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Chronic heat stress affects the photosynthetic apparatus of *Solanum lycopersicum* L. cv Micro-Tom

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Highlights

- Heat stress affects the photosynthetic apparatus of tomato plant.
- Heat stress induces changes in ATP, ADP and sugar concentrations of tomato leaves.
- Rubisco enzyme’s isoforms are differently accumulated in tomato leaves during heat stress.
- Heat stress causes morphological and histological variations in tomato leaves.

Abstract

Tomato (*Solanum lycopersicum* L.) is one of the most widely cultivated crops in the world. Tomato is a plant model and the relationship between yield and biotic/abiotic stress has attracted increasing scientific interest. Tomato cultivation under sub-optimal conditions usually has a negative impact on growth and development; in particular, heat stress affects several cellular and metabolic processes, such as respiration and photosynthesis. In this work, we studied the effects of chronic heat stress on various cytological and biochemical aspects using the Micro-Tom cultivar as a model. Photosynthetic efficiency decreased during heat stress while levels of post-photosynthetic sugars (sucrose, fructose, glucose and glucose 6-phosphate) oscillated during stress. Similarly, photosynthetic pigments (lutein, chlorophyll a, chlorophyll b and β-carotene) showed an oscillating downward trend with partial recovery during the stress-free phase. The energetic capacity of leaves (*e.g.* ATP and ADP) was altered, as well as the Reactive Oxygen Species (ROS) profile; the latter increased during stress. Important effects were also found on the accumulation of Rubisco isoforms, which decreased in number. Heat stress also resulted in a decreased accumulation of lipids (oleic and linoleic acid). Photosynthetically alterations were accompanied by cytological changes in leaf structure, particularly in the number of lipid bodies and starch granules. The collected data indicate that the metabolism of tomato leaves is progressively compromised as the duration of heat stress increases. The present study reports multi-approach information on metabolic and photosynthetic injuries and responses of tomato plants to chronic heat stress, highlighting the plant’s ability to adapt to stress.

Abbreviations:

DAB	Diaminobenzidine
HPLC	High-Performance Liquid Chromatography
HPLC-MS	High-Performance Liquid Chromatography Mass Spectrometry
HSP	Heat Shock Protein
LB	Lipid Bodies
Pi	Performance Index
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive Oxygen Species
RT	Room Temperature
Rubisco	Ribulose 1,5-bisphosphate carboxylase
SuSy	Sucrose Synthase
TEM	Transmission Electron Microscope

1. Introduction

Tomato (*Solanum lycopersicum* L.) is an important fruit plant widely cultivated worldwide and a model plant for studies on the effects of heat stress. Many studies have demonstrated the drastic impacts of heat (*i.e.* 25–30 °C during the daytime and 20 °C at night) on tomato physiology (Nankishore & Farrell, 2016). For example, when the environmental temperature exceeds 35 °C, tomato seed germination, seedling and vegetative growth, flowering and fruit set, and fruit ripening are adversely affected (Foolad, 2005). Several studies have focused on the reproductive process, mainly on meiosis in both male and female organs, pollen germination and pollen tube growth, ovule viability, stigma and style positions, number of pollen grains retained by the stigma, fertilization and post-fertilization processes, growth of endosperm, proembryo and fertilized embryo (Golam et al., 2012). In addition, heat stress can disrupt the relationships between tomato leaves and hydraulic conductivity of roots (Morales et al., 2003). Many studies have evaluated heat tolerance in tomato using various parameters, such as phenotypic index, physiological and biochemical stress indexes, and microscopic observation index (Ayenan et al., 2019). Different cellular and metabolic aspects of tomato plants are the focus of many studies, trying to figure out physiological responses and tolerance mechanisms to heat stress, an imperative goal to maintain crop production. These studies are particularly important in a perspective where the effects of climate change on agricultural productivity could be severe.

The measurement of photochemical efficiency of PSII, based on chlorophyll *a* fluorescence and expressed, as reduction in Fv/Fm, is an effective and non-invasive technique to detect damage in PSII and thus the genotypic differences to heat stress (Zhou et al., 2015). The reduction in Fv/Fm rate does neither take into account a possible decrease of photosynthesis due to a reduced amount of antenna pigments (Camejo et al., 2006), nor possible alterations in the chloroplast ultrastructure (Zhang et al., 2014). Moreover, photosynthesis decrease and carbohydrate accumulation were correlated to a direct damage of leaf ultrastructure (Zhou et al., 2015). As a result, not only chlorophylls and carotenoids in heat-stressed plants undergo variations in content, but also downstream photosynthetic products, *i.e.* carbohydrates, may undergo increased accumulation (Zhou et al., 2020) and transport and/or accumulation to the sink, as recently shown for sucrose (Zhou et al., 2015) and starch.

In particular, heat stress perturbs the sink-source relations by altering the carbon balance. The decrease in leaf photosynthetic efficiency under heat stress leads to an increased carbohydrate demand, which increases dark respiration and photorespiration as a final result (Sharma et al., 2015). However, the effect of heat stress on leaf photosynthesis and carbohydrate metabolism differs between genotypes, allowing its use as a key indicator for the detection of heat susceptibility in plants (Upchurch, 2008). General responses to heat stress include the accumulation of heat shock proteins

(HSPs) and the remodeling of membrane fluidity thus the release of membrane lipids. Increased improper folding of newly synthesized proteins and denaturation of existing proteins leads to their accumulation; the resulting activation of HSPs, expressed in many plant tissues in response to heat stress, can provide a molecular tool for the development of thermo-tolerance (Nover et al., 2001). An increased production of HSPs occurs when plants experience either unexpected or gradual increases in temperature resulting in heat stress, representing one part of complex defense mechanisms (Gupta & Kaur, 2005; Piterková et al., 2013).

Heat stress results in the production of Reactive Oxygen Species (ROS) and invokes oxidative stress responses (Xu et al. 2006). Generating activated oxygen species under heat stress is a symptom of cellular damage, because peroxidation of membrane lipids and pigments compromise membrane permeability and function. ROS cause damage to a wide range of cellular components such as the photosynthetic apparatus and, at the whole plant level, these results in limiting metabolic flux activities thus affecting plant growth and yield by (Foyer & Noctor 2009). However, new evidence showed that oxidative stress and related signaling accompanied heat stress by. Increased protection from heat stress, as mediated by oxidative stress, might be a component of the acquired thermotolerance trait because the activities of ROS scavengers such as ascorbate peroxidase (APX) increase under heat stress conditions (Frank et al., 2009).

