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Abscisic acid promotes plant acclimation to the combination of salinity and high light stress

Clara Segarra-Medina [a], Saleh Alseekh [b,c], Alisdair R Fernie [b,c], José L. Rambla [a], Rosa M. Pérez-Clemente [a], Aurelio Gómez-Cádenas [a,**], Sara I Zandalinas [a,*]

[a] Department of Biology, Biochemistry and Natural Sciences, Universitat Jaume I, 12071, Castellón, Spain
[b] Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476, Potsdam, Germany
[c] Center of Plant Systems Biology and Biotechnology, 4000, Plovdiv, Bulgaria

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ABSTRACT

Plants encounter combinations of different abiotic stresses such as salinity (S) and high light (HL). These environmental conditions have a detrimental effect on plant growth and development, posing a threat to agricultural production. Metabolic changes play a crucial role in enabling plants to adapt to fluctuations in their environment. Furthermore, hormones such as abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) have been previously identified as regulators of plant responses to different abiotic stresses. Here we studied the response of Arabidopsis wild type (Col and Ler) plants and mutants impaired in hormone biosynthesis (*aba2-11* and *aba1-1* in ABA, *aos* in JA and *sid2* in SA) to the combination of S and HL (S + HL). Our findings showed that *aba2-11* plants displayed reduced growth, impaired photosystem II (PSII) function, increased leaf damage, and decreased survival compared to Col when subjected to stress combination. However, *aos* and *sid2* mutants did not display significant changes in response to S + HL compared to Col, indicating a key role for ABA in promoting plant tolerance to S + HL and suggesting a marginal role for JA and SA in this process. In addition, we revealed differences in the metabolic response of plants to S + HL compared to S or HL. The analysis of altered metabolic pathways under S + HL suggested that the accumulation of flavonoids is ABA-dependent, whereas the accumulation of branched-chain amino acids (BCAAs) and proline is ABA-independent. Therefore, our study uncovered a key function for ABA in regulating the accumulation of different flavonoids in plants during S + HL.

1. Introduction

Climate change is a global phenomenon characterized by long-term shifts in temperature patterns, precipitation levels, and extreme climate events. These changes have significant effects on several ecosystems and species, including plant and crop species (Pascual et al., 2022). Plants are highly vulnerable to the impact of climate change-associated abiotic stresses, decreasing plant growth, development, and productivity. Among some of the most impactful environmental constraints affecting plants, salinity is considered a growing concern in agricultural systems due to factors such as irrigation with brackish water, coastal intrusion, and improper soil management practices. Salt stress was additionally shown to disrupt plant water relations and osmotic balance, leading to cellular damage and impaired metabolic activities (Dinneny, 2015). Furthermore, salt stress may trigger an increased production of reactive oxygen species (ROS) that can potentially cause oxidative damage to lipids, proteins, and DNA, further compromising plant health and function. The imbalance in ion homeostasis caused by salt stress was also demonstrated to disrupt essential nutrient uptake and transport, particularly potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}), further impairing plant growth and development (Munns, 2005; Obata and Fernie, 2012). Consequently, plants readjust their metabolism to cope with the osmolarity and ionic disorders caused by salinity (Gong et al., 2005; Obata and Fernie, 2012). These metabolic changes include the accumulation of compatible solutes including sugars, alcohols, and amino acids such as proline, to maintain osmotic balance and protect

Abbreviations: ABA, abscisic acid; BCAAs, branched-chain amino acids; CT, control; HL, high light; JA, jasmonic acid; PSII, photosystem II; S, salinity; SA, salicylic acid; S + HL, the combination of salinity and high light; TCA, tricarboxylic acid.

* Corresponding author.
** Corresponding author.
E-mail addresses: aurelio.gomez@uji.es (A. Gómez-Cádenas), sizquier@uji.es (S.I. Zandalinas).

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cellular structures (Batista-Silva et al., 2019; Munns and Gilliham, 2015; Munns and Tester, 2008). Additionally, plants activate antioxidant defense systems to scavenge ROS and minimize oxidative damage (Bose et al., 2014). In addition to salt stress, plants exposed to direct sunlight experience light intensities that surpass their photosynthetic capacity (Ort, 2001). It was previously shown that under conditions of high-light stress, the reaction centers in chloroplasts are saturated, causing the excessive excitation energy to become detrimental as it can inflict irreversible damage on photosystem II (PSII). This harmful situation results in photoinhibition, a prolonged reduction in photosynthetic efficiency caused by the imbalance between the rate of photodamage to PSII and the rate of PSII repair (Balfagón et al., 2019a; Murata et al., 2007; Ruban, 2015).

In addition to activating specific physiological and molecular responses to stress, plants undergo adjustments in multiple metabolic pathways to alleviate the impact of stress on their growth and development. These adaptations enable them to cope with the increased energy requirements imposed by changing environmental constraints (Dusenge et al., 2019; Fernie et al., 2020). Among the metabolites produced by plants in response to different developmental or environmental stimuli, primary metabolites such as carbohydrates, amino acids, polyols or polyamines, are known to be directly required for plant growth, whereas specialized metabolites such as phenolics, terpenes, and nitrogen-containing compounds are involved in regulating plant–environment interactions (Zandalinas et al., 2022).

In addition to primary and specialized metabolites, hormones play an important role in regulating plant growth and development as well as plant responses to fluctuating environmental conditions. Among them, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) are essential for plants to respond to abiotic stresses and different combinations of them (Fang et al., 2019). For example, previous studies have demonstrated the important role of ABA in mitigating the negative effects of the combination of salinity and heat stress (Suzuki et al., 2016) or drought and heat stress in Arabidopsis plants (Zandalinas et al., 2016). In turn, JA was shown to be required for plant tolerance to the combination of high light and heat stress (Balfagón et al., 2019a).

The combination of different abiotic stresses was previously described as a novel and distinct challenge for plants with respect to single stresses (Zandalinas and Mittler, 2022). Despite substantial research efforts dedicated to investigating plant responses to combinations of two or three different stresses in the last decade, a comprehensive analysis of the metabolic adjustments that plants undergo when facing multiple stressors at the same time is vital for enhancing stress tolerance in different crops. Therefore, the aim of this work was to investigate the influence of different phytohormones, namely ABA, JA and SA, in plant tolerance to the combination of salinity and high light stress (S + HL), and to dissect metabolic responses in Arabidopsis plants subjected to this stress combination. To identify promising metabolic markers associated with plant tolerance to the combined effect of salinity and high light, we examined the effects of S + HL on the levels of different metabolites in both Arabidopsis wild type and ABA-deficient plants. Our findings reveal that the metabolic response of Arabidopsis plants to the combination of salinity and high light is different from that of plants subjected to each individual stress. We further identify ABA as a hormone that specifically accumulates in plants in response to S + HL. Furthermore, by using the ABA-deficient mutant *aba2-11*, we propose a potential role for ABA in regulating the acclimation of plants to this stress combination, potentially by activating different metabolic pathways including the biosynthesis of flavonoids.

