the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2024) doi: 10.1111/tpj.16642

RESOURCE

Specific ABA-independent tomato transcriptome reprogramming under abiotic stress combination

Miriam Pardo-Hernández[1], Vicent Arbona[2], Inmaculada Simón[3] and Rosa M. Rivero[1,*]

[1]*Department of Plant Nutrition, Center of Edaphology and Applied Biology of Segura (CEBAS-CSIC), Campus Universitario Espinardo, Ed 25, 30100 Murcia, Spain,*

[2]*Departament de Biologia, Bioquímica i Ciències Naturals, Universitat Jaume I, Castelló de la Plana 12071, Spain, and*

[3]*Centro de Investigación e Innovación Agroalimentaria y Agroambiental (CIAGRO-UMH), Miguel Hernández University, Orihuela, Spain*

Received 29 March 2023; revised 8 January 2024; accepted 11 January 2024.
*For correspondence (e-mail rmrivero@cebas.csic.es).

SUMMARY

Crops often have to face several abiotic stresses simultaneously, and under these conditions, the plant's response significantly differs from that observed under a single stress. However, up to the present, most of the molecular markers identified for increasing plant stress tolerance have been characterized under single abiotic stresses, which explains the unexpected results found when plants are tested under real field conditions. One important regulator of the plant's responses to abiotic stresses is abscisic acid (ABA). The ABA signaling system engages many stress-responsive genes, but many others do not respond to ABA treatments. Thus, the ABA-independent pathway, which is still largely unknown, involves multiple signaling pathways and important molecular components necessary for the plant's adaptation to climate change. In the present study, ABA-deficient tomato mutants (*flacca, flc)* were subjected to salinity, heat, or their combination. An in-depth RNA-seq analysis revealed that the combination of salinity and heat led to a strong reprogramming of the tomato transcriptome. Thus, of the 685 genes that were specifically regulated under this combination in our *flc* mutants, 463 genes were regulated by ABA-independent systems. Among these genes, we identified six transcription factors (TFs) that were significantly regulated, belonging to the R2R3-MYB family. A protein–protein interaction network showed that the TFs SlMYB50 and SlMYB86 were directly involved in the upregulation of the flavonol biosynthetic pathway-related genes. One of the most novel findings of the study is the identification of the involvement of some important ABA-independent TFs in the specific plant response to abiotic stress combination. Considering that ABA levels dramatically change in response to environmental factors, the study of ABA-independent genes that are specifically regulated under stress combination may provide a remarkable tool for increasing plant resilience to climate change.

Keywords: transcriptome, salinity, heat, stress combination, ABA-deficient mutant, tomato, TFs, ABA-independent genes.

INTRODUCTION

Changes in the Earth's climate, primarily driven by the escalating anthropogenic emissions of heat-trapping greenhouse gases, are having widespread detrimental effects on the environment. The frequency and severity of extreme weather events, such as hurricanes, heatwaves, wildfires, droughts, and floods, have increased dramatically in the last few years and are expected to continue doing so in the coming decades. These extreme events are directly impinging global agricultural production, resulting in annual losses amounting to billions of dollars/euros (Mariani & Ferrante, 2017) and leading to an inevitable surge in food prices. The scientific community has long recognized the serious impact of abiotic stresses on plant growth and production, and over many decades, extensive efforts have been dedicated toward the identification of new molecular markers that can aid plants thrive and produce under various abiotic stresses.

It is well-demonstrated that in the field, plants must often confront multiple abiotic stresses simultaneously. Under such conditions, the plant's response significantly differs from that traditionally observed under individual stresses (Liu et al., 2023; Mittler, 2006; Rivero et al., 2014; Zandalinas et al., 2021). For example, combining salinity with heat induces a unique stress response in tomato plants, such as the enhancement of some plant protection mechanisms, as compared to plants exposed to salinity alone (Rivero et al., 2014). Similarly, when tomato plants were subjected to the combination of salinity and heat, the metabolic, biochemical, and molecular profiles obtained were different from that observed when these stresses were applied individually (Martinez et al., 2016). Moreover, the combination of heat and salinity granted tomato plants with a significant tolerance to salinity through the specific accumulation of glycine betaine and trehalose. The accumulation of these compounds under stress combination played a crucial role in maintaining a high K^+ concentration, which consequently reduced the Na^+/K^+ ratio. As a result, the cell's water status and photosynthetic processes showed a significant improvement when compared to exposure to salinity alone (Rivero et al., 2014). On the contrary, some studies conducted in Arabidopsis grown under the combination of salinity and heat showed an overall negative impact on plants development (Suzuki et al., 2016). Furthermore, Nahar et al. (2022) demonstrated a significant water limitation and a crucial role for Na^+ and K^+ uptake and transport, osmoprotectant accumulation, and the expression of ROS-scavenging enzymes when rice plants were exposed to the combined stresses of heat and salinity. These findings cannot be inferred from the specific responses of rice plants to each of these individual stresses when applied alone (Nahar et al., 2022). Accordingly, photosynthesis is more sensitive to the combination of soil waterlogging and high temperatures than to individual stressors (Liu et al., 2023). Elevated CO_2 levels have been found to mitigate the adverse effects of water deficit stress on plants, indicating a cooperative effect in safeguarding plants against water stress (Shanker et al., 2022). Additionally, *Eucalyptus globulus* exposed to a combination of heat and drought stresses triggered a specific protective response not observed when individual stresses were applied (Correia et al., 2018). In *Arabidopsis*, a comparative differential expression analysis and gene ontology enrichment after the application of drought and cold stresses revealed the presence of shared and unique components between them. In this light, it was found that several transcription factor families common to drought and cold stress specifically regulated several common ABA-dependent stress-responsive genes (Sharma et al., 2018).

Plants have evolved intricate signaling pathways to facilitate their adaptation to changing environmental conditions, and the phytohormone abscisic acid (ABA) is the critical regulator of plant responses to abiotic stresses. When plants experience abiotic stress, ABA accumulates in various organs and tissues (Larkindale & Knight, 2002; Muhammad Aslam et al., 2022; Popova et al., 1995), activating ABA-dependent signaling mechanisms that involve numerous stress-responsive genes (Nakashima & Yamaguchi-Shinozaki, 2013). ABA is synthesized from β-carotene, and the final step in ABA biosynthesis involves the oxidation of abscisic aldehyde to ABA, catalyzed by the molybdenum-containing aldehyde oxidase (AO; EC 1.2.3.1) (Leydecker et al., 1995; Walker-Simmons et al., 1989). In turn, MoCo-containing aldehyde oxidase must be sulfurated by MoCo sulfurase, which activates AO for ABA synthesis. Several mutants with defects in any of the enzymes belonging to the β-carotenoid biosynthesis and the downstream pathway have been generated in many plant species, and they are characterized by their ABA deficiency (Giraudat et al., 1994; Koornneef et al., 1982; Neill et al., 1986; Parry et al., 1991; Quarrie, 1982; Robertson, 1955; Rousselin et al., 1992; Tal & Nevo, 1973; Taylor et al., 1988; Wang et al., 1984). Among these mutants, *aba3* in Arabidopsis and *flacca* (*flc*) in tomato plants are characterized by mutations in the MoCo sulfurase gene (Min et al., 2000; Sagi et al., 1999, 2002) resulting in a strong ABA deficiency. These plants have served scientists to delve into the involvement of ABA cellular signaling processes. ABA-dependent responses encompass stomatal closure, growth inhibition, and the induction of stress-related genes. For instance, ABA-induced stomatal closure is mediated by the binding of ABA to PYR/PYL/RCAR receptors in guard cells, which activate ion channels and pumps, and ultimately lead to stomatal closure (Dittrich et al., 2019; Fujii et al., 2007).

Nevertheless, it has been established that a significant number of genes induced under various abiotic stress conditions do not respond to ABA treatments. For example, Du et al. (2018) discovered that in rice, certain flowering-related genes (*OsGL, OsELF3, OsPRR37*, and *OsMADS50*) were induced by water-deficit stress but remained unresponsive to ABA (Du et al., 2018). Moreover, their expression levels were unaffected in ABA-deficient mutants, indicating that these genes are involved in the drought regulation escape mechanism via an ABA-independent route (Du et al., 2018). In this context, an ABA-independent cell signaling transduction pathway has been elucidated, which operates independently of ABA receptors or ABA signaling components (Agarwal & Jha, 2010; Liu et al., 2018; Yoshida et al., 2014). However, the molecular intricacies governing ABA-dependent gene regulation and its potential interaction with ABA-dependent pathway in controlling gene expression under abiotic stress conditions remain largely uncharted due to the inherent complexities involved. Transcription factors, such as DREB2A/2B, AREB1, RD22BP1, and MYC/MYB, regulate ABA-responsive gene expression

by interacting with their corresponding cis-acting elements DRE/CRT, ABRE, and MYCRS/MYBRS (Tuteja, 2007). It has been demonstrated that DREB1 is activated by drought, salt stress, and exogenous ABA (Yang et al., 2011). Intriguingly, the overexpression of DREB1 upregulates both ABA-independent and ABA-dependent stress-induced genes (*COR15a* and *rd29B*, respectively) in *Arabidopsis*, suggesting a potential interaction between the ABA-dependent and independent pathways (Yang et al., 2011). Another study conducted by Lee et al. (2017) revealed that the *OsERF71* gene in rice participates in an ABA-independent pathway, enhancing drought resistance through the regulation of cell wall modifications (Lee et al., 2017). Similarly, a mutant harboring a T-DNA insertion in *AtMYB60* exhibited a reduced stomatal opening, with observable effects on water loss and transpiration rates during drought stress (Cominelli et al., 2005). Consequently, the regulation of this gene has been characterized as ABA-independent. Numerous studies have demonstrated the crucial roles played by R2R3-MYB transcription factors in plant responses to both biotic and abiotic stresses by following an ABA-dependent regulation, as well as their involvement in the regulation of secondary metabolite biosynthesis (Abe et al., 2003; Stracke et al., 2007). Thus, flavonoid levels are regulated through different and specific MYB factors, depending on the plant species. In this sense, *SlMYB72* in tomato (Wu et al., 2020), CmMYB8 in maize (Zhu et al., 2020), *AtMYB4* in *Arabidopsis* (Wang et al., 2020), *CsMYB2* and *CsMYB26* in tea (Wang et al., 2018), and *NtMYB3* in Narcissus (Anwar et al., 2019), have been successfully identified as the main regulators of the biosynthesis of flavonoids. It has to be noted that the majority of the research conducted on these R2R3-MYB transcription factor families has traditionally been associated with the ABA-dependent pathway. In a study by Zhao et al. (2014), which aimed to identify and characterize the R2R3-MYB family in tomato, transcript abundances of 51 R2R3-MYB genes were scrutinized under different stress treatments, including NaCl (100 mM), low temperature (4°C), and ABA (100 μM) (Zhao et al., 2014). Notably, only three genes (*SlMYB62, SlMYB74, and SlMYB102*) responded to all three treatments, and interestingly, nine genes (*SlMYB2, SlMYB20, SlMYB21, SlMYB53, SlMYB78, SlMYB92, SlMYB108, SlMYB113, and SlMYB114*) did not exhibit a response to the ABA treatment (Zhao et al., 2014).

Generally, the existing literature has primarily focused on the identification of genes involved in either ABA-dependent or ABA-independent pathways under single stress conditions. Given that plant gene regulation under abiotic stresses combination is highly specific and unique, it is imperative to conduct studies that explore the plant's transcriptome regulation under different abiotic stress combinations. Additionally, considering the significant fluctuations in ABA levels in response to environmental changes, identifying ABA-independent genes involved in stress signaling transduction pathways can be a valuable tool for the development of plants with an enhanced resilience to climate change.

In pursuit of this objective, the present study used tomato ABA-deficient mutants (*flacca, flc*, Solyc07g066480) subjected to single or combined stresses. The *flc* mutation results in a complete loss of AO and xanthine dehydrogenase (XDH) activities in the shoot, while a minor but measurable activity is retained in the roots, where low although detectable levels of ABA accumulate (Sagi et al., 1999). Through an extensive RNA-seq analysis of these *flc* mutants, this study revealed that the combination of salinity and heat strongly reprogrammed the tomato transcriptome. Notably, among the 685 genes that were specifically regulated under the combination of salinity and heat found in *flc* mutants, 463 genes exhibited an ABA-independent regulation under these specific conditions. Within this group of genes, six transcription factors belonging to the R2R3-MYB family were significantly upregulated. An analysis of protein–protein interactions further demonstrated that *SlMYB50* and *SlMYB86* were directly involved in the upregulation of genes related to the flavonol biosynthetic pathway. These findings provide evidence that the upregulation of *SlMYB50* and *SlMYB86* follows an ABA-independent pathway, and that this regulation is uniquely induced under the combination of salinity and heat stress in ABA-deficient plants. This resource article analyzes the complete transcriptome of wild-type tomato plants and ABA-deficient mutants grown under individual or combined stresses. It provides an important and novel reference for scientists working in this field. Future exploration of the data presented in this study could lead to the identification of new pathways and genes associated with ABA signaling processes. These findings may be utilized to enhance crop resilience to heat waves, salinity, and their combination, contributing to addressing food security challenges in a climate change scenario.

