

Title: Synchronization and interaction of proline, ascorbate and oxidative stress pathways under abiotic stress combination in tomato plants

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HIGHLIGHTS:

- The combination of salinity and heat causes a unique reprogramming of tomato metabolic pathways by changing the expression of specific genes and metabolic features.
- Proline and ascorbate pathways act synchronously to maintain cellular redox homeostasis
- Key transcription factors from the basic Leucine Zipper Domain (bZIP), Zinc Finger Cysteine-2/Histidine-2 (C2H2) and Trihelix families were identified as putative regulators of the identified up-regulated genes under salinity and heat combination.

ABSTRACT

Adverse environmental conditions have a devastating impact on plant productivity. In nature, multiple abiotic stresses occur simultaneously, and plants have evolved unique responses to cope against this combination of stresses. Here, we coupled genome-wide transcriptional profiling and untargeted metabolomics with physiological and biochemical analyses to characterize the effect of salinity and heat applied in combination on the metabolism of tomato plants. Our results demonstrate that this combination of stresses causes a unique reprogramming of metabolic pathways, including changes in the expression of 1,388 genes and the accumulation of 568 molecular features. Pathway enrichment analysis of transcript and metabolite data indicated that the proline and ascorbate pathways act synchronously to maintain cellular redox homeostasis, which was supported by measurements of enzymatic activity and oxidative stress markers. We also identified key transcription factors from the basic Leucine Zipper Domain (bZIP), Zinc Finger Cysteine-2/Histidine-2 (C2H2) and Trihelix families that are likely regulators of the identified up-regulated genes under salinity+heat combination. Our results expand the current understanding of how plants acclimate to environmental stresses in combination and unveils the synergy between key cellular metabolic pathways for effective ROS detoxification. Our study opens the door to elucidating the different signaling mechanisms for stress tolerance.

Keywords: salinity, heat, protein oxidation, lipid peroxidation, reactive oxygen species, plant stress.

1 INTRODUCTION

2 Multiple environmental factors such as salinity, high temperatures, cold, or drought
3 cause abiotic stresses in plants, which result in large agricultural losses worldwide,
4 estimated to be around \$14–19 million (Rivera *et al.*, 2017). Under field conditions,
5 different abiotic stressors usually occur at the same time; for example, it is common that
6 high temperatures coexist with highly saline soils or water scarcity. Studies in the last
7 decade have shown that plant responses to combined abiotic stresses are unique and
8 cannot be deduced from the study of plants subjected to each stress separately (Mittler,
9 2006; Miller *et al.*, 2010; Rivero *et al.*, 2014; Anjum *et al.*, 2019; Sehgal *et al.*, 2019;
10 Lopez-Delacalle *et al.*, 2020; Zandalinas *et al.*, 2020).

11 Many metabolic mechanisms act in concert during the plant's response to abiotic stress,
12 including rapid changes in gene expression, ionic adjustment, and activation and
13 inactivation of proteins that carry out the synthesis and degradation of compounds used
14 for cell signaling and protection (e.g., osmoprotectants and antioxidants), among others
15 (Rivero *et al.*, 2014; Zushi *et al.*, 2014; Zandalinas *et al.*, 2020). Proline has been widely
16 reported to act as an osmoprotectant in plant defense against certain stress conditions,
17 such as drought and salinity (Rivero *et al.*, 2004b, 2014)(Rivero *et al.*, 2004b, 2014;
18 Martinez *et al.*, 2018). Under heat stress, plants synthesize proline, as reported by the
19 induction of pyrroline-5-carboxylate synthase (P5CS) and the subsequent accumulation
20 of the amino acid (Rivero *et al.*, 2004b; Torres *et al.*, 2006). Shalata & Neumann (2001)
21 have also reported that under salinity, proline accumulation can improve plant salt
22 tolerance and reduce oxidative damage by decreasing lipid peroxidation in tomato
23 plants.

24 Stress conditions cause the accumulation of reactive oxygen species (ROS), which are
25 known to induce oxidative stress (e.g., lipid peroxidation) and serve as signaling
26 molecules in plants (Suzuki *et al.*, 2012; Kollist *et al.*, 2019). Plants accumulate
27 antioxidants, such as ascorbate (ASC), glutathione (GSH), carotenoids, tocopherols, and
28 flavonoids, and activate enzymatic reactions to maintain cell homeostasis under
29 increasing oxidative conditions. The ASC/GSH cycle is critical for detoxifying ROS
30 from plant cells. Briefly, the hydrogen peroxide (H₂O₂) generated by detoxification of
31 superoxide radicals is further converted to H₂O and O₂ by ASC peroxidase (APX), the
32 first enzyme of the ASC/GSH cycle, using ASC as an electron donor (Noctor and Foyer,
33 1998). Because ASC is considered the first antioxidant line of defense in H₂O₂

34 detoxification (Foyer and Noctor, 2011; Akram *et al.*, 2017), it is expected that plants
35 with a high cellular accumulation of this compound will have a greater tolerance to
36 oxidative stress. In fact, tomato seeds treated with ascorbic acid have been shown to
37 have better tolerance to salinity stress, improved germination, and better growth
38 parameters (Sayed, 2013). We have previously reported (Rivero *et al.* 2004) that
39 enzymes that belong to the ASC/GSH cycle were inhibited in tomato plants under high
40 temperature, leading to H₂O₂ accumulation and inhibition of plant growth and yield. In
41 addition to its importance in ROS detoxification, the cellular content of GSH and ASC
42 improves osmoregulation, efficient use of water, photosynthetic activity, and general
43 parameters of plant productivity (Noctor and Foyer, 1998; Meyer, 2008; Foyer and
44 Noctor, 2011).

45 Plants need to rapidly regulate and fine-tune their responses to stress in order to
46 maximize energy expenditure in adverse conditions. Transcription factors (TFs) are
47 considered key components in the control of abiotic stress signaling (Schmidt *et al.*,
48 2012; Castelán-Muñoz *et al.*, 2019); however, little is known about their role in stress
49 combination. Just recently, a report by Zandalinas *et al.* (Zandalinas *et al.*, 2020) found
50 that *Arabidopsis* plants induced a unique set of TFs when subjected to different abiotic
51 stress combinations, and that those genes were relatively unique across stress
52 conditions.

53 Here we hypothesize that the combination of salinity and heat induces a unique
54 physiological response in tomato plants by activating specific regulatory and metabolic
55 pathways that act synergistically to maintain cell redox homeostasis. In this work, we
56 analyze how the combination of salinity and heat affects the transcriptome and
57 metabolome of tomato plants in order to find the unique elements that are differentially
58 regulated under these stress conditions and that may be key in ROS detoxification and,
59 thus, plant tolerance to abiotic stress combination.

60 MATERIALS AND METHODS

61 Plant material, experimental design, and growth conditions

62 *Solanum lycopersicum* L cv. Boludo (Monsanto) seeds were germinated in vermiculite
63 under optimal and controlled conditions in a growth chamber (chamber A). These
64 conditions were: a photoperiod of 16/8 hours of day/night with a light intensity of 500
65 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a relative humidity (RH) between 60 and 65% and a temperature of 25 °C.
66 Subsequently, when the plants had at least two true leaves, they were transplanted to an
67 aerated hydroponic system containing a modified Hoagland solution and grown under
68 these conditions for one week. The nutrient solution had the following composition:
69 KNO_3 (3 mM), $\text{Ca}(\text{NO}_3)_2$ (2 mM), MgSO_4 (0.5 mM), KH_2PO_4 (0.5 mM), Fe-EDTA (10
70 μM), H_3BO_3 (10 μM), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (1 μM), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (2 μM), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.5
71 μM), and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.5 μM) (Hoagland and Arnon, 1950). The electric
72 conductivity (EC) and pH of the nutrient solution were measured and maintained within
73 1.4–1.7 $\text{mS} \cdot \text{cm}^{-1}$ and 5.2–5.6, respectively. After acclimatization, half of the plants were
74 transferred to a twin chamber whose temperature was previously set at 35 °C (chamber
75 B). In both twin chambers, a saline concentration in the nutrient solution of 75 mM
76 NaCl was added to half of the plants. Therefore, four different conditions were
77 obtained: control (25 °C 0 mM NaCl), salinity (25 °C and 75 mM NaCl), heat (35 °C 0
78 mM NaCl), and salinity and heat (35 °C and 75 mM NaCl). Plants were kept under
79 these conditions for a period of 14 days. After this time, six plants from each treatment
80 were sampled for subsequent analysis.

81

82 Measurements of photosynthetic parameters

83 Photosynthetic parameters were determined on a fully-expanded, metabolically-mature
84 middle leaf in all plants. These data were taken with a gas exchange system (LI-COR
85 6400, Li-Cor) at the beginning (day 0), the middle (day 7), and at the end of the
86 experiment (day 14). The conditions established in the LI-COR were: 1000 μmol
87 $\text{photons m}^{-2} \text{s}^{-1}$ and 400 $\mu\text{mol mol}^{-1} \text{CO}_2$. The leaf temperature was maintained at 25 °C
88 for control and salinity treatment plants, and at 35 °C for plants in the high temperature,
89 and the combination of high temperature and salinity treatments. The leaf-air vapor
90 pressure deficit was maintained between 1-1.3 kPa. With this analysis, the device
91 reported data on CO_2 assimilation, stomatal conductance, and transpiration rate. At the

92 end of the experiment, the leaves, stem, and root were separated, and the fresh weight
93 (FW) of each part of the plant was determined separately.

