**Unusual tolerance to high temperatures in a new herbicide resistant D1 mutant
from *Glycine max* (L.) Merr. cell cultures deficient in fatty acid desaturation**

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Abstract. The unusual tolerance to heat stress found in STR7, an atrazine resistant mutant isolated from photosynthetic cell suspension cultures of soybean (*Glycine max* L. Merr. var. Corsoy) and previously characterised (Alfonso et al. 1996, Plant Physiol 112: 1499-1508) has been studied.

The STR7 mutant maintained normal growth and fluorescence parameters at temperatures higher than the WT. The temperature for 50% inactivation of the oxygen evolving activity of STR7 thylakoids was 13 °C higher than in the WT. SDS-PAGE and immunoblot analysis with specific antibodies revealed that integrity of PSII in the STR7 mutant was maintained at higher temperatures than in WT. This unusual-intrinsic tolerance to high temperatures contrasted with the higher sensitivity to heat-stress reported as a feature linked to the triazine resistance trait. The chloroplast membrane of STR7 accumulated an unusually high content of saturated C16:0 and reduced levels of C16:1 and C18:3 unsaturated fatty acids with respect to the WT. Between all the lipid classes, chloroplastic lipids synthesized via the prokariotic pathway (MGDG, PG and DGDG) that represented more than 75% of the total lipid classes, showed the most significant differences in C16:0 and C18:3 levels. In addition, changes in the physico-chemical properties of the thylakoid membrane and chloroplast ultrastructure were also detected.

Key words: Cell cultures - Fatty acid unsaturation - *Glycine* - Heat-stress - Herbicide resistance - *psbA* mutant

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Abbreviations: BHT = butylated hydroxytoluene; Chl = chlorophyll; DCBQ = 2,6 dichloro benzoquinone; DGDG = di-galactosyl-diacyl-glycerol; DSC = differential scanning calorimetry; FDA = fluorescein diacetate; HSP = heat-shock protein; LHC = light harvesting complex; LMW = low molecular weight; MGDG = mono-galactosyl-diacyl-glycerol; OEE = oxygen evolving extrinsic protein; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PI = phosphatidylinositol; PS = photosystem; Q_A = primary quinone acceptor; Q_B = secondary quinone acceptor; RC = reaction center; SL = sulpholipid

Introduction

Exposure of photosynthetic organisms to heat stress induces inactivation of photosynthesis whether the stress is applied to whole plants, intact tissues or isolated organelles (for review see Berry and Björkman 1980). The photosystem II (PSII) complex is the most susceptible to heat among the components of the chloroplast membrane (Berry and Björkman 1980). Substantial inactivation of PSI is not detected until temperatures are reached that cause complete inactivation of PSII activity (Berry and Björkman 1980). Within PSII, the oxygen-evolving system is most susceptible to heat stress, although both the reaction center (RC) and the light harvesting complexes (LHCs) can be damaged by high temperatures as well (Berry and Björkman 1980; Takeuchi and Thornber 1994; Enami et al. 1994; Shutilova et al. 1995).

Thermotolerance of PSII and the adaptation of the photosynthetic machinery to heat-stress vary widely among species (Berry and Björkman 1980) and several protective mechanisms have been reported. Xanthophyll-cycle carotenoids (Havaux et al. 1996) or isoprene (Sharkey and

Singaas 1995) were reported to have a protective effect. A role of LHCII in the stabilization of PSII against heat has also been described (Havaux and Tardy 1997). Stabilization of the oxygen-evolving machinery *via* small proteins located on the thylakoid membranes seemed to play an important role in protecting the entire photosynthetic system from heat inactivation in cyanobacteria (Nishiyama et al. 1997) and higher plants (Heckathorn et al. 1998). Since most of the photosynthetic complexes are integrated within the thylakoid membrane, changes with temperature in the structure of these membranes are expected to have effects on PSII function and integrity (Berry and Björkman 1980). The physical and biochemical characteristics of membrane lipids depend on the unsaturation level of their fatty acids and the physical state of lipids in biological membranes influences the physiological function of these membranes (Nishida and Murata 1995; Tasaka et al. 1996). The role of membrane lipids on low- and high-temperature tolerance is still not well understood. Genetic manipulation of fatty acid unsaturation modifies the chilling sensitivity of prokaryotes and higher plants (for review see Nishida and Murata 1995; Moon et al. 1995). However, elimination of trienoic and dienoic lipid molecules did not enhance PSII activity against heat stress in *Synechocystis* (Gombos et al. 1994). In contradiction with this, Pearcy (1978) and Raison et al. (1982) observed that increases in growth temperature increased the level of saturated fatty acids in membrane lipids and enhanced the heat stability of photosynthesis. Similarly, highly saturated lipids were isolated from thermophilic cyanobacteria (Mannock et al. 1985). An increased thermal stability of higher plant thylakoids following catalytic hydrogenation of membrane lipids has also been described (Thomas et al. 1986). This hypothesis was supported by results obtained in *Arabidopsis* mutants demonstrating an enhanced thermal tolerance of photosynthesis in the *fadC* mutant deficient in 16:1 and 18:1 unsaturation (no 16:3 and 18:3; Hugly et al. 1989) and in the *fabB* mutant from *Arabidopsis* deficient in palmitic acid unsaturation (high 16:0 levels and no 16:1; Kunst et al. 1989). More recently, decreases in trienoic fatty acids with concomitant increases in dienoic 16:2 and 18:2 provoked an increased tolerance to high temperatures in tobacco transgenic lines (Murakami et al. 2000).

