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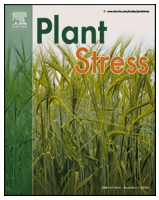

Integrated physiological and genetic data reveal key-traits for heat tolerance in tomato

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ABSTRACT

As global temperatures continue to rise, high summer temperatures severely affect crop growth, reducing yield and quality. The projections of annual declines in crop yield require more in-depth multidisciplinary studies on plant tolerance to abiotic stresses. As tomato is a major crop in the Mediterranean region, its response to heat stress has become important to be addressed in order to identify those traits affecting stress tolerance. In this study, physiological and genomic analyses were performed on two heat-tolerant genotypes (LA3120 and E42) grown at high temperatures during the entire life cycle. The results showed that heat stress diversely affected gas exchange and fluorescence parameters in the two genotypes. In particular, E42 regulated the photosynthetic machinery under heat stress by modulating the electron transport chain, whereas LA3120 was less affected by the applied stress. Genotyping data obtained from a GBS (genotyping by sequencing) analysis were used to explore the genetic variability of both genotypes with the aim of identifying candidate genes that might regulate their stress response. These results further deepen our understanding of the physiological mechanisms activated in response to heat stress and allowed to select key traits that could be used in breeding program to select thermotolerant tomato genotypes.

1. Introduction

Mediterranean agriculture faces major challenges associated to climate change and sustainability. While climate change can improve temperature and rainfall patterns in some regions of the globe, the opposite is expected in the case of the Mediterranean region, identified as one of the most prominent hotspots in future climate-change projections, with 4–5 °C rising temperatures and extreme rainfall patterns (www.fao.org/3/i3084e/i3084e16.pdf). In the European Mediterranean region, increases in the frequency of extreme climate events during specific plant developmental stages (e.g. heat stress during flowering period), together with higher rainfall intensity and longer dry spells, are likely to reduce yield of many plant species, including tomato (*Solanum lycopersicum* L.) (Alcamo et al., 2007). Indeed, the cultivation of tomato is deeply affected by high temperatures, since optimal reproduction and fruit set are limited in this species when the day's maximum temperatures exceed 32 °C and the night's minimum falls below 21 °C (Janni et al., 2020; Singh et al., 2022; Farinon et al., 2022; Graci and Barone, 2024). This is particularly worrisome considering that tomato is one of the most extensively cultivated species in temperate regions worldwide; indeed, according to FAOSTAT, globally 186.821 million tons of tomatoes were produced on 5,051,983 hectares in 2020 (FAO, 2022). Heat stress (HS) can cause different and often irreversible damages to plants altering biochemical, morphological, and physiological processes and leading to reductions in growth, early senescence, leaf abscission, damage to reproductive organs and pollen, fruit discoloration and reduced fruit quality (Singh et al., 2022; Gonzalo et al., 2022). Yield-related traits associated with the reproductive stages (such as total flower and fruit number, pollen viability, fruit set and final yield) are generally the main focus of the majority of the studies aimed at identifying elite tolerant lines (Gonzalo et al., 2021; Ruggieri et al., 2019). For this reason, high number of studies focusing on the genetic basis of these traits are reported (Graci and Barone, 2024). However, it is important to consider that heat tolerance is also dependent on the physiological status of vegetative organs. Indeed, physiological parameters are dramatically affected by HS and this can ultimately influence the final fruit production (Ahammed et al., 2018; Zahra et al., 2023). Plants subjected to high temperatures show a decrease in the rate of photosynthesis and in the accumulation of photosynthetic pigments in leaves (Shanmugam et al., 2013), water loss in aerial parts and alterations of membrane integrity

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(Li et al., 2015, 2016). Both photosystems I and II (PSI and PSII) are affected by elevated temperatures due to the inhibition of light energy absorption, electron transport and energy distribution (Li et al., 2016), with PSII being more sensitive to HS (Yoshioka et al., 2006; Yan et al., 2013). However, the photosynthesis-related traits associated to HS are still not known and very few studies focused on these traits. In the present study, in-depth physiological analyses were carried out on two different tolerant genotypes grown under prolonged high temperatures in order to identify key physiological traits related to HS tolerance. One genotype (LA3120, Malintka) is a well-known heat-tolerant genotype (Tomato Genetics Resource Centre, TGRC, University of California, CA, USA, www.tgrc-mvc.plantsciences.ucdavis.edu). The second used (coded E42) is a genotype that was selected in southern Italy and has been recently identified as heat-tolerant following many open field trials carried out over several years and in different locations (Ruggieri et al., 2019; Olivieri et al., 2020). The physiological responses to HS in E42 and LA3120 were previously investigated in a study from Francesca et al. (2022) that demonstrated the activation of different adaptive mechanisms in the two genotypes in young plants in response to a 3-week stress. However, it has been demonstrated that age-related eco-physiological mechanisms may be activated in response to abiotic stress that are deeply dependent on plant size and developmental stage (Zotz et al., 2001; Zhang et al., 2023). Therefore, in the present study a more in-depth physiological analysis was conducted on the same genotypes, which were here subjected to a prolonged HS over a period of 5-weeks. Our experimental design allowed us to identify different morpho-physiological traits related to HS tolerance. Moreover, these physiological data were combined with genotyping data in order to get further insights into the genetic basis of the identified tolerance-related traits. The emergence of Next Generation Sequencing (NGS) technologies able to identify millions of genome-wide single-nucleotide polymorphisms (SNPs) for high-throughput genotyping can allow the selection of candidate genes associated to specific phenotypic traits (Phan and Sim, 2017). Here the genomic analyses were conducted by using Reduced Representation Sequencing (RRS) data, a technology that uses one or more restriction enzymes to construct reduced representative libraries for the Illumina platform, resulting highly cost-effective (Peterson et al., 2012; Chung et al., 2017; Scheben et al., 2017).