94

95 **Quantification of oxidative stress-related markers**

96 *H₂O₂ accumulation*

97 H₂O₂ was extracted as described by Yang et al. (2007), with some modifications, which
98 are fully described in García-Martí et al. (García-Martí *et al.*, 2019). The samples were
99 used for the future determination of H₂O₂ concentration and lipid peroxidation. H₂O₂
100 was quantified as described by MacNevin and Urone (1953).

101 *Lipid peroxidation*

102 For lipid peroxidation determination, malondialdehyde (MDA) was used, which is a
103 product of the peroxidation of membrane lipids. The same enzyme extract as the one
104 utilized for the determination of H₂O₂ was used. The procedure was described by Fu
105 and Huang (2001).

106 *Antioxidant capacity*

107 Regarding antioxidant capacity, it was carried out according to the protocol by Koleva
108 *et al.* (2002). The remaining amount of 2,2-diphenyl-1-picrylhydrazyl (DPPH),
109 measured at a certain time, is inversely proportional to the antioxidant capacity of the
110 substances present in the sample. Results are expressed as % Radical Scavenging
111 Activity (RSA), or percentage of free radical scavenging activity.

112 *Protein oxidation*

113 Protein oxidation was assayed as according to Reznick and Packer (1994). PCO groups
114 react with 2,4-dinitrophenylhydrazine (DNPH) to generate chromophoric
115 dinitrophenylhydrazones, which can be recorded with a spectrophotometer. The
116 absorbance was measured at 360 nm, using the molar extinction coefficient of DNPH
117 $2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

118 **RNA extraction and sequencing**

119 Total RNA was extracted from 1 g of frozen tomato leaves using TRI-Reagent (Sigma-
120 Aldrich) and following the manufacturer's instruction. The quantity and quality of RNA
121 were determined using a NanoDrop 3300 fluorospectrometer (Thermo Scientific
122 Instruments, USA). Three biological replications for each treatment were used for total
123 RNA extraction and sequencing. For each RNA sample, mRNA was enriched using a
124 Dynabeads mRNA purification kit (Invitrogen), then the samples were sent to BGI-

125 Shenzhen (hereafter ‘BGI’, China) for RNA sequencing. Sequencing was carried out on
126 a HiSeq2000 according to the Illumina protocols for 90××2 pair-end sequencing
127 covering a read length of 100 bp. An average of 10 Gb clean data per sample was
128 generated after filtering to ensure a complete set of expressed transcripts with sufficient
129 coverage and depth for each sample.

130

131 **Bioinformatics pipeline**

132 *RNA sequencing and data processing*

133 Raw sequencing reads were trimmed for quality and adapter sequences using
134 Trimmomatic v0.33 (Bolger *et al.*, 2014) were used with the following parameters:
135 maximum seed mismatches = 2, palindrome clip threshold = 30, simple clip threshold =
136 10, minimum leading quality = 3, minimum trailing quality = 3, window size = 4,
137 required quality = 15, and minimum length = 36. Trimmed reads were mapped using
138 Bowtie2 (Langmead and Salzberg, 2012) to the tomato transcriptome (SL4.0 release;
139 <http://solgenomics.net>). Count matrices were made from the Bowtie2 results using
140 sam2counts.py v0.91 (<https://github.com/vsbuffalo/sam2counts/>). A summary of the
141 quality assessment and mapping results can be found in **Supplemental Table S1**. The
142 raw sequencing reads and the read mapping count matrices are available in the National
143 Center for Biotechnology Information Gene Expression Omnibus database under the
144 accession GSE152620.

145 *Differential expression analysis*

146 The Bioconductor package DESeq2 (Love *et al.*, 2014) was used to perform
147 normalization of read counts and differential expression analyses for various treatment
148 comparisons. Differentially expressed (DE) genes for each comparison were those with
149 an adjusted p-value of less than or equal to 0.05.

150 *Functional annotation and enrichment analyses*

151 Basic functional annotations for genes were determined with the Automated
152 Assignment of Human Readable Descriptions (AHRD) provided in the SL4.0 build of
153 the tomato genome. KEGG annotations were determined using the KEGG Automatic
154 Annotation Server (Moriya *et al.*, 2007). Enrichments were conducted via Fisher’s exact
155 test with p-values adjusted using the Benjamini and Hochberg method (Benjamini and
156 Hochberg, 1995).

157 *Promoter motif analysis*

158 Binding motifs for tomato transcription factors were obtained from the Plant
159 Transcription Factor Database (<http://planttfdb.cbi.pku.edu.cn/>). Promoter sequences
160 defined as 1000 base pairs upstream from the transcription start site of each gene were
161 obtained using the ‘flank’ function in bedtools v2.29.2
162 (<https://bedtools.readthedocs.io/en/latest/>). Enrichment of transcription factor binding
163 motifs on the promoter sequences of up-regulated genes was performed using the
164 Analysis of Motif Enrichment tool in MEME-Suite (McLeay and Bailey, 2010) using
165 all non-up-regulated tomato genes as the control sequences, the average odds score
166 scoring method and Fisher's exact test.

167

168 **Metabolomics analysis**

169 Six biological replications of frozen tomato leaves per treatment were used for the
170 metabolomics analysis. One gram of this frozen plant material was extracted in
171 methanol: water (3:1 v/v) as described previously in Martinez *et al.* (2016). Agilent
172 MassHunter Qualitative analysis software v 6.00 (Agilent Technologies, Palo Alto,
173 USA) was used to obtain an initial peak processing (**Supplemental Fig. S1**). Then,
174 XCMS online software (www.xcmsonline.scripts.edu) which incorporates CAMERA (a
175 Bioconductor package to extract spectra, annotate isotopes and adduct peaks, among
176 other functions) in its analysis, was implemented in our curated raw data
177 (**Supplemental Table S2**). A second level of statistical analysis was carried out,
178 consisting of data normalization of the peaks obtained for each treatment against the
179 control, and a t-test followed by an ANOVA analysis. Then, \log_2 of the fold-change was
180 calculated and all the molecular features with a P_{adj} adjusted greater than 0.05 and a \log_2
181 fold change (FC) greater than -1 or smaller than 1 were eliminated from the analyses
182 (**Supplemental Table S3**). All the molecular features that remained after these
183 restricted statistical analyses were compared among the different treatments applied
184 (supplemental Table S3; Euler diagram Fig 3B).

185 The metabolite identification of the molecular features of interest for this study was
186 performed using a mathematical search based on the predicted elemental composition
187 through some of the most important open-source databases (MOTO, KNApSAcK,
188 KOMOCS, MassBank, ARMeC and METLIN) within a mass tolerance of 10 ppm.
189 Then, the isotope ratio (IR) and retention time (rt) from the different metabolites
190 identified unequivocally were checked again across the different databases mentioned

191 above. Identified metabolites that remained after this filtering were labeled accordingly
192 and highlighted in yellow in **Supplemental Table S4**. The concentration of the
193 compounds that showed significant differences ($P_{adj} < 0.05$ and $\log_2 FC > 2$) under
194 salinity and heat combination as compared to control plants and which were of interest
195 in this study were plotted in a box-and-whisker type plot using XCMS online
196 (**Supplemental Figs. S2 and S3**).

197

198 **Enzymatic activities**

199 *Crude extract*

200 All enzymatic activities were extracted according to the procedure described by Torres
201 *et al.* (2006).

202 *Superoxide dismutase (SOD)*

203 SOD activity was assayed as described previously by McCord JM (1969). SOD activity
204 was expressed as units of SOD ($\text{mg prot}^{-1} \text{min}^{-1}$), a unit which indicates the amount of
205 enzyme needed to neutralize one unit of xanthine oxidase.

206 *Catalase (CAT)*

207 CAT activity was calculated using the extinction coefficient of $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ as
208 described by Aebi (1984). CAT activity was expressed as μmol of reduced H_2O_2 (mg
209 $\text{prot}^{-1} \text{min}^{-1}$).

210 *Ascorbate peroxidase (APX) Dehydroascorbate reductase (DHAR) and* 211 *Monodehydroascorbate reductase (MDHAR)*

212 APX, DHAR and MDHAR activities were assayed as described by Miyake and Asada
213 (1992). The rate of reaction was calculated using a molar extinction coefficient of 2.8
214 $\text{mM}^{-1} \text{ cm}^{-1}$. APX activity was expressed as μmol of reduced ascorbic acid (mg prot^{-1}
215 min^{-1}).

216 *Glutathione peroxidase (GPX)*

217 GPX activity was carried out using the Glutathione Peroxidase Assay Kit (Abcam, Ref.
218 ab102530, Cambridge, UK) considering the decrease of NADPH at 340 nm, using an
219 extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$.

220 *Glutathione reductase (GR)*

221 GR activity was assayed through the non-enzymatic NADPH oxidation (Halliwell and
222 Foyer, 1976). The activity was determined by measuring the decrease in the reaction
223 rate at 340nm and was calculated from the 6.22 mM^{-1} extinction coefficient.

224 *Protein concentration in the enzyme extract*

225 Proteins were quantified with the Bradford method (Bradford, 1976), in which a volume
226 of Bradford Reagent reagent (BioRAD, Catalog No. 30214) was added to an aliquot of
227 the enzyme extract. The absolute values as well as the calculated \log_2 of the data
228 normalized against control plants of all the enzymatic activities assayed can be found in
229 **Supplementary Table S5**.