Herbicide resistant mutants have provided an excellent tool to study the PSII structure and function or stress factors affecting PSII activity. Such mutants from higher plants present a single

mutation in the *psbA* gene coding for the D1 RC subunit that results in an altered herbicide binding and a decrease in the rate of $Q_A \rightarrow Q_B$ electron transport rate (Hirschberg and McIntosh 1983). Atrazine resistant mutants usually present additional pleiotropic effects such as an increased sensitivity to strong illumination (Sundby et al. 1993; Alfonso et al. 1996), changes in antenna size and chloroplast ultrastructure (Burke et al. 1982; Lemoine et al. 1986; De Prado et al. 1992) or changes in lipid/fatty acid composition (Pillai and St John, 1981; Burke et al. 1982; Lemoine et al. 1986; De Prado et al. 1992). Interestingly, a higher sensitivity to heat-stress has also been reported as a feature linked to the triazine resistance trait (Ducruet and Lemoine 1985; Ducruet and Ort 1988; Havaux 1989; Dulai et al. 1998).

We have previously described a new atrazine resistant mutant, STR7, isolated from soybean photosynthetic cell suspension cultures with a single new mutation in the chloroplastic *psbA* gene (Alfonso et al. 1996). This mutation induced the substitution of Ser268 to Pro in the D1 subunit of the PSII reaction center. The S268P substitution was different to the S264G or T reported in other higher plant resistant biotypes. In fact, the STR7 mutant belongs to a new class of atrazine resistant mutants affected in the redox couples involving Q_A and Q_B (Alfonso et al. 1996). In the present work we have used WT and STR7 cell lines as a tool to study the effect of heat stress on PSII function. A complete series of biochemical and physiological experiments has been performed, indicating that the STR7 mutant presented an intrinsic and unusual tolerance to heat stress. The possible causes of this unexpected phenotype will be discussed.

Materials and methods

Cell suspension culture conditions and temperature experiments. Photosynthetic cell suspension cultures from soybean (*Glycine max* L. var Corsoy) WT and STR7 mutant were grown as previously described (Alfonso et al. 1996). The inoculum from which WT cultures were started was kindly provided by Dr. Jack M. Widholm (University of Illinois at Urbana-Champaign, Ill., USA). Both cell suspensions were cultured at 25 °C under continuous light ($75 \mu\text{mol m}^{-2} \text{s}^{-1}$) on a rotatory shaker at 130 rpm in a 5% CO₂ atmosphere. For high temperature treatment, cells were placed in a similar incubator, under the same growth conditions except that the temperature was increased to 40 °C for short-time (hours) or long time (days) analyses.

Cell growth and viability. Cell growth rates were determined by measuring the “packed cell volume” (Alfonso et al. 1996). Cell viability was determined by the fluorescein-diacetate method (FDA). Viable cells were quantified under an optical fluorescence microscope (American Optical AO111, Buffalo, NY, USA). The analysis of the microscope images was performed using the public software domain NIH Image.

Chlorophyll fluorescence measurements. Chlorophyll fluorescence induction curves were recorded as previously described (Alfonso et al. 1996). The sample ($15 \mu\text{g Chl mL}^{-1}$) was stirred in a thermostated Clark-type cuvette and the actinic light intensity was $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ on the surface of the cuvette.

Measurement of photosynthetic activity. Photosynthetic activity of thylakoid membranes isolated from WT and STR7 cells grown at control temperature was measured at 25 °C with a Clark-type oxygen electrode fitted with a thermostated water jacket (Hansatech, Norfolk, UK) as previously described (Alfonso et al. 1996). The standard assay medium consisted of 300 mM sucrose, 10 mM NaCl and 25 mM Mes-NaOH (pH 6.5). 2,6 Dichloro benzoquinone (DCBQ) was used as an

artificial electron acceptor. Thylakoids from WT and STR7 were incubated in a thermostated bath in the dark at different increasing temperatures for 30 min before measuring photosynthetic activity.