Altogether, in this study we investigated the establishment of the different physiological and molecular mechanisms controlling stress adaptation in two genotypes known to be tolerant to elevated temperatures. The combination of physiological and genomic analyses was here able to provide key physiological parameters and related candidate genes, which could help breeders to select resilient genotypes that can cope with the current challenges of climate changes.

2. Materials and methods

2.1. Experimental design and plant growth conditions

This study was laid out at the Department of Agricultural Science of the University of Naples Federico II during the year 2023. The heat-tolerant tomato genotype LA3120 (Tomato Genetics Resource Centre, TGRC, University of California, CA, USA) and the tomato genotype E42 (University of Naples Federico II, Department of Agricultural Sciences) were used in the trial. The experiment was conducted with 10 biological replicates for each genotype per treatment. Plants were grown using a completely randomized block design. Seeds were sown in seed trays and after 20 days the seedlings were transferred to plastic pots (21 cm diameter) with commercial substrate. All pots were subjected to equivalent management throughout the experiment. One week after transplant, tomato seedlings were transferred to two growth chambers with a 16/8 h photoperiod, PAR of 350 μmol m^{-1} s^{-1}, RH of 65–75 % and with climate settings of 26/18 °C day/night temperatures in the control chamber and 35/18 °C day/night temperatures in the hot chamber The treatments were applied on 20-day-old seedlings and lasted for 5 weeks.

2.2. Assessment of growth parameters and leaf functional traits

Five plants per genotype were randomly selected from each treatment to assess variability in growth traits. Leaf area (cm^2), specific leaf area (cm^2/g), fresh and dry shoot weight (g) were assessed. Leaf area was measured after scanning the leaves with the image analyzer ImageJ (Schneider et al., 2012). Specific leaf area (SLA) was measured as the ratio of leaf area to leaf dry mass. Leaf functional traits were determined on five leaves *per* genotype *per* treatment. The third leaf from the apex in each plant was chosen and the sampled leaves were in good condition, without wilting or disease. The tissues (leaves and shoot biomass) were dried in an oven at 80 °C for 72 h to obtain dry biomass (DW).

2.3. Gas exchange and fluorescence analyses

Gas exchange and fluorescence measurements were conducted on five healthy, young, fully expanded leaves (typically the 3rd leaf counting from the apical meristem) *per* genotype *per* treatment. Leaf gas exchange and chlorophyll fluorescence were simultaneously measured by using the Li6400 portable photosynthesis system (LiCor, Lincoln, NE, USA) integrated with Li6400-40 Leaf Chamber Fluorometer, which acts as both a leaf cuvette and light source/pulse-amplitude modulated fluorometer. Measurements were carried out in the morning with the following environmental parameters: photosynthetic photon flux density of 1200 μmol photons m^{-2} s^{-1}, 400 μmol CO_2 mol^{-1}, relative humidity 50–55 %, and two fixed temperature regimens: 25 °C (considered as ambient control) and 35 °C (considered as heat treatment). Net photosynthesis (A_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration (T_r) were calculated with the software of the Li6400. Steady-state fluorescence (F_s) and maximum fluorescence ($F'm$) upon illumination were measured after a 0.8-s saturating flash of 7000 μmol photons m^{-2} s^{-1}. Quantum yield of PSII electron transport (Φ_{PSII}) was calculated as reported in Maxwell and Johnson (2000). Intrinsic water use efficiency ($_i$WUE) was calculated as A_N/g_s ratio (μmol/mol). Photochemical quenching (qP) was calculated according to Kramer et al. (2004) while the non-photochemical quenching of excitation in PSII-associated antenna complexes (NPQ_T) was obtained as proposed by Tietz et al. (2017). The electron transport rate (ETR) was calculated as: $ETR = 0.5 \times 0.84 \times I \times \Phi_{PSII,}$ where 0.5 is the fraction of absorbed quanta used by PSII, 0.84 is the leaf absorbance, and I is the incident PPFD (μmol m^{-2} s^{-1}).

2.4. Determination of carotenoids and chlorophylls content

Carotenoid and chlorophyll assessment was performed following Wellburn (1994) and Zouari et al. (2014), as modified by Rigano et al. (2016). To obtain the lipophilic extract, 0.30 g of sample was extracted with 24 ml of acetone/hexane (40/60, v/v). The mixture was centrifuged at 20,000 g for 5 min at 4 °C. The supernatants were collected and stored at −20 °C until analysis. For the determination of carotenoids and chlorophyll a and b, the absorbance of the lipophilic extracts was read at 470, 663 and 645 nm, respectively. Results were expressed as mg 100 g^{-1} FW. Five separate biological replicates for each sample and three technical assays for each biological replicate were measured.

2.5. Hydrogen peroxide and malondialdehyde determination

The H_2O_2 content was quantified by a colorimetric method (Sergiev et al., 1997). Briefly, 500 mg of frozen leaf powder was extracted with 5 ml ice-cold 0.1 % trichloroacetic acid (TCA), the mixture was incubated on ice for 15 min and centrifuged at 9400 g for 15 min at 4 °C. To 500 μl of the supernatant, 500 μl of phosphate buffer 10 mM (pH 7.0) and 1 ml of potassium iodide (1 M) were added. The mixtures were then incubated in the dark for 40 min and measured at 525 nm using a nano

photometer (Implen, Munich, Germany). Concentrations were expressed as mmol g^{-1} FW. For the determination of malondialdehyde (MDA), 0.2 g of leaf sample was ground with 1 ml ice-cold 0.1 % TCA. Samples were incubated on ice for 15 min and centrifuged at 9400 g for 10 min at 4 °C. Then 0.25 ml supernatant was mixed with 1250 ml reaction solution (TCA 20 % + thiobarbituric acid [TBA] 0.5 %), placed in a water bath at 95 °C for 30 min and measured at 532 and 600 nm with a Nano Photometer TM (Implen). The concentration was expressed as the amount of MDA-TBA complex (Zhang and Kirkham, 1996). For both analyses, five separate biological replicates were measured for each sample and three technical assays were measured for each biological replicate.

