bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **Short title:** Light and heat stress combination

2 [*]**Corresponding author:**
3 Sara I Zandalinas
4 Email: izquierdozandalins@missouri.edu
5 Bond Life Sciences Center, Division of Plant Sciences, College of Agriculture, Food and Natural
6 Resources. University of Missouri, Columbia, Missouri, 65211 USA, and Departamento de
7 Ciencias Agrarias y del Medio Natural, Universitat Jaume I, Castelló de la Plana, 12071 Spain

8 GABA plays a key role in plant acclimation to a combination 9 of high light and heat stress

10 Damián Balfagón[1], Aurelio Gómez-Cadenas[1], José L. Rambla[1], Antonio Granell[2], Carlos de
11 Ollas[1], Ron Mittler[3,ǂ], Sara I Zandalinas[1,3,ǂ,*]

12 [1]Departamento de Ciencias Agrarias y del Medio Natural, Universitat Jaume I, Castelló de la
13 Plana, 12071 Spain

14 [2]Instituto de Biología Molecular y Celular de Plantas, CSIC-Universidad Politécnica de Valencia,
15 Valencia, 46022 Spain

16 [3]Bond Life Sciences Center, Interdisciplinary Plant Group, and Division of Plant Sciences, College
17 of Agriculture, Food and Natural Resources, University of Missouri, Columbia, Missouri, 65211
18 USA

19 [ǂ] Senior author

20 Author Contributions

21 D.B. and S.I.Z performed the research; S.I.Z., R.M. and A.G-C designed and supervised the
22 research; R.M. and A.G-C provided laboratory infrastructure and funding; J.L.R., A.G., D.B. and
23 C.d.O. performed the metabolomics analysis; D.B., S.I.Z. and R.M. wrote the manuscript and
24 prepared figures. All authors read and approved the final version of the manuscript.

25 One sentence summary

26 The non-proteinogenic amino acid γ-aminobutyric acid (GABA) is required for plant acclimation
27 to a combination of high light and heat stress in Arabidopsis.

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

28 **Funding information**

29 This work was supported by funding from the National Science Foundation (IOS-1353886, MCB-
30 1936590, and IOS-1932639), the Bond Life Sciences Early Concept Grant, the University of
31 Missouri, Ministerio de Ciencia e Innovación (Spain, PID2019-104062RB-I00) and Plan GenT
32 2020 from Generalitat Valenciana (CDEIGENT/2020/013). DB was recipient of a predoctoral
33 contract funded by Generalitat Valenciana (FEDEGENT/2018/001). JLR was supported by the
34 Spanish Ministry of Economy and Competitiveness through a "Juan de la Cierva-Formación" grant
35 (FJCI-2016-28601).

36

37 **ABSTRACT**

38 Plants are frequently subjected to different combinations of abiotic stresses, such as high light
39 intensity and elevated temperatures. These environmental conditions pose an important threat to
40 agriculture production, affecting photosynthesis and decreasing yield. Metabolic responses of
41 plants, such as alterations in carbohydrates and amino acid fluxes, play a key role in the successful
42 acclimation of plants to different abiotic stresses, directing resources towards stress responses and
43 suppressing growth. Here we show that the primary metabolic response of *Arabidopsis thaliana*
44 plants to high light or heat stress is different than that of plants subjected to a combination of high
45 light and heat stress. We further demonstrate that a combination of high light and heat stress results
46 in a unique metabolic response that includes increased accumulation of sugars and amino acids,
47 coupled with decreased levels of metabolites participating in the tricarboxylic acid (TCA) cycle.
48 Among the amino acids exclusively accumulated during a combination of high light and heat
49 stress, we identified the non-proteinogenic amino acid γ-aminobutyric acid (GABA). Analysis of
50 different mutants deficient in GABA biosynthesis, in particular two independent alleles of
51 glutamate decarboxylase 3 (*gad3*), reveal that GABA plays a key role in the acclimation of plants
52 to a combination of high light and heat stress. Taken together, our findings identify a new role for
53 GABA in regulating plant responses to stress combination.

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

54 **INTRODUCTION**

55 Plants growing under natural conditions are exposed to different abiotic and biotic stresses that
56 impact plant growth and development. Among these, high light intensities that exceed the plant
57 photosynthetic capacity often occur in native habitats (Ort, 2001; Roeber et al., 2020). Because
58 light plays a key role in the life of photosynthetic organisms, plants evolved many different
59 acclimation and adaptation mechanisms to counteract the effect of high light stress, including
60 paraheliotropic movements, pathways for adjusting the size of the antenna complexes, quenching
61 mechanisms, and pathways to scavenge excess reactive oxygen species (ROS; Asada, 2006; Li et
62 al., 2009; Dietz, 2015). The excess excitation energy produced at the antennas of the
63 photosynthetic apparatus during high light stress is potentially dangerous and could lead to
64 irreversible damage to the reaction centers. Consequently, a sustained decrease in efficiency and
65 electron transport rates could occur, leading to photoinhibition (Ruban, 2015). In addition to high
66 light stress, heat stress can also compromise PSII electron transport due to the increase in fluidity
67 of the thylakoid membranes, dislodging of PSII light harvesting complexes and decreasing the
68 integrity of PSII (Mathur et al., 2014). Moreover, because CO_2 fixation is dependent on stomatal
69 regulation and temperature, high light stress may cause a more severe hazard to plants when
70 combined with other stresses that already limit the rates of CO_2 fixation (Mittler, 2006; Roeber et
71 al., 2020). It was recently reported that a combination of high light and heat stress displayed unique
72 transcriptomic, physiological and hormonal responses in *Arabidopsis thaliana* plants (Balfagón et
73 al., 2019). In addition, this abiotic stress combination was found to have a severe impact on PSII
74 performance and to decrease the ability of plants to repair PSII (Balfagón et al., 2019). Lipophilic
75 antioxidant molecules were previously shown to contribute to the protection of PSII against
76 photodamage and enhance tolerance of tomato plants to high light and heat stress combination
77 (Spicher et al., 2017). In sunflower, changes in the steady-state level of transcripts associated with
78 energy metabolism were found in response to this stress combination (Hewezi et al., 2008). The
79 specific physiological and molecular responses observed in different plant species in response to
80 a combination of high light and heat stress (Hewezi et al., 2008; Spicher et al., 2017; Balfagón et
81 al., 2019), could in turn lead to changes in plant metabolism that would minimize stress-induced
82 damages (Balfagón et al., 2020).

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

83 Metabolites play an essential role in plant growth and development, as well as modulate different
84 environmental responses of plants. The plant metabolome consists of a wide variety of low
85 molecular weight compounds with many different biological functions, such as carbohydrates that
86 are direct products of photosynthesis and substrates of energy metabolism; tricarboxylic acid
87 (TCA) cycle intermediates; and amino acids involved in protein synthesis and/or other cellular
88 processes such as osmotic readjustments. Increased levels of different polar compounds in plants
89 subjected to different abiotic stresses, including drought, salinity, high light, and extreme
90 temperatures, are thought to play a key role in plant acclimation (Kaplan et al., 2004; Cramer et
91 al., 2007; Maruyama et al., 2009; Caldana et al., 2011). For example, under osmotic stress, TCA
92 cycle, gluconeogenesis and photorespiration are activated to increase glucose, malate and proline
93 levels in order to cope with ROS production and photoinhibition (Cramer et al., 2007). A
94 comparative metabolite analysis of Arabidopsis plants responding to heat or cold shock suggested
95 that a metabolic network consisting of proline, monosaccharides (glucose and fructose), galactinol
96 and raffinose has an important role in tolerance to temperature stress (Kaplan et al., 2004; Urano
97 et al., 2010). Rizhsky et al. (2004) reported that different sugars and amino acids could play a key
98 role in the response of Arabidopsis plants to a combination of drought and heat stress. A study in
99 citrus plants subjected to a combination of drought and heat stress further revealed that the ability
100 of a tolerant citrus genotype to retain a high photosynthetic activity and to cope with oxidative
101 stress was directly linked to its ability to maintain primary metabolic activity (Zandalinas et al.,
102 2016). Moreover, metabolomic analysis of maize plants subjected to drought, heat, and their
103 combination revealed a direct relationship between metabolism and grain yield, highlighting the
104 importance of photorespiration and raffinose family oligosaccharide metabolism for grain yield
105 under drought conditions (Obata et al., 2015).

106 In general, abiotic stress conditions result in the accumulation of free amino acids in different
107 plants (*e.g.,* Rizhsky et al., 2004; Lugan et al., 2010; Aleksza et al., 2017; Huang and Jander, 2017;
108 Batista-Silva et al., 2019). Several amino acids can act as precursors for the synthesis of secondary
109 metabolites and signaling molecules. For example, polyamines are derived from arginine (Alcázar
110 et al., 2010), and the plant hormone ethylene is synthesized from methionine (Amir, 2010). In
111 addition, a wide range of secondary metabolites with different biological functions are derived
112 from the aromatic amino acids phenylalanine, tyrosine and tryptophan, or from intermediates of
113 their biosynthesis pathways (Tzin and Galili, 2010).

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

114 To dissect different primary metabolic responses and to identify promising metabolic markers for
115 a combination of high light and heat stress in plants, we studied the effect of this stress combination
116 on the levels of different primary metabolites in *Arabidopsis thaliana* plants. Both high light and
117 heat stress conditions impacted PSII performance when occurring individually, and their
118 combination displayed unique transcriptomic and physiological responses in plants (Balfagón et
119 al., 2019). We therefore hypothesized that this stress combination would have a unique
120 metabolomic response, leading to the accumulation of metabolites unique to the state of stress
121 combination. Our findings indicate that the primary metabolic response of Arabidopsis plants to a
122 combination of high light and heat stress is different than that of plants subjected to high light or
123 heat stress. We further identified γ-aminobutyric acid (GABA) as a metabolite that specifically
124 accumulates in plants during a combination of high light and heat stress. Using different mutants
125 deficient in GABA accumulation, we further reveal that GABA plays a key role in the acclimation
126 of plants to this stress combination.

