the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2024) doi: 10.1111/tpj.16658

WRKY48 negatively regulates plant acclimation to a combination of high light and heat stress

Damián Balfagón[1,2], Lidia S. Pascual[1], Soham Sengupta[3], Karen J. Halliday[2], Aurelio Gómez-Cadenas[1], María Ángeles Peláez-Vico[4], Ranjita Sinha[4], Ron Mittler[4] and Sara I. Zandalinas[1,*]

[1]*Department of Biology, Biochemistry and Natural Sciences, Universitat Jaume I, 12071 Castellón, Spain,*
[2]*Institute of Molecular Plant Sciences, School of Biological Sciences, University of Edinburgh, Edinburgh 3H9 3BF, UK,*
[3]*St. Jude Children's Research Hospital, Memphis, TN 38105, USA, and*
[4]*Division of Plant Science and Technology, College of Agriculture Food and Natural Resources, University of Missouri, Columbia, MO 65211, USA*

Received 22 September 2023; accepted 22 January 2024.
*For correspondence (e-mail sizquier@uji.es).

SUMMARY

Plants growing under natural conditions experience high light (HL) intensities that are often accompanied by elevated temperatures. These conditions could affect photosynthesis, reduce yield, and negatively impact agricultural productivity. The combination of different abiotic challenges creates a new type of stress for plants by generating complex environmental conditions that often exceed the impact of their individual parts. Transcription factors (TFs) play a key role in integrating the different molecular signals generated by multiple stress conditions, orchestrating the acclimation response of plants to stress. In this study, we show that the TF WRKY48 negatively controls the acclimation of *Arabidopsis thaliana* plants to a combination of HL and heat stress (HL + HS), and its expression is attenuated by jasmonic acid under HL + HS conditions. Using comparative physiological and transcriptomic analyses between wild-type and *wrky48* mutants, we further demonstrate that under control conditions, WRKY48 represses the expression of a set of transcripts that are specifically required for the acclimation of plants to HL + HS, hence its suppression during the HL + HS stress combination contributes to plant survival under these conditions. Accordingly, mutants that lack WRKY48 are more resistant to HL + HS, and transgenic plants that overexpress WRKY48 are more sensitive to it. Taken together, our findings reveal that WRKY48 is a negative regulator of the transcriptomic response of Arabidopsis to HL + HS and provide new insights into the complex regulatory networks of plant acclimation to stress combination.

Keywords: stress combination, heat stress, high light, WRKY, *Arabidopsis thaliana*, transcription factor, climate change.

INTRODUCTION

Plants growing under natural conditions or in agricultural settings encounter different environmental conditions that challenge their growth and development. In the field, the combination of two or more environmental stress conditions can generate a complex scenario that may significantly differ from the cumulative impact of individual stresses on the plant (Mittler, 2006; Pascual et al., 2022; Zandalinas et al., 2018, 2021; Zandalinas & Mittler, 2022). High irradiation (*e.g.*, high light, HL) and high temperatures (*e.g.*, heat stress, HS) are abiotic stresses that often occur simultaneously in nature. These stressors pose a significant threat to photosynthetic function, trigger oxidative damage, and under extreme conditions, negatively impact plant survival (Pospíšil, 2016; Szymańska et al., 2017; Takahashi & Murata, 2008). In *Arabidopsis thaliana*, the combination of HL and HS (HL + HS), at moderate intensities, causes an impairment in photosystem II (PSII) repair, as well as induces extensive structural damage to chloroplasts, negatively impacting the photosynthetic capacity of the plant (even after the stress period is over), and increasing plant mortality (Balfagón et al., 2019). Our previous work demonstrated that simultaneous application of HL and HS is associated with specific metabolic responses required for acclimation (Balfagón et al., 2019; Balfagón, Gómez-Cadenas, et al., 2022). A buildup of jasmonic acid (JA) in response to this stress combination increased plant survival and was associated with elevated expression of the transcription factors

(TFs) *ZAT6* and *ZAT10*, and of the antioxidative enzymes *ASCORBATE PEROXIDASE* 1 (*APX1*) and *APX2* (Balfagón et al., 2019). Similarly, the specific accumulation of gamma-aminobutyric acid in response to HL + HS was associated with the activation of different autophagy-responsive genes. This, in addition to physiological adjustments such as stomatal regulation, contributed to a decrease in plant mortality under HL + HS (Balfagón, Gómez-Cadenas, et al., 2022).

The role of transcriptional regulation is key for plant adaptation to stress. Several studies emphasized the uniqueness of the transcriptomic response of plants to stress combination, which may be crucial for the general acclimation process of plants (Balfagón et al., 2019, 2023; Prasch & Sonnewald, 2013; Rizhsky et al., 2004; Suzuki et al., 2016; Zandalinas et al., 2020). Transcription factor families such as HEAT SHOCK FACTORS (HSF), v-MYB MYELOBLASTOSIS VIRAL ONCOGENE HOMOLOG (MYB), APETALA2/ETHYLENE RESPONSE ELEMENT BINDING PROTEIN (AP2-EREBP), or WRKY, and the transcriptional networks they control, have been reported to play a major role in plant acclimation to different abiotic stress combinations (Rivero et al., 2022; Zandalinas et al., 2020). WRKY TFs constitute one of the most extensive families of transcriptional regulators in plants, and they are known to play an important role in biotic and abiotic stress responses (Rushton et al., 2010). WRKY proteins can function as both repressors and activators of gene expression, and were shown to function in the repression and de-repression of key signaling pathways (Chen, Zhang, et al., 2010; Rushton et al., 2010; Xing et al., 2008). For example, Arabidopsis WRKY40 was shown to enhance abscisic acid (ABA)-mediated salt and osmotic stress tolerance by antagonizing WRKY18 and WRKY60 functions (Chen, Lai, et al., 2010). In *Medicago truncatula* plants, the combination of ozone and drought stress led to enhanced expression of five WRKY genes, suggesting a signaling role during the stress combination response (Iyer et al., 2013). A meta-analysis of the transcriptomic response to drought, salt, or HL stress in combination with high temperatures showed a significantly altered regulation of 12, 3, and 29 WRKYs, respectively, in response to these stress combinations (Zandalinas et al., 2020). There is, therefore, mounting evidence that WRKY TFs display important roles in regulating plant responses to complex stress conditions.

In the current study, we investigated the function of WRKY48 in the acclimation process of Arabidopsis plants to the combination of HL and HS. *WRKY48* expression is downregulated during HL + HS, and we therefore hypothesized that it could play a suppressive role in regulating the acclimation of Arabidopsis to HL + HS. To test this hypothesis, we conducted physiological and transcriptomic analyses of wild-type plants and *wrky48* knockout mutants (Salk_066438C and Salk_144719C) subjected to control (CT), HL, HS, and HL + HS conditions. Our results indicate that WRKY48 negatively affects the tolerance of Arabidopsis plants to HL + HS. In addition, we show that JA could repress WRKY48 expression under HL + HS, and that under control conditions, WRKY48 could potentially act to suppress the expression of a set of genes that are specifically required for the acclimation of Arabidopsis plants to the stress combination.

RESULTS

WRKY48 negatively impacts plant acclimation to high light and heat stress combination

To investigate the role of WRKY48 in plant acclimation to combined HL and HS, we determined the responses of wild-type (Col) and two independent WRKY48 T-DNA insertion mutants (*wrky48-1* and *wrky48-3*; Figure 1a) to HL stress (900 μmol m^{-2} sec^{-1}; 8 h), HS (40°C; 8 h), or the combination of HL and HS (HL + HS; 900 μmol m^{-2} sec^{-1} + 40°C; 8 h). Control plants were maintained at 50 μmol m^{-2} sec^{-1} and 23°C throughout the entire experimental period (Figure S1). Gene expression analyses using reverse transcription-quantitative PCR (RT-qPCR) revealed that *WRKY48* transcript levels were significantly downregulated in wild-type plants in response to HS and more markedly under HL + HS conditions (Figure 1a; Table S1). Survival rate and Leaf Damage Index (LDI; Balfagón et al., 2019) were measured in wild-type as well as in *wrky48-1* and *wrky48-3* plants subjected to HL, HS, and HL + HS (Figure 1b,c). Under control conditions, *wrky48-1* and *wrky48-3* plants did not show any differences in terms of growth (Figure S2), indicating that this TF did not play a role in affecting plant growth and development. As shown in Figure 1b and Figure S2, only HL + HS caused a significant impact on plant survival in wild-type and both *wrky48-1* and *wrky48-3* plants. We however noted that plant survival exhibited a significantly higher decline in wild-type plants (69%) compared with *wrky48-1* and *wrky48-3* (81% and 83%, respectively; Figure 1b; Figure S2). As plant survival was only affected by HL + HS, we next measured LDI in response to HL + HS in wild-type and the *wrky48-1* and *wrky48-3* mutants (Figure 1c). Our findings revealed that the percentage of dead leaves was significantly higher in the wild-type compared with both *wrky48* mutants in response to HL + HS. These results were accompanied by a greater decrease in the relative water content (RWC) caused by HL + HS in wild-type compared with that of both *wrky48* mutants, whereas the RWC in response to any of the individual stresses was comparable to that of wild-type plants (Figure 1d). PSII performance in terms of quantum yield of PSII (Φ_{PSII}) was determined immediately after the stress treatments (Figure 1e) and 24 h following a stress recovery period (Figure 1f). The HL