Isolation of chloroplasts and SDS-PAGE of chloroplastic proteins. Chloroplasts from WT and STR7 cell lines were isolated from 14 d-old cultures as described in Alfonso et al. (1996). Chloroplasts were routinely stored at -80 °C in buffer B2 (150 mM NaCl, 5 mM MgCl₂, 300 mM sucrose, 10 mM Hepes-NaOH pH 7.5) until use. SDS-PAGE of chloroplastic proteins from WT and STR7 was carried out in a 12-22% acrylamide gradient gel containing 4 M urea in both resolving and stacking gels. Samples were denatured at room temperature in sample buffer containing 50 mM Tris-HCl, pH 7.2, 2% SDS, 2 M urea, and 40 mM dithiothreitol. Gels were stained with Coomassie Brilliant Blue R-250.

Immunoblot detection of specific PSII proteins. Chloroplast proteins were separated under denaturing conditions in 15% acrylamide gels in the presence of 6 M urea. Gels were electroblotted onto nitrocellulose membranes (BioRad transfer system) and probed with antiserum against specific PSII proteins. The antibody used to detect the D1 protein was raised against a synthetic peptide homologous to the N-terminus of the D1 protein, and it was a kind gift from Dr. Autar K. Matoo (Plant Molecular Biology Laboratory, US Department of Agriculture, Beltsville, Md., USA). Antibodies used to detect the LHCII and the 33 kDa extrinsic protein were raised in rabbit against the same proteins from spinach and were a kind gift from Dr. Matilde Barón (Estación Experimental del Zaidín, CSIC, Granada, Spain).

Lipid analysis. Total lipids were extracted from thylakoid suspensions with chloroform-methanol (2:1, v:v) as described by Bligh and Dyer (1959). Lipid classes were separated by thin-layer chromatography on silica gel G60 plates which were developed with chloroform:acetone:methanol:acetic acid:water (50:20:10:10:5; by vol.). Amounts of the total lipids or the different lipid classes were determined gravimetrically as reported by Herbes and

Allen (1983). The bands corresponding with total SL, PI, PE, PC, DGDG, PG and MGDG were visualised under exposure to iodine vapour. Bands corresponding to total lipid classes were scrapped from chromatographic plates and transmethylated with a 80 °C treatment with 2.5% (w/v) H₂SO₄ in methanol for 90 min. In order to avoid oxidation of fatty acids during the separation and fractionation procedure, 3 µg mL⁻¹ of BHT as antioxidant were added to the lipid extracts. Fatty acid methyl esters were analysed by gas-liquid chromatography using a Shimadzu GC70 equipped with a flame ionization detector. A 300 x 0.25 cm stainless steel column (Cromosorb W-AW 80-100 mesh, Supelco, Bellefonte, Pa., USA) was used. Both injector and detector temperature were maintained at 250 °C. For quantitative determination of fatty acids, a known amount of heptadecanoic acid was added as an internal standard to each lipid sample prior to the transesterification reaction.

Differential scanning calorimetry (DSC). Freshly isolated thylakoid membranes from WT and STR7 mutant cells were resuspended in 50 mM Hepes-NaOH buffer, pH 7.5 at a chlorophyll concentration of 1.5 mg Chl mL⁻¹. Buffer alone was used in the reference cuvette for baseline corrections. Differential scanning calorimetry was performed in a Perkin-Elmer microcalorimeter (DSC-2) operating at a scanning rate of 1 °C/min as previously reported in other calorimetric studies with thylakoid membranes (Nolan et al. 1992; Tasaka et al. 1996). Temperature and enthalpy calibration was achieved using Indium standards. The same volume of sample or buffer alone were used in both sample and reference cells, respectively. A buffer alone baseline was also run at the same scan rate (1 °C/min). Data were collected from -10 to 70 °C. Immediately upon completion of the first heating scan, the calorimeter was cooled and a second upscan was performed following equilibration. Normally, 1 h was needed before starting the second upscan, but a longer waiting time was occasionally used.

Transmission electron microscopy of chloroplasts. Cell suspension cultures (small aggregates) fixed with 3% (w/v) glutaraldehyde in 25 mM sodium cacodylate buffer, pH 7.0 for 3 h and postfixed with 1% (w/v) osmium tetra-oxyde in the same buffer for 2 h, were dehydrated with

acetone and embedded in Araldite. Ultrathin sections (Reichert Ultracut Leica ultra-microtome) were observed with a TEM 910 (Zeiss, Jena, GE). Quantitative measurements of membrane profiles on electron micrographs were made on sections of several chloroplasts from both WT and mutant lines. Granal and stromal assignments were made as described in Hugly et al. (1989).

Results

Effect of high temperatures on cell growth and viability

To study the long-term effects of high temperatures on our soybean cell cultures, we examined the effect of increasing temperature on cell growth. Under normal growth conditions, the photomixotrophic cell suspension cultures are morphologically and physiologically (photosynthesis, CO₂ fixation rate, fatty acid composition) similar to photoautotrophic cultures or meristematic leaf cells (Martin et al., 1984). The WT and STR7 cell lines were grown in liquid medium at 25 °C (normal growth conditions) and 40 °C (heat stress conditions) under the same light and CO₂ conditions. After 2-3 days, the STR7 mutant grew slightly slower than the WT at 25 °C; the doubling time was 5 d compared to 3 d for WT (Fig. 1A). When the temperature was increased to 40 °C the growth profile of WT was seriously affected. The WT cells grew very slowly for a period of time and then failed to grow at all. On the contrary, STR7 was able to grow at levels only slightly lower than those obtained at 25 °C (Fig. 1B).