230

231 **Statistical analysis**

232 Statistical analysis for FW, photosynthetic parameters, H_2O_2 concentration, MDA
233 content, protein oxidation, and enzymatic activities were performed with an analysis of
234 variance with p-value < 0.05 set as the cut-off value, as indicative of significant
235 differences, followed by a Duncan test and a t-test when necessary. Transcriptomics and
236 metabolomics statistical analysis was performed as described above.

237

238 **RESULTS**

239 **Tomato plants grown under the combination of salinity showed a better** 240 **performance in the photosynthetic parameters as compared to salinity alone**

241 Eighty-four tomato plants were grown in two independent chambers using four different
242 environmental conditions: 25 °C and 0 mM NaCl (control), 25 °C and 75 mM NaCl
243 (salinity), 35 °C and 0 mM NaCl (heat), and 35 °C and 75 mM NaCl (salinity + heat)
244 for 14 days (**Fig. 1A**). Fresh weight was recorded at the end of the experiment (**Fig. 1B**).
245 Salinity and the salinity + heat resulted in a significant reduction of biomass when
246 compared to control plants, whereas the heat-treated plants did not differ significantly
247 from the controls. Interestingly, when salinity and heat were applied simultaneously, the
248 growth was significantly improved with respect to salinity up to about 18%.
249 Photosynthetic parameters were also measured at 0 days, and after 7 and 14 days after
250 the start of the treatments, as stress physiological markers (**Figs. 1C-F**). In our
251 experiments, CO_2 assimilation rate was highly inhibited after 7 days under salinity as
252 compared to control plants, with an inhibition of 50% at 7 days, and 70% at 14 days
253 with respect to control plants (**Fig. 1C**). The other stress treatments applied (heat and
254 salinity + heat) did not differ significantly with respect to the values obtained in control
255 plants during the entire experiment, contrary to that observed under salinity. Control
256 plants had a transpiration rate and a stomatal conductance that were practically constant

257 during the entire experiment, whereas plants subjected to heat and salinity + heat
258 treatments showed a significant increase in transpiration rate (41%) and stomatal
259 conductance (29%) at 7 days, which was maintained until the end of the experiment
260 (**Figs. 1D and 1E**). Contrarily, the salinity treatment led to a significant reduction in the
261 transpiration rate and the stomatal conductance at day 7 from the start of the treatment
262 until the end. In this regard, the salinity + heat treatment showed a significant
263 improvement in the photosynthetic parameters as compared to salinity alone. Curiously,
264 no differences were found between salinity, heat, and the combination of both stresses
265 for water use efficiency (WUE, **Fig. 1F**), but all the treatments showed a significant
266 decrease in this parameter as compared to control plants.

267

268 **Salinity and heat combination induced a specific transcriptional response and** 269 **pathway activation**

270 An RNAseq study was performed to identify specific biochemical pathways or
271 molecular functions that could explain the different physiological responses of the
272 tomato plants to the salinity, heat, and salinity + heat treatments. RNA was sequenced
273 from three biological replicates from each treatment, including control plants. A
274 principal component analysis of the normalized reads revealed that all samples clustered
275 according to treatment, which validated the unique transcriptional reprogramming caused
276 by each stress condition (**Fig. 2A**). Then, a differential expression analysis was
277 performed to determine the individual genes affected by each treatment when compared
278 to the control. A total of 15,852 genes were found to be differentially expressed ($P_{adj} <$
279 0.05) across all three treatments (**Supplemental Table S6**). A comparison of both up-
280 and down-regulated genes from each of the three treatments further confirmed that each
281 treatment resulted in a high number of unique differentially expressed genes (**Fig. 2B**).
282 Most notably, it was found that 1,388 (7.32% of the total) were differentially expressed
283 only for salinity + heat, with 923 genes up-regulated and 465 genes down-regulated by
284 this stress combination (**Fig. 2B**).

285 To identify important functions activated in response to each stress, an enrichment
286 analysis of the significantly up-regulated genes was conducted using pathway
287 annotations from the Kyoto Encyclopedia of Genes and Genomes (KEGG;
288 **Supplemental Table S7**). A total of 27 pathways were found to be enriched ($P_{adj} <$
289 0.05) with these up-regulated genes across the three treatments (**Fig. 2C**). In line with

290 the genes themselves, enriched pathways were largely unique, with all but three
291 pathways (glutathione metabolism (sly00480), protein processing in the ER (sly04141),
292 and spliceosome (sly03040)) being enriched in just one of the three treatments. Most
293 interestingly, the salinity + heat treatment resulted in the upregulation of genes
294 belonging to two main metabolic pathways, ASC and aldarate metabolism (sly00053)
295 and arginine and proline metabolism (sly00330), which were not enriched in either of
296 the individual stress treatments, suggesting that the combination of stresses induced
297 specific changes in plant metabolism that in turn led to variation in the physiological
298 responses of the plants.

299 **The salinity and heat combination showed a unique metabolic profile with the** 300 **enrichment of specific pathways**

301 A metabolomics study was carried out to identify molecular features that were common
302 or unique to the simple or combined stresses and to validate the RNAseq results. Our
303 main interest mainly resided in those that were specifically accumulated under the
304 combination of salinity and heat. A total of 3,338 molecular features showed significant
305 ($P_{adj} < 0.05$ and a $\log_2 < -1$ or $\log_2 > 1$) changes across the three stress conditions.
306 Similar to the RNAseq analyses, each stress condition showed a unique metabolic
307 profile (**Fig. 3A; Supplemental Tables S3 and S4**). Only 208 molecular features were
308 commonly altered by all the treatments, which represented 6.30% of the total. When the
309 combination of salinity and heat was applied, a total of 568 molecular features (17.19%
310 of the total) were significantly and specifically accumulated with respect to the control
311 (**Fig. 3B**). Salinity + heat caused reprogramming of multiple metabolic pathways,
312 observed as a similar number of molecular features that were up- or down-regulated,
313 337 and 208, respectively, when compared to the control (**Fig. 3C**). Pathway enrichment
314 analysis of the up-regulated molecular features revealed that four biochemical pathways
315 (i.e., ASC and aldarate metabolism, purine metabolism, arginine and proline
316 metabolism and arginine biosynthesis) were significantly altered in tomato under the
317 combination of salinity and heat (**Fig. 3D and Supplementary Table S4,**
318 **Supplementary Figs. S2 and S3**). In agreement with the RNAseq data, the ASC and
319 aldarate metabolism and the arginine and proline metabolism were among the most
320 significantly enriched pathways.

321

322 **The integration of transcriptomics and metabolomics revealed that the proline and**
323 **ASC pathways are interconnected for ROS homeostasis**

324 The RNAseq and metabolomics data was combined with measurements of enzymatic
325 activity to obtain a detailed picture of the changes in the ASC and aldarate, and arginine
326 and proline metabolic pathways caused by the combination of salinity and heat stresses
327 (**Fig. 4**). The first observation was that proline appears to be degraded in favor of 4-
328 hydroxyproline and L-glutamate-5-semialdehyde accumulation, with the concomitant
329 up-regulation of prolyl 4-hydroxylase (P4HA) and pyrroline-5-carboxylate reductase
330 (PROC), as well as the down-regulation of proline dehydrogenase (PRODH). The
331 accumulation of L-glutamate-5-semialdehyde was also likely derived from ornithine
332 through the up-regulation of arginase (ARG) and ornithine aminotransferase (ROCD).
333 In summary, proline was not differentially accumulated under the combination of
334 salinity + heat as compared to controls. Instead, 4-hydroxyproline and L-glutamate-5-
335 semialdehyde, two proline-derivative compounds, significantly accumulated in tomato
336 leaves under stress combination.

337 ASC significantly accumulated under combined salinity and heat stress in tomato. Its
338 synthesis from UDP-glucose or myo-inositol results in the precursor D-glucuronate,
339 which also increased under salinity + heat, in part due to the down-regulation of
340 glucuronokinase transcript (GLCAK) through glucuronate-1P synthesis and to the up-
341 regulation of one copy of the inositol oxygenase (MIOX). The levels of L-gulose and L-
342 gulonate also increased under the combination of stresses, which seemed to favor ASC
343 accumulation. The high ASC levels observed in tomato plants under stress combination
344 could also be due to the degradation of the GDP-L-galactose and L-galactose-1P, since
345 GDP-L-galactose phosphorylase (VTC2-5) and L-galactose 1-phosphate phosphatase
346 (VTC4) were up-regulated under these conditions (**Fig. 4**). ASC is known to detoxify
347 ROS through the Halliwell-Asada cycle. Remarkably, this pathway was highly
348 represented among the differentially expressed genes and the significant molecular
349 features altered by salinity + heat (**Figs. 2-3**). These results were also supported by the
350 enzymatic activities of the proteins encoded by those transcripts (**Fig. 4**). Superoxide
351 dismutases (SOD1 and SOD2), involved in cell ROS detoxification, were up-regulated
352 at the transcript and activity levels, leading to the conversion of $O_2^{\cdot -}$ to H_2O_2 . Then,
353 H_2O_2 can be detoxified by catalase (CAT) or by ASC peroxidase (APX) through the
354 ASC/GSH pathway. CAT was not differentially expressed in the RNAseq analysis and

355 the enzyme activity was inhibited by stress combination. Several APX homologs were
356 up-regulated at the transcript level and, more importantly, its enzymatic activity was
357 very high ($\log_2 = 1.97$, **Supplemental Table S5**) under salinity + heat. The APX
358 activity generates monodehydroascorbate, which accumulated significantly in our
359 experiments. Monodehydroascorbate spontaneously forms dehydroascorbate, which is
360 reduced to ASC, once again through the action of dehydroascorbate reductase (DHAR),
361 using glutathione (GSH) as a reducing agent. Tomato plants showed a significant
362 accumulation of glutathione and monodehydroascorbate under combined salinity and
363 heat stress, with a non-significant dehydroascorbate accumulation or DHAR activity.
364 However, the MDAR enzyme, responsible for the regeneration of ASC, was up-
365 regulated at the transcript and enzymatic levels. Lastly, the glutathione peroxidases
366 GPX and PhGPX, responsible for the recovery of lipid peroxidation, were also up-
367 regulated under stress combination. Our results are indicative of a connection between
368 ASC synthesis and oxidative stress-proline metabolism, with the intersection between
369 these pathways found at the 1-pyrroline-5-carboxylate level (**Fig. 4**).