2. Methods

2.1. Plant material, growth conditions and stress treatments

Arabidopsis thaliana Columbia-0 (Col) and Landsberg *erecta* (Ler) plants, along with mutants deficient in hormone biosynthesis (JA, *aos*, Park et al., 2002; SA, *sid2*, Nawrath and Métraux, 1999; and ABA, *aba2-11*, González-Guzmán et al., 2002, and *aba1-1*, Koornneef et al., 1982) were grown in peat pellets (Jiffy7; http://www.jiffygroup.com/) under long-day growth conditions, with temperatures maintained at 23 °C during the light period and 18 °C during the dark period. The growth conditions included a 16-h light cycle (from 7 a.m. to 11 p.m.) with a light intensity of 75 μmol m^{-2} s^{-1}, followed by an 8-h dark cycle (from 11 p.m. to 7 a.m.). Fifteen-day-old plants from each genotype were subjected to the different individual and combined stress treatments in parallel: long-term salinity (S), short-term high light (HL), and a combination of long-term salinity and short-term high light (S + HL) (refer to Supplementary Fig. S1A for details). The salinity treatment involved watering 15-day-old plants with a solution containing 150 mM NaCl for ten days. For the high light treatment, 25-day-old plants were exposed to a light intensity of 650 μmol m^{-2} s^{-1} for 7 h (from 8 a.m. to 3 p.m.) at the end of the experimental period. To apply the stress combination, plants were simultaneously watered with a solution containing 150 mM NaCl for ten days, and then exposed to 650 μmol m^{-2} s^{-1} for 7 h (from 8 a.m. to 3 p.m.) at the end of the salinity treatment, creating conditions that mimic the long-term growth of plants in harsh soil conditions and then subjecting them to a sudden and intense, short-term environmental stress (high light; Supplementary Fig. S1A). Control (CT) plants were maintained under the regular growth conditions explained above until the end of the experiments. The temperature was recorded with a portable USB datalogger (OM-EL-USB-2-LCD-PLUS, OMEGA; Supplementary Fig. S1B). At the conclusion of the stress applications, percentage of damaged leaves, rosette diameter and plant survival were recorded in each experimental group of plants as explained in Balfagón et al. (2019a). All experiments were repeated at least three times with 30 plants per genotype, stress treatment and biological repeat.

2.2. PSII fluorescence

Quantum yield of PSII was assessed at the end of the stress period (Supplementary Fig. S1A) on at least two fully expanded young leaves of three different plants per genotype and stress treatment by using a portable fluorometer (FluorPen FP 110/S, Photon Systems Instruments, Czech Republic).

2.3. Hormone profiling

Hormone extraction and analysis were carried out as described in Šimura et al. (2018) with a few modifications. Briefly, a mixture containing 50 ng of [$^2H_6$]-ABA, [^{13}C]-SA (Sigma-Aldrich), and dehydrojasmonic acid (DHJA) was added to 50 mg of frozen leaf tissue. The tissue was immediately homogenized in 1 mL of cold acetonitrile solution (1:1) in a ball mill (MillMix20; Domel). After 5 min of ultrasonication, a centrifugation at 8000 *g*, 4 °C, was performed and supernatants were recovered. Before hormone extraction, columns (Oasis SPE; Waters) were activated by passing 1 mL of ultrapure methanol followed by 1 mL of ultrapure water. After activation, to equilibrate the column, 1 mL of cold acetonitrile solution (1:1) was added. Supernatants were then added to individual columns and the flow was discarded. Finally, 0.3 mL of cold 30% acetonitrile solution was added to the column to elute the extracts. Extracts were directly transferred to HPLC vials and injected into an ultra-performance LC system (Acquity SDS; Waters). Chromatographic separations were carried out on a reversed-phase C18 column (gravity, 50 × 2.1 mm, 1.8-μm particle size; Macherey-Nagel) using an acetonitrile:H_2O (both supplemented with 0.1% [v/v] formic acid) gradient at a flow rate of 300 mL min^{-1}. Hormones were quantified with a TQ-S Triple Quadrupole Mass Spectrometer (Micromass) as previously described (Balfagón et al., 2019a).

2.4. Extraction of metabolites

Metabolite extraction was performed according to Salem et al.

(2020) using 50 mg of pulverized freeze-dried leaves from three independent biological repeats. Three technical repetitions were performed for each biological replicate. Metabolites were extracted in 1 mL of methyl-*tert*-butyl-ether:methanol (3:1) supplemented with 0.3 μg mL^{-1} isovitexin and 0.5 μg mL^{-1} of ribitol as internal standards for secondary and primary metabolites, respectively. Samples were agitated for 10 min on an orbital shaker at 4 °C and subsequently sonicated for 10 min on an ice-cooled sonication bath. Then, 0.5 mL of a H_2O:MeOH (3:1 v/v) solution was added, and the samples were quickly vortexed and centrifuged for 5 min at 10000 *g* at 4 °C. The polar fraction was aliquoted, dried overnight and kept at −80 °C until use. Three independent extractions were performed per each biological replicate.

2.5. Gas chromatography coupled with mass spectrometry (GC/MS) analysis

For primary metabolite analysis, pellets from 130 μL polar fraction were derivatized with 40 μL of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine for 120 min at 37 °C. Subsequently, 70 μL of *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was added and samples were incubated shaking for 30 min at 37 °C (Lisec et al., 2006). Autosampler Gerstel Multi-Purpose system (Gerstel GmbH & Co.KG, Mülheim an der Ruhr, Germany) was used to inject the samples into a gas chromatograph coupled to a time-of-flight mass spectrometer (GC/MS) (Leco Pegasus HT TOF-MS; LECO Corporation, St. Joseph, MI, USA). Mass chromatograms were analyzed using Chroma TOF 4.5 (Leco) and TagFinder 4.2 software. Peak areas were corrected to the internal standard (ribitol) area in each chromatogram, and also to the exact sample weight.

2.6. Liquid chromatography coupled with mass spectrometry (LC/MS)

For specialized metabolite analysis, dry pellets from 260 μL polar fraction obtained before were resuspended in 400 μL of 50% MeOH vortexing for 5 min. Resuspended samples were sonicated for 3 min and centrifuged for 5 min at 4000 g. Then, samples were loaded on a UPLC-MS equipped with an HSS T3 C18 reverse-phase column (100 × 2.1 mm internal diameter, 1.8 μm particle size; Waters) at 40 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Processing of chromatograms, peak detection, and integration were performed using RefinerMS (version 5.3; GeneData). Metabolite identification and annotation were performed as per recent reporting standards (Alseekh et al., 2021). On using our in-house reference compounds library, 10 ppm mass error, and a dynamic retention-time shift of 0.1 were allowed (Alseekh et al., 2021). Peak areas were corrected to the internal standard (isovitexin) area in each chromatogram, and also to the exact sample weight.

