic lines studied [8]. When the transgene overburdens the chloroplast expression machinery, a poisoning of the endogenous gene expression occurs, and it would lead to such phenotype. Several studies showed that over-expression of recombinant proteins in chloroplasts is usually accompanied by a significant reduction in the accumulation of plastid-encoded proteins, most notably the accumulation of the RuBisCO large (RbcL) and small (RSSU) subunits because of a diminish of transcription rate of endogenous genes [26,27]. Since pale plants contain reduced RbcL protein, we aimed to determine whether the low RbcL accumulation observed in LiHsp83-SAG1 line is due to a decrease of its gene expression. We evaluated the levels of *RSSU* and other photosynthesis-related genes, but unexpectedly, none of the genes studied in LiHsp83-SAG1 plants showed statistic differences compared to WT plants. The results showed that the *RSSU* mRNA levels do not correlate with the low levels of RbcL protein accumulated, though stable *RSSU* mRNA level does not preclude an effect on the accumulation and translation of the RbcL mRNA. Likewise, there was no significant down-regulation of *LHCa/b* and *CHL1* genes as it was expected given the reduction of Chl *a* and Chl *b* pigments previously observed. These findings are consistent with other studies carried out by Waheed et al. [8]. They demonstrated that even though recombinant protein yields obtained were under to 2%, the chlorosis effects were directly related with the presence of the recombinant protein. This suggests that the presence of the recombinant protein would modify the chloroplast metabolism affecting photosynthetic mechanisms and, in consequence, the correct plant development [10]. We believe that the reduction of RuBisCO amount in LiHsp83-SAG1 line was probably due to an impairment of photosynthesis.

The significance of Hsp90C for chloroplast biogenesis has been previously reported [20,28,29]. In fact, the lack of Hsp90C in chloroplasts either by Hsp90C co-suppression, by a point mutation in Hsp90C or Hsp90C silencing by VIGS displayed a yellow-green phenotype and impaired growth in *Arabidopsis* as well as in tobacco [28–32]. Considering that LiHsp83-SAG1 is constitutively over-expressed in chloroplasts and bearing in mind that LiHsp83 shares a 65 % of similarity with *N. tabacum* Hsp90C, we considered the possibility that the accumulation of Hsp90C might be impeded due to the high expression levels of the recombinant protein. To rule out this possibility, we examined the expression levels of *Hsp90C* gene, and it did not present significant differences between LiHsp83-SAG1 and WT plants. Therefore, we can conclude that overexpression of LiHsp83-SAG1 is not disturbing endogenous *Hsp90C* gene expression. Other studies associated the impairment of plastid development to the carbohydrate-binding activity of the recombinant protein or to the binding of the recombinant protein to thylakoids [33,34]. Since no putative thylakoid- or carbohydrate-binding motifs were identified in the LiHsp83-SAG1 sequence, our results support the idea that the expression of LiHsp83-SAG1 protein could be affecting the amount of RuBisCO and/or other proteins involved in photosynthetic processes rather than affecting the transcription rate of photosynthesis-related genes or the plastid development due to its binding to thylakoids.