127

128 **RESULTS**

129 **Physiological responses of Arabidopsis plants to high light, heat stress and their combination**

130 To study the physiological responses of Arabidopsis plants to high light, heat stress and their
131 combination, we subjected wild-type (Col-0) plants to high light intensity (600 µmol m^{-2} s^{-1}; HL),
132 high temperature (42ºC; HS), or to the combination of HL and HS (600 µmol m^{-2} s^{-1} and 42ºC;
133 HL+HS) for 7 hours. Control (CT) plants were maintained at 50 µmol m^{-2} s^{-1} and 23ºC during the
134 entire experimental period (Supplemental Fig. S1; Balfagón et al., 2019). Gas exchange
135 parameters, including leaf photosynthetic rate (A), transpiration (E) and stomatal conductance (gs),
136 were determined in Arabidopsis plants subjected to HL, HS and HL+HS (Fig. 1). Photosynthesis,
137 transpiration, and stomatal conductance significantly decreased following the application of HL
138 compared to CT values. These results are in agreement with previous reports showing stomata to
139 close during light stress (Devireddy et al., 2018; Balfagón et al., 2019), limiting transpiration and
140 negatively affecting photosynthetic rates. In contrast, the application of HS increased transpiration
141 and stomatal conductance, maintaining stomata open to cool down the leaf surface via transpiration
142 (in agreement with stomatal aperture measurements; Balfagón et al., 2019). However, HS did not

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

143 affect photosynthesis compared to CT. Interestingly, the HL+HS combination induced a
144 significant decrease in photosynthesis compared to CT, whereas transpiration and stomatal
145 conductance dramatically increased by about 8-fold compared to CT, or 4-fold compared to HS
146 (Fig. 1). These findings demonstrate that although leaf temperature and stomatal aperture were
147 similar between plants subject to HS or HL+HS (Balfagón et al., 2019), compared to plants
148 subjected to HS, transpiration and stomatal conductance were much higher in plants subjected to
149 the stress combination (HL+HS; Fig. 1). Stomatal aperture measurements and transpiration rates
150 may therefore not always correlate with each other, and it may take higher transpiration rates to
151 cool a leaf during HL+HS combination, potentially a result of heat generated due to dissipation of
152 excess light energy by non-photochemical quenching (NPQ) and/or other protective processes
153 (Czarnocka and Karpiński, 2018; Murchie and Ruban, 2020). Taken together, the results presented
154 in Fig. 1 suggest that during HL+HS combination, HS-associated transpiration and stomatal
155 conductance responses prevailed over those induced by HL (stomatal closure), and that reduction
156 in leaf temperature was more important for plants subjected to HL+HS, than HL-induced stomatal
157 closure that could minimize water loss (Fig. 1; Balfagón et al., 2019).

158 **Metabolomic responses of Arabidopsis plants to high light, heat stress and their combination**

159 To study the accumulation of stress-associated metabolites in Arabidopsis plants subjected to HL,
160 HS or HL+HS, a gas chromatography-mass spectrometric (GC-MS) analysis of polar compounds
161 extracted from leaves of plants subjected to the different stresses was performed (Supplemental
162 Fig. S1). Principal Component Analysis (PCA) revealed that the main source of variation in the
163 data was due to metabolic changes associated with the stress combination, as the first principal
164 component, accounting for 56.6% of total variance, was defined by the characteristic profile of
165 HL+HS samples. In turn, principal component 3, explaining 11.5% of total variation, clearly
166 separated the samples based on the metabolic profile of plants subjected to HS (Fig. 2A). Analysis
167 of variance revealed a total of 25 polar metabolites with levels significantly altered in response to
168 HL (21 and 4 over- and under-accumulated, respectively), levels of 23 metabolites significantly
169 altered in response to HS (19 and 4 over- and under-accumulated, respectively), and levels of 38
170 metabolites changed compared to CT under HL+HS (28 and 10 over- and under-accumulated,
171 respectively) (Fig. 2B; Table 1). Moreover, of the 28 metabolites with levels significantly elevated
172 in response to HL+HS, 3 metabolites (10.7%) were common with HL-induced metabolites, other

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

173 3 metabolites (10.7%) were common with HS-induced metabolites, and 7 metabolites (25.0%)
174 were found to be specifically accumulated in response to HL+HS. Similarly, levels of 1 metabolite
175 (10.0%) commonly decreased in response to either HL or HS, and levels of 7 metabolites (70.0%)
176 were reduced in response to HL+HS (Fig. 2B). These results indicated that a substantial portion of
177 polar metabolites with altered levels in plants subjected to HL+HS was specific for the stress
178 combination. As shown in Table 1, metabolites that exclusively accumulated in plants in response
179 to HL+HS were glycerol, succinic acid, GABA, rhamnose, arginine, gluconic acid and tyrosine.
180 In contrast, levels of threonic acid, urea, fumaric acid, nicotinic acid, citric acid, pyroglutamic acid
181 and putrescine specifically decreased in response to HL+HS (Table 1).

182 **The impact of high light and heat stress combination on sugar metabolism, TCA cycle**
183 **intermediates, and amino acid levels**

184 Further analysis of metabolites involved in glycolysis, TCA cycle and amino acid biosynthesis
185 during stress combination, was conducted (Fig. 3). The soluble sugars glucose and fructose, as
186 well as raffinose and maltose strongly accumulated in response to HL+HS whereas their
187 accumulation, in general, was less pronounced in response to HL or HS. In addition, trehalose and
188 erythritol accumulated in response to the different treatments and particularly during HL+HS. In
189 contrast, sucrose, the major form of carbohydrates transported from photosynthetically active
190 tissues, slightly increased in its level in response to the individual and combined stresses (Fig. 3A;
191 Table 1; Supplemental Table S1). Analysis of TCA-cycle intermediates revealed that HL+HS
192 perturbed the TCA cycle and reduced the levels of the TCA-cycle-derived amino acids aspartate
193 and glutamate. Aromatic amino acids are synthesized in plants through the shikimate pathway. In
194 our study, levels of tryptophan and phenylalanine significantly increased during HL+HS, whereas
195 individual stresses had a marginal effect on their accumulation. In addition, tyrosine was also
196 accumulated under HL+HS combination, while no change in its levels was found under HL or HS.
197 Amino acids synthesized from pyruvate including alanine, leucine, valine, and isoleucine
198 significantly accumulated under all stress conditions, although more noticeably under HL+HS
199 conditions (Fig. 3A; Table 1; Supplemental Table S1). The reduction in aspartate levels under
200 HL+HS was accompanied by accumulation of asparagine, methionine, threonine, and especially
201 lysine, whose accumulation was especially high in response to HL+HS (Fig. 3A; Table 1;
202 Supplemental Table S1). Analysis of the expression of genes encoding for enzymes that participate

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

203 in different reactions of the TCA cycle revealed different patterns of transcript accumulation
204 among the individual and combined stresses (Fig. 3B; Supplemental Table S2). In general,
205 although TCA-related metabolites were suppressed in response to a combination of HL+HS,
206 expression of transcripts encoding TCA cycle-related enzymes increased in response to the stress
207 combination (Fig. 3B; Supplemental Table S2), possibly as a response to counteract the low
208 metabolite accumulation.

209 **Impact of a combination of high light and heat stress on glutamate metabolism**

210 Glutamate has a central role in amino acid metabolism in plants, and is also a substrate for the
211 synthesis of arginine, ornithine, proline, glutamine and GABA (Forde and Lea, 2007). As shown
212 in Fig. 4A and Supplemental Table S3, the observed decline in glutamate levels in response to
213 HL+HS was associated with proline, glutamine, and GABA accumulation. In contrast, levels of
214 arginine and urea, as well as levels of the polyamine putrescine, decreased or did not change in
215 response to the application of stress (Fig. 4A; Supplemental Table S3). It was reported that under
216 abiotic stress conditions, oxidation of putrescine contributes to GABA production (Shelp et al.,
217 2012), suggesting that the specific decrease in putrescine under HL+HS conditions could lead to
218 GABA accumulation in response to this stress combination. Indeed, as shown in Fig. 4A and
219 Supplemental Table S3, GABA accumulated exclusively in response to HL+HS. To further dissect
220 GABA metabolism in plants in response to a combination of HL and HS, we analyzed the
221 expression of transcripts involved in GABA biosynthesis (*GAD1*, *GAD2*, *GAD3* and *GAD4*) as
222 well as the expression of transcripts related to GABA catabolism (*POP2* and *ALDH5F1*; using
223 RNA-Seq data obtain by Balfagón et al., 2019). As shown in Fig. 4B and Supplemental Table S4,
224 the expression of *GAD1* and especially *GAD3* remarkably increased only in response to HL+HS.
225 In contrast, expression of *GAD2* was repressed in response to HS and transcript accumulation of
226 *GAD4* slightly increased in plants subjected to the individual HL or HS treatments. The expression
227 of *POP2* decreased in response to HL and HL+HS and all stresses reduced the expression of
228 *ALDH5F1* (Fig. 4B; Supplemental Table S4). The findings presented in Fig. 4 and Supplemental
229 Table S4 suggest therefore a possible role for GABA in regulating plant responses to HL+HS.

230 **Involvement of GABA in plant tolerance to the combination of high light and heat stress**

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

231 To further study the role of GABA in the response of plants to HL+HS, we analyzed the response
232 of two independent lines of the GABA-deficient mutant *gad3* (SALK_138534C and
233 SALK_033307C) to HL, HS and HL+HS combination (Fig. 5A). Accumulation of GABA was
234 repressed or did not change in *gad3* mutants subjected to HL or HS, as well as in wild type plants
235 in response to HL. In contrast, HL+HS induced a pronounced increase in GABA levels in Col-0
236 plants, whereas both *gad3* mutants slightly accumulated GABA, probably due to the action of
237 *GAD1* (Figs. 4B, 5B). The reduced accumulation of GABA in *gad3* plants in response to HL+HS
238 compared to Col-0 (Fig. 5B) was accompanied by a significant decrease in the survival of *gad3*
239 mutants in response to a combination of HL+HS (Fig. 5A, C). Whereas all *gad3* plants survived
240 the individual HL or HS, the survival rate of both *gad3* mutants subjected to HL+HS combination
241 decreased by about 40%. Furthermore, analysis of Leaf Damage Index (LDI; Balfagón et al., 2019)
242 of Col-0 and *gad3* mutants subjected to the different stresses (Fig. 5D) revealed that HL+HS
243 negatively impacted leaf appearance of both *gad3* lines, with 51.2% and 50.2% of leaves dead,
244 28.2% and 32.7% of leaves injured, and only 20.6% and 16.9% of leaves appearing healthy, in
245 SALK_138534C and SALK_033307C, respectively (Fig. 5D). Compared to Col-0 plants, *gad3*
246 mutants were therefore more sensitive to HL+HS combination.