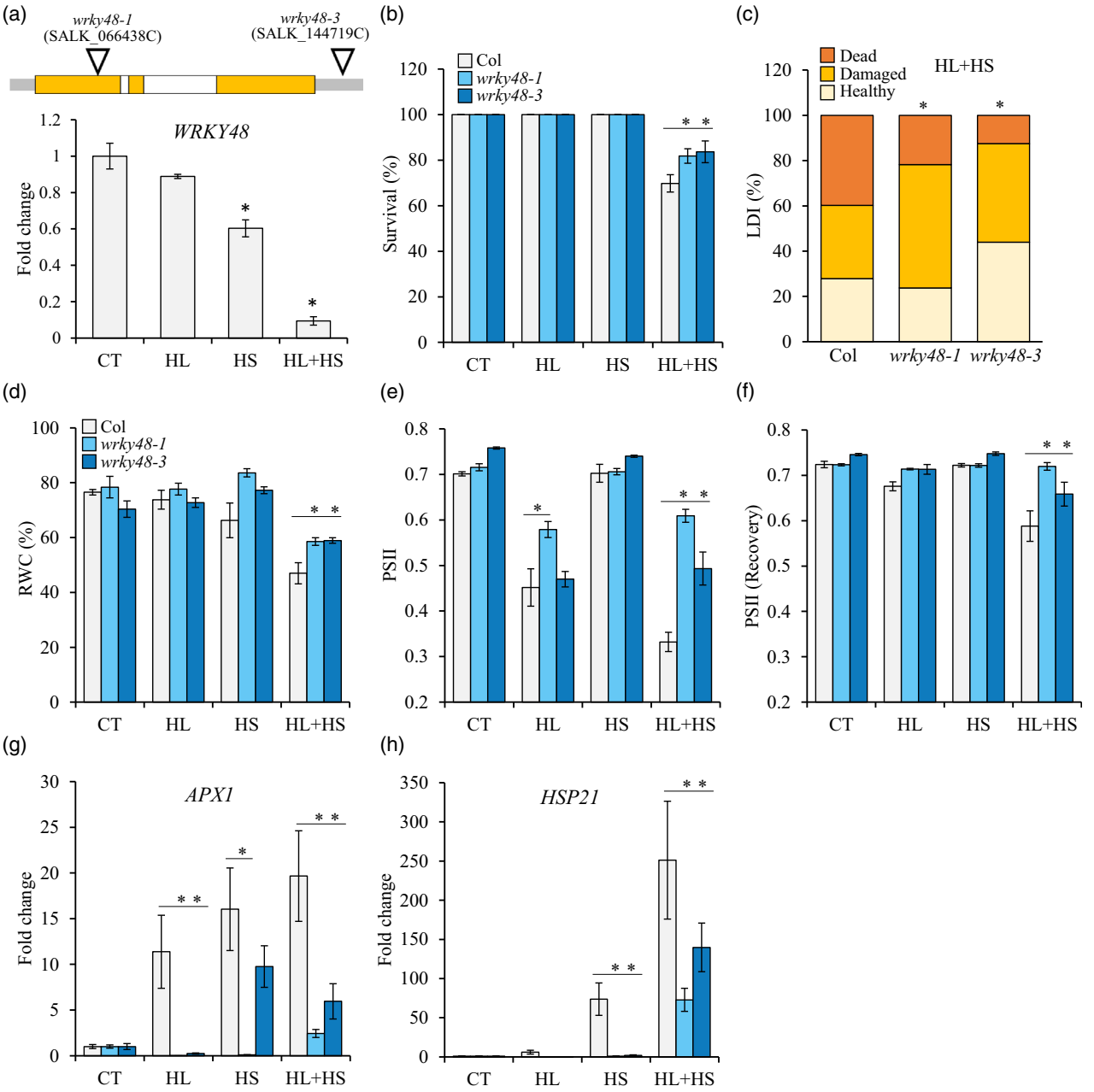

Figure 1. WRKY48 mutants display enhanced tolerance to the combination of high light and heat stress. (a) The two independent T-DNA insertion mutants used in this study. The *wrky48-1* insertion (SALK_066438C) is located in the first exon and *wrky48-3* insertion (SALK_144719C) is located in the 3′-UTR. T-DNA insertions are marked with triangles, white areas are introns, gray areas are UTRs, and yellow areas are exons (top). Reverse transcription-quantitative PCR (RT-qPCR) expression analysis of *WRKY48* in wild-type plants in response to HL, HS, and HL + HS (bottom). (b) Survival of wild-type, *wrky48-1*, and *wrky48-3* plants in response to HL, HS, and HL + HS. (c) Leaf Damage Index (LDI) of wild-type, *wrky48-1*, and *wrky48-3* plants in response to HL, HS, and HL + HS. (d) Relative water content of wild-type, *wrky48-1*, and *wrky48-3* plants in response to HL, HS, and HL + HS. (e, f) Quantum yield of PSII immediately after the application of each stress (e) and 24 h following recovery from the stress treatments (f). (g, h) RT-qPCR steady-state transcript expression level analyses of *APX1* (g) and *HSP21* (h) in wild-type, *wrky48-1*, and *wrky48-3* plants grown under CT conditions, or subjected to HL, HS, or HL + HS. Error bars represent SE. Asterisks denote statistical significance at $P < 0.05$. APX1, Ascorbate Peroxidase 1; CT, control; HL, high light; HL + HS, the combination of high light and heat stress; HS, heat stress; HSP21, Heat Shock Protein 21; LDI, Leaf Damage Index; PSII, photosystem II; Φ_{PSII}, quantum yield of PSII; RWC, relative water content.

treatment led to a decrease in Φ_{PSII} in wild-type and *wrky48* mutants, and a further reduction was observed in response to HL + HS, but only in wild-type plants. These results indicate the *wrky48-1* and *wrky48-3* confer some level of Φ_{PSII} protection. In addition, increased recovery of Φ_{PSII} following the 24 h of stress recovery period was observed in both *wrky48* mutants, compared with wild-type (Figure 1f). The relative expression of *WRKY48* in Col plants following

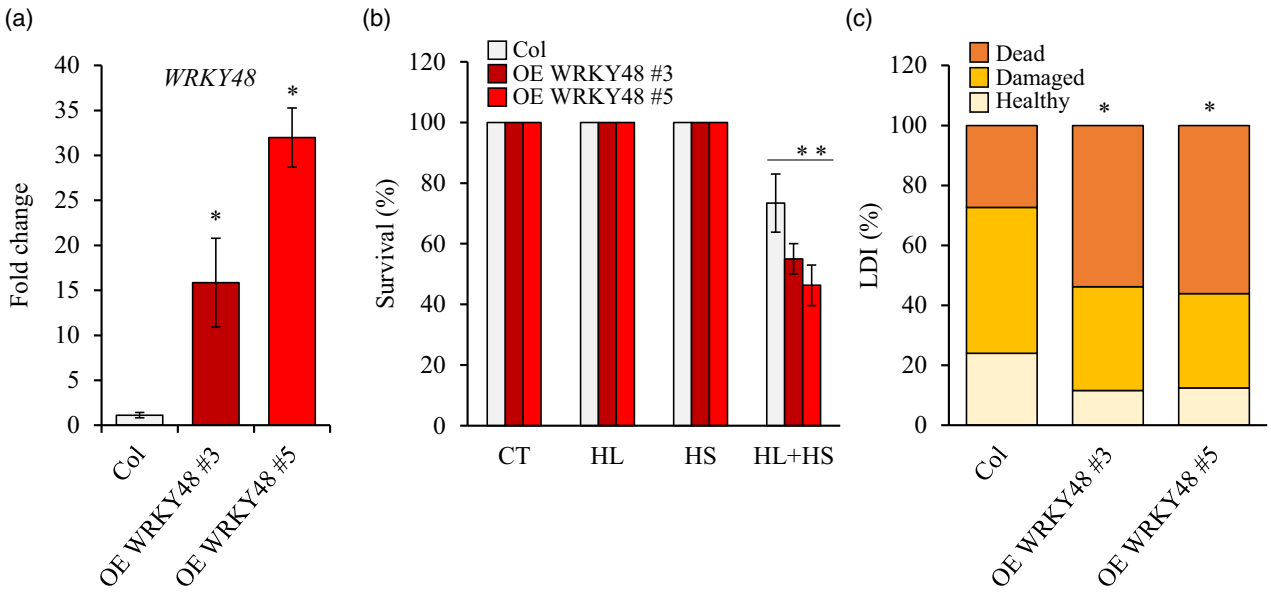

Figure 2. WRKY48-overexpressing lines display decreased tolerance to the combination of high light and heat stress. (a) Reverse transcription-quantitative PCR (RT-qPCR) expression analysis of WRKY48 in the two independent WRKY48-overexpressing lines (OE WRKY48 #3 and #5) under control conditions used in this study. (b) Survival of wild-type and the two independent WRKY48-overexpressing lines (OE WRKY48 #3 and #5) in response to HL, HS, and HL + HS. (c) Leaf Damage Index (LDI) of wild-type and the two independent WRKY48-overexpressing lines (OE WRKY48 #3 and #5) in response to HL + HS. Error bars represent SE. Asterisks denote statistical significance at $P < 0.05$. CT, control; HL, high light; HL + HS, the combination of high light and heat stress; HS, heat stress; LDI, Leaf Damage Index.

24 h of recovery from the stress combination showed a similar pattern to the expression immediately after the stress (Figure 1a; Figure S3), supporting the Φ_{PSII} results and indicating a persistent involvement of WRKY48 during the recovery period from HL + HS. The steady-state transcript level of stress and oxidative stress markers was also measured in both wild-type and mutant plants in response to each of the stress conditions (Figure 1g,h). The upregulation of *APX1* expression, observed in Col plants in response to all stress conditions, was only evident in *wrky48-1* plants subjected to HL + HS as well as in *wrky48-3* plants subjected to HS and HL + HS, albeit to a lesser degree compared with Col (Figure 1g). In turn, the expression of HEAT SHOCK PROTEIN 21 (*HSP21*) increased in Col plants when exposed to HS, and this upregulation was even more pronounced under HL + HS conditions. In contrast, the expression of this transcript was noticeably reduced in both mutants when subjected to HL + HS compared with Col (Figure 1h). Taken together, our findings suggest that WRKY48 could play a negative role in mediating plant acclimation to HL + HS combination.