4.2. Heat treatment partially reverts the pleiotropic effects observed in LiHsp83-SAG1 transplastomic line

Unlike most chaperones, Hsp90s are constitutively expressed at much higher concentrations than required to fulfill their normal functions and they are essential proteins required for the growth of cells at high temperatures [35,36]. Considering this, we aimed to know whether LiHsp83-SAG1 line which has higher levels of Hsp90 since over-expresses LiHsp83 would improve its fitness at 37 °C. Interestingly, LiHsp83-SAG1 plants showed a partial alleviation of their phenotype. They grew greener and bigger, their Fv/Fm was close to WT and RbcL protein amount increased. The explanation for this could be that Hsp90s are induced by heat shock and Hsp90 network facilitates the adaptation to new environments [37]. As endogenous Hsp90C are also induced by heat shock [29,31,38], we first evaluated if the Hsp90C mRNA was newly synthesized after the heat treatment. Besides, we assessed whether the reason of the phenotype alleviation was due to an up-regulation of *RSSU* and/or other photosynthetic-related genes. The results demonstrated that there were no differences in *Hsp90C* expression between LiHsp83-SAG1 heat-treated plants and WT heat-treated plants. On the other hand, *CHLI* and *RSSU* expression were down-regulated in LiHsp83-SAG1 plants. Similar to observed previously, the higher RbcL accumulation and the improvement of photosynthesis after heat treatment do not correlate with gene expression. Therefore, we hypothesize that as LiHsp83 is a heat shock protein, the excess of this molecular chaperone benefits the plant in a possible heat shock and prevents the expected denaturation of proteins. Some reports showed that the heterologous expression of Hsp90 proteins was demonstrated to be beneficial for the organism expressing them [37,39,40]. For instance, *Escherichia coli* showed enhanced thermotolerance by expressing heterologous rice Hsp90 (OsHsp90) [39]. Besides, Hsp90.3 isoform from *Arabidopsis thaliana* supported the yeast growth under heat stress [40]. Moreover, when *Saccharomyces cerevisiae* Hsp90 was replaced by an ortholog from *Yarrowia lipolytica*, yeasts exhibited an improved growth in hypersaline environments [37]. In this context, we conducted a co-immunoprecipitation assay to investigate the interactors of LiHsp83-SAG1 protein at 37 °C. We found that LiHsp83-SAG1 is interacting with remarkable proteins involved in photosynthesis, like *RSSU*, RbcL, RuBisCO activase, Chlorophyll a/b binding protein, ATP synthase subunit alpha and oxygen-dependent coproporphyrinogen-III oxidase suggesting that LiHsp83-SAG1 present in stroma could bind to these key proteins chaperoning and/or stabilizing their structures during the heat treatment. However, further studies should be done to shed light on the interaction among LiHsp83-SAG1 and those proteins. Finally, several approaches were explored to reduce the pleiotropic effect in transplastomic plants in order to increase the recombinant proteins yields [26,41,42]. In our study, the heat treatment applied help LiHsp83-SAG1 plants to alleviate their phenotype, although the LiHsp83-SAG1 content was weakly decreased in heat treatment (1 µg per g FW at 37 °C vs 2.4 µg per g FW at 25 °C). In this context, we cannot rule out that the alleviation observed of the phenotype may be due by a reduction of the accumulation levels of the recombinant protein at 37 °C. In conclusion, the results described here suggest that the pleiotropic effect observed in the LiHsp83-SAG1 plants are mainly due to a malfunction of the PSII and a significant reduction of RbcL abundance. In addition, we demonstrated that heat stress alleviates the phenotype of this transplastomic line suggesting that this strategy could help us to discern, in future experiments, the specific mechanisms that would be influencing the metabolic and

photosynthetic processes under this condition. As far as we know, this is the first report where demonstrated that a transplastomic line performs much better at higher temperatures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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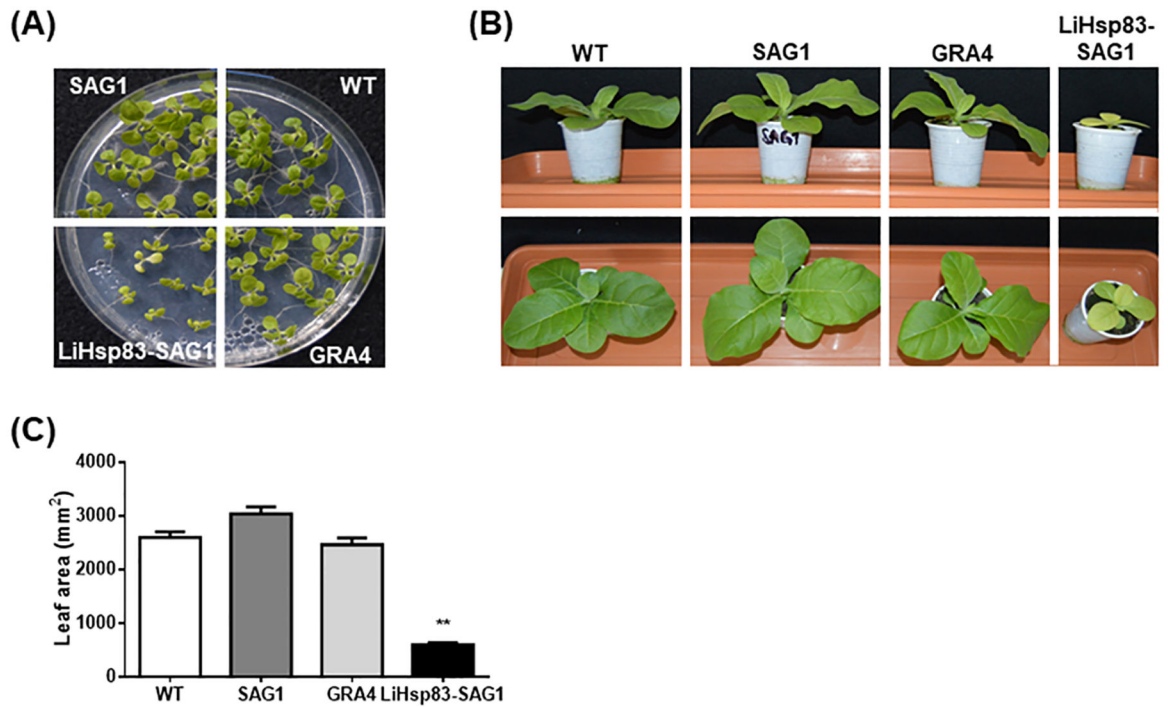