247

248 DISCUSSION

249 The ability of plants to sense and react to different adverse conditions in their environment by
250 modulating physiological responses, gene expression and metabolism, is crucial for plant
251 adaptation and survival during stress. Due to the frequent occurrence of HL+HS combination in
252 nature, and its impact on crops (Yamamoto et al., 2008; Suzuki et al., 2014; Roeber et al., 2020),
253 as well as its impact on plant survival (Balfagón et al., 2019), the study of metabolic changes
254 during this stress combination is of particular interest. A recent study of the physiological and
255 transcriptomic responses of Arabidopsis plants subjected to HL, HS and their combination
256 (HL+HS) revealed that the HL+HS combination was accompanied by irreversible damage to PSII,
257 decreased D1 (PsbA) protein levels, enhanced accumulation of the hormones jasmonic acid (JA)
258 and JA-isoleucine (JA-Ile), elevated expression of over 2,200 different transcripts unique to the
259 stress combination, distinctive structural changes to chloroplasts and a decreased survival rate

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

260 (Balfagón et al., 2019). In the present study, we show that HL+HS combination has a detrimental
261 effect on photosynthetic rates, and that the effects of HS on stomatal responses and transpiration
262 (opening of stomata and increasing transpiration) prevails over the effects of HL (closing of
263 stomata and decreasing transpiration; Balfagón et al., 2019) (Fig. 1). This result is different than
264 the response of plants to a combination of drought and heat stress, in which the effects of drought
265 prevailed over the effects of heat on stomatal regulation (Rizhsky et al., 2002; Rizhsky et al., 2004).
266 The observed decrease in photosynthetic rates under HL+HS (Fig. 1) prompted us to analyze the
267 primary metabolism of plants subjected to this stress combination to unravel specific patterns of
268 sugar, amino acid and polyamine accumulation (Figs. 2-4; Table 1; Supplemental Tables S1-4).
269 Individual and combined HL and HS displayed different polar accumulation patterns (Fig. 2; Table
270 1), suggesting that the different stress conditions alter the primary metabolism in different ways,
271 reinforcing the idea that metabolic changes due to stress combination are unique and not a mere
272 additive combination of the effects of each individual stress.

273 In our study, the levels of several metabolites appeared to be correlated with plant sensitivity to
274 HL+HS combination (Balfagón et al., 2019). Plants subjected to this stress combination
275 accumulated sugars such as glucose, fructose, raffinose, maltose and trehalose, whereas the levels
276 of sucrose slightly increased in response to individual and combined stresses (Fig. 3; Table 1;
277 Supplemental Table S1). The source of sugars in plants subjected to a combination of HL and HS
278 is unknown. Taking into consideration that photosynthesis is suppressed in plants subjected to
279 HL+HS combination (Fig. 1), sugars could be synthesized by way of starch degradation, as
280 proposed to occur during a combination of drought and heat stress (Rizhsky et al., 2004). Indeed,
281 high accumulation of maltose, a major sugar associated with starch degradation (Thalmann and
282 Santelia, 2017), and of its derived sugars were observed in HL+HS-stressed plants (Fig. 3; Table
283 1; Supplemental Table S1). Additional studies are, however, required to examine this possibility.
284 The increased accumulation of sugars participating in glycolysis under HL+HS (Fig. 3;
285 Supplemental Table S1) suggests that this pathway could provide an alternative source of ATP in
286 plants subjected to HL+HS stress combination, to counteract the negative effects of the stress
287 combination on PSII and photosynthetic rates (Fig. 1; Balfagón et al., 2019), as well as to function
288 as compatible solutes (Krasensky and Jonak, 2012; Shaar-Moshe et al., 2019). Moreover, the levels
289 of glycolysis-derived aromatic amino acids produced through the shikimate pathway, tryptophan,

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

290 phenylalanine and tyrosine, as well as amino acids synthesized from pyruvate, including alanine,
291 leucine, valine and isoleucine significantly increased during a combination of HL+HS (Fig. 3;
292 Table 1; Supplemental Table S1). Although glycolysis appeared to be activated under HL+HS
293 combination, a concomitant activation of the TCA cycle was not observed (Fig. 3; Table 1;
294 Supplemental Table S1), similar to the findings of Shaar-Moshe et al. (2019), demonstrating that
295 organic acids produced by the TCA cycle were reduced under the combination of salinity, drought
296 and heat. Therefore, depletion of metabolites related to the TCA cycle under stress combination
297 could indicate that respiration might be compromised in plants subjected to HL+HS combination.
298 The reduction in oxalacetate-derived aspartate levels under HL+HS conditions was accompanied
299 by an increase in aspartate-related amino acids, especially lysine (Fig. 3; Table 1; Supplemental
300 Table S1). Increased accumulation of amino acids has been shown in plants subjected to different
301 abiotic stresses (*e.g.*, Kaplan et al., 2004; Rizhsky et al., 2004; Kempa et al., 2008; Sanchez et al.,
302 2008; Usadel et al., 2008; Lugan et al., 2010; Krasensky and Jonak, 2012), and could be a result
303 of amino acid biosynthesis and/or enhanced stress-induced protein degradation. In this sense, the
304 higher impact of HL+HS combination on plant physiology and survival (Fig. 1; Balfagón et al.,
305 2019) could lead to an increase in protein degradation and therefore, higher amino acid content.
306 Further studies elucidating this possibility are needed. The decreased levels of TCA-derived
307 glutamate in response to HL and especially in response to HL+HS combination (Figs. 3, 5; Table
308 1; Supplemental Table S1) was further accompanied by a concomitant decrease in putrescine (Fig.
309 4; Table 1; Supplemental Table S3). These results indicate that the role of polyamines under
310 HL+HS as osmoprotectants might be marginal, and that other metabolites including sugars (Fig.
311 3) and/or proline (Fig. 4) could have a key role as osmoprotective elements under this stress
312 combination. In addition, as a compatible solute, proline is involved in the stabilization of proteins
313 and protein complexes in the chloroplast and cytosol, protection of the photosynthetic apparatus
314 and enzymes involved in detoxification of ROS, as well as redox balance stabilization (Szabados
315 and Savouré, 2010). The high accumulation of proline observed in plants subjected to HL+HS
316 could therefore suggest that the stress combination imposes a stronger pressure on plant
317 metabolism, as indicated by the decrease in survival rates and values of LDI (Fig. 5; Balfagón et
318 al., 2019).

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

319 Interestingly, GABA levels were specifically elevated in plants subjected to HL+HS combination
320 (Fig. 4A Table 1; Supplemental Table S3), and GABA-deficient mutants (*gad3*) showed a
321 significant decline in their ability to acclimate to this stress combination (Fig. 5), suggesting that
322 GABA could be required for plant acclimation to a combination of high light and heat stress.
323 GABA is a key non-proteinogenic amino acid that displays important physiological functions
324 involved in plant growth regulation (Seifikalhor et al., 2019). Exogenous GABA application to
325 plants was reported to improve tolerance to different environmental stresses (*e.g.,* Shi et al., 2010;
326 Shang et al., 2011; Li et al., 2016; Salvatierra et al., 2016; Priya et al., 2019; Seifikalhor et al.,
327 2020). Furthermore, GABA levels increased in response to different abiotic stress combinations,
328 namely, salt and drought, as well as salt, drought and heat (Shaar-Moshe et al., 2019). GABA was
329 further proposed to act as a signaling molecule during stress (Bouché and Fromm, 2004; Yu et al.,
330 2014; Fromm, 2020). Another potential function of GABA in plant survival during stress could be
331 linked to its role in regulating autophagy (Supplemental Fig. S2; Signorelli et al., 2019),
332 contributing to the recycling of damaged cellular components during stress. Taken together, the
333 results presented here indicate that GABA plays a key role in the response of plants to HL+HS
334 stress combination, and that genes involved in GABA metabolism could be used as potential
335 breeding markers for HL+HS-tolerant crops.

336

337 **MATERIALS AND METHODS**

338 **Plant material and growth conditions**

339 *Arabidopsis thaliana* Col-0 (var. Columbia-0) and *gad3* (SALK_138534C and SALK_033307C)
340 plants were grown in peat pellets (Jiffy-7, http://www.jiffygroup.com/) at 23ºC under long day
341 growth conditions (12-hour light from 7 AM to 7 PM; 50 µmol m^{-2} s^{-1}/12-hour dark from 7 PM to
342 7 AM).

343 **Stress treatments**

344 Individual HL and HS, and a combination of HL and HS were applied in parallel as described in
345 (Balfagón et al., 2019) and shown in Supplemental Fig. S1, using 30-day-old *Arabidopsis thaliana*
346 plants (wild type Col-0 and the SALK_138534C and SALK_033307C *gad3* mutants). HL was
347 applied by exposing plants to 600 µmol m^{-2} s^{-1} (Philips, F54T5/TL84/HO/ALTO) at 23ºC for 7

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

348 hours. HS was applied by subjecting plants to 42ºC, 50 µmol m^{-2} s^{-1}, for 7 hours. HL+HS
349 combination was performed by simultaneously subjecting plants to 600 µmol m^{-2} s^{-1} of light stress
350 and 42ºC for 7 hours. Control plants were maintained at 50 µmol m^{-2} s^{-1}, 23ºC during the entire
351 experiment. Following the stress treatments, control plants and plants subjected to HL, HS and
352 HL+HS combination were divided into two groups: a group used for sampling leaves for
353 metabolomics analysis as described below; and a group allowed to recover under controlled
354 conditions until flowering time to score for survival. 24 hours following the stress treatments, Leaf
355 Damage Index (LDI; Gallas and Waters, 2015; Balfagón et al., 2019) was recorded (Supplemental
356 Fig. S1). All experiments were carried out at the same time-of-day during the light cycle (from 9
357 AM to 4 PM) and were repeated at least three times with 30 plants per biological repeat.