WRKY48 overexpression impairs HL + HS acclimation

To further investigate the potential role of WRKY48 in inhibiting the acclimation process of Arabidopsis to HL + HS, we studied how constitutive WRKY48 overexpression could impact plant survival and leaf damage under HL, HS, and HL + HS conditions. For this purpose, we generated two independent WRKY48 overexpression lines (OE WRKY48 #3 and #5) that exhibited a 15- (OE WRKY48 #3) and 31- (OE WRKY48 #5) fold higher WRKY48 expression compared with wild-type under control conditions (Figure 2a). As shown in Figure 2b and Figure S2, the survival rate of the WRKY48 #3 and #5 OE plants following HL + HS was significantly lower (55% and 46.3%, respectively) compared with wild-type (73.4%). Similarly, the percentage of dead leaves increased in WRKY48 #3 and #5 OE lines to 53.7% and 56.1%, respectively, whereas it remained significantly lower in wild-type plants (27.3%; Figure 2c). These findings reinforced the idea that WRKY48 functions as a repressor of plant survival under conditions of combined HL and HS.

Jasmonic acid signaling represses WRKY48 expression in response to high light and heat stress combination

To determine the accumulation of different plant hormones in wild-type and *wrky48* mutants subjected to individual and combined HL and HS, we analyzed their content of JA, ABA, and salicylic acid (SA; Figure 3). JA levels increased in response to HL and more markedly in response to HL + HS conditions in wild-type plants, whereas in the *wrky48* mutants, JA increase was only evident after HL + HS application and was significantly lower than that of wild-type plants (Figure 3a). Previous reports demonstrated that JA signaling negatively regulates the expression of *WRKY48* in response to pathogen-induced stress, acting as a negative regulator of gene expression (Xing et al., 2008). In this study, to analyze whether JA

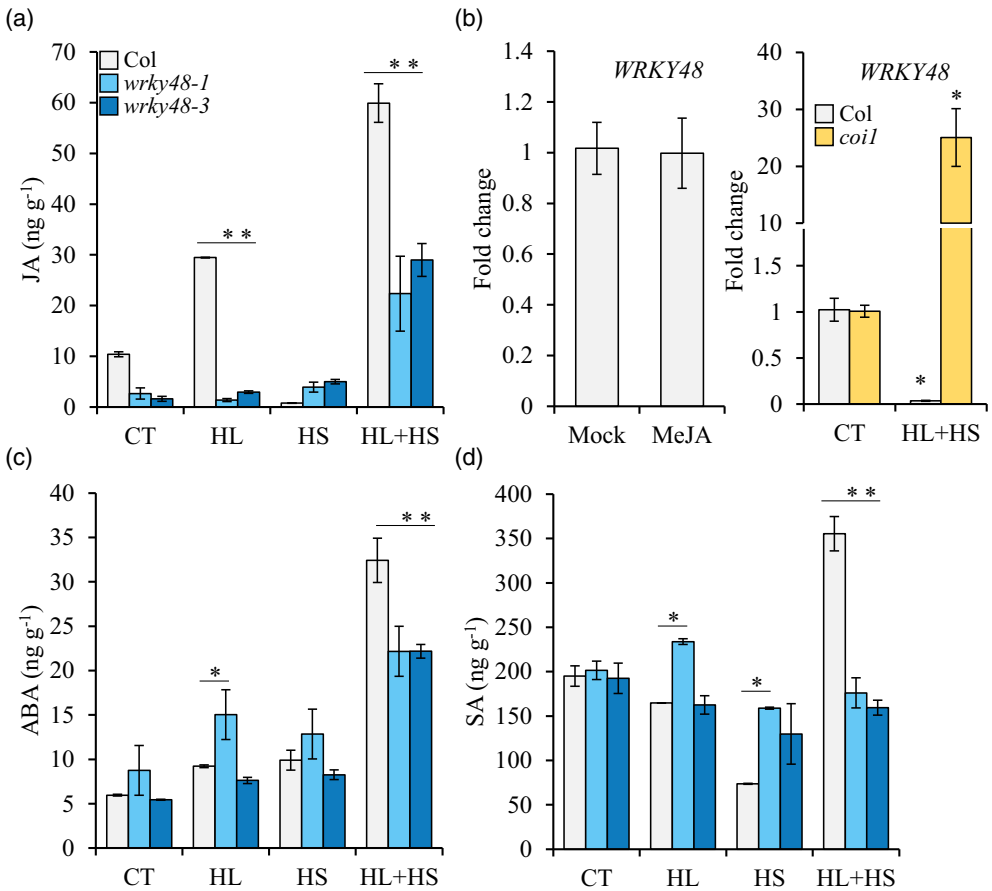

Figure 3. Levels of JA, ABA, and SA in Arabidopsis plants subjected to high light, heat stress, and combined high light and heat stress. (a) Levels of JA in wild-type, *wrky48-1*, and *wrky48-3* plants in response to HL, HS, and HL + HS. (b) Expression of WRKY48 in wild-type plants after spraying with MeJA (0.1 mM) (left), and in wild-type and *coi1* plants subjected to HL + HS (right). (c) Levels of ABA in wild-type, *wrky48-1*, and *wrky48-3* plants in response to HL, HS, and HL + HS. (d) Levels of SA in wild-type, *wrky48-1*, and *wrky48-3* plants in response to HL, HS, and HL + HS. Error bars represent SE. Asterisks denote statistical significance at $P < 0.05$. ABA, abscisic acid; CT, control; HL, high light; HL + HS, the combination of high light and heat stress; HS, heat stress; JA, jasmonic acid; MeJA, methyl jasmonate; SA, salicylic acid.

could be involved in regulating *WRKY48* expression, we applied methyl jasmonate (MeJA) to wild-type plants under control conditions (Figure 3b). In agreement with Xing et al. (2008) and as shown in Figure 3b (left panel), no change in WRKY48 expression was observed in wild-type plants treated with MeJA compared with mock-sprayed plants. However, in response to HL + HS stress, *coi1* mutants, that are impaired in JA signaling, exhibited a marked increase in *WRKY48* levels, whereas *WRKY48* expression was repressed in wild-type plants, as also shown in Figure 1a (Figure 3b, right panel). Similar to JA, the increase in ABA and SA content in response to HL + HS was significantly higher in wild-type than in *wrky48-1* and *wrky48-3* plants subjected to HL + HS (Figure 3c,d). These findings suggest that, compared with wild-type plants, the hormonal response of the *wrky48* mutants to HL + HS conditions is diminished. Furthermore, our results suggest that under HL + HS, JA likely represses *WRKY48* expression through the COI1 signaling pathway.

RNA-Seq analysis of wild-type and *wrky48-1* plants subjected to high light and heat stress combination

To study the relative effect of WRKY48 on the transcriptomic response of Arabidopsis plants subjected to HL, HS, and HL + HS conditions, we conducted an RNA-Seq analysis on wild-type and *wrky48-1* plants subjected to these treatments. We opted to use this mutant for RNA-Seq analysis instead of the *wrky48-3* mutant due to its prior validation and use (Xing et al., 2008), and because this mutant harbors a T-DNA insertion at the first exon of the gene, disrupting the WRKY DNA-binding domain located in the third exon.

Principal component analysis (PCA) revealed distinct transcriptomic responses under control conditions and in response to each stress treatment in both genotypes (Figure 4a). Transcriptomic changes associated with HS were the primary source of variation in the data. This was evident from the first principal component (PC1),

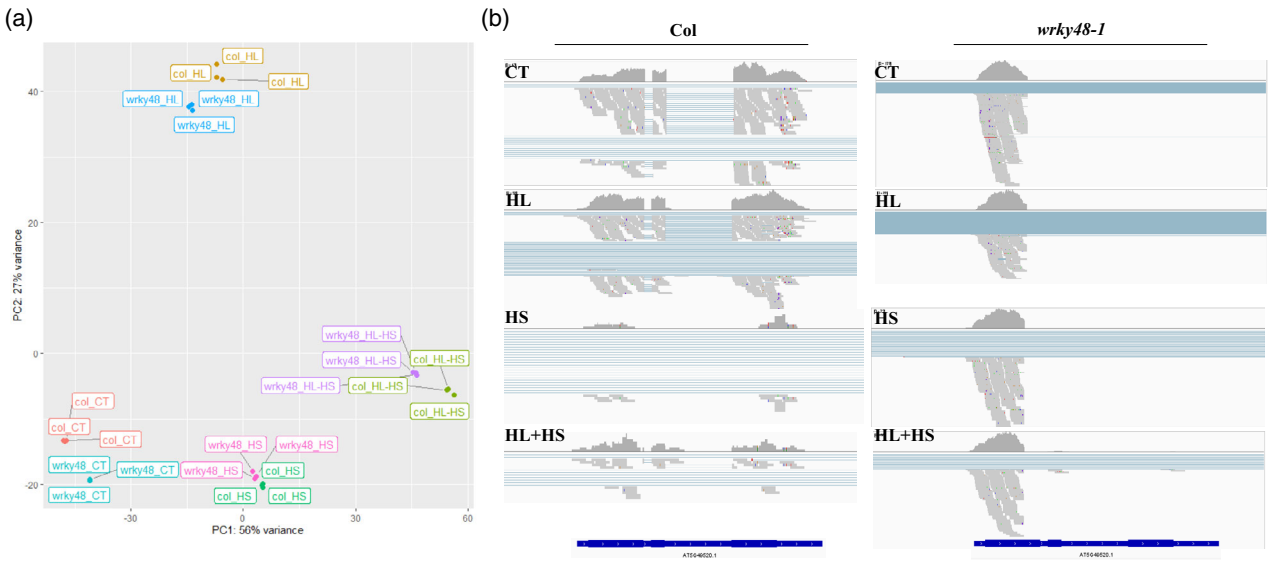

Figure 4. Transcriptomic study of wild-type and *wrky48-1* plants subjected to high light, heat stress, and combined high light and heat stress. (a) PCA plot of transcriptomic profiles obtained from control wild-type and *wrky48-1* plants, and wild-type and *wrky48-1* plants in response to HL, HS, and HL + HS. (b) Standardized RNA sequencing read maps for WRKY48 transcript in wild-type and *wrky48-1* plants subjected to CT, HL, HS, and HL + HS. CT, control; HL, high light; HL + HS, the combination of high light and heat stress; HS, heat stress; PC, principal component; PCA, principal component analysis.

explaining 56% of the total variance, which represented the distinctive profile of HS and HL + HS samples. In turn, principal component 2 (PC2), accounting for 27% of the total variation, effectively distinguished the samples based on the transcriptomic profile of plants exposed to HL (Figure 4a). Changes in transcript accumulation, visualized by RNA-Seq read alignment maps, were analyzed for *WRKY48* transcript across the entire length of its corresponding gene in Col and *wrky48-1* plants in response to CT and each stress condition (Figure 4b). The sequencing reads of the *WRKY48* gene (AT5G49520.1) indicated that its transcription in *wrky48-1* mutant was disrupted by the T-DNA insertion in the first exon as expected (Figure 4b), occurring before the WRKY DNA-binding domain located in the third exon, and confirming the loss-of-function state of the TF.