The visible effects of WT and STR7 mutant exposed to high temperatures are shown in Fig. 2. The WT and STR7 mutant cells were grown in plates for two weeks under control conditions (25 °C, 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 5% CO₂). The cells were then cultured under similar growth

conditions except that temperature inside the incubator was 40 °C. After 3 d at 40 °C, WT cells showed a brownish colour indicating that chloroplasts had degenerated and were almost dead (Fig. 2). On the contrary, STR7 cells were less affected maintaining a green healthy colour (Fig. 2).

To corroborate the results, we determined the viability of the cultures at 25 °C and 40 °C using the FDA (fluorescein diacetate) method. Special care was taken to reduce the cell aggregate size by carefully passing the cultures through a syringe prior to the addition of the FDA reagent. At 25 °C, both cell lines showed a high percentage of viability up to 95% (Table 1). After 3 d of treatment at 40°C, WT cells were almost dead; the percentage of viability was less than 5 % (Table 1). On the contrary, STR7 cells maintained their viability in more than 65% (Table 1). All these results indicated that STR7 has an increased tolerance to high temperatures.

Long-term (days) and short-term (hours) temperature effects on PSII fluorescence induction kinetics

We also examined the long-term effect of high temperatures on the fluorescence parameters. Cells in the early stationary phase were maintained at 25 and 40 °C during 3 d and fluorescence induction curves were recorded. The initial fluorescence level (F_0) and the maximum fluorescence level (F_m) after illumination were measured. Variable fluorescence ($F_m - F_0$) was obtained and the F_v/F_m ratio (reflecting the quantum yield of PSII primary photochemistry) was calculated. Both WT and STR7 showed normal F_v/F_m values at 25 °C (Table 1). However, when cells were maintained at 40 °C the F_v/F_m ratios decreased about a 85% in WT cells while the STR7 mutant maintained about a 60% of the initial F_v/F_m values (Table 1).

The PSII complex is considered the primary site for damage induced by heat stress. We examined therefore the short-term effects of temperature on PSII activity in both cell lines in vivo by measuring PSII fluorescence induction kinetics. WT and STR7 cells were exposed to higher temperatures for 2 h. No significant differences in the F_v/F_m ratio were found between 20 to 30 °C (Fig. 3A). However, at temperatures higher than 30 °C, the WT cells showed a small but continuous decrease of the F_v/F_m ratio after 2-h exposure to high temperature. The F_v/F_m ratio decreased about 20% after 2-h incubation at 40 °C. Larger differences were not expected since a

2-h treatment should not be long enough to induce severe damage to the photosynthetic machinery. On the contrary, Fv/Fm values in STR7 cells remained quite stable and close to normal control values (0.73-0.75) in the same temperature range (Fig. 3A).

The initial fluorescence level, F_0 , has been commonly used as an indicator of the sensitivity of PSII to heat-stress (Schreiber and Berry 1977; see also Buckov et al., 1980). Changes in the F_0 level with temperature were monitored in both cell lines (Fig. 3B). The WT showed an increase of the F_0 value in the 30-40 °C range after 2-h incubation at higher temperatures. This result indicated that the effect of temperature on PSII was very rapid and specific in the WT. On the contrary, F_0 values for STR7 were not modified in the same range of temperatures (Fig. 3B), indicating that the temperature causing damage of PSII is higher than 40 °C in the mutant. Most intriguing are the differences found in the F_0 level between both cell lines at control temperatures. These differences could be due to a larger antenna size or a higher amount of Q_A^- in the dark as a result of the stabilization of Q_A^- and Q_B^- states reported in STR7 (Alfonso et al. 1996).

Thermal tolerance of isolated thylakoid membranes

The effects of incubation at high temperatures on the oxygen evolving activity of PSII, i.e. the transport of electrons from water to DCBQ, of WT and STR7 thylakoid membranes were also compared (Fig. 4). Thylakoids were isolated from WT and STR7 cells grown at 25 °C, incubated for 30 min at different increasing temperatures and then photosynthetic activity was measured. The inactivation profiles of photosynthetic oxygen evolution with higher temperatures of WT and STR7 thylakoids were similar. However, the temperature for 50% inactivation of the oxygen-evolving activity of WT was 34 °C while the same parameter for the STR7 mutant was about 47 °C, 13 °C higher than the WT.