358 **Photosynthetic parameters**

359 Photosynthetic rate (A), transpiration (E) and stomatal conductance (gs) were measured using a
360 LCpro+ portable infrared gas analyzer (ADC BioScientific Ltd., Hoddesdon, UK) under ambient
361 CO_2 and moisture conditions. Supplemental light was provided by a PAR lamp at 50 or 600 µmol
362 m^{-2} s^{-1} photon flux density, and air flow was set at 150 µmol mol^{-1}. After instrument stabilization,
363 at least 10 measurements were taken on three fully expanded leaves of three plants immediately
364 after the 7 hours of individual and combined stress treatments (Supplemental Fig. S1). All
365 experiments were repeated at least three times.

366 **Determination of primary metabolites**

367 The relative levels of polar metabolites were determined as described in Zanor et al. (2009). Fifteen
368 mg of freeze-dried plant tissue were extracted in 1.4 mL of methanol and 60 µL of an aqueous
369 solution with 0.2 mg mL^{-1} of ribitol, which was used as internal standard. Extraction was
370 performed at 70ºC for 15 min in a water bath. The extract was centrifuged at 14,000 rpm for 10
371 min, and the supernatant was recovered and fractionated adding chloroform and Milli-Q water.
372 After vigorous vortexing and 15 min and centrifugation at 4,000 rpm, 50 µL of the aqueous phase
373 were recovered and dried overnight in a speed-vac. The dry residue was subjected to a double
374 derivatization procedure with methoxyamine hydrochloride (20 mg mL^{-1} in pyridine, Sigma) and
375 *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (Macherey-Nagel). Fatty acid methyl esters (C_8-
376 C_{24}) were added and used as retention index (RI) markers. Analyses were performed on a 6890N
377 gas chromatograph (Agilent Technologies, USA) coupled to a Pegasus 4D TOF mass spectrometer

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

378 (LECO, St. Joseph, MI). Chromatography was performed with a BPX35 (30 m, 0.32 mm, 0.25
379 μm) capillary column (SGE Analytical Science Pty Ltd., Australia) with a 2 mL min^{-1} helium flow.
380 Oven programming conditions were as follows: 2 min of isothermal heating at 85°C, followed by
381 a 15°C min^{-1} temperature ramp up to 360°C. Injection temperature was set at 230°C, and the ion
382 source was adjusted to 250°C. Data were acquired after EI ionization at 70 eV, and recorded in the
383 70–600 m/z range at 20 scans s^{-1}. Chromatograms were analyzed by means of the ChromaTOF
384 software. Metabolites were identified by comparison of both mass spectra and retention time with
385 those of pure standards injected under the same conditions. Peak area of each identified compound
386 was normalized to the internal standard area (ribitol) and sample dry weight. All experiments were
387 repeated four times.

388 **γ-aminobutyric acid quantification**

389 About 5 mg of freeze-dried plant tissue were transferred to a 1.5-mL microcentrifuge tube. Three
390 glass beads and 50 μL of deuterium labelled internal standard γ-aminobutyric acid (GABA-d2) at
391 concentration of 20 ppm was added. Then, 300 μL of cold MeOH:H_2O (80:20) was added,
392 following sonication in an ultrasound bath with ice for 10 min and centrifugation at 10,000 rpm
393 for 5 min. 250 μL of supernatant were recovered and 250 μL of acetonitrile were added, following
394 filtration through a PTFE 0.2 μm pore size cellulose filter. Final concentration of the deuterated
395 standard (GABA-d2) was 200 ppb. GABA was quantified in plant extracts using a UPLC system
396 (Waters Acquity SDS, Waters Corp., Milford, MA, USA) interfaced to a TQD triple quadrupole
397 (Micromass Ltd, Manchester, UK) mass spectrometer through an orthogonal Z-spray electrospray
398 ion source. Separations were carried out on 2.1 mm × 150 mm ACQUITY UPLC 1.7 m BEH
399 amide Column using a linear gradient of (A) acetonitrile-water 95:5 (v:v), 0.1% ammonium
400 formate and (B) acetonitrile-water 2:98 (v:v), 0.1% ammonium formate at a flow rate of 300 μL
401 min^{-1}. Chromatographic run started at 0% B; after 1 min a linear gradient increased A to 75% for
402 3 min; finally, mobile phase composition returned to the initial conditions for 2 min. Transitions
403 for GABA (104>87) and GABA-d2 (106>89), were monitored in positive ionization mode. GABA
404 was identified by comparing both mass spectra and retention time with those of pure standards
405 injected in the same conditions. Peak area of GABA was normalized to internal standard area
406 (GABA-d2) and sample dry weight. All experiments were repeated at least three times.

407 **Statistical analysis**

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

408 Results are presented as the mean ± SD. Statistical analysis were performed by two-way ANOVA
409 followed by a Tukey post hoc test when a significant difference was detected (different letters
410 denote statistical significance at $p < 0.05$), or by two-tailed Student's t-test (asterisks denote
411 statistical significance at $p < 0.05$). Principal component analysis (PCA) was performed by means
412 of the SIMCA version 13.0.3.0 software, using the $\log_2$ transformed data and unit variance
413 normalization.

414 **ACKNOWLEDGMENTS**

415 Metabolite measurements were carried out at Instituto de Biología Molecular y Celular de Plantas,
416 CSIC-Universidad Politécnica de Valencia, and Servei Central d'Instrumentació Científica of the
417 Universitat Jaume I.

418

419 **SUPPLEMENTAL MATERIAL**

420 **Table S1.** Levels of metabolites involved in glycolysis, TCA cycle, and amino acid metabolism in
421 Col-0 plants subjected to high light (HL), heat stress (HS) and the combination of HL and HS
422 (HL+HS). Metabolite levels are expressed as the fold change compared to control conditions.
423 *Abbreviations used*: CT, control; HL, high light; HS, heat stress; HL+HS, a combination of high
424 light and heat stress.

425 **Table S2**. Expression level of transcripts involved in TCA cycle in Col-0 plants subjected to high
426 light (HL), heat stress (HS) and the combination of HL and HS (HL+HS). Significant transcript
427 levels ($p < 0.05$) are expressed as the fold change compared to control conditions. Data was
428 obtained from the RNA-Seq analysis conducted by Balfagón et al. (2019). *Abbreviations used*:
429 CT, control; HL, high light; HS, heat stress; HL+HS, a combination of high light and heat stress;
430 n.s., not significant.

431 **Table S3.** Level of metabolites involved in glutamate metabolism in Col-0 plants subjected to high
432 light (HL), heat stress (HS) and the combination of HL and HS (HL+HS). Metabolite levels are
433 expressed as the fold change compared to control conditions. *Abbreviations used*: CT, control;
434 GABA, γ-aminobutyric acid; HL, high light; HS, heat stress; HL+HS, a combination of high light
435 and heat stress.

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

436 **Table S4.** Expression level of transcripts involved in GABA metabolism in Col-0 plants subjected
437 to high light (HL), heat stress (HS) and the combination of HL and HS (HL+HS). Significant
438 transcript levels ($p < 0.05$) are expressed as the fold change compared to control conditions. Data
439 was obtained from the RNA-Seq analysis conducted by Balfagón et al. (2019). *Abbreviations used*:
440 ALDH5F1, aldehyde dehydrogenase 5F1; CT, control; GAD, glutamate decarboxylase; HL, high
441 light; HS, heat stress; HL+HS, a combination of high light and heat stress; n.s., not significant;
442 POP2, pollen-pistil incompatibility 2.

443 **Fig. S1.** The experimental design used for the metabolomic study of high light (HL, yellow), heat
444 stress (HS, orange) and a combination of high light and heat stress (HL+HS, grey) using
445 Arabidopsis plants. HL was applied by exposing 30-day-old plants to 600 µmol m^{-2} s^{-1} (Philips,
446 F54T5/TL84/HO/ALTO) at 23°C. HS was applied by transferring 30-day-old plants to 42°C.
447 HL+HS was performed by simultaneously subjecting plants to 600 µmol m^{-2} s^{-1} and 42°C. Stress
448 treatments were performed in parallel during 7 h. Following the stress treatments, plants were
449 sampled for metabolomic analysis and gas exchange parameters were recorded. Another group of
450 plants was allowed to recover under controlled conditions until flowering time to score for
451 survival. 24 hours following the stress treatments, Leaf Damage Index (LDI) was also determined.
452 All experiments were carried out at the same time-of-day during the light cycle (from 9 AM to 4
453 PM) and were repeated at least three times using Col-0 and *gad3* plants. *Abbreviations used*: CT,
454 control; HL, high light; HS, heat stress; HL+HS, a combination of high light and heat stress; LDI,
455 Leaf Damage Index.

456 **Fig. S2.** Enrichment of autophagy-related transcripts in the response of Arabidopsis plants to a
457 combination of high light and heat stress. (A) Venn diagrams depicting the overlap between
458 transcripts altered in Col-0 plants in response to a combination of high light and heat stress
459 (HL+HS) and transcripts related to autophagy. (B) Heat map showing the expression levels of
460 transcripts involved in autophagy in Col-0 plants subjected HL, HS and HL+HS combination.
461 Non-significant expression levels compared to controls are shown in grey. Data was obtained from
462 the RNA-Seq analysis conducted by Balfagón et al. (2019). *Abbreviations used*: HL, high light;
463 HS, heat stress; HL+HS, a combination of high light and heat stress.

