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Reduction of heat stress pressure and activation of photosystem II repairing system are crucial for citrus tolerance to multiple abiotic stress combination

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Abstract

Drought, heat and high irradiance are abiotic stresses that negatively affect plant development and reduce crop productivity. The confluence of these three factors is common in nature, causing extreme situations for plants that compromise their viability. Drought and heat stresses increase the saturation of the photosystem reaction centers, increasing sensitivity to high irradiance. In addition, these stress conditions affect photosystem II (PSII) integrity, alter redox balance of the electron transport chain and decrease the photosynthetic rate. Here, we studied the effect of the stress combinations on the photosynthetic apparatus of two citrus genotypes, Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*) and Cleopatra mandarin (*Citrus reshni*). Results obtained showed that physiological responses, such as modulation of stomatal aperture and transpiration rate, aimed to reduce leaf temperature, are key to diminishing heat impact on photosynthetic apparatus and increasing tolerance to double and triple combinations of drought, high irradiance and high temperatures. By using transcriptomic and proteomic analyses, we have demonstrated that under these abiotic stress combinations, Carrizo plants were able to increase expression of genes and proteins related to the photosystem repairing machinery (which better maintained the integrity of PSII) and other components of the photosynthetic apparatus. Our findings reveal crucial physiological and genetic responses in citrus to increase tolerance to the combination of multiple abiotic stresses that could be the basis for breeding programs that ensure a sustainable citrus production.

1 | INTRODUCTION

Small variations in environmental conditions, above or below optimum levels for plant growth, constantly occur in cultivated fields. These small changes, such as short periods of drought or heatwaves, may not be enough to cause large impacts on plant health or crop productivity. However, the co-occurrence of two or more stress situations is common in nature and in agroecosystems and may have additive impacts (Zandalinas, Fritschi, & Mittler, 2021; Zandalinas & Mittler, 2022). Studies carried out in recent years have shown that the combination of two or three simultaneous adverse conditions causes a new stress situation in plants, resulting in synergistic deleterious interactions that exceed the sum of the effects of each individual stress (Balfagón et al., 2019, 2022; Fábián et al., 2019; Shaar-Moshe

et al., 2017; Suzuki et al., 2016; Zandalinas et al., 2018; Zandalinas, Sengupta, et al., 2021; Zhao et al., 2017). The damage caused by the combination of stresses, due to its effects on the vegetative development and on the reproductive processes of crops, threatens agricultural production (Balfagón et al., 2020; Elferjani & Soolanayakanahally, 2018; Fábián et al., 2019; Mahalingam & Bregitzer, 2019; Sinha et al., 2021).

The Mediterranean basin, one of the most sensitive regions to climate change (Korres et al., 2016), is threatened by progressive decreases in rainfall together with increases in temperatures, which affect both ecosystems and agriculture production. Consequently, the citrus industry, with a high economic and social importance in the Mediterranean area (Zhong & Nicolosi, 2020), is likely to be severely affected by these changing environmental conditions, causing a strong impact in the region (De Ollas et al., 2019). Therefore, it is important to study how the combination of abiotic stress factors affects citrus physiology to establish a basis for new techniques and breeding programs that ensure a sustainable citrus production.

Drought and high temperatures are among the most detrimental abiotic stresses for agricultural production (Balfagón et al., 2020; Lesk et al., 2016; Li et al., 2009; Suzuki et al., 2014; Zhao et al., 2017). In addition, drought and high temperatures together with high irradiance often co-occur in the same location (Teuling, 2018). These three stresses induce different physiological responses in plants that lead to reduced photosynthesis. Under water stress conditions, this decline is mainly produced by an impaired CO_2 diffusion from the atmosphere caused by stomatal closure (Chaves et al., 2009). On the other hand, high temperatures trigger stomatal opening to increase transpiration rate and cool the leaves. This response may induce an increase in the photosynthetic rate due to an enhanced CO_2 exchange with the atmosphere. However, extreme temperatures or long periods of heat negatively affect PSII integrity due to the destacking of thylakoid membranes, reducing therefore the photosynthetic efficiency (Pospíšil, 2016). High irradiance causes energy saturation in the reaction centers of photosystems, leading to the reduction of the energy fraction utilized in photosynthesis (Ruban, 2009). In addition, all these stress factors cause an increase in reactive oxygen species (ROS) production derived from the photochemical reactions imbalance. Over accumulation of ROS, such as $^1O_2$ or H_2O_2 (which leads to the formation of the highly reactive hydroxyl free radical $OH^{\cdot}$), causes oxidative damage in the PSII, affecting especially the D1 protein, and hinders its reparation (Nishiyama et al., 2006). As a consequence, other components of the photosynthetic electron transport chain (PETC; Cyt-b_6f, PSI or F-type ATPase) become more exposed to photooxidative reactions (Takahashi & Murata, 2008; Tikkanen et al., 2014). This situation leads to a reduction of the photosynthetic proteins, an impairment of the PETC functioning and the inability to accomplish photosynthesis (Nishiyama et al., 2011).

Previous studies on Carrizo citrange and Cleopatra mandarin, two citrus genotypes with contrasting sensitivity to abiotic stress conditions, showed that Carrizo is more tolerant to drought and heat stress combination than Cleopatra due to a proper metabolic rearrangement, a higher activation of the antioxidant system (Balfagón et al., 2018; Zandalinas et al., 2017) and an improved physiological response to alleviate the effect of high temperatures (Zandalinas et al., 2016). To further investigate the influence of multiple stress factors on the photosynthetic apparatus, in this work, we studied the physiological, transcriptomic and proteomic responses of these citrus genotypes to combined conditions of drought, heat and high light intensity. Data showed that, in citrus plants subjected to environmental conditions where drought, high irradiance and/or high temperatures converge, mechanisms to reduce heat stress pressure on the photosynthetic apparatus and activation of PSII repairing genes are crucial for plant survival.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Carrizo citrange (*Poncirus trifoliata* L. Raf. × *Citrus sinensis* L. Osb.) and Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) plants were purchased from an authorized nursery (Beniplant S.L., Penyíscola, Spain). One-year-old seedlings of both citrus genotypes were transferred to 0.6 L plastic pots filled with perlite and watered three times a week with 0.5 L of a half-strength Hoagland solution in greenhouse conditions (natural photoperiod and day and night temperature averaging 25.0 ± 3.0°C and 18.0 ± 3.0°C, respectively). Plants were allowed to acclimate for 2 weeks in growth chambers at 23°C, 16 h/8 h light/dark photoperiod cycle (from 8:00 to 24:00 h), 312 μmol m^{-2} s^{-1} of light intensity and 80% of relative moisture.

2.2 | Stress treatments

Seven different stress treatments were applied in parallel to plants of both citrus genotypes: water stress (WS), high irradiance/light (HL), heat stress (HS), high irradiance and heat stress combination (HL + HS), water stress and high irradiance combination (WS + HL), water stress and heat stress combination (WS + HS) and the combination of water stress, high light and heat stress (WS + HL + HS; Figure 1A). Water stress was imposed by transplanting plants to dry perlite for 24 h. High light was applied by increasing light intensity to 1000 μmol m^{-2} s^{-1}, 8 h per day (from 12:00 to 20:00 h) during 5 days. Heat stress was achieved by increasing temperature to 40°C during 5 days. HL + HS was performed by simultaneously subjecting plants to high light and heat stress treatments during 5 days. WS + HL was applied by subjecting plants to high light and transplanting them to dry perlite for the last 24 h of the stress treatments. WS + HS was performed by increasing temperature to 40°C during 5 days and transplanting plants to dry perlite for the last 24 h of the stress treatments. WS + HL + HS was applied by simultaneously subjecting plants to HL and HS stress treatments during 5 days and transplanting them to dry perlite for the last 24 h of the stress treatments. Another group of plants was grown under control conditions (CT). The recovery period consisted of returning plants to control