2.7. Statistical analysis

Metabolite data were normalized as ratio to control and subjected to statistical analysis using MetaboAnalyst (Pang et al., 2021). Statistical significance was denoted by * for $P < 0.05$. For leaf damage, survival, hormone profiling, rosette diameter, and PSII efficiency data, the mean values of three biological replicates were reported along with their standard error (mean ± SE). A two-way ANOVA was performed, followed by a Tukey post hoc test ($P < 0.05$) when a significant difference was detected. Different letters denoted significant differences at $P < 0.05$.

3. Results

3.1. Role of hormones in plant tolerance to the combination of salinity and high light stress

To investigate the potential roles of SA, JA, and ABA in plant tolerance to S, HL, and their combination (S + HL), we conducted experiments on Arabidopsis wild-type plants (Col and Ler) as well as mutants deficient in ABA (*aba2-11* and *aba1-1*), JA (*aos*), and SA (*sid2*). Plants were exposed to 150 mM NaCl for 10 days (S), 650 μmol m^{-2} s^{-1} light intensity for 7 h (HL), or a combination of both stresses (S + HL; Supplementary Fig. S1A). S + HL increased the percentage of leaf damage compared to CT conditions in wild-type plants (Col and Ler), whereas leaf appearance was not significantly affected by S. The increase of damaged leaves was more evident in *aba2-11* plants subjected to S + HL compared to Col, whereas *aba1-1*, *sid2* and *aos* plants did not exhibit significant changes in response to any individual or combined stress compared to wild-type plants (Fig. 1A and Supplementary Figs. S2, S3A, S4A). As shown in Fig. 1B and Supplementary Figs. S2, S3B and S4B, the survival of Col, *sid2* and *aos* plants decreased in response to S + HL, and *aba2-11* and *aba1-1* mutants displayed reduced survival under both S and S + HL compared to wild-type plants. Additionally, S treatment (S and S + HL) hindered plant growth measured as rosette diameter in Col, Ler, *sid2*, *aba1-1* and *aba2-11* (Fig. 1C and Supplementary Figs. S3C and S4C), and HL stress, either alone or combined with S, reduced PSII efficiency in Ler, Col, *sid2*, *aba1-1*, and *aos* (Supplementary Figs. S3D and S4D). However, *aba2-11* and *aba1-1* mutants showed reduced values of PSII efficiency in response to individual HL stress, with a more prominent effect observed under S + HL conditions (Fig. 1D and Supplementary Fig. S3D). Considering the significant impact of ABA absence and the marginal role of JA and SA in plant tolerance to S + HL, our subsequent analysis focused on *aba2-11* plants subjected to S, HL, and S + HL.

3.2. Impact of ABA absence on JA and SA accumulation in plants subjected to the combination of salinity and high light stress

To investigate the regulatory role of ABA on other hormones including JA and SA, hormone profiles were compared between Col and *aba2-11* mutant plants under different stress conditions (S, HL and S + HL; Fig. 2). Results showed significant increases in JA and SA levels in *aba2-11* plants exposed to HL and S + HL stresses compared to both Col and CT conditions. In contrast, Col plants did not exhibit any significant changes in the levels of JA and SA in response to any stress condition (Fig. 2A and B). Interestingly, Col plants showed a significant increase in ABA levels in response to S + HL, whereas in *aba2-11* plants subjected to individual or combined S and HL, no significant change in ABA content was observed as expected (Fig. 2C). These findings suggest that ABA may play a role in regulating the accumulation of JA and SA in response to S + HL.

3.3. Differential metabolic responses of Col and aba2-11 plants subjected to the combination of salinity and high light stress

In order to investigate variations in the accumulation of different primary metabolites such as sugars, alcohols, amino acids, tricarboxylic acid (TCA) cycle intermediates, and polyamines between Arabidopsis wild type and *aba2-11* plants under the different stress conditions, a comprehensive analysis of polar compounds using gas chromatography-mass spectrometry was conducted (Figs. 3 and 4; Supplementary Tables S1 and S2). Fig. 3A shows the results of the Principal Component Analysis (PCA) plot, revealing that the dissimilarities between genotypes accounted for a cumulative variance of 33.6% (PC1). Moreover, PC2 accounted for an additional 20.9% of the total variance, effectively segregating the samples based on the specific stress treatments applied. Notably, the metabolic changes were significantly more pronounced in the presence of salt stress (S and S + HL), indicating a stronger impact on the overall metabolic profile in both Arabidopsis genotypes. As shown in Fig. 3B, differences in significant metabolite accumulation between Col and *aba2-11* plants in response to different stress treatments were found. In contrast to the synergistic trend observed in the physiological responses to stress combination (Fig. 1), both genotypes exhibited similar