Involvement of protein factors in heat-stress tolerance

To analyze the intrinsic characteristics of the heat-tolerant mutant we have chosen a heat-exposure period of 6 h exceeding the shorter induction periods. We examined whether the differences found in the thermal behaviour of both WT and STR7 cell lines were due to changes in

the chloroplastic proteins like in cyanobacteria (Nishiyama et al. 1997) or higher plants (Heckathorn et al. 1998). The polypeptide composition from chloroplasts isolated from WT and STR7 cell lines grown at 25 °C (control temperature) and cells incubated at 40 °C for 6 h was compared. At control temperatures no major differences in the polypeptide composition were found between both cell lines (Fig. 5A, lanes 1 and 2). On the contrary, exposure of WT cells to 40 °C during 6 h affected many of the proteins of the thylakoid membrane. The most remarkable effect of high temperatures on chloroplast membranes from both WT and STR7 cells was the appearance of a strong band around 15-17 kDa and other band around 23 kDa in both cell lines (Fig. 5A, lanes 3 and 4). These proteins were heat-specific and had a molecular mass similar to several HSP previously reported in the literature (Vierling 1991; Nishiyama et al. 1997; Heckathorn et al. 1998). The amounts of these low molecular weight proteins were similar in both cell lines under the same temperature treatment, suggesting that factors other than the heat-induced proteins are responsible of the increased tolerance to heat-stress observed in STR7.

Analysis of the SDS-PAGE profile showed that several thylakoid proteins were also affected by the heat treatment. Thus, the amount of proteins ranging from 24 to 27 kDa decreased in WT cells after a 6 h of heat incubation (Fig. 5A, lane 3). These proteins may correspond to the major LHCII complex proteins and other minor antenna complexes. In addition, a decrease of protein levels in the region of 32-33 kDa was also observed in WT (Fig. 5A, lane 3). These differences were not found in STR7 mutant cells (Fig. 5A, lane 4). Specific antibodies against D1, the LHCII complex and the 33 kDa extrinsic protein were used to assess the changes in these thylakoid protein levels during the heat treatment. To that end, cells were incubated at 40 °C during 2 or 6 h, and then thylakoids were extracted, proteins separated in polyacrylamide gels and blotted against specific antibodies. The results are shown in Fig. 5B. Incubation of WT cells at 40 °C resulted in progressive loss of the extrinsic 33 kDa protein, the LHCII proteins and also the D1 RC subunit. These results were consistent with previous observations (Enami et al. 1994; Takeuchi and Thornber 1994; Shutilova et al. 1995). Loss of the 33 kDa extrinsic protein was slightly faster than LHCII polypeptides, that remained as prominent thylakoid membrane components. This fact may correlate with the fast inactivation and release of the OEE proteins at

high temperatures (Enami et al. 1994). Interestingly, thylakoids from stressed STR7 mutant cells did not show the same behaviour and levels of these proteins were essentially similar to those observed under control conditions (Fig. 5B). These data indicated that the integrity of the PSII in the STR7 mutant was maintained at higher temperatures than in WT.

Lipid and fatty acid composition from WT and STR7 thylakoid membranes

Thylakoid membrane lipids isolated from the WT and STR7 mutant cells were analyzed to determine possible differences in fatty acid or glycerolipid composition. Thylakoid lipids included seven types of glycerolipids (Table 2). The galactolipids (MGDG and DGDG) were the main lipids, representing about 75 mol % of total glycerolipid composition, whereas the remaining 25 mol % was attributed to a mixture of five different phospholipids. In general, there was no significant difference in the proportion of each glycerolipid component between WT and STR7 mutant (data not shown). On a chlorophyll basis, the thylakoids from both cell lines also contained a similar amount of total lipids. On a protein basis, the total lipid to protein ratio (w/w) was also similar in both cell lines obtaining values of **1.02 and 1.21 for WT and STR7**, respectively. However, remarkable differences were found between individual fatty acids of the respective glycerolipids from both cell lines (Table 2). The most abundant fatty acid detected were linolenic acid (18:3), linoleic acid (18:2) and palmitic acid (16:0) that represented around 70, 14 and 10% of total fatty acids respectively in WT cells cultured at 22 °C for 3 weeks. These data are in agreement with previous analysis carried out in the same soybean photoautotrophic cell line (Martin et al. 1984). Compared with WT, the STR7 mutant presented increased levels of 16:0 (around 20% of total fatty acids) while levels of 16:1 were nearly undetectable (< 0.05%; Table 2). The differences were particularly high in chloroplastic MGDG, PG and DGDG lipids (about 10-12% higher in STR7 thylakoids). The C18:3 levels also decreased (around 13-14%) in the MGDG, PG and DGDG fractions of STR7 mutant cells and levels of 18:2, 18:1 and 18:0 concomitantly increased (Table 2). These differences were less apparent for phospholipids (PE, PI, PC) **of non-chloroplastic origin**. The ratio of saturated to unsaturated fatty acids for all lipid

classes was always higher in the STR7 mutant (Table 2). Again, the most substantial differences were obtained for MGDG, DGDG and PG fractions (Table 2).