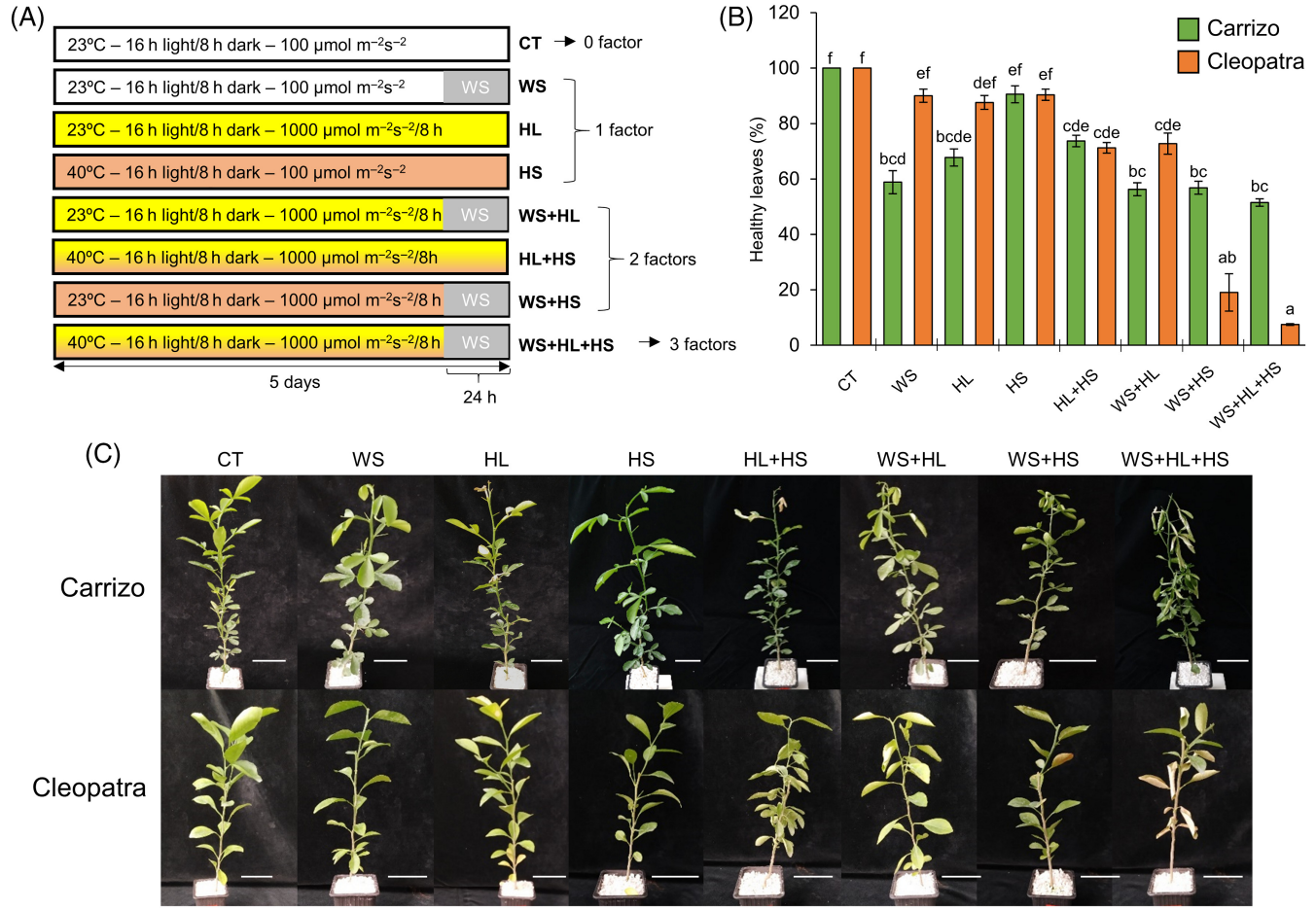

FIGURE 1 (A) The experimental design used for the study of water stress (WS), high light (HL) and heat stress (HS), high light and heat stress combination (HL + HS), water stress and high light stress combination (WS + HL), water stress and heat stress combination (WS + HS), and water stress, high light and heat stress combination (WS + HL + HS) using Carrizo and Cleopatra plants. (B) Percentage of healthy leaves in Carrizo and Cleopatra plants after the stress treatments. (C) Representative images of Carrizo and Cleopatra plants after the stress treatments. Scale bar = 8 cm. Bars represent mean of 15 plants ± SD. Different letters denote statistical significance among treatments at $p \leq 0.05$ (Kruskal–Wallis test followed by a Conover's post hoc test).

conditions for 24 h after the stress treatments. The number of plants per treatment was 24 and the experiment was repeated three times.

2.3 | Leaf damage index, quantum yield of photosystem II (φPSII) and photosynthetic parameters

Leaf damage index (LDI) was evaluated after each stress treatment similar to Balfagón et al. (2019, 2022). Chlorotic or curved leaves were considered as damaged leaves, and wilted leaves were considered as dead leaves. φPSII was measured with a portable fluorometer (FluorPen FP-MAX 100, Photon Systems Instruments). Measurements were taken from five different plants, taking measurements on three mature leaves from a middle position in the stem per plant, for each stress treatment. In this case, measurements were made at the end of the stress treatment, maintaining light conditions of the treatment (100 or 1000 µmol m^{-2} s^{-1}) and after the recovery period (24 h following the end of each stress; 100 µmol m^{-2} s^{-1} of light intensity). Electron transport rate (ETR) of PSII was obtained similarly to Chen et al. (2021) and Wong et al. (2014) and was calculated as $ETR = \varphi PSII \times PPFD \times 0.5 \times 0.84$.

Net carbon assimilation rate (A), transpiration rate (E), stomatal conductance (gs) and leaf temperature were measured with an LI-6800 Portable Photosynthesis System (Lincoln) under ambient CO_2 and moisture conditions. Supplemental light was provided by a PAR lamp at 100 or 1000 µmol m^{-2} s^{-1} photon flux density, and air flow was set at 150 µmol mol^{-1}. After instrument stabilization, at least 10 measurements were taken on three leaves in five replicate plants from each stress treatment. All experiments were repeated three times.

2.4 | Protein extraction and digestion

Comparative proteomic analyses were performed with three biological replicate leaf samples (300 mg of fresh weight per sample) of Carrizo

citrange and Cleopatra mandarin plants subjected to individual stresses (WS, HL or HS) and to their factorial combinations (HL + HS, WS + HL, WS + HS and WS + HL + HS). Total protein extraction was performed according to Oliveira et al. (2022).

2.5 | Proteomic data analysis

Spectral processing and database searching were performed using the ProteinLynx Global Server (PLGS; version 3.0.2; Waters) as described in Oliveira et al. (2022). For protein identifications, the sweet orange (*C. sinensis*) protein sequence database from National Center for Biotechnology Information Search database was used (NCBI; https://www.ncbi.nlm.nih.gov). The mass spectrometry proteomic data have been deposited with ProteomeXchange (Deutsch et al., 2020) consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD034419. To ensure the quality of the results after data processing, only the proteins that were present or absent (for unique proteins) in all three runs of biological replicates were considered in the differential accumulation analysis using Student's *t*-test (two-tailed; $p < 0.05$). The protein sequences were submitted to a BLAST search against the NCBI nonredundant green plant protein database (taxa: 33090, Viridiplantae).

2.6 | RNA-Seq analysis

Briefly, frozen (−80°C) and grounded leaf tissue from at least 10 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. RNA extraction and purification, preparation of RNA libraries, sequencing, mapping, expression profile, differential gene expression and functional annotation analyses were processed at Macrogen Inc. (Seoul, Korea; https://dna.macrogen.com/). RNA extraction and purification were performed using Maxwell® 16 LEV plant RNA Purification Kit protocol and automated Maxwell® 16 MDx Instrument. RNA integrity was assessed using an Agilent Technologies 2100 Bioanalyzer (or 2200 TapeStation) with an RNA Integrity Number (RIN) value greater than or equal to 7. mRNA libraries were prepared using the Illumina TruSeq Stranded mRNA HT Library Prep Kit (Illumina, https://www.illumina.com/). To verify the size of PCR-enriched fragments, the template size distribution was checked by running on an Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip. mRNA libraries were quantified using qPCR according to the Illumina qPCR Quantification Protocol Guide. To generate a standard curve of fluorescence readings and calculate the library sample concentration, Qubit standard Quantification solution and calculator were used (Invitrogen). Sequencing was performed on a Novaseq 6000 system sequencer (Illumina) and produced total read bases >6.67 Gbp with a GC ≥43.36% and a Q30 score ≥94.7%.