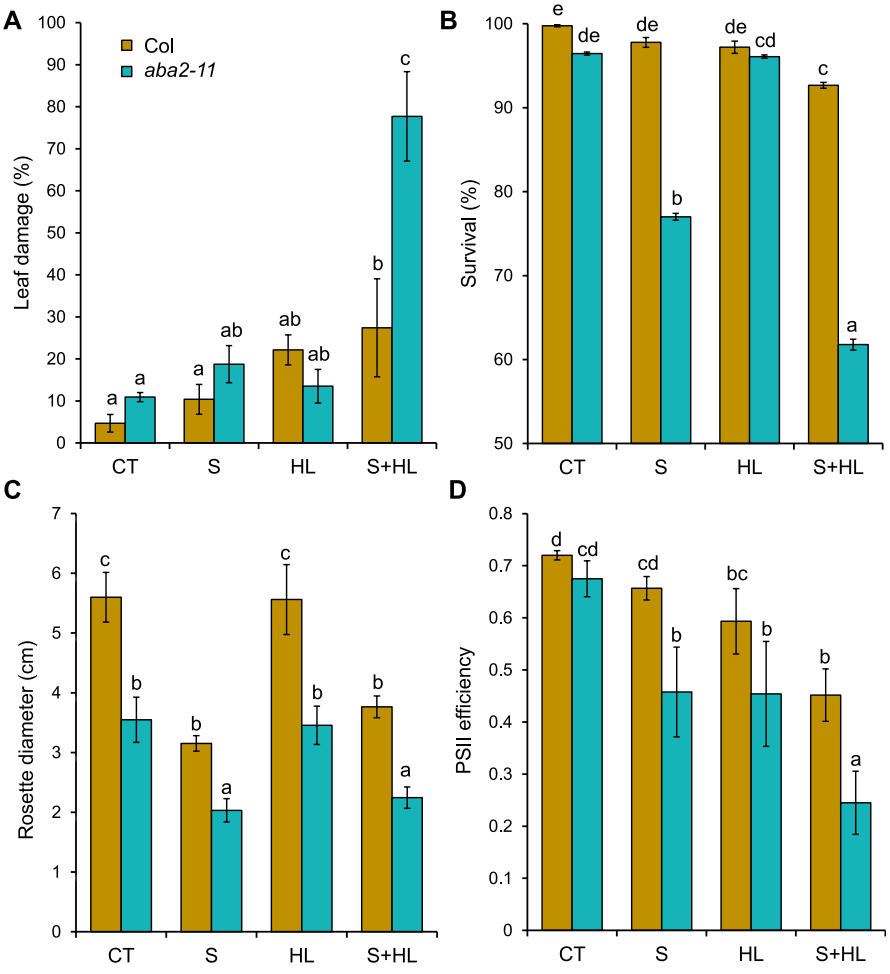

Fig. 1. Leaf damage, survival, growth, and PSII efficiency of Col and *aba2-11* plants subjected to the combination of salinity (S) and high light (HL) stress. Percentage of leaf damage (A), survival (B), rosette diameter (C), and PSII efficiency (D) of Col and *aba2-11* plants subjected to S, HL and S + HL. Error bars represent SE. Different letters denote statistical significance at $P < 0.05$. CT, control; HL, high light; PSII, photosystem II; S, salinity; S + HL, the combination of salinity and high light.

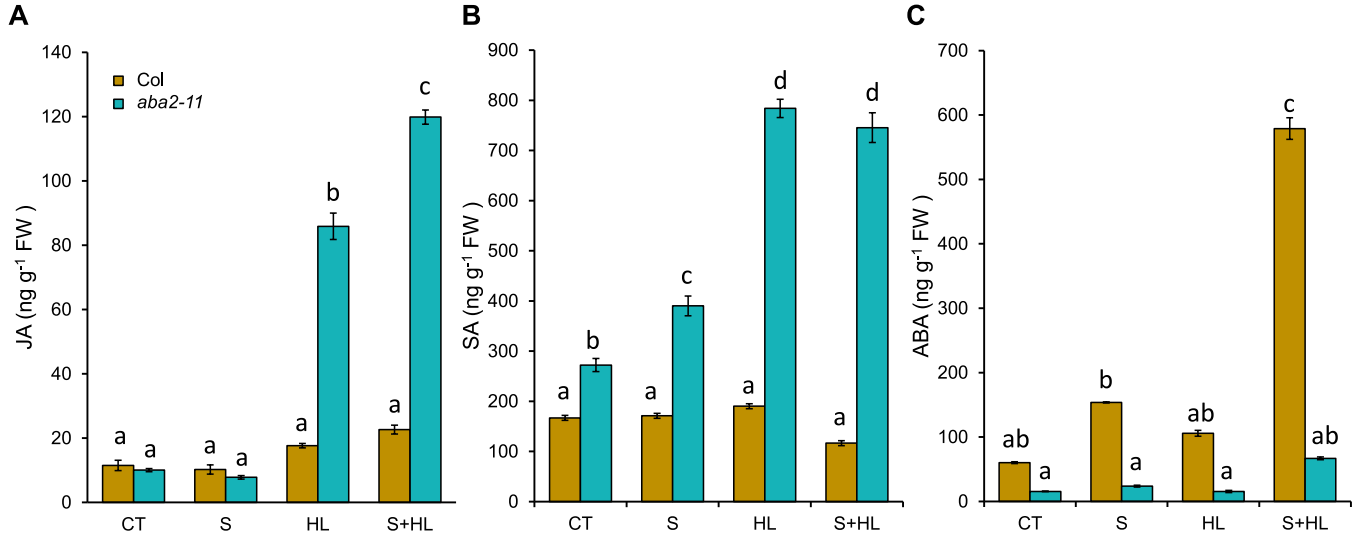

Fig. 2. Hormonal levels in Col and *aba2-11* plants subjected to the combination of salinity (S) and high light (HL) stress. (A–C) JA (A), SA (B), and ABA (C) levels in Col and *aba2-11* plants subjected to S, HL and S + HL. Error bars represent SE. Different letters denote statistical significance at $P < 0.05$. ABA, abscisic acid; CT, control; HL, high light; JA, jasmonic acid; S, salinity; SA, salicylic acid; S + HL, the combination of salinity and high light.

alterations (over- or under-accumulation) in the number of primary metabolites in response to individual and combined stress conditions Specifically, in Col plants, 49 metabolites were altered under S, 46 under HL, and 49 under S + HL. Similarly, in *aba2-11* plants, 44 metabolites were altered under S, 46 under HL, and 48 under S + HL. Going into more depth, S induced the over-accumulation of 19 metabolites and the

