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Research Article

# γ-Aminobutyric acid plays a key role in plant acclimation to a combination of high light and heat stress

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## Abstract

Plants are frequently subjected to different combinations of abiotic stresses, such as high light (HL) intensity, and elevated temperatures. These environmental conditions pose a threat to agriculture production, affecting photosynthesis, and decreasing yield. Metabolic responses of plants, such as alterations in carbohydrates and amino acid fluxes, play a key role in the successful acclimation of plants to different abiotic stresses, directing resources toward stress responses, and suppressing growth. Here we show that the primary metabolic response of Arabidopsis (*Arabidopsis thaliana*) plants to HL or heat stress (HS) is different from that of plants subjected to a combination of HL and HS (HL + HS). We further demonstrate that the combined stress results in a unique metabolic response that includes increased accumulation of sugars and amino acids coupled with decreased levels of metabolites participating in the tricarboxylic acid cycle. Among the amino acids exclusively accumulated during HL + HS, we identified the nonproteinogenic amino acid γ-aminobutyric acid (GABA). Analysis of different mutants deficient in GABA biosynthesis (GLUTAMATE DESCARBOXYLASE 3 [*gad3*]) as well as mutants impaired in autophagy (autophagy-related proteins 5 and 9 [*atg5* and *atg9*]), revealed that GABA plays a key role in the acclimation of plants to HL + HS, potentially by promoting autophagy. Taken together, our findings identify a role for GABA in regulating plant responses to combined stress.

## Introduction

Plants growing under natural conditions are subjected to different abiotic and biotic stresses that impact plant growth and development. Among these, light intensities that exceed the plant photosynthetic capacity often occur in native

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habitats (high light [HL] stress; Ort, 2001; Roeber et al., 2021). Because light plays a key role in the life of photosynthetic organisms, plants evolved many different acclimation and adaptation mechanisms to counteract the effect of HL stress, including paraheliotropic movements, pathways for adjusting the size of the antenna complexes, quenching mechanisms, and pathways to scavenge excess reactive oxygen species (ROS; Asada, 2006; Li et al., 2009; Dietz, 2015). The excess excitation energy produced at the antennas of the photosynthetic apparatus during HL stress is potentially dangerous and could lead to irreversible damage to the reaction centers. Consequently, a sustained decrease in efficiency and electron transport rates could occur, leading to photoinhibition (Ruban, 2015). In addition to HL stress, heat stress (HS) can also compromise photosystem II (PSII) electron transport due to the increase in fluidity of the thylakoid membranes, dislodging of PSII light-harvesting complexes, and decreasing the integrity of PSII (Mathur et al., 2014). Moreover, because $CO_2$ fixation is dependent on stomatal regulation and temperature, HL stress may have a higher negative impact on plants when combined with other stresses that already limit the rates of $CO_2$ fixation (Mittler, 2006; Roeber et al., 2021). It was recently reported that a combination of HL and HS (HL + HS) caused unique transcriptomic, physiological, and hormonal responses in *Arabidopsis thaliana* plants (Balfagón et al., 2019). In addition, this abiotic stress combination was found to have a severe impact on PSII performance and to decrease the ability of plants to repair PSII (Balfagón et al., 2019). Lipophilic antioxidant molecules were previously shown to contribute to the protection of PSII against photodamage and enhance tolerance of tomato (*Solanum lycopersicum*) plants to HL + HS (Spicher et al., 2017). In sunflower (*Helianthus annuus*), changes in the steady-state level of transcripts associated with energy metabolism were found in response to this stress combination (Hewezi et al., 2008). The specific physiological and molecular responses observed in different plant species in response to HL + HS (Hewezi et al., 2008; Spicher et al., 2017; Balfagón et al., 2019) could, in turn, lead to changes in plant metabolism that would minimize stress-induced damages (Balfagón et al., 2020).

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

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

To identify promising metabolic markers for HL + HS in plants, we studied the effects of this stress combination on the levels of different metabolites in Arabidopsis plants. Our findings indicate that the primary metabolic response of Arabidopsis plants to HL + HS is different from that of plants subjected to HL or HS. We further identified γ-aminobutyric acid (GABA) as a metabolite that specifically accumulates in plants during HL + HS. The use of different mutants deficient in GABA biosynthesis revealed a potential role for GABA in regulating the acclimation of plants to this stress combination. In addition, because it was suggested that GABA regulates autophagy (Signorelli et al., 2019; Li et al., 2020; Wang et al., 2021), we used mutants deficient in autophagy to show a role for this important process in plant acclimation to HL + HS. Taken together, our findings suggest that GABA, together with autophagy, play a key role in plant responses to combined stress.

## Results

### Physiological responses of Arabidopsis plants to HL, HS, and HL + HS

To study the physiological responses of Arabidopsis plants to HL, HS, and HL + HS, we subjected wild-type (Columbia-0 [Col-0]) plants to HL intensity (600 µmol $m^{-2}$ $s^{-1}$), high temperature (42°C), or HL + HS (600 µmol $m^{-2}$ $s^{-1}$ and 42°C) for 7 h. Control (CT) plants were maintained at 50 µmol $m^{-2}$ $s^{-1}$ and 23°C during the entire experimental period (Supplemental Figure S1; Balfagón et al., 2019). Gas exchange parameters, including leaf photosynthetic rate (A), transpiration (E), and stomatal conductance (gs), were determined in Arabidopsis plants subjected to HL, HS, and HL + HS (Figure 1). Photosynthesis, E, and gs significantly decreased following the application of HL compared to CT values. These results are in agreement with previous reports showing stomata to close during light stress (Devireddy et al., 2018; Balfagón et al., 2019), limiting E and negatively affecting photosynthetic rates. In contrast, the application of HS increased E and gs, maintaining stomata open to cool down the leaf surface via E (in agreement with stomatal aperture measurements; Balfagón et al., 2019). However, HS did not affect photosynthesis compared to CT. Interestingly, HL + HS induced a significant decrease in photosynthesis compared to CT, whereas E and gs dramatically increased by about eight-fold compared to CT, or four-fold compared to HS (Figure 1). Taken together, the results presented in Figure 1 suggest that during HL + HS, HS-associated increases in E and gs prevailed over those induced by HL (stomatal closure and decreased E), and that a reduction in leaf temperature (Leaf T) took precedence in plants subjected to HL + HS, over HL-induced stomatal closure that would minimize water loss (Figure 1; Balfagón et al., 2019).