Paired-ends sequenced reads obtained from the Novaseq 6000 system were quality-tested using FastQC v0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), trimmed reads were mapped to the reference genome with HISAT2 v2.1.0 (Kim et al., 2015) and transcripts were assembled by StringTie v2.1.3b (Pertea et al., 2015, 2016) with aligned reads. *C. sinensis* (GCF_000317415.1_Csi_valencia_1.0) obtained from the NCBI genome database (https://www.ncbi.nlm.nih.gov/genome) was used as genome of reference. Differential gene expression analysis was carried out using the R-based package DESeq2 v1.20.0 that is available in Bioconductor (Love et al., 2014). Transcripts differentially expressed in the stress treatments compared to control were identified by examining the difference in their abundance under the different conditions. Read count data were normalized with relative log expression (RLE) method in DESeq2 R library. Then, statistical test was performed with the normalized data. log2(read count + 1) and regularized log (rlog) transformed values were used for data visualization. Statistical analysis was performed using fold change (fc), nbinomWaldTest using DESeq2 per comparison pair. The significant results are selected on conditions of $|fc| \geq 2$ and nbinomWaldTest raw *p*-value <0.05.

Raw and processed RNA-seq data files were deposited in GEO (https://www.ncbi.nlm.nih.gov/geo/) under the following accession number GSE203331.

2.7 | Statistical analysis

All experiments were repeated at least three times. Results are presented as the mean ± SD. Statistical analyses of LDI were performed using Kruskal–Wallis test followed by a Conover post hoc test. Statistical analyses of A, E, gs, leaf temperature, φPSII and ETR parameters were performed using ANOVA followed by a Tukey post hoc test. In both statistical tests, different letters denote statistical significance $p < 0.05$ (Table S10). Statistical analyses of protein relative levels were performed by two-tailed Student's *t*-test (asterisks denote statistical significance at $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$ comparing the stress treatments with respect to control values within the same genotype).

3 | RESULTS

3.1 | Tolerance of citrus plants to WS, HL and HS stress conditions applied individually or in combination

To study the responses of citrus plants to individual and multiple combined abiotic stress conditions, Carrizo citrange and Cleopatra mandarin plants were subjected to individual stresses (WS, HL or HS) and to their factorial combinations (HL + HS, WS + HL, WS + HS and WS + HL + HS; Figure 1A). Leaf damage caused on Carrizo and Cleopatra plants gradually increased as stress factors were added (Figure 1B,C and Figure S1). In Carrizo plants, stress combinations increased the number of damaged leaves and, more markedly, of dead leaves. HL + HS, WS + HL and WS + HL + HS increased the percentage of dead leaves to 10.8%, 6.1% and 15.2%, respectively. The

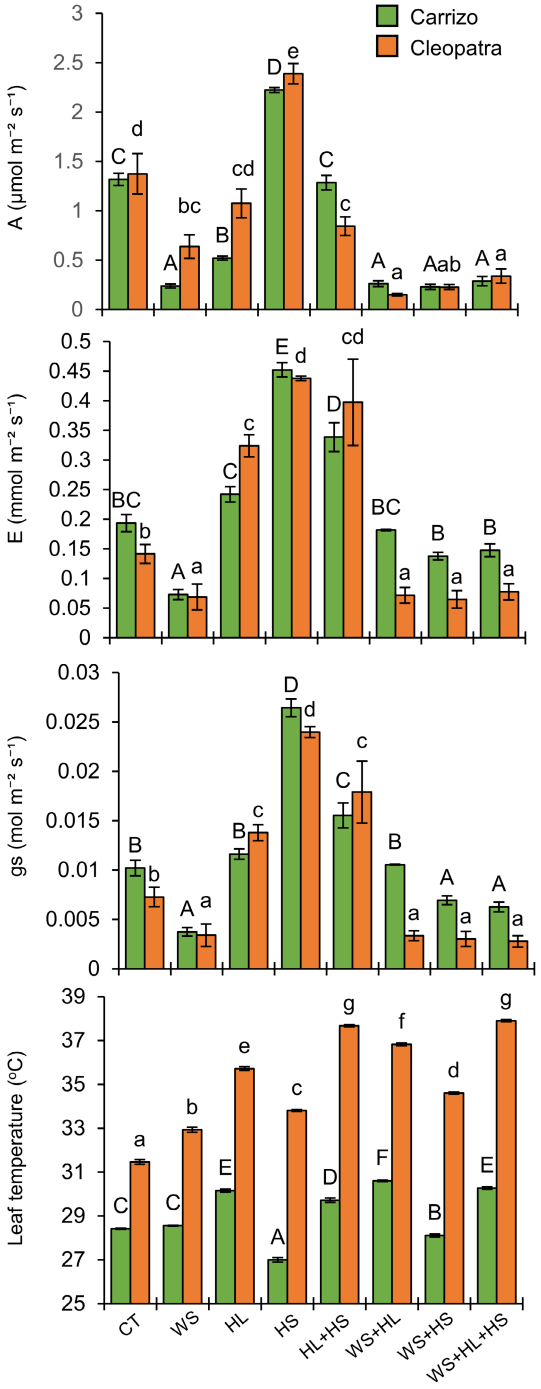

FIGURE 2 Net rate of carbon assimilation (A), transpiration rate (E), stomatal conductance (gs) and leaf temperature of Carrizo (green) and Cleopatra (orange) plants subjected to WS, HL, HS, HL + HS, WS + HL, WS + HS and WS + HL + HS. Bars represent mean of 10 measurements taken on three leaves in five plants ± SD. Uppercase letters denote statistical significance among treatments at $p \leq 0.05$ (ANOVA followed by a Tukey's post hoc test) for Carrizo plants. Lowercase letters denote statistical significance among treatments at $p \leq 0.05$ (ANOVA followed by a Tukey's post hoc test) for Cleopatra plants.

percentage of healthy leaves decreased to 56.2%, 56.8% and 51.4% in Carrizo plants subjected to WS + HL, WS + HS and WS + HL + HS, respectively. In Cleopatra, the stresses hit harder, HL + HS and WS + HL reduced the percentage of healthy leaves to 71.2% and 72.7%, respectively, and only 19% of healthy leaves with the combined WS + HS stress. Furthermore, the triple stress damaged 92.5% of Cleopatra leaves with 23% of completely dead leaves (Figure 1B,C and Figure S1).

3.2 | Physiological responses of citrus plants to WS, HL and HS stress conditions applied individually or in combination

Net rate of carbon assimilation (A), transpiration rate (E), stomatal conductance (gs) and leaf temperature of Carrizo and Cleopatra plants were measured under individual and combined stresses (Figure 2). In Carrizo plants, HL and, to a greater extent, WS induced a reduction of A, whereas HS significantly increased this parameter. WS effect on A prevailed when combined with other stresses, as observed in plants subjected to WS +- HL, WS + HS and WS + HL + HS. However, HL + HS did not modify this parameter with respect to control. Net rate of carbon assimilation values decreased in Cleopatra plants subjected to individual and combined stress conditions, except for those under individual HL (with no significant changes) or under HS (with a significant increase). In contrast to Carrizo plants, the combination of both HL and HS (HL + HS) caused a reduction of A in plants of this genotype.