370

371 **Plants subjected to salinity and heat combination showed lower oxidative damage** 372 **than those under salinity alone**

373 The proposed coordination between the proline, ASC, and redox pathways may improve
374 the ability of the tomato plants to deal with ROS detoxification. Markers of oxidative
375 stress were evaluated to determine if tomato plants subjected to stress combination
376 displayed a more efficient antioxidant system than those plants grown under individual
377 stresses (**Fig. 5**). Tomato plants under salinity had the highest levels of H_2O_2 , with a
378 significant 4-fold increase compared to control plants. However, when salinity and heat
379 were applied in combination, it was found that the H_2O_2 content was about 50% lower
380 than in the salinity treatment (**Fig. 5A**). A similar trend was observed for lipid
381 peroxidation, an indicator of oxidative damage to cell membranes, with a maximum
382 value found for salinity stress, and an intermediate value found for the salinity + heat
383 stress combination (**Fig. 5B**). Thus, the stress combination appears to reduce oxidative
384 damage with respect to the salinity treatment. In neither case, the differences between
385 heat stress and control were statistically significant. Interestingly, the antioxidant
386 capacity (**Fig. 5C**) obtained for plants subjected to salinity was the lowest among all
387 treatments, with a reduction of up to 90% as compared to the controls. When salinity

388 and heat were combined, the antioxidant capacity index was significantly lower than the
389 control but 6-fold higher than salinity. Again, the heat treatment did not show
390 significant differences with respect to the control. Protein oxidation values obtained for
391 the four stress conditions were directly related to H₂O₂ and lipid peroxidation, with a
392 positive and significant correlation (H₂O₂-protein oxidation: $r = 0.992$, $P_{adj} < 0.001$;
393 lipid peroxidation-protein oxidation: $r = 0.996$, $P_{adj} < 0.001$). In short, our results
394 indicated that ROS levels were lower when salinity and heat were applied jointly as
395 compared to the salinity treatment alone, which was directly observed as a lower
396 damage to the membrane lipids and to the cellular proteins under abiotic stress
397 combination.

398

399 **The combined salinity and heat responses are associated with the upregulation of** 400 **unique transcription factors families**

401 The high specificity of the tomato plant responses to salinity + heat suggests that a tight
402 regulatory control must be in place to rapidly and efficiently cope with the oxidative
403 damage caused by these conditions. TFs are known to be key players in modulating the
404 expression of genes involved in abiotic stress responses. TFs that may regulate the
405 transcriptional responses to salinity, heat, and/or salinity + heat were identified by
406 evaluating the promoter regions (1000 bp upstream from the transcription start site) of
407 up-regulated genes from each stress condition for overrepresented cis-element motifs
408 (**Fig. 6A**). Binding sites from a total of 46 TFs belonging to multiple gene families were
409 found to be enriched ($P_{adj} < 0.05$) across all treatments. Of these, only 9 TFs were
410 associated with all stress conditions (salinity, heat, and salinity + heat), including three
411 Homeobox-Homeodomain-Leucine Zipper Protein (HB-HD-ZIP) TFs identified. The
412 salinity + heat treatment exhibited five unique TFs, including three from the stress-
413 related Zinc Finger Cysteine-2/Histidine-2 (C2H2) family.

414 In contrast to the diverse enrichment results, most enriched TFs did not exhibit
415 significant up-regulation themselves under their associated stress condition. For salinity
416 + heat, only three TFs had significant ($P_{adj} < 0.05$) expression levels when compared to
417 the controls (**Fig. 6B**). These TFs, one each from the basic Leucine Zipper Domain
418 (bZIP), C2H2, and HB-HD-ZIP families, were also all differentially expressed in the
419 salinity treatment, although the bZIP TF (*Solyc04g078840*) was down-regulated under
420 this treatment, and up-regulated exclusively for salinity + heat. None of these three were

421 differentially expressed under the heat stress alone. The sequences of the differentially
422 expressed genes from the proline, ASC, and redox pathways (**Fig. 4**) were evaluated to
423 determine which of the overrepresented cis-element motifs associated with salinity +
424 heat were present in their promoters (**Fig. 6A**). Most of these genes include binding sites
425 for TFs from the Apetala 2 (AP2), Dof zinc finger protein (C2C2-Dof), and Cysteine-
426 rich Polycomb-like Protein (CPP) families, among others. Binding sites for the single
427 CPP TF, which were highly enriched across all three stress conditions, matched to the
428 promoters of genes from the proline, ASC, and oxidative metabolism pathways,
429 including all four up-regulated copies of *APX* genes. Remarkably, binding sites for the
430 single-enriched Trihelix TF, *Solyc11g012720*, were found only in the promoters of
431 proline metabolism genes. Ultimately, these results suggest that specific sets of TFs
432 coordinate the modulation of proline, ASC, and redox metabolism under salinity + heat
433 stress, which should be further studied to validate their direct or indirect regulatory
434 roles.

435

436 **DISCUSSION**

437 In the present study, we demonstrated that the combination of heat stress with moderate
438 salinity in tomato plants induced a specific physiological, biochemical, and molecular
439 response that could not be deduced from a single stress application. From the
440 physiological stand point, tomato plants under the combination of salinity and heat grew
441 better than when salinity was applied as a sole stress, showing a significant increase in
442 plant biomass. At the same line, plants under stress combination showed better
443 photosynthetic performance and lower cellular oxidation than those growing under
444 salinity, with the balance between these two processes necessary for both plant growth
445 and adaptation to abiotic stress (Considine and Foyer, 2013; Woehle *et al.*, 2017).
446 Under salinity stress, ROS accumulation (measured as H_2O_2) likely induced damage to
447 membranes and an increase in protein oxidation, which translated into a lower cell
448 antioxidant capacity. These oxidative stress-associated processes may have caused the
449 strong inhibition of photosynthesis and reduction of growth observed in plants subjected
450 to the salinity treatment. Interestingly, when salinity was combined with heat, ROS
451 were accumulated to a lesser extent, with the damage to membranes and proteins being
452 also lower and maintaining an antioxidant capacity of over 60%, which was observed as
453 plants with better growth rates than in the salinity conditions alone. These observations

454 indicate that ROS could be produced in a lower quantity under stress combination than
455 under salinity, and that ROS is being produced at the same level than under salinity,
456 although their detoxification may be more efficient and/or effective under stress
457 combination. Our results mainly support the latter possibility, in which the combination
458 of salinity and heat induced the reprogramming of some important stress-related
459 pathways, such as proline and ASC metabolism, facilitating their interconnectivity for a
460 more efficient cellular ROS detoxification through the activation of oxidative
461 metabolism.

462 Tomato plant responses to salinity and heat combination involved complex
463 transcriptional networks and changes in metabolic fluxes. However, the modulation of
464 the proline and ASC pathways was shown to be a strong and unique response to this
465 stress combination. Interestingly, these metabolic pathways were not found to be
466 significantly induced under salinity or heat when applied individually. Proline can
467 protect cells from damage by acting as an osmoprotectant but also as a ROS scavenger
468 (Szabados and Saviouré, 2010; Narayanan and Govindarajan, 2012). Although proline
469 metabolism was induced under the combination of salinity and heat, proline levels did
470 not increase under these conditions, and instead, the derivatives 4-hydroxyproline and
471 L-glutamate-5-semialdehyde were significantly accumulated. Several studies have
472 pointed out that during stress recovery, proline is oxidized to provide the cell with a
473 large amount of energy (one molecule of proline captures 30 ATP equivalents)
474 (Verbruggen and Hermans, 2008; Zhang and Becker, 2015). Jaspers and Kangasjärvi
475 (2010) showed that when salinity levels were increased, proline was used as a source of
476 energy by plants, providing ATP and NADPH through its catalysis by the enzyme
477 PRODH. This oxidation process increased the formation of ROS, activating the
478 response signaling cascade generated by the oxidative stress (Jaspers and Kangasjärvi,
479 2010), and thus relating proline with the stress response mechanisms found in plants.