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

TABLES

Table 1. List of metabolites over- and under-accumulated in Col-0 plants subjected to high light (HL), heat stress (HS) and a combination of HL and HS (HL+HS). Values represent fold changes compared to control. Bold values represent fold changes > 10 for over-accumulated metabolites, and fold changes < 0.5 for under-accumulated metabolites. Metabolites shown are all significant (N=4, t-test, $p < 0.05$; see Tables S1, S3). *Abbreviations used*: GABA, γ-aminobutyric acid; HL, high light; HS, heat stress; HL+HS, a combination of high light and heat stress.

		Over-accumulated (Fold change)				**Under-accumulated (Fold change)**		
Stress	**Metabolite**	**HL**	**HS**	**HL+HS**	**Metabolite**	**HL**	**HS**	**HL+HS**
HL	Fumaric acid	1.90			GABA	0.65		
	Malic acid	2.60			Rhamnose	0.69		
	α-ketoglutaric acid	2.11						
HL & HL+HS	Proline	**10.35**		**27.35**	Glutamic acid	0.74		**0.36**
	Methionine	1.81		2.37				
	Threalose	2.13		3.92				
HS	Putrescine	1.49			Methionine		0.64	
					Arginine		0.52	
HS & HL+HS	Asparagine		3.18	2.71	Myoinositol		0.53	**0.44**
	Lysine		2.57	**23.90**				
	Tryptophan		1.95	**16.72**				
HL+HS	Glycerol			2.48	Threonic acid			0.69
	Succinic acid			2.33	Urea			0.55
	GABA			**59.43**	Fumaric acid			0.58
	Rhamnose			2.32	Nicotinic acid			0.60
	Arginine			1.31	Citric acid			**0.47**
	Gluconic acid			2.32	Pyroglutamic acid			0.68
	Tyrosine			2.11	Putrescine			**0.38**
HL & HS & HL+HS	Alanine	**43.61**	**287.70**	**593.02**	Aspartic acid	0.62	0.61	**0.25**
	Valine	**12.35**	**16.14**	**73.63**				
	Leucine	7.82	8.10	**174.49**				
	Isoleucine	7.41	**12.56**	**148.53**				
	Glycine	26.28	9.14	**12.78**				
	Threonine	2.86	2.75	3.21				
	Erythritol	1.48	1.74	3.86				
	4-hydroxyproline	1.54	1.95	2.05				
	Phenylalanine	1.78	3.64	**15.04**				
	Glutamine	6.64	3.56	**15.18**				
	Fructose	1.68	1.93	7.73				
	Glucose	2.77	3.31	9.42				
	Sucrose	1.50	2.06	1.69				
	Maltose	3.72	1.83	**50.28**				
	Raffinose	3.07	**42.54**	**31.74**				

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

472 **FIGURE LEGENDS**

473 **Fig. 1.** Physiological measurements of Arabidopsis plants subjected to high light, heat stress and
474 their combination. Leaf photosynthetic rate (A), transpiration (E), and stomatal conductance (gs)
475 of Col-0 plants subjected to high light (HL), heat stress (HS) and the combination of HL and HS
476 (HL+HS). Error bars represent SD (N=9). Different letters denote statistical significance at $p <$
477 0.05. *Abbreviations used*: A, photosynthetic rate; E, transpiration; gs, stomatal conductance; CT,
478 control; HL, high light; HS, heat stress; HL+HS, a combination of high light and heat stress.

479 **Fig. 2.** Metabolic analysis of Arabidopsis Col-0 plants subjected to high light, heat stress and their
480 combination. (A) Principal Component Analysis (PCA) score plot of metabolite profiles obtained
481 from control Col-0 plants (CT), and Col-0 plants subjected to high light (HL), heat stress (HS) or
482 a combination of HL and HS (HL+HS). (B) Venn diagrams showing the overlap between
483 metabolites over-accumulated (left) or under-accumulated (right) in response to HL, HS and
484 HL+HS combination. *Abbreviations used*: CT, control; HL, high light; HS, heat stress; HL+HS, a
485 combination of high light and heat stress.

486 **Fig. 3.** Levels of amino acids and metabolites involved in glycolysis and TCA cycle in Arabidopsis
487 plants subjected to high light, heat stress and their combination. (A) Levels of metabolites
488 participating in glycolysis, TCA cycle, and amino acid metabolism in Col-0 plants subjected to
489 high light (HL), heat stress (HS) or a combination of HL and HS (HL+HS). Significant metabolite
490 levels ($p < 0.05$) are expressed as fold change compared to control conditions and are shown as a
491 color scale (Table S1). Non-significant accumulation compared to control is shown in white. (B)
492 Heat map showing the expression levels of transcripts involved in TCA cycle in Col-0 plants
493 subjected HL, HS and HL+HS combination. Significant transcript levels ($p < 0.05$) are expressed
494 as fold change compared to control conditions and are shown as a color scale. Non-significant
495 expression levels compared to control are shown in grey. Transcript expression data was obtained
496 from the RNA-Seq analysis conducted by Balfagón et al. (2019) (Table S2). *Abbreviations used*:
497 HL, high light; HS, heat stress; HL+HS, a combination of high light and heat stress; PEP,
498 phosphoenolpyruvate.

499 **Fig. 4.** Glutamate metabolism in Arabidopsis plants subjected to high light, heat stress and their
500 combination. (A) Level of metabolites involved in glutamate metabolism in Col-0 plants subjected
501 to high light (HL), heat stress (HS) or a combination of HL and HS (HL+HS). Significant

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

502 metabolite levels ($p < 0.05$) are expressed as fold change compared to control conditions and are
503 shown as a color scale (Table S3). Non-significant accumulation compared to controls are shown
504 in white. (B) Heat map showing the expression levels of transcripts involved in GABA metabolism
505 in Col-0 plants subjected HL, HS and HL+HS combination. Non-significant expression levels
506 compared to controls are shown in grey. Transcript expression data was obtained from the RNA-
507 Seq analysis conducted by Balfagón et al. (2019) (Table S4). *Abbreviations used*: ALDH5F1,
508 aldehyde dehydrogenase 5F1; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; HL,
509 high light; HS, heat stress; HL+HS, a combination of high light and heat stress; POP2, pollen-
510 pistil incompatibility 2.

511 **Fig. 5.** Involvement of GABA in the response of Arabidopsis plants to high light, heat stress and
512 their combination. (A) Representative images of Col-0 and the GABA mutant *gad3* (two
513 independent knockout lines; SALK_138534C and SALK_033307C) subjected to high light (HL),
514 heat stress (HS) and a combination of HL and HS (HL+HS). (B) Levels of GABA in Col-0 and
515 the GABA knockout mutant *gad3* (two independent lines) subjected to HL, HS and HL+HS
516 combination. (C) Survival of Col-0 and the GABA mutant *gad3* (two independent lines) subjected
517 to HL, HS and HL+HS combination. (D) Leaf Damage Index (LDI) of Col-0 and the GABA
518 mutant *gad3* (two independent lines) subjected to HL, HS and HL+HS combination. Asterisks
519 denote Student's t-test significance at $p < 0.05$ compared to wild type (C) or to control (B and D).
520 Error bars represent SD. *Abbreviations used*: CT, control; GAD, glutamate decarboxylase; HL,
521 high light; HS, heat stress; HL+HS, a combination of high light and heat stress; LDI, Leaf Damage
522 Index.

523

524

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

525 **REFERECES**

526 **Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Carrasco P, Tiburcio**
527 **AF** (2010) Polyamines: Molecules with regulatory functions in plant abiotic stress tolerance.
528 Planta **231**: 1237–1249

529 **Aleksza D, Horváth G V., Sándor G, Szabados L** (2017) Proline accumulation is regulated by
530 transcription factors associated with phosphate starvation. Plant Physiol **175**: 555–567

531 **Amir R** (2010) Current understanding of the factors regulating methionine content in vegetative
532 tissues of higher plants. Amino Acids **39**: 917–931

533 **Asada K** (2006) Production and Scavenging of Reactive Oxygen Species in Chloroplasts and
534 Their Functions. Plant Physiol **141**: 391–396

535 **Balfagón D, Sengupta S, Gómez-Cadenas A, Fritschi FBFB, Azad R, Mittler R, Zandalinas**
536 **SISI** (2019) Jasmonic acid is required for plant acclimation to a combination of high light
537 and heat stress. Plant Physiol **181**: 1668–1682

538 **Balfagón D, Zandalinas SI, Mittler R, Gómez-Cadenas A** (2020) High temperatures modify
539 plant responses to abiotic stress conditions. Physiol Plant doi:10.1111/ppl.13151

540 **Batista-Silva W, Heinemann B, Rugen N, Nunes-Nesi A, Araújo WL, Braun H, Hildebrandt**
541 **TM** (2019) The role of amino acid metabolism during abiotic stress release. Plant Cell
542 Environ **42**: 1630–1644

543 **Bouché N, Fromm H** (2004) GABA in plants: Just a metabolite? Trends Plant Sci **9**: 110–115

544 **Caldana C, Degenkolbe T, Cuadros-Inostroza A, Klie S, Sulpice R, Leisse A, Steinhauser D,**
545 **Fernie AR, Willmitzer L, Hannah MA** (2011) High-density kinetic analysis of the
546 metabolomic and transcriptomic response of Arabidopsis to eight environmental conditions.
547 Plant J **67**: 869–884

548 **Cramer GR, Ergül A, Grimplet J, Tillett RL, Tattersall EAR, Bohlman MC, Vincent D,**
549 **Sonderegger J, Evans J, Osborne C, et al** (2007) Water and salinity stress in grapevines:
550 Early and late changes in transcript and metabolite profiles. Funct Integr Genomics **7**: 111–
551 134

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

552 **Czarnocka W, Karpiński S** (2018) Friend or foe? Reactive oxygen species production,
553 scavenging and signaling in plant response to environmental stresses. Free Radic Biol Med
554 **122**: 4–20