In Carrizo, WS reduced E and gs values. However, E reduction was not observed in plants subjected to WS + HL, WS + HS and WS + HL + HS conditions, and only slight reductions of gs were observed under WS + HS and WS + HL + HS. Transpiration rate values increased in Carrizo plants subjected to HL, HS and HL + HS conditions with respect to control, but gs values only increased significantly under HS and HL + HS. Under WS, applied individually or in combination with HL and/or HS, Cleopatra plants had decreased E and gs values. In this genotype, HL, HS and HL + HS induced higher levels of E and gs than control (Figure 2).

Finally, the leaf temperature of Carrizo plants showed small variations (±2°C) upon the different stress conditions, whereas these variations were higher in Cleopatra plants (±7°C). HL, HL + HS, WS + HL and WS + HL + HS induced significant increases of leaf temperature in both genotypes. However, under WS, HS and WS + HS, leaf temperature increased significantly in Cleopatra plants but remained as control or even decreased in Carrizo (Figure 2).

3.3 | PSII efficiency of plants subjected to stress combinations

PSII efficiency in terms of quantum yield (φPSII) was measured in Carrizo and Cleopatra plants subjected to individual stresses and to their factorial combinations. φPSII was also measured 24 h after releasing the stress conditions to examine the photochemical efficiency of PSII (averaged over closed and open PSII traps) when leaves were illuminated at a standard irradiance of 100 μmol m^{-2} s^{-1} (Balfagón et al., 2019; Manaa

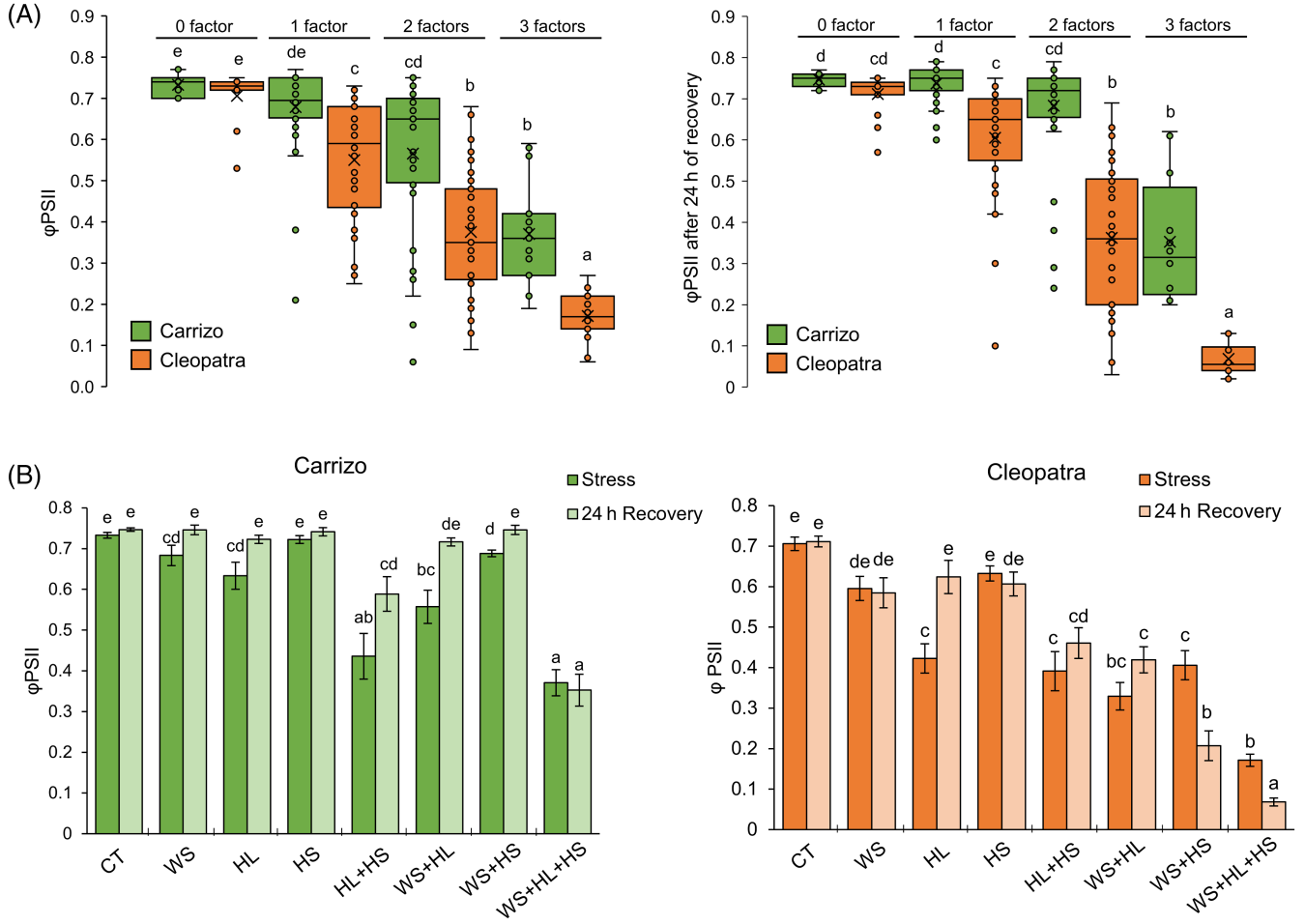

FIGURE 3 (A) The impact of one, two or three stress factors applied simultaneously on φPSII of Carrizo and Cleopatra plants, at the end of the stress period (left) and 24 h after recovery from the stress treatments (right). (B) φPSII values immediately before the end of each stress (dark) and 24 h after recovery from the stress treatments (pale) of Carrizo and Cleopatra plants subjected to WS, HL, HS, HL + HS, WS + HL, WS + HS and WS + HL + HS. Box plots show the median (horizontal line), the lower and upper bounds of each box plot denote the first and third quartiles (the 25th and 75th percentiles, respectively), and whiskers above and below the box plot indicate 1.5 times the interquartile range. Bars represent mean of 15 measurements taken in 5 plants ± SD. Different letters denote statistical significance among treatments at $p \leq 0.05$ (ANOVA followed by a Tukey's post hoc test).

et al., 2021). As shown in Figure 3A, Carrizo and Cleopatra plants subjected to one stress factor showed φPSII values similar to control plants during and after the stress treatments. φPSII values slightly decreased in Carrizo plants subjected to WS or in plants of both genotypes subjected to HL (Figure 3B). Applying two or three simultaneous stress factors negatively altered PSII efficiency in both genotypes, showing a more pronounced reduction in Cleopatra, especially in response to 3-factor stress combination (Figure 3A). Furthermore, Cleopatra φPSII values were lower than those of Carrizo after the recovery period in response to 2- or 3-factor combinations. HL + HS and WS + HL + HS conditions deeply affected φPSII in Carrizo plants during the stress and prevented recovery to control values after 24 h of stress release. WS + HS and WS + HL + HS were extremely damaging to Cleopatra plants since φPSII values continued decreasing even when the control conditions were reestablished (Figure 3).

Electron transport rate (ETR) values were not significantly affected by WS, HS, HL + HS and WS + HS during or after the stress treatments given to Carrizo (Figure S2). HL and WS + HL induced an increase in ETR levels with respect to CT during stress but not after the recovery period. In plants under WS + HL + HS, ETR levels were similar to CT during the stress treatment but significantly decreased after the recovery period. In Cleopatra, individual stresses did not alter ETR. However, plants under HL + HS and WS + HL showed a decrease in this parameter after the recovery period. Finally, WS + HS and WS + HL + HS significantly reduced the ETR values of Cleopatra plants during the stress treatment and after the recovery period (Figure S2).