Gene expression is altered in the *wrky48-1* mutant under control conditions

The enhanced tolerance of the *wrky48* mutants to HL + HS (Figure 1b–f), the decreased tolerance to HL + HS of plants overexpressing WRKY48 (Figure 2), and the suppressed accumulation of JA (required for tolerance to HL + HS conditions; Balfagón et al., 2019) under HL + HS conditions in the *wrky48* mutants (Figure 3a), support the hypothesis that WRKY48 functions as a negative regulator of key genes involved in plant resilience to HL + HS. One possibility could be that *wrky48* mutant exhibits increased HL + HS tolerance due to the enhanced expression of certain genes, that are typically suppressed by WRKY48, under control or stress conditions. To determine potential WRKY48-regulated genes altered under control conditions in *wrky48-1* plants, we identified upregulated transcripts in *wrky48-1* compared with wild-type in the absence of stress (*i.e.*, CT conditions; Figure 5). As shown in Table S2, 995 transcripts were found to be upregulated in *wrky48-1* compared with wild-type plants. Among these, 178 were also found to be upregulated in both wild-type and *wrky48-1* in response to HL + HS, and their expression was more pronounced in wild-type compared with *wrky48-1* (Figure 5a). According to Gene Ontology (GO) term enrichment analysis, a high representation of transcripts encoding light, heat, and other abiotic stress-response transcripts, as well as transcripts involved in different stress-impacted processes such as photosynthesis, chloroplast organization, and protein refolding, was found in this group of transcripts (Figure 5b). These findings demonstrate that these potential WRKY48-regulated genes, with enhanced expression in wild-type compared with the *wrky48-1* mutant under HL + HS, included genes and transcripts involved in the response of plants to conditions of abiotic stresses, such as HS and HL conditions. In addition, the different molecular functions associated with these transcripts included protein, mRNA, or unfolded protein binding (Figure 5b). Overall, these findings suggest that increased expression of a set of transcripts in the *wrky48-1* mutant compared with Col under control conditions could provide a preconditioned higher tolerance that is specific to the HL and HS combination.

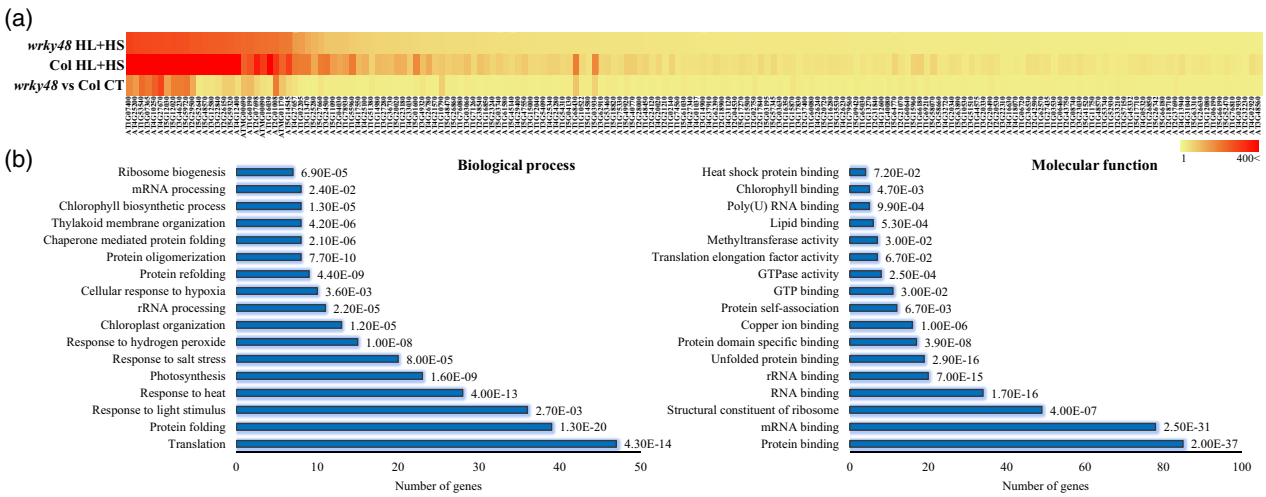

Figure 5. Transcriptomic analysis of *wrky48-1* mutant under control conditions. (a) Heat map showing changes in the expression of transcripts significantly upregulated in *wrky48-1* mutant compared with wild-type under control conditions, that are upregulated in wild-type and *wrky48-1* plants subjected to HL + HS. (b) Biological process (left) and molecular function (right) GO annotations for transcripts shown in the heat map in (a). Numbers above each bar represent *P*-value for statistical significance. CT, control; GO, Gene Ontology; HL + HS, the combination of high light and heat stress.

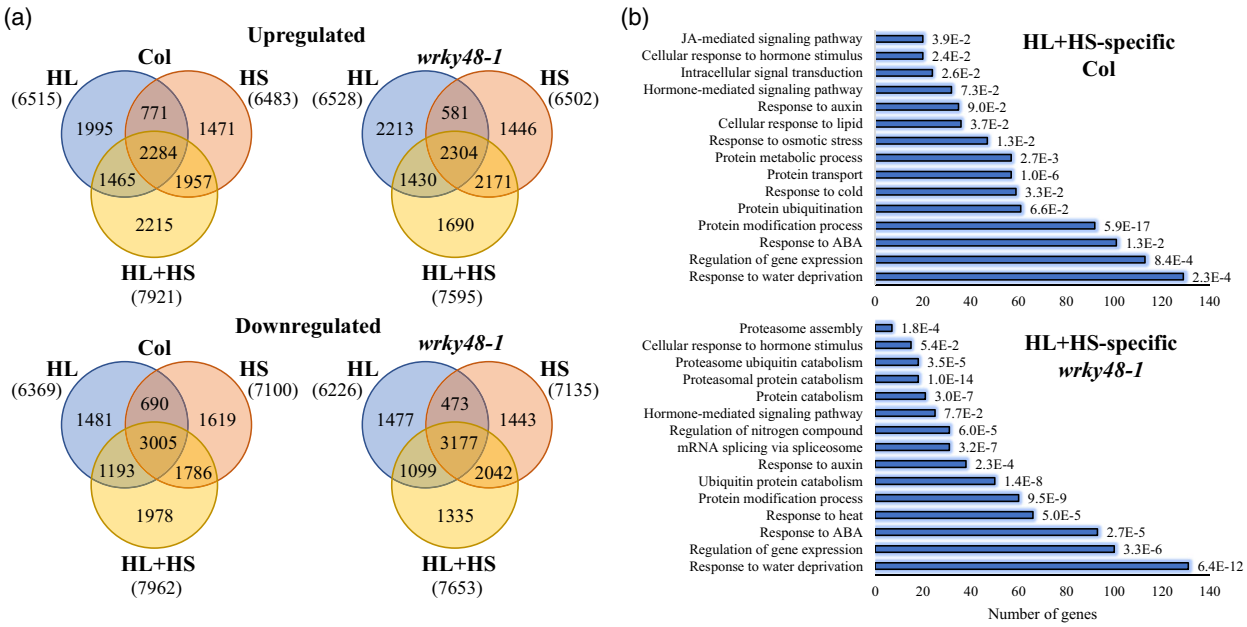

Figure 6. Combination of high light and heat stress is accompanied by a unique transcriptomic response in both wild-type and *wrky48-1* mutant plants. (a) Venn diagrams showing the overlap among the upregulated (top) and downregulated (bottom) transcripts in wild-type (left) and *wrky48-1* (right) plants subjected to HL, HS, and HL + HS. (b) Biological process (GO) annotations for transcripts specifically upregulated in wild-type (up) and *wrky48-1* (bottom) plants subjected to HL + HS (numbers above each bar represent *P*-value for statistical significance). ABA, abscisic acid; CT, control; HL, high light; HL + HS, the combination of high light and heat stress; HS, heat stress; JA, jasmonic acid.