The remodeling of membrane fluidity often leads to the release of α–linolenic acid (18:3) from membranes. Changes in unsaturated fatty acid levels in chloroplast membranes, usually due to upregulation of fatty acid desaturase enzymes, has been shown to strongly enhance high-temperature tolerance in plants (Li et al., 2015). The effects of elevated temperature on fatty acid composition of storage lipids have been examined extensively in developing seeds. Changes in glycerolipid composition of soybean seed exposed at high temperatures consisted of an increase in oleic acid (18:1) and a decrease in polyunsaturated fatty acids (18:2 + 18:3), a pattern similar to that of plant leaves acclimatizing to rising temperatures (Dornbos & Mullen, 1992). Thus, increasing the saturation level of fatty acids appears to be critical for maintaining membrane stability and enhancing heat tolerance (Bita & Gerats, 2013).

Heat stress in plants was also correlated to alteration of chloroplast ultrastructure, which directly affects the state of the photosynthetic apparatus and the photosynthesis rate (Zhang et al., 2014). Heat stress damaged the chloroplast structure by disordering the lamellae in the chloroplast and increasing the plastoglobulus number (Gao et al., 2010). Zhou et al., (2015) suggest that heat stress negatively affected the photosynthesis and carbohydrate accumulation by both decreasing the leaf pigment contents and damaging the leaf ultrastructure.

In this work, we investigated the effects of chronic heat stress on selected cytological and biochemical aspects of tomato plants cv Micro-Tom in relation to the mechanism of photosynthesis.

Micro-Tom represents the smallest tomato variety in the world, with a maximum height about 20 cm. This peculiarity is due to the presence of two recessive mutations: one in the *dwarf* gene and the other one in the *miniature* gene (Marti et al., 2006). Availability of sequenced genome, the high density growth capacity (1357 plants / m^2) and the short life cycle (70-90 days) (Sun et al., 2006), make the Micro-Tom an effective model system for the study of plant biology (Shikata & Ezura 2016). High temperature chronic stress was chosen because it mirrors a typical situation where temperatures persist at high levels for several days. This is reasonably expected for a plant such as tomato, which is usually grown in areas with medium to high temperatures. The cv Micro-Tom has been chosen because it allows to process simultaneously an adequate number of plants under controlled conditions. The aim of the work was to evaluate the damage induced to photosynthesis and the responses that tomato could implement. For this purpose, we initially evaluated photosynthetic efficiency as a general parameter, and the concentration of four main sugars produced post photosynthesis (sucrose, fructose, glucose and glucose 6-phosphate) as well as the level of photosynthetic pigments and the concentrations of ATP and ADP. The induction of oxidative stress was evaluated at the entire leaf level. On the protein side, we analyzed the accumulation of sucrose synthase (SuSy), a sucrose-metabolizing enzyme involved in sugar metabolism during heat stress; HSP70, a chaperone stress-relieving protein whose increased level indicates stress damage, and isoforms of Rubisco (large subunit, hereafter indicated as Rubisco), the key enzyme in the Calvin cycle. Finally, the effects of heat treatment on the content of specific fatty acids were examined and compared to possible cytological and ultrastructural changes of tomato leaves. Although several effects of heat stress have been investigated in tomato, an integrated view that simultaneously takes into account metabolic, physiological and protein aspects of photosynthesis is missing. Here we looked for possible effects and responses ranging from thylakoid membranes to the production of specific sugars. We use a multi-approach information to investigate metabolic and photosynthetic injuries and responses of tomato plants to chronic heat stress. A broad overview of plant responses can allow a better understanding of the ability to recover from stress while highlighting how tomato plants are able to adapt to stress.

2. Materials and Methods

2.1. Reagents

Unless differently mentioned, all chemicals used in this work were purchased from Merck Life Science (Milan, Italy).

2.2. Tomato plants growth and stress

Seeds of tomato plants cv Micro-Tom (*Solanum lycopersicum* L.), purchased from JustSeed Ltd, (Wrexham, UK), were first germinated in Petri dishes with filter paper soaked with distilled water at a constant temperature of 25 °C in the dark. Afterwards, seedlings were transferred to a plant growth chamber, equipped with a set of SON-T and HPI-T Plus lamp (Philips, Amsterdam, NE) in a tray with wells (each well 4 x 5 x 6 cm) at a constant temperature of 25 °C with a 16 h/8 h light/darkness photoperiod with a PPFD (photosynthetic photon-flux density) of 350 µmol m^{-2} s^{-1}, with relative humidity of 60 ± 10% and ambient CO_2 concentration. At the stage of two-leaf seedlings, plants were moved into larger pots (9 × 9 × 10 cm) at the same growth conditions previously described. The substrate used for repotting operations was the Vigor Plant® Growing Medium, Professional Mix. Tomato plants were grown in greenhouses at temperature of 25 °C. Heat stress, corresponding to 40 °C as reported in literature (Camejo et al., 2005), was applied chronically for 8 h for 6 consecutive days in a thermostatic chamber (Bertagnin, Bologna, Italy) to plants grown for 3 weeks. Light intensity and humidity were the same as described above. Leaves samples were taken before heat stress induction (H0, hereinafter referred to as the reference sample), after 4 h of heat stress (H4) and after 8 h of heat stress (H8) only for the first day of treatment. During the following days, samples at H0 were analyzed, to evaluate a night-recovery, and after additional 8 h of stress (H8). After each daily stress phase, plants were left at RT (room temperature, around 18-20 °C in the dark), throughout the night. For each treatment, at least 12 plants were used. Heat stress treatment and specific point of analysis were schematically reported in supplementary material 1.

2.3. Analysis of Photosynthetic Efficiency (Fv/Fm and Performance Index)

Photosynthetic Efficiency was estimated with induction of chlorophyll fluorescence using a Handy PEA 2000 fluorometer (Hansatech Instruments, King's Lynn, Norfolk, UK). The instrument performs a fluorometric analysis of the chlorophyll on leaves and measures changes in the level of fluorescence emission, in order to obtain data on the effectiveness of the exploitation of light in the photosynthetic process (Conti et al., 2019). Parameters used: peak at 650 nm, 3000 µmol m^{-2} s^{-1}. Recorded parameters: Fv/Fm= (Fm-F0)/Fm (Fm= maximum fluorescence value, F0= fluorescence value at origin, Fv= F0 value minus Fm value), and Performance Index (Pi) a more sensitive multiparameter, covering the main photochemical processes (www.hansatech-instruments.com/product/handy-pea/).

Measurement of the maximum photochemical efficiency requires the sample to be fully dark-adapted prior to measurement. Every leaf/clip were dark adapted for 20 minutes. Photosynthetic efficiency was estimated for 5 consecutive days of treatment on different plant leaves randomly selected. At least sixty different measurement were performed for each sample points.

2.4. HPLC analysis of photosynthetic pigments

Photosynthetic pigments were analyzed by high-performance liquid chromatography (HPLC) (LC Module I Plus, Waters) as previously described (Parrotta et al., 2016a), for the first 4 days of treatment. A total of 10 mg of tomato leaves were lysed in 1 mL of ethanol using an Ultra-Turrax® T-25 basic (IKA®-Werke GmbH & Co. KG, Staufen im Breisgau, Germany) homogenizer for 2 min. The homogenate was centrifuged at 13,000 g for 5 min and supernatants with pigments were collected. A volume of 20 µL of each sample were injected into a C18 (Supelco Sigma-Aldrich) HPLC column (15 cm × 4 mm, particle size of 5 µm). Identification of different components was achieved by comparing the retention times with those of standards. The CSW- 32 software (Clarity-DataAPEX) was used for pigments' quantification.