3.4 | Accumulation of transcripts and proteins related to photosynthetic processes

To further study the ability of citrus plants to tolerate additive abiotic stress factors and maintain their photosynthetic capacity,

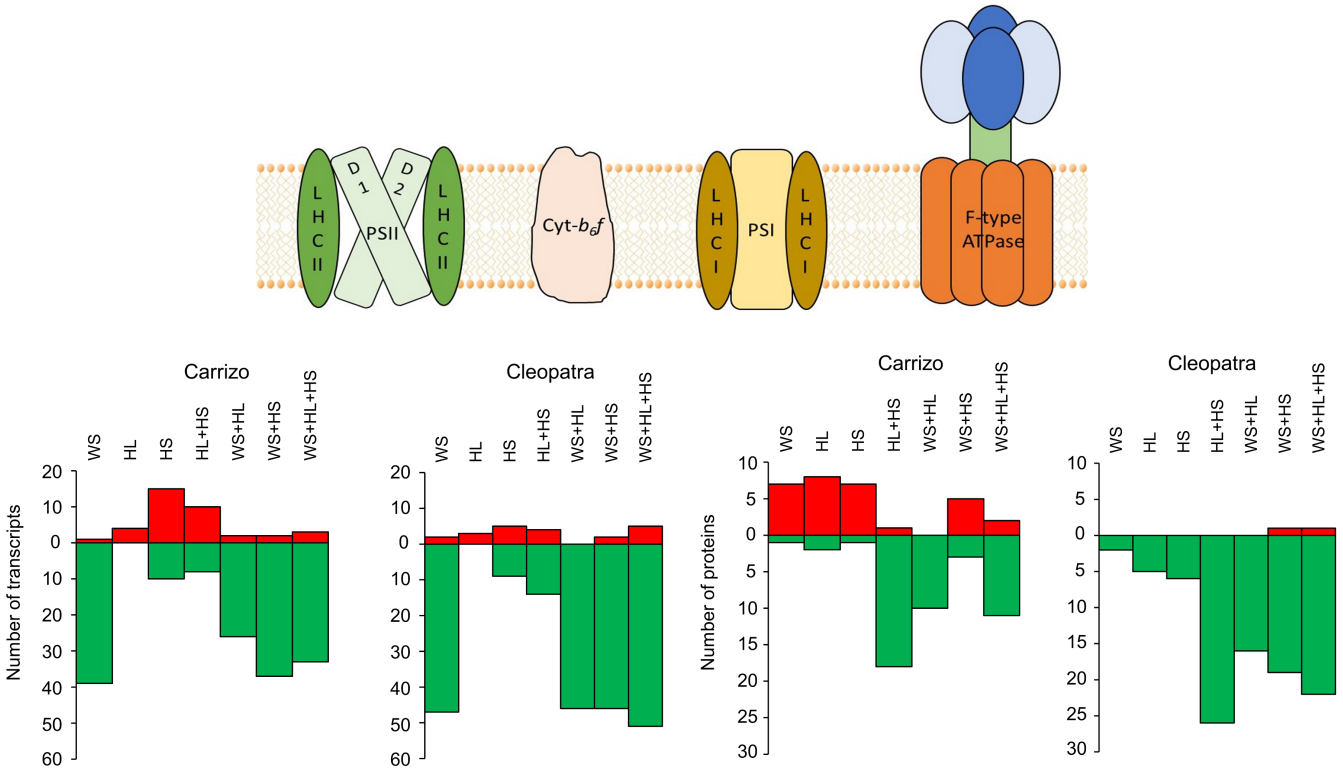

FIGURE 4 Number of up- and downregulated transcripts (left) and proteins (right), with respect to CT, from the photosynthetic apparatus in leaves of Carrizo and Cleopatra plants subjected to WS, HL, HS, HL + HS, WS + HL, WS + HS and WS + HL + HS. Red bars represent upregulated transcripts or proteins and green bars represent downregulated transcripts or proteins. A picture depicting the protein complexes of the different structures of the photosynthetic apparatus is shown on top.

we evaluated the number of transcripts and proteins of the photosynthetic system that were up- or down-regulated in plants subjected to individual and combined stresses (Figure 4; Tables S1 and S2). As shown in Figure 4, a clear downregulation of transcripts in plants of both genotypes under WS conditions (WS, WS + HL, WS + HS and WS + HL + HS) was observed. However, the number of repressed transcripts was higher in Cleopatra than in Carrizo, especially under WS + HL, WS + HS and WS + HL + HS conditions. In addition, only a few transcripts related to the photosynthetic apparatus were upregulated under these stress conditions, highlighting the induction of three and five transcripts under WS + HL + HS in Carrizo and Cleopatra, respectively (Figure 4 and Table S1). HL only induced the upregulation of four transcripts in Carrizo and three in Cleopatra. The number of upregulated transcripts induced by HS was higher in Carrizo (15) than in Cleopatra (5). Similarly, HL + HS induced the accumulation of a higher number of transcripts in Carrizo (10) than in Cleopatra (4), and transcript downregulation was lower in Carrizo (8) than in Cleopatra (14) (Figure 4 and Table S1).

The relative protein accumulation pattern was different between individual and combined stresses, particularly in Carrizo plants. Individual WS, HL and HS induced the accumulation of different proteins of the photosynthetic complexes (7, 8 and 7, respectively) in Carrizo, whereas only one (in response to WS and HS) or two (in response to HL) proteins were less abundant in Cleopatra (Figure 4 and Table S2). In Carrizo, 18 proteins related to the photosynthetic apparatus were less abundant under HL + HS conditions, 10 under WS + HL and 11 under WS + HL + HS. However, WS + HS induced an accumulation of five proteins and a reduction of three. Relative levels of photosynthetic proteins of Cleopatra plants decreased in all stress conditions, especially under stress combinations. In addition, only one protein increased its content in plants subjected to WS + HS or WS + HL + HS in this genotype (Figure 4 and Table S2).

The number of differentially regulated transcripts and proteins from the KEGG Pathway "Carbon fixation in photosynthetic organisms" was analyzed in Carrizo and Cleopatra plant subjected to individual and combined stresses (Figure S3A; Tables S3 and S4). More transcripts were upregulated than downregulated under WS, HS, WS + HL, WS + HS and WS + HL + HS in both genotypes. The number of upregulated transcripts increased in Carrizo under HL + HS, while a general downregulation was observed in Cleopatra. HL did not affect the accumulation of these transcripts in Carrizo and induced the upregulation of one transcript in Cleopatra. Proteins from the "Carbon fixation in photosynthetic organisms" pathway were more abundant in Carrizo leaves under HL and in Cleopatra under WS. However, in both genotypes, the other stress conditions induced a reduction in the relative amount of proteins of this category. Finally, as shown in Figure S3B, any stress condition caused significant variations in the content of the two RuBisCo subunits.