RESULTS AND DISCUSSION

ABA-deficient *flc* mutants showed a dramatic phenotype due to an impairment in stomatal regulation caused by ABA deficiency

ABA has multiple functions in plants, including those related to growth and development (Cutler et al., 2010). Consequently, a basal amount of ABA is essential for the development of plant tissues and organs. In our experiments, ABA-deficient tomato plants (*flc*) exhibited a significantly weaker phenotype as compared to the wild type (Wt) under control conditions (Figure 1a), with 70% less biomass than the latter (Figure 1b), which was primarily attributed to the deficiency in endogenous ABA content in leaves (Figure 1c; Imber & Tal, 1970). Interestingly, when

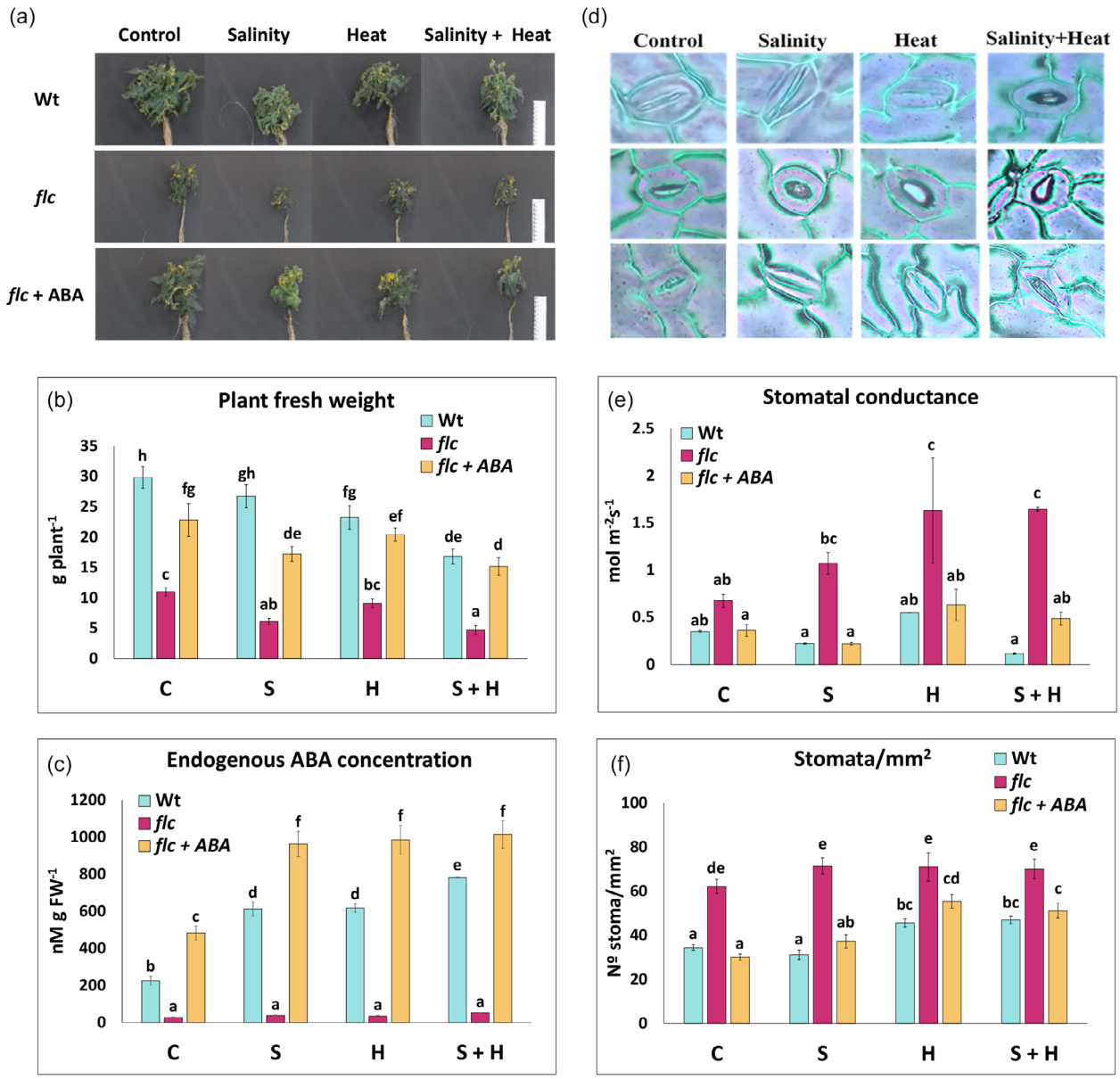


Figure 1. Physiological characterization in tomato wild-type (Wt) and *flacca* mutants (*flc*) plants under control (C), salinity (S, NaCl 100 mM), heat (H, 35°C), and combination of salinity and heat (S + H). (a) Pictures of tomato plants at the sampling time (b) Whole plant fresh weight (FW) of tomato plants (c) ABA endogenous concentration (d) Representative stoma of each of the genotype under the single or combined stress treatments. Whole micrographics for adaxial leaf surfaces can be found in Figures S3 and S4 (e) Stomatal conductance (f) N° stomata per mm^2. Values represent means ± SE ($n = 6$ in Figure 1b,f, $n = 3$ in Figure 1c,e,f). Letters within each panel are significantly different at $P < 0.05$ according to Duncan's test.

flc mutants were treated with 100 μM ABA sprayed to the leaves (a dose selected from a preliminary experiment, Figure S2), a substantial phenotype recovery was observed (Figure 1a,b). Under individual stress conditions, Wt showed a slight reduction in biomass, which became much more pronounced when salinity and heat were combined (Figure 1b). On the other hand, *flc* mutants biomass obtained in plants grown under heat stress was not significantly different to those plants grown under control conditions. However, *flc* mutants grown under salinity or stress combination showed a significant decrease in plant biomass as compared to the control conditions (Figure 1b). Thus, our results showed that ABA is required for plant growth under stress conditions.

Figure 1c shows that the ABA endogenous concentration increased under all the stress conditions applied to both, Wt and *flc* + ABA plants. Furthermore, the stress combination induced the highest accumulation of endogenous ABA levels in Wt plants, as compared to the levels found under single stress conditions in these plants

(Figure 1c). In *flc* mutants supplemented with ABA, whether exposed to single or combined stresses, a significant increase in the endogenous concentration of ABA was also observed, with no discernible differences between the different stress treatments. On the other hand, in *flc* mutants that were not treated with exogenous ABA, the endogenous concentration of this phytohormone remained very low, with no significant changes observed under the different stress conditions when compared to control plants (Figure 1c). These results confirm the ABA deficiency in our *flc* plants, and the effectiveness of ABA application (Figure 1c). However, it should be noted that despite these findings, a complete phenotypic recovery as compared to the Wt control was not obtained (Figure 1a,b), which points to the involvement of other ABA-independent factors in the plant's development and stress response.

ABA is directly associated with stomatal regulation processes (Hsu et al., 2021; Yoshida et al., 2019; Zandalinas, Balfagón, et al., 2016), and it is therefore important to study how the stomatal conductance in *flc* mutants behaves, and if an application of exogenous ABA has any effect on these mutants. Figure 1d shows a representative single stoma from both genotypes grown under control and under every stress condition applied (Figure S3 and S4). Wt and *flc* + ABA plants showed a clear stomata closure under salinity or heat applied individually, although the stress combination induced stomata opening in these plants (Figure 1d), which indicated the specificity of abiotic stress combination in the stomatal regulation of Wt and *flc* + ABA plants (Figure 1e). In fact, in *flc* plants, the stomatal conductance under heat stress and the combination of salinity and heat was significantly higher as compared to *flc* under control conditions (Figure 1e). It is remarkable that in Wt plants, the salinity and salinity+heat combination treatments induced a significant decrease ($P < 0.001$ ***), whereas heat induced a significant increase ($P < 0.001$***) in the stomatal conductance as compared to Wt plants, although these statistical differences were diluted when plotted with the data obtained in *flc* mutants, with values up to three times higher than Wt and *flc* + ABA. Similar results were previously obtained by our research group (Lopez-Delacalle et al., 2020, 2021; Rivero et al., 2014). When trying to elucidate if the stomatal conductance was stress- or stomatal number-related, we observed that stomata number did not follow the same trend as stomatal conductance in every genotype studied. The stomata number in *flc* plants was not significantly different between the different conditions applied, although it was observed that it was significantly higher as compared to Wt and *flc* + ABA plants under any condition tested (Figure 1f). However, a significant increase was observed in the number of stomata in Wt and *flc* + ABA plants under heat stress, and the combination of heat and salinity stresses, as compared to their respective controls (Figure 1f). In addition, ABA deficiency induced an increase in the stomata number, while heat and the combination of salinity and heat increased the stomata number in plants without an ABA deficiency. In short, ABA deficiency significantly increased the stomata number under all the conditions tested; however, in plants without this ABA deficiency (Wt and *flc* + ABA) the stomata number was only significantly higher under heat or the combination of salinity and heat. Our research group previously showed that in tomato plants, the transpiration rate increased under heat or the combination of salinity and heat as compared to the salinity treatment alone or in control plants (Lopez-Delacalle et al., 2020, 2021; Rivero et al., 2014). In this light, it can be hypothesized that heat stress (applied as a single stress or in combination with salinity) may be inducing an increase in the stomata number to increase the leaf transpiration index, thus reducing leaf temperature without affecting the stomatal conductance. On the other hand, the stomatal response of leaves is oftentimes different from that observed in flowers and fruits during stress combination (Sinha et al., 2022, 2023). These authors demonstrated that during a combination of drought and heat stress, soybean plants prioritized transpiration through flowers rather than leaves. This was achieved by stomata opening in the flowers, while maintaining those on the leaves closed. This adaptive approach, known as 'differential transpiration', results in a reduction of the inner temperature of the flowers by approximately 2–3°C. While this protects reproductive processes, it comes at the cost of the well-being of vegetative tissues (Sinha et al., 2023).

In summary, our study demonstrates that plant biomass is dependent on both ABA levels and stress conditions. Previous research has extensively documented the significant roles of ABA and stress conditions in plant growth and development (Segarra-Medina et al., 2023; Zandalinas, Rivero, et al., 2016). Consequently, as our results indicated, it was expected that the ABA deficiency imposed on *flc* mutants would result in a poor control of stomatal closure, which is consistent with previous findings (Tal et al., 1970). Our studies also demonstrated that an ABA deficiency leads to an increase in stomatal density (Figure 1f). Caine et al. (2023), in their work on several traditionally bred rice varieties, observed that a high stomatal density was associated with increased stomatal conductance and decreased biomass production (Caine et al., 2023). These observations support our findings in *flc* mutants (Figure 1b,c,e,f), as we showed a positive correlation between stomatal conductance and stomatal number (Figure 1c,e,f), which was negatively correlated with plant biomass.

Additionally, our results revealed a specific trend in plant biomass and ABA concentration in plants subjected to combined stress conditions across all genotypes tested, indicating the specificity of stress combination in the

plant's response to abiotic stress combination (Figure 1b,c) (Zandalinas, Balfagón, et al., 2016; Segarra-Medina et al., 2023). Finally, the application of 100 μM ABA did not lead to a complete recovery of the Wt phenotype, suggesting the existence of ABA-independent regulatory mechanisms (Li & Liu, 2021).

ABA-deficient *flc mutants* showed a specific regulation of 685 transcripts under the combination of salinity and heat, mostly related to cell catalytic, transcription regulation, and transporter activities

To further investigate how ABA deficiency can affect the transcriptional machinery of the plants tested, an RNA-seq analysis was performed on Wt and *flc* mutants after 10 days of growth under control, single, or combined stress conditions. For these analyses, metabolically active and fully expanded leaves were processed for RNA extraction, and RNA sequencing was performed by Macrogen Inc. (Seoul, Rep. of Korea) (Figure S5; Tables S1-S3). Wt control or *flc* control samples were used to normalize and obtain differentially expressed transcripts (DEGs) from Wt and *flc* plants, under a single or combined stress (Table S4). Considering all the stress conditions, our results showed that ABA-deficient *flc* mutants had a lower total number of DEGs (5691), as compared to Wt (6451) (Figure 2). This may indicate that *flc* mutants have a defective transcriptional machinery, which may be affected by ABA deficiency. It has been previously shown that ABA-dependent stress signaling first adjusts the constitutively expressed TFs, leading to the expression of early response transcriptional activators, which activate downstream stress tolerance effector genes (Hussain et al., 2021). Therefore, ABA-deficient mutants could be defective in this transcriptional adjustment, which is consistent with the stronger growth inhibition observed in *flc* mutants under any stress condition applied (Figure 1a,b). Salinity applied as an individual stress induced the lowest number of DEGs in both *flc* (1613) and Wt (975) plants (Figure 2a), although it was noted that only 10% of the DEGs found in the *flc* mutant under this stress were common to Wt. This observation, together with the fact that Wt plants grown under salinity stress shared only 40 (22 upregulated and 18 downregulated) transcripts with Wt plants grown under heat, and only 85 transcripts (18 upregulated and 67 downregulated) with Wt plants grown under the combination of salinity+heat, indicate that Wt plants were more tolerant to salinity stress than to heat or the combination of stresses, and that the plant's metabolism needs less transcriptome readjustment under salinity stress alone (Rivero et al., 2014). On the other hand, in *flc* mutants grown under salinity, 1288 transcripts out 1431 DEGs were specifically upregulated by this condition (~90%) (Figure 2a). This indicates that ABA-deficient *flc* mutants are more susceptible to salinity than Wt plants, and that under this condition, the ABA-dependent pathway plays an important role in the development of the salinity stress response (Hussain et al., 2021; Mathan et al., 2021). Under heat applied as an individual stress or under the combination of salinity and heat, *flc* mutants shared 65% and 47% of the DEGs with Wt plants under each condition, respectively, so half of the transcriptome response was heat- or salinity+heat-dependent (Figure 2a). But more importantly, the other half of the DEGs were genotype-dependent, highlighting the importance of the ABA-independent pathway in the plant's response to heat and salinity+heat.