Thermotropic properties of the thylakoid membranes from WT and STR7

We studied the influence of changes in the saturation level of fatty acids from membrane lipids in the thermotropic behaviour of thylakoid membranes isolated from WT and STR7 mutant cells that had been grown at 25 °C using differential scanning calorimetry (DSC). During the first temperature scan one major endothermic peak was observed reproducibly in the low temperature range (0-30 °C). Several endothermic peaks appeared in the high temperature range (30-70 °C). These transitions peaked at about 48, 54, 60 and 67 °C (data not shown). These T_m values fitted well with previous calorimetric studies carried out in thylakoid membranes from higher plants (Nolan et al. 1992). Sample was cooled again and after equilibration a second temperature upscan was carried out. The DSC curves of the second upscan for both WT and STR7 thylakoid membranes and for buffer alone used for baseline subtraction are presented in Fig. 6. Only one major endothermic peak in the low temperature range was observed in both WT and mutant thylakoids. This transition was, within the limits of reproducibility, similar to that from the first scan. The endothermic transitions observed in the 45-70 °C temperature range during the first scan were absent, suggesting that these peaks corresponded to irreversible denaturation of photosynthetic proteins. The reversible endothermic peak in the low temperature range corresponded to the phase transitions of membrane lipids as previously reported (Sanderson and Williams 1992; Tasaka et al. 1996). The temperatures for the onset of phase transition were 8 °C and 13 °C for thylakoid membranes isolated from WT and STR7 cells, respectively, grown at 25 °C (Fig. 6). The temperature neccessary to reach the lipid-crystalline phase in STR7 mutant was 5 °C higher than in WT.

Ultrastructural changes in WT and STR7 mutant chloroplasts

The ultrastructure of chloroplasts from both WT and STR7 mutant cells grown at 25 °C was studied by transmission electron microscopy. Morphometric analysis of chloroplasts from the

STR7 mutant and WT cells is shown in Table 3. The STR7 mutant had grana of smaller size but were more numerous than in WT and less organized (Table 3). The STR7 mutant presented an almost double (93%) increase in the number of grana per plastid. This higher grana content in the mutant was compensated with a smaller granal width (about 47% with respect to WT) and reduced number of thylakoids per granum (Table 3). Another **striking** difference between chloroplasts from both cell lines was the stromal thylakoid length, that was about 83 μm longer in WT chloroplasts. In STR7, small grana stacks (2-3 lamellae) connected the larger grana stacks together, as would stroma lamellae in the WT chloroplast (Lemoine et al. 1986; Hugly et al. 1989). Finally, even if chloroplasts from the STR7 mutant were slightly bigger in size with respect to the WT, the ratio of granal surface to total chloroplast surface was very similar in both cell lines. This resulted from the decreased number of thylakoids per grana and the smaller width of granae in chloroplasts from the STR7 mutant. Concomitantly with the higher grana content found in the STR7 mutant, an increase in the amount of the Chl a/b light harvesting complex and a decrease in the Chl a/b ratio was also observed (Alfonso et al. 1996).

Discussion

In this paper we report the unusual tolerance to heat-stress observed in STR7, a new atrazine resistant D1 mutant from soybean previously characterised in our laboratory. Tolerance to high temperatures determined as cell growth and viability, as maintenance of the PSII function and integrity, reflected by the fluorescence induction parameters and oxygen measurements was higher for STR7 than the WT. The SDS-PAGE and Western blot analysis of thylakoid proteins showed that the loss of specific PSII proteins in WT did not occur in the STR7 mutant after high temperature treatment. This phenotype is unusual as all the resistant biotypes studied up to now presented an increased susceptibility to heat-stress (Ducruet and Lemoine 1985; Ducruet and Ort 1988; Havaux 1989; Dulai et al. 1998).

Several different mechanisms related to protection against high temperatures have been reported (Berry and Bjorkman 1980; Havaux et al. 1996; Havaux and Tardy 1997; Heckathorn et al. 1998). Levels of heat-shock proteins (HSPs) increased in both WT and STR7 mutant cells after exposure to high temperatures. This increase was particularly relevant for the LMW-HSPs (approximately 17-30 kDa) that in many plant species are the most abundant proteins induced by heat-stress (Vierling 1991; Heckathorn et al. 1998). However, as their levels were similar in both cell lines, involvement of HSPs in the thermal tolerance found in the STR7 mutant seems to be **highly** unlikely. Our data indicate that the thylakoid membranes from the STR7 mutant accumulated an unusually high content of saturated C16:0 and reduced levels of C16:1. In parallel, a slight but significant reduction of trienoic C18:3 with a concomitant increase in 18:0, 18:1 and 18:2 fatty acids with respect to the WT was also detected. The role of lipids on low and high temperature tolerance has been **a** subject of considerable experimentation, but it still remains **unclear subject of much debate**. Our results are supported by physiological studies (Percy 1978; Raison et al. 1982; Thomas et al. 1986), *Arabidopsis* mutants (Hugly et al. 1989; Kunst et al. 1989) or more recent observations obtained in tobacco transgenic lines (Murakami et al. 2000) deficient in trienoic fatty acids. However, they contradict those reported in cyanobacteria (Gombos et al. 1994) or transgenic tobacco plants (Moon et al. 1995). Reasons for these