555 **Devireddy AR, Zandalinas SI, Gómez-Cadenas A, Blumwald E, Mittler R** (2018)
556 Coordinating the overall stomatal response of plants: Rapid leaf-to-leaf communication
557 during light stress. Sci Signal **11**: 518

558 **Dietz K-J** (2015) Efficient high light acclimation involves rapid processes at multiple mechanistic
559 levels. J Exp Bot **66**: 2401–2414

560 **Forde BG, Lea PJ** (2007) Glutamate in plants: Metabolism, regulation, and signalling. J Exp Bot
561 **58**: 2339–2358

562 **Fromm H** (2020) GABA signaling in plants: targeting the missing pieces of the puzzle. J Exp Bot
563 **71**: 6238–6245

564 **Gallas G, Waters ER** (2015) Boechera species exhibit species-specific responses to combined
565 heat and high light stress. PLoS One **10**: e0129041

566 **Hewezi T, Léger M, Gentzbittel L** (2008) A comprehensive analysis of the combined effects of
567 high light and high temperature stresses on gene expression in sunflower. Ann Bot **102**: 127–
568 140

569 **Huang T, Jander G** (2017) Abscisic acid-regulated protein degradation causes osmotic stress-
570 induced accumulation of branched-chain amino acids in Arabidopsis thaliana. Planta **246**:
571 737–747

572 **Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N, Sung DY, Guy CL** (2004)
573 Exploring the temperature-stress metabolome of Arabidopsis. Plant Physiol **136**: 4159–4168

574 **Kempa S, Krasensky J, Dal Santo S, Kopka J, Jonak C** (2008) A Central Role of Abscisic Acid
575 in Stress-Regulated Carbohydrate Metabolism. PLoS One **3**: e3935

576 **Krasensky J, Jonak C** (2012) Drought, salt, and temperature stress-induced metabolic
577 rearrangements and regulatory networks. J Exp Bot **63**: 1593–1608

578 **Li Z, Wakao S, Fischer BB, Niyogi KK** (2009) Sensing and responding to excess light. Annu

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

579 Rev Plant Biol **60**: 239–260

580 **Li Z, Yu J, Peng Y, Huang B** (2016) Metabolic pathways regulated by γ-aminobutyric acid
581 (GABA) contributing to heat tolerance in creeping bentgrass (Agrostis stolonifera). Sci Rep
582 **6**: 30338

583 **Lugan R, Niogret M-FF, Leport L, Guégan J-PP, Larher FR, Savouré A, Kopka J,**
584 **Bouchereau A** (2010) Metabolome and water homeostasis analysis of Thellungiella
585 salsuginea suggests that dehydration tolerance is a key response to osmotic stress in this
586 halophyte. **64**: 215–229

587 **Maruyama K, Takeda M, Kidokoro S, Yamada K, Sakuma Y, Urano K, Fujita M,**
588 **Yoshiwara K, Matsukura S, Morishita Y, et al** (2009) Metabolic pathways involved in
589 cold acclimation identified by integrated analysis of metabolites and transcripts regulated by
590 DREB1A and DREB2A. Plant Physiol **150**: 1972–1980

591 **Mathur S, Agrawal D, Jajoo A** (2014) Photosynthesis: Response to high temperature stress. J
592 Photochem Photobiol B Biol **137**: 116–126

593 **Mittler R** (2006) Abiotic stress, the field environment and stress combination. Trends Plant Sci
594 **11**: 15–19

595 **Murchie EH, Ruban A V.** (2020) Dynamic non-photochemical quenching in plants: from
596 molecular mechanism to productivity. Plant J **101**: 885–896

597 **Obata T, Witt S, Lisec J, Palacios-Rojas N, Florez-Sarasa I, Yousfi S, Araus JL, Cairns JE,**
598 **Fernie AR** (2015) Metabolite profiles of maize leaves in drought, heat, and combined stress
599 field trials reveal the relationship between metabolism and grain yield. Plant Physiol **169**:
600 2665–2683

601 **Ort DR** (2001) When there is too much light. Plant Physiol **125**: 29–32

602 **Priya M, Sharma L, Kaur R, Bindumadhava H, Nair RM, Siddique KHM, Nayyar H** (2019)
603 GABA (γ-aminobutyric acid), as a thermo-protectant, to improve the reproductive function
604 of heat-stressed mungbean plants. Sci Rep **9**: 1–14

605 **Rizhsky L, Liang H, Mittler R** (2002) The combined effect of drought stress and heat shock on

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

606 gene expression in tobacco. Plant Physiol **130**: 1143–1151

607 **Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R** (2004) When defense
608 pathways collide. The response of Arabidopsis to a combination of drought and heat stress.
609 Plant Physiol **134**: 1683–1696

610 **Roeber VM, Bajaj I, Rohde M, Schmülling T, Cortleven A** (2020) Light acts as a stressor and
611 influences abiotic and biotic stress responses in plants. Plant Cell Environ pce.13948

612 **Ruban A V.** (2015) Evolution under the sun: optimizing light harvesting in photosynthesis. J Exp
613 Bot **66**: 7–23

614 **Salvatierra A, Pimentel P, Almada R, Hinrichsen P** (2016) Exogenous GABA application
615 transiently improves the tolerance to root hypoxia on a sensitive genotype of Prunus
616 rootstock. Environ Exp Bot **125**: 52–66

617 **Sanchez DH, Siahpoosh MR, Roessner U, Udvardi M, Kopka J** (2008) Plant metabolomics
618 reveals conserved and divergent metabolic responses to salinity. Physiol Plant **132**: 209–219

619 **Seifikalhor M, Aliniaeifard S, Bernard F, Seif M, Latifi M, Hassani B, Didaran F, Bosacchi**
620 **M, Rezadoost H, Li T** (2020) γ-Aminobutyric acid confers cadmium tolerance in maize
621 plants by concerted regulation of polyamine metabolism and antioxidant defense systems. Sci
622 Rep **10**: 3356

623 **Seifikalhor M, Aliniaeifard S, Hassani B, Niknam V, Lastochkina O** (2019) Diverse role of γ-
624 aminobutyric acid in dynamic plant cell responses. Plant Cell Rep **38**: 847–867

625 **Shaar-Moshe L, Hayouka R, Roessner U, Peleg Z** (2019) Phenotypic and metabolic plasticity
626 shapes life-history strategies under combinations of abiotic stresses. Plant Direct **3**: e00113

627 **Shang H, Cao S, Yang Z, Cai Y, Zheng Y** (2011) Effect of exogenous γ-aminobutyric acid
628 treatment on proline accumulation and chilling injury in peach fruit after long-term cold
629 storage. J Agric Food Chem **59**: 1264–1268

630 **Shelp BJ, Bozzo GG, Trobacher CP, Zarei A, Deyman KL, Brikis CJ** (2012)
631 Hypothesis/review: Contribution of putrescine to 4-aminobutyrate (GABA) production in
632 response to abiotic stress. Plant Sci **193–194**: 130–135

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

633 **Shi SQ, Shi Z, Jiang ZP, Qi LW, Sun XM, Li CX, Liu JF, Xiao WF, Zhang SG** (2010) Effects
634 of exogenous GABA on gene expression of Caragana intermedia roots under NaCl stress:
635 Regulatory roles for H2O2 and ethylene production. Plant, Cell Environ **33**: 149–162

636 **Signorelli S, Tarkowski ŁP, Van den Ende W, Bassham DC** (2019) Linking Autophagy to
637 Abiotic and Biotic Stress Responses. Trends Plant Sci **24**: 413–430

638 **Spicher L, Almeida J, Gutbrod K, Pipitone R, Dörmann P, Glauser G, Rossi M, Kessler F**
639 (2017) Essential role for phytol kinase and tocopherol in tolerance to combined light and
640 temperature stress in tomato. J Exp Bot **68**: 5845–5856

641 **Suzuki N, Rivero RM, Shulaev V, Blumwald E, Mittler R** (2014) Abiotic and biotic stress
642 combinations. New Phytol **203**: 32–43

643 **Szabados L, Savouré A** (2010) Proline: a multifunctional amino acid. Trends Plant Sci **15**: 89–
644 97

645 **Thalmann M, Santelia D** (2017) Starch as a determinant of plant fitness under abiotic stress. New
646 Phytol **214**: 943–951

647 **Tzin V, Galili G** (2010) New Insights into the shikimate and aromatic amino acids biosynthesis
648 pathways in plants. Mol Plant **3**: 956–972

649 **Urano K, Kurihara Y, Seki M, Shinozaki K** (2010) "Omics" analyses of regulatory networks in
650 plant abiotic stress responses. Curr Opin Plant Biol **13**: 132–138

651 **Usadel B, Bläsing OE, Gibon Y, Poree F, Höhne M, Günter M, Trethewey R, Kamlage B,**
652 **Poorter H, Stitt M** (2008) Multilevel genomic analysis of the response of transcripts, enzyme
653 activities and metabolites in Arabidopsis rosettes to a progressive decrease of temperature in
654 the non-freezing range. Plant, Cell Environ **31**: 518–547

655 **Yamamoto Y, Aminaka R, Yoshioka M, Khatoon M, Komayama K, Takenaka D, Yamashita**
656 **A, Nijo N, Inagawa K, Morita N, et al** (2008) Quality control of photosystem II: impact of
657 light and heat stresses. Photosynth Res **98**: 589–608

658 **Yu GH, Zou J, Feng J, Peng XB, Wu JY, Wu YL, Palanivelu R, Sun MX** (2014) Exogenous
659 γ-aminobutyric acid (GABA) affects pollen tube growth via modulating putative Ca2+-

bioRxiv preprint doi: https://doi.org/10.1101/2021.02.13.431103; this version posted February 14, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

660 permeable membranechannels and is coupled to negative regulation on glutamate
661 decarboxylase. J Exp Bot **65**: 3235–3248

662 **Zandalinas S, Sales C, Beltrán J, Gómez-Cadenas A, Arbona V** (2016) Activation of
663 Secondary Metabolism in Citrus Plants Is Associated to Sensitivity to Combined Drought and
664 High Temperatures. Front Plant Sci **7**: 1954