### Metabolomic responses of Arabidopsis plants to HL, HS, and HL + HS

To study the accumulation of stress-associated metabolites in Arabidopsis plants subjected to HL, HS, or HL + HS, a gas chromatography–mass spectrometric analysis of polar compounds extracted from leaves of plants subjected to the different stresses was performed (Supplemental Figure S1). Principal component analysis (PCA) revealed that the main source of variation in the data was due to metabolic changes associated with the stress combination, as the first principal component, accounting for 56.6% of total variance, was defined by the characteristic profile of HL + HS samples. In turn, principal component three, explaining 11.5% of total variation, clearly separated the samples based on the metabolic profile of plants subjected to HS (Figure 2A). Analysis of variance revealed a total of 25 polar metabolites significantly altered in their levels in response to HL (21 and 4 over- and under-accumulated, respectively), 23 metabolites significantly altered their levels in response to HS (19 and 4 over- and under-accumulated, respectively), and 38 metabolites significantly altered their levels in response to HL + HS (28 and 10 over- and under-accumulated, respectively) (Figure 2B and Table 1). Moreover, of the 28 metabolites with significantly elevated levels in response to HL + HS, 3 metabolites (10.7%) were common with HL-induced metabolites, another 3 metabolites (10.7%) were common with HS-induced metabolites, and 7 metabolites (25.0%) were found to be specifically accumulated in response to HL + HS. Similarly, levels of one metabolite (10.0%) commonly decreased in response to either HL or HS, and levels of seven metabolites (70.0%) were reduced only in response to HL + HS (Figure 2B). These results indicated that a substantial portion of the polar metabolites with altered levels in plants subjected to HL + HS was specific for the stress combination. As shown in Table 1, metabolites that exclusively accumulated in plants in response to HL + HS included glycerol, succinic acid, GABA, rhamnose, arginine, gluconic acid, and tyrosine. In contrast, the levels of threonic acid, urea, fumaric acid, nicotinic acid, citric acid, pyroglutamic acid, and putrescine specifically decreased in response to HL + HS (Table 1).

### The impact of HL + HS on sugar metabolism, TCA cycle intermediates, and amino acid levels

Further analysis of metabolites involved in glycolysis, TCA cycle, and amino acid biosynthesis during stress

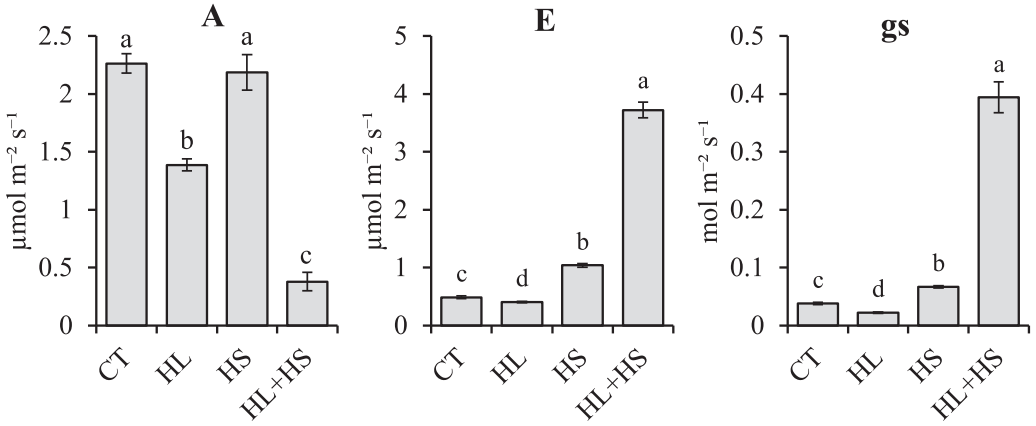


**Figure 1** Physiological measurements of Arabidopsis plants subjected to HL, HS, and HL + HS. Leaf A, E, and gs of Col-0 plants subjected to HL, HS, and HL + HS. Error bars represent standard error (SE) ($N = 9$). Different letters denote statistical significance at $P < 0.05$ (two-way ANOVA followed by a Tukey's post hoc test).

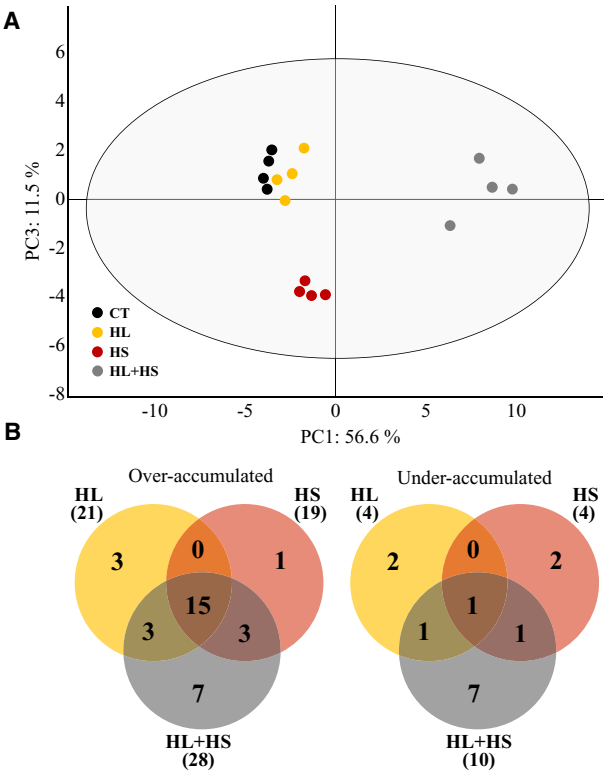


**Figure 2** Metabolic analysis of Arabidopsis Col-0 plants subjected to HL, HS, and HL + HS. A, PCA score plot of metabolite profiles obtained from CT Col-0 plants, and Col-0 plants subjected to HL, HS, or HL + HS. B, Venn diagrams showing the overlap between metabolites over-accumulated (left) or under-accumulated (right) in response to HL, HS, and HL + HS.

combination, was conducted (Figure 3). The soluble sugars glucose and fructose, as well as raffinose and maltose strongly accumulated in response to HL + HS whereas their accumulation, in general, was less pronounced in response to HL or HS. In addition, trehalose and erythritol accumulated in response to the different treatments and particularly during HL + HS. In contrast, sucrose, the major form of carbohydrates transported from photosynthetically active tissues, slightly increased in its level in response to the individual and combined stresses (Figure 3A and Table 1; Supplemental Table S1). Analysis of TCA cycle intermediates revealed that HL + HS perturbed the TCA cycle and reduced the levels of the TCA cycle-derived amino acids aspartate and glutamate. Aromatic amino acids are synthesized in plants through the shikimate pathway. In our study, the levels of tryptophan and phenylalanine significantly increased during HL + HS, whereas individual stresses had a marginal effect on their accumulation. In addition, tyrosine was also accumulated under HL + HS, while no change in its levels was found under HL or HS. Amino acids synthesized from pyruvate including alanine, leucine, valine, and isoleucine significantly accumulated under all stress conditions, although more noticeably under HL + HS conditions (Figure 3A and Table 1; Supplemental Table S1). The reduction in aspartate levels under HL + HS was accompanied by accumulation of asparagine, methionine, threonine, and especially lysine, whose accumulation was especially high in response to HL + HS (Figure 3A and Table 1; Supplemental Table S1). Analysis of the expression of genes encoding for enzymes that participate in different reactions of the TCA cycle revealed different patterns of transcript accumulation among the individual and combined stresses (Figure 3B; Supplemental Table S2). In general, although TCA-related metabolites were reduced in response to HL + HS, expression of transcripts encoding TCA cycle-related enzymes increased in response to the stress combination (Figure 3B; Supplemental Table S2), possibly as a response to counteract the low metabolite accumulation.