2.5. Determination of ATP and ADP content

ATP and ADP analysis were performed using HPLC as previously reported (Liu et al., 2006). Briefly, 50 mg of leaves were collected and suspended in boiling water (1 mL). A Potter-Elvehjem homogenizer with 40 strokes per sample produced complete disintegration and rupture of tissues. The homogenate was centrifuged at 15,000 g for 15 min at RT. The supernatants were transferred to vials and 20 µL of samples were injected into a solid stationary phase C18 column (Supelco Sigma-Aldrich) (75 mm × 4.6 mm, particle size of 5 µm). The mobile phase was a binary mobile phase gradient (solvent A: 10 mM phosphate buffer pH 7; solvent B: acetonitrile) working in accordance to the following gradient: 0 min, 100% solvent A, 0% solvent B; 2 min, 95% A, 5% B; 4 min, 80% A, 20% B; 5.3 min, 75% A, 25% B; 6 min, 100% A, 0% B. The following parameters were used: flow rate of 0.3 ml/min; RT; the approximate elution times were 6 min for ATP and 7 min for ADP. Identification of different components was obtained by programming the spectrophotometric detector DAD 235C (Perkin Elmer, Shelton, CT, USA) with excitation wavelength at 254 nm. Treatment was conducted for the first 4 days.

2.6. HPLC analysis of sugars

HPLC sugar analysis was performed during the first 4 days of treatment. Leaves tissues were lysed as described above and supernatants were examined by Waters Sugar-Pak I ion-exchange column (6.5 × 300 mm) at a temperature of 90 °C using a Waters 2410 refractive index detector. MilliQ grade water (pH 7) was used as a mobile phase with a flow rate of 0.5 mL/min; an injection loop of 20 µL was used for all samples.

2.7. Determination of linoleic and oleic acids

About 200 mg of tomato leaves were extracted with 1.5 mL of a mixture of chloroform and methanol (2: 1 v / v). Samples were homogenized for 3 min using Ultra-Turrax® T-25 basic (IKA®-Werke GmbH & Co. KG, Staufen im Breisgau, Germany) homogenizer, until complete disintegration. The homogenate obtained was centrifuged at 5,000 g for 5 min at RT and subsequently filtered (0.45 µm). To 0.5 mL of extract were added 5 mL of NaOH and samples were put at 100 °C for 1 h in a speed-vac concentrator (RC1010; Jouan, Winchester, Va.). 5 mL of petroleum ether were added to the solution and mixed. After separation in two clearly visible phases, the upper phase was discarded. These steps were repeated twice. The remaining solution was acidified to pH 2.9 with 1 M HCl, dried using a speed-vac and finally resuspended in 400 µL of methanol. Lipid analysis was carried out using a high-performance liquid chromatography-mass spectrometry (HPLC-MS). An HP 1100 autosampler equipped with a 100 µL loop and an HP 1090A LC pump, both from Hewlett-Packard (Palo Alto, CA, USA) were used. A C18 reversed-phase column (5 µm, 250 × 4 mm i.d., LiChrospher 100 RP-18) was used for the chromatographic separation. The mobile phases applied were composed by methanol and ammonium acetate (25 mM pH 7), using following gradient, from 0 min 70% methanol + 30% ammonium acetate to 65 min 100% of methanol. Analysis of fatty acid was conducted only for the first 2 days of treatment, to correlate this data with the microscopy analysis of lipid bodies.

2.8. Visualization of ROS

ROS localization was performed for the first 2 days of treatment, using Diaminobenzidine (DAB) according to literature (Aloisi et al., 2015). Briefly, fresh material was incubated with 0.5 mg/mL DAB pH 3.8 for 30 min under vacuum and left for 24 h at RT. Samples were washed with 95% ethanol for 15 min at 70 °C prior to image analysis performed with the ImageJ software (https://imagej.nih.gov/ij/index.html). All images of leaves were photographed under exactly the same exposure parameters, and then individual images were imported into ImageJ and threshold-processed with the same settings to highlight the leaf surface damaged by oxidative stress. The area as detected by thresholding was highlighted in red and measured.

2.9. Protein extraction, 1-D electrophoresis, western blotting and image analysis

Proteins for mono-dimensional electrophoresis were extracted according to a protocol developed for protein extraction from recalcitrant tissues protocol (Wang et al., 2006). Samples from the first 4 days of the time-course experiment were processed simultaneously to minimize the experimental variability. Protein concentration of samples was determined using a commercial kit (2-D Quant Kit, GE HealthCare) and a UV-160 spectrophotometer at 480 nm (Shimadzu Italia S.r.l. Milan Italy). Separation of proteins by 1-D electrophoresis was performed as previously reported (Parrotta et al., 2010). After SDS-PAGE separation, proteins were electrotransferred to nitrocellulose membranes using a Trans-Blot Turbo Transfer System (Bio-Rad) according to the manufacturer's instructions. Membranes were blocked overnight at 4 °C in 5% ECL Blocking Agent (GE HealthCare) in TBS (20 mM Tris pH 7.5, 150 mM NaCl) plus 0.1% Tween-20. After washing with TBS, membranes were incubated for 1 h at RT with following primary antibodies:

- Rabbit polyclonal AS03 anti-Rubisco (Agrisera), diluted 1:3500,
- Mouse monoclonal anti-HSP70 (Enzo Life Science), diluted 1:3000,
- Rabbit polyclonal K2 anti-SuSy (Heinlein & Starlinger 1989), diluted 1:1000.

Subsequently, membranes were washed several times with TBS and then incubated for 1 h with secondary antibodies: a goat anti-rabbit IgG conjugated with peroxidase (Bio-Rad) and a goat anti-mouse IgG conjugated with peroxidase (Bio-Rad), diluted 1:3000. Images of gels and blots were acquired using a Fluor-S apparatus (Bio-Rad) and analyzed with the QuantityOne software (Bio-Rad). Exposure times were 30–60 s for blots and 5–7 s for Coomassie-stained gels. Analysis of relative quantitation of blots was performed by the QuantityOne software (Bio-Rad).