480 Our results pointed out to an interconnection between proline catalysis, ROS generation
481 (due to stress conditions and proline degradation) and an upregulation of the oxidative
482 metabolism. ASC metabolism was also up-regulated, as ASC is a necessary substrate to
483 maintain oxidative metabolism active. It can be suggested that proline accumulation
484 occurs early during the acclimation to stress combination and that its oxidation is a sign
485 of stress recovery in these plants. However, we have previously reported that proline
486 does not preferentially accumulate during the first 72 hours after tomato plants were

487 subjected to the combination of salinity and heat stress (Rivero *et al.*, 2014), which
488 contradicts this idea. Instead, glycine-betaine was the osmolyte that was preferentially
489 accumulated in tomato under these conditions. Thus, in this study, we propose and
490 provide evidence that proline oxidation may be interconnected with glutathione redox
491 homeostasis for efficient ROS scavenging. Recent publications have demonstrated that
492 proline catabolism to P5C is induced in animal cells during cell infection (Tang and
493 Pang, 2016). These authors proposed that PRODH and PROC act together to raise P5C
494 levels and thus, govern ROS homeostasis. This mechanism is largely unknown in
495 plants, although a similar hypothesis was proposed in *Arabidopsis thaliana* as a
496 response to pathogen attack (Qamar *et al.*, 2015). In a previous study by our research
497 group (Rivero *et al.*, 2014) we have also shown that PRODH and PROC were
498 differentially up-regulated at the gene and protein levels under the combination of
499 salinity and heat, whereas under salinity or heat applied individually these enzymes
500 were down-regulated, thereby favoring proline accumulation. The significant
501 enrichment of proline metabolism found in the analysis of the transcriptomics and
502 metabolomics data and the potential role of proline intermediaries in ROS homeostasis,
503 such as P5C, provide a strong argument for the role of proline oxidation in ROS
504 signaling mechanisms under stress combination; however, we recognize that more
505 research is needed to confirm this hypothesis.

506 ASC is one of the main compounds involved in plant oxidative metabolism through the
507 Halliwell-Asada cycle, and genes and compounds found in this pathway were
508 significantly induced under salinity and heat combination. Activities of the oxidative
509 metabolism-related enzymes were determined to confirm the upregulation of the
510 oxidative metabolism at the protein level, as well as to find whether or not this pathway
511 was specifically regulated under the combination of salinity and heat, as previously
512 shown for proline and ASC metabolism. Our research group, as well as other authors,
513 have reported on the high activation of oxidative metabolism-related enzymes through a
514 specific upregulation under the combination of salinity in tomato plants (Rivero *et al.*,
515 2014; Martinez *et al.*, 2018; García-Martí *et al.*, 2019). The enzymatic activities
516 assayed, together with the gene expression and the metabolites identified in our study,
517 indicate the efficient detoxification of H₂O₂ through the Halliwell-Asada cycle, and a
518 very active lipid recovery from oxidation thanks to PhGPX activity. These observations
519 could explain that under salinity and heat combination, the oxidative markers measured

520 (H₂O₂, lipid peroxidation, protein oxidation, and antioxidant capacity) in tomato plants
521 were lower than under salinity as the sole stress.

522 Zandalinas *et al.* (2020) recently described that different combinations of abiotic
523 stresses applied to *A. thaliana* plants resulted in unique transcriptional profiles and that
524 their regulation by different TF families was also characteristic of each stress
525 combination. In this report, the bHLH, MYB and bZIP TFs families were significantly
526 induced under the combination of salinity and heat. Our results showed that some genes
527 belonging to the bZIP TF family were differentially and uniquely regulated under the
528 combination of salinity and heat in tomato plants (e.g., *Solyc10g081350*). Other TFs
529 belonging to other stress-related families, such as C2H2 (e.g., *Solyc02g085580*,
530 *Solyc03g121660*, *Solyc07g053570*) and Trihelix (e.g., *Solyc11g012720*), also showed
531 this particularity under our experimental conditions. Most of these TFs families have
532 been reported to be involved in the control of plant development, cell division, different
533 physiological process, but also in abiotic responses of plants (Kaplan-Levy *et al.*, 2014;
534 Agarwal *et al.*, 2019). For example, Agarwal *et al.* (2019) reported that the bZIP family
535 was involved in the mitigation of several abiotic stresses (e.g., salinity, drought, heat or
536 oxidative stress) and the increase in plant productivity under adverse conditions. The
537 Trihelix TF family has been shown to be involved in the response to salinity and
538 pathogen-related stresses, and in the development of trichomes, stomata, and the seed
539 abscission layer (Kaplan-Levy *et al.*, 2014). Numerous members of the C2H2-type zinc
540 finger family have been shown to play a significant role in the plant's response to
541 different abiotic stresses and in plant hormonal transduction signals (Kiełbowicz-Matuk,
542 2012). Most of the information found in the literature regarding the C2H2 family has
543 been for *Arabidopsis*, and very little is known about other plant species, including
544 tomato. Hu *et al.* (2019) found that this family regulates many genes in response to
545 some abiotic stress, and especially in response to heat stress in tomato plants. Most of
546 the C2H2 genes that were up-regulated under heat stress in the report by Hu *et al.*
547 (2019) were also differentially expressed in our transcriptomic analysis when heat was
548 applied as the sole stress. However, the C2H2 identified in our study that was
549 specifically up-regulated under salinity + heat was not listed in the study by Hu *et al.*
550 (2019), again demonstrating the importance of studying stresses in combination. The
551 TFs identified as being up-regulated under the combination of salinity and heat aligned

552 with the promoter regions of many genes studied in this report, including those
553 belonging to the proline, ASC, and oxidative metabolisms.

554 In summary, we showed that proline, ASC and oxidative metabolism are
555 interconnected, with a tight coordination to maintain not only an optimal cellular redox
556 balance, but also to trigger the proper signaling mechanisms responsible for inducing
557 the plant's acclimation to the combination of salinity and heat. In this process, proline
558 oxidation is suggested to be used as the energy source needed for triggering the stress
559 response, with subsequent ROS formation. At this point, oxidative metabolism enters
560 the stage, with the upregulation of its main enzymes to maintain ROS at basal levels.
561 One of the main limiting factors for maintaining the activity of the redox metabolic
562 pathways is ASC abundance, which suggests the presence of a connection between ASC
563 biosynthesis with oxidative metabolism and, most likely, with proline oxidation.
564 Cellular basal levels of ROS could trigger downstream signaling mechanisms through
565 the activation of particular TFs families, such as the trihelix, C2H2 and bZIP families,
566 which in turn, may regulate the expression of genes involved in the reprogramming of
567 different metabolic pathways, including those involved in proline, ASC, and redox
568 metabolism (i.e., positive feedback loops). Future validation of the role of specific TFs
569 families in the successful acclimation of plants to heat + salinity is necessary for
570 developing breeding strategies for more resilient crops against abiotic stresses.

571

572 **Supplementary data**

573 **Figure S1.** Total ion chromatogram extracted from UPLC-QTOF performed in 6
574 biological replications of tomato leaves subjected to control, salinity heat or the
575 combination of salinity + heat.

576 **Figure S2.** Box-and-whisker plots of the compounds belong to the Ascobate, Alderate
577 and oxidative metabolism with significant differences between salinity + heat treatment
578 respect to control.

579 **Figure S3.** Box-and-whisker plots of the compounds belong to the Proline metabolism
580 with significant differences between salinity + heat treatment respect to control.

581 **Table S1.** Raw, parsed and mapped reads of mRNA of all samples.

582 **Table S2 - Sheet 1-** Comparison of salinity treated tomato plants against control plants.

583 **Sheet 2-** Comparison of heat treated tomato plants against control plants. **Sheet 3-**

584 **Comparison of the salinity combined with heat treatment against control plants.**

585 **Table S3.** Comparison of the peaks of each independent analysis with the aim of find
586 common and specific peaks among all the treatments.

587 **Table S4.** Identified compounds in the Control vs Salinity+Heat peaks comparison
588 related to the enriched pathway analysis results.

589 **Table S5.** Activities of the oxidative metabolism-related enzymes.

590 **Table S6.** Differential expression output from DESeq2 (Love et al., 2014).

591 **Table S7.** Enrichment of KEGG pathways in upregulated genes for each treatment.

592

593 **Data availability:** The raw sequencing reads and the read mapping count matrices are
594 available in the National Center for Biotechnology Information Gene Expression
595 Omnibus database under the accession GSE152620. All data supporting the findings of
596 this study are available within the paper and within its supplementary materials
597 published online.

598

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609

610 **Author contribution**

611 RMR conceived, designed and supervised the experiments; ML-D, CJS and BB-U
612 performed the RNAseq analysis. VM and RMR performed the metabolomic analysis.
613 ML-D, TCM and RMR performed the biochemical analysis. ML-D and RMR
614 performed the photosynthetic measurements. ML-D, BB-U and RMR wrote the paper.
615 The authors declare that they have no competing interests.

616

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765

766

767 **Figure Legends**

768 **Figure 1. (A)** Pictures of tomato plants at the end of the control or stress treatments. **(B)**

769 Whole plant fresh weight (FW) of tomato plants grown under control, heat, salinity
770 or the combination of salinity and heat. **(C-F)** Photosynthetic parameters measured
771 in the third and four fully mature expanded leaves of tomato plants grown under
772 control or stress conditions measured at the beginning (0 days), during (7 days) or
773 at the end (14 days) of the treatments. Values represent means \pm SE (n = 9). Bars
774 with different letters within each panel are significantly different at $p < 0.05$
775 according to Tukey's test.

776 **Figure 2.** RNAseq analysis performed in tomato leaves after 14 days of growing under
777 control or stress (salinity, heat or the combination of salinity and heat) conditions.

778 **(A)** Principal component analysis (PCA) of the normalized reads obtained for each
779 treatment. **(B)** Euler diagram representing up- and down-regulated genes (adjusted
780 $P < 0.05$) of tomato plants grown under control, simple (salinity or heat) or
781 combined (salinity + heat) stress. **(C)** Pathway enrichment analysis performed
782 within the up-regulated genes under the different stress conditions applied. More

783 details on these analyses can be found in the Materials and Method section.