2.6. Leaf pigment index

Leaf pigment indexes such as chlorophyll content index, anthocyanin index, flavonol index, and nitrogen flavonol index (NFI, ratio of chlorophyll to flavonoids) were non-destructively measured on five fully expanded leaves *per* genotype *per* treatments using a portable instrument (multi-PigmentMeter MPM-100, Paris, France). This instrument uses ratio fluorescence to measure anthocyanin and flavonol content (ratios F660nm/ F525nm and F660nm/F325nm respectively) and leaf transmittance in the near and far and far infrared to measure chlorophyll content (T850/ T720). NFI was calculated as the ratio between chlorophyll (T850/T720) and flavonol (F660nm/F325nm) content.

2.7. Statistical analyses

Data were subjected to analysis of variance using a two-way ANOVA test. To separate means within each parameter, the student's *t*-test was performed. Differences at $p < 0.05$ were considered to be significant. To explore the overall data, we used the R environment for statistical computing and graphics R Core Team (2018). The PCA plot was obtained using R packages *factoMineR* version 2.9 (Le et al., 2008) and *factoextra* version 1.0.7 (Kassambara and Mundt, 2020).

2.8. Genome analyses

Genotyping analyses were conducted on the E42 and LA3120 genome data (available at https://data.mendeley.com/datasets/hcr5fwtpf3/1) obtained by using the Genotyping-By-Sequencing (GBS) platform through the double digest restriction-site associated (ddRAD) Illumina technology (Olivieri et al., 2020). Raw FASTQ files were processed in accordance with Graci et al. (2023). The number and distribution of homozygous variants for the alternative allele compared to Heinz were evaluated through a single-nucleotide polymorphism (SNP) density analysis by using the snpden function of VCFtools (1 Mb non-overlapping windows). Variants were annotated through the snpEff software (Cingolani et al., 2012) as reported by Graci et al. (2023). Finally, a list of genes potentially involved in the HS response of the two genotypes was prioritized by selecting those: I) showing HIGH and/or MODERATE impact on the translated protein and II) presenting Gene Ontology terms related to photosynthesis (GO:0015979), identified on the QuickGO database (Binns et al., 2009).

3. Results

3.1. Morpho-anatomical changes in tomato heat stressed plants

Morpho-anatomical changes in the two genotypes LA3120 and E42 were determined during exposure to HS in controlled chambers. Two-way ANOVA test evidenced that leaf area, specific leaf area and fresh weight biomass were significantly affected by the single factors heat stress and genotype, while the dry weight biomass was influenced only by the treatment factor (Table S1). The GxT interaction was not significant for all morpho-anatomical traits. Leaf area differed between genotypes under control condition, and the largest leaf area (154.25 ± 35.66 cm^2) was recorded in LA3120 (Table 1). In both genotypes a significant reduction in both leaf area and specific leaf area occurred under high temperatures. A strong decrease (approximately 50 %) in fresh and dry biomass weight was also observed in both genotypes exposed to high temperatures compared to control plants.

Table 1
Plant growth parameters and leaf traits (leaf area, specific leaf area, shoot and dry biomass weight) of two tomato genotypes under control (CTRL) and high temperatures conditions (HEAT). The data are mean ± standard deviation of five biological replicates. Within each tomato genotypes, asterisks indicate significant differences between treatments based on Student's *t*-test ($p < 0.05$*; $p < 0.01$**; $p < 0.001$***).

Parameters	E42		LA3120	
	CTRL	HEAT	CTRL	HEAT
Leaf area (cm^2)	87.83 ± 11.42	44.47 ± 15.76**	154.25 ± 35.66	107.81 ± 25.34*
Specific leaf area (cm^2 g^{-1})	314.97 ± 23.08	191.81 ± 41.45**	257.53 ± 41.84	142.62 ± 27.73**
Fresh weight biomass (g)	436.57 ± 86	218.8 ± 34.9**	505.2 ± 44.98	263.3 ± 63.05**
Dry weight biomass (g)	84.25 ± 24.36	39 ± 4.50*	96.98 ± 25.828	49.28 ± 5.16**

3.2. Heat stress modulate photochemical and fluorescence parameters in tomato plants

After exposure to thermal stress at 36 °C, both genotypes exhibited physiological and photochemical changes compared to non-treated plants (Fig. 1). ANOVA test indicated that genotypes, heat treatment and their interactions affected stomatal conductance (gs), intrinsic water use efficiency (iWUE) and quantum yield of PSII electron transport (ΦPSII). For the other photosynthetic parameters, there were no significant interactions between the factors, but only an effect of the single factor (genotype and treatment). On the contrary, internal CO_2 concentration (Ci) and non-photochemical quenching (NPQ_t) were only influenced by heat treatment and genotype, respectively (Table S1). Interestingly, in the absence of HS, significant differences were observed for all photosynthetic parameters between the two genotypes. The basal net photosynthetic values (A_N) under control conditions were significantly different between E42 and LA3120, presenting values of 27.58 and 14.20 μmol CO_2 m^{-2} s^{-1}, respectively (Fig. 1a). The CO_2 assimilation rate measured immediately after HS was significantly reduced (−20.34 %) in E42, while in LA3120 no modifications after stress were observed. Analyses of stomatal conductance (g_s) showed that it was different between genotypes (Fig. 1b) when no heating was applied, and g_s values were 0.79 and 0.26 mol m^{-2} s^{-1} in E42 and LA3120, respectively. After HS the stomatal conductance increased in both genotypes (+206 % in E42 and +246 % in LA3120). Under control conditions E42 displayed a higher leaf intercellular CO_2 concentration (C_i) compared to LA3120 (Fig. 1c); however, and consistent with g_s values, both genotypes showed a significant increase in C_i after HS. The intrinsic water use efficiency ($_i$WUE) data are shown in Fig. 1d and demonstrated that LA3120 had a 48 % higher water use efficiency than E42 under control conditions. Nevertheless, $_i$WUE decreased significantly in both genotypes under HS, with LA3120 showing a more dramatic decrease compared to the control (−76.42 %).