2.10. Rubisco purification and Two-Dimensional Electrophoresis (2DE)

Purification of Rubisco was achieved following the methods previously described by (Sudhani & Moreno 2015), with modifications. In detail, leaves were taken during the first 2 days of treatment, then were powdered in liquid nitrogen and suspended in cold extraction buffer (100 mM Tris-HCl, 10 mM $MgCl_2$, 20 mM 2-mercaptoethanol pH 8, supplemented with protease inhibitors). Insoluble polyvinylpolypyrrolidone (PVPP) was added (2% final concentration) and mixed with a magnetic stirrer for 5 min in a cold chamber (4 °C). Subsequently samples were centrifuged at 25,000 g for 15 min at 4 °C and supernatants were collected. Then, while still mixing with a magnetic stirrer, ammonium sulfate was added (final concentration 35%) to supernatants. Samples were stirred for 30 min in a cold room (4 °C), thus centrifuged at 25,000 g for 10 min at 4 °C. Ammonium sulfate was added (final concentration 60%) to supernatants and samples stirred for 30 min in a cold room (4 °C). Thereafter, samples were further centrifuged at 25,000 g for 10 min at 4 °C and the supernatant was discarded. The obtained protein pellets were resuspended in Sucrose Gradient Buffer (SGB: 10 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM β-mercaptoethanol) and desalted through a gel filtration chromatography using HiTrap Desalting columns (GE HealthCare) previously equilibrated with SGB. The column was eluted with SGB and the Rubisco positive fractions identified by dot-blot with the anti-Rubisco antibody, were pooled and loaded over a linear gradient of 0.2-0.8 M sucrose in SGB. Subsequently samples were ultracentrifuged at 132,000 g for 4 h in a fixed angle rotor (Beckmann, rotor SW41) at 4 °C, then pooled and fractionated by anion exchange chromatography using a Mono-Q HR5/5 column and an AKTA Purifier System (GE HealthCare); the column was equilibrated with Column Buffer A (CBA: 20 mM Tris-HCl pH 7.5). A linear gradient at 1 mL/min for 20 ml from 0% to 100% of Column Buffer B (CBB: 20 mM Tris-HCl pH 7.5, 1 M NaCl) was used, monitoring the absorbance at 280 nm. After chromatography, positive fractions were collected and confirmed by dot-blot. Rubisco-containing fractions were concentrated with VivaSpin-2 (GE HealthCare), and samples were further fractionated by gel permeation chromatography using a Superdex 200 column (GE Health Care), previously equilibrated with Activation Buffer (AB: 100 mM Tris-HCl pH 8.2, 10 mM $MgCl_2$, 10 mM $NaHCO_3$). Elution was carried out at 0.8 mL/min, monitoring the absorbance at 280 nm. Positive fractions were analyzed by dot-blot with the Rubisco antibody. Finally, the pool of positive fractions was precipitated by cold TCA-acetone, the pellet was washed twice with cold acetone and resuspended with Rehydration Buffer (40 mM Tris, 8 M urea, 2 M thiourea, 2% CHAPS, bromophenol blue) for 2-D electrophoresis as previously described (Parrotta et al., 2019).

2.11. Transmission electron microscopy (TEM) and optical microscope analysis

Leaves from 2 days treatment were prepared for optical and electron microscopy following the protocol previously described (Behr et al., 2019). Samples were sectioned using the ultra-microtome NOVA LKB (Leica Microsystems) to obtain sections of about 2-3 μm for light microscopy observation and sections of 600 Å for TEM observations. Samples, collected on copper grids and counterstained with a solution of 2% uranyl acetate and lead citrate, were observed with a Morgagni 262 transmission electron microscope (Philips) and an Axiophot phase contrast optical microscope (Zeiss).

2.12. Statistical analysis

All the experimental analyses have been carried out in triplicate and for each single analysis; different plants have been selected at random. Row data were analyzed using student-test of GraphPad Prism. Differences among sample sets were determined by analysis of variance with a threshold P-value of 0.05 and P-value of 0.01.

3. Results

3.1. Heat stress affects photosynthetic efficiency of tomato plants

Photosynthetic efficiency in tomato plants stressed at 40 °C for an 8-hour period over 6 days was measured with a Handy PEA fluorometer. The Fv/Fm and Pi parameters are considered ideal indicators of the functioning of photosynthesis mechanism in plants. The Fv/Fm parameter showed significant changes during the time of treatment (Figure 1A). Statistical analysis revealed significant differences after the first 8 hours of stress between reference samples and stressed samples, clearly indicating that heat stress affects photosynthesis. Similar differences were found after analyzing the Pi parameter. Data showed a general decrease after heat stress, with a partial recovery within day 2. After the first 4 h of treatment at 40 °C (D1H4), the Pi value decreased significantly at the onset of treatment and then increased again at the end of the first day of heat stress (D1H8). The measurement of Pi carried out after a night-time recovery at 25 °C (D2H0) showed that Pi was partially restored. During the next days of stress, the values of Pi remained lower than reference sample and the night-time recovery was not equally evident, indicating a damage of the photosynthetic apparatus (Figure 1B).

3.2. Concentration of photosynthetic pigments oscillates during heat stress

The concentration of the various photosynthetic pigments present in tomato leaves was analyzed during the first 4 days of stress. Proper amount of pigments is required to ensure that photosynthesis proceeds optimally. The data indicate that, as stress increased, the concentration of all four pigments analyzed (lutein, Figure 2A; chlorophyll b, Figure 2B; chlorophyll a, Figure 2C; β-carotene, Figure 2D) decreased during stress. After the first night-time recovery (D2H0), pigment concentrations increased significantly but, after one more round of stress, concentrations decreased again (D2H8). We have observed this trend for the rest of treatment, although with a less pronounced recovery.

3.3. Levels of ATP and ADP change during heat treatment

We measured the concentration of adenosine triphosphate (ATP, Figure 3A) and adenosine diphosphate (ADP, Figure 3B) in samples treated at high temperatures. The concentration of ATP in leaf cells is the result of various metabolic activities of synthesis and hydrolysis and can be a good candidate to indicate the general metabolic status. After the first 4 hours of stress (D1H4), the concentration of ATP decreased slightly and then increased again at the end of the first day of heat treatment (D1H8) achieving a value similar to that of reference plants. From the second day of treatment, ATP values increased as exposure time extended, except for the D4H8 sample, which showed a reduced concentration of ATP. In terms of ADP values, we found a peculiar trend; in fact, the concentration of ADP was low during the first days of treatment, while in the following days of treatment ADP levels remained below the detection limit of HPLC spectrophotometric detector.