Transcriptomic responses of wild-type and *wrky48-1* plants to high light and heat stress combination

The RNA-Seq analysis of Arabidopsis plants subjected to HL, HS, and HL + HS revealed that the steady-state level of 6515, 6483, and 7921 was significantly enhanced in response to HL, HS, and HL + HS, respectively, in wild-type plants (Figure 6a; Tables S3–S5). In comparison, the expression of 6528, 6502, and 7595 transcripts increased in response to HL, HS, and HL + HS, in *wrky48-1* plants compared with CT (Figure 6a; Tables S6–S8). The steady-state level of 6369, 7100, and 7962 was significantly downregulated in response to HL, HS, and HL + HS, respectively, in wild-type plants (Figure 6a; Tables S9–S11). In turn, *wrky48-1* plants subjected to HL, HS, and HL + HS

downregulated 6226, 7135, and 7653 transcripts, compared with CT, respectively (Figure 6a; Tables S12–S14). Of the 7921 transcripts significantly elevated in response to HL + HS in wild-type plants, 2215 transcripts (28%) were found to be specifically upregulated by the stress combination, whereas 1690 transcripts (out of 7595; 22%) were exclusively accumulated under HL + HS in *wrky48-1* plants. Similarly, 1978 (out of 7962; 25%) and 1335 (out of 7653; 17%) transcripts were found to be downregulated specifically by HL + HS in wild-type and *wrky48-1* plants, respectively (Figure 6a). These findings indicate that a notable proportion of the alterations in gene expression patterns observed in both genotypes is uniquely attributed to HL + HS. GO term enrichment analysis of upregulated HL + HS-specific transcripts in both Arabidopsis genotypes revealed their potential involvement in different biological processes including 'response to water deprivation', 'regulation of gene expression', 'response to ABA', 'cellular response to hormone stimulus', and 'hormone-mediated signaling pathway' (Figure 6b). The upregulated HL + HS-specific transcripts also included transcripts associated with the JA-mediated signaling pathway in wild-type plants, as well as with different proteasome processes in *wrky48-1* plants (Figure 6b).

Identification of potential transcripts regulated by WRKY48 in the response of Arabidopsis to high light and heat stress combination

To identify potential transcripts controlled by WRKY48 in response to HL + HS, we determined the fraction of upregulated transcripts specific to HL + HS conditions in wild-type (in which WRKY48 expression is suppressed under the stress combination; Figure 1a) that are also common to those specifically upregulated under HL + HS conditions in the *wrky48-1* mutant (experiencing loss of WRKY48 function during HL + HS; Figure 4b). We then investigated how many of these common transcripts were also among the 995 transcripts upregulated in *wrky48-1* mutant compared with wild-type under CT conditions (Figure 7). Interestingly, 13 transcripts displayed specific upregulation in response to HL + HS stress in both the wild-type plants and *wrky48-1* mutant and were also upregulated in *wrky48-1* mutant under CT conditions compared with wild-type (Figure 7a). As shown in Figure 7b, these 13 transcripts exhibited a more significant upregulation in wild-type plants exposed to HL + HS when compared with *wrky48-1*, further supporting the idea that the increased expression of these transcripts in the mutant under control conditions could contribute to its enhanced tolerance to HL + HS. In addition, a promoter analysis of this set of transcripts revealed that four transcripts (AT1G26850, AT1G66180, AT3G23030, and AT4G31040) contained WRKY-binding motifs in their promoters, suggesting their potential regulation by WRKY48 (Figure 7b). To determine the potential involvement of these genes in plant responses to stress combination, a survival analysis of two independent mutant knockout lines for each of these genes, encoding potential WRKY48-dependent transcripts in response to HL, HS, and HL + HS, was conducted (Figure 7c). Our results indicated that mutant lines for AT1G66180, AT3G23030, and AT4G31040 but not for AT1G26850, showed reduced survival rate under HL + HS compared with Col plants (Figure 7c). These findings support the hypothesis that, compared with the wild-type, the *wrky48* mutant has a higher pre-conditioned acclimation state to HL + HS, due to the expression of genes required for acclimation to this stress combination under CT conditions.

DISCUSSION

Plants often encounter complex combinations of stress conditions in their natural environment that challenge their growth and development (Mittler, 2006; Zandalinas et al., 2021). For example, conditions of HL stress during HS periods can aggravate the oxidative damage generated by both individual stress conditions and severely affect the photosynthetic apparatus of plants growing in full sunlight (Balfagón et al., 2019; Balfagón, Gómez-Cadenas, et al., 2022; Balfagón, Zandalinas, et al., 2022; Ort, 2001; Pospíšil, 2016; Roeber et al., 2021; Suzuki et al., 2014). In a previous study, we demonstrated that the response of Arabidopsis plants to HL and HS combination results in specific physiological, metabolic, and transcriptomic changes that cannot be inferred from the response to each of these individual stress conditions (Balfagón et al., 2019; Balfagón, Gómez-Cadenas, et al., 2022). In addition, different studies have shown the important role of TFs in integrating different stress signaling pathways during stress combination (Prasch & Sonnewald, 2015; Rivero et al., 2022; Zandalinas et al., 2020). In this study, we focused on the role of WRKY48 in the acclimation response of Arabidopsis plants to a combination of HL and HS. Interestingly, our findings indicate that WRKY48 knockout mutants (-*wrky48-1* and *wrky48-3*) exhibited enhanced survival rates and reduced leaf damage when subjected to HL + HS compared with wild-type plants (Figure 1a–c). Additionally, both mutants maintained higher RWC values under the stress combination, exhibited a greater recovery of Φ_{PSII} following a stress recovery period, and displayed decreased expression of stress/oxidative stress marker transcripts, compared with wild-type, further indicating their improved ability to cope with the stress combination (Figure 1d–f). It was previously reported that the photosynthetic apparatus is highly affected by HL + HS combination due to an impairment in the PSII repair process (Balfagón et al., 2019). We further report that, in contrast to *wrky48* mutants that displayed enhanced tolerance to HL + HS, WRKY48 OE lines were more sensitive to this stress

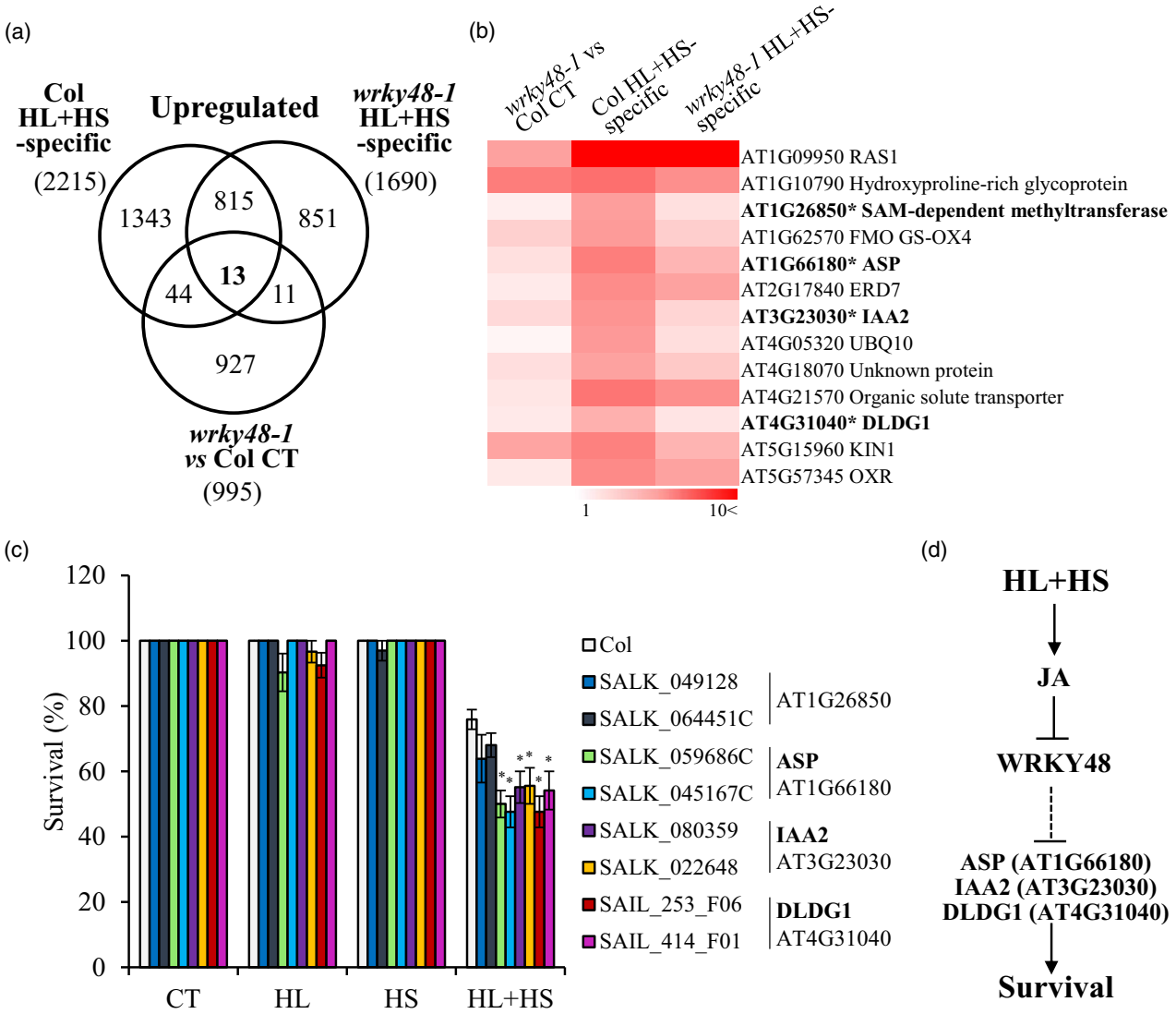

Figure 7. Identification of WRKY48-dependent transcripts significantly upregulated in response to HL + HS. (a) Venn diagram showing the overlap between transcripts specifically accumulated in wild-type and *wrky48-1* plants subjected to HL + HS, and transcripts significantly upregulated in *wrky48-1* plants with respect to wild-type under control conditions. (b) Heat map showing changes in the expression of the 13 transcripts commonly upregulated in wild-type and *wrky48-1* plants subjected to HL + HS, and in *wrky48-1* plants with respect to wild-type under control conditions. Transcripts in bold and marked with an asterisk contain WRKY-binding motives in their promoter. (c) Survival of two independent knockout mutants for genes encoding potential WRKY48-dependent transcripts in plants grown under CT conditions or subjected to HL, HS, or HL + HS conditions. (d) A model for the putative function of WRKY48 in negatively regulating plant acclimation to a combination of high light and heat stress. See text for more details. Error bars represent SE. Asterisks denote statistical significance at $P < 0.05$ compared with Col. CT, control; HL, high light; HL + HS, the combination of high light and heat stress; HS, heat stress JA, jasmonic acid.

combination (Figures 1 and 2). These findings highlight an important role for WRKY48 in regulating plant tolerance to a specific set of stress combination conditions (*i.e.*, to HL + HS, but not to HL or HS).