The parameters of Chl fluorescence were measured under control and HS conditions (Fig. 2). Interestingly, it was possible to note a constitutive difference in all the Chl fluorescence parameters between the two genotypes, with E42 presenting under control conditions higher values for Φ_{PSII} (quantum yield of PSII electron transport), qP (photochemical quenching) and ETR (electron transport rate) (Fig. 2a, b, d), and lower values for NPQ_t (non-photochemical quenching) (Fig. 2c) compared to LA3120. This confirmed the higher photosynthetic

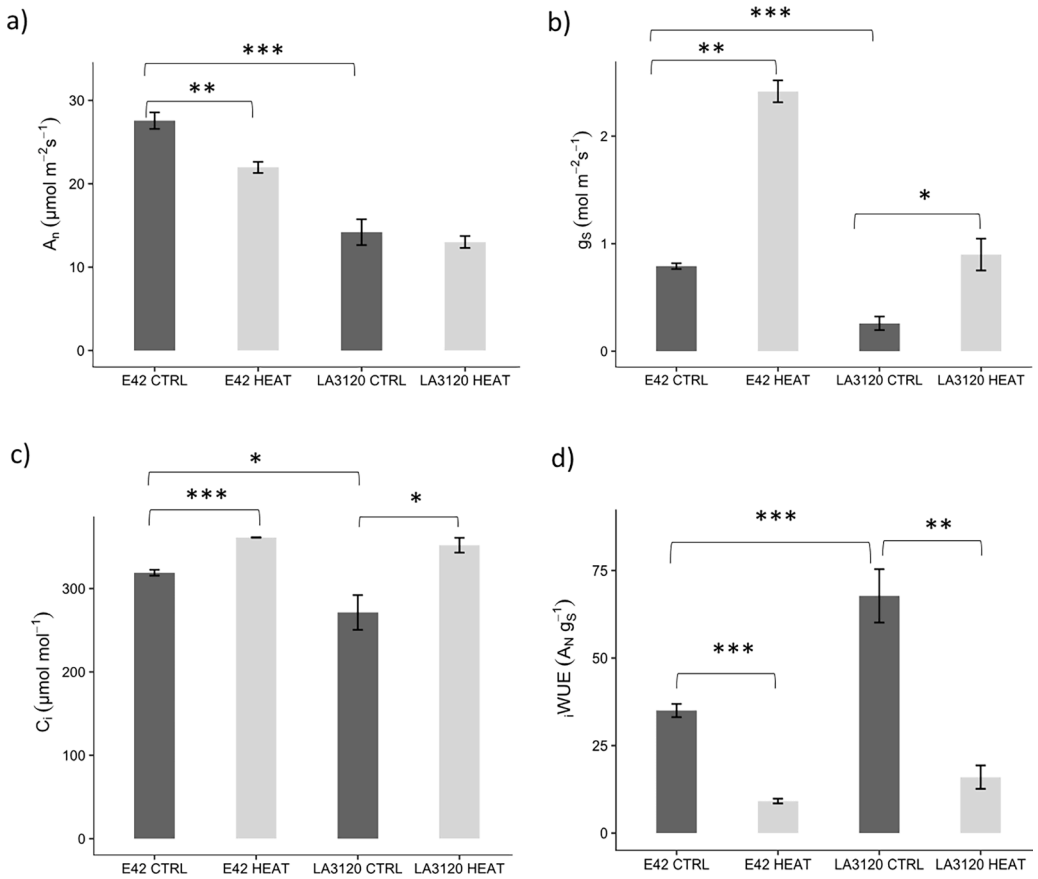

Fig. 1. Gas exchange in leaves of two tomato genotypes under control (CTRL) and high temperatures conditions (HEAT). (a) Net photosynthesis (A_N), (b) stomatal conductance (g_s), (c) intracellular CO_2 concentration (C_i), (d) intrinsic water use efficiency (iWUE). Data are mean ± standard error (SE) ($n = 5$). Asterisks indicate significant differences between genotypes and between treatments based on Student's *t*-test ($p < 0.05$*; $p < 0.01$**; $p < 0.001$***).

performance of E42 (see Fig. 1a). The parameters Φ_{PSII} and qP showed a significant decrease with the increasing temperatures only in E42 (Fig. 2a,b). Moreover, as expected, HS caused a reduction in ETR that was statistically significant only in E42 (Fig. 2d), with a reduction of 30.41 % relative to its basal value.

3.3. Effects of high temperature stress on photosynthetic pigment content, oxidative markers and secondary metabolites

Usually, HS alters chlorophylls and carotenoid contents in plants. In this study, carotenoid content decreased (−27.34 %) under high temperature only in LA3120, while chlorophylls *a* and *b* content decreased under heat in both genotypes (Table 2) according with ANOVA test which indicated a significant effect only of the treatment (Table S1). That said, E42 subjected to heat showed a slight reduction of chlorophylls *a* and *b* (−18.52 % and −18.87 %, respectively) compared to the control, whereas a higher reduction in Chl *a* and *b* levels was observed in LA3120 treated plants (−28.96 % and −33.66 %, respectively). However, these differences in Chl *a* and *b* levels did not significantly affect the Chl *a/b* ratio in both genotypes. In order to check whether the stress conditions were responsible for oxidative damage in the two genotypes, the concentrations of H_2O_2 and MDA were determined. As expected, the levels of the two oxidative markers used were higher in heat-stressed plants than in control plants, being significantly affected by the heat treatment (Table S1). No significant changes were observed in the production of flavonols and anthocyanins among treated and control plants in E42. On the contrary, an increase in flavonol content was observed in LA3120 after HS. Similar to the flavonol index, no significant differences in nitrogen-flavonol index (NFI) were found in E42 after HS. However, it was observed that LA3120 under HS exhibited lower NFI compared to control plants.