Our research interest was centered on the DEGs present in Wt and *flc* mutants under the combination of salinity and heat, and not under single stress condition, as the latter have already been widely studied and published in previous decades. With this aim, the DEGs obtained from both genotypes under single and combined stresses were plotted in Venn diagrams (Figure 2b,c, Table S6–S9). Our results demonstrated that the combination of salinity and heat induced a specific transcriptomic response, in both Wt and *flc* mutants, which was different from that observed under salinity or heat applied individually (Figure 2b,c). It was observed that the number of genes specifically upregulated in *flc* mutants under the combination of salinity and heat were significantly higher (817) than those found in Wt (547) (Tables S6 and S7). This observation has been extensively reported by other researchers and highlights the importance of studying abiotic stress in combination (Lopez-Delacalle et al., 2021; Mikołajczak et al., 2022; Rivero et al., 2014; Zandalinas, Rivero, et al., 2016). A GO enrichment analysis based on the biological processes affected showed that in both genotypes, genes related to RNA modification and protein folding were significantly enriched (Table S10). Adverse environmental conditions, such as heat stress, can disrupt protein folding, which is an error-prone process, leading to endoplasmic reticulum stress, and consequently, triggering the unfolded protein response (UPR) that results in the accumulation of misfolded proteins (Li et al., 2020). Additionally, in *flc* mutants, many of these 817 genes that were specifically upregulated under the combination of salinity and heat were related to the transcription process, which revealed that there are many specific ABA-independent genes engaged to the transcriptional machinery in response to salinity and heat combination (Figure 2b; Table S11).

In addition, with respect to the number of genes that were specifically downregulated under the combination of salinity and heat (Figure 2c), both genotypes presented a similar number of DEGs (450 in Wt and 445 in *flc*) (Tables S8 and S9). However, a GO enrichment analysis revealed that in Wt plants, most of the important processes related to primary (photosynthesis, amino acid synthesis, pyruvate and TCA cycle, glyoxylate metabolism) and

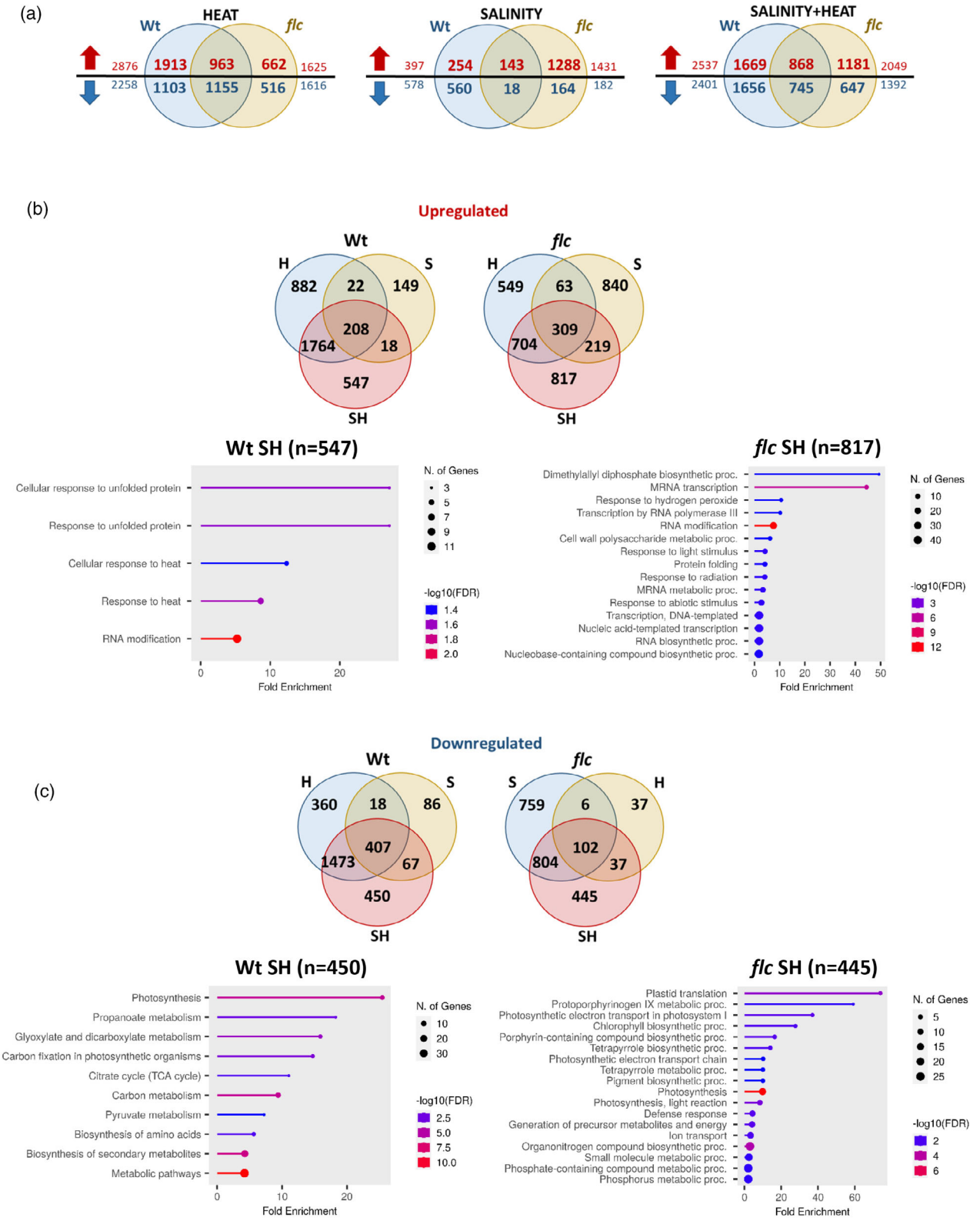


Figure 2. Differentially expressed genes (DEGs) in Wt and *flc* mutants under salinity, heat or the combination of salinity and heat. (a) Venn diagrams of the overlap between Wt and *flc* mutants in each stress treatment (b, c) Venn diagrams representing the upregulated (b) and downregulated (c) DEGs in Wt under salinity, heat and the combination of salinity+heat and the same for *flc* mutants. GO enrichment analysis for the specific DEGs upregulated (b) or downregulated (c) under the combination of salinity+heat in Wt and *flc* mutants.

The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd.,
The Plant Journal, (2024), doi: 10.1111/tpj.16642

secondary metabolism were significantly downregulated (Table S12). In *flc* mutants, these downregulated genes were mainly associated with the chlorophyll biosynthetic pathway (plastid translation, protoporphyrin synthesis, tetrapyrrole synthesis), electron transport in PSI, and phosphorous metabolic process. Under stress conditions, the decrease in chlorophyll production results in the accumulation of these intermediates alongside reactive oxygen species (ROS) (Table S13). It has been suggested that ROS, produced by porphyrin mediators, function as signaling molecules within the stress response pathway. Thus, it has been previously postulated that the ROS increase activates the stress response network, ultimately leading to the downregulation of genes associated with porphyrin metabolism and chlorophyll metabolism inhibition (Karami et al., 2023; Yun et al., 2013).

ABA-independent transcriptome reprogramming under the combination of salinity and heat induced the specific regulation of oxidative metabolism-, transcription-, and transport activity-related genes

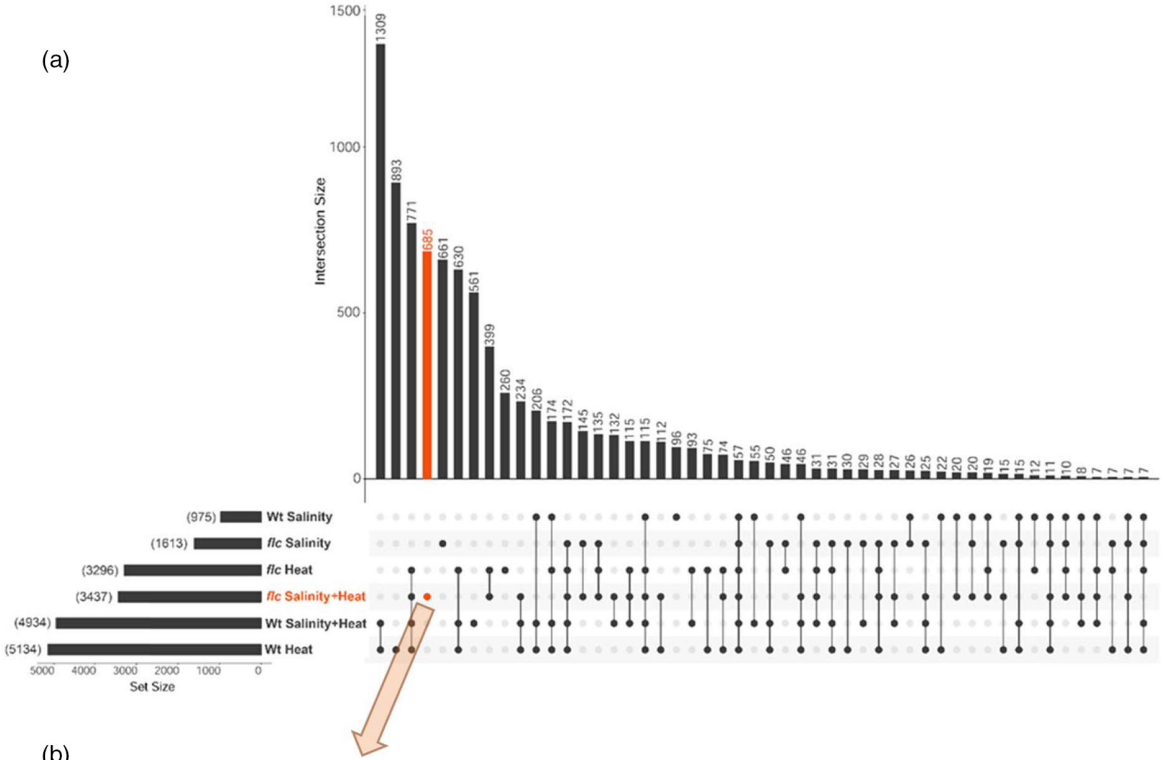
Given that studies on abiotic stress combination can provide a better insight on specific molecular markers involved in plant stress tolerance, and as our transcriptomic data showed a clear involvement of the ABA-independent pathway in this specific response, we were particularly interested in those DEGs that were specifically regulated in ABA-deficient *flc* mutants grown under stress combination, and at the same time, those that were not shared by any other stress condition or with Wt plants. Thus, we constructed an Upset Plot (Figure 3a), where the DEGs found in *flc* mutants under the combination of salinity and heat (2047 upregulated, 1390 downregulated, total 3437) were compared against all the DEGs found in Wt under any of the stress conditions used or in *flc* mutants under a single stress. A total of 685 DEGs were extracted (Figure 3a, marked in orange, Table S14). Interestingly, this stress combination yielded the second-highest number of DEGs (685), following Wt-Heat (893). These DEGs were not shared with any other genotype or condition used in our experiments, underscoring the specificity of this stress combination in the transcriptome reprogramming of ABA-deficient tomato plants. Suzuki et al. (2016) showed that *Arabidopsis* mutants deficient in abscisic acid metabolism and signaling were more susceptible to a combination of salt and heat stresses than wild-type plants, which supports our findings and demonstrates the significant plant transcriptional reprogramming under an ABA deficiency (Suzuki et al., 2016). We further extracted these 685 transcripts and a GO enrichment analysis was performed (http://www.pantherdb.org/geneListAnalysis.do), showing that most of them were related to catalytic (46%), transcription (40%), and transport activity (9%) (Figure 3b, Tables S15–S17). Among the DEGs with GO enrichment for catalytic activity, those related to oxidative metabolism almost comprised 20% of the total (Table S18), with most of them showing an upregulated expression under the combination of salinity and heat (Figure 3c; Table S13). Considering that all of these genes are only specifically regulated in *flc* mutants under the combination of salinity and heat, and that they were not found among the DEGs in Wt under any stress condition, the existence of an ABA-independent pathway associated with some oxidative metabolism-, transcription-, and transport activity-related genes is underlined here. It has been shown that ABA deficiency can trigger the expression of ROS-related genes to induce an ROS wave necessary for stomata control under abiotic stress in coordination with Ca^{2+} signaling waves (Mittler & Blumwald, 2015; Postiglione & Muday, 2020). Recent advances in the study of ROS signaling in plants involve the discovery of ROS receptors and crucial regulatory hubs. These components establish connections between ROS signaling and other significant pathways related to stress response and hormones (revised by Mittler et al., 2022). An ABA deficiency in our *flc* mutants specifically induced the expression of some ROS-related transcripts under the combination of salinity+heat, but the absence in stomata control (Figure 1d,e) may indicate that ABA levels are also fundamental for the Ca^{2+} waves and ROS signaling mechanisms needed to induce stomata closure (Mittler & Blumwald, 2015). DEGs related to binding and transcription regulation activity were also significantly enriched among the transcripts that were specifically regulated under the combination of salinity+heat (40%), confirming that ABA deficiency directly affects the transcriptional machinery reprogramming of tomato under the combination of salinity and heat.