Since JA accumulation was previously found to be required for plant acclimation to HL + HS and was correlated with the overexpression of key JA-regulated genes involved in this acclimation process (Balfagón et al., 2019), we subsequently explored whether *WRKY48* expression could be regulated by JA in response to HL + HS. As MeJA treatment did not affect *WRKY48* expression under controlled conditions, as shown previously (Xing et al., 2008), and the *coi1* mutant, impaired in JA signaling, showed increased levels of *WRKY48* expression under HL + HS (Figure 3a,b), our results support a model in which JA accumulation and signaling represses *WRKY48* expression under conditions of HL + HS (Figure 7d). These results are also consistent with previous studies suggesting that JA signaling negatively impacts *WRKY48* expression in response to pathogen-induced stress (Xing et al., 2008), and is further supported by our previous transcriptomic analysis that showed the effects of HL + HS on

JA-regulated transcript expression (Balfagón et al., 2019). In addition to *WRKY48*, other WRKYs were shown to be regulated by JA in response to different stimuli (Jiang et al., 2014, 2017; Li et al., 2010). For example, during leaf senescence, *WRKY57* was found to be negatively regulated by JA at the transcriptomic level through the action of the JASMONATE ZIM-DOMAIN 4 and 8 (JAZ4 and JAZ8), and at the protein level via the 26S proteasome pathway (Jiang et al., 2014). Similarly, our findings suggest a potential mechanism by which *WRKY48* expression is negatively regulated by JA under HL + HS combination, and this regulation contributes to the overall acclimation response of plants to this stress combination (Figure 7d). While we focused our analysis on JA, we cannot rule out the possibility that other hormones such as ABA or SA are involved in regulating *WRKY48* expression during abiotic stresses. Further studies are therefore needed to determine the potential of the other hormones to regulate *WRKY48*.

By analyzing the primary transcriptomic response of *wrky48-1* plants to the different short-period stresses, compared with wild-type, our findings revealed that a considerable number of transcripts were specifically up- and downregulated by HL + HS in both genotypes (Figure 6a; Tables S3–S14). These results were consistent with previous transcriptomic studies in which different stress combinations resulted in specific transcriptomic changes, different from those displayed by each of the individual stresses (Balfagón et al., 2023; Osthoff et al., 2019; Rizhsky et al., 2004; Sharma et al., 2018; Suzuki et al., 2016). Interestingly, the absence of WRKY48 resulted in the elevated expression of several transcripts in plants grown under CT conditions, and the expression of some of these transcripts was also found to be elevated in wild-type plants in response to HL + HS (Figure 5; Table S2). Included among these transcripts were transcripts associated with different biological processes including translation, protein folding, and responses to light stimulus and heat (Figure 5b), indicating a potential pre-adaptation, or pre-conditioning, to HL + HS in *wrky48-1* plants in the absence of stress. Moreover, when exposed to HL + HS conditions, wild-type plants exhibited a more pronounced upregulation in these transcripts compared with *wrky48-1* plants (Figure 5a). Taken together, our results support the hypothesis that JA plays a crucial role in the suppression of *WRKY48* expression which, in turn, enables the elevated expression of different acclimation transcripts that could be negatively regulated by this TF under control and HL + HS conditions. Interestingly, we found 13 genes that were specifically upregulated under HL + HS in both genotypes but potentially repressed by WRKY48 under control conditions (Figure 7). Among them, AT1G26850, AT1G66180, AT3G23030, and AT4G31040 contained WRKY-binding domains in their promoters and could be directly regulated by WRKY48. AT1G66180 is a predicted *A1 FAMILY ASPARTYL PROTEASE* (*ASP*) in Arabidopsis (Beers et al., 2004) and it was reported to be induced by HL in the singlet oxygen overproducer mutant *flu* (Op Den Camp et al., 2003). AT3G23030 (*INDOLE-3-ACETIC ACID INDUCIBLE 2*; *IAA2*) expression increases under hypoxic conditions and is associated with higher tolerance to this stress condition (Lin et al., 2017). AT4G31040 encodes a Non-Photochemical Quenching (NPQ) regulatory protein identified as *DAY-LENGTH-DEPENDENT DELAYED-GREENING 1* (*DLDG1*). *DLDG1* associates with the chloroplast envelope membrane and interacts with other proteins to control H^+ homeostasis in chloroplasts, which is important for the light-acclimation response, by optimizing the extent of NPQ (Harada et al., 2019). Due to the importance of photoprotection in the tolerance of plants to HL + HS (Balfagón et al., 2019), *DLDG1* can have an important role in plant tolerance to this stress combination. A study of the survival of two independent mutant knockout lines for each of the genes described above in response to CT, HL, HS, and HL + HS revealed an important role of ASP, IAA2, and DLDG1 in plant tolerance, specifically, to this stress combination (Figure 7c). As *wrky48* and *asp1*, *iaa2*, and *dldg1* mutants displayed a specific survival phenotype only in response to HL + HS (Figures 1 and 7), and their expression pattern is linked (Figures 5–7), they could be functioning in the same signal transduction pathway that is specific to the stress combination (Figure 7d).

Taken together, our findings suggest that WRKY48 is a negative regulator of plant acclimation to HL + HS conditions. *WRKY48* downregulation under HL + HS in wild-type plants is further correlated with accumulation of JA (counteracted in the *coi1* mutant, impaired in JA sensing), indicating a regulatory role for JA in *WRKY48* expression. Transcriptional analysis of wild-type and *wrky48-1* plants revealed a higher expression of transcripts associated with responses to HL + HS in *wrky48-1* under control conditions that could confer a pre-conditioned higher tolerance to the stress combination. These transcripts include *ASP*, *IAA2*, *DLDG1*, which contain WRKY-binding DNA elements in their promoters (Figure 7b), and a survival analysis of mutants impaired in these transcripts suggested that *ASP*, *IAA2*, and *DLDG1* could play an important role in plant acclimation to the HL and HS combination (Figure 7c,d). Our findings shed therefore new light on the intricate regulatory networks that plants employ to cope with complex stress conditions and highlight the importance of understanding the role of different TFs in these responses. Future studies should explore the downstream targets of WRKY48 and its interactions with other transcriptional regulators to unravel the full complexity of the plant response to abiotic stress combinations. Exploring the role of WRKY48 homologs in different commercially important plant species, spanning both monocots and dicots, is also important for unraveling its impact on fruit or grain production. Understanding the multifaceted role of WRKY48

across plant species and under complex stress combinations holds promise for tailoring agricultural strategies to optimize fruit and grain production, fostering resilience in the face of challenging environmental conditions.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana wild-type Col (var Columbia-0) and homozygous knockout mutants (Col background) *wrky48-1* (Salk_066438C), *wrky48-3* (Salk_144719C), *coi1* (Salk_045434C), SALK_049128, SALK_064451C, SALK_059686C, SALK_045167C, SALK_080359, SALK_022648, SAIL_253_F06, and SAIL_414_F01, as well as two independent lines overexpressing WRKY48 (AT5G49520; OE WRKY48 #3 and OE WRKY48 #5), were grown in peat pellets (Jiffy-7; http://www.jiffygroup.com/) at 23°C under long-day growth conditions (12-h light from 7 AM to 7 PM; 50 μmol m^{-2} sec^{-1}/12-h dark from 7 PM to 7 AM). To generate OE WRKY48 lines, Mobius protocol, a Golden Gate assembly system, was used (Andreou et al., 2021; Andreou & Nakayama, 2018). The cDNA fragment that contained the full coding sequence and 3′ untranslated region of WRKY48 was cloned into mUAV vector (Cai et al., 2020) and assembled with mUAV vectors containing CaMV 35S promotor and NOS terminator sequences. *Agrobacterium tumefaciens* strain AGL1 was transformed with the construct and used to obtain the overexpressing lines (OE WRKY48 #3 and OE WRKY48 #5 from *Arabidopsis thaliana* Columbia-0) using the floral-dip procedure (Zhang et al., 2006). Homozygous plants were obtained by selecting transformed lines using BASTA resistance, and WRKY48 overexpression was confirmed through RT-qPCR using gene-specific primers (Table S1).

Stress treatments

Individual HL stress and HS, and combined HL and HS (HL + HS) were applied in parallel using 30-day-old Arabidopsis plants. HL was applied by exposing plants to 900 μmol m^{-2} sec^{-1} at 23°C for 8 h. HS was applied by subjecting plants to 40°C, 50 μmol m^{-2} sec^{-1}, for 8 h. HL + HS was performed by simultaneously subjecting plants to 900 μmol m^{-2} sec^{-1} of light stress and 40°C for 8 h. Control (CT) plants were maintained at 50 μmol m^{-2} sec^{-1} and 23°C throughout the entire experiment. Following the stress treatments, the plants were divided into two groups: one group was sampled and immediately frozen in liquid nitrogen, while the other group was allowed to recover under controlled conditions until flowering, at which point survival rates were recorded. Additionally, 24 h after the stress treatments, LDI (Balfagón et al., 2019) and the relative expression pattern of *WRKY48* by reverse transcription-quantitative PCR (RT-qPCR) were determined. For MeJA treatment, 30-day-old Col plants grown under CT conditions as detailed above were sprayed with 0.1 mM MeJA or mock (deionized water supplemented with 0.2% ethanol) solutions, and leaves were collected 2 h after the treatment. All experiments were conducted during the light cycle, from 9 AM to 5 PM, and were repeated at least three times with 45 plants per stress treatment and biological repeat.