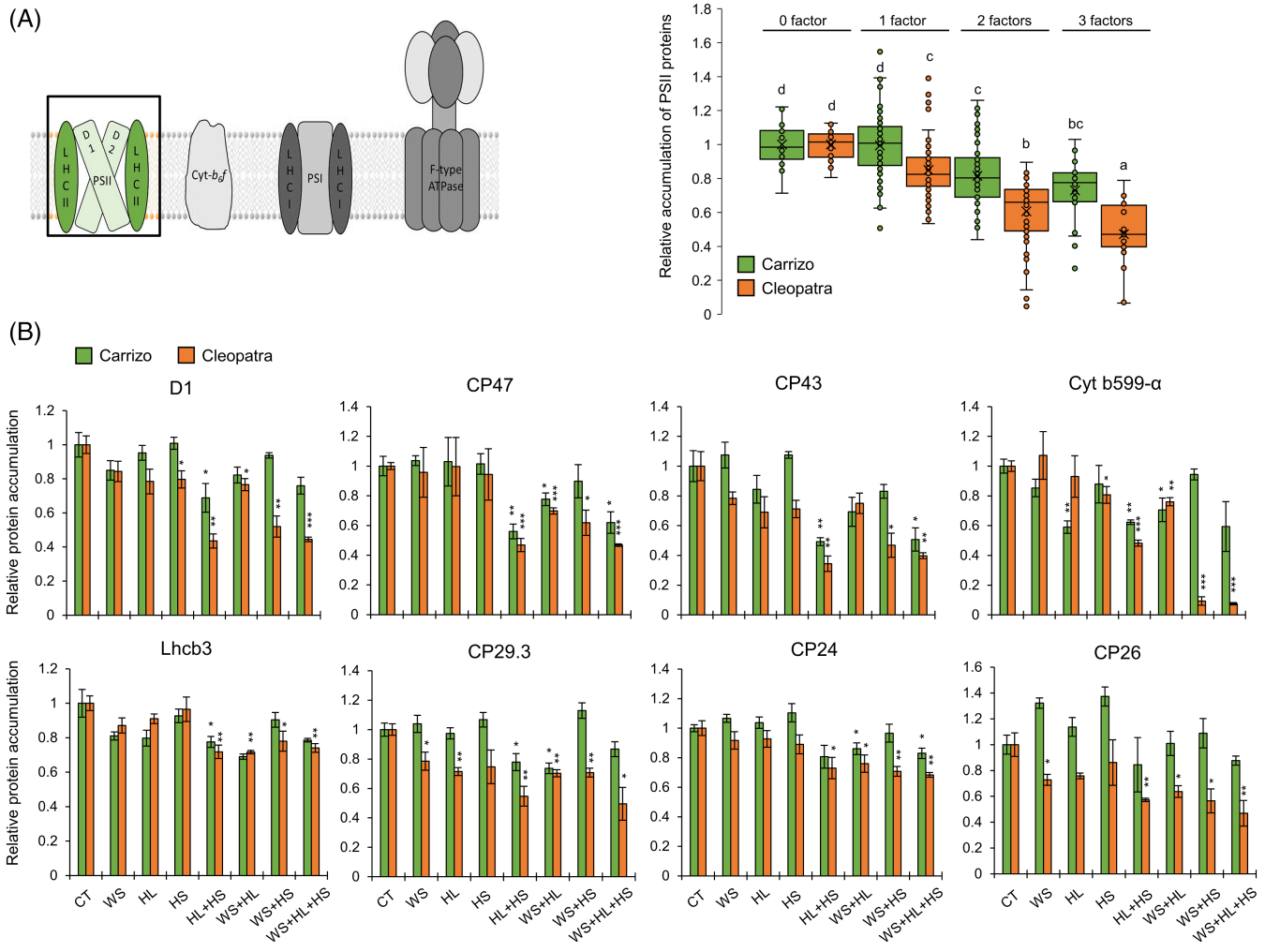

FIGURE 5 (A) The impact of one, two or three stress factors applied simultaneously on content of PSII proteins in leaves of Carrizo and Cleopatra plants. A picture depicting the protein complexes of the different structures of the photosynthetic apparatus is shown (left), and the PSII complex is represented in color. (B) Accumulation of PSII proteins (D1, CP47, CP43, Cyt b599-α, Lhcb3, CP29.3, CP24 and CP26) in leaves of Carrizo and Cleopatra plants subjected to WS, HL, HS, HL + HS, WS + HL, WS + HS and WS + HL + HS; $n = 3$. Box plots show the median (horizontal line), the lower and upper bounds of each box plot denote the first and third quartiles (the 25th and 75th percentiles, respectively), and whiskers above and below the box plot indicate 1.5 times the interquartile range. Different letters denote statistical significance among treatments at $p < 0.05$ (ANOVA followed by a Tukey's post hoc test). Asterisks denote Student's *t*-test statistical significance at $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ with respect to control values within the same genotype.

3.5 | Accumulation of transcripts and proteins involved in PSII structure and repairing

PSII is the first component of the photosynthetic apparatus affected by abiotic stresses (Tikkanen et al., 2014; Yi et al., 2022). To examine the tolerance of Carrizo and Cleopatra to the additive effect of stresses, we evaluated the content of proteins forming the PSII (Figure 5 and Table S2) and that of transcripts and proteins related to the activation of the PSII repairing system (Figure 6; Tables S5 and S6) in both genotypes under conditions of individual WS, HL, HS and their factorial combinations. As shown in Figure 5A, the addition of abiotic stress factors decreased the levels of PSII proteins in both Carrizo and Cleopatra in a directional trend. However, levels of proteins involved in the PSII in Carrizo were higher than those in Cleopatra under individual or combined stresses (Figure 5A). As shown in Figure 5B, HL + HS combination significantly affected the content of six and eight PSII proteins in Carrizo and Cleopatra, respectively. Levels of proteins CP47, CP29.3, CP24 and Cyt b599-α in Carrizo and Cleopatra plants subjected to WS + HL were similarly reduced with respect to their controls, whereas D1, Lhcb3 and CP26 levels decreased in Cleopatra but not in Carrizo plants. In addition, WS + HS and WS + HL + HS affected a higher number of PSII proteins in Cleopatra than in Carrizo. Therefore, D1, CP47, CP43, Cyt b599-α, Lhcb3, CP29.3, CP24 and CP26 were significantly less abundant under these stress combinations. In contrast, none of these proteins was affected by WS + HS in Carrizo plants, and only CP47, CP43 and CP24 contents were reduced by WS + HL + HS (Figure 5B and Table S2).