The specific ABA-independent transcriptome reprogramming that occurs under the combination of salinity and heat induced the regulation of transcription – related and serine/threonine kinase – related genes

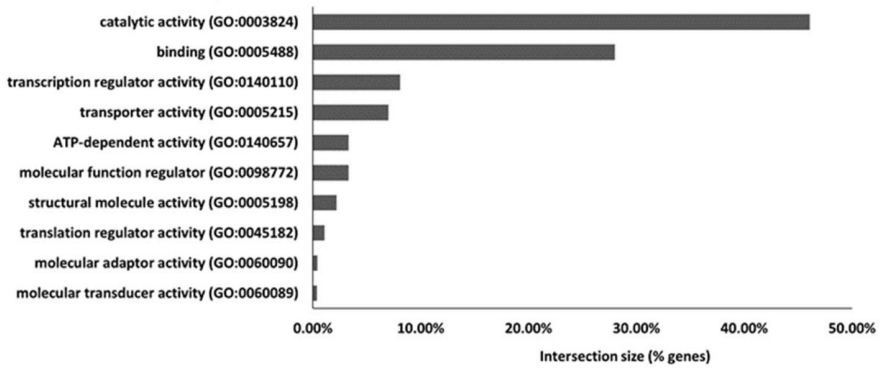
ABA-deficient *flc* mutants showed a basal ABA concentration (very low, but with some ABA synthesis and accumulation, Figure 1c), and therefore, it cannot be absolutely confirmed that the 685 DEGs found to be specifically regulated in *flc* under salinity and heat combination (Figure 3a), were also subjected to ABA-independent regulation.

Figure 3. (a) Upset plot displaying the intersections between the sets of DEGs found under single or combined stresses. Upregulated and downregulated genes for each genotype and condition were put in the same set for the specific DEGs subtraction for the *flc* mutant under the combination of salinity and heat. (b) GO enrichment analysis of the specific 685 DEGs found under the combination of salinity and heat. (c) Heatmap of the specific oxidative metabolism-related genes found to be differentially expressed in *flc* mutants under salinity+heat.

(a)



(b)



(c)

CATALYTIC ACTIVITY (GO:003824): Oxidative metabolism-related genes

Gene	Gene name	*flc* S	*flc* H	*flc* S+H	Wt S	Wt H	Wt S+H
SOLYC06G005750.3	METHYLSTEROL MONOOXYGENASE 2-2-LIKE ISOFORM X1 (PTHR11863:SF137)						
SOLYC07G043590.3	AMINO_OXIDASE DOMAIN-CONTAINING PROTEIN (PTHR10742:SF411)						
SOLYC04G083160.2	ENT-KAURENE OXIDASE, CHLOROPLASTIC (PTHR47283:SF1)						
SOLYC08G068420.3	L-GULONOLACTONE OXIDASE 3 (PTHR13878:SF125)						
SOLYC01G091320.3	METHYLSTEROL MONOOXYGENASE 1-1-LIKE (PTHR11863:SF121)						
SOLYC01G108020.3	THIOREDOXIN M3, CHLOROPLASTIC (PTHR45663:SF21)						
SOLYC02G084930.3	CYTOCHROME P450 FAMILY PROTEIN (PTHR24286:SF235)						
SOLYC09G082650.4	1,2-DIHYDROXY-3-KETO-5-METHYLTHIOPENTENE DIOXYGENASE (PTHR23418:SF0)						
SOLYC04G007960.4	INACTIVE PEROXYGENASE-LIKE PROTEIN-RELATED (PTHR31495:SF1)						
SOLYC05G021390.4	BETA-AMYRIN 28-MONOOXYGENASE (PTHR24286:SF331)						
SOLYC01G108210.4	ABSCISIC ACID 8'-HYDROXYLASE 4-LIKE (PTHR24286:SF375)						
SOLYC05G011940.4	CYTOCHROME P450 (PTHR24282:SF20)						
SOLYC01G080900.4	ENT-KAURENOIC ACID OXIDASE 2 (PTHR24286:SF356)						
SOLYC03G006810.3	PEROXIDASE 66 (PTHR31235:SF247)						
SOLYC04G082460.4	CATALASE-2 (PTHR11465:SF23)						
SOLYC02G077300.2	PEROXIDASE 19 (PTHR31235:SF56)						
SOLYC12G096780.2	TRANS-2-ENOYL-COA REDUCTASE, MITOCHONDRIAL-RELATED (PTHR43981:SF7)						
SOLYC02G069490.4	DELTA(24)-STEROL REDUCTASE-LIKE (PTHR10801:SF11)						
SOLYC09G097980.4	ALDO-KETO REDUCTASE YAKC [NADP(+)] (PTHR43625:SF40)						
SOLYC02G065590.3	Cytochrome b561_LD36721P (PTHR10106:SF0)						
SOLYC06G074090.3	7-DEHYDROCHOLESTEROL REDUCTASE (PTHR21257:SF38)						

The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd.,
The Plant Journal, (2024), doi: 10.1111/tpj.16642

Therefore, we performed an RNA-seq analysis on these *flc* mutants supplemented with 100 μM ABA (Figure 1; Tables S1–S3), to remove the DEGs that could be subject to ABA-dependent regulation.

After normalizing the data obtained in *flc* + ABA under single or combined stresses, against *flc* + ABA plants grown under control conditions, the DEGs obtained were matched with the 685 DEGs that were specifically expressed under the combination of salinity+heat found in our previous experiment (Figure 3a) through a Venn Diagram (Figure 4a, Table S19). *flc* + ABA plants grown under salinity+heat or under heat alone obtained the highest number of DEGs (6583 and 5978 respectively). From these, 1386 transcripts were specifically regulated in *flc* + ABA under the combination of salinity and heat, which again underlined the specificity of this stress combination on the plant's transcriptional response. In this light, 37 genes were common to the previously extracted 685 genes that were specifically regulated under the combination of salinity and heat (Figure 3a), indicating that these genes were perhaps subjected to ABA regulation. Similarly, another 185 genes in total were shared with the other abiotic stress conditions applied to *flc* + ABA, which were not considered for future analyses as they were considered to be ABA-dependent. Thus, a total of 222 transcripts were removed from the initial list of 685, and the remaining 463 genes were considered to be specific to the combination of salinity and heat and, most importantly, with an ABA-independent regulation (Figure 4a). These 463 DEGs were subjected to an enriched network molecular function analysis (http://bioinformatics.sdstate.edu/go/; Figure 4b) using a FDR cutoff of 0.02. A narrow and enriched network containing a total of 124 transcription-related genes was found (Figure 4b,c, Table S20). Additionally, a group of 17 genes related to serine/threonine kinase activity proteins were also enriched among these 463 DEGs (Figure 4b,c, Table S20). Previously (Figure 2b), the GO enrichment analysis performed with the 685 DEGs that were specifically regulated in *flc* mutants grown under the combination of salinity+heat showed that a high percentage of these transcripts had a binding/transcription activity (~40%). In the literature, several publications were found that demonstrated the transcriptional regulation of gene expression governed by various key TF pathways that operate in ABA-dependent and independent signaling pathways under abiotic stress (Hussain et al., 2021), which are regulated by AREB/ABFs (bZIP TFs) and DREB2A (AP2/ERF family of plant-specific TFs), respectively (Yoshida et al., 2014). DREB2 has been identified as the main TFs in ABA-independent pathways in *Arabidopsis* and soybean under single drought and/or salinity stresses (Chen et al., 2016; Yoshida et al., 2014). In addition, some studies have dealt with the functionality of these DREBs and the genes they may regulate under single abiotic stress conditions. However, as far as we know, this is the first evidence that shows an enriched list of 124 transcription-related genes under ABA-independent regulation, which are specifically regulated under the combination of salinity and heat. And more importantly, these TFs were not significantly regulated under heat or salinity applied as individual stresses.

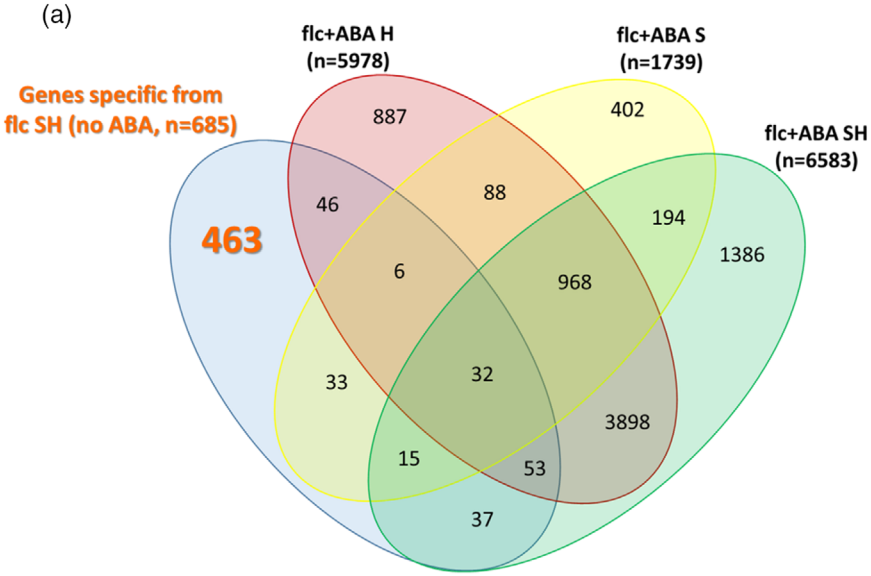
Of these 124 transcription-related genes (Figure 4c), some of them were repeated among the different GO molecular functions groups. Thus, only 49 true transcription-related transcripts were extracted and subjected to further analysis (Table S21).

SlMYB50 and *SlMYB86* TFs were ABA-independently upregulated under the combination of salinity and heat and were found to be related to flavonoid biosynthesis

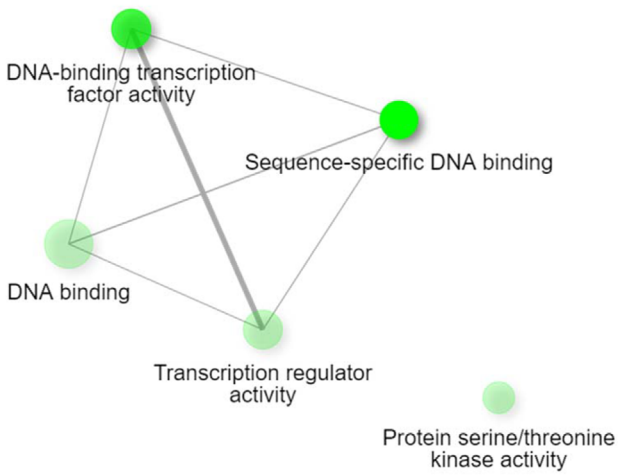
Forty-nine transcription-related DEGs were found to be specifically regulated under the combination of salinity and heat, and they were also selected for being under ABA-independent regulation. These 49 transcription factors (TFs) were grouped under the different known TF families, and it was found that most of these families were represented, under our specific conditions, by two to three TFs, except for the MYB family, which was represented by nine of them (Figure 5a, Table S21). Also, it was remarkable that most of the TFs found among all the TF families were upregulated, with only four TFs downregulated, indicating the need in *flc* mutants for transcriptional reprogramming within the ABA-independent pathway under the combination of salinity and heat. While it is true that the MYB family is the largest one, comprising a total of 140 TFs in tomato (PlantTFDB http://planttfdb.gao-lab.org/), it should also be noted that in our analysis, after screening out all the TFs that were specific to the combination of salinity and heat, and that followed an ABA-independent regulation, having nine TFs from the MYB family on this list, with almost all of them being upregulated, was something we considered of the upmost importance. Members of the MYB family are involved in many cell events, including plant growth (Oppenheimer et al., 1991), plant responses

Figure 4. Identification of ABA-independent genes specifically regulated by the combination of salinity and heat. (a) Venn diagram displaying the intersections between the sets of specific DEGs found in *flc* mutants under the combination of salinity and heat (n = 685, Figure 3) and DEGs found in *flc* mutants grown with ABA supplementation under single or combined stress. 463 DEGs were found for being specifically regulated under the combination of salinity and heat and with an ABA-independent regulation (b) Molecular functions pathway enrichment of ABA-independent DEGs specific to the combination of salinity+heat (FDR cutoff: 0.02). The size of the circles is proportional to the number of genes related to that pathway and the color intensity is related to the pathway P-value significance level ($P < 0.05$) (c) Number of genes representative of each pathway. The raw data used for this figure can be found in Table S20.

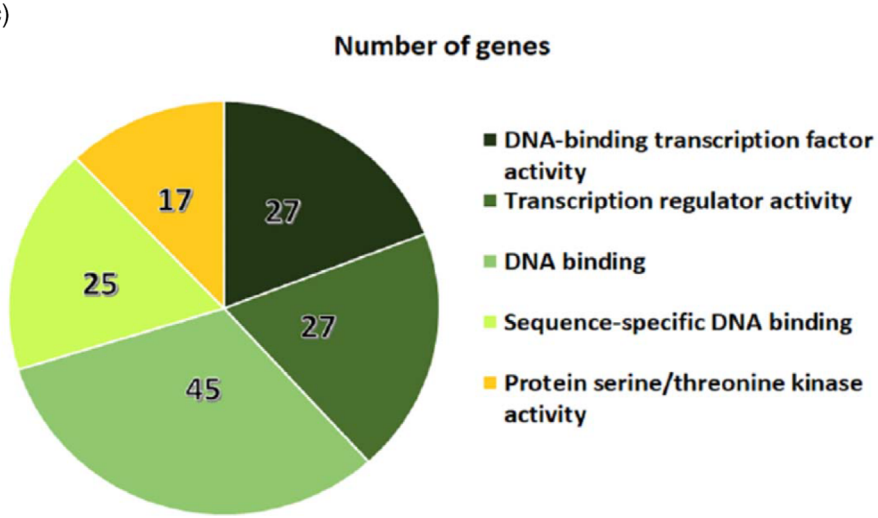
(a)



(b)



(c)



to environmental factors and hormones (Jin & Martin, 1999; Lee et al., 2007; Magaraggia et al., 1997; Urao et al., 1993), signal transduction processes (Gubler et al., 1995; Hamaguchi et al., 2008; Li et al., 2019), and pathogen defense (Noman et al., 2019; Shan et al., 2016), among others. It is also known that the regulation of TFs from the MYB family may be ABA-dependent or ABA-independent (Zhao et al., 2014).