## Impact of HL + HS on glutamate metabolism

Glutamate has a central role in amino acid metabolism in plants, and is also a substrate for the synthesis of arginine, ornithine, proline, glutamine, and GABA (Forde and Lea, 2007). As shown in Figure 4A and Supplemental Table S3, the observed decline in glutamate levels in response to HL + HS was associated with proline, glutamine, and GABA accumulation. In contrast, levels of arginine and urea, as well as levels of the polyamine putrescine, decreased or did not change in response to the application of stress (Figure 4A; Supplemental Table S3). It was reported that under abiotic stress conditions, oxidation of putrescine contributes to GABA production (Shelp et al., 2012), suggesting that the specific decrease in putrescine under HL + HS conditions could lead to GABA accumulation in response to this stress combination. Indeed, as shown in Table 1 and Figure 4A, and Supplemental Table S3, GABA accumulated exclusively in response to HL + HS. To further dissect GABA metabolism in plants in response to HL + HS, we analyzed the expression of transcripts involved in GABA biosynthesis (GLUTAMATE DESCARBOXYLASE 1, 2, 3, and 4; *GAD1*, *GAD2*, *GAD3*, and *GAD4*) as well as the expression of transcripts related to GABA catabolism (POLLEN-PISTIL INCOMPATIBILITY 2 [*POP2*], and ALDEHYDE DEHYDROGENASE 5F1 [*ALDH5F1*]; using RNA-Seq data obtain by Balfagón et al., 2019). As shown in Figure 4B and Supplemental Table S4, the expression of *GAD1* and especially *GAD3* specifically increased 4.64- and 29.03-fold, respectively, in response to HL + HS compared to CT, suggesting that GAD3 could be the main enzyme contributing to GABA accumulation under this stress combination. A positive association could therefore be found between the expression level of several TCA cycle transcripts (Figure 3B) and transcripts encoding regulators of the GABA shunt (e.g. *GAD1* and *GAD3*; Figure 4B; Fait et al., 2008). In contrast, expression of *GAD2* was repressed in response to HS and transcript accumulation of *GAD4* slightly increased in plants subjected to the individual HL or HS treatments. The expression of *POP2* decreased in response to HL and HL + HS and all stresses reduced the expression of *ALDH5F1* (Figure 4B; Supplemental Table S4). The findings presented in Figure 4 and Supplemental Table S4 suggest, therefore, a possible role for GABA in regulating plant responses to HL + HS.

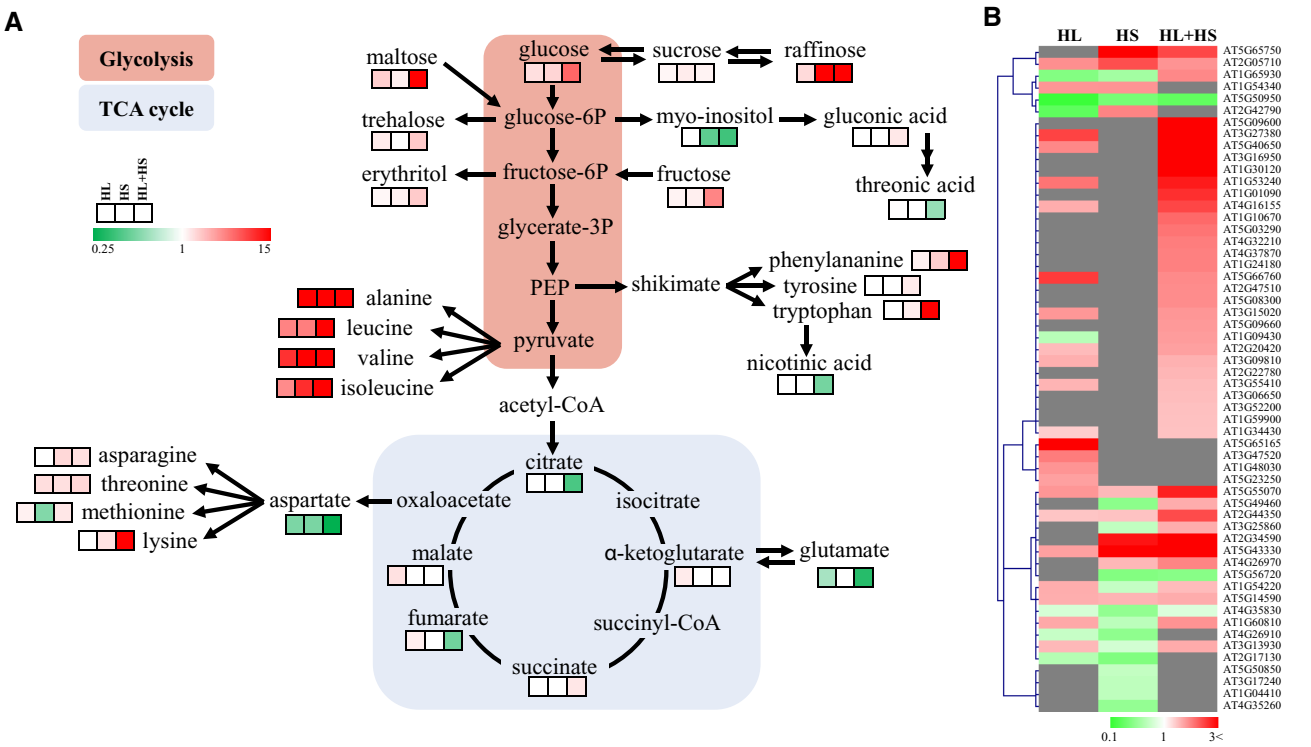


**Figure 3** Levels of amino acids and metabolites involved in glycolysis and TCA cycle in Arabidopsis plants subjected to HL, HS, and HL + HS. A, Levels of metabolites participating in glycolysis, TCA cycle, and amino acid metabolism in Col-0 plants subjected to HL, HS, or HL + HS. Significant metabolite levels ($P < 0.05$; Student's $t$ test) are expressed as fold change compared to CT conditions and are shown as a color scale (Supplemental Table S1). Nonsignificant accumulation compared to CT 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. Significant transcript levels ($P < 0.05$; negative binomial Wald's test followed by Benjamini–Hochberg correction) are expressed as fold change compared to CT conditions and are shown as a color scale. Nonsignificant expression levels ($P < 0.05$; negative binomial Wald's test followed by Benjamini–Hochberg correction) compared to CT are shown in gray. Transcript expression data were obtained from the RNA-Seq analysis conducted by (Balfagón et al. 2019; Supplemental Table S2). PEP, phosphoenolpyruvate.