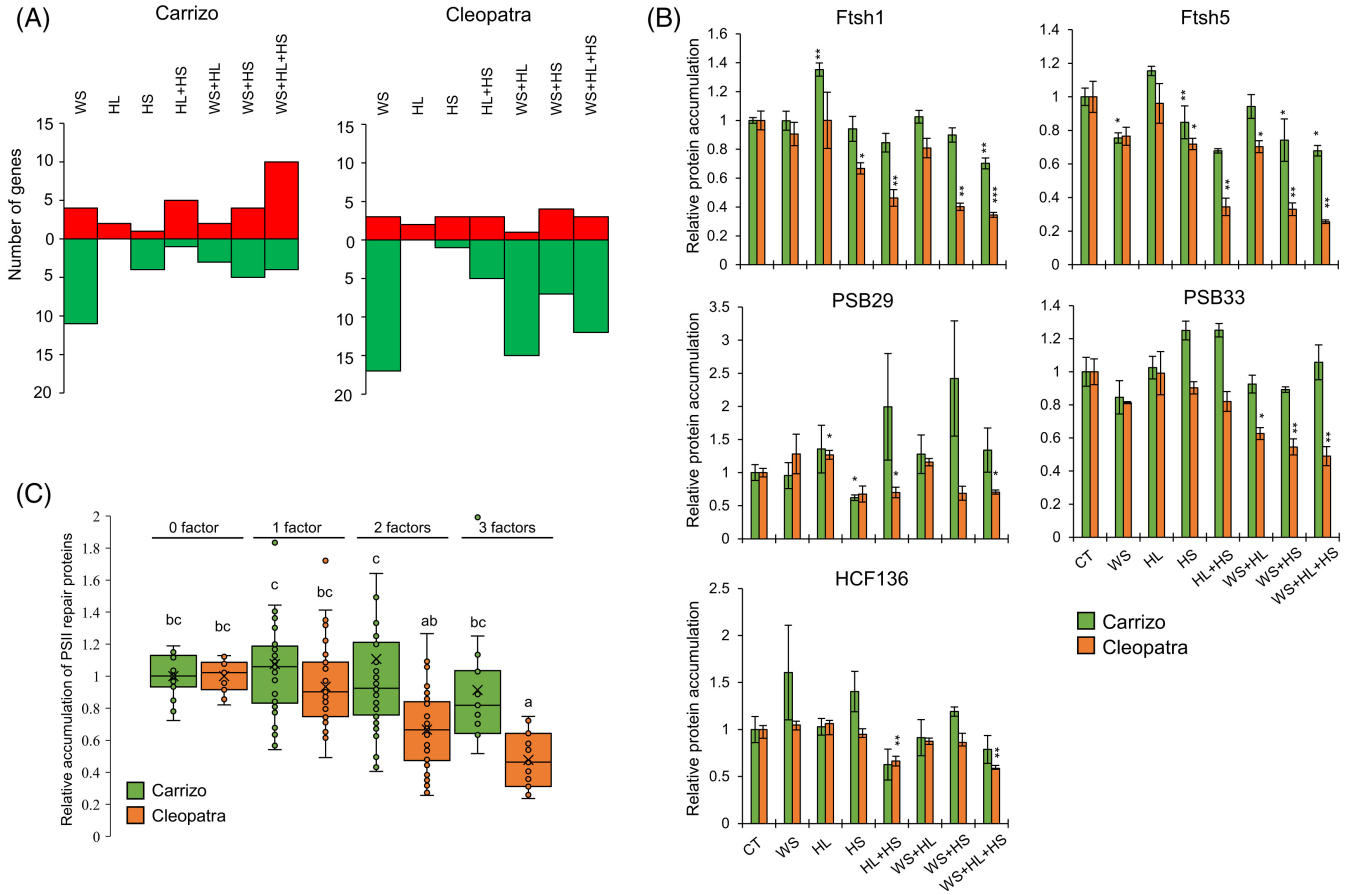

FIGURE 6 (A) Number of up- and down-regulated transcripts, with respect to CT, involved in the PSII-repairing system in leaves of Carrizo and Cleopatra plants subjected to WS, HL, HS, HL + HS, WS + HL, WS + HS and WS + HL + HS. (B) Relative accumulation of PSII-repairing system proteins (Ftsh1, Ftsh5, PSB29, PSB33 and HCF136) in leaves of Carrizo and Cleopatra plants subjected to WS, HL, HS, HL + HS, WS + HL, WS + HS and WS + HL + HS with respect to CT values; $n = 3$. (C) The impact of one, two or three stress factors applied simultaneously on the content of the PSII-repairing system proteins in leaves of Carrizo and Cleopatra plants. Box plots show the median (horizontal line), the lower and upper bounds of each box plot denote the first and third quartiles (the 25th and 75th percentiles, respectively), and whiskers above and below the box plot indicate 1.5 times the interquartile range. Different letters denote statistical significance among treatments at $p < 0.05$ (ANOVA followed by a Tukey's post hoc test). Asterisks denote Student's *t*-test statistical significance at $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ with respect to control values within the same genotype.

Figure 6 shows the number of genes and proteins related to the PSII repairing system that were significantly up- or down-regulated under each stress condition in both citrus genotypes. Cleopatra showed a higher number of downregulated transcripts than Carrizo in response to WS and combined stress conditions, especially WS + HL and WS + HL + HS. In turn, Carrizo showed a higher number of upregulated genes in response to HL + HS and WS + HL + HS compared to Cleopatra (Figure 6A and Table S5). Relative protein content decreased due to the addition of stresses in Cleopatra plants but remained as control in Carrizo (Figure 6B and Table S6). Thus, HL + HS and WS + HS reduced the relative levels of Ftsh1 in Cleopatra plants, whereas they have no effect on Carrizo. In addition, WS + HL + HS reduced Ftsh1 abundance to a higher extent in Cleopatra than in Carrizo (Figure 6B and Table S6). Similarly, Ftsh5 relative concentration decreased in response to all stress combinations in Cleopatra plants and only in response to WS + HS and WS + HL + HS in Carrizo. Relative accumulation of PSB29, PSB33 and HCF136 proteins was mostly reduced in Cleopatra plants subjected to two or three stress factors, which indicates that this genotype had a lower availability of the PSII repairing system proteins than Carrizo (Figure 6C).

3.6 | Accumulation of proteins involved in other photosynthetic complexes

The relative contents of PsaF, PsaH, Lhca1 and Lhca4 proteins, which belong to the PSI complex, were differentially altered in plants of Carrizo and Cleopatra subjected to the different stress conditions (Figure 7A and Table S2). In Carrizo, protein levels were similar to control or even increased in response to all stress conditions.

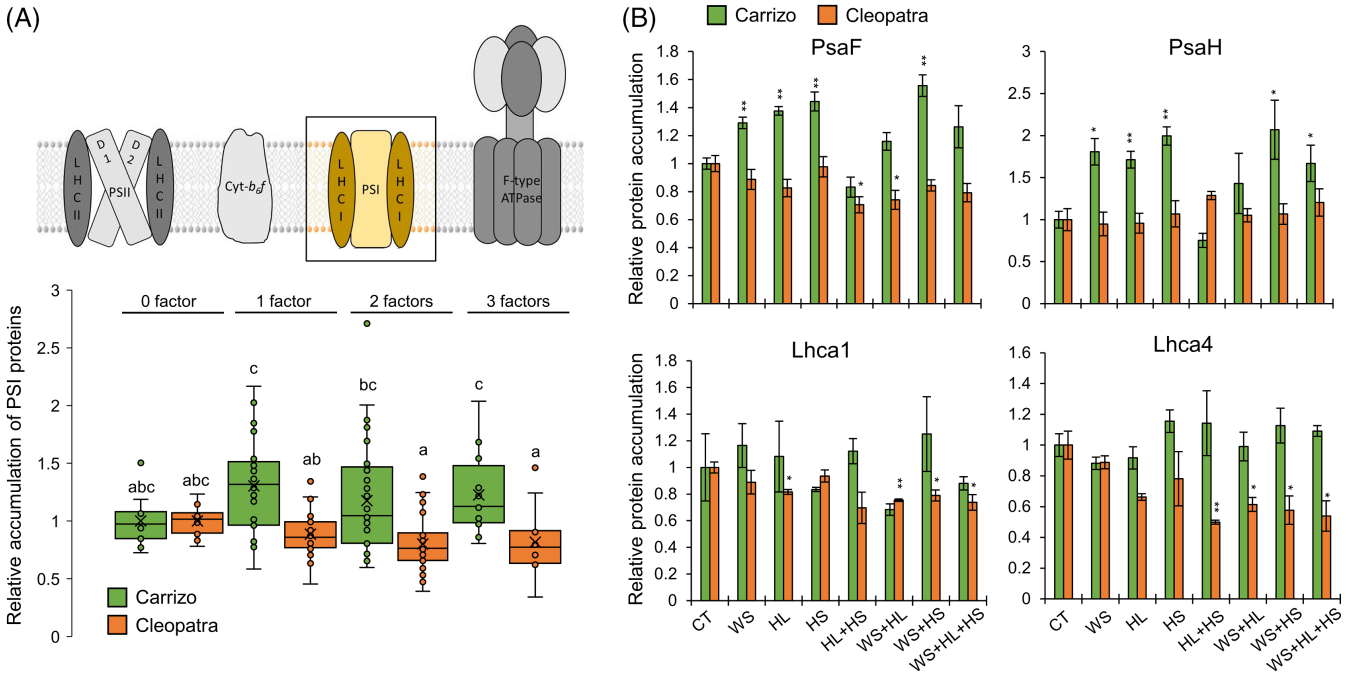

FIGURE 7 (A) The impact of one, two or three stress factors applied simultaneously on content of PSI proteins in leaves of Carrizo and Cleopatra plants. A picture depicting the protein complexes of the different structures of the photosynthetic apparatus is shown (left), and the PSI complex is represented in color. (B) Relative accumulation of PSI proteins (PsaF, PsaH, Lhca1 and Lhca4) in leaves of Carrizo and Cleopatra plants subjected to WS, HL, HS, HL + HS, WS + HL, WS + HS and WS + HL + HS with respect to CT values; $n = 3$. Box plots show the median (horizontal line), the lower and upper bounds of each box plot denote the first and third quartiles (the 25th and 75th percentiles, respectively), and whiskers above and below the box plot indicate 1.5 times the interquartile range. Different letters denote statistical significance among treatments at $p < 0.05$ (ANOVA followed by a Tukey's post hoc test. Asterisks denote Student's *t*-test statistical significance at $^{*}p < 0.05$ or $^{**}p < 0.01$ with respect to control values within the same genotype.

However, levels of PsaF, Lhca1 and Lhca4 decreased in Cleopatra under some of the two- and/or three-factor stress combinations (Figure 7B).