There are four classes of MYB proteins depending on the number of adjacent repeats (one, two, three, or four). The three repeats of the prototypic MYB protein c-Myb are referred to as R1, R2, and R3 (Dubos et al., 2010; Kirik & Bäumlein, 1996). The most heterogeneous class comprises proteins with a single or a partial MYB repeat, collectively designated 'MYB-related', with the R2R3- MYB class being the largest (Dubos et al., 2010). Our results showed that six genes containing the R2R3 MYB domain (*SlMYB2*, *SlMYB102*, *SlMYB10*, *SlMYB50*, *SlMYB32*, and *SlMYB86*) were found to be upregulated, with the seventh (*SlMYB84*) repressed under our specific conditions (Figure 5b). R2R3MYB proteins play regulatory roles in developmental processes and defense responses in plants (Zhao et al., 2014), with a total of 121 R2R3MYB genes identified in the tomato genome. Moreover, the transcript abundance level under abiotic conditions identified a group of R2R3MYB genes that responded to one or more stresses, suggesting that SlR2R3MYBs played major roles in the plant's response to abiotic conditions, being involved in signal transduction pathways (Zhao et al., 2014). It has been shown that the *Arabidopsis AtMYB2* was under ABA-dependent regulation after the application of drought and salinity stress as individual stresses (Abe et al., 2003). Also in rice, Yang et al. (2012) demonstrated that *OsMYB2*-overexpressing plants were more tolerant to salt, cold, and dehydration stresses, and more sensitive to abscisic acid than wild-type plants (Yang et al., 2012). However, our results indicate an ABA-independent regulation of this *SlMYB2* under the combination of salinity and heat. On the other hand, Liao et al. (2008) showed that the transgenic *Arabidopsis* plants overexpressing soybean *GmMYB76*, *GmMYB92*, or *GmMYB177* showed a reduced sensitivity to ABA and exhibited better tolerance to salt and freezing treatments when compared with the Wt plants, following an ABA-independent regulation (Liao et al., 2008). Similarly, *OsMYB3R2* was also found to be under ABA-independent regulation under cold, drought, or salinity stress (Dai et al., 2007). The results from that study suggested an ABA-independent regulation of some R2R3MYB transcription factors under abiotic stress, but these experiments were only assayed under the application of a single stress and, nothing was shown about their specific regulation when abiotic stresses were applied in combination.

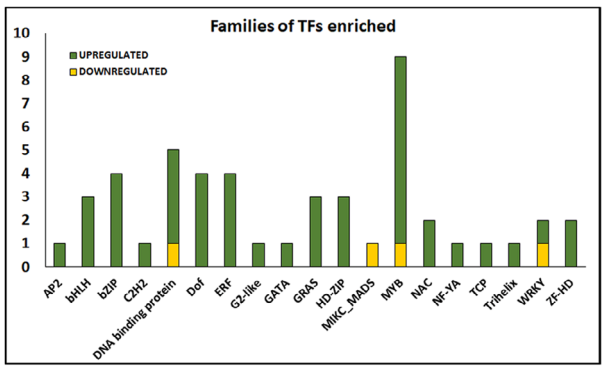
To positively verify an ABA-independent regulation of these selected MYB TFs, we analyzed all their promoter sequences. An ABA-responsive element (ABRE; ACGTGG/TC) was not found in any of them, except for *SlMYB84* (Solanaceae Genome Network database; https://solgenomics.net/). Since *SlMYB84* was the only gene from this TFs list that was downregulated, it can be hypothesized that an ABA deficiency governed its differential regulation.

To further delve into the putative role of the upregulation of these SlMYB-related transcription factors on the plant's response to the combination of salinity and heat, a predicted functional protein interaction network analysis was performed using STRING (https://string-db.org/) with these seven R2R3MYB genes (Figure S6). These predictive protein networks may help us to elucidate the potential targets of these TFs and their specific regulation. *SlMYB2* and *SlMYB102* were related to the spliceosome; *SlMYB32* were related to the biosynthesis of amino acids, and *SlMYB84* was related to DNA-binding function (Figure S6). Surprisingly, *SlMYB50* and *SlMYB86* showed an identical protein interaction network (Figure S7; Table S22), with a GO KEGG enrichment in the flavonoid synthesis pathway. The first hypothesis that emerged was that these two genes could perhaps be the same gene. However, after an identity check performed in the Solanaceae Genome Network database (https://solgenomics.net/tools/blast/) through a blast analysis (Figure S7), these two transcripts only shared 58.68% of identity (at the nucleotide and protein levels), and were therefore considered different and independent genes with an identical function. Thus, and due to the interest in studying the upregulation observed in these two transcription factors (*SlMYB50* and *SlMYB86*), which in turn may regulate the same genes, these two protein interaction networks were kept for further analyses. After performing a k-means clustering analysis within these two networks (Tables S23 and S24), as well as a GO enrichment analysis, two specific pathways were found to be enriched in them: flavonoids biosynthetic process (green) and phospholipid translocation (yellow) (Figure 5c).

Flavonoids comprise a large group of secondary metabolites with a broad range of biological functions,

Figure 5. Identification of SlMYB50 and SlMYB86 transcription factors (TFs) with an ABA-independent regulation and specifically induced under the combination of salinity and heat.(a) Number of upregulated and downregulated transcription factors (TFs) identified for having an ABA-independent and specific regulation under the combination of S + H (b) MYB-related TFs identified in Figure 5a (c) Predicted functional protein interaction networks for SlMYB50 and SlMYB86 (d) Fold-change expression values (log_2FC) of the genes represented within the SlMYB50 and SlMYB86 protein interaction networks in Wt and *flc* mutants under single and combined stresses. sf3a602: splicing factor subunit 3a, DF4R: dihydroflavonol 4-reductase, CHS: chalcone synthase, F3H: flavanone 3-hydroxylase and F3′H: flavonoid 3′-hydroxylase.

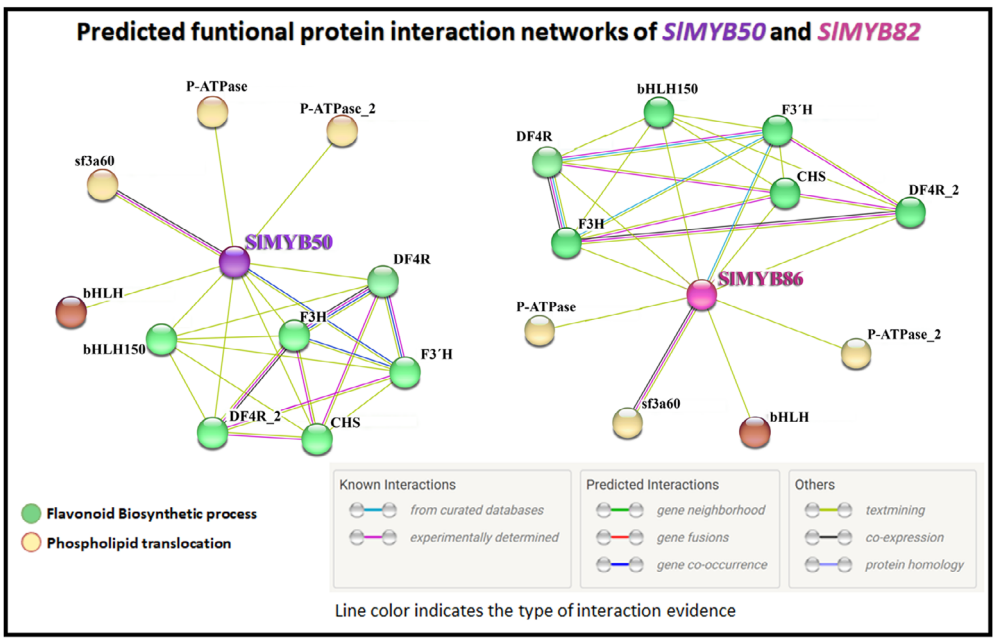
(a)



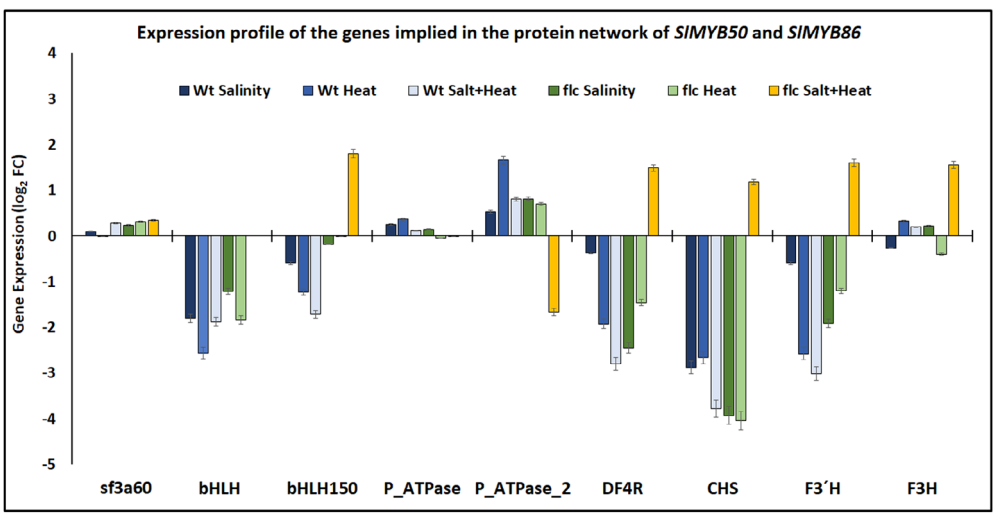
(b)

TF FAMILY	Gene	Annotation	Expression (Log2)	*p-adj*
MYB	Solyc01g057910.3	R2R3MYB transcription factor 2	1.50815967	0.00194724
MYB	Solyc05g055030.2	R2R3MYB transcription factor 10	1.53656306	0.000110984
MYB	Solyc10g055410.2	R2R3MYB transcription factor 32	1.47040198	0.008374574
MYB	Solyc06g071690.3	R2R3MYB transcription factor 50	1.34874388	0.004661306
MYB	Solyc03g112390.3	R2R3MYB transcription factor 86	1.17780703	0.000449706
MYB	Solyc02g091980.2	R2R3MYB transcription factor 84	-1.07388131	0.000272064
MYB	Solyc02g079280.3	R2R3MYB transcription factor 102	1.64056619	0.000799074
MYB_related	Solyc06g076270.3	Telomere repeat-binding protein 2	1.72629363	6.01E-05
MYB_related	Solyc03g121840.3	Telomere repeat-binding factor 4	1.12065279	0.003486189

(c)



(d)



including stress protection (Winkel-Shirley, 2002). The flavonoid biosynthesis pathway is a branch of the general phenylpropanoid pathway, with the first step catalyzed by chalcone synthase (CHS). In this pathway, the correlated activities of chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), and

flavonol synthase (FLS) result in the synthesis of the flavonols kaempferol and quercetin, which are further glycosylated by a number of glycosyltransferases (Winkel-Shirley, 2002). A few years ago, our research group showed that most of the genes that belong to the flavonoid biosynthetic pathway were differentially and specifically upregulated under the combination of salinity and heat in tomato plants, and that flavonols were accumulated over hydroxycinnamic acids (Martinez et al., 2016). The fold-change expression values (log_2) of the genes contained in these *SlMYB50* and *SlMYB86* protein interaction networks were measured in Wt and *flc* mutants under single and combined stresses (Figure 5d; Table S25). Surprisingly, those belonging to the flavonoids biosynthetic branch (DF4R, CHS, F3H, and F3′H) were significantly upregulated in *flc* mutants grown under the combination of salinity and heat, whereas they were downregulated or not significant in Wt (under any stress condition applied), or in *flc* mutants grown under single stress conditions. These results confirm that the overexpression of *SlMYB50* and *SlMYB86* followed an ABA-independent plus a salinity+-heat specific regulation and that those TFs may control the upregulation of the flavonoids biosynthetic-related genes. Thus, in tomato plants, *SlMYB50* and *SlMYB86* may be targeted as potential molecular markers for enhancing flavonoid biosynthesis and the plant's resilience to the combination of salinity and heat.