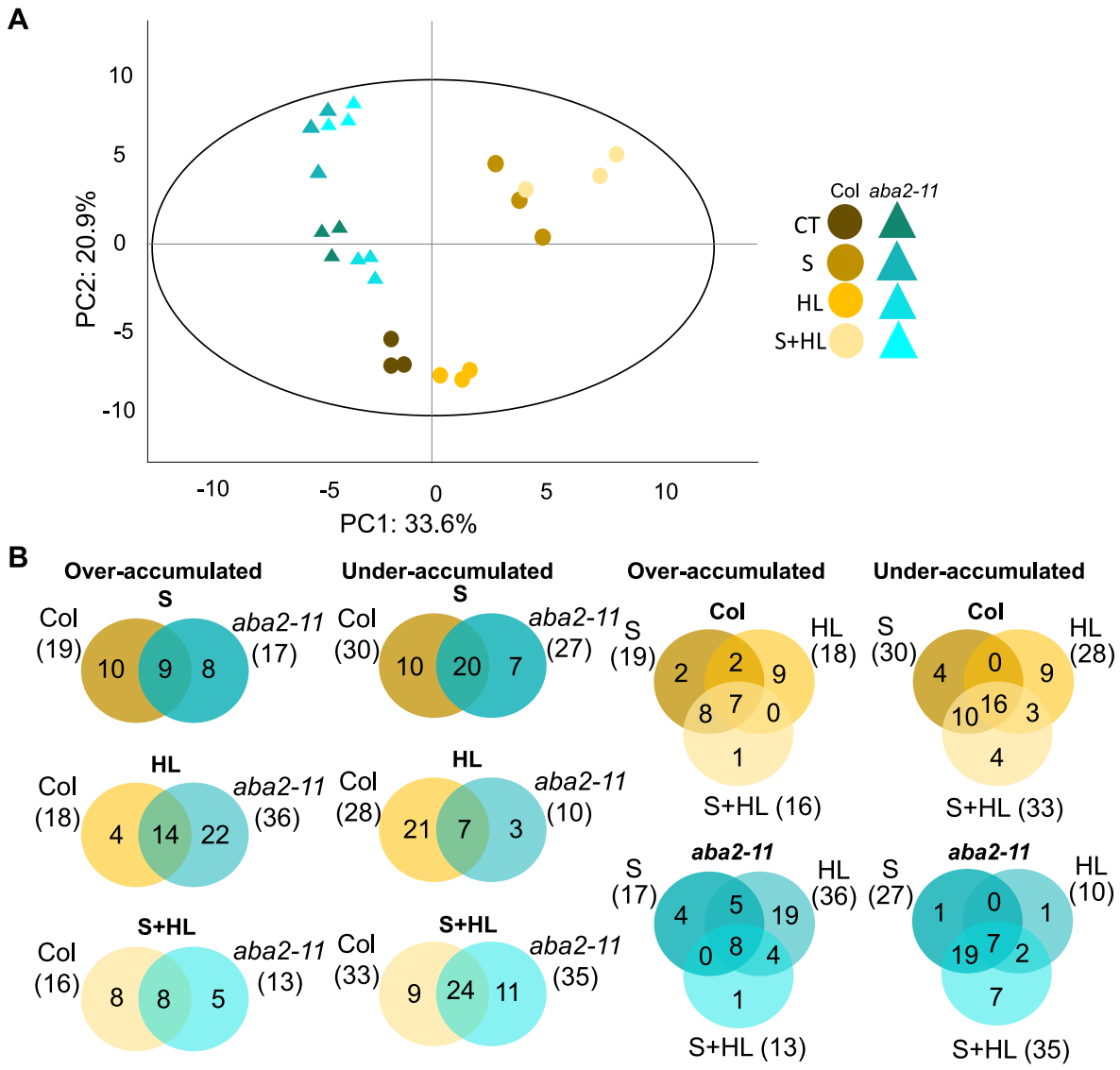

Fig. 3. Differential accumulation of primary metabolites in Col and *aba2-11* plants subjected to the combination of salinity (S) and high light (HL) stress. (A) Principal Component Analysis (PCA) score plot of metabolite profiles obtained from CT Col and *aba2-11* plants, and Col and *aba2-11* plants subjected to S, HL and S + HL. (B) Venn diagrams depicting the overlap among over-accumulated and under-accumulated metabolites in Col and *aba2-11* plants subjected to S, HL and S + HL. A comparison of the metabolite changes between both genotypes in each stress condition is shown on the left and a comparison of the metabolite changes among the different stress treatments (S, HL and S + HL) in each genotype is shown on the right. CT, control; HL, high light; PC, principal component; S, salinity; S + HL, the combination of salinity and high light.

under-accumulation of 30 metabolites in Col plants. In contrast, the *aba2-11* mutant exhibited the over-accumulation of 17 metabolites and the under-accumulation of 27 metabolites under salt treatment. Under HL, Col plants triggered the over-accumulation of 18 metabolites and the under-accumulation of 28 metabolites, whereas in *aba2-11* plants, the number of metabolites over-accumulated increased to 36, and only 10 metabolites were under-accumulated. Furthermore, S + HL resulted in the increased levels of 16 metabolites and the attenuation of 33 metabolites in Col plants. Similarly, in the *aba2-11* mutant, 13 metabolites were over-accumulated, but 35 metabolites were under-accumulated in response to stress combination. Interestingly, in Col but not in *aba2-11* plants, 8 and 9 metabolites exhibited over- and under-accumulated, respectively, under S + HL, indicating a potential involvement of ABA regulating the accumulation of these metabolites in response to the combined stress conditions (Table 1). Metabolites over-accumulated in Col but not in *aba2-11* in response to S + HL included urea, glycerol-3-phosphate, citric acid, glucuronic acid lactone, galactonic acid, tryptophan and galactinol, and levels of lactic acid, succinic acid, alanine, spermidine, tyramine, nicotinic acid, rhamnose, threonine and adenine were attenuated in Col plants but not in *aba2-11* under stress combination compared to CT (Table 1).

A more detailed comparison between metabolites that were over- and under-accumulated in both Arabidopsis genotypes (Col and *aba2-11*) under individual or combined stress conditions of S and HL, revealed that 7 and 16 metabolites were over- and under-accumulated, respectively, in Col under all stress conditions (S, HL and S + HL). In *aba2-11* plants, in turn, 8 and 7 metabolites exhibited over- and under-accumulation, respectively, in response to all three stress treatments (S, HL, and S + HL) (Fig. 3B; Supplementary Tables S1 and S2). Furthermore, glycerol-3-phosphate and histidine were exclusively over-accumulated in Col and *aba2-11*, respectively, under S + HL (Table 2). Conversely, 4 and 7 metabolites were specifically under-accumulated in Col and *aba2-11*, respectively, under S + HL (Fig. 3B; Table 2). Therefore, the metabolic response of plants subjected to S + HL was, to some extent, different from that of plants subjected to S or HL, harboring metabolites that specifically responded to the stress combination. At