Fig. 1.

Phenotype characterization of transplastomic lines. (A) Twenty-days-old after germination in MS medium. (B) Forty-days-old after germination of non-transformed (WT) plants and SAG1, GRA4 and LiHsp83-SAG1 transplastomic lines grown in sterile sand: soil: perlite mixture. (C) Leaf area analysis. The area of the youngest fully developed leaf from 40-days-old plants was calculated with Gel-Pro Analyzer software. Results are the mean of 10 biological replicates \pm SEM. Statistical analysis was performed by one-way non-parametric analysis of variance (ANOVA) using the Dunn's multiple comparison test. LiHsp83-SAG1 vs WT, SAG1 and GRA4: ** $p < 0.01$.

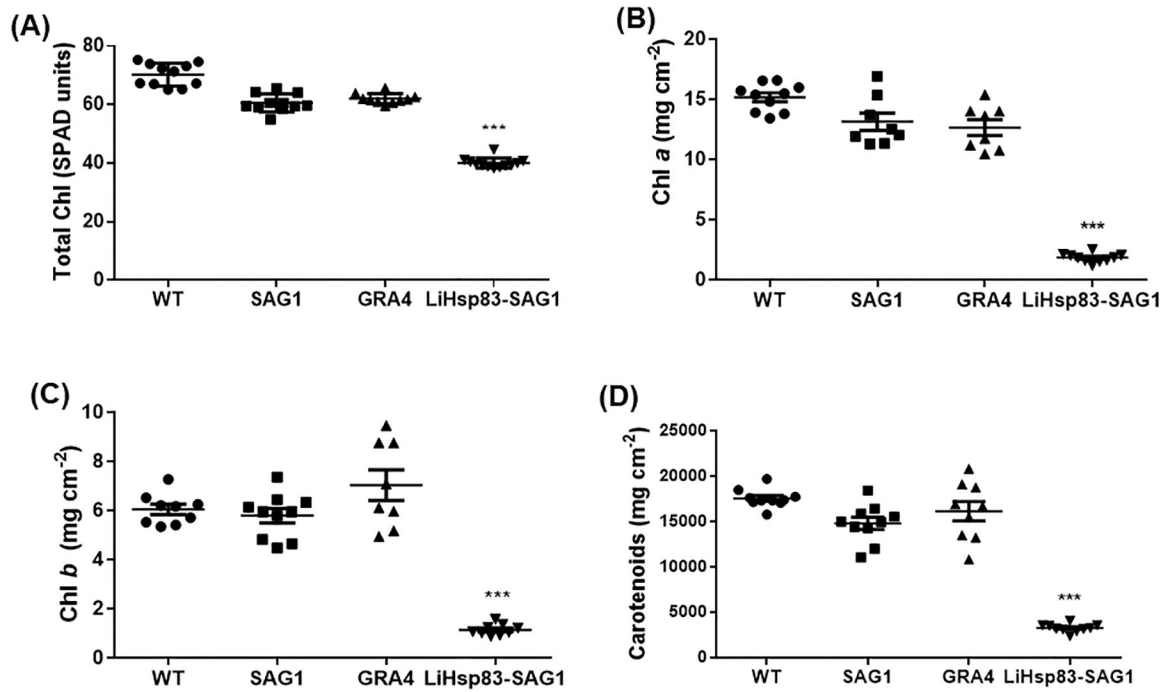


Fig. 2. Chlorophyll (Chl) and accessory pigments content in the transplastomic lines. (A) Total Chl, (B) Chl *a*, (C) Chl *b*, (D) Carotenoids present in the youngest fully developed leaf in 40-days-old plants were determined. Non-transformed (WT) plants and SAG1, GRA4 and LiHsp83-SAG1 transplastomic lines were assessed. Results are the mean of 10 biological replicates \pm SEM. Statistical analysis was performed by Kruskal-Wallis analysis using the Dunn's multiple comparison test. WT vs LiHsp83-SAG1: ** $p < 0.01$.