## Involvement of GABA in plant tolerance to the HL + HS

To further study the role of GABA in the response of plants to HL + HS, we studied the response of two independent lines of the GABA-deficient mutant *gad3* (SALK_138534C and SALK_033307C) (Figure 5A), as well as a *gad1-5* mutant (Supplemental Figure S2) to HL, HS, and HL + HS. Accumulation of GABA was repressed or did not change in *gad3* mutants subjected to HL or HS, as well as in wild-type plants in response to HL. In contrast, HL + HS induced a pronounced increase in GABA levels in Col-0 plants, whereas the two *gad3* mutants slightly accumulated GABA, probably due to the action of *GAD1* (Figures 4, B and 5, B). The reduced accumulation of GABA in *gad3* plants in response to HL + HS compared to Col-0 (Figure 5B) was accompanied by a significant decrease in the survival of *gad3* mutants subjected to HL + HS (Figure 5, A and C). Whereas all *gad3* plants survived the individual HL or HS, the survival rate of the two *gad3* mutants under conditions of HL + HS decreased by about 40%. In contrast, the *gad1-5* mutant did not show significant differences (Student's t test, $P < 0.05$) in survival rate when subjected to the individual or combined stresses compared to Col-0 plants (Supplemental Figure S2). Furthermore, analysis of Leaf Damage Index (LDI; Balfagón et al., 2019) of Col-0 and *gad3* mutants subjected to the different stresses (Figure 5D) revealed that HL + HS negatively impacted leaf appearance of both *gad3* lines, with 51.2% and 50.3% of leaves dead, 28.2% and 32.8% of leaves injured, and only 20.6% and 16.9% of leaves appearing healthy, in SALK_138534C and SALK_033307C, respectively. In contrast, Col-0 plants showed 34.9%, 38.1%, and 26.8% of dead, damaged, and healthy leaves, respectively (Figure 5D). Compared to Col-0 plants, *gad3* mutants were, therefore, more susceptible to the HL + HS. Physiological characterization of the *gad3* mutants subjected to HL, HS, and HL + HS (Figure 5E) further revealed that photosynthesis, E, and gs were significantly lower in the *gad3* mutants compared to Col-0, following the application of HS and HL + HS. The decreased gs and E of the *gad3* mutants under these conditions led to an increase in Leaf T, compared to Col-0 plants (Figure 5E). These results suggest that GABA depletion in the *gad3* mutants resulted in altered stomatal responses that in turn caused higher Leaf T and reduced photosynthesis, potentially impairing the ability of *gad3* mutants to acclimate to the stress combination.

**Table 1** List of metabolites over- and under-accumulated in Col-0 plants subjected to HL, HS, and HL + HS

| | 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 and 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 and 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 and HS and 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** | – | – | – | – |

Values represent fold changes compared to CT. 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 Supplemental Tables S1 and S3).

## Involvement of autophagy in plant acclimation to HL + HS

In addition to its potential role in regulating stomatal responses (Mekonnen et al., 2016; Xu et al., 2021), GABA was recently proposed to regulate autophagy in response to environmental stress in plants (Signorelli et al., 2019; Li et al., 2020; Wang et al., 2021). To determine whether autophagy is essential for plant tolerance to HL + HS, we subjected two autophagy-related mutants, *autophagy-related protein* 5 and 9 (*atg5* and *atg9*; Le Bars et al., 2014; Zhuang et al., 2017), to HL, HS, and HL + HS (Figure 6A). In contrast to Col-0, *atg5* and *atg9* plants displayed a reduced survival rate under conditions of HL + HS (Figure 6B). In addition, LDI analysis of Col-0 and *atg5* and *atg9* plants subjected to the different stresses indicated that HL + HS had a significantly higher negative impact on leaf appearance of both *atg5* and *atg9* mutants compared to Col-0 (Figure 6C). In contrast to Col-0 (with 35.0% of leaves dead), 55.9% and 52.3% of leaves were dead in *atg5* and *atg9* mutants, respectively, following HL + HS treatment. In addition, only 17.9% and 16.6% of leaves appeared healthy in *atg5* and *atg9* plants, respectively, whereas Col-0 plants showed 35.0% of leaves healthy under this stress combination (Figure 6C). Similar to the *gad3* mutants (Figure 6), both autophagy-related mutants (*atg5* and *atg9*) were therefore more susceptible to HL + HS compared to Col-0 plants, suggesting that GABA and autophagy contribute to HL + HS tolerance. Interestingly, similar to the *gad3* lines (Figure 6), photosynthesis, E, and gs of the *atg5* and *atg9* mutants were also significantly lower compared to Col-0 in response to HS and HL + HS, leading to higher Leaf Ts under these conditions (Figure 6D). These findings suggest that the two autophagy mutants were also impaired in

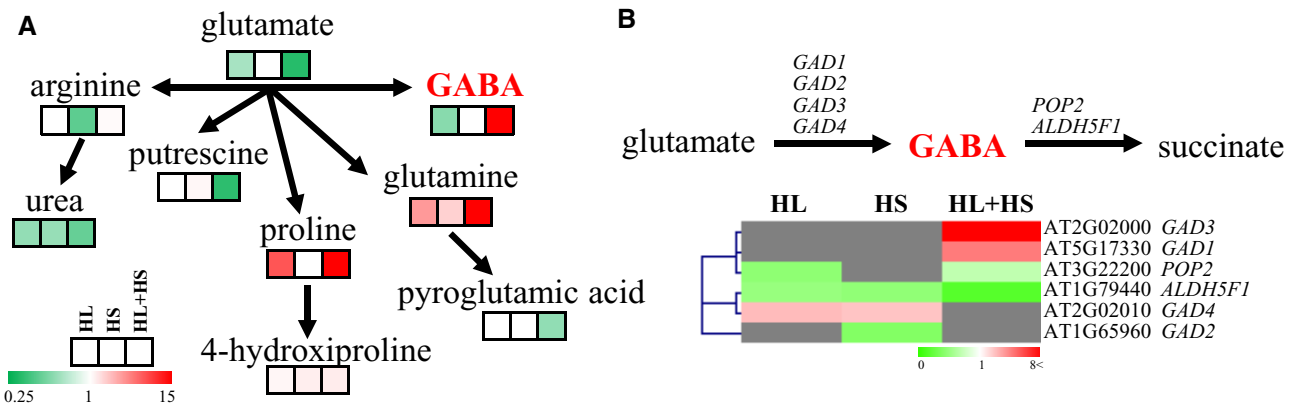


**Figure 4** Glutamate metabolism in Arabidopsis plants subjected to HL, HS, and HL + HS. A, Level of metabolites involved in glutamate metabolism in Col-0 plants subjected to HL, HS, or HL + HS. Significant metabolite levels ($P < 0.05$; Student's $t$ test) are expressed as fold change compared to CT conditions and are shown as a color scale (Supplemental Table S3). Nonsignificant accumulation compared to CTs is shown in white. B, Heat map showing the expression levels of transcripts involved in GABA metabolism in Col-0 plants subjected to HL, HS, and HL + HS. Nonsignificant expression levels ($P < 0.05$; negative binomial Wald's test followed by Benjamini–Hochberg correction) compared to CTs are shown in gray. Transcript expression data were obtained from the RNA-Seq analysis conducted by (Balfagón et al. 2019; Supplemental Table S4).

their stomatal responses under conditions of HL + HS. A role for autophagy was previously proposed in regulating stomatal and abscisic acid responses (Yamauchi et al., 2019; Tarnowski et al., 2020).