Proteins of the Cytochrome b_6/f complex, PetA and PetB, were affected by the combination of stress conditions in both citrus genotypes. As shown in Figure S4, these proteins were significantly downregulated in Cleopatra plants subjected to the combination of two and three stress factors. On the contrary, relative levels of these proteins remained similar to controls in Carrizo plants despite the effect of stress combination. Only HL + HS caused a decrease in PetA and PetB contents in this genotype (Figure S4).

Finally, F-type ATP synthase protein subunits, ATP-α, ATP-δ and ATP-ε were differentially accumulated in Carrizo and Cleopatra plants subjected to the different abiotic stresses and their combination (Figure S5). In Carrizo, no drastic differences in levels of these proteins were found in response to stress, and only ATP-α levels slightly decreased under HL + HS, WS + HS and WS + HL + HS. In turn, ATP-δ content increased under WS + HS and ATP-ε content increased under individual HL or HS (Figure S5). Conversely, Cleopatra plants showed a decrease in the content of these proteins concomitant to the addition of stress conditions. Thus, the combination of three stress factors caused a more marked reduction of these proteins, with respect to control, in this citrus genotype (Figure S5).

3.7 | Accumulation of transcripts and proteins involved in ROS and chloroplast retrograde signal pathways

The number of differentially regulated ROS-responsive transcripts and proteins was represented in Figure S6. WS and WS + HL + HS induced the highest variation in the accumulation pattern of ROS-responsive transcripts in both genotypes. On the contrary, HL and HS induced the least variation in the accumulation of these transcripts with respect to CT (Figure S6 and Table S7). Regarding the accumulation of ROS-responsive proteins in both genotypes, WS and WS + HL were the conditions that induced the fewest changes, while HL + HS, WS + HS and WS + HL + HS had a higher impact on plants (Figure S6 and Table S8).

The expression levels of TFs involved in the chloroplast retrograde signaling pathway (Yurina & Odintsova, 2019) in Carrizo and Cleopatra plants subjected to individual and combined stresses were analyzed (Table S9). The expression of five TFs was altered by some of the stress conditions in both genotypes. WS + HL + HS was the condition that induced more changes in the regulation of these transcripts. Among these TFs, ANACO17 and WRKY40 were upregulated in Carrizo and Cleopatra plants subjected to WS + HL + HS and WS + HS. WRKY63 expression was induced in Cleopatra plants under

WS + HL + HS but repressed in Carrizo ones. WS + HL + HS induced the up-regulation of PTM in Carrizo and the downregulation of GLK1 in Cleopatra (Table S9).

4 | DISCUSSION

Abiotic stresses affect PSII integrity, destabilizing the electrochemical balance of the electron transport chain, and increasing oxidative damage in chloroplasts and cells (Murata et al., 2007; Pospíšil, 2016; Wang et al., 2018). The combination of abiotic stresses aggravates the damage caused to the photosynthetic apparatus and can even result in plant death (Awasthi et al., 2014; Balfagón et al., 2019; Sehgal et al., 2017). Results obtained in this work indicate that the confluence of two or three abiotic stress factors that often co-occur in nature (WS, HL and HS), even at a moderate intensity or during a short period, caused a significant leaf damage on citrus plants, disrupted photosynthetic apparatus and compromised photosynthetic performance. Thus, triple stress was the most damaging condition to both studied genotypes. In addition, the increasing number of combined stress factors caused a unique and genotype-dependent response. Previous studies showed that the combination of two stress factors caused specific responses on plants (Balfagón et al., 2019, 2022; Lopez-Delacalle et al., 2021; Ravi et al., 2020; Shaar-Moshe et al., 2017; Zandalinas et al., 2019; Zhang & Sonnewald, 2017). This work shows how a factorial combination of three abiotic stresses differently affects the photosynthetic capacity and gene regulation of two citrus genotypes with contrasting tolerance to abiotic stresses (Zandalinas et al., 2016, 2017).

Differences in tolerance to stress combinations were noticeable between the analyzed genotypes. Cleopatra is sensitive to high temperatures (Zandalinas et al., 2016) and, as shown in this work, the addition of WS or WS + HL to HS increased the damage caused to the plant (Figure 1). Moreover, Carrizo, which is a genotype more tolerant than Cleopatra to WS + HS (Zandalinas et al., 2016, 2017), was more affected in terms of photosynthetic parameters (φPSII and ETR; Figure 3 and Figure S2) by the addition of a new stress factor, such as HL. Stress combinations modified stomatal conductance and transpiration rate, downregulated several photosynthetic proteins, caused a significant impact on carbon assimilation rate and, importantly, increased leaf damage on plants (Figures 1 and 2; Figure S1). The three stress factors had a synergistic effect in both genotypes, as observed in leaf damage or photosynthetic efficiency (Figures 1B and 3; Figure S2 and Table S10). HL + HS affected Carrizo and Cleopatra similarly in terms of LDI and φPSII; WS + HL caused a more drastic effect on φPSII in Cleopatra; and WS + HS was remarkably harmful to Cleopatra but it did not importantly affect Carrizo (Figures 1B and 3B; Figure S1). The combination of stress factors considerably increased the damages in both genotypes, but Cleopatra was more affected than Carrizo (Figure 1 and Figure S1). A recent report in *Arabidopsis thaliana* showed that the multifactorial accumulation of low-intensity stresses, with negligible individual effect on plant growth and survival, was detrimental for plants and became lethal when a high number of stresses (i.e., six stress factors) were combined (Zandalinas, Sengupta, et al., 2021). Here, it is demonstrated that individual drought, high light intensity or heat stress conditions slightly affected citrus plants, but their two- and/or three-stress combinations caused increasing damages in citrus plants, especially on the photosynthetic apparatus (Figures 3–7; Figures S4 and S5). Also, it was shown that the harmful effect of the stress combination, especially when three factors co-occur, depends on plant genotype (Figures 1 and 3; Figure S1 and Table S10).

Photosynthetic rate is affected by abiotic stresses due to biochemical changes that occur in chloroplasts, among other physiological perturbations (Biehler & Fock, 1996; Pospíšil, 2016; Takahashi & Murata, 2008; Tamburino et al., 2017). In our study, WS applied individually or in combination, was a key factor limiting A in both genotypes (Figure 2). Under individual WS, A decline seems to be induced by the stomatal closure that limits CO_2 uptake (Flexas et al., 2002). Under conditions of WS in combination with HL and/or HS (WS + HL, WS + HS and WS + HL + HS), a similar situation was observed in Cleopatra plants (with clear gs reductions associated to A declines). However, in Carrizo, reductions of gs were negligible (WS + HL) or very moderate (WS + HS and WS + HL + HS). Thus, A decline was not only caused by a reduction of CO_2 uptake but also by a reduction of the photosynthetic apparatus. In this sense, downregulation of photosynthetic proteins under these stress conditions (Figure 4) and PSII efficiency loss (Figure 3) would impair photosynthesis. Zandalinas et al. (2016) demonstrated that, under a combination of drought and heat stress, the ability of Carrizo to cool their leaves was an acclimation response that conferred an advantage, with respect to Cleopatra, to tolerate this stress combination. Here, we showed that Carrizo is able to maintain a higher transpiration rate and lower leaf temperature than Cleopatra under WS + HL and WS + HL + HS conditions, which would reduce stress pressure on the photosynthetic apparatus. On the other hand, under WS or HS, non-stomatal limitations can also affect the net rate of carbon assimilation (Flexas et al., 2006; Grassi & Magnani, 2005). Specifically, carbon-fixing reactions may account for part of the decline in the net rate of carbon assimilation (Grassi & Magnani, 2005). In this sense, HL + HS and WS + HS in Carrizo and WS + HL + HS in both genotypes caused a general reduction in the content of proteins involved in this pathway with respect to CT (Figure S3A and Table S4). However, the decrease in A under the different WS situations does not correlate with a reduction in the levels of these proteins. In addition, RuBisCo levels were not affected in leaves of Carrizo or Cleopatra plants under any stress condition (Figure S3B). Although these results do not show a clear effect of carbon-fixing reactions in our