665 **Zanor MI, Rambla J-LL, Chaïb J, Steppa A, Medina A, Granell A, Fernie AR, Causse M**
666 (2009) Metabolic characterization of loci affecting sensory attributes in tomato allows an
667 assessment of the influence of the levels of primary metabolites and volatile organic contents.
668 J Exp Bot **60**: 2139–2154

669

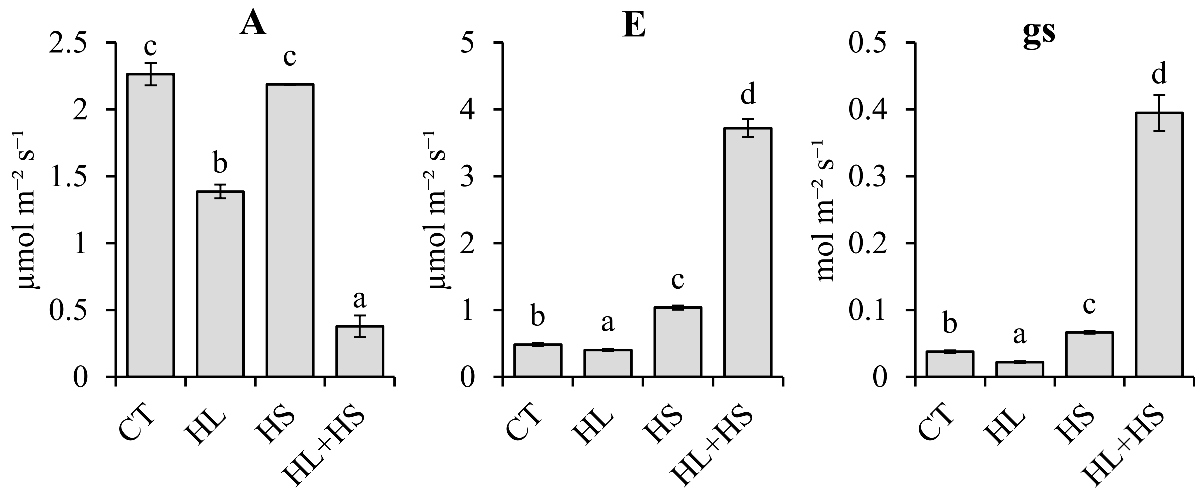
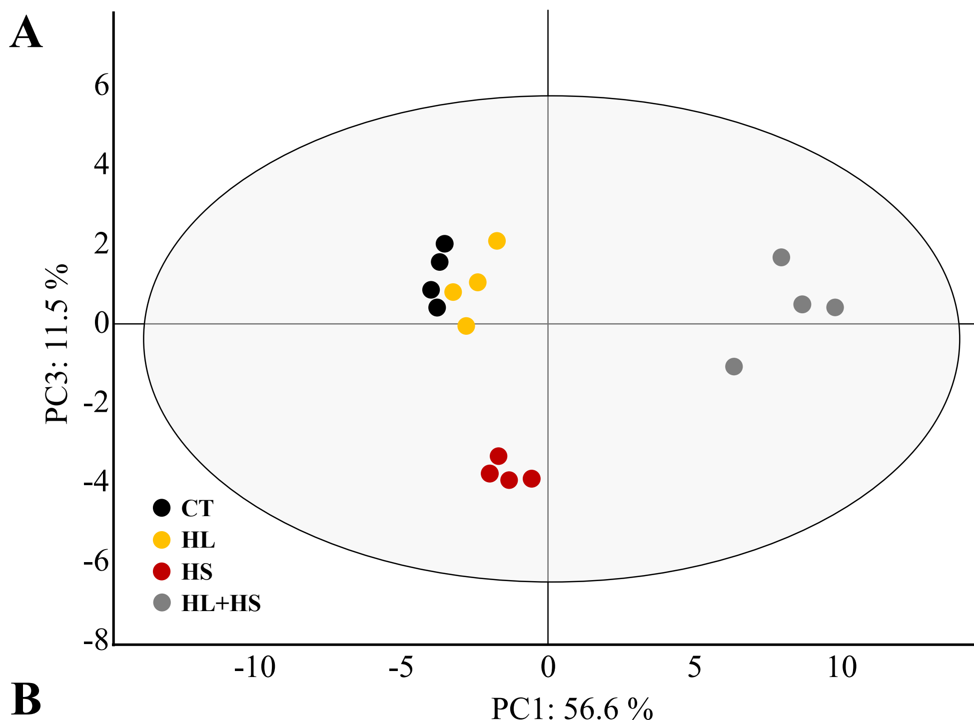


Fig. 1. Physiological measurements of Arabidopsis plants subjected to high light, heat stress and their combination. Leaf photosynthetic rate (A), transpiration (E), and stomatal conductance (gs) of Col-0 plants subjected to high light (HL), heat stress (HS) and the combination of HL and HS (HL+HS). Error bars represent SD (N=9). Different letters denote statistical significance at $p < 0.05$. *Abbreviations used*: A, photosynthetic rate; E, transpiration; gs, stomatal conductance; CT, control; HL, high light; HS, heat stress; HL+HS, a combination of high light and heat stress.



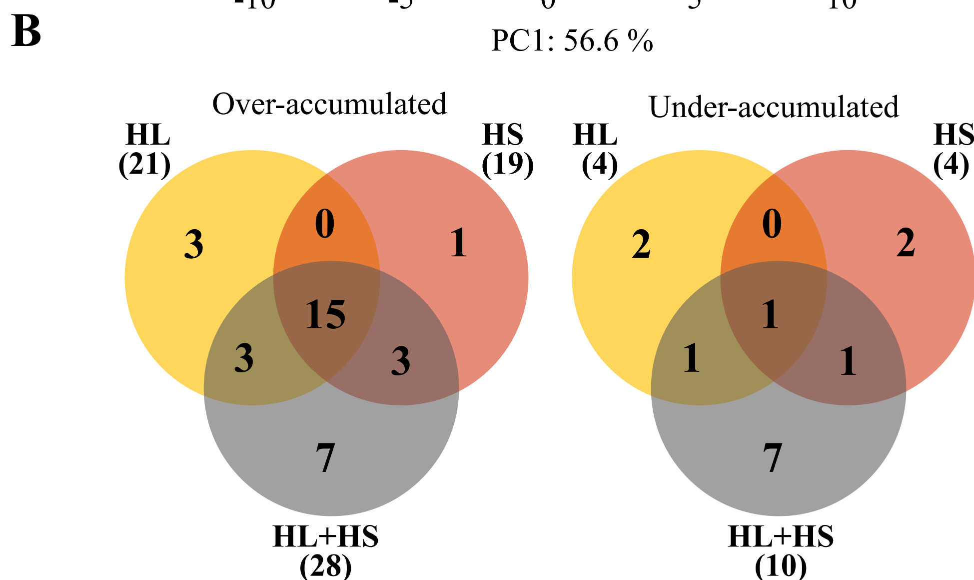


Fig. 2. Metabolic analysis of Arabidopsis Col-0 plants subjected to high light, heat stress and their combination. (A) Principal Component Analysis (PCA) score plot of metabolite profiles obtained from control Col-0 plants (CT), and Col-0 plants subjected to high light (HL), heat stress (HS) or a combination of HL and HS (HL+HS). (B) Venn diagrams showing the overlap between metabolites over-accumulated (left) or under-accumulated (right) in response to HL, HS and HL+HS combination. *Abbreviations used*: CT, control; HL, high light; HS, heat stress; HL+HS, a combination of high light and heat stress.

A

Glycolysis
TCA cycle
HL HS HL+HS
0.25 1 15

maltose
glucose
sucrose
raffinose
trehalose
glucose-6P
myo-inositol
gluconic acid
erythritol
fructose-6P
fructose
threonic acid
glycerate-3P
phenylananine
PEP
shikimate
tyrosine
alanine
tryptophan
leucine
pyruvate
valine
nicotinic acid
isoleucine
acetyl-CoA
asparagine
citrate
threonine
aspartate
oxaloacetate
isocitrate
methionine
lysine
malate
α-ketoglutarate
glutamate
fumarate
succinyl-CoA
succinate