3.4. Heat stress affects the concentrations of sugars in tomato leaves

We analyzed the concentration of four sugars present in tomato leaves (*i.e.* sucrose, fructose, glucose and glucose 6-phosphate) by HPLC. The four sugars act in steps following the Calvin cycle; glucose 6-phosphate is an intermediate in the synthesis of sucrose while glucose and fructose derive from the hydrolysis of sucrose. The analysis was performed for the first four days of stress. As shown in figure 4A, the reference sample (D1H0) had a relatively low concentration of sucrose, do not showed a significant change after 4 h of stress (D1H4), however D1H8 sample reached a concentration almost three times higher than the initial concentration. At the beginning of the second day of stress, sucrose levels dropped dramatically (D2H0) indicating high consumption during the night; when plants were stressed for the second day, the amount of sucrose increased again (D2H8) but it was lower than after the first 8 hours of stress. On the third day of stress sucrose levels were similar to the previous day, in both day samples (D3H0 and D3H8). During the fourth day, sucrose levels were lower than on the previous days, but remained stable during the 8 hours of heat stress. Glucose concentrations increased after the first 4 hours of treatment but at the end of the next four hours of stress, the value of glucose decreased drastically (Figure 4B). Glucose concentrations remained low and constant for the rest of treatment, except in D3H0, when concentrations increased significantly. A similar trend was found for fructose (Figure 4C). Even in this case, the concentrations of fructose decreased as the heat treatment advances. In particular, fructose levels decreased slightly and negligibly, except for the D3H0 sample. Finally, glucose 6-phosphate concentrations, unlike the other sugars analyzed, showed a slight decrease after the first 8 h of stress (D1H8) but, in any case, it exhibited a constant and practically unchanged trend during all phases of treatment (Figure 4D).

3.5. Oleic and linoleic acid decrease in concentration at the beginning of heat treatment

The concentration of two main fatty acids (oleic acid and linoleic acid) present in leaves of tomato plants was investigated. Fatty acids are not only a reservoir of energy but also a pool of molecules for the synthesis of compounds with a protective action. The analysis was conducted for the first two days of stress, and the values are reported in Figure 5. The starting concentration of oleic acid (D1H0) appeared to decrease during the first day of stress (D1H8), without an evident recovery during the night (D2H0). When plants were subjected to a new heat stress cycle, the values decreased significantly (D2H8) (Figure 5A). Linoleic acid concentrations decreased meaningfully during the first day of stress (D1H4 and D1H8). The values continuously decreased during the second day of exposure (both in D2H0 and D2H8) (Figure 5B).

3.6. High temperatures increase ROS production

By analyzing the pattern of DAB staining in tomato leaves (Figure 6A), we have highlighted the distribution of ROS. ROS production is one of the most evident effects following heat stress and is therefore an index of the physiological state of leaves. Whereas scattered but small areas of ROS production could be observed in reference samples (D1H0), the area highlighted by DAB increased significantly in heat-stressed samples. In some cases, the area enriched in ROS was concentrated along the central vein (as in D1H8) or along the lateral veins (in D2H0). In the final sample (D2H8) the areas affected by ROS production were evident at various areas. We observed a more significant increase in samples treated with 8 h of heat stress (D1H8) compared to reference samples (D1H0) and those obtained after night-time recovery (D2H0). The data also showed a higher amount of ROS (defined as the leaf area labelled by DAB) positively correlated to the increase in chronic exposure to high temperatures (Figure 6B).

3.7. HSP70, SuSy and Rubisco accumulate differently during heat stress

Protein samples from reference and stressed plants were analyzed by one-dimensional SDS-PAGE. The resulting gel (Figure 7A) highlighted the absence of significant variations in protein intensity between treatments. Following one-dimensional electrophoresis, an immunoblotting analysis was performed to detect any changes in specific protein levels during the first 3 days of chronic stress (Figure 7B). Specifically, we have analyzed Rubisco because it is the key enzyme during the Calvin cycle and because it is often targeted for environmental stress conditions. In addition, we analyzed the stress-related protein HSP70 and the sucrose-metabolizing enzyme SuSy.

As reported in Figure 8A, accumulation levels of Rubisco decreased after the first 8 h of heat stress (D1H8) and, even after a slight night-time recovery to ambient temperature (D2H0), protein accumulation levels decreased again. Between the second and third day of treatment, protein levels (even if low) remained constant and the night recovery was not evident. Despite partial night-time recoveries, the amount of Rubisco never reached the reference levels after stress treatment. Accumulation of HSP70 (Figure 8B) showed a slight increase after the first eight hours of stress, the highest level of accumulation was found after the night-recovery during all stress treatment (D2H0 and D3H0). The immunoblotting analysis of SuSy (Figure 8C) showed a constant trend during the first days of treatment but we found a significant increase in D3H0 sample.

Rubisco was purified and separated by two-dimensional electrophoresis in order to detect changes in protein isoforms. Figure 9 shows the two-dimensional gels of reference samples (D1H0) and of samples taken after the first day of stress (D1H8) and after the next day of treatment (D2H0 and D2H8). Rubisco spots ranged in a pH between 5.5 and 6.5. During the first day of stress, both reference (D1H0) and stressed samples (D1H8) showed eight protein spots; despite the same number of spots, they had a different distribution, both in terms of molecular weight and, more clearly in terms of isoelectric point (pI). In the reference sample (D1H0, Figure 9A), spots were very close to each other, focusing in a relatively small pH range; not only they were separated by isoelectric point, some spots also exhibited the same isoelectric point but a slight different molecular weight (~ 55 kDa) (spots numbered as 5, 6 and 7). In the D1H8 sample (Figure 9B) spots showed almost the same molecular weight and differentiated exclusively by the isoelectric point. In the first sample of the second day (D2H0, Figure 9C), 8 total spots were identified, which were separated only by isoelectric point. Compared to the D1H8 treatment, spots were more compressed towards the basic region of the pH gradient, with the two most basic spots being quantitatively larger. Finally, in the last sample (D2H8, Figure 9D), only 3 spots were highlighted, focusing in a very narrow pH range around 6.5. These data were confirmed by the corresponding signal quantification graphs obtained with the QuantityOne software, by which the relative density percentage was evaluated. The graphs show how the first day of stress returns a significant prevalence of isoforms numbered as 6 and 7, both in D1H0 and D1H8. On the contrary, a different situation occurred on the second day, because in sample D2H0 isoforms 7 and 8 were prevalent while in sample D2H8 isoform 1 was more expressed. What was clear, however, was the progressive shift of the accumulation region of Rubisco spots, which cluster towards the basic region. In addition to this, the last stage of sampling showed a drastic reduction in the spots numbers.