Plant flavonoids exhibit not only potent antioxidant properties but also function as regulators of cell metabolism. It is hypothesized that they can influence cell signaling pathways by interacting with receptors on the cell surface or by inserting themselves into the lipid bilayer of membranes. Certain flavonoids have the capability to enhance lipid viscosity and reduce the cooperativity of hydrocarbon chain melting, while others can significantly lower the lipid melting temperature, thereby allowing greater freedom for lipid diffusion. As lipid rafts play crucial roles in cellular signal transduction, endocytosis, and the transmembrane movement of various compounds, flavonoids may modulate cell metabolism by influencing the state of the lipid bilayer (Tarahovsky et al., 2008; Karonen, 2022). Our GO clustering analysis on *SlMYB50* and *SlMYB86* showed and significant enrichment on flavonoids biosynthetic process (green) but also on phospholipid translocation (yellow) (Figure 5c), pointing to the putative role of flavonoids on controlling phospholipid metabolism under the combination of salinity and heat in an ABA-independent manner. Further studies with tomato plants that either overexpress or have these two R2R3MYB TFs silenced will yield valuable insights into their role in conferring plant abiotic stress tolerance. Furthermore, CHIP-sequencing experiments performed in ABA-deficient plants would help to identify other novel genes specifically regulated under abiotic stress combination, which could be key in breeding crops with an enhanced tolerance to climate change.

MATERIAL AND METHODS

Plant growth and stress treatments

Wild-type (Wt) and the ABA-deficient mutants *flacca* (*flc*) (generously donated by TGRC, UC Davis, CA, USA, accession LA4479) of tomato (*Solanum lycopersicum* L. cv MicroTom) were used in all our experiments. *Flc* mutants are defective in the synthesis and/or maturation of the molybdenum cofactor (MoCo) sulfurylase gene (Solyc07g066480), with these mutants deficient in ABA synthesis. Seeds were sterilized and germinated in vermiculite under optimal and controlled conditions (25/20°C day/night, light intensity of 400 μmol m^{-2} sec^{-1}, 16/8 h day/night photoperiod, and 60–80% relative humidity). When the plants had at least two true leaves, 24 Wt plants and 48 *flc* mutants were transplanted into an aerated hydroponic system containing a modified Hoagland solution, and grown under controlled conditions until all of them developed four true leaves. The modified Hoagland solution had the following composition: KNO_3 (6 mM), $Ca(NO_3)_2$ (4 mM), $MgSO_4$ (1 mM), KH_2PO_4 (1 mM), KCl (50 μM), Fe-EDTA (20 μM), H_3BO_3 (25 μM), $MnSO_4{\cdot}H_2O$ (2 μM), $ZnSO_4{\cdot}7H_2O$ (2 μM), $CuSO_4{\cdot}5H_2O$ (0.5 μM), and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.5 μM) (Hoagland & Arnon, 1950). The pH of the nutrient solution was measured and maintained within 5.2–5.7, and the nutrient solution was renewed every 3 days.

When all the plants had developed four true leaves, half of the plants of each genotype (24 *flc* and 12 Wt) were transferred from Chamber A (where the plants were germinated and transplanted) to Chamber B, with the light, photoperiod, and humidity parameters set identical to Chamber A, except for the environmental temperature variable, which was set to 35°C, while the temperature remained at 25°C in Chamber A. At the same time, half of the plants (12 *flc* and 12 Wt) of each genotype received 100 mM NaCl in their nutrient solution. Therefore, the experiment consisted in four treatments (Figure S1): control (25°C and 0 mM NaCl), salinity (25°C and 100 mM NaCl), heat (35°C and 0 mM NaCl), and salinity + heat (35°C and 100 mM NaCl). In parallel, half of our *flc* mutants from each treatment group (24 plants, with 6 plants per treatment) received an exogenous application of 100 μM ABA through a foliar spray of 10 ml of this solution. (based on a preliminary experiment, described below). After this time, all the plants were separated into roots and leaves, and fresh weight (FW) was recorded. The leaves of each plant were immediately stored at −80°C for future determinations.

ABA-exogenous application experimental design

Forty-eight *flc* mutant plants were germinated, transplanted, and grown under control conditions (25/20°C day/night, light intensity of 400 μmol m^{-2} sec^{-1}, 16/8 h day/night photoperiod, and 60–80% relative humidity), as described previously. When the plants had developed four true leaves, different concentrations of exogenous ABA (0, 50, 100, 150 μM) were applied to a group of 12 plants per ABA treatment. These treatments were repeated three times a week. Plants were kept under these conditions for 1 month, and were physiologically characterized (Figure S2). The application of 100 μM of exogenous ABA was chosen as the best for the stress combination experiments described previously.

Stomatal conductance and stomata number determination

The stomatal conductance was measured every 3 days, as the stress treatments began in three single and fully expanded leaves

of each plant, using a LI-6400XT photosynthesis system (LICOR, Inc., Lincoln, NE, USA). Leaf gas exchange was measured in a 2 cm^2 leaf cuvette. During these measurements, the conditions set in the LICOR were 1000 μmol photons m^{-2} sec^{-1}, and 400 μmol mol^{-1} CO_2. The leaf-air vapor pressure deficit was maintained between 1 and 1.3 kPa. The data reported are the mean six biological replicates per treatment and genotype.

Alternatively, the stomata number was determined by leaf epidermal impressions on 50 × 75 mm slides fashioned from 4.74 mm (3/16 in) cellulose acetate butyrate (CAB)/acetone. The impression of the adaxial (upper) part of the tomato leaf epidermis was performed as described by (Gitz & Baker, 2009) and photographed in a microscope (Olympus CKX41, Olympus Life Science, Tokio, Japan).

ABA quantification

Endogenous ABA concentration was quantified in three biological samples per treatment and genotype in the Scientific and Technological Research Area (ACTI) of the University of Murcia. The extraction was carried out on 100 mg of frozen fresh leaf following the Müller and Munné-Bosch protocol (Müller & Munné--Bosch, 2011) with minor modifications (Müller & Munné-Bosch, 2011). Then, the organic extracts were injected into high-resolution UPLC-QToF-MS/MS equipment (Waters I-Class UPLC and Bruker Daltonics' maXis impact Series QToF-MS with a resolution of ≥55 000 FWHM). Ionization by electrospray (ESI) was used as the source MS with both positive and negative polarities. Broad Band Collision Induced Dissociation (±bbCID) was used for MS/MS analysis with collision energies applied between 20 and 24 eV. Other parameters included a voltage source of 4.0–4.5 kV, a nebulizer pressure of 2.0 bar, drying temperature of 200°C, a N_2 flow rate of 9 L/min, and a mass range of 50–1200 Da. Sodium Formate (HCOONa) was used as a reference standard. An ABA standard curve (2–1000 ppm) was used for calculating the endogenous concentration of ABA in leaf samples.

Total RNA extraction

Total RNA was isolated from 100 mg of frozen tomato leaves using the NucleoSpin® RNA kit (Reference no. 740949.50) in three biological replicates for each treatment and genotype. Total RNA was quantified and RNA integrity number (RIN) was checked in a bioanalyzer (2100 Expert Plant RNA Nano_DE13806178). 2 μg of total RNA with a RIN ≥8 of each genotype and treatment were sent to Macrogen (Seoul) to proceed with the RNA-sequencing analysis.

RNA sequencing, data analysis, functional annotation, and GO enrichment analysis

RNA sequencing was performed in an Illumina Hlseq (151 read-length paired-ends) analyzer. The raw reads obtained were filtered (Illumina passed-filter call) and further checked for sequence contaminants with fastQC (Table S1, S2, Figure S5). Contaminant-free, filtered reads for each sample were mapped with Bowtie/TopHat to the tomato genome sequence SL3.0 (ITAG4.0) (Hosmani et al., 2019) using HISAT2, which is known to handle spliced read mapping through the Bowtie2 aligner. After mapping, StringTie was used for transcript assembly. The expression profile was calculated for each sample and transcript/gene as read count, FPKM (Fragment per Kilobase of transcript per Million mapped reads) and TPM (Transcripts Per Kilobase Million) with a cutoff value of 0.1 DEG (Differentially Expressed Genes) analysis was performed on a comparison *t*-test pair using edgeR, where Wt and *flc* grown under the different stress treatments were compared with their respective control. The statistical analysis was performed using Fold Change, and exactTest per comparison pair. The significant results were selected on conditions of |fc| > =2 and exactTest raw *P*-value <0.05 (Table S3–S9). The DEG list was further analyzed with gProfiler (https://biit.cs.ut.ee/gprofiler/orth) and the Panther Classification System (http://www.pantherdb.org/geneListAnalysis.do; [Mi et al., 2019]) for gene set enrichment analysis (*q*-value 0.05) per biological process (BP), cellular component (CC), and molecular function (MF) (Table S10–S18). Upset plots were created in UpSetR (https://gehlenborglab.shinyapps.io/upsetr).

Network enrichment analyses

Specific DEGs found in *flc* mutants under the combination of salinity and heat (Table S20) were submitted to the platform ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go/) (Ge et al., 2020) with a FRD cutoff of 0.02 and a minimum pathway size of 5. The source code is available at https://github.com/iDEP-SDSU/idep/tree/master/shinyapps/go61.

For the construction of the protein–protein interaction network, the web-based tool STRING v11.5 (Szklarczyk et al., 2015), which is based on a large database of protein–protein interactions (PPI), was used. It also provides functionality for enrichment analysis of GO and protein domains in 5090 species (version 11), including tomato. The protein network analysis was performed based on the protein sequences as high confidence (0.7) with a maximum number of interaction of 10.

Statistical analysis

All the experiments were performed in at least three biological repeats using the IBM SPSS 26 Statistics program. An analysis of variance (ANOVA) with a *P*-value <0.05 was performed for fresh weight, stomatal conductance, stoma number per mm^2, and ABA concentration, followed by Duncan's test. Standard error (SE) values for the different treatments and genotypes was calculated and added as shown in the figures. Significant changes in transcript expression as compared to the control (DEGs) were defined as $\log_2$ FC < −2 or >2 and adjusted $P < 0.05$ (negative binomial Wald test followed by Benjamini–Hochberg correction) (Ferreira & Zwinderman, 2006).

ACKNOWLEDGEMENTS

This research was supported by the Ministry of Economy and Competitiveness from Spain (MCIU/AEI/FEDER, UE, Grant No. PGC2018-09573- B-100) to RMR; by the Ministry of Science and Innovation of Spain (Grant No. FPU20/03051) to MP-H. We sincerely acknowledge Mario G. Fon for proofreading the manuscript. We thank ACTI (Scientific and Technological Research Area for University of Murcia) for the assistance with the analysis. All authors declare no commercial, industrial links, or affiliations.

AUTHOR CONTRIBUTIONS

MP-H did the experiments, data management, statistical analyses, analyzed RNA-seq data, wrote the manuscript, and designed the figures and tables. IS contributed to the writing, editing, data analysis, and literature updating. VA analyzed ABA endogenous concentration. RMR conceived the project, supervised, analyzed RNA-seq data, corrected, and supervised with the writing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

DATA AVAILABILITY STATEMENT

The RNA-seq reads are available National Center for Biotechnology Information (NCBI) database under the Sequence Read Archive (SRA, https://submit.ncbi.nlm.nih.gov/subs/sra/), under the BioProject identification number PRJNA947059 (Submission ID: SUB12991832; direct link to datasets: https://www.ncbi.nlm.nih.gov/sra/PRJNA947059). Additionally, all the analyses are provided as raw data as supplementary material (Figure S1-S7, Tables S1-S25).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Experimental design followed in our experiments with Wt and *flc* mutants subjected to single or combined stresses.

Figure S2. Preliminary experiment in *flc* mutant for the establishment of the exogenous ABA concentration.

Figure S3. Light microscopy micrograph of adaxial leaf surface of tomato Wt and *flc* mutants grown under control, salinity, heat stress, or their combination. Scale bars: 100 μm.

Figure S4. Light microscopy micrograph of adaxial leaf surface of tomato *flc* mutants grown with an exogenous ABA supplementation (100 μM) under control, salinity, heat stress, or their combination. Scale bars: 100 μm.

Figure S5. Heatmap of the gene expression obtained for each sample and genotype in Wt and *flc* mutants grown under every stress condition applied. C: Control; S: Salinity; H: Heat; SH: Salinity+Heat. 1, 2, and 3 represent the respective biological replication.

Figure S6. Protein interaction network for (1) SIMYB2, (2) SIMYB102, (3) SIMYB10, (4) SIMYB50, (5) SIMYB32, (6) SIMYB86, (7) SIMYB84. The networks were constructed using STRING (https://string-db.org/) for *Solanum lycopersicum* L., with a minimum interaction score of 0,7 (high confidence) and using UniProt protein sequencing for each SIMYB transcription factor studied.

Figure S7. SIMYB50 and SLMYB86 identity check (https://solgenomics.net/tools/blast/) Proteins SIMYB50 and SLMYB86 showed a 58,68% identity, therefore they were considered as two different TFs with similar functions. They can regulate identical proteins in tomato metabolism, as shown in Figure 5c.

Table S1. Quality check of the reads obtained in the raw RNA-seq dataset for trimmed sequences.

Table S2. Mapping data statistics obtained in the RNA-seq analysis.

Table S3. Gene expression profile obtained for Wt and *flc* mutants. For the *flc* mutants, the presented samples are without or with 100 μM ABA exogenous application. Reads are shown as counts, Fragments Per Kilobase Million (FPKM) and Transcripts Per Kilobase Million (TPM).

Table S4. Differentially expressed genes (DEGs) obtained in Wt and *flc* mutants under salinity, heat or the combination of salinity and heat. Samples were normalized against control condition.

Table S5. Differentially expressed genes for Wt and *flc* as shown in the Venn diagram of Figure 2a.

Tables S6–S9. Differentially expressed genes for Wt and *flc* mutants as shown in the Venn diagram of Figure 2b,c.