784 **Figure 3.** Metabolomic analysis performed in tomato leaves after 14 days of growing
785 under control, simple (salinity or heat) or combined (salinity + heat) stress
786 conditions. **(A)** Principal component analysis (PCA) of the normalized molecular
787 features found under each treatment applied ($n = 6$). **(B)** Euler diagram of the
788 common and uniquely molecular features with a differential and significant
789 accumulation in each treatment ($P_{adj} < 0.05$). **(C)** Bubble diagram representing the
790 up- and down-regulated molecular features found among the 568 molecular
791 features uniquely and significantly changing under the combination of salinity +
792 heat. Each bubble (i.e. molecular feature) is positioned in the chromatogram by its
793 mass-to-charge (y-axis) and retention time (x-axis) and the size and darkness of
794 one bubble represented the \log_2 and p-value, respectively of this molecular feature.
795 The raw data of Figure 3C can be found in the supplemental material
796 (Supplementary Table S2 and S3). **(D)** KEGG pathway enrichment analysis
797 performed with the significantly up-regulated molecular features identified under
798 the combination of salinity + heat. More details on these analyses can be found in
799 the Materials and Methods section.

800 **Figure 4.** Schematic diagram of the metabolic interconnection between ascorbate,
801 proline and oxidative metabolism in tomato plants. \log_2 (fold change) of the
802 metabolite concentration (\circ), gene expression ($\square \square$) or enzymatic activity (\diamond)
803 obtained in tomato plants grown under the combination of salinity + heat after
804 RNAseq, metabolomics or biochemical analyses were represented.

805 **Figure 5.** Oxidative metabolism-related markers measured in tomato leaves grown for
806 14 days under control, simple (salinity +heat) or combined (salinity+heat) stress.
807 Values represent means \pm SE ($n = 9$). Bars with different letters within each panel
808 are significantly different at $p < 0.05$ according to Tukey's test.

809 **Figure 6.** Cis-element enrichment results for up-regulated genes from each stress
810 treatment. **(A)** Enrichment p-values for binding motifs corresponding to 46 TFs in
811 each of the stress treatments. TFs are grouped and color coded by family, and a
812 consensus diagram for the binding motif and gene accession is given for each. **(B)**
813 \log_2 (fold change) of expression of three selected TFs in each stress treatment. **(C)**
814 Counts of TF families overrepresented in genes up-regulated in the salinity + heat
815 treatment from the ascorbate metabolism, oxidative metabolism, and proline

816 metabolism families. Accessions and common abbreviations are given for each
817 gene. Numbers in boxes refer to the count of TFs in that family with a match to
818 that gene based on Analysis of Motif Enrichment results.
819

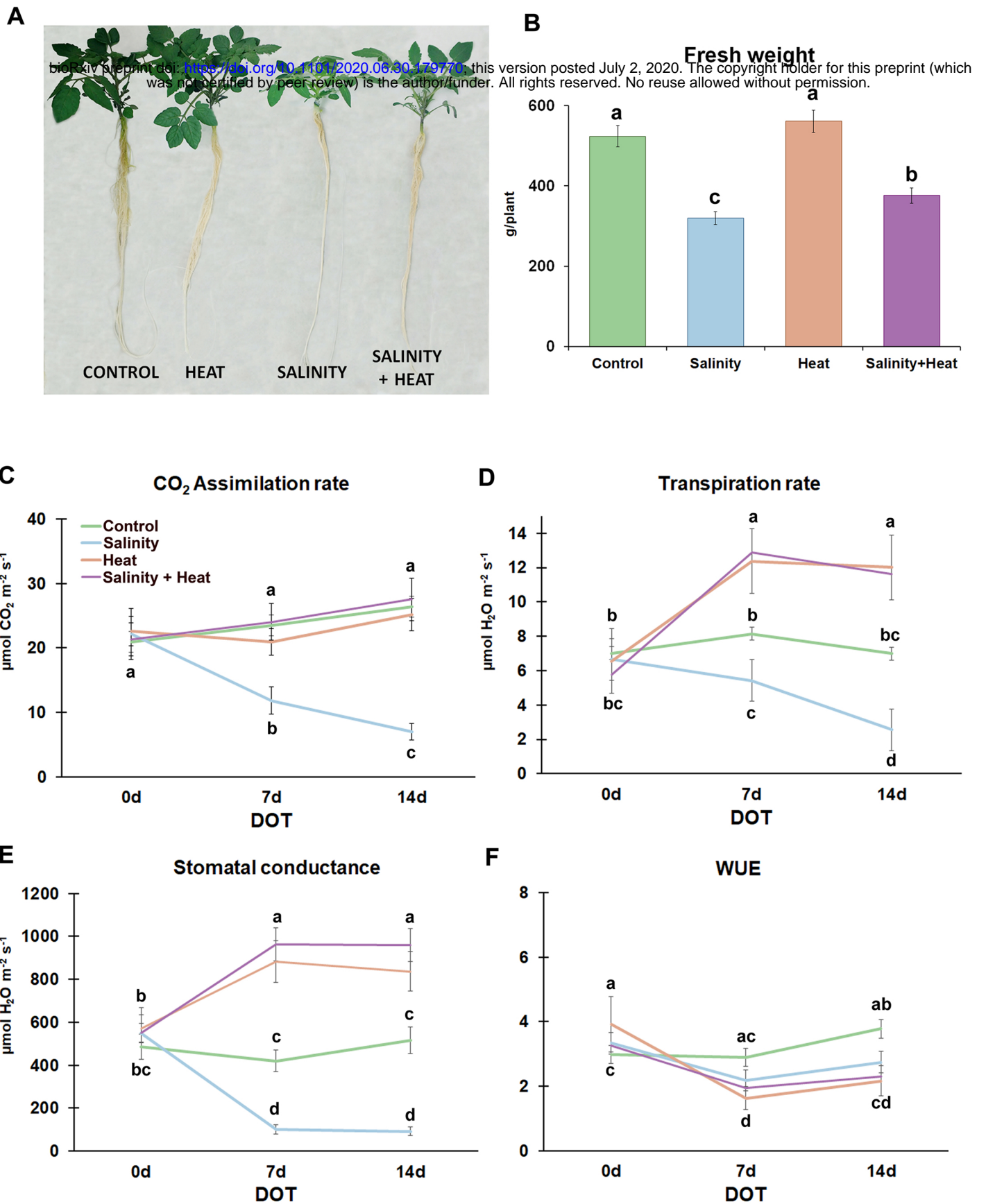


Figure 1. (A) Pictures of tomato plants at the end of the control or stress treatments. (B) Whole plant fresh weight (FW) of tomato plants grown under control, heat, salinity or the combination of salinity and heat. (C-F) Photosynthetic parameters measured in the third and four fully mature expanded leaves of tomato plants grown under control or stress conditions measured at the beginning (0 days), during (7 days) or at the end (14 days) of the treatments. Values represent means \pm SE (n = 9). Bars with different letters within each panel are significantly different at $p < 0.05$ according to Tukey's test.