## Discussion

The ability of plants to sense and react to adverse conditions in their environment is crucial for plant adaptation and survival during stress. Due to the frequent occurrences of HL + HS in nature, and its impact on crops (Yamamoto et al., 2008; Suzuki et al., 2014; Roeber et al., 2021) and plant survival (Balfagón et al., 2019), the study of metabolic changes during this stress combination is of particular interest. A recent study of the biochemical and transcriptomic responses of Arabidopsis plants to HL, HS, and HL + HS revealed that the HL + HS was accompanied by irreversible damage to PSII, decreased D1 (PsbA) protein levels, enhanced accumulation of the hormones jasmonic acid (JA), and JA-isoleucine, elevated expression of over 2,200 different transcripts unique to the stress combination, distinctive structural changes to chloroplasts and a decreased survival rate (Balfagón et al., 2019). In this study, we show that HL + HS has a detrimental effect on A, and that during HL + HS the effects of HS on stomatal responses and E (opening of stomata and increasing E) prevails over the effects of HL (closing of stomata and decreasing E; Figure 1). Both E and gs markedly increased under HL + HS (Figure 1), suggesting that it may take higher E rates to cool a leaf during HL + HS, potentially a result of heat generated due to dissipation of excess light energy by nonphotochemical quenching and/or other protective processes (e.g. Czarnocka and Karpiński, 2018; Murchie and Ruban, 2020). This result is different from the response of plants to a combination of drought and HS, in which the effects of drought prevailed over the effects of heat on stomatal regulation (Rizhsky et al., 2002, 2004). The observed decrease in A under conditions of HL + HS (Figure 1) prompted us to analyze the primary metabolism of plants subjected to this stress combination to unravel specific patterns of sugar, amino acid and polyamine accumulation (Figures 2–4 and Table 1; Supplemental Tables S1–S4). Individual and combined HL and HS displayed different polar metabolite accumulation patterns (Figure 2 and Table 1), suggesting that different stress conditions and their combination alter plant primary metabolism in different ways. This reinforces the idea that metabolic changes due to stress combination are unique and not a mere additive response to the effects of each individual stress.

In our study, the levels of several metabolites were associated to plant sensitivity to HL + HS. Plants subjected to this stress combination accumulated sugars such as glucose, fructose, raffinose, maltose, and trehalose, whereas the levels of sucrose slightly increased in response to individual and combined stresses (Figure 3 and Table 1; Supplemental Table S1). The source of sugars in plants subjected to HL + HS is unknown. Considering that photosynthesis is suppressed in plants subjected to this stress combination (Figure 1), sugars could be synthesized by way of starch degradation, as proposed to occur during a combination of drought and HS (Rizhsky et al., 2004). Indeed, high accumulation of maltose, a major sugar associated with starch degradation (Thalmann and Santelia, 2017), and of its derived sugars were observed in HL + HS-stressed plants (Figure 3 and Table 1; Supplemental Table S1). Additional studies are, however, required to examine this possibility. The increased accumulation of sugars participating in glycolysis under HL + HS (Figure 3; Supplemental Table S1) suggests that this pathway could provide an alternative source of ATP in plants subjected to HL + HS, to counteract the negative effects of the stress combination on PSII and A (Figure 1; Balfagón et al., 2019), as well as to function as compatible solutes (Krasensky and Jonak, 2012; Shaar-Moshe et al., 2019). Moreover, the levels of glycolysis-derived aromatic amino