Photosystem II activity

PSII activity (Φ_{PSII}) was measured using a portable fluorometer (model no.: 110/S FluorPen; Photon Systems Instruments, Czech Republic). Measurements were taken at two time points: immediately after the 8 h of individual and combined stress treatments, and 24 h after the stress treatments (recovery period). Measurements for CT and each stress treatment were taken in at least 10 plants in two fully expanded leaves per plant, and each experiment was repeated at least three times.

RWC

The relative water content of at least three whole rosettes was calculated in CT plants and after each stress treatment. Immediately after the stress treatments, rosettes were weighed to obtain fresh mass (M_f). Rosettes were allowed to rehydrate for 24 h in an opaque beaker filled with distilled water. Then, samples were reweighed to obtain a turgid mass (M_t). Finally, samples were dried at 80°C for 24 h to obtain dry mass (M_d). RWC was calculated as $[(M_f - M_d) \times (M_t - M_d) - 1] \times 100$ according to Morgan (1984).

RNA-Seq analysis

Fully expanded leaves pooled from at least 20 different plants subjected to each of the control and stress treatments were used for each biological repeat for RNA-Seq analysis, and three biological repeats were performed. Total RNA was isolated using RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA libraries for sequencing were prepared using standard Illumina protocols and RNA sequencing was performed using NovaSeq 6000 by Novogene Co. Ltd (Cambridge, UK).

The Illumina platform was used to generate sequenced reads, which underwent quality assessment using FastQC v0.11.7 (Andrews, 2010). Subsequently, these reads were aligned to the Arabidopsis reference genome (genome build 10) acquired from TAIR (https://www.arabidopsis.org/) utilizing STAR aligner v2.4.0.1 (Dobin et al., 2013). Default mapping parameters were employed, allowing for 10 mismatches per read and permitting nine multi-mapping locations per read, in accordance with the methodology outlined in (Zandalinas et al., 2019). Differential gene expression analysis was carried out using the R-based DESeq2 v1.20.0 package available in Bioconductor (Love et al., 2014). To identify transcripts with differential expression compared with the control (CT), we analyzed the variance in transcript abundance across various stress conditions (HL, HS, and HL + HS). The difference in expression was quantified as the logarithm of the ratio of mean normalized counts between two conditions, referred to as the log fold change. Differentially expressed transcripts were defined as those with an adjusted *P*-value of less than 0.05 in their fold change, determined through a negative binomial Wald test followed by Benjamini–Hochberg correction. Functional annotations and over-representation of GO terms ($P < 0.05$) were performed using DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/) (Huang et al., 2009). The generation of RNA sequencing read maps for the *WRKY48* transcript in both wild-type and *wrky48-1* plants exposed to the different conditions (CT, HL, HS, and HL + HS) was conducted using the Integrated Genomics Viewer (IGV) platform (https://igv.org/). The alignment file (BAM) containing the aligned reads was imported into IGV, utilizing the Arabidopsis TAIR 10 genome as the reference genome build. The representation of coverage as gray bars accompanied the aligned reads. IGV automatically loaded annotations, and snapshots were captured at the AT5G49520 gene locus, subsequently saved as PNG files. This was repeated for all the different conditions and genotypes.

Promoter analysis

Promoter sequences (1000-bp upstream of gene start) for potential WRKY48-regulated genes in leaves of Col and *wrky48-1* plants in

response to HL + HS treatment were downloaded from TAIR. Transcription factor binding sites for WRKY48 (AT5G49520) were obtained from the Arabidopsis Gene Regulatory Information Server (AGRIS; http://agris-knowledgebase.org/). The occurrence of DNA-binding elements of the above-mentioned TF in promoters was determined using an in-house Perl script.

Hormonal content analysis

Hormone extraction and analysis were performed as described in Balfagón et al. (2019). Briefly, a mixture containing 50 ng of [$^2H_6$]-ABA, [^{13}C]-SA, and dehydrojasmonic acid (DHJA) was added to 0.1 g of grounded, frozen leaf tissue. The tissue was homogenized in 2 ml of ultrapure water in a ball mill (MillMix20, Domel, Železniki, Slovenija). After centrifugation at 10 000 ***g*** at 4°C for 10 min, supernatants were recovered, and pH adjusted to 3 with 80% acetic acid. The water extract was partitioned twice against 2 ml of diethyl ether and the organic layer recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). Then, samples were resuspended in a 90:10 (v/v) H_2O:MeOH solution by using a sonicator (Elma S30, Elmasonic, Singen, Germany). Samples were filtered through 0.22-μm polytetrafluoroethylene membrane syringe filters (Albet S.A., Barcelona, Spain), and extracts were directly injected into an ultra-performance UPLC system (Xevo TQ-S, Waters Corp., Milford, MA, USA). Chromatographic separations were performed on a reversed-phase C18 column (Gravity, 50 × 2.1 mm, 1.6-μm particle size, Luna Omega, Phenomenex, Torrance, CA, USA) using a H_2O:MeOH (both supplemented with 0.1% formic acid) gradient at a flow rate of 300 μl min^{-1}. Hormones were quantified with a triple quadrupole mass spectrometer connected online to the output of the column through an orthogonal Z-spray electrospray ion source. Results were processed using Masslynx v. 4.1 software, and the phytohormone content was quantified with a standard curve prepared with commercial standards as described in Balfagón et al. (2019).

RT-qPCR analysis

Relative expression analysis by RT-qPCR was performed according to Zandalinas et al. (2016) by using a StepOne Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) and gene-specific primers (Table S1).

Statistical analysis

Results are presented as the mean ± SE. Statistical differences were discriminated by a two-tailed Student's *t*-test (asterisks denote statistical significance at $P < 0.05$ with respect to wild-type or CT). Differentially expressed transcripts were defined as those that had a fold change with an adjusted *P*-value < 0.05 (negative binomial Wald test followed by a Benjamini–Hochberg correction).

AUTHOR CONTRIBUTIONS

DB and LSP performed experiments and analyzed the data. AG-C, RM, and SIZ designed and supervised the research. SS performed the transcriptomic bioinformatics analysis. DB, KH, AG-C, MAP-V, RS, RM, and SIZ provided funding and/or wrote the manuscript. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

Hormone measurements were carried out at the central facilities (Servei Central d'Instrumentació Científica, SCIC) of the Universitat Jaume I, Spain. DB was the recipient of a post-doctoral contract financed by the European Union—NextGenerationEU and the Spanish Ministerio de Universidades (MGS/2021/17). LSP was supported by the contract PRE2022-101650 funded by MCIN/AEI/10.13039/501100011033 and FSE+.

FUNDING INFORMATION

This research was supported by the project PID2021-128198OA-I00 funded by Ministerio de Ciencia e Innovación/Agencia Estatal de Investigación MCIN/AEI/10.13039/501100011033 and FEDER Una manera de hacer Europa, Universitat Jaume I (UJI-A2022-06), Generalitat Valenciana (CIAICO/2021/063) and Ramón y Cajal program (RYC2020-029967-I).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw and processed RNA-Seq data files were deposited in GEO (https://www.ncbi.nlm.nih.gov/geo/) under the following accession nos.: GSE242435. WRKY48 (AT5G49520), COI1 (AT2G39940), AT1G26850, ASP (AT1G66180), IAA2 (AT3G23030), and DLDG1 (AT4G31040).

SUPPORTING INFORMATION

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

Figure S1. Experimental design used for the study of high light (HL), heat stress (HS), and combined high light and heat stress (HL + HS) using *Arabidopsis* plants.

Figure S2. Representative images of wild-type, *wrky48-1*, *wrky48-3* and two independent WRKY48-overexpressing lines (OE WRKY48 #3 and #5) grown under CT conditions or subjected to HL, HS, or HL + HS.

Figure S3. RT-qPCR expression analysis of *WRKY48* in wild-type plants grown under CT conditions or subjected to HL, HS, or HL + HS, and allowed to recover for 24 h.

Table S1. Transcript-specific primers used for relative expression analysis by RT-qPCR.

Table S2. List of transcripts significantly upregulated ($P < 0.05$) in *wrky48-1* plants under control conditions with respect to Col.

Table S3. List of transcripts significantly upregulated ($P < 0.05$) in Col plants subjected to high light (HL).

Table S4. List of transcripts significantly upregulated ($P < 0.05$) in Col plants subjected to heat stress (HS).

Table S5. List of transcripts significantly upregulated ($P < 0.05$) in Col plants subjected to the combination of high light and heat stress (HL + HS).

Table S6. List of transcripts significantly upregulated ($P < 0.05$) in *wrky48-1* plants subjected to high light (HL).

Table S7. List of transcripts significantly upregulated ($P < 0.05$) in *wrky48-1* plants subjected to heat stress (HS).

Table S8. List of transcripts significantly upregulated ($P < 0.05$) in *wrky48-1* plants subjected to the combination of high light and heat stress (HL + HS).

Table S9. List of transcripts significantly downregulated ($P < 0.05$) in Col plants subjected to high light (HL).

Table S10. List of transcripts significantly downregulated ($P < 0.05$) in Col plants subjected to heat stress (HS).

Table S11. List of transcripts significantly downregulated ($P < 0.05$) in Col plants subjected to the combination of high light and heat stress (HL + HS).

Table S12. List of transcripts significantly downregulated ($P < 0.05$) in *wrky48-1* plants subjected to high light (HL).

Table S13. List of transcripts significantly downregulated ($P < 0.05$) in *wrky48-1* plants subjected to heat stress (HS).