3.8. Heat stress causes cytological and ultrastructural variations of tomato leaves

In order to verify the possible cytological and ultrastructural variations of tomato leaf cells, we have carried out analyses by light microscope (Figure 10) and transmission electron microscope (Figure 11). Data on the cytological structure of leaf cells are reported in figure 10, for reference samples (D1H0, Figure 10A), and stressed samples (D1H8, Figure 10B; D2H0, Figure 10C; D2H8, Figure 10D). The cuticle (C) was relatively evident in all the samples while the epidermis (E) was composed of a simple monocellular layer without intercellular spaces. Both the underlying palisade parenchyma and spongy parenchyma were easily discernible. Within each cell, the vacuole (V), chloroplasts (P for plastids), thylakoid grana and starch granules (S) could be identified. When comparing the various cases, the most evident variation observed was a higher accumulation of rounded lipid bodies (LB) in the stressed tomato leaves (D1H8 and D2H8) compared to both the reference (D1H0) and the night-recovery sample (D2H0). In addition, the same samples were analyzed by TEM (Figure 11) confirming data obtained by optical microscope. In particular, the D1H0 sample contained very few lipid bodies, mainly observable in the plastids (Figure 11A). In D1H8 sample, lipid bodies were more abundant and localized also in the cell cytoplasm of the spongy parenchyma, of the palisade parenchyma and also in the epidermis (Figure 11B). In the D2H0 sample, lipid bodies (LB) could be observed within chloroplasts, however, their number was not comparable to that observed in the previous D1H0 and D1H8 samples (Figure 11C). The D2H8 sample was characterized by a significant increase in the amount and size of lipid bodies (Figure 11D). In particular, the insert in Figure 11D shows large lipid bodies (LB) distributed in the cell cytoplasm. Stage D2H8 clearly shows that the synthesis of lipid bodies resumed after they had been consumed on the recovery night. Nevertheless, even though they could be observed inside plastids, their size and number were small. In summary, the main changes observed at both cytological and ultrastructural level concern the amount of stored energy, such as lipid bodies and starch granules. Since we did not detect any clear damage to the leaf structure, the data indicate that the response of leaf cells to heat stress is in the metabolic system.

4. Discussion

Rising global average temperature can affect the whole plant world (Saidi et al., 2011). Biomass loss, reduction in plant growth and development and ultimately death, are only few examples of the effects of heat stress on plants (Timperio et al., 2008). Heat stress affects several cellular processes and it is particularly severe when high temperatures occur in conjunction with the critical stages of plant development, particularly during the reproductive period (Teixeira et al., 2013). Being sessile, plants have evolved strategies to cope with different biotic and abiotic stresses, allowing cellular homeostasis and contributing to plant survival (Kotak et al., 2007).

In this work, we examined how chronic heat stress (6 days, 40 °C) affects *Solanum lycopersicum* L. cv Micro-Tom. The proposed experimental design allows the comparison of heat stressed samples with a reference point in order to highlight changes in the analyzed parameters. We focused on photosynthesis, as it can be completely inhibited by heat stress, probably by direct inhibition of the PSII activity, which has been shown to be a thermally labile system (Camejo et al., 2005). It is likely that thermal radiations can alter the conformations of PSII proteins, causing protein and degradation (Nath et al., 2013). The reduction of photosynthetic activity may also be due to alteration of the electron flow (Oukarroum et al., 2012) and to the inhibition of the de novo synthesis of proteins involved in PSII repair. This phenomenon resulting from the production of reactive oxygen species (ROS) (Nishiyama et al., 2011, Ahammed et al., 2018) as well as the loss of thylakoid membrane integrity (Allakhverdiev et al., 2008). One of the questions to be clarified is whether chronic stress over several days can produce a linear response or whether plants can show a progressive adaptation; in our case study, while the Fv/Fm ratio remained constant over the treatment, the Pi multi-parameter significantly fluctuated during stress, confirming photosynthetic alterations. Fluctuations of Pi characterized both stress and recovery stages during the first days of treatment; however, Pi values were constantly lower compared to reference sample after heat stress from day 1 to 5, suggesting that plants' homeostasis buffers only short periods of heat stress. Similar reductions of these parameters have been observed in two heat-sensitive tomato varieties (Zhou et al., 2015) and during tomato growth under water deficit (Nankishore & Farrell 2016). The decrease in photosynthetic efficiency could be due to a reduction in the concentration of photosynthetic pigments (Khan et al., 2015, Nankishore & Farrell, 2016). Indeed, our results showed that levels of photosynthetic pigments, *i.e.* chlorophyll a and b, lutein and β-carotene partially mirrored the decrease of photosynthetic efficiency; in particular, during night recovery both photosynthetic efficiency and the amount of pigments increased, however, photosynthetic pigments never reached suboptimal concentrations, especially after prolonged periods of stress. All these evidences are in according with a the recent evidence that the photosynthetic pigment content and light absorption flux decrease in melatonin deficient by silencing of a melatonin biosynthetic gene COMT1-silenced (*Caffeic acid O-Methyltrasferase 1*) tomato plants under heat stress (Ahammed et al., 2018).

Sugars, the products of photosynthesis, also underwent significant alterations. Sucrose levels drastically increased during the first day of stress, probably due to an enhanced degradation of starch, the reserve energy of plants when photosynthesis is compromised during stress (Rizhsky et al., 2004) or to an increase in the activity of enzymes responsible for sucrose synthesis. In our case, sucrose levels gradually decrease after accumulating during the first day; this could be due to the catabolism of starch initially available for sucrose synthesis or to an increased activity of enzymes responsible for its synthesis. When stress conditions then reoccur, plants lose the ability to recover adequate levels of sucrose, possibly decrease in starch stocks, reduces the availability of sucrose. A decrease in sucrose levels following an initial increase has already been reported in tomato leaves stressed by moderately high temperatures (Jie et al., 2012). Like sucrose, a decrease in the levels of fructose and glucose was found, probably because they are metabolized to produce energy for survival; the stress conditions to which plants are subjected require high energy investments in an attempt to increase protection. Characteristically, the levels of fructose and glucose showed a peak at the beginning of the third day of treatment; this conditions, although peculiar, could be related to the accumulation of SuSy, which peaked exactly at the same day. The increase in levels of SuSy could actually lead to a higher cleavage of sucrose molecules thereby raising fructose and (indirectly) glucose levels. In other cases, levels of fructose, glucose and sucrose were observed to decrease almost constantly during heat stress of tomato leaves. Changes in the accumulation of Susy have been observed in different plants or plant cells subjected to heat stress (Kaushal et al., 2013, Parrotta et al., 2016b, Pressman et al., 2006). Arabidopsis plants subjected to a combination of drought and heat stress for 6 hours showed accumulation of sucrose and other sugars such as glucose (Rizhsky et al., 2004); this suggests that the metabolic alterations of sucrose-metabolizing enzymes in response to unfavorable environmental conditions may be common to different plant species. However, it is not clear how plants under heat stress can maintain appropriate levels of starch and soluble sugars and it is not equally clear whether resistant varieties modulate starch and soluble sugar levels through either increased biosynthesis, or reduced degradation, or increased uptake (Sangu et al., 2015). The concentration of glucose 6-phosphate, an intermediate in the synthesis of sucrose and UDP-glucose (the precursor of cellulose) were constant during heat stress. This might be achieved by a reduced production of UDP-glucose and therefore cellulose, coherently with the lower growth rate of plants under heat stress.