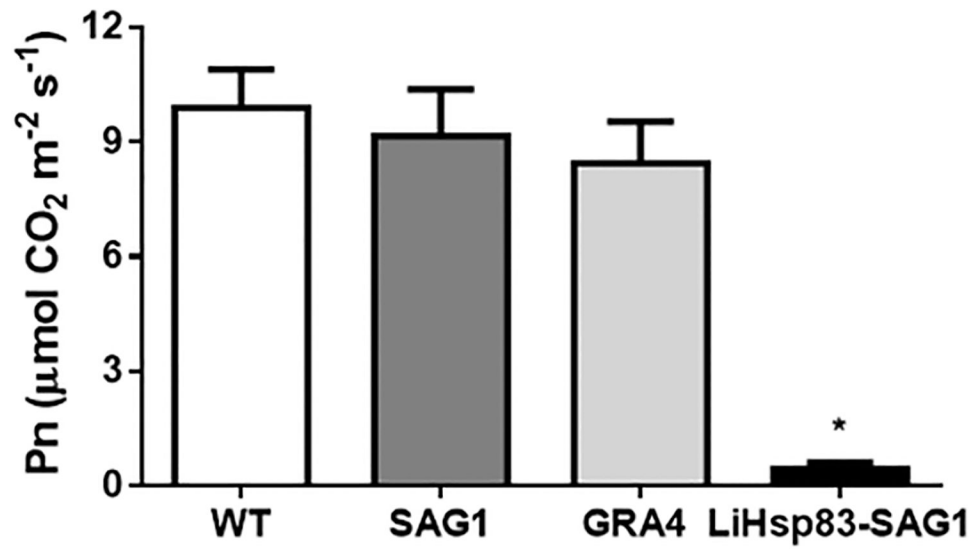


Fig. 3. Determination of net photosynthesis (Pn). Pn was measured in the youngest completely developed leaf at light saturation. Results are the mean of 6 biological replicates \pm SEM. Statistical analysis was performed by Kruskal-Wallis analysis using the Dunn's multiple comparison test. LiHsp83-SAG1 vs WT, SAG1 and GRA4: * $p < 0.05$.

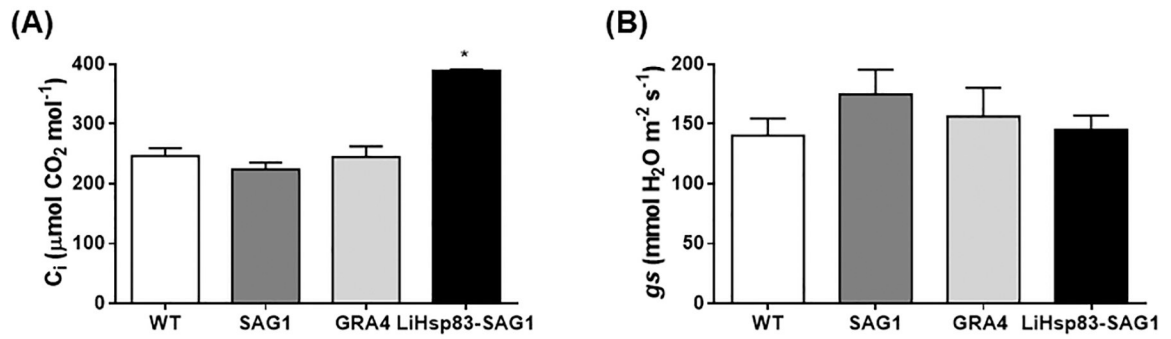


Fig. 4. Gas Exchange determination in the transplastomic lines. (A) Concentration of CO₂ in the substomatal cavity (C_i). (B) stomatal conductance (g_s). C_i and g_s were measured in the youngest completely developed leaf at light saturation. Results are the mean of 6 biological replicates ± SEM. Statistical analysis was performed by Kruskal-Wallis analysis using the Dunn's multiple comparison test. LiHsp83-SAG1 vs WT, SAG1 and GRA4: * p < 0.05.