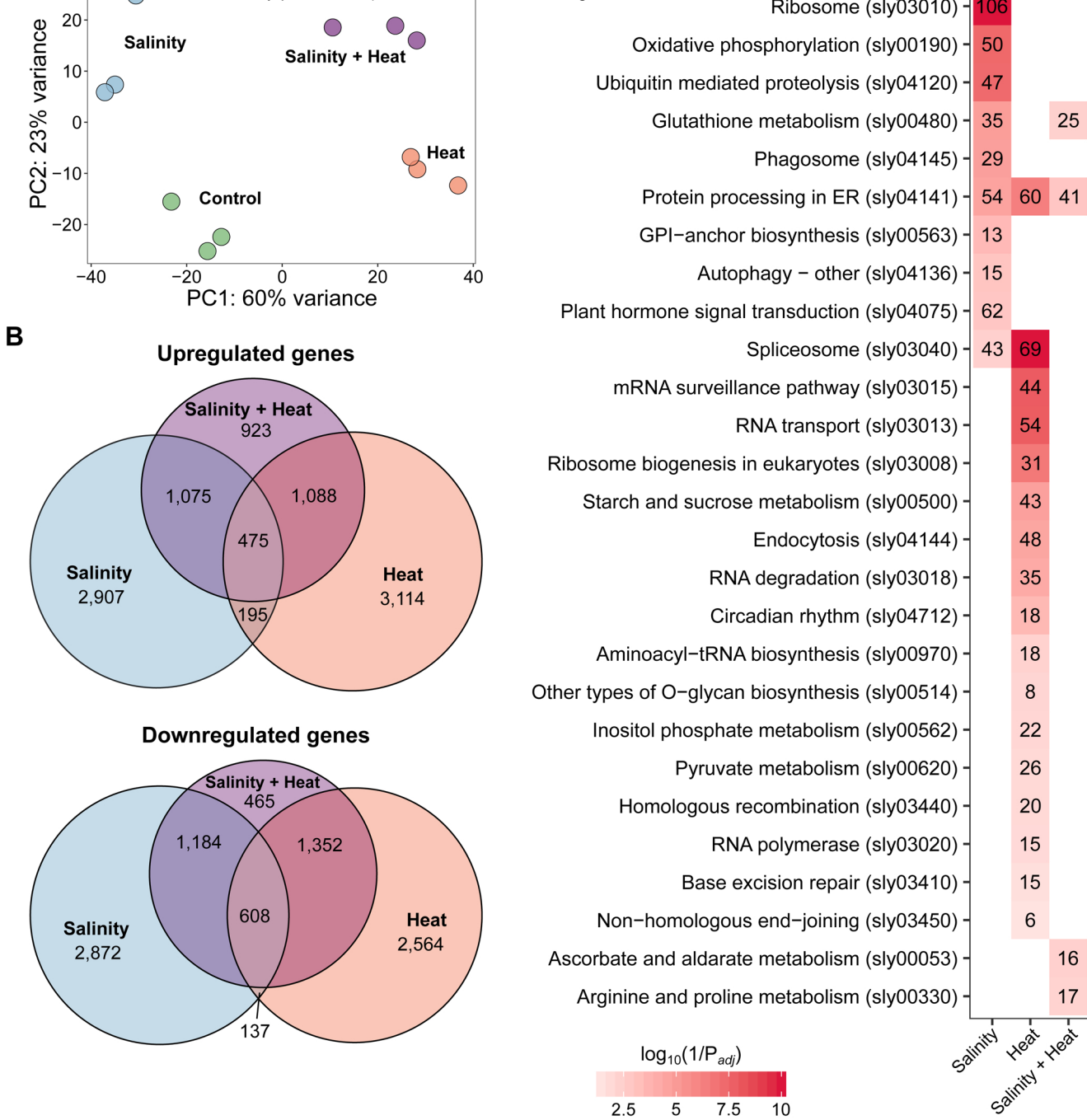


Figure 2. RNAseq analysis performed in tomato leaves after 14 days of growing under control or stress (salinity, heat or the combination of salinity and heat) conditions. **(A)** Principal component analysis (PCA) of the normalized reads obtained for each treatment. **(B)** Euler diagram representing up- and down-regulated genes (adjusted $P < 0.05$) of tomato plants grown under control, simple (salinity or heat) or combined (salinity + heat) stress. **(C)** Pathway enrichment analysis performed within the up-regulated genes under the different stress conditions applied. More details on these analyses can be found in the Materials and Method section.

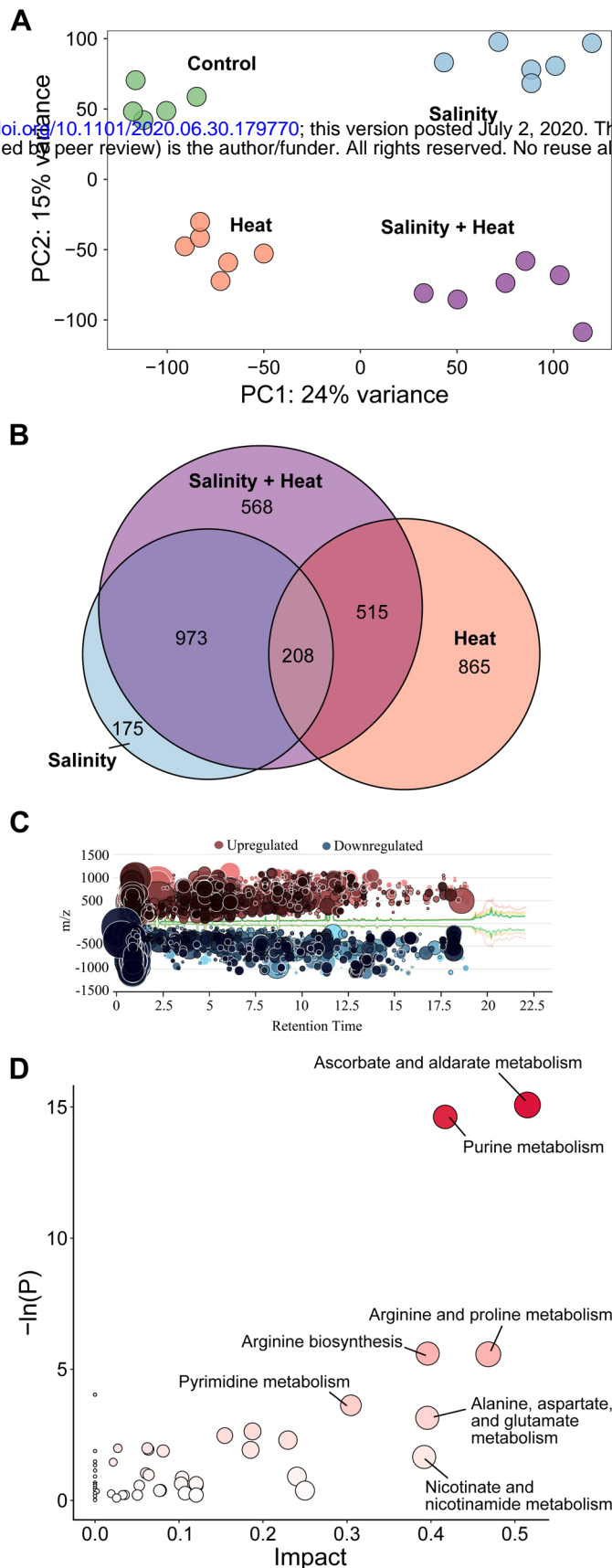


Figure 3. Metabolomic analysis performed in tomato leaves after 14 days of growing under control, simple (salinity or heat) or combined (salinity + heat) stress conditions. **(A)** Principal component analysis (PCA) of the normalized molecular features found under each treatment applied ($n = 6$). **(B)** Euler diagram of the common and uniquely molecular features with a differential and significant accumulation in each treatment ($P_{adj} < 0.05$). **(C)** Bubble diagram representing the up- and down-regulated molecular features found among the 568 molecular features uniquely and significantly changing under the combination of salinity + heat. Each bubble (i.e. molecular feature) is positioned in the chromatogram by its mass-to-charge (y-axis) and retention time (x-axis) and the size and darkness of one bubble represented the \log_2 and p-value, respectively of this molecular feature. The raw data of Figure 3C can be found in the supplemental material (Supplementary Table S2 and S3). **(D)** KEGG pathway enrichment analysis performed with the significantly up-regulated molecular features identified under the combination of salinity + heat. More details on these analyses can be found in the Materials and Methods section.

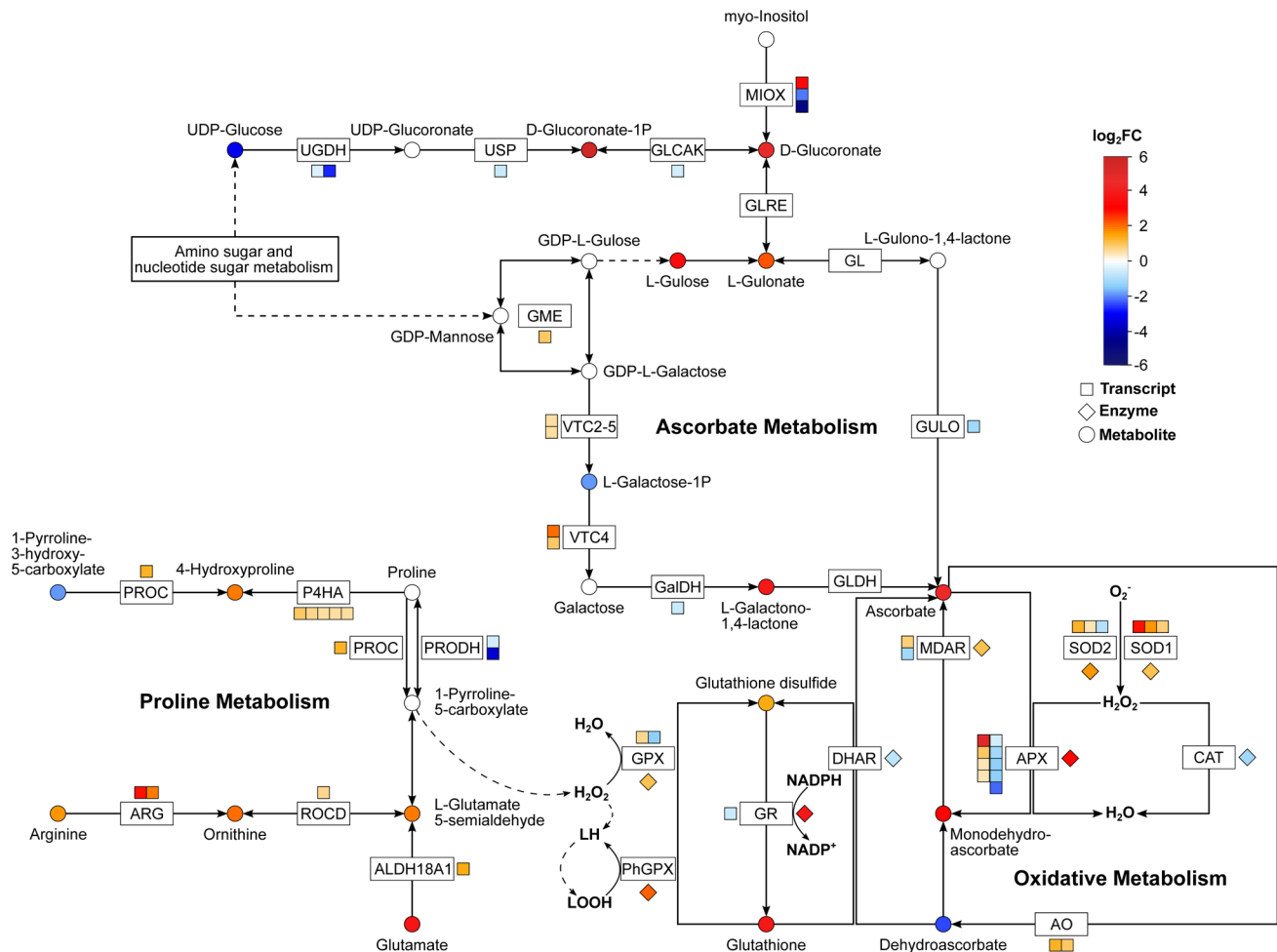


Figure 4. Schematic diagram of the metabolic interconnection between ascorbate, proline and oxidative metabolism in tomato plants. Log₂(fold change) of the metabolite concentration (○), gene expression (□) or enzymatic activity (◇) obtained in tomato plants grown under the combination of salinity + heat after RNAseq, metabolomics or biochemical analyses were represented.