In plants, ROS production is a normal consequence of aerobic metabolism. Hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and the hydroxyl radical (• OH) affect various cellular components such as membrane lipids, proteins and nucleic acids. Despite the potential harmful effects of ROS, these molecules are necessary for several physiological processes (Mittler, 2017). Hence, the need to finely tune and regulate ROS concentration by an efficient ROS scavenging machinery based on enzymes and secondary metabolites (D'Autréaux & Toledano, 2007). This appears to be in line with our data as ROS were detectable also in reference samples; however, their concentration increased during heat stress. Accumulation of ROS is linked to the reduction of photosynthetic efficiency, which concerns the two photosystems and that could free up more electrons capable of combining with oxygen to generate ROS (Asada, 2006, Kim & Portis, 2004). Finally, oxidative stress can have a feedback on the photosynthetic efficiency, exacerbating the damage (Murata et al., 2007, Nishiyama et al., 2005).

The accumulation of hydrogen peroxide in plant tissues is a critical step as it leads downstream to the activation of defense genes, including genes coding for HSPs (Volkov et al., 2006). The role of HSPs in maintaining the correct folding of proteins and in avoiding their aggregation during stress is well known (Parrotta et al., 2013, Usman et al., 2017). This is in line with the increase of HSP70 during stress as observed in this paper. It should be noted, however, that the highest levels of HSP70 were found in samples collected in the morning, after a theoretical recovery of plants at night. In addition, we observed that HSPs decrease during 8 hours of heat stress. These data suggest that the higher expression of HSPs occurs during the night in response to daily stress.

The general decrease in sugar levels may explain the increase in ATP concentration. The amount of ATP available results from the respiration process and is therefore not an index of photosynthetic efficiency. However, the production of ATP is clearly linked to the availability of sugars, mainly monosaccharides. A decrease in the latter can imply an increase in ATP, which is necessary to sustain the response to heat stress. The increase in ATP observed at the end of treatment (parallel to a drop in ADP, they appear inversely correlated) could therefore be linked both to the decrease in sucrose and to the consumption of glucose and fructose. This data suggest that plants address their energy resources to cope with the damage caused by high temperatures (Hemme et al., 2014). Changes in ATP concentration could also be related to the reduced growth rate of plants (a parameter that we have not measured), therefore to a diminished synthesis of the cell wall so that the energy normally directed to the synthesis of cellulose is deviated to ATP production. Wheat plants tolerant to heat stress manage to maintain adequate respiration levels (and therefore ATP production) (Almeselmani et al., 2012) and the ability of heat stress-tolerant varieties to maintain adequate respiratory levels seems to be a common strategy (Hu et al., 2010).

Various stresses, such as high temperature, salinity and ultraviolet irradiation, can have effects on Rubisco (Galmes et al., 2013). Our results indicate that a few hours of stress are enough to trigger a clear reduction in the Rubisco amount. The data is supported by literature, which suggests that Rubisco inactivation is closely related to the inhibition and acclimatization of photosystems to heat stress (Demirevska-Kepova et al., 2005). In this work, we focused on large subunit Rubisco isoforms because this analysis might detect a different use of Rubisco under stress conditions. It is not known whether differences in Rubisco isoforms are due to either new protein synthesis or altered/differential degradation or post-translational modifications. A study conducted on chlorophytes and cyanobacteria, in the Lake Bonney in Antarctica showed how climate and light variations influence the accumulation of Rubisco isoforms (Kong et al., 2012). Furthermore, the research conducted on cyanobacteria, brown and red algae and C3-C4 plants, confirmed how different Rubisco isoforms have undergone natural variations and evolved to cope with and adapt to changes in the levels of atmospheric CO_2 and O_2 over time (Parry et al., 2013). In this work we have purified and analyzed Rubisco by 2-D electrophoresis enabling a quick direct visualization of the different Rubisco isoforms expressed under stress conditions when plants decrease the number of Rubisco isoforms, the latter being also characterized by a more basic isoelectric point. All this suggests that during heat treatment tomato plants attempt to use a small number of Rubisco isoforms, probably those that are more stable or work better at high temperatures. It is also likely that post-translational modifications may help to stabilize the enzyme and consequently to promote photosynthetic process under harsh conditions. We are inclined to assume the absence of proteolytic events that would damage specific Rubisco isoforms having never observed such occurrence during enzyme purification (Supplementary Material 2).

Cytological analysis using both optical and electron microscopy with the aim of revealing any structural changes and cell damage after heat stress showed an accumulation of lipid bodies inside the cytoplasm of spongy and palisade parenchyma. The formation of lipid bodies starts from sucrose and includes the synthesis of fatty acids (we specifically quantified oleic acid and linoleic acid by HPLC-MS) in plastids from Acil-CoA followed by their export to the endoplasmic reticulum where synthesis of lipid bodies takes place (van der Schoot et al., 2011). In heat-stressed tomato plants, accumulation of lipid bodies could start after the increase in sucrose at D1H8 and could be either a defense mechanism or the attempt to store energy in the form of lipids. We found that the number of lipid bodies was not comparable to that of samples after night-recovery, suggesting that they were dismantled overnight. We also noted the almost total absence of starch granules, suggesting that this polysaccharide was completely consumed during the night recovery. Lipid bodies are involved in temperature stress responses, and lipid droplets characteristically proliferate in Arabidopsis leaves under stress (Gidda et al., 2016). Another hypothesis is that plants under heat stress also invest their energy for the synthesis of protective lipid compounds, such as oxylipins, a group of secondary metabolites produced by oxidation of polyunsaturated and monounsaturated fatty acids. The process is catalyzed by lipoxygenase and includes linoleic and oleic acid respectively, as a defense system against environmental changes (Mosblech et al., 2009). The study carried out in Arabidopsis (Upchurch, 2008) suggested that plants release α-linolenic acid from the chloroplast membrane in the attempt to adapt to abiotic stress suggesting that modulation of oleic acid and linoleic acid levels is essential for stress defense.

Tomato exhibited a variety of responses to high temperatures, which are depicted by symptomatic and quantitative changes in morphology and growth. The ability of plants to manage or adapt to heat stress varies both between species and in relation to different developmental stages (Ahmad et al., 2013). The data presented in this work (mainly at the photosynthetic level) indicate that tomato plants suffer damage from heat treatment, but also try to respond by adapting to new conditions. Adaptation covers various metabolic aspects, including sugar levels, ATP production and accumulation of specific Rubisco isoforms. All these data, corroborated by previous studies, suggest that high temperatures primarily induce a reduction in photosynthetic efficiency, which is a consequence of reduced photosynthetic pigment concentrations and Rubisco accumulation. During the night, plants might activate a recovery system to partially restore energy production in leaf cells. However, recovery is not yet enough to bring the parameter values back to initial levels. Leaf cells could therefore respond to heat stress with a different use of those Rubisco isoforms more resistant to stress. The normal metabolism of ATP is also affected by changes to sugar metabolism and the data are supported by different accumulation of SuSy, and by fatty acid and lipid bodies. In conclusion, this study advances our understanding on the mechanisms of response to heat stress in tomato plants and can potentially be useful for breeding and selection of heat-tolerant varieties in the time of climate change.