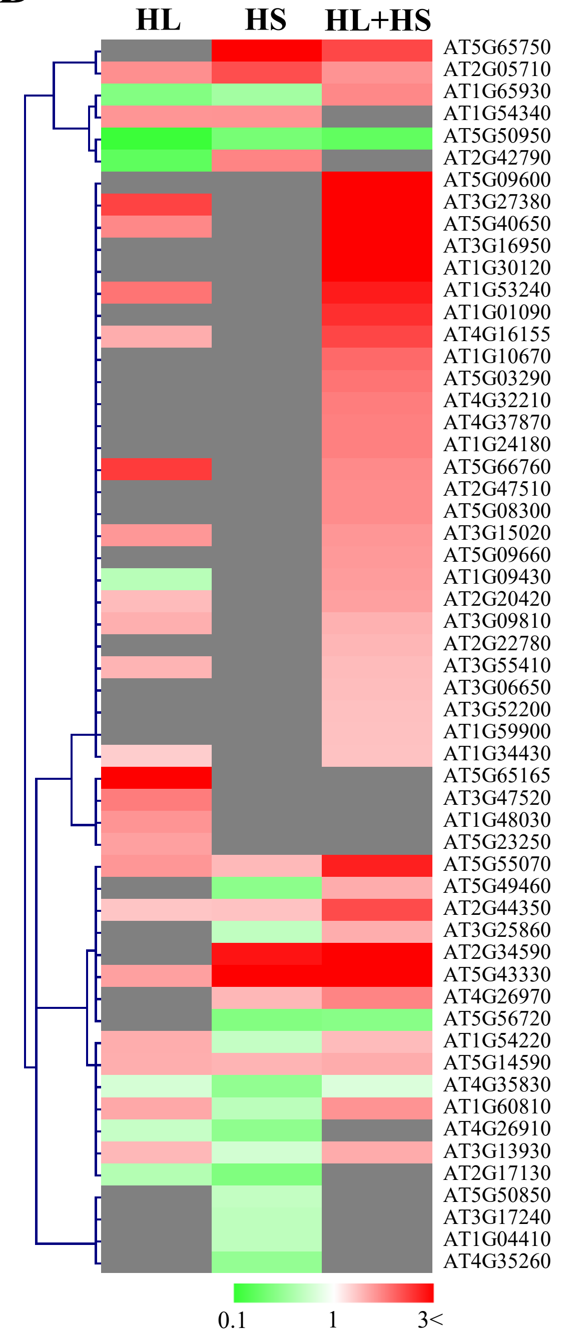


Fig. 3. Levels of amino acids and metabolites involved in glycolysis and TCA cycle in Arabidopsis plants subjected to high light, heat stress and their combination. (A) Levels of metabolites participating in glycolysis, TCA cycle, and amino acid metabolism in Col-0 plants subjected to high light (HL), heat stress (HS) or a combination of HL and HS (HL+HS). Significant metabolite levels ($p < 0.05$) are expressed as fold change compared to control conditions and are shown as a color scale (Table S1). Non-significant accumulation compared to control is shown in white. (B) Heat map showing the expression levels of transcripts involved in TCA cycle in Col-0 plants subjected HL, HS and HL+HS combination. Significant transcript levels ($p < 0.05$) are expressed as fold change compared to control conditions and are shown as a color scale. Non-significant expression levels compared to control are shown in grey. Transcript expression data was obtained from the RNA-Seq analysis conducted by Balfagón et al. (2019) (Table S2). *Abbreviations used*: HL, high light; HS, heat stress; HL+HS, a combination of high light and heat stress; PEP, phosphoenolpyruvate.

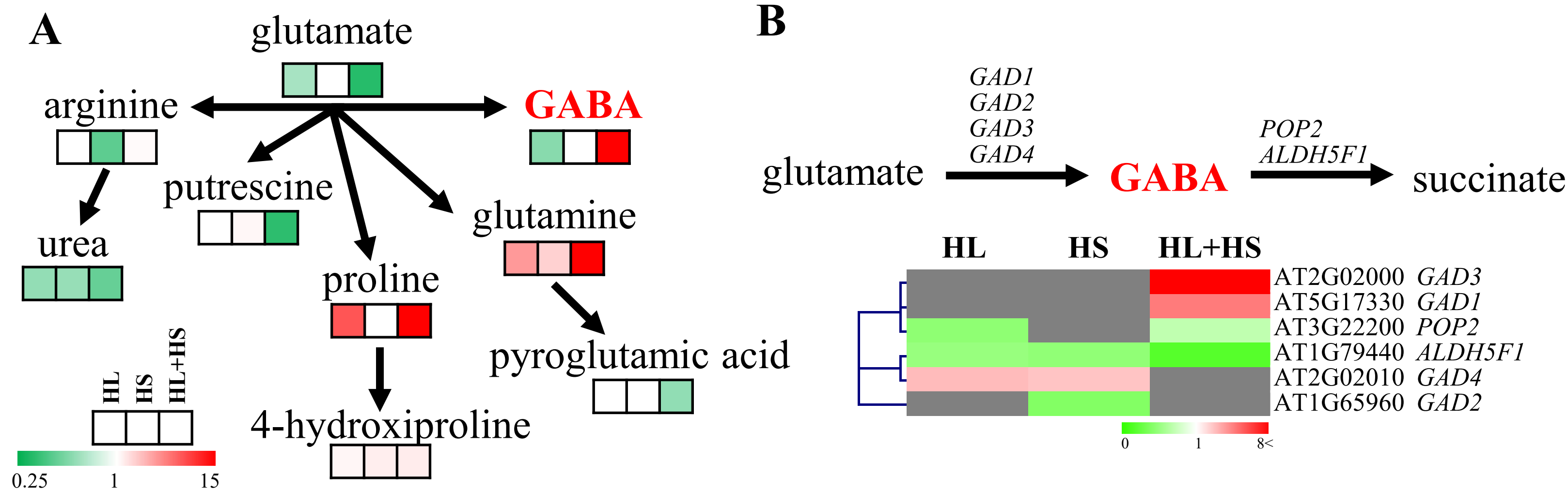


Fig. 4. Glutamate metabolism in Arabidopsis plants subjected to high light, heat stress and their combination. (A) Level of metabolites involved in glutamate metabolism in Col-0 plants subjected to high light (HL), heat stress (HS) or a combination of HL and HS (HL+HS). Significant metabolite levels ($p < 0.05$) are expressed as fold change compared to control conditions and are shown as a color scale (Table S3). Non-significant accumulation compared to controls are shown in white. (B) Heat map showing the expression levels of transcripts involved in GABA metabolism in Col-0 plants subjected HL, HS and HL+HS combination. Non-significant expression levels compared to controls are shown in grey. Transcript expression data was obtained from the RNA-Seq analysis conducted by Balfagón et al. (2019) (Table S4). *Abbreviations used*: ALDH5F1, aldehyde dehydrogenase 5F1; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; HL, high light; HS, heat stress; HL+HS, a combination of high light and heat stress; POP2, pollen-pistil incompatibility 2.

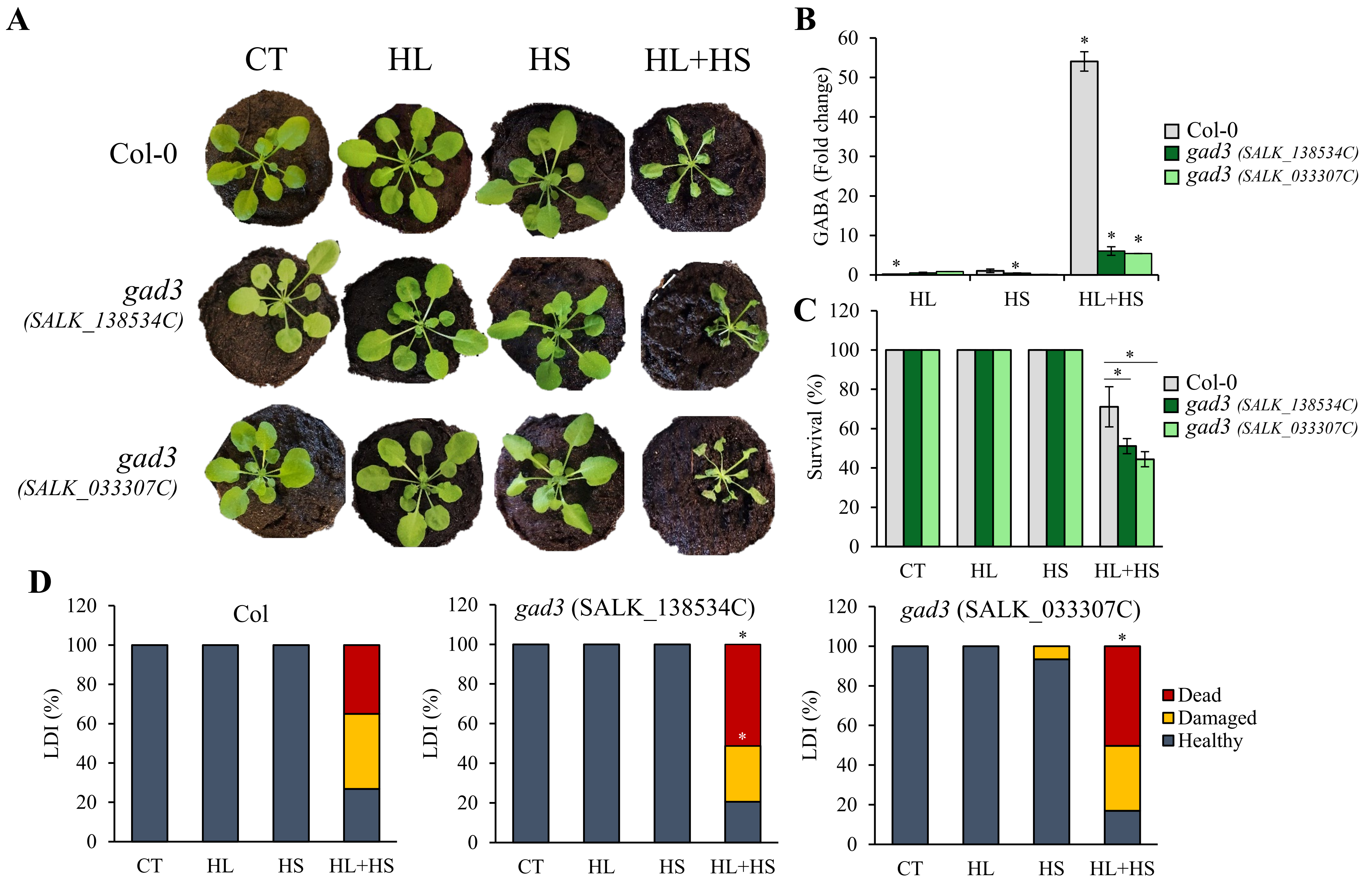


Fig. 5. Involvement of GABA in the response of Arabidopsis plants to high light, heat stress and their combination. (A) Representative images of Col-0 and the GABA mutant *gad3* (two independent knockout lines; SALK_138534C and SALK_033307C) subjected to high light (HL), heat stress (HS) and a combination of HL and HS (HL+HS). (B) Levels of GABA in Col-0 and the GABA knockout mutant *gad3* (two independent lines) subjected to HL, HS and HL+HS combination. (C) Survival of Col-0 and the GABA mutant *gad3* (two independent lines) subjected to HL, HS and HL+HS combination. (D) Leaf Damage Index (LDI) of Col-0 and the GABA mutant *gad3* (two independent lines) subjected to HL, HS and HL+HS combination. Asterisks denote Student's t-test significance at $p < 0.05$ compared to wild type (C) or to control (B and D). Error bars represent SD. *Abbreviations used*: CT, control; GAD, glutamate decarboxylase; HL, high light; HS, heat stress; HL+HS, a combination of high light and heat stress; LDI, Leaf Damage Index.