contradictions are not clear. With the exception of the tobacco mutants described by Moon et al. (1995), genetic manipulation of the extent of fatty acid unsaturation (decrease of unsaturated fatty acids) in higher plants resulted in a thermotolerant phenotype (Kunst et al. 1989; Hugly et al. 1989; Murakami et al. 2000). Transgenic tobacco plants with increased 16:0 levels in PG lipid class presented no differences in PSII activity against high or low temperature (Moon et al. 1995). However, total % of PG in their transformed plants was 7% (Moon et al. 1995) of total lipid species so even a 14% increase in 16:0 levels in PG may not be enough to induce changes in heat tolerance. Interestingly, our STR7 mutant from soybean or those from *Arabidopsis* with enhanced thermal tolerance (Hugly et al. 1989; Kunst et al. 1989) presented increased 16:0 levels with very small amounts of monoinsaturated 16:1 in MGDG, PG and DGDG chloroplastic lipids that represent > 75% of the total lipid classes. Thus, a modification of the unsaturation level of major lipid classes like MGDG, should induce significant changes affecting function and integrity of the photosynthetic membranes.

It has been proposed that increased saturation of fatty acids from membrane lipids may raise the temperature at which lipids such as MGDG phase-separate into non-bilayer structures, which disrupt membrane organization (Thomas et al. 1986; Tasaka et al. 1996; Murakami et al. 2000). According to this hypothesis, plant tolerance to higher temperatures might be improved by reducing the content of lower unsaturated fatty acids such as dienoic or monoic fatty acids (Hugly et al. 1989; Kunst et al. 1989). Our data indicate that, in fact, the thermotropic properties of the thylakoid membranes from STR7 are modified with respect to the WT. However, stability of certain protein-lipid interactions that can be directly affected by fatty-acid unsaturation, could also play a role in the stabilization of photosynthetic proteins against heat (Thomas et al. 1986; Kunst et al. 1989).

Many herbicide resistant biotypes present several other changes (like alterations in the chloroplast ultrastructure) that probably develop as a compensatory mechanism to maintain a normal photosynthetic function (Burke et al. 1982; Lemoine et al. 1986; De Prado et al. 1992). The thermal behaviour seems to be a pleiotropic effect of the mutation (Ducruet and Ort 1988; Havaux 1989; Dulai et al. 1998). The STR7 mutant (containing the S268P change) presented an

increase in saturated fatty acids and at the same time a higher thermal tolerance was observed. Other atrazine resistant mutants (containing the S264G change) that showed an increased sensitivity to heat-stress presented an increase in unsaturated fatty acids (Pillai and St. John 1981; Burke et al. 1982). This observation may suggest that different substitutions have different effects on the tolerance/sensitivity to heat stress. Whether or not there is a direct relationship between a single substitution in the D1 RC subunit and the unsaturation degree of fatty acids from membrane lipids and why there seems to be a correlation with specific mutations is still unknown. Interestingly, chloroplastic lipids like MGDG, PG, or DGDG, synthesized in the chloroplast by the prokaryotic pathway, presented a higher degree of saturation while other glycerolipids such as PC, PE or PI, synthesized in the endoplasmic reticulum by the eukaryotic pathway (Miquel and Browse 1998) presented only small changes. These data may hint to a relationship between the photosynthetic reactions and the extent of unsaturation of chloroplastic fatty acids. In chloroplasts from leaves, fatty-acid synthesis is light dependent and regulated (Sasaki et al. 1997; Klyachko-Gurvich et al. 1999). The expression and activity of the FAD7 gene, encoding a ω 3-desaturase was light regulated in *Arabidopsis* (Nisiuchi et al. 1995). Furthermore, Kis et al. (1998) demonstrated that in the cyanobacterium *Synechocystis* PCC 6803, light regulated the expression of the desaturase genes and desaturase activity *via* photosynthetic electron transport. Their results indicated that any factor affecting photosynthetic electron transport may ultimately influence the activity of fatty acid desaturases and then the capability of adaptation to an environmental stress such as temperature. This problem and how a higher saturation of fatty acids from chloroplastic membrane lipids influence the synthesis and assembly of the photosystem components will be the object of future studies in our laboratory.