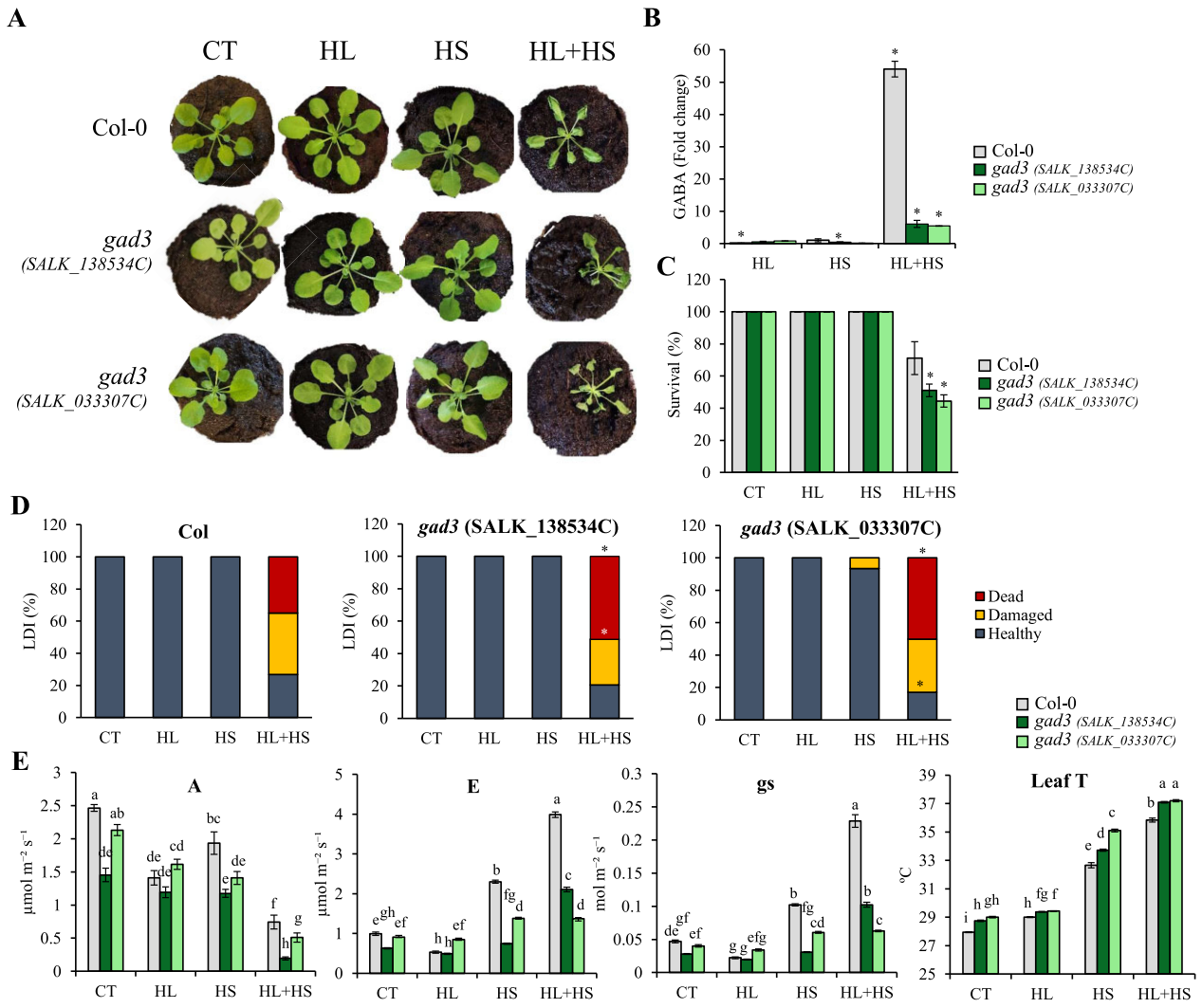


**Figure 5** Involvement of GABA in the response of Arabidopsis plants to HL, HS, and HL + HS. A, Representative images of Col-0 and the GABA mutant *gad3* (two independent knockout lines; SALK_138534C and SALK_033307C) subjected to HL, HS, and HL + HS. Images were digitally extracted for comparison. B, Levels of GABA in Col-0 and the GABA knockout mutant *gad3* (two independent lines) subjected to HL, HS, and HL + HS. C, Survival of Col-0 and the GABA mutant *gad3* (two independent lines) subjected to HL, HS, and HL + HS. D, LDI of Col-0 and the GABA mutant *gad3* (two independent lines) subjected to HL, HS, and HL + HS. E, Measurements of leaf A, E, gs and Leaf T of Col-0 and the GABA mutant *gad3* (two independent lines) subjected to HL, HS, and HL + HS. Different letters denote statistical significance at $P < 0.05$ (two-way ANOVA followed by a Tukey's post hoc test). Asterisks denote Student's *t* test significance at $P < 0.05$ compared to wild-type (C) or to CT (B and D). Error bars represent SE ($N = 9$).

acids produced through the shikimate pathway, tryptophan, phenylalanine, and tyrosine, as well as amino acids synthesized from pyruvate, including alanine, leucine, valine, and isoleucine significantly increased during HL + HS (Figure 3 and Table 1; Supplemental Table S1). Although glycolysis appeared to be activated under HL + HS, a concomitant activation of the TCA cycle was not observed (Figure 3 and Table 1; Supplemental Table S1), similar to the findings of Shaar-Moshe et al. (2019), demonstrating that organic acids produced by the TCA cycle were reduced under a combination of salinity, drought, and heat. Therefore, depletion of metabolites related to the TCA cycle under stress combination could indicate that respiration might be compromised in plants subjected to HL + HS. The reduction in oxalacetate-derived aspartate levels under HL + HS conditions was accompanied by an increase in aspartate-related amino acids, especially lysine (Figure 3 and Table 1; Supplemental Table S1). Increased accumulation of amino acids was shown in plants subjected to different abiotic stresses (e.g. Kaplan et al., 2004; Rizhsky et al., 2004; Kempa et al., 2008; Sanchez et al., 2008; Usadel et al., 2008; Lugan et al., 2010; Krasensky and Jonak, 2012), and could be a result of amino acid biosynthesis and/or enhanced stress-induced protein degradation. In this respect, the higher impact of HL + HS on plant physiology and survival (Figure 1; Balfagón et al., 2019) could lead to an increase in protein degradation and therefore, higher amino acid content. Further studies elucidating this possibility are needed. The

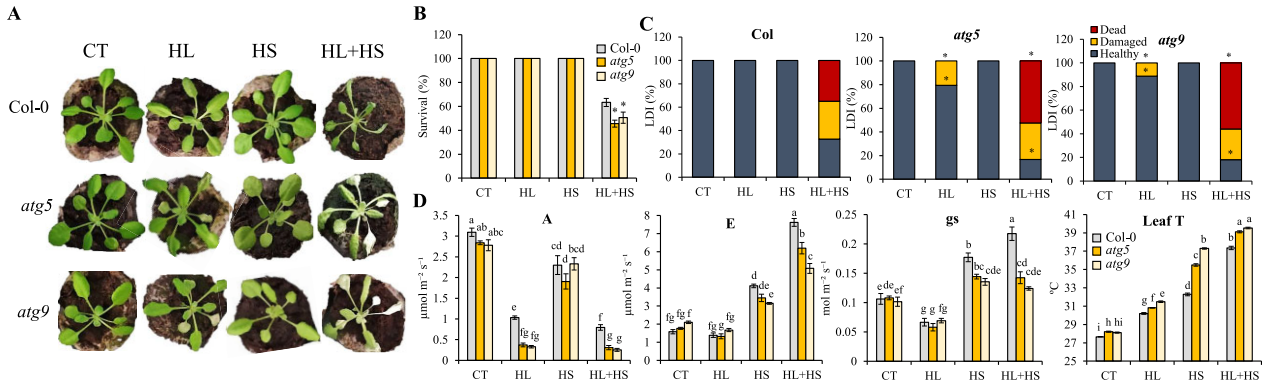


**Figure 6** Involvement of autophagy in the response of Arabidopsis plants to HL, HS, and HL + HS. A, Representative images of Col-0 and the autophagy mutants *atg5* and *atg9* subjected to HL, HS, and HL + HS. Images were digitally extracted for comparison. B, Survival of Col-0 and the autophagy mutants *atg5* and *atg9* subjected to HL, HS, and HL + HS. C, LDI of Col-0 and the autophagy mutants *atg5* and *atg9* subjected to HL, HS, and HL + HS. D, Measurements of leaf A, E, gs, and Leaf T of Col-0 and the autophagy mutants *atg5* and *atg9* subjected to HL, HS, and HL + HS. Asterisks denote Student's *t* test significance at $P < 0.05$ compared to wild-type. Different letters denote statistical significance at $P < 0.05$ (two-way ANOVA followed by a Tukey's post hoc test). Error bars represent SE ($N = 9$).

decreased levels of TCA-derived glutamate in response to HL and especially in response to HL + HS (Figures 3 and 5; Table 1; Supplemental Table S1) were further accompanied by a concomitant decrease in putrescine (Figure 4 and Table 1; Supplemental Table S3). These results indicate that the role of polyamines as osmoprotectants under HL + HS might be marginal, and that other metabolites including sugars (Figure 3) and/or proline (Figure 4) could have a key role as osmoprotective elements under this stress combination. In addition, as a compatible solute, proline is involved in the stabilization of proteins and protein complexes in the chloroplast and cytosol, protection of the photosynthetic apparatus, and enzymes involved in detoxification of ROS, as well as redox balance stabilization (Szabados and Savouré, 2010). The high accumulation of proline observed in plants subjected to HL + HS could therefore suggest that the stress combination imposes a stronger pressure on plant metabolism, as indicated by the decrease in survival rates and values of LDI (Figure 5; Balfagón et al., 2019).