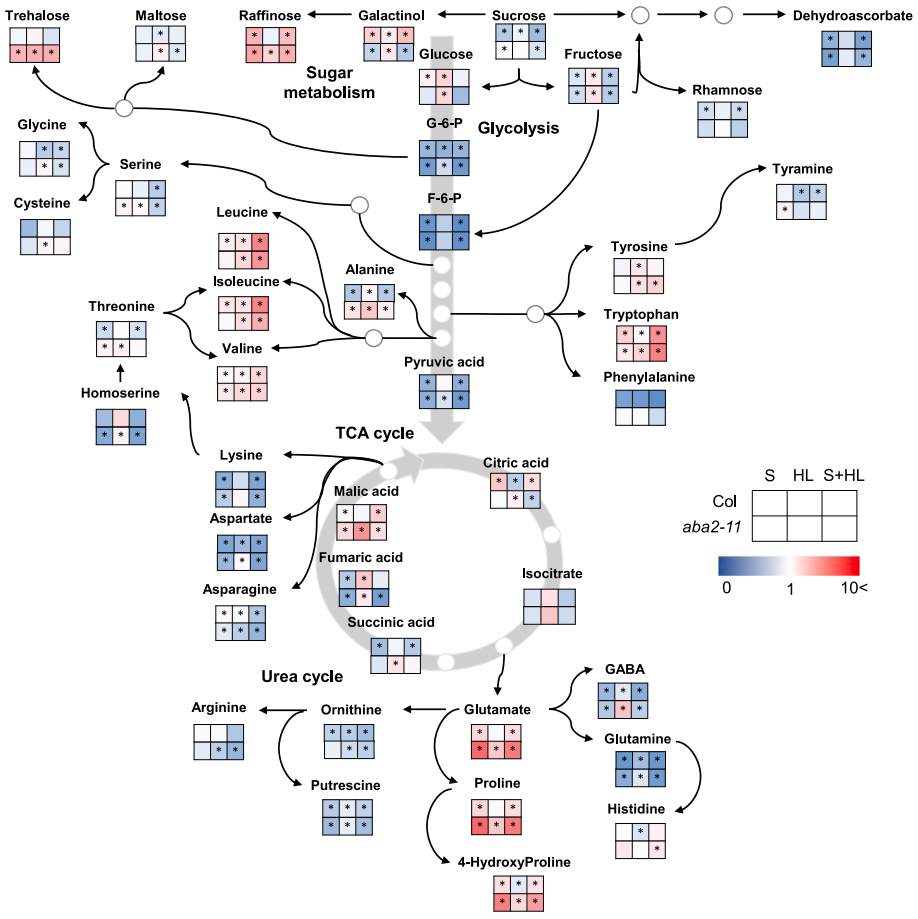

Fig. 4. Levels of primary metabolites in Col and *aba2-11* plants subjected to the combination of salinity (S) and high light (HL) stress. Level of sugars, amino acids, polyamines and TCA-related metabolites in Col and *aba2-11* plants subjected to S, HL and S + HL. Metabolite levels are expressed as fold change compared to control values and are shown as a color scale. Asterisks denote significant metabolite level (*P < 0.05) compared to control conditions. F-6-P, fructose-6-phosphate; GABA, gamma-aminobutyric acid; G-6-P, glucose-6-phosphate; HL, high light; S, high salinity; S + HL, the combination of salinity and high light, TCA, tricarboxylic acid.

Table 1
List of potential ABA-dependent primary metabolites altered under the combination of salinity and high light (S + HL). Metabolite levels are expressed as fold change compared to control values. SE, standard error.

Metabolite name	Fold change	SE	*t*-test
Lactic acid	0.40	0.077	3.82E-02
Succinic acid	0.56	0.089	6.01E-03
Alanine	0.57	0.023	8.09E-06
Spermidine	0.64	0.149	1.76E-02
Tyramine	0.66	0.119	1.71E-02
Nicotinic acid	0.69	0.040	3.12E-03
Rhamnose	0.72	0.061	4.34E-03
Threonine	0.77	0.041	6.81E-04
Adenine	0.80	0.110	4.00E-02
Urea	1.26	0.112	3.15E-02
Glycerol-3-phosphate	1.41	0.097	1.71E-02
Citric acid	1.88	0.311	6.77E-03
Glucuronic acid lactone	2.78	0.368	7.80E-04
Galactonic acid	2.85	0.378	7.80E-04
Tryptophan	4.08	0.629	7.51E-04
Galactinol	6.03	0.571	5.06E-04

Table 2
List of metabolites specifically altered in Col and *aba2-11* plants in response to the combination of salinity and high light (S + HL). Metabolite levels are expressed as fold change compared to control values. SE, standard error.

Metabolite name	Fold change	SE	*t*-test
Col			
Lysine	0.24	0.005	0.001
Lactic acid	0.40	0.077	0.038
Succinic acid	0.56	0.089	0.006
Glyoxylic acid	0.72	0.047	0.007
Glycerol-3-phosphate	1.41	0.097	0.017
aba2-11			
Homoserine	0.23	0.045	0.000
Guanidine	0.59	0.066	0.023
Glyoxylic acid	0.63	0.019	0.005
Serine	0.65	0.018	0.000
Citric acid	0.65	0.033	0.001
Glycine	0.78	0.029	0.003
Maltose	0.83	0.055	0.044
Histidine	1.63	0.115	0.020

least when it comes to the role of ABA in these responses, our results support a role of ABA in determining metabolite over- or under-accumulation under stress combination. Overall, these findings provide valuable insights into the specific metabolites that contribute to the differential responses observed between Col and *aba2-11* plants under the different stress conditions.

3.4. Alteration of primary metabolic pathways of Col and aba2-11 plants subjected to the combination of salinity and high light stress

Differences in the levels of metabolites participating in various metabolic pathways were observed between Col and *aba2-11* plants exposed to S, HL, and S + HL conditions (Fig. 4; Supplementary Tables S1 and S2). Sugar metabolism, in particular, exhibited distinct alterations in both Col and *aba2-11* plants. In response to S + HL, Col

plants redirected sugar metabolism towards the accumulation of raffinose and galactinol, whereas *aba2-11* plants accumulated trehalose and raffinose while reducing levels of galactinol and maltose. Furthermore, both plant genotypes exhibited decreased levels of fructose and sucrose in response to S and S + HL. Certain glycolysis intermediates, such as glucose-6-phosphate (G-6-P) decreased its levels in response to all stresses, whereas fructose-6-phosphate (F-6-P) decreased its content under conditions of S and S + HL in both genotypes. Interestingly, the levels of branched-chain amino acids (BCAAs) such as leucine, isoleucine, and valine exhibited similar fluctuations in both Arabidopsis wild type and *aba2-11* mutants, with the highest levels observed in response to the combined stress conditions. In contrast, levels of serine and glycine showed a decrease in both genotypes under S + HL conditions and, contrarily, levels of both serine and glycine increased in *aba2-11* plants under HL. In both genotypes, lysine levels decreased in response S and S + HL, whereas aspartate and asparagine contents decreased under all stress conditions.

The metabolic pathway of glutamate, which plays a crucial role in certain amino acid and polyamine metabolism (Forde and Lea, 2007), was altered in response to individual and combined stresses in Arabidopsis plants (Fig. 4). Overall, a significant accumulation of glutamate in *aba2-11* plants upon stress was found, accompanied by increased levels of its derivatives proline and 4-hydroxyproline. In Col plants, in turn, glutamate and proline were accumulated in response to S and S + HL, whereas 4-hydroxyproline increased its levels in response to all stresses. In contrast, levels of various glutamate derivatives, including polyamines (ornithine and putrescine), as well as GABA and glutamine, displayed a decrease in response to all stresses in Col plants, whereas in *aba2-11* plants, levels of putrescine and glutamine decreased under all stresses, ornithine content decreased under HL and S + HL, and gamma-aminobutyric acid (GABA) levels decreased under salinity treatments (S and S + HL) but increased under HL (Fig. 4). Since certain primary metabolites were similarly altered in response to both S and S + HL, these results suggest that salinity stress responses could potentially play a dominant role, at least partially, in influencing the alteration of primary metabolites in Arabidopsis plants.