Tables S10–S13. GO enrichment analysis in Wt and *flc* mutants specifically regulated under the combination of salinity and heat as shown in Figure 2b,c.

Tables S14–S17. GO enrichment analysis of DEGs with catalytic activity, transcription and transporter activity specifically regulated in *flc* mutants under the combination of salinity and heat.

Table S18. Oxidative metabolism-related genes contained in Table S15 (Catalytic activity). Expression data was taken from Table S4.

Table S19. Data for Venn diagram as shown in Figure 3a using specific DEGs found in *flc* Salt + Heat (without ABA application) and DEGs in *flc* (with ABA application) under salinity, heat and Salt+Heat. Only 463 DEGs were foundthat were not shared with any *flc* stress treatment with ABA application.

Table S20. Network enrichment analysis with the DEGs specifically regulated in *flc* mutants under the combination of salinity and heat and with an ABA-independent regulation.

Table S21. Transcription factor classification and fold-change found in *flc* mutants specifically regulated under the combination of salinity and heat and ABA-independent.

Table S22. K-means clustering analysis of SIMYB50 protein interaction network (http://string-db.org).

Table S23. K-means clustering analysis of SIMYB86 protein interaction network (http://string-db.org).

Table S24. Process, Component, Clusters, KEGG, Compartments, Pfam and InterPro Enrichment in SIMYB50 and SLMYB86 protein interaction network analysis found in *flc* mutants.

Table S25. Gene expression of the genes contained in SIMYB50 and SIMYB86 protein interaction network. Data extracted from Table S4.

REFERENCES

Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K. & **Yamaguchi-Shinozaki, K.** (2003) *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *The Plant Cell*, **15**, 63–78.

Agarwal, P.K. & **Jha, B.** (2010) Transcription factors in plants and ABA dependent and independent abiotic stress signalling. *Biologia Plantarum*, **54**, 201–212.

Anwar, M., Yu, W., Yao, H., Zhou, P., Allan, A.C. & **Zeng, L.** (2019) NtMYB3, an R2R3-MYB from narcissus, regulates flavonoid biosynthesis. *International Journal of Molecular Sciences*, **20**, 5456.

Caine, R.S., Harrison, E.L., Sloan, J., Flis, P.M., Fischer, S., Khan, M.S. *et al.* (2023) The influences of stomatal size and density on rice abiotic stress resilience. *New Phytologist*, **237**, 2180–2195.

Chen, H., Liu, L., Wang, L., Wang, S. & **Cheng, X.** (2016) VrDREB2A, a DREB-binding transcription factor from *Vigna radiata*, increased drought and high-salt tolerance in transgenic *Arabidopsis thaliana*. *Journal of Plant Research*, **129**, 263–273.

Cominelli, E., Galbiati, M., Vavasseur, A., Conti, L., Sala, T., Vuylsteke, M. *et al.* (2005) A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. *Current Biology*, **15**, 1196–1200.

Correia, B., Hancock, R.D., Amaral, J., Gomez-Cadenas, A., Valledor, L. & **Pinto, G.** (2018) Combined drought and heat activates protective responses in *Eucalyptus globulus* that are not activated when subjected to drought or heat stress alone. *Frontiers in Plant Science*, **9**, 819.

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R. & **Abrams, S.R.** (2010) Abscisic acid: emergence of a Core signaling network. *Annual Review of Plant Biology*, **61**, 651–679.

Dai, X., Xu, Y., Ma, Q., Xu, W., Wang, T., Xue, Y. *et al.* (2007) Overexpression of an R1R2R3 MYB gene, OsMYB3R-2, increases tolerance to freezing, drought, and salt stress in transgenic *Arabidopsis*. *Plant Physiology*, **143**, 1739–1751.

Dittrich, M., Mueller, H.M., Bauer, H., Peirats-Llobet, M., Rodriguez, P.L., Geilfus, C.M. *et al.* (2019) The role of *Arabidopsis* ABA receptors from the PYR/PYL/RCAR family in stomatal acclimation and closure signal integration. *Nature Plants*, **5**, 1002–1011.

Du, H., Huang, F., Wu, N., Li, X., Hu, H. & **Xiong, L.** (2018) Integrative regulation of drought escape through ABA-dependent and-independent pathways in rice. *Molecular Plant*, **11**, 584–597.

Dubos, C., **Stracke, R.**, **Grotewold, E.**, **Weisshaar, B.**, **Martin, C.** & **Lepiniec, L.** (2010) MYB transcription factors in *Arabidopsis*. *Trends in Plant Science*, **15**, 573–581.

Ferreira, J.A. & **Zwinderman, A.H.** (2006) On the Benjamini–Hochberg method. *The Annals of Statistics*, **34**, 1827–1849.

Fujii, H., **Verslues, P.E.** & **Zhu, J.-K.** (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. *The Plant Cell*, **19**, 485–494.

Ge, S.X., **Jung, D.** & **Yao, R.** (2020) ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics*, **36**, 2628–2629.

Giraudat, J., **Parcy, F.**, **Bertauche, N.**, **Gosti, F.**, **Leung, J.**, **Morris, P.C.** ***et al.*** (1994) Current advances in abscisic acid action and signalling. *Plant Molecular Biology*, **26**, 1557–1577.

Gitz, D.C. & **Baker, J.T.** (2009) Methods for creating stomatal impressions directly onto archivable slides. *Agronomy Journal*, **101**, 232–236.

Gubler, F., **Kalla, R.**, **Roberts, J.K.** & **Jacobsen, J.V.** (1995) Gibberellin-regulated expression of a myb gene in barley aleurone cells: evidence for Myb transactivation of a high-pI alpha-amylase gene promoter. *The Plant Cell*, **7**, 1879–1891.

Hamaguchi, A., **Yamshino, T.**, **Koizumi, N.**, **Kiba, T.**, **Kojima, M.**, **Sakakibara, H.** ***et al.*** (2008) A small subfamily of *Arabidopsis* RADIALIS-LIKE SANT/-MYB genes: a link to HOOKLESS1-mediated signal transduction during early morphogenesis. *Bioscience, Biotechnology, and Biochemistry*, **72**, 2687–2696.

Hoagland, D.R. & **Arnon, D.I.** (1950) The water-culture method for growing plants without soil. In: *Circular. California Agricultural Experiment Station*, **347**, pp. 1–7. Available from: https://archive.org/download/waterculturemeth347hoag/waterculturemeth347hoag.pdf [Accessed March 6, 2023].

Hosmani, P.S., **Flores-Gonzalez, M.**, **van de Geest, H.** ***et al.*** (2019) An improved de novo assembly and annotation of the tomato reference genome using single-molecule sequencing, Hi-C proximity ligation and optical maps, 767764. https://doi.org/10.1101/767764v1 [Accessed March 9, 2023].

Hsu, P.-K., **Dubeaux, G.**, **Takahashi, Y.** & **Schroeder, J.I.** (2021) Signaling mechanisms in abscisic acid-mediated stomatal closure. *The Plant Journal*, **105**, 307–321.

Hussain, Q., **Asim, M.**, **Zhang, R.**, **Khan, R.**, **Farooq, S.** & **Wu, J.** (2021) Transcription factors interact with ABA through gene expression and signaling pathways to mitigate drought and salinity stress. *Biomolecules*, **11**, 1159.

Imber, D. & **Tal, M.** (1970) Phenotypic reversion of Flacca, a Wilty mutant of tomato, by abscisic acid. *Science*, **169**, 592–593.

Jin, H. & **Martin, C.** (1999) Multifunctionality and diversity within the plant MYB-gene family. *Plant Molecular Biology*, **41**, 577–585.

Karami, S., **Shiran, B.**, **Ravash, R.** & **Fallahi, H.** (2023) A comprehensive analysis of transcriptomic data for comparison of plants with different photosynthetic pathways in response to drought stress. *PLoS One*, **18**, e0287761.

Karonen, M. (2022) Insights into polyphenol-lipid interactions: chemical methods, molecular aspects and their effects on membrane structures. *Plants (Basel)*, **11**, 1809.

Kirik, V. & **Bäumlein, H.** (1996) A novel leaf-specific myb-related protein with a single binding repeat. *Gene*, **183**, 109–113.

Koornneef, M., **Jorna, M.L.**, **Brinkhorst-van der Swan, D.L.C.** & **Karssen, C.M.** (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) heynh. *Theoretical and Applied Genetics*, **61**, 385–393.

Larkindale, J. & **Knight, M.R.** (2002) Protection against heat stress-induced oxidative damage in *Arabidopsis* involves calcium, abscisic acid, ethylene, and salicylic acid. *Plant Physiology*, **128**, 682–695.

Lee, D.-K., **Yoon, S.**, **Kim, Y.S.** & **Kim, J.-K.** (2017) Rice OsERF71-mediated root modification affects shoot drought tolerance. *Plant Signaling & Behavior*, **12**, e1268311.

Lee, T.G., **Jang, C.S.**, **Kim, J.Y.**, **Kim, D.S.**, **Park, J.H.**, **Kim, D.Y.** ***et al.*** (2007) A Myb transcription factor (TaMyb1) from wheat roots is expressed during hypoxia: roles in response to the oxygen concentration in root environment and abiotic stresses. *Physiologia Plantarum*, **129**, 375–385.

Leydecker, M.T., **Moureaux, T.**, **Kraepiel, Y.**, **Schnorr, K.** & **Caboche, M.** (1995) Molybdenum cofactor mutants, specifically impaired in xanthine dehydrogenase activity and abscisic acid biosynthesis, simultaneously overexpress nitrate reductase. *Plant Physiology*, **107**, 1427–1431.

Li, J., **Han, G.**, **Sun, C.** & **Sui, N.** (2019) Research advances of MYB transcription factors in plant stress resistance and breeding. *Plant Signaling & Behavior*, **14**, 1613131.

Li, S. & **Liu, F.** (2021) Exogenous Abscisic acid priming modulates water relation responses of two tomato genotypes with contrasting endogenous abscisic acid levels to progressive soil drying under elevated CO2. *Frontiers in Plant Science*, **12**, 733658.

Li, Z., **Tang, J.**, **Srivastava, R.**, **Bassham, D.C.** & **Howell, S.H.** (2020) The transcription factor bZIP60 links the unfolded protein response to the heat stress response in maize. *The Plant Cell*, **32**, 3559–3575.

Liao, Y., **Zou, H.-F.**, **Wang, H.-W.**, **Zhang, W.-K.**, **Ma, B.**, **Zhang, J.-S.** ***et al.*** (2008) Soybean GmMYB76, GmMYB92, and GmMYB177 genes confer stress tolerance in transgenic *Arabidopsis* plants. *Cell Research*, **18**, 1047–1060.

Liu, S., **Lv, Z.**, **Liu, Y.**, **Li, L.** & **Zhang, L.** (2018) Network analysis of ABA-dependent and ABA-independent drought responsive genes in *Arabidopsis thaliana*. *Genetics and Molecular Biology*, **41**, 624–637.

Liu, S., **Sun, B.**, **Cao, B.**, **Lv, Y.**, **Chen, Z.** & **Xu, K.** (2023) Effects of soil waterlogging and high-temperature stress on photosynthesis and photosystem II of ginger (Zingiber officinale). *Protoplasma*, **260**, 405–418.

Lopez-Delacalle, M., **Camejo, D.M.**, **García-Martí, M.**, **Nortes, P.A.**, **Nieves-Cordones, M.**, **Martínez, V.** ***et al.*** (2020) Using tomato recombinant lines to improve plant tolerance to stress combination through a more efficient nitrogen metabolism. *Frontiers in Plant Science*, **10**, 1702. Available from: https://doi.org/10.3389/fpls.2019.01702 [Accessed February 24, 2023].

Lopez-Delacalle, M., **Silva, C.J.**, **Mestre, T.C.**, **Martinez, V.**, **Blanco-Ulate, B.** & **Rivero, R.M.** (2021) Synchronization of proline, ascorbate and oxidative stress pathways under the combination of salinity and heat in tomato plants. *Environmental and Experimental Botany*, **183**, 104351.

Magaraggia, F., **Solinas, G.**, **Valle, G.**, **Giovinazzo, G.** & **Coraggio, I.** (1997) Maturation and translation mechanisms involved in the expression of a myb gene of rice. *Plant Molecular Biology*, **35**, 1003–1008.

Mariani, L. & **Ferrante, A.** (2017) Agronomic management for enhancing plant tolerance to abiotic stresses—drought, salinity, hypoxia, and lodging. *Horticulturae*, **3**, 52.

Martinez, V., **Mestre, T.C.**, **Rubio, F.**, **Girones-Vilaplana, A.**, **Moreno, D.A.**, **Mittler, R.** ***et al.*** (2016) Accumulation of flavonols over hydroxycinnamic acids favors oxidative damage protection under abiotic stress. *Frontiers in Plant Science*, **7**, 838. Available from: https://doi.org/10.3389/fpls.2016.00838 [Accessed March 13, 2023].

Mathan, J., **Singh, A.** & **Ranjan, A.** (2021) Sucrose transport in response to drought and salt stress involves ABA-mediated induction of OsSWEET13 and OsSWEET15 in rice. *Physiologia Plantarum*, **171**, 620–637.

Mi, H., **Muruganujan, A.**, **Huang, X.**, **Ebert, D.**, **Mills, C.**, **Guo, X.** ***et al.*** (2019) Protocol update for large-scale genome and gene function analysis with the PANTHER classification system (v.14.0). *Nature Protocols*, **14**, 703–721.