Interestingly, GABA levels were specifically elevated in plants subjected to HL + HS (Figure 4A and Table 1; Supplemental Table S3), and GABA-deficient mutants (*gad3*) showed a significant decline in their ability to acclimate to this stress combination (Figure 5), suggesting that GABA could be required for plant acclimation to HL + HS. GABA is a key nonproteinogenic amino acid that displays important physiological functions involved in plant growth regulation and stress responses (Bouché and Fromm, 2004; Yu et al., 2014; Seifikalhor et al., 2019; Fromm, 2020). Exogenous GABA application to plants was reported to improve tolerance to different environmental stresses (e.g. Shi et al., 2010; Shang et al., 2011; Li et al., 2016; Salvatierra et al., 2016; Priya et al., 2019; Seifikalhor et al., 2020). Furthermore, GABA levels increased in response to different abiotic stress combinations, namely, salt and drought, as well as salt, drought, and heat (Shaar-Moshe et al., 2019). A potential function for GABA in plant survival during stress was recently linked to its role in regulating autophagy (Figure 7; Supplemental Figure S3; Signorelli et al., 2019; Li et al., 2020; Wang et al., 2021) and stomatal responses (Mekonnen et al., 2016; Xu et al., 2021). Autophagy is considered a conserved mechanism for the degradation and recycling of damaged cellular components in plants and is induced in response to different environmental stresses as well as developmental stimuli (Tang and Bassham, 2018; Avin-Wittenberg, 2019). For example, autophagy plays an important role in plant tolerance to osmotic and salt stresses (Liu et al., 2009), submergence (Chen et al., 2015), and HS (Zhou et al., 2014). In addition, it was reported that plants with an impaired autophagy show early senescence, reduced yield, and hypersensitivity to carbon and nitrogen starvation (reviewed in Avin-Wittenberg, 2019). Autophagy-related proteins (i.e. ATGs) function during the induction of autophagy and the formation of autophagosomes (Tang and Bassham, 2018), and, among them, ATG5 and ATG9 are key for autophagosome formation (Le Bars et al., 2014; Zhuang et al., 2017). ATG5 defines a phagophore domain connected to the endoplasmic reticulum during autophagosome formation in plants (Le Bars et al., 2014), whereas ATG9 regulates autophagosome progression from the endoplasmic reticulum (Zhuang et al., 2017). Here, we show that compared to Col-0 plants, Arabidopsis plants deficient in ATG5 or ATG9 (*atg5* or *atg9*) are more susceptible to HL + HS (Figure 6). In addition, the expression of some autophagy-related transcripts was altered in Col-0 plants in response to HL + HS (Supplemental Figure S3, A and B), and both *gad3* mutants showed a decreased expression of some autophagy markers, including TYPE 2A-PHOSPHATASE-ASSOCIATED PROTEIN 46 kDa (*TAP46*), PROTEIN PHOSPHATASE 2A-4 (*PP2A-4*), *ATG3*, *ATG6*, *ATG8B*, and *ATG8C* in response to HL + HS compared to Col-0 (Supplemental Figure S3C). Taken together with our findings that GABA-deficient mutants (*gad3*) are susceptible to the HL + HS (Figure 5) and the proposed role of GABA in

regulating autophagy (Signorelli et al., 2019; Li et al., 2020; Wang et al., 2021), these findings suggest that GABA could promote autophagy under conditions of HL + HS, contributing to plant acclimation and survival (Figure 7; Supplemental Figure S3). Because GABA and autophagy were also found to play an important role in stomatal regulation (Mekonnen et al., 2016; Xu et al., 2021), it is also possible that these two processes promote plant survival during stress combination through the regulation of stomata (Figure 7).

## Materials and methods

### Plant materials and growth conditions

Arabidopsis (*A. thaliana*) Col-0, *gad3* (SALK_138534C and SALK_033307C), *gad1-5* (CS860069), and *atg5* and *atg9* (Thompson et al., 2005; Floyd et al., 2015) plants were grown in peat pellets (Jiffy-7; http://www.jiffygroup.com/) at 23°C under long-day growth conditions (12-h light from 7 a.m. to 7 p.m.; 50 µmol $m^{-2}$ $s^{-1}$/12-h dark from 7 p.m. to 7 a.m.).

### Stress treatments

Individual HL and HS, and HL + HS were applied in parallel as described in Balfagón et al. (2019) and shown in Supplemental Figure S1, using 30-d-old Arabidopsis plants (wild-type Col-0, the SALK_138534C, and SALK_033307C *gad3* mutants, the *gad1-5* mutant and two autophagy-related mutants *atg5* and *atg9*). HL was applied by exposing plants to 600 µmol $m^{-2}$ $s^{-1}$ (Philips, F54T5/TL84/HO/ALTO) at 23°C for 7 h. HS was applied by subjecting plants to 42°C, 50 µmol $m^{-2}$ $s^{-1}$, for 7 h. HL + HS was performed by simultaneously subjecting plants to 600 µmol $m^{-2}$ $s^{-1}$ of light stress and 42°C for 7 h. CT plants were maintained at 50 µmol $m^{-2}$ $s^{-1}$, 23°C during the entire experiment. Following the stress treatments, CT plants and plants subjected to HL, HS, and HL + HS were divided into two groups: a group used for sampling leaves for metabolomics analysis as described below; and a group allowed to recover under controlled conditions until flowering time to score for survival. About 24 h following the stress treatments, LDI (Gallas and Waters, 2015; Balfagón et al., 2019) was recorded (Supplemental Figure S1). All experiments were carried out at the same time of the day during the light cycle (from 9 a.m. to 4 p.m.) and were repeated at least 3 times with 30 plants per biological repeat.

### Photosynthetic parameters

A, E, gs, and Leaf T were measured using a LI-6800 Portable Photosynthesis System (Dilus, Alcobendas, Spain) under ambient $CO_2$ and moisture conditions. Supplemental light was provided by a PAR lamp at 50 or 600 µmol $m^{-2}$ $s^{-1}$ photon flux density, and air flow was set at 150 µmol $mol^{-1}$. After instrument stabilization, at least 10 measurements were taken on three fully expanded leaves of three plants immediately after the 7 h of individual and combined stress treatments (Supplemental Figure S1). All experiments were repeated at least 3 times.