3.4. Principal components analysis

To provide a broad overview of the different analyses conducted on the two tomato genotypes in response to HS, a PCA was conducted. Based on our experimental data, three principal components (PCs) were associated with Eigenvalues >1 and accounted for 100 % of the total variance, with PC1, PC2, PC3 accounting for 58.6 %, 36.1 %, and 5.4 %, respectively (Table S2). PC1 was the primary drive for differences between treatment (Fig. 3, red light box) and the main parameters leading to this separation were oxidative markers and plant growth indices. There was also a genotype-dependent clustering, with the primary differences driven by PC2 (Fig. 3, blue light). The main parameters of PC2 were linked to primary and secondary metabolism.

3.6. Genome analyses

The VCF file of the two genotypes obtained from GBS sequencing data evidenced a final dataset of 30,046 and 9562 variants for E42 and LA3120, respectively, respect to the reference genome of Heinz. The highest number of homozygous alternative polymorphisms compared to Heinz was detected in E42 (22,150), while LA3120 showed 7474 homozygous polymorphisms. When looking at the distribution of the homozygous alternative variants compared to Heinz across the chromosomes (Table S3), E42 showed a high number of polymorphisms on chromosomes 1, 4, 7 and 12. On the other hand, LA3120 displayed a slightly higher number of variants on chromosome 12 compared to the others. In order to estimate the probable impact on proteins of homozygous SNPs and InDels detected in the two genotypes compared to

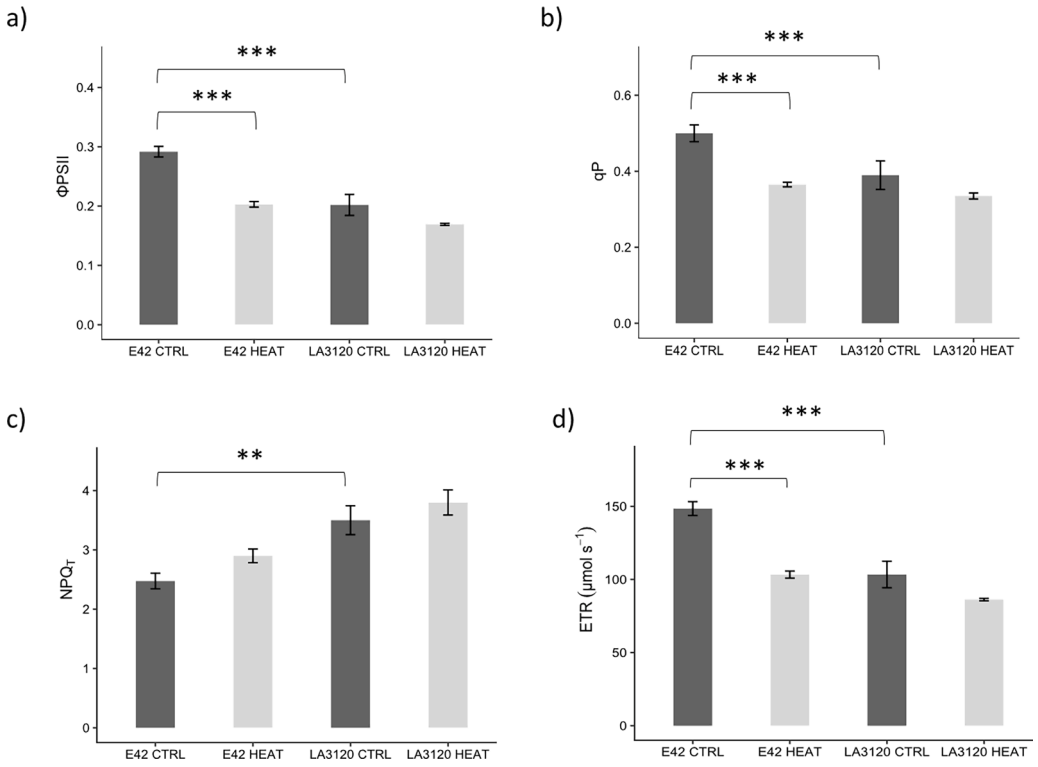

Fig. 2. Fluorescence measurements in leaves of two tomato genotypes under control (CTRL) and high temperatures conditions (HEAT). (a) quantum yield of PSII (Φ_{PSII}), (b) photochemical quenching (qP), (c) nonphotochemical quenching (NPQ_T), (d) electron transport rate (ETR). Data are mean ± standard error (SE) ($n = 5$). Asterisks indicate significant differences between genotypes and between treatments based on Student's *t*-test ($p < 0.05$*; $p < 0.01$**; $p < 0.001$***).

Table 2
Pigments content, oxidative markers and secondary metabolites of two tomato genotypes under control (CTRL) and high temperatures conditions (HEAT). Data are means ± standard deviation ($n = 5$). Within each tomato line, asterisks indicate significant differences between treatments based on Student's *t*-test ($p < 0.05$*; $p < 0.01$**; $p < 0.001$***).