Mikołajczak, K., **Kuczyńska, A.**, **Krajewski, P.**, **Kempa, M.** & **Nuc, M.** (2022) Transcriptome profiling disclosed the effect of single and combined drought and heat stress on reprogramming of genes expression in barley flag leaf. *Frontiers in Plant Science*, **13**, 1096685.

Min, X., **Okada, K.**, **Brockmann, B.**, **Koshiba, T.** & **Kamiya, Y.** (2000) Molecular cloning and expression patterns of three putative functional aldehyde oxidase genes and isolation of two aldehyde oxidase pseudogenes in tomato. *Biochimica et Biophysica Acta (BBA)—Gene Structure and Expression*, **1493**, 337–341.

Mittler, R. (2006) Abiotic stress, the field environment and stress combination. *Trends in Plant Science*, **11**, 15–19.

Mittler, R. & **Blumwald, E.** (2015) The roles of ROS and ABA in systemic acquired acclimation. *The Plant Cell*, **27**, 64–70.

Mittler, R., **Zandalinas, S.I.**, **Fichman, Y.** & **Van Breusegem, F.** (2022) Reactive oxygen species signalling in plant stress responses. *Nature Reviews. Molecular Cell Biology*, **23**, 663–679.

Muhammad Aslam, M., **Waseem, M.**, **Jakada, B.H.**, **Okal, E.J.**, **Lei, Z.**, **Saqib, H.S.A.** ***et al.*** (2022) Mechanisms of abscisic acid-mediated drought stress responses in plants. *International Journal of Molecular Sciences*, **23**, 1084.

Müller, M. & **Munné-Bosch, S.** (2011) Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. *Plant Methods*, **7**, 37.

Nahar, L., **Aycan, M.**, **Hanamata, S.**, **Baslam, M.** & **Mitsui, T.** (2022) Impact of single and combined salinity and high-temperature stresses on agro-physiological, biochemical, and transcriptional responses in rice and stress-release. *Plants (Basel)*, **11**, 501.

Nakashima, K. & **Yamaguchi-Shinozaki, K.** (2013) ABA signaling in stress-response and seed development. *Plant Cell Reports*, **32**, 959–970.

Neill, S.J., **Horgan, R.** & **Parry, A.D.** (1986) The carotenoid and abscisic acid content of viviparous kernels and seedlings ofZea mays L. *Planta*, **169**, 87–96.

Noman, A., **Hussain, A.**, **Adnan, M.**, **Khan, M.I.**, **Ashraf, M.F.**, **Zainab, M.** ***et al.*** (2019) A novel MYB transcription factor CaPHL8 provide clues about evolution of pepper immunity againstsoil borne pathogen. *Microbial Pathogenesis*, **137**, 103758.

Oppenheimer, D.G., **Herman, P.L.**, **Sivakumaran, S.**, **Esch, J.** & **Marks, M.D.** (1991) A myb gene required for leaf trichome differentiation in *Arabidopsis* is expressed in stipules. *Cell*, **67**, 483–493.

Parry, A.D., **Blonstein, A.D.**, **Babiano, M.J.**, **King, P.J.** & **Horgan, R.** (1991) Abscisic-acid metabolism in a wilty mutant of *Nicotiana plumbaginifolia*. *Planta*, **183**, 237–243.

Popova, L.P., **Stoinova, Z.G.** & **Maslenkova, L.T.** (1995) Involvement of abscisic acid in photosynthetic process in *Hordeum vulgare* L. during salinity stress. *Journal of Plant Growth Regulation*, **14**, 211–218.

Postiglione, A.E. & **Muday, G.K.** (2020) The role of ROS homeostasis in ABA-induced guard cell signaling. *Frontiers in Plant Science*, **11**. Available from: https://doi.org/10.3389/fpls.2020.00968 [Accessed March 13, 2023].

Quarrie, S.A. (1982) Droopy: a wilty mutant of potato deficient in abscisic acid. *Plant, Cell & Environment*, **5**, 23–26.

Rivero, R.M., **Mestre, T.C.**, **Mittler, R.**, **Rubio, F.**, **Garcia-Sanchez, F.** & **Martinez, V.** (2014) The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. *Plant, Cell & Environment*, **37**, 1059–1073.

Robertson, D.S. (1955) The genetics of vivipary in maize. *Genetics*, **40**, 745–760.

Rousselin, P., **Kraepiel, Y.**, **Maldiney, R.**, **Miginiac, E.** & **Caboche, M.** (1992) Characterization of three hormone mutants of *Nicotiana plumbaginifolia*: evidence for a common ABA deficiency. *Theoretical and Applied Genetics*, **85**, 213–221.

Sagi, M., **Fluhr, R.** & **Lips, S.H.** (1999) Aldehyde oxidase and xanthine dehydrogenase in a flacca tomato mutant with deficient abscisic acid and Wilty Phenotype1. *Plant Physiology*, **120**, 571–578.

Sagi, M., **Scazzocchio, C.** & **Fluhr, R.** (2002) The absence of molybdenum cofactor sulfuration is the primary cause of the flacca phenotype in tomato plants. *The Plant Journal*, **31**, 305–317.

Segarra-Medina, C., **Alseekh, S.**, **Fernie, A.R.**, **Rambla, J.L.**, **Pérez-Clemente, R.M.**, **Gómez-Cádenas, A.** ***et al.*** (2023) Abscisic acid promotes plant acclimation to the combination of salinity and high light stress. *Plant Physiology and Biochemistry*, **203**, 108008.

Shan, T., **Rong, W.**, **Xu, H.**, **Du, L.**, **Liu, X.** & **Zhang, Z.** (2016) The wheat R2R3-MYB transcription factor TaRIM1 participates in resistance response against the pathogen *Rhizoctonia cerealis* infection through regulating defense genes. *Scientific Reports*, **6**, 28777.

Shanker, A.K., **Gunnapaneni, D.**, **Bhanu, D.**, **Vanaja, M.**, **Lakshmi, N.J.**, **Yadav, S.K.** ***et al.*** (2022) Elevated CO_2 and water stress in combination in plants: brothers in arms or partners in crime? *Biology (Basel)*, **11**, 1330.

Sharma, R., **Singh, G.**, **Bhattacharya, S.** & **Singh, A.** (2018) Comparative transcriptome meta-analysis of *Arabidopsis thaliana* under drought and cold stress. *PLoS One*, **13**, e0203266.

Sinha, R., **Shostak, B.**, **Induri, S.P.**, **Sen, S.**, **Zandalinas, S.I.**, **Joshi, T.** ***et al.*** (2023) Differential transpiration between pods and leaves during stress combination in soybean. *Plant Physiology*, **192**, 753–766.

Sinha, R., **Zandalinas, S.I.**, **Fichman, Y.**, **Sen, S.**, **Zeng, S.**, **Gómez-Cadenas, A.** ***et al.*** (2022) Differential regulation of flower transpiration during abiotic stress in annual plants. *New Phytologist*, **235**, 611–629.

Stracke, R., **Ishihara, H.**, **Huep, G.**, **Barsch, A.**, **Mehrtens, F.**, **Niehaus, K.** ***et al.*** (2007) Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. *The Plant Journal*, **50**, 660–677.

Suzuki, N., **Bassil, E.**, **Hamilton, J.S.**, **Inupakutika, M.A.**, **Zandalinas, S.I.**, **Tripathy, D.** ***et al.*** (2016) ABA is required for plant acclimation to a combination of salt and heat stress. *PLoS One*, **11**, e0147625.

Szklarczyk, D., **Franceschini, A.**, **Wyder, S.**, **Forslund, K.**, **Heller, D.**, **Huerta-Cepas, J.** ***et al.*** (2015) STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Research*, **43**, D447–D452.

Tal, M., **Imber, D.** & **Itai, C.** (1970) Abnormal stomatal behavior and hormonal imbalance in flacca, a wilty mutant of tomato: I. Root effect and kinetin-like activity. *Plant Physiology*, **46**, 367–372.

Tal, M. & **Nevo, Y.** (1973) Abnormal stomatal behavior and root resistance, and hormonal imbalance in three wilty mutants of tomato. *Biochemical Genetics*, **8**, 291–300.

Tarahovsky, Y.S., **Muzafarov, E.N.** & **Kim, Y.A.** (2008) Rafts making and rafts braking: how plant flavonoids may control membrane heterogeneity. *Molecular and Cellular Biochemistry*, **314**, 65–71.

Taylor, I.B., **Linforth, R.S.T.**, **Al-Naieb, R.J.**, **Bowman, W.R.** & **Marples, B.A.** (1988) The wilty tomato mutants flacca and sitiens are impaired in the oxidation of ABA-aldehyde to ABA. *Plant, Cell & Environment*, **11**, 739–745.

Tuteja, N. (2007) Abscisic acid and abiotic stress signaling. *Plant Signaling & Behavior*, **2**, 135–138.

Urao, T., **Yamaguchi-Shinozaki, K.**, **Urao, S.** & **Shinozaki, K.** (1993) An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *The Plant Cell*, **5**, 1529–1539.

Walker-Simmons, M., **Kudrna, D.A.** & **Warner, R.L.** (1989) Reduced accumulation of ABA during water stress in a molybdenum cofactor mutant of barley 1. *Plant Physiology*, **90**, 728–733.

Wang, T.L., **Donkin, M.E.** & **Martin, E.S.** (1984) The physiology of a wilty pea: abscisic acid production under water stress. *Journal of Experimental Botany*, **35**, 1222–1232.

Wang, W.-L., **Wang, Y.-X.**, **Li, H.**, **Liu, Z.-W.**, **Cui, X.** & **Zhuang, J.** (2018) Two MYB transcription factors (CsMYB2 and CsMYB26) are involved in flavonoid biosynthesis in tea plant [*Camellia sinensis* (L.) O. Kuntze]. *BMC Plant Biology*, **18**, 288.

Wang, X.-C., **Wu, J.**, **Guan, M.-L.**, **Zhao, C.-H.**, **Geng, P.** & **Zhao, Q.** (2020) *Arabidopsis* MYB4 plays dual roles in flavonoid biosynthesis. *The Plant Journal*, **101**, 637–652.

Winkel-Shirley, B. (2002) Biosynthesis of flavonoids and effects of stress. *Current Opinion in Plant Biology*, **5**, 218–223.

Wu, M., **Xu, X.**, **Hu, X.**, **Liu, Y.**, **Cao, H.**, **Chan, H.** ***et al.*** (2020) SlMYB72 regulates the metabolism of chlorophylls, carotenoids, and flavonoids in tomato fruit. *Plant Physiology*, **183**, 854–868.

Yang, A., **Dai, X.** & **Zhang, W.-H.** (2012) A R2R3-type MYB gene, OsMYB2, is involved in salt, cold, and dehydration tolerance in rice. *Journal of Experimental Botany*, **63**, 2541–2556.

Yang, W., **Liu, X.-D.**, **Chi, X.-J.**, **Wu, C.A.**, **Li, Y.Z.**, **Song, L.L.** ***et al.*** (2011) Dwarf apple MbDREB1 enhances plant tolerance to low temperature, drought, and salt stress via both ABA-dependent and ABA-independent pathways. *Planta*, **233**, 219–229.

Yoshida, T., **Christmann, A.**, **Yamaguchi-Shinozaki, K.**, **Grill, E.** & **Fernie, A.R.** (2019) Revisiting the basal role of ABA – roles outside of stress. *Trends in Plant Science*, **24**, 625–635.

Yoshida, T., **Mogami, J.** & **Yamaguchi-Shinozaki, K.** (2014) ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Current Opinion in Plant Biology*, **21**, 133–139.

Yun, Y.B., **Park, J.I.**, **Choi, H.S.**, **Jung, H.**, **Jang, S.J.**, **Back, K.** ***et al.*** (2013) Protoporphyrinogen oxidase–overexpressing transgenic rice is resistant to drought stress. *Crop Science*, **53**, 1076–1085.

Zandalinas, S.I., **Balfagón, D.**, **Arbona, V.**, **Gómez-Cadenas, A.**, **Inupakutika, M.A.** & **Mittler, R.** (2016) ABA is required for the accumulation of APX1 and MBF1c during a combination of water deficit and heat stress. *Journal of Experimental Botany*, **67**, 5381–5390.

Zandalinas, S.I., **Rivero, R.M.**, **Martínez, V.**, **Gómez-Cadenas, A.** & **Arbona, V.** (2016) Tolerance of citrus plants to the combination of high temperatures and drought is associated to the increase in transpiration modulated by a reduction in abscisic acid levels. *BMC Plant Biology*, **16**, 105.

Zandalinas, S.I., **Sengupta, S.**, **Fritschi, F.B.**, **Azad, R.K.**, **Nechushtai, R.** & **Mittler, R.** (2021) The impact of multifactorial stress combination on plant growth and survival. *New Phytologist*, **230**, 1034–1048.

Zhao, P., **Li, Q.**, **Li, J.**, **Wang, L.** & **Ren, Z.** (2014) Genome-wide identification and characterization of R2R3MYB family in *Solanum lycopersicum*. *Molecular Genetics and Genomics*, **289**, 1183–1207.

Zhu, L., **Guan, Y.**, **Zhang, Z.**, **Song, A.**, **Chen, S.**, **Jiang, J.** ***et al.*** (2020) CmMYB8 encodes an R2R3 MYB transcription factor which represses lignin and flavonoid synthesis in chrysanthemum. *Plant Physiology and Biochemistry*, **149**, 217–224.