### Determination of primary metabolites

The relative levels of polar metabolites were determined as described in Zanor et al. (2009). Fifteen milligrams of freeze-dried plant tissue were extracted in 1.4 mL of methanol and 60 µL of an aqueous solution with 0.2 mg $mL^{-1}$ of ribitol, which was used as an internal standard. Extraction was performed at 70°C for 15 min in a water bath. The extract was centrifuged at 14,000 rpm for 10 min, and the supernatant was recovered and fractionated by adding chloroform and Milli-Q water. After vigorous vortexing and centrifugation at 4,000 rpm for 15 min, 50 µL of the aqueous phase were recovered and dried overnight in a speed-vac. The dry residue was subjected to a double derivatization procedure with methoxyamine hydrochloride (20 mg $mL^{-1}$ in pyridine; Sigma, Macherey-Nagel, Düren, Germany) and *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (Macherey-Nagel, Düren, Germany). Fatty acid methyl esters ($C_8$–$C_{24}$) were added and used as retention index markers. Analyses were performed on a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to a Pegasus 4D TOF mass spectrometer (LECO, St Joseph, MI, USA). Chromatography was performed with a BPX35 (30 m, 0.32 mm, and 0.25 µm) capillary column (SGE Analytical Science Pty Ltd., Melrose Park, Australia) with a 2 mL $min^{-1}$ helium flow. Oven programming conditions were as follows:

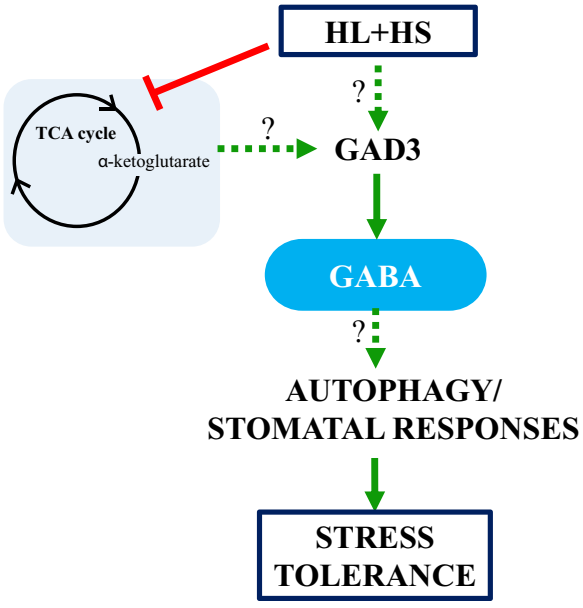


**Figure 7** A model showing the proposed role of GABA in plant acclimation to HL + HS. HL + HS is proposed to cause the inhibition of the TCA cycle and the accumulation of GABA through the function of GAD3. GABA in turn is proposed to regulate autophagy and stomatal responses promoting plant tolerance to combined stress. Green arrows indicate promotion and the red T-bar indicates inhibition. Dashed arrows and question marks indicate links to be confirmed and solid arrows indicate confirmed links.

2 min of isothermal heating at 85°C, followed by a 15°C $min^{-1}$ temperature ramp up to 360°C. Injection temperature was set at 230°C, and the ion source was adjusted to 250°C. Data were acquired after EI ionization at 70 eV, and recorded in the 70–600 *m/z* range at 20 scans $s^{-1}$. Chromatograms were analyzed by means of the ChromaTOF software. Metabolites were identified by comparison of both mass spectra and retention time with those of pure standards injected under the same conditions. Peak area of each identified compound was normalized to the internal standard area (ribitol) and sample dry weight. All experiments were repeated 4 times.

### GABA quantification

About 5 mg of freeze-dried plant tissue were transferred to a 1.5-mL microcentrifuge tube. Three glass beads and 50 µL of deuterium-labeled internal standard GABA (GABA-$d_2$) at a concentration of 20 ppm were added. Then, 300 µL of cold MeOH:$H_2O$ (80:20) was added, following sonication in an ultrasound bath with ice for 10 min and centrifugation at 10,000 rpm for 5 min. An aliquot of 250 µL of supernatant was recovered and 250 µL of acetonitrile was added, following filtration through a polytetrafluoroethylene (PTFE) 0.2-µm pore size cellulose filter. Final concentration of the deuterated standard (GABA-$d_2$) was 200 ppb. GABA was quantified in plant extracts using a ultra-performance liquid chromatography (UPLC) system (Waters Acquity SDS, Waters Corp., Milford, MA, USA) interfaced to a triple quadrupole detector (TQD) (Micromass Ltd, Manchester, UK) mass spectrometer through an orthogonal Z-spray electrospray ion source. Separations were carried out on 2.1 × 150 mm ACQUITY UPLC 1.7-m ethylene bridged hybrid (BEH) amide column using a linear gradient of (1) acetonitrile-water 95:5 (v:v), 0.1% ammonium formate (w:v) and (2) acetonitrile-water 2:98 (v:v), 0.1% ammonium formate (w:v) at a flow rate of 300 µL $min^{-1}$. Chromatographic run started at 0% B; after 1 min a linear gradient increased A to 75% for 3 min; finally, mobile phase composition returned to the initial conditions for 2 min. Transitions for GABA (104 > 87) and GABA-$d_2$ (106 > 89), were monitored in positive ionization mode. GABA was identified by comparing both mass spectra and retention time with those of pure standards injected in the same conditions. Peak area of GABA was normalized to internal standard area (GABA-$d_2$) and sample dry weight. All experiments were repeated at least 3 times.

### RT-qPCR analysis

Relative expression analysis by reverse transcription-quantitative PCR (RT-qPCR) was performed according to Balfagón et al. (2019) by using a StepOne Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) and gene-specific primers (Supplemental Table S5).

### Statistical analysis

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

## Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *GAD3* (AT2G02000), *GAD1* (AT5G17330), *GAD2* (AT1G65960), *GAD4* (AT2G02010), *POP2* (AT3G22200), *ALDH5F1* (AT1G79440), *ATG3* (AT5G61500), *ATG5* (AT5G17290), *ATG6* (AT3G61710), *ATG8B* (AT4G04620), *ATG8C* (AT1G62040), *ATG9* (AT2G31260), *PP2A-4* (AT3G58500), and *TAP46* (AT5G53000).

## Supplemental data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** The experimental design used for the metabolomic study of

HL (yellow), HS (orange), and HL + HS (gray) using Arabidopsis plants.

**Supplemental Figure S2.** Survival of Col-0 and the GABA mutant *gad1-5* subjected to HL, HS, and HL + HS.

**Supplemental Figure S3.** Enrichment of autophagy-related transcripts in the response of Arabidopsis plants to HL + HS.

**Supplemental Table S1.** Levels of metabolites involved in glycolysis, TCA cycle, and amino acid metabolism in Col-0 plants subjected to HL, HS, and HL + HS.

**Supplemental Table S2.** Expression level of transcripts involved in TCA cycle in Col-0 plants subjected to HL, HS, and HL + HS.

**Supplemental Table S3.** Level of metabolites involved in glutamate metabolism in Col-0 plants subjected to HL, HS, and HL + HS.

**Supplemental Table S4.** Expression level of transcripts involved in GABA metabolism in Col-0 plants subjected to HL, HS, and HL + HS.

**Supplemental Table S5.** Transcript-specific primers used for relative expression analysis by RT-qPCR.

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