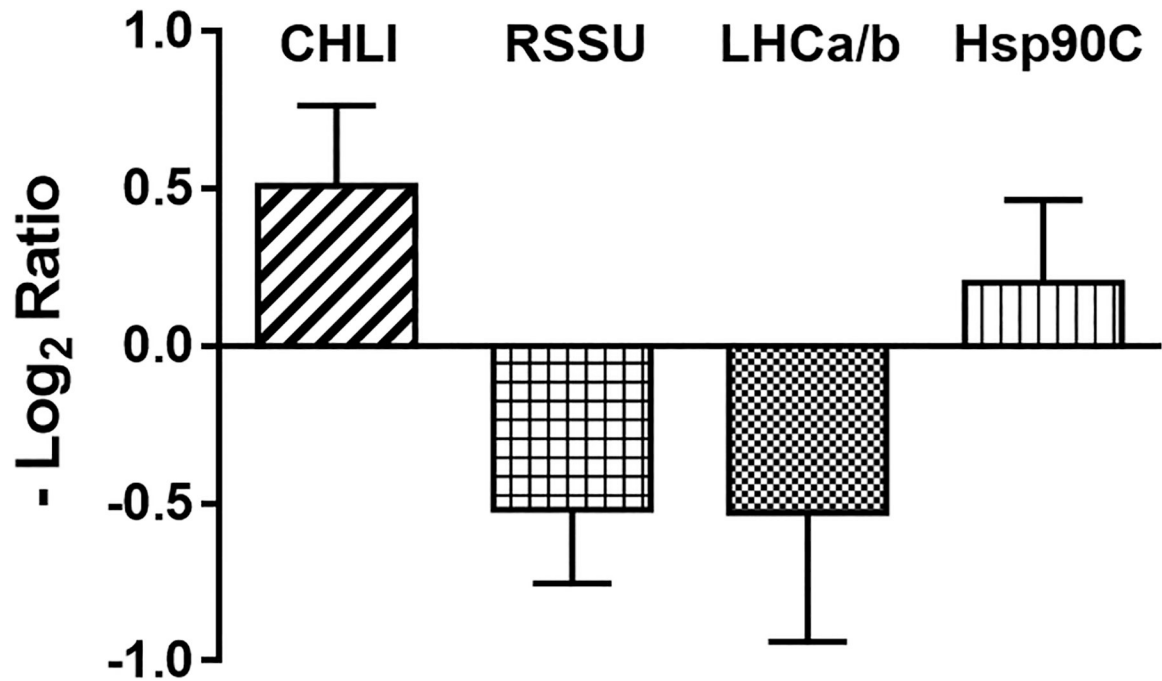


Fig. 5.

Analysis of the expression profile of photosynthesis- and heat-related genes. qRT-PCR was used to analyze the abundance of CHLI; RSSU; LHCa/b; Hsp90C transcripts in the LiHsp83-SAG1 plants. The transcript levels were normalized to WT transcript level. *NtEFa* gene was used as an internal control. Three technical replicates were performed per experiment, and three independent experiments were performed. The expression analysis was assessed in 47-day-old plants.

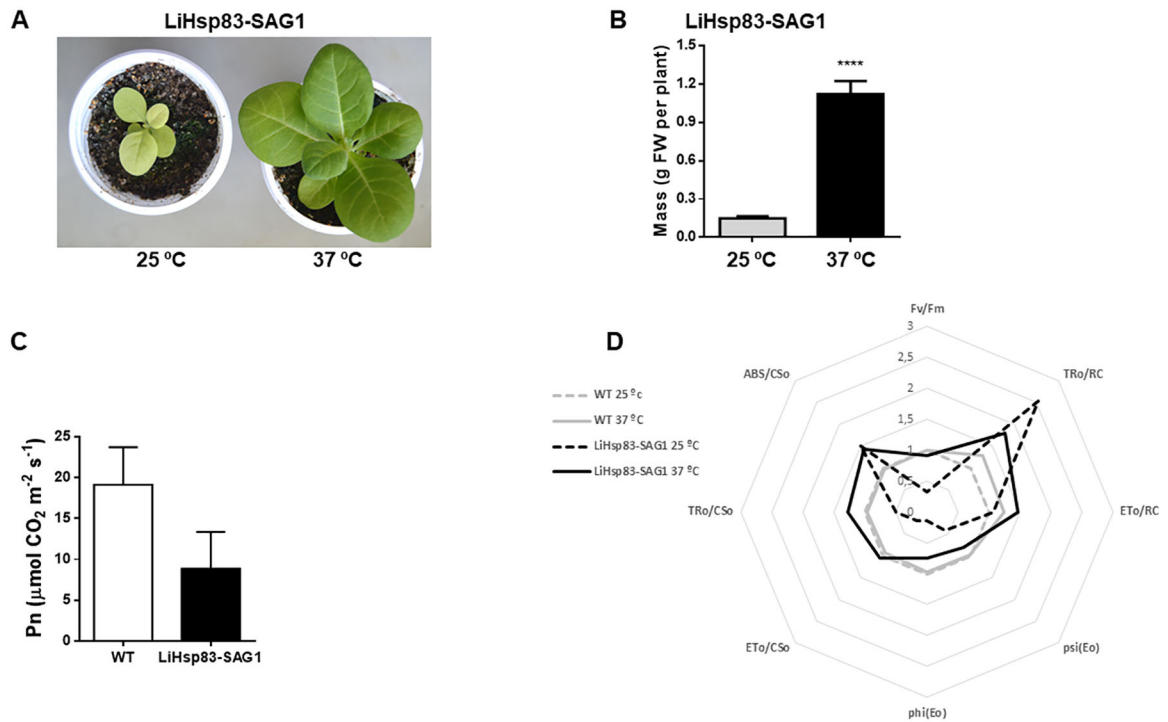


Fig. 6. Analysis of the reversion of pleiotropic phenotype in 60-days-old LiHsp83-SAG1 plants. (A) Image of LiHsp83-SAG1 plants grown at 25 °C or 37 °C for 3 weeks. (B) Aerial mass of LiHsp83-SAG1 plants grown at 25 °C (grey bar) or heat-treated at 37 °C for 3 weeks (black bar). Results are the mean of 10 biological replicates \pm SEM. Statistical analysis was performed using the unpaired t-test with Welch's correction. **** $p < 0.0001$. (C) Quantification of the net photosynthesis (Pn) in WT and LiHsp83-SAG1 heat-treated plants at 37 °C for 3 weeks. Pn was measured in the youngest completely developed leaf at light saturation. Statistical analysis was performed using Mann-Whitney test. (D) Analysis of photosynthetic parameters in WT and LiHsp83-SAG1 plants grown at 25 °C or 37 °C for 3 weeks. Mean values of 8 OJIP parameters are shown in radar charts for WT at 25 °C (grey dotted line), LiHsp83-SAG1 at 25 °C (grey solid line) and LiHsp83-SAG1 at 37 °C (black solid line). Results are expressed as relative to WT, which was assigned to 1. The definition of each parameter is provided in Table S2.

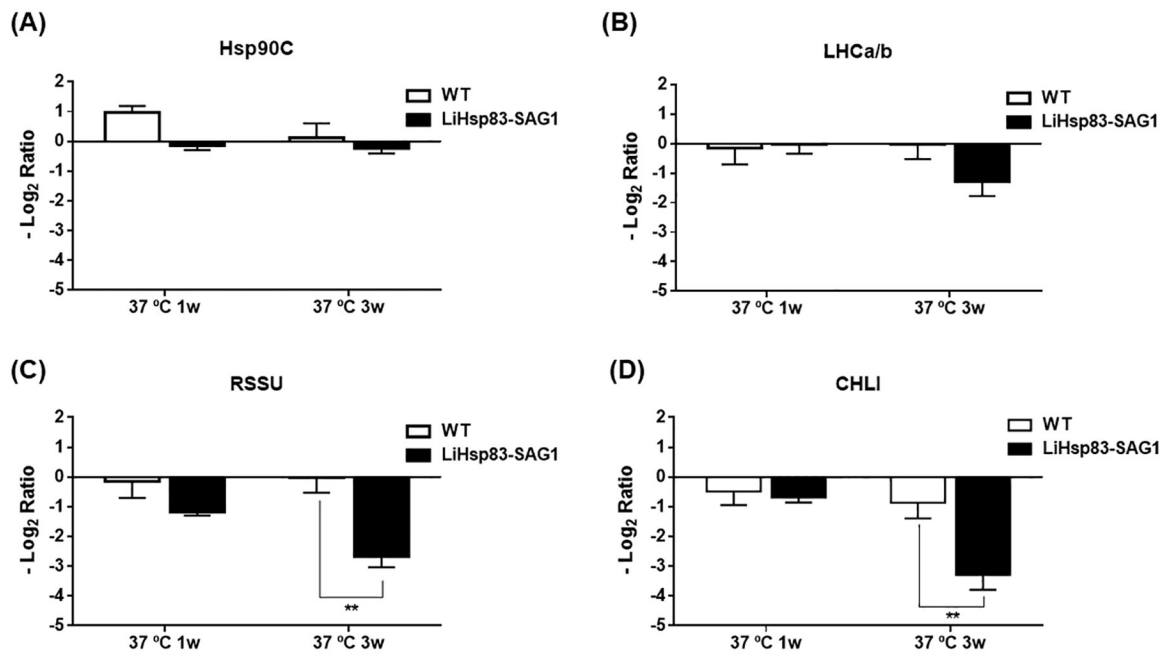
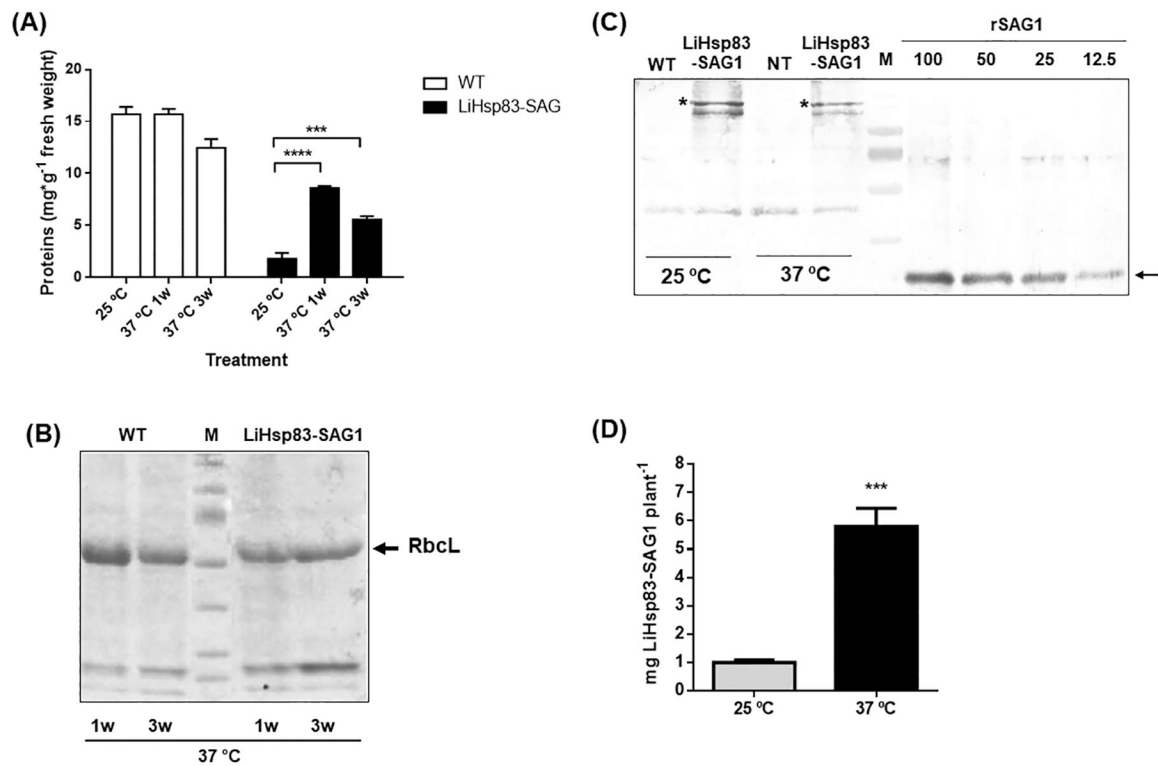


Fig. 7.

Analysis of the expression profile of photosynthesis- and heat-related genes. qRT-PCR was used to analyze the abundance of (A) Hsp90C; (B) LHCa/b; (C) RSSU; (D) CHLI transcripts in WT and LiHsp83-SAG1 plants exposed at 37 °C for 1 week (1w) or 3 weeks (3w). The transcript levels at 37 °C were normalized to transcript level at 25 °C. *NtEFa* gene was used as an internal control. Three technical replicates were performed per experiment, and three independent experiments were performed. The expression analysis was assessed in 60-day-old WT and LiHsp83-SAG1 plants. ** $p < 0.01$.

**Fig. 8.**

Analysis of the heat treatment effect on WT and LiHsp83-SAG1 plants. (A) Content of total protein in WT and LiHsp83-SAG1 plants maintained either at 25 °C, 37 °C 1 week (1w) or 37 °C 3 week (3w) quantified by RC-DC method. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's Multiple Comparison Test. LiHsp83-SAG1 25 °C vs LiHsp83-SAG1 37 °C 1w and 3w. *** $p < 0.001$; **** $p < 0.0001$. (B) Analysis of RbcL protein in WT and LiHsp83-SAG1 grown at 37 °C for 1w or 3w. Total soluble proteins (25 μg) extracted from WT or LiHsp83-SAG1 plants electrophoresed on SDS-PAGE gels and stained with Coomassie brilliant blue. Arrow points Ribulose biphosphate carboxylase large subunit (RbcL). (C) Quantification of LiHsp83-SAG1 by Western blot analysis in 60-days-old transplastomic plants grown at 37 °C for 3 weeks. Ten micrograms of total protein extracts from WT and LiHsp83-SAG1 plants maintained at 25 °C or at 37 °C for 3 weeks (3w) were immunoblotted with an anti-SAG1 polyclonal antibody. Serial dilution of rSAG1 (100, 50, 25 and 12.5 ng/μl) was used as reference [12]. (D) Estimation of LiHsp83-SAG1 yields per plant. Results are the mean of 4 biological replicates \pm SEM. Statistical analysis was performed using unpaired t test with Welch's correction. *** $p < 0.001$.

Table 1

Analysis of PSII efficiency (Fv/Fm and Phi(Eo)), energy flux (ABS/RC, ETo/RC, TRo/RC) and PSII global state (PI_{ABS}) parameters.

LINES	Fv/Fm	Phi(Eo)	ABS/RC	TRo/RC	PI _{ABS}
WT	0.824 ± 0.003 ^a	0.405 ± 0.006 ^a	1.81 ± 0.03 ^a	0.502 ± 0.02 ^a	2.524 ± 0.129 ^a
GRA4	0.808 ± 0.012 ^a	0.327 ± 0.009 ^a	2.18 ± 0.11 ^a	0.482 ± 0.01 ^a	1.258 ± 0.198 ^a
SAG1	0.773 ± 0.008 ^a	0.303 ± 0.007 ^a	2.37 ± 0.06 ^a	0.474 ± 0.02 ^a	1.014 ± 0.072 ^a
LiHsp83-SAG1	0.199 ± 0.007 ^b	0.021 ± 0.003 ^b	16.89 ± 0.59 ^b	1.083 ± 0.08 ^b	0.002 ± 0.001 ^b

Results are expressed as the mean ± SD. Statistical analysis was performed by Kruskal-Wallis analysis using the Dunn's multiple comparison test. Different letters indicate a significance of 0.05. Ten plants from each line were used.

Table 2

Content of RbcL, total proteins, soluble sugars and starch.

LINES	PROTEINS		SUGARS	
	RbcL relative levels (% RbcL band / total bands)	Total proteins (mg g ⁻¹ FW)	Soluble Sugars (mg g ⁻¹ FW)	Starch (mg Glc g ⁻¹ FW)
WT	65.71	11.72 ± 0.89 ^a	9.24 ± 1.34 ^a	84.53 ± 11.84 ^a
GRA4	60.85	9.05 ± 0.74 ^a	7.95 ± 1.34 ^a	82.93 ± 11.64 ^a
SAG1	59.27	9.45 ± 0.59 ^a	6.62 ± 2.02 ^a	78.02 ± 13.33 ^a
LiHsp83-SAG1	26.17	4.17 ± 0.25 ^b	<i>Non-detectable</i>	3.91 ± 1.27 ^b

Results are expressed as the mean ± SD. Statistical analysis was performed by Kruskal-Wallis analysis using the Dunn's multiple comparison test. Different letters indicate a significance of 0.05. Ten plants from each line were used.