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Author contributions

L.P., I.A., C.F. performed the investigation and methodological analysis; L.P. and I.A. wrote the original draft; M.R., G.C. S.D.D. design the research, reviewed and edited the text. All authors read and approved the manuscript.

Figure legend:

Figure 1. Photosynthetic efficiency of reference and stressed tomato plants. Analysis of Fv/Fm (**A**) and Performance Index (**B**) parameters. Data collection was performed using the HandyPea Fluorimeter. Reported values are the average of at least 20 different measurements. Samples were analyzed in triplicate and compared with the reference sample by a t-student test. Asterisks indicate statistically significant differences (one asterisk for $P < 0.05$, two asterisks for $P < 0.01$).

Figure 2. HPLC analysis of photosynthetic pigments of tomato leaves subjected to chronic stress. The main photosynthetic pigments, *e.g.* lutein (**A**), chlorophyll b **(B**), chlorophyll a (**C**) and β-carotene (**D**), were quantified during heat stress. Reported values are the means of at least three replicate. The Lod (detection level) of the HPLC was 0.0285 μg / mL for lutein, 0.0722 μg / mL for chlorophyll b, 0.013 μg / mL for chlorophyll a and 0.217 μg / mL for β-carotene. Samples were analyzed in triplicate and compared with the reference sample (D1H0) by a t-student test. Asterisks indicate statistically significant differences (one asterisk for $P < 0.05$, two asterisks for $P < 0.01$).

Figure 3. HPLC analysis of ATP and ADP concentration of tomato leaves subjected to chronic stress. ATP (**A**) and ADP (**B**) concentrations were quantified by HPLC. At least three replicate were analyzed. Lod (detection level) were 0.015855 μg / mL for the ATP and 0.009527 μg / mL for the ADP. Samples were compared with the reference sample (D1H0) by a t-student test. Asterisks indicate statistically significant differences (one asterisk for $P < 0.05$, two asterisks for $P < 0.01$).

Figure 4. HPLC analysis of sugar concentrations present in tomato leaves subjected to chronic heat stress. Sucrose (**A**), glucose (**B**), fructose (**C**) and 6-P glucose (**D**) concentrations were quantified during heat stress. Samples were analyzed in triplicate and compared with the reference sample (D1H0) by a t-student test. Asterisks indicate statistically significant differences (one asterisk for $P < 0.05$, two asterisks for $P < 0.01$).

Figure 5. HPLC-MS of fatty acid concentrations present in tomato leaves during the first two days of chronic heat stress. Oleic acid (**A**) and linoleic acid (**B**) concentrations are respectively reported. At least three replicate were performed for each sample and samples were compared with reference sample (D1H0) by a t-student test. Asterisks indicate statistically significant differences (one asterisk for $P < 0.05$, two asterisks for $P < 0.01$).

Figure 6. ROS analysis in tomato leaves during the first two days of chronic heat stress. ROS distribution in leaves of heat stressed plants stained with DAB (**A**). Images were converted to

grayscale and leaf area percentage calculated (**B**). At least three replicate were analyzed. Means of treated samples were compared with reference sample (D1H0) by a t-student test. Asterisks indicate statistically significant differences (one asterisk for P < 0.05).

Figure 7. Electrophoretic analysis and immunoblotting of samples subjected to chronic heat stress. (**A**) Coomassie colored SDS-PAGE containing the leaf proteins. Molecular weights markers in the first lane (STD - lane 1) and their values in kDa on the left. Samples analyzed are three on the first day of stress (D1H0, D1H4 and D1H8), two samples on the second day (D2H0 and D2H8) and the third day of stress (D3H0 and D3H8). (**B**) Immunoblotting carried out of each sample to check the amount of the Rubisco, HSP70 and SuSy. The samples analyzed are the same as the one-dimensional electrophoresis gel.

Figure 8. Analysis of bands volume made with Quantity One software on the blot images. Densitometric quantification after immunoblotting against Rubisco (**A**), HSP70 (**B**) and SuSy (**C**). Intensity of bands is reported in Y-axis as Integrated Density. Values are average of three independent measurements.

Figure 9. Two-dimensional electrophoresis carried out following the purification of the Rubisco. 2D-electrophoresis (first dimension isoelectrofocusing, second dimension SDS-PAGE). The area containing the spots stained by Coomassie have been quantified as reported by graphs on the left. (**A**) D1H0 sample, (**B**) D1H8 sample, (**C**) D2H0 sample and (**D**) D2H8 sample. Analysis of spot volume made with Quantity One software Intensity is reported in Y-axis as Integrated Density. Values are average of three independent measurements with their standard deviation.

Figure 10. Cytological analysis of tomato leaves using a light microscope. In D1H0 (**A**) only a few lipid bodies are detectable. D1H8 sample (**B**) showed a more abundant lipid bodies localized in the cytoplasm of the spongy parenchyma, of the palisade parenchyma and in the epidermis. Data collected in D2H0 (**C**) indicated that the number of lipid bodies is very low and not comparable with other samples. While D2H8 (**D**) sample reported a remarkable increased number of lipid bodies. Leaves structure is visible. C: cuticle: E: epidermis; V: vacuole; P: plastid; S: starch; LB: lipid bodies. For each images, bar scale is reported

Figure 11. Cytological analysis of tomato leaves using a TEM. In D1H0 (**A**) only a few lipid bodies are detectable. D1H8 sample (**B**) showed a more abundant lipid bodies localized in the cytoplasm of the spongy parenchyma, of the palisade parenchyma and in the epidermis. Data collected in D2H0

(**C**) indicated that the number of lipid bodies is very low and not comparable with other samples. While D2H8 (**D**) sample reported a remarkable increased number and size of lipid bodies. Magnification of lipid body was reported in the insert. Leaves structure is visible. C: cuticle: E: epidermis; V: vacuole; P: plastid; S: starch; LB: lipid bodies. For each images, bar scale is reported

Figure 12. Schematically representation of photosynthetic and metabolic pathways involved in response to chronic heat stress in tomato leaves. The schematic reports the hypothetically involved organelles and metabolic pathways, according to the obtained data. Flash of lightning indicate main cellular process studied.

SM 1. Heat stress treatment schematically reported. The phases of heat stress and the different points of sampling are indicated with red x. Time-course analysis are reported on the top of image.

SM2. Electrophoretic analysis of different Rubisco purification steps. (**A**) SDS-PAGE gel obtained of reference sample (D1H0) purification protocol and (**B**) gel of D1H8 sample (only two gels were reported). Lane 1 = standards of known molecular weight. Lane 2 = S1, first supernatant. Lane 3 = S2, supernatant after ammonium sulphate precipitation. Lane 4 = P2, precipitate after ammonium sulphate precipitation. Lane 5 = PS, positive fractions after sucrose gradient separation. Lane 6 = PS2, positive fractions following ion exchange chromatography. Lane 7 = PF-GF, positive fractions post second gel filtration chromatography. No protein degradation is highlighted.