Parameters	E42		LA3120	
	CTRL	HEAT	CTRL	HEAT
Carotenoids (mg 100 g FW^{-1})	17.07 ± 2.55	16.40 ± 3.30	21.14 ± 2.21	15.36 ± 1.42***
Chla (mg 100 g FW^{-1})	94.94 ± 5.64	77.35 ± 11.87**	100.67 ± 7.78	71.51 ± 6.27***
Chlb (mg 100 g FW^{-1})	48.57 ± 5.95	39.40 ± 5.11*	53.85 ± 10.21	35.72 ± 3.90***
Chla/b (mg 100 g FW^{-1})	1.92 ± 0.10	1.95 ± 0.07	1.96 ± 0.19	2.00 ± 0.05
Hydrogen peroxide (mmol g^{-1})	0.086 ± 0.03	0.168 ± 0.03*	0.086 ± 0.01	0.156 ± 0.01**
Lipid peroxidation (nmol MDA-TBA g FW^{-1})	0.032 ± 0.002	0.052 ± 0.004***	0.035 ± 0.004	0.054 ± 0.001***
Flavonol content	0.26 ± 0.13	0.26 ± 0.08	0.08 ± 0.03	0.17 ± 0.07*
Anthocyanins content	0.073 ± 0.005	0.076 ± 0.010	0.050 ± 0.020	0.053 ± 0.02
Nitrogen flavonol index (NFI)	2.24 ± 0.83	2.14 ± 0.87	3.54 ± 1.28	1.67 ± 0.57**

Heinz, snpEff analysis was performed. Results showed that most of the variants had a putative MODIFIER impact (98 % and 96.6 %), followed by those with MODERATE (1.1 % and 1.6 %), LOW (0.8 % and 1.5 %) and HIGH (0.1 % and 0.3 %) impact in E42 and LA3120, respectively. E42 presented 31 HIGH and 288 MODERATE variants affecting 29 and 207 genes, respectively, while LA3120 showed 22 HIGH and 117 MODERATE polymorphisms affecting 21 and 94 genes, respectively.

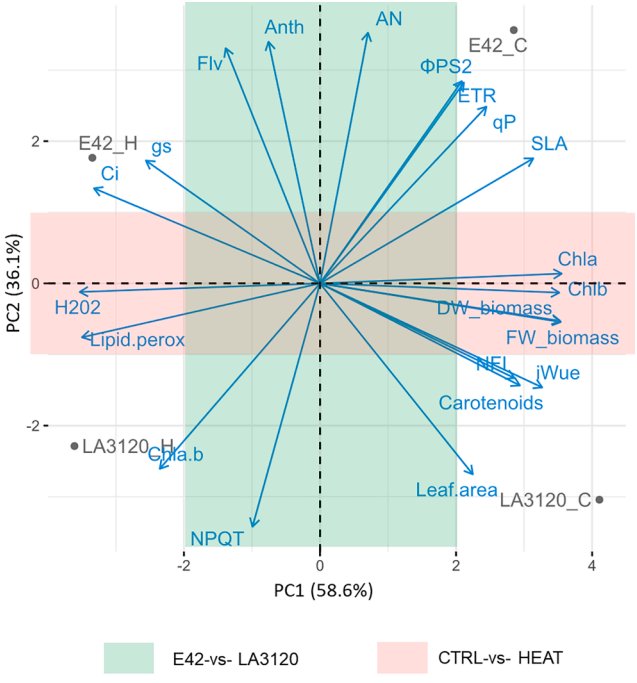

Fig. 3. Principal component loading plot and scores of principal components analysis (PCA) in two tomato genotypes under control (C) and heat (H) stress.

A list of 101 GO terms (Table S4) was retrieved from the QuickGO database by selecting the GO:0015979 biological process term annotated for "photosynthesis" and the first 100 co-occurring GO terms, that were co-annotated to the GO:0015979 for the highest number of times. These features were combined and a list of 187 genes (Table S5) were

Table 3
List of the 22 E42 and LA3120 candidate genes showing HIGH and/or MODERATE variants in the gene coding sequences and also exhibiting GO terms related to photosynthesis.

Gene	Position (SL4.0)	Impact	Effect	Genotype	Annotation ITAG4.1
Solyc01g056890	50,654,068	MODERATE	missense_variant	E42	protein CURVATURE THYLAKOID 1A, chloroplastic-like
	50,654,069	MODERATE	missense_variant	E42	
Solyc01g090710	76,596,312	MODERATE	missense_variant	E42	Malate dehydrogenase
Solyc02g011900	12,636,271	MODERATE	missense_variant	E42	NAD(P)H-quinone oxidoreductase subunit 2, chloroplastic
Solyc02g090890	50,411,604	MODERATE	missense_variant	E42	zeaxanthin epoxidase
Solyc03g124068	65,296,482	MODERATE	missense_variant	LA3120	Ent-kaurene synthase
	65,296,500	MODERATE	missense_variant	LA3120	
	65,296,533	MODERATE	missense_variant	LA3120	
	65,296,789	MODERATE	missense_variant	LA3120	
Solyc04g014210	4,534,561	MODERATE	missense_variant	E42	RNA helicase DEAH-box12
Solyc04g039820	12,288,603	MODERATE	missense_variant	E42	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic
Solyc04g076870	59,787,258	MODERATE	missense_variant	E42, LA3120	Glutamyl-tRNA reductase
Solyc05g055000	64,162,774	MODERATE	missense_variant	LA3120	Cysteine desulfurase
Solyc07g021200	16,851,870	MODERATE	missense_variant	E42	Ribulose bisphosphate carboxylase large chain
Solyc07g021205	16,852,040	MODERATE	missense_variant	E42	DNA-directed RNA polymerase subunit beta
	16,852,059	MODERATE	missense_variant	E42	
Solyc07g056020	63,838,955	MODERATE	missense_variant	E42	Translation initiation factor IF-2
Solyc07g062930	65,398,593	MODERATE	missense_variant	E42	Ribosomal L.1
Solyc08g076480	58,602,801	HIGH	stop_gained	LA3120	Plastid lipid-associated protein chloroplastic-like
Solyc11g007780	2,058,238	MODERATE	missense_variant	E42	SEC12-like protein.1
Solyc11g019860	9,795,826	MODERATE	missense_variant	LA3120	Adenylate isopentenyltransferase
Solyc11g020507	11,424,152	MODERATE	missense_variant	E42, LA3120	Adenylate isopentenyltransferase
Solyc11g065930	49,569,438	MODERATE	missense_variant	E42	Xanthine dehydrogenase
Solyc12g009990	3,175,319	MODERATE	missense_variant	E42	Signal recognition particle receptor subunit alpha
	3,175,321	MODERATE	missense_variant	E42	
	3,175,323	MODERATE	missense_variant	E42	
Solyc12g014250	5,109,796	MODERATE	missense_variant	LA3120	Phosphoenolpyruvate carboxylase
Solyc12g044450	59,073,114	MODERATE	missense_variant	LA3120	Protein trichome birefringence-like 42
Solyc12g056940	62,629,174	MODERATE	missense_variant	LA3120	Acetyl CoA carboxylase
	62,629,532	MODERATE	missense_variant	LA3120	