3.5. *Alteration of specialized metabolites in Col and aba2-11 plants subjected to the combination of salinity and high light stress*

In plants, phenylalanine, tryptophan and tyrosine play pivotal roles as precursors for specialized metabolites, such as phenylpropanoids and flavonoids (Barros and Dixon, 2020). In our study, differences in the accumulation of different flavonoids between Col and *aba2-11* in

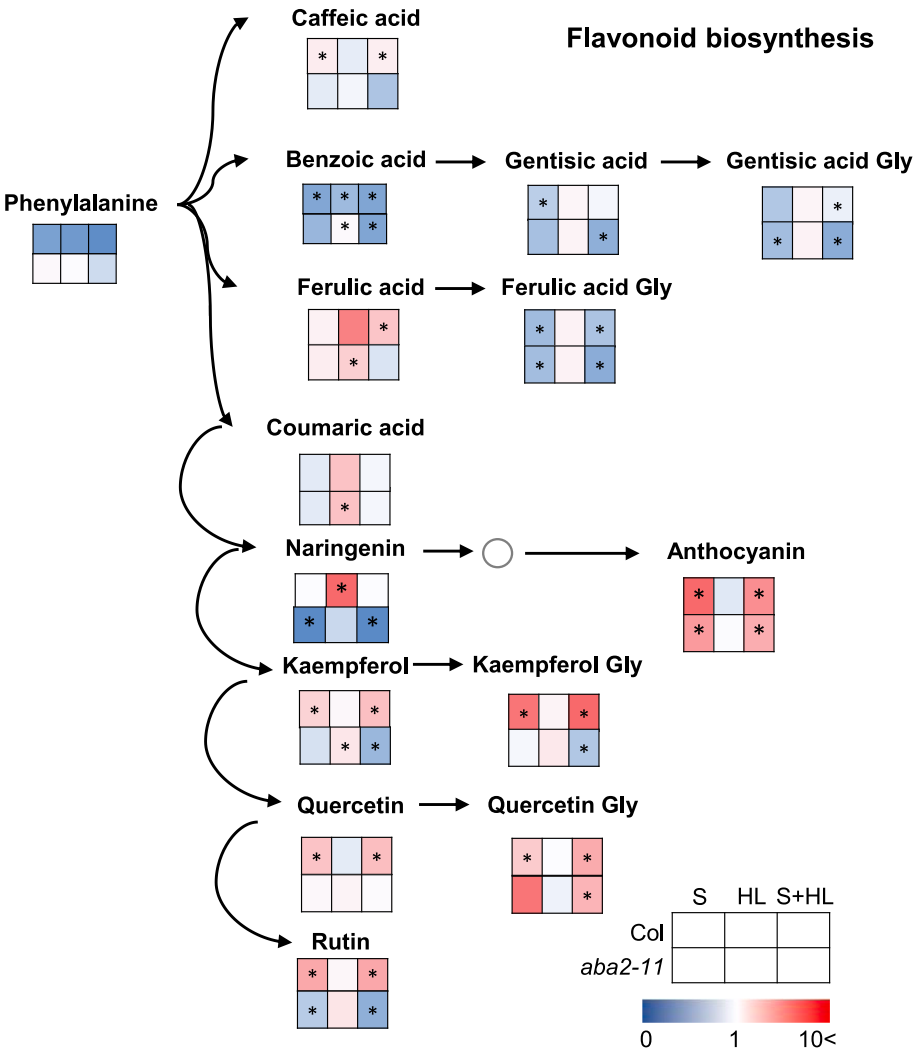

Fig. 5. Levels of metabolites related to flavonoid biosynthesis pathway in Col and *aba2-11* plants subjected to the combination of salinity (S) and high light (HL) stress. Level of flavonoids and flavonoid-related metabolites in Col and *aba2-11* plants subjected to S, HL and S + HL. Metabolite levels are expressed as fold change compared to control values and are shown as a color scale. Asterisks denote significant metabolite level (*P < 0.05) compared to control conditions. Gly, glycoside; HL, high light; S, high salinity; S + HL, the combination of salinity and high light.

response to S + HL were observed (Fig. 5; Supplementary Tables S3 and S4). Notably, *aba2-11* plants showed significant under-accumulation of naringenin, kaempferol, rutin, as well as phenolic and phenylpropanoid compounds, including benzoic acid, gentisic acid and ferulic acid, under stress combination. Conversely, Col plants exposed to S + HL stress displayed an over-accumulation of kaempferol, quercetin, and rutin (Fig. 5; Supplementary Tables S3 and S4).

4. Discussion

Over the past few decades, numerous studies have examined the changes in metabolic profile in *Arabidopsis thaliana* plants exposed to different individual or combined abiotic stresses (Balfagón et al., 2022; Fàbregas and Fernie, 2019; Kaplan et al., 2004; Kusano et al., 2011; Maruyama et al., 2009; Nishizawa et al., 2008; Rizhsky et al., 2004; Schmitz et al., 2014; Wulff-Zottele et al., 2010; Zinta et al., 2018). Among key metabolites altered in response to different abiotic stresses or their combinations, hormones such as ABA, SA and JA, were previously shown to exhibit distinct accumulation patterns depending on the specific stress or combination of stresses. Furthermore, the capacity of plants to modulate specific and unique hormonal responses has been demonstrated to be key in plant acclimation to combined stresses (Zandalinas et al., 2022). For example, mutants impaired in ABA biosynthesis (*aba1-1*) and signaling (*abi1-1*) showed reduced survival rates when exposed to a combination of drought and heat stress compared to wild-type plants (Zandalinas et al., 2016), highlighting the role of ABA in enhancing the tolerance of plants subjected to this stress combination. In addition, ABA was involved in the acclimation of Arabidopsis to heat combined with drought by regulating the accumulation of Ascorbate Peroxidase 1 (APX1) and Multiprotein Bridging Factor 1c (MBF1c) proteins (Zandalinas et al., 2016). Moreover, studies by Balfagón et al. (2019b) in citrus and Xue et al. (2021) in tomato have determined significant accumulations of ABA in response to combined conditions of salinity and high temperatures, as well as salinity and drought, respectively. Additionally, studies on poplar revealed an increased transcription of ABA-responsive genes such as Responsive to Desiccation 26 (RD26) and ABA Repressor 1 (ABR1) when plants were exposed to the combination of salinity and heat stress (Jia et al., 2017). Taking together, these findings suggest that ABA plays a central role in signaling pathways that contribute to the ability of plants to adapt to different combinations of abiotic stresses. However, the influence of ABA in plant tolerance to the combination of salinity and high light stress (S + HL) and its role in regulating metabolic responses under this stress combination has not been deeply evaluated. In this study, we examined the effects of S + HL on the growth, survival rates and metabolite levels of both Arabidopsis wild type and ABA-deficient (*aba2-11*) plants to identify potential metabolic markers associated with plant tolerance to the combined effect of salinity and high light. Our findings indicate that both Arabidopsis genotypes differed in their ability to tolerate S + HL, showing *aba2-11* plants decreased survival rates, growth, and PSII efficiency, as well as increased leaf damage compared to Col plants (Fig. 1 and Supplementary Fig. S2). In addition, these divergences were accompanied by distinct metabolic responses, and significant differences in the accumulation of plant hormones (Fig. 2), primary metabolites (Figs. 3 and 4), and flavonoids (Fig. 5) were observed between both Col and *aba2-11* plants subjected to S, HL and S + HL. Under conditions of S + HL, a significant and unique increment in ABA content was observed in Col plants subjected to S + HL (Fig. 2C), further reinforcing the key role of this phytohormone in plant responses to stress combination. In the absence of ABA, however, JA and SA levels increased compared to CT in response to S + HL (Fig. 2A and B), indicating the importance of ABA as a key regulator in mediating crosstalk between different hormone signaling pathways during plant responses to S + HL. The intricate interplay between these hormones have been also shown during responses of stress combination involving different abiotic stresses (Suzuki, 2016). For example, the occurrence of both drought and heat stress led to a simultaneous increase in JA levels, which counteracted the effects of SA in the ABA-insensitive mutants *abi1-1* (Zandalinas et al., 2016).