Table S14. List of transcripts significantly downregulated ($P < 0.05$) in *wrky48-1* plants subjected to the combination of high light and heat stress (HL + HS).

REFERENCES

Andreou, A.I. & **Nakayama, N.** (2018) Mobius assembly: a versatile Golden-Gate framework towards universal DNA assembly. *PLoS One*, **13**, e0189892.

Andreou, A.I., **Nirkko, J.**, **Ochoa-Villarreal, M.** & **Nakayama, N.** (2021) Mobius Assembly for plant systems highlights promoter-terminator interaction in gene regulation. *bioRxiv*, 2021.03.31.437819.

Andrews, S. (2010) FastQC: a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Balfagón, D., **Gómez-Cadenas, A.**, **Rambla, J.L.**, **Granell, A.**, **Ollas, C.d.**, **Bassham, D.C.** ***et al.*** (2022) γ-Aminobutyric acid plays a key role in plant acclimation to a combination of high light and heat stress. *Plant Physiology*, **188**, 2026–2038.

Balfagón, D., **Sengupta, S.**, **Gómez-Cadenas, A.**, **Fritschi, F.B.**, **Azad, R.**, **Mittler, R.** ***et al.*** (2019) Jasmonic acid is required for plant acclimation to a combination of high light and heat stress. *Plant Physiology*, **181**, 1668–1682.

Balfagón, D., **Zandalinas, S.I.**, **Reis de Oliveira, T.d.**, **Santa-Catarina, C.** & **Gómez-Cadenas, A.** (2023) Omics analyses in citrus reveal a possible role of RNA translation pathways and unfolded protein response regulators in the tolerance to combined drought, high irradiance, and heat stress. *Horticulture Research*, **10**, uhad107.

Balfagón, D., **Zandalinas, S.I.**, **Reis de Oliveira, T.d.**, **Santa-Catarina, C.** & **Gómez-Cadenas, A.G.** (2022) Reduction of heat stress pressure and activation of photosystem II repairing system are crucial for citrus tolerance to multiple abiotic stress combination. *Physiologia Plantarum*, **174**, e13809.

Beers, E.P., **Jones, A.M.** & **Dickerman, A.W.** (2004) The S8 serine, C1A cysteine and A1 aspartic protease families in Arabidopsis. *Phytochemistry*, **65**, 43–58.

Cai, Y.M., **Carrasco Lopez, J.A.** & **Patron, N.J.** (2020) Phytobricks: manual and automated assembly of constructs for engineering plants. *Methods in Molecular Biology*, **2205**, 179–199.

Chen, H., **Lai, Z.**, **Shi, J.**, **Xiao, Y.**, **Chen, Z.** & **Xu, X.** (2010) Roles of arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress. *BMC Plant Biology*, **10**, 281.

Chen, L., **Zhang, L.** & **Yu, D.** (2010) Wounding-Induced WRKY8 is involved in basal defense in arabidopsis. *Molecular Plant-Microbe Interactions*, **23**, 558–565.

Dobin, A., **Davis, C.A.**, **Schlesinger, F.**, **Drenkow, J.**, **Zaleski, C.**, **Jha, S.** ***et al.*** (2013) STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, **29**, 15–21.

Harada, K., **Arizono, T.**, **Sato, R.**, **Trinh, M.D.L.**, **Hashimoto, A.**, **Kono, M.** ***et al.*** (2019) DAY-LENGTH-DEPENDENT DELAYED-GREENING1, the Arabidopsis homolog of the cyanobacterial H+-extrusion protein, is essential for chloroplast pH regulation and optimization of non-photochemical quenching. *Plant and Cell Physiology*, **60**, 2660–2671.

Huang, D.W., **Sherman, B.T.** & **Lempicki, R.A.** (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, **4**, 44–57.

Iyer, N.J., **Tang, Y.** & **Mahalingam, R.** (2013) Physiological, biochemical and molecular responses to a combination of drought and ozone in Medicago truncatula. *Plant, Cell and Environment*, **36**, 706–720.

Jiang, J., **Ma, S.**, **Ye, N.**, **Jiang, M.**, **Cao, J.** & **Zhang, J.** (2017) WRKY transcription factors in plant responses to stresses. *Journal of Integrative Plant Biology*, **59**, 86–101.

Jiang, Y., **Liang, G.**, **Yang, S.** & **Yu, D.** (2014) Arabidopsis WRKY57 functions as a node of convergence for jasmonic acid– and auxin-mediated signaling in jasmonic acid–induced leaf senescence. *The Plant Cell*, **26**, 230–245.

Li, S., **Zhou, X.**, **Chen, L.**, **Huang, W.** & **Yu, D.** (2010) Functional characterization of Arabidopsis thaliana WRKY39 in heat stress. *Molecules and Cells*, **29**, 475–483.

Lin, I.S., **Wu, Y.S.**, **Chen, C.T.**, **Chen, G.H.**, **Hwang, S.G.**, **Jauh, G.Y.** ***et al.*** (2017) AtRBOH I confers submergence tolerance and is involved in auxin-mediated signaling pathways under hypoxic stress. *Plant Growth Regulation*, **83**, 277–285.

Love, M.I., **Huber, W.** & **Anders, S.** (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, **15**, 550.

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

Morgan, J.A. (1984) Interaction of water supply and N in wheat. *Plant Physiology*, **76**, 112–117.

Op Den Camp, R.G.L., **Przybyla, D.**, **Ochsenbein, C.**, **Laloi, C.**, **Kim, C.**, **Danon, A.** ***et al.*** (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. *The Plant Cell*, **15**, 2320–2332.

Ort, D.R. (2001) When there is too much light. *Plant Physiology*, **125**, 29–32.

Osthoff, A., **Donà dalle Rose, P.**, **Baldauf, J.A.**, **Piepho, H.-P.** & **Hochholdinger, F.** (2019) Transcriptomic reprogramming of barley seminal roots by combined water deficit and salt stress. *BMC Genomics*, **20**, 325.

Pascual, L.S., **Segarra-Medina, C.**, **Gómez-Cadenas, A.**, **López-Climent, M.F.**, **Vives-Peris, V.** & **Zandalinas, S.I.** (2022) Climate change-associated multifactorial stress combination: a present challenge for our ecosystems. *Journal of Plant Physiology*, **276**, 153764.

Pospíšil, P. (2016) Production of reactive oxygen species by photosystem II as a response to light and temperature stress. *Frontiers in Plant Science*, **7**, 1950.

Prasch, C.M. & **Sonnewald, U.** (2013) Simultaneous application of heat, drought, and virus to Arabidopsis plants reveals significant shifts in signaling networks. *Plant Physiology*, **162**, 1849–1866.

Prasch, C.M. & **Sonnewald, U.** (2015) Signaling events in plants: stress factors in combination change the picture. *Environmental and Experimental Botany*, **114**, 4–14.

Rivero, R.M., **Mittler, R.**, **Blumwald, E.** & **Zandalinas, S.I.** (2022) Developing climate-resilient crops: improving plant tolerance to stress combination. *The Plant Journal*, **109**, 373–389.

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

Roeber, V.M., **Bajaj, I.**, **Rohde, M.**, **Schmülling, T.** & **Cortleven, A.** (2021) Light acts as a stressor and influences abiotic and biotic stress responses in plants. *Plant, Cell and Environment*, **44**, 645–664.

Rushton, P.J., **Somssich, I.E.**, **Ringler, P.** & **Shen, Q.J.** (2010) WRKY transcription factors. *Trends in Plant Science*, **15**, 247–258.

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.

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.

Suzuki, N., **Rivero, R.M.**, **Shulaev, V.**, **Blumwald, E.** & **Mittler, R.** (2014) Abiotic and biotic stress combinations. *New Phytologist*, **203**, 32–43.

Szymańska, R., **Ślesak, I.**, **Orzechowska, A.** & **Kruk, J.** (2017) Physiological and biochemical responses to high light and temperature stress in plants. *Environmental and Experimental Botany*, **139**, 165–177.

Takahashi, S. & **Murata, N.** (2008) How do environmental stresses accelerate photoinhibition? *Trends in Plant Science*, **13**, 178–182.

Xing, D.H., **Lai, Z.B.**, **Zheng, Z.Y.**, **Vinod, K.M.**, **Fan, B.F.** & **Chen, Z.X.** (2008) Stress- and pathogen-induced Arabidopsis WRKY48 is a transcriptional activator that represses plant basal defense. *Molecular Plant*, **1**, 459–470.

Zandalinas, S.I., **Fritschi, F.B.** & **Mittler, R.** (2020) Signal transduction networks during stress combination. *Journal of Experimental Botany*, **71**, 1734–1741.

Zandalinas, S.I., **Fritschi, F.B.** & **Mittler, R.** (2021) Global warming, climate change, and environmental pollution: recipe for a multifactorial stress combination disaster. *Trends in Plant Science*, **26**, 588–599.

Zandalinas, S.I. & **Mittler, R.** (2022) Plant responses to multifactorial stress combination. *New Phytologist*, **234**, 1161–1167.

Zandalinas, S.I., **Mittler, R.**, **Balfagón, D.**, **Arbona, V.**, **Gómez-Cadenas, A.**, **Balfagon, D.** ***et al.*** (2018) Plant adaptations to the combination of drought and high temperatures. *Physiologia Plantarum*, **162**, 2–12.

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.**, **Burks, D.**, **Azad, R.K.** & **Mittler, R.** (2019) Identification and characterization of a core set of ROS wave-associated transcripts involved in the systemic acquired acclimation response of Arabidopsis to excess light. *The Plant Journal*, **98**, 126–141.

Zhang, X., **Henriques, R.**, **Lin, S.S.**, **Niu, Q.W.** & **Chua, N.H.** (2006) Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. *Nature Protocols*, **1**, 641–646.