prioritized since they showed 245 HIGH and/or MODERATE impact variants in the coding sequence of E42 and/or LA3120 and also exhibited annotation for GO terms related to photosynthesis. Among these genes, seven HIGH and 152 MODERATE polymorphisms affected 116 E42 genes, while five HIGH and 63 MODERATE mutations interested 54 LA3120 genes. In addition, both the genotypes displayed 17 genes exhibiting nine HIGH and nine MODERATE variants. Finally, from the list of 187 genes, a subset of 22 genes (Table 3) was highlighted, including those candidate genes that may explain the different physiological response to HS in the two genotypes.

Interestingly, the highest number of variants (17 MODERATE) were exclusively found in the E42 genome, followed by one HIGH and ten MODERATE mutations in LA3120, and two MODERATE polymorphisms common to both genotypes. Out of the 19 E42 variants, 14 mapped in the four most polymorphic chromosomes (1, 4, 7 and 12), while eight out of 13 LA3120 polymorphisms mapped on the other chromosomes.

4. Discussions

In this study, two tomato genotypes already identified as heat-tolerant based on yield-related traits were exposed to prolonged HS from the seedling stage up to the adult stage. Although high temperatures have been shown to affect all plant physiological processes, it remains unclear why high temperatures suppress plant growth and why some plants appear to be more heat tolerant than others. Over the past decade, several research studies focused on the effects of HS on specific physiological mechanisms, such as photosynthesis (Zahra et al., 2023). The majority of these studies were performed on plants at the seedling or vegetative stage, while little effort has been made to integrate the effects of high temperatures on these mechanisms in adult plants. A previous work (Francesca et al., 2021) carried out on the HS tolerant genotypes E42 and LA3120 on 20-day-old plants revealed the presence of valuable physiological traits for adaptation to stressful environments in both genotypes. However, since temperature is the main determinant of plant phenology, here we wondered how prolonged HS for almost the entire plant cycle might alter stress adaptation mechanisms. Looking at the overview provided by the PCA analysis, it can be observed that the experimental setup used in this study allowed us to better identify the physiological traits affected by HS in both genotypes and, at the same time, to highlight the distinctive strategy adopted by them.

Generally, plants respond to changing environmental conditions by adjusting a suite of morphological traits, such as specific leaf area (SLA, the ratio of leaf area to leaf dry mass) and leaf size (Legner et al., 2014). In particular, given the direct relationship between SLA and light interception (Reich et al., 1998), SLA is intimately connected to resource acquisition and photosynthetic performance (Guo et al., 2022; Wright et al., 2004). In this work, we were able to decouple the effect of heat on leaf morphology from the genotype. Indeed, and contrary to what was observed previously on young plants (Francesca et al., 2021), here a general reduction in leaf area and SLA after HS was observed in both genotypes. This is in accordance with previous studies that demonstrated that leaves with lower SLA are better able to withstand and recover photosynthetic electron transport after high temperature stresses compared with leaves with greater SLA (Knight et al., 2003). It is generally recognized that the photosynthetic rate is associated with SLA; indeed, by increasing the light capture area per mass, high SLA improves photosynthesis (Goorman et al., 2011). In the present study both a higher SLA and a higher photosynthetic rate (A_N) were observed in E42 under control condition compared to LA3120. The A_N values observed in the present study were not observed previously when the same analyses were carried out on younger plants, stressing the fact that size and developmental stage deeply influence physiological traits. This observed high A_N could be the reason behind the high number of flowers and the high and stable final fruit yield observed in E42 in all the field trials previously conducted (Olivieri et al., 2020). In the present work, A_N was affected by the heat treatments in a genotype-dependent way, as it was negatively affected by stress only in E42, reaching the same A_N values observed in LA3120 both under stressful and non-stressful conditions. Under HS, generally, stomatal conductance (g_s) rises to increase intracellular CO_2 concentration and facilitate water loss by transpiration (Dos

Santos et al., 2022). Evaporative cooling through open stomata mitigates the negative effects of supra-optimal temperatures and have a positive effect on photosynthesis, yield, and plant survival (Ameye et al., 2012; Urban et al., 2017). Accordingly, under HS an increase in stomatal conductance (g_s) and intracellular CO_2 concentration (C_i) was here observed, as expected. A stable photosynthetic rate combined with an increased stomatal conductivity, as shown here in LA3120, has been previously reported in heat-tolerant tomato and pepper cultivars (Zhou et al., 2015). On the contrary, the A_N decrease accompanied by the increases in gs and C_i values observed in E42 after HS could indicate biochemical limitations (Zhou et al., 2015).

Fluorescence parameters measured under non-stressed condition confirmed the higher photosynthetic performances of E42, which showed higher levels of quantum yield of PSII (Φ_{PSII}), photochemical quenching (qP) and electron transport rate (ETR), and lower levels of non-photochemical quenching (NPQ) compared to LA3120. After HS, only in E42 a decrease in qP, Φ_{PSII} and ETR was demonstrated. This observation, which well fits with the gas exchange data previously discussed, confirmed that the photosynthetic apparatus of E42 was more sensible than LA3120 to HS.