The presence of a common set of metabolites between both genotypes that consistently exhibited over- or under-accumulation in response to the different stress conditions suggests potential ABA-independent core metabolic pathways that are regulated in response to each particular stress (Figs. 3 and 4). Among them, different osmolytes that could contribute to osmotic adjustments (Bartels and Sunkar, 2007; Fàbregas and Fernie, 2019) were found to be similarly regulated in response to S + HL in both Col and *aba2-11* plants. In this sense, proline and its derivative 4-hydroxyproline, as well as raffinose were over-accumulated in both Col and *aba2-11* plants subjected to S + HL (Fig. 4), suggesting that these metabolites could act as osmoprotectants in an ABA-independent manner under this stress combination. Similarly, under cold, osmotic stress or low water potential, the proline biosynthetic pathway was shown to be activated independently of ABA levels in Arabidopsis plants (Savouré et al., 1997; Sharma and Verslues, 2010). However, since levels of proline and 4-hydroxyproline were higher in *aba2-11* compared to Col in response to stress, a possible role of ABA in negatively modulating the levels of these metabolites cannot be ruled out. In addition to proline, levels of different BCAAs were altered in wild type as well as in *aba2-11* plants in response to stress combination, suggesting a potential ABA-independent regulation of these metabolites upon stress.

Different primary metabolites were, by contrast, accumulated in Col subjected to S + HL but not in *aba2-11* plants (Figs. 3 and 4), indicating potential ABA-dependent regulatory mechanisms. A metabolic pathway that was correlated with ABA signaling under S + HL was the biosynthesis of flavonoids. Different flavonoids including kaempferol, quercetin or rutin were accumulated in Col but not in *aba2-11* in response to stress combination (Fig. 5). Flavonoids are shown to accumulate in response to many abiotic stresses (Nakabayashi and Saito, 2015) and their antioxidant properties due to their intracellular free radical scavenging capacities have been experimentally confirmed under different stress conditions (Hernández et al., 2004; Kusano et al., 2011; Nakabayashi et al., 2014; Nakabayashi and Saito, 2015). Therefore, the highest sensitivity of *aba2-11* plants to S + HL could be partially attributed to the absence of potential ABA-regulated antioxidants that play a role in mitigating oxidative stress under S + HL conditions. In agreement with these findings, different reports have demonstrated that ABA-regulated flavonoids enhance the tolerance of several plant species to drought stress (Gai et al., 2020; Gao et al., 2021; Yang et al., 2021). For example, the application of exogenous ABA significantly increased the biosynthesis of flavonoids in tea leaves (Gai et al., 2020) or in pigeon pea (Yang et al., 2021), resulting in enhanced drought tolerance. In addition, the interplay between ABA and flavonoid signaling played a significant role in determining the contrasting levels of drought resistance between two subspecies of sea buckthorn (Gao et al., 2021).

Reconfiguration of metabolic profile is one of the main mechanisms that plants use as a response to adverse conditions (Zandalinas et al., 2022). Our results show that, at least when it comes to changes in primary metabolism (Fig. 3), the plant response to stress combination does not exhibit a synergistic trend as observed in physiological responses. This reinforces the idea that the stress combination induces a novel state of stress, rather than simply being the sum of individual stresses. Nevertheless, it is important to note that synergistic changes might still occur in other aspects, such as transcriptomic or other biochemical responses. In addition, our findings also suggest that ABA could play an important role for plant tolerance to S + HL by promoting flavonoid accumulation, indicating a potential molecular regulatory relationship between ABA, flavonoid metabolism and Arabidopsis ability to tolerate the combined effect of salinity and high light (Fig. 6). Of course, the role of other ABA-dependent physiological and molecular changes in promoting plant tolerance to S + HL cannot be ruled out. In addition to ABA-dependent responses, our findings revealed common metabolic

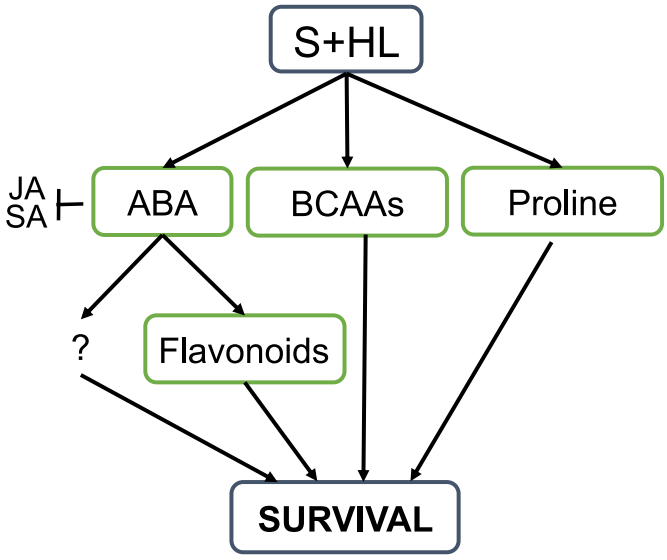

Fig. 6. ABA could play an important role in enhancing plant tolerance to the combination of salinity and high light by promoting the accumulation of flavonoids along with other potential ABA-dependent physiological and molecular changes (indicated by a question mark). Furthermore, the presence of common metabolic changes, such as the accumulation of BCAAs and proline, in both wild type plants and ABA-deficient plants exposed to S + HL, suggests the involvement of potential ABA-independent responses that may also contribute to plant adaptation and survival under this specific stress combination. ABA, abscisic acid; BCAAs, branched chain amino acids; JA, jasmonic acid; SA, salicylic acid; S + HL, the combination of salinity and high light.

changes to S + HL between wild type plants and plants deficient in ABA such as the accumulation of BCAAs or proline, indicating that potential ABA-independent responses may also contribute to plant adaptation and survival under this stress combination (Fig. 6).

Contributions

CS-M performed the research; SIZ, SA, ARF and AG-C designed and supervised the research; SIZ, ARF and AG-C provided laboratory infrastructure and funding; JLR, SA, CS-M and ARF performed the metabolomics analysis; SIZ and CS-M wrote the manuscript and prepared Figures. All authors read and approved the final version of the manuscript.

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2023.108008.

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