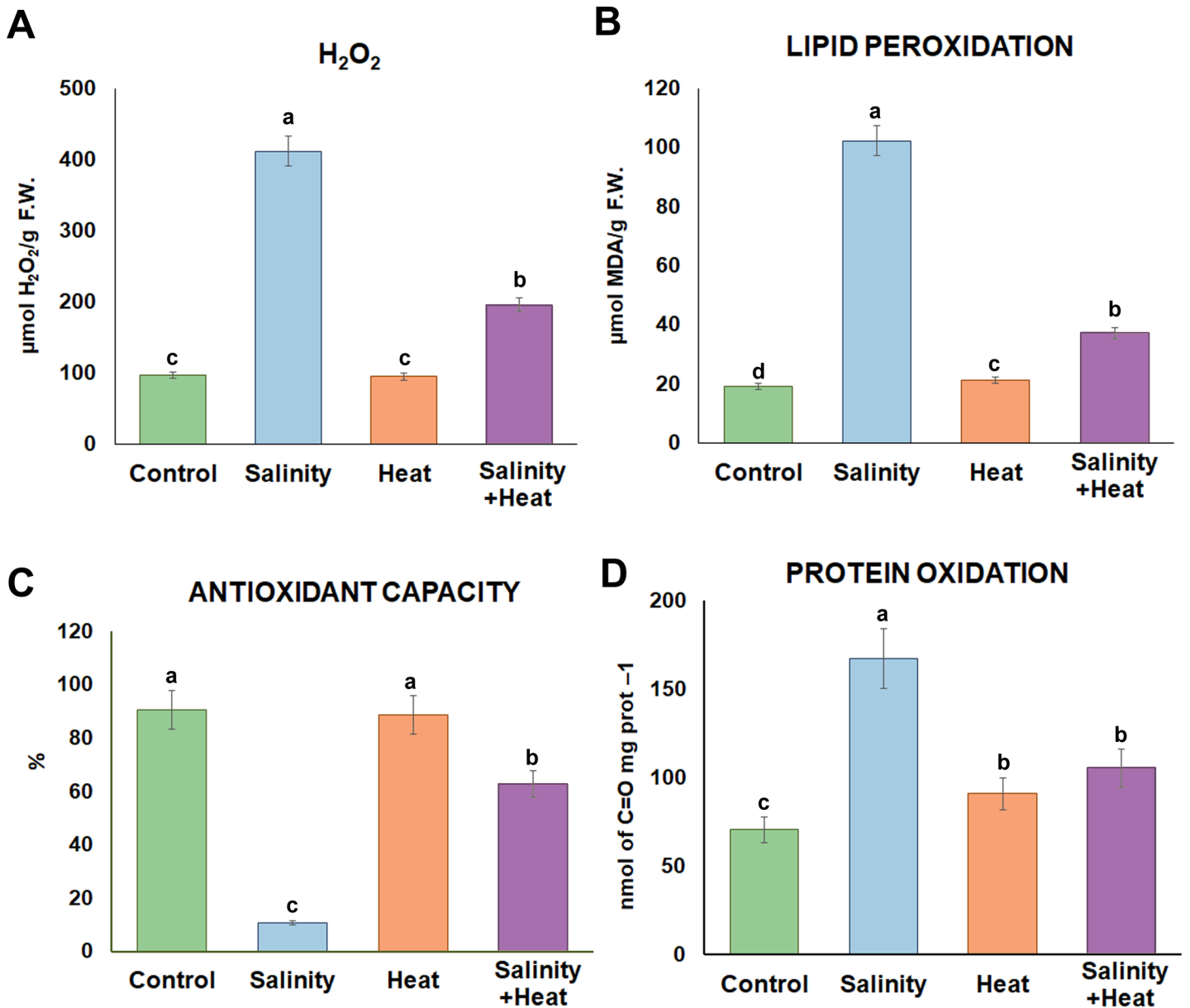


Figure 5. Oxidative metabolism-related markers measured in tomato leaves grown for 14 days under control, simple (salinity +heat) or combined (salinity+heat) stress. Values represent means \pm SE (n = 9). Bars with different letters within each panel are significantly different at $p < 0.05$ according to Tukey's test.

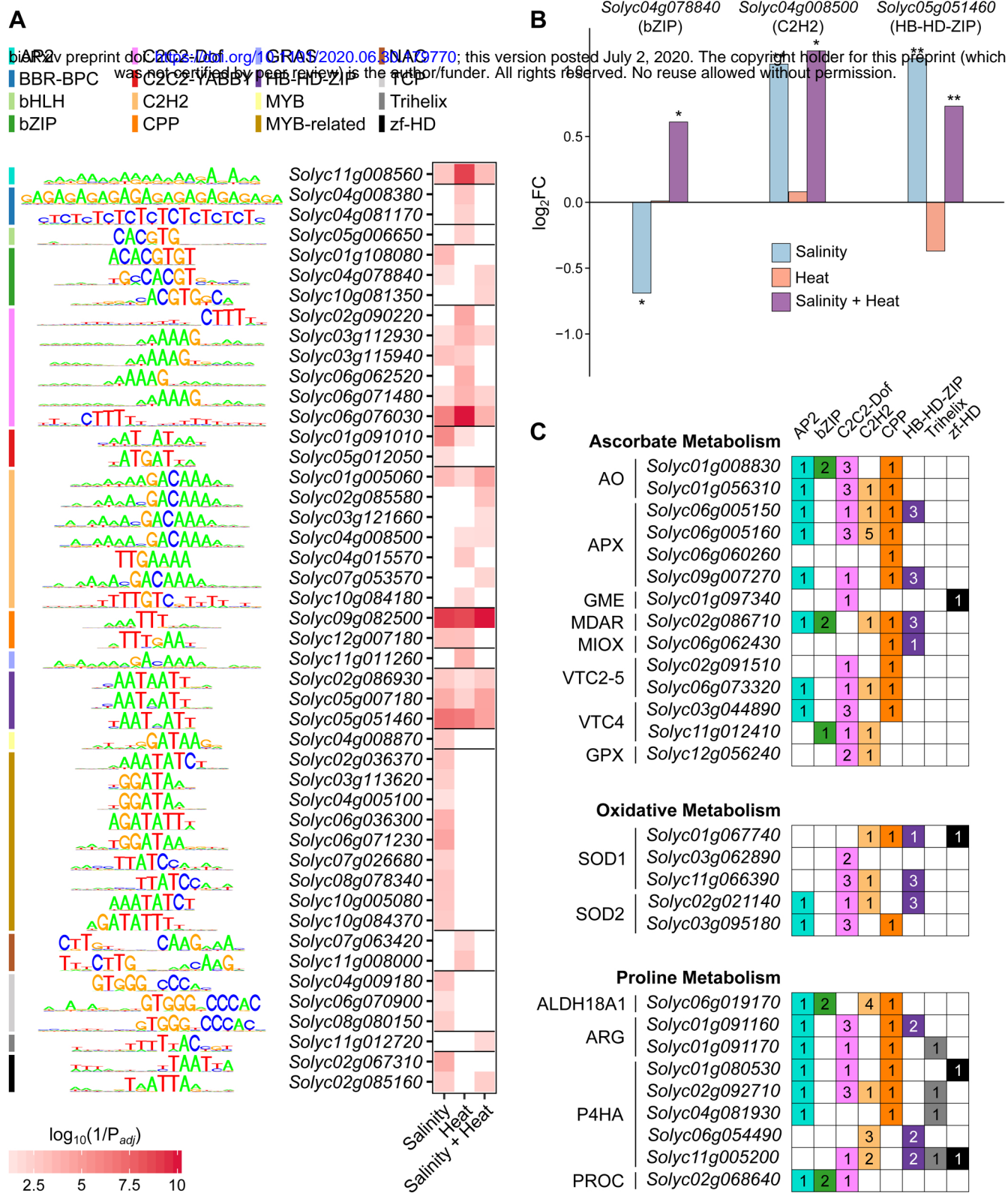


Figure 6. Cis-element enrichment results for up-regulated genes from each stress treatment. **(A)** Enrichment p-values for binding motifs corresponding to 46 TFs in each of the stress treatments. TFs are grouped and color coded by family, and a consensus diagram for the binding motif and gene accession is given for each. **(B)** $\log_2(\text{fold change})$ of expression of three selected TFs in each stress treatment. **(C)** Counts of TF families overrepresented in genes up-regulated in the salinity + heat treatment from the ascorbate metabolism, oxidative metabolism, and proline metabolism families. Accessions and common abbreviations are given for each gene. Numbers in boxes refer to the count of TFs in that family with a match to that gene based on Analysis of Motif Enrichment results.