Another consequence of abiotic stress in plants is the oxidative damage. This leads to the overproduction of reactive oxygen species (ROS), which are primarily directed against the lipids of the membrane (Francesca et al., 2022). Accordingly, an increase in H_2O_2 content and lipid peroxidation under HS was here observed in stressed plants. These changes were accompanied by an increase in flavonol content only in LA3120, while E42 did not alter its already high basal levels of flavonols and anthocyanins.

In order to identify the genetic basis of the identified physiological traits involved in the response mechanism to HS, a genomic analysis was here performed. Data obtained from GBS high-throughput genotyping technology were investigated to identify candidate genes involved in HS-tolerance mechanisms in E42 and LA3120. Francesca et al. (2021) already used the same dataset thus highlighting five genes showing E42 private variants with HIGH impact on the translated proteins, while no genes were previously discussed to explain the LA3120 response. Moreover, the analysis carried out in that study did not focus on all the MODERATE polymorphisms, which contribute to change the protein function by altering the amino acid sequence. Herein, attention was focused on all the genes showing HIGH and MODERATE polymorphisms known to be involved in the photosynthetic process by using their gene ontology (GO) annotation. This strategy allowed us to identify 22 candidate genes among which 13 presented variants exclusively in E42 and seven in LA3120. The genes Solyc01g056890 coding for the protein CURVATURE and the Solyc07g021200 coding for the Ribulose bisphosphate carboxylase large chain were already highlighted by Francesca et al. (2021) in E42. The presence of these variants is in accordance with physiological data here observed. Indeed, the A_N decrease observed in E42 after HS could have been caused by modification in the gene Solyc07g021200.

Interestingly, the analyses carried out in the present study allowed the identification of another gene in E42, the gene Solyc02g090890, coding for a zeaxanthin epoxidase showing one missense variant. Zeaxanthin is a crucial carotenoid and, as a free pigment in thylakoids, plays a key role in antioxidation and stabilizing the membrane structure by inhibiting and scavenging reactive oxygen species and suppressing lipid peroxidation (Tang et al., 2021). One hypothesis is that the MODERATE polymorphism in this gene may have blocked the zeaxanthin epoxidase activity, limiting the conversion of violaxanthin to zeaxanthin via the so-called xanthophyll cycle. Experiments have shown that NPQ cannot be activated when the xanthophyll cycle is blocked (Gilmore, 1997; Dreuw et al., 2003). Accordingly, our data showed lower NPQ values in E42 compared to LA3120 under all conditions. The block of the xanthophyll cycle could also have promoted in E42 the synthesis of the 9-cis-epoxycarotenoid dioxygenase (NCED) enzyme which is reported to positively regulate plant tolerance to abiotic stresses (Zhang et al., 2022). Considering LA3120, GBS analysis evidenced the presence of four missense variants in the Solyc03g124068 coding for the Ent-kaurene synthase. This is the key enzyme for the biosynthesis of gibberellins (GAs), which catalyze the formation of ent-kaurene, the precursor for GAs (Yang et al., 2021). This hormone is involved in flowering, pollen formation and pollen tube growth and promotes tomato fruit set and fruit initiation (Sponsel, 2016; Graci and Barone, 2024). It is also involved in leaf expansion, dry matter accumulation, photosynthesis, and transpiration rate. A clear correlation between this hormone and tolerance to drought and HS has also been found (Shohat et al., 2021). Recently, Guo et al. (2022) used gibberellic acid as a foliar treatment to alleviate HS in tomato plants. The polymorphisms present in the Ent-kaurene synthase gene may have had an impact on GA synthesis and accumulation. This well correlates with the physiological data observed here and in previous paper on LA3120 (Francesca et al., 2021). This is also in accordance with the LA3120 phenotypic data recorded in different years and environments, that showed a reduction of the time required in this genotype for flower and fruit formation (Ayenan et al., 2021; Olivieri et al., 2021). This could be a key mechanism in LA3120 that allows it to precociously form flower and fruit before the onset of the high summer temperatures typical of the Mediterranean area. Other two genes (Solyc12g014250 coding for the Phosphoenolpyruvate carboxylase and Solyc12g056940 coding for the Acetyl CoA carboxylase) were here identified that showed one and two missense variants in LA3120, respectively. Also, these two genes may play pivotal role in plants during carbon metabolism in photosynthesis, physicochemical processes, and tolerance to various abiotic stresses (Waseem and Ahmad, 2019; Upadhyay et al., 2020).

Altogether, in the present study a more in-depth physiological analysis was conducted on two thermotolerant genotypes, which were here subjected to a prolonged HS over a period of 5-weeks. The genotypes were able to employ different defense mechanisms in response to HS revealing key tolerance traits. The candidate genes here identified by combining the phenotypic and genotypic analyses carried out in this work, helped to dissect these complex traits, and could explain the different physiological response to stress observed. These genes should be further studied and could be used in future breeding programs in order to improve tolerance to abiotic stress in modern cultivars.

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CRediT authorship contribution statement

S. Francesca: Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **L. Vitale:** Writing – review & editing, Methodology, Data curation. **S. Graci:** Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **M. Addonizio:** Methodology, Data curation. **A. Barone:** Writing – review & editing, Funding acquisition, Conceptualization. **M.M. Rigano:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The GBS raw data that support the findings of this study are available here: https://nam11.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdata.mendeley.com%2Fdatasets%2Fhcr5fwtpf3%2F1&data=05%7C02%7Ce.j%40elsevier.com%7C68d7f1d2814643d92b9408dcb59b49ce%7C9274ee3f94254109a27f9fb15c10675d%7C0%7C0%7C638584925771685462%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=gP%2F3yUQj3EZydmahdhwN1bCAriTrJfDX%2Bjy%2BjLBsJfU%3D&reserved=0.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100555.

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