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FIGURE LEGENDS

Fig. 1. Cell growth curves of WT (●) and STR7 (□) at 25 °C (A) and 40 °C (B). Mean values $\pm$ SE (n=4) of two different determinations from two independent experiments are shown

Fig. 2. Visible damage to WT and STR7 mutant cell lines exposed to high temperatures (40 °C) for 3 d. WT and STR7 cells growing in plates were placed under control conditions ($75 \mu\text{mol m}^{-2} \text{s}^{-1}$) except that temperature was 40 °C

Fig. 3. Short-term effect (2 h) of temperature on the fluorescence induction parameters F_v/F_m (A) and F_0 (B). WT (●) and STR7 (□) cells were grown at 25 °C and incubated at different temperatures for 2 h before measuring the fluorescence induction parameters. The values are the average of three independent experiments

Fig. 4. Profiles of high-temperature inactivation of oxygen evolution in WT and STR7 thylakoid membranes isolated from cells grown at 25 °C. Thylakoids were incubated at designated temperatures in darkness for 30 min. The oxygen-evolving activity was measured at the growth temperature with DCBQ as electron acceptor. Control activity values were 203 ± 23 and $143 \pm 18 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{h}^{-1}$ for WT and STR7, respectively. (●) WT thylakoids; (□) STR7 thylakoids. Values were obtained from three independent experiments

Fig. 5. SDS-PAGE profile (12-22% acrylamide gradient) of chloroplastic proteins from WT and STR7 grown at different temperatures (A). Cells were grown at 25 °C (lane 1, WT; lane 2, STR7). Cells were incubated at 40 °C for 6 h prior to chloroplast isolation (lane 3, WT; lane 4, STR7). Proteins were stained with Coomassie Brilliant-Blue. 25 μg Chl were loaded in each well. (B) Immunoblot of thylakoid samples isolated from control cells and treated during 2 or 6 h at 40 °C. Thylakoids were separated and blotted against specific antibodies. Lane 1, WT control; lane 2,

STR7 control; lane 3, WT 40 °C 2 h; lane 4, STR7 40 °C 2 h; lane 5, WT 40 °C 6 h; lane 6, STR7 40 °C 6 h

Fig. 6. Differential scanning calorimetry of thylakoid membranes isolated from WT and STR7 mutant cells grown at 25 °C. The traces have been displaced in the Y-axis to avoid overlap of the thermograms. DSC curve for WT thylakoids (a), for STR7 mutant thylakoids (b), and buffer baseline (c). Scan rate 1 °C/min. Traces correspond to the 2nd scan. The temperature of phase transition is also indicated

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Table 1. Effect of temperature on the fluorescence induction kinetic and viability of WT and STR7 cells after 3 d of heat treatment. Data represent the average of three independent experiments

	25°C		40°C	
	Fv/Fm	% of viability	Fv/Fm	% of viability
WT	0.75	> 95 %	0.11	5 %
STR7	0.79	> 95 %	0.51	> 65 %

Table 2. Fatty acid composition of the total glycerolipids from thylakoids of WT and STR7 mutant soybean cell lines. Cells were grown at 25 °C. Data represent means of three independent experiments from two different cultures (n = 6). The dash means that the relative amount of fatty acid was less than 0.05%

Lipid Class	Strain	Fatty acid composition (mol %)						
		16:0	16:1	18:0	18:1	18:2	18:3	Rsat/insat
SL	WT	9.1	3.1	1.4	2.5	13.4	70.3	0.12
	STR7	11.4	0.7	2.1	3.4	15.6	64.7	0.16
PI	WT	7.5	2.9	1.5	2.3	14.1	71.7	0.11
	STR7	12.8	0.4	3.4	4.3	15.9	63.2	0.20
PE	WT	8.3	3.6	1.9	2.6	14.6	69.0	0.12
	STR7	13.2	0.6	4.1	5.2	15.6	61.3	0.21
PC	WT	9.7	1.9	1.4	2.8	13.7	70.5	0.13
	STR7	12.9	0.4	3.8	4.1	15.3	63.5	0.20
DGDG	WT	10.2	2.3	1.4	2.4	13.6	70.1	0.13
	STR7	20.1	0.1	2.8	3.9	15.4	57.7	0.30
PG	WT	10.5	2.1	1.6	2.1	14.0	69.7	0.14
	STR7	21.3	-	3.4	4.6	14.9	55.8	0.33
MGDG	WT	7.4	3.0	1.7	2.2	14.3	71.4	0.10
	STR7	19.9	-	3.1	4.3	15.2	57.5	0.30

Table 3. Morphometric analysis of chloroplasts from STR7 mutant and WT soybean cells. Values represent means of five independent determinations from three different micrographs. ^a LSD, least significant difference, $P < 0.05$

Measurement	WT	STR7	LSD (0.05) ^a
Grana/plastid	28.8 ± 5.6	55.8 ± 5.7	8.30
Thylakoids/granum	4.6 ± 1.6	3.22 ± 0.89	0.78
Stromal length (μm)	101 ± 4.5	17.8 ± 7.7	0.18
Granal width (μm)	0.76 ± 0.18	0.36 ± 0.08	0.07
Granal/plastid surface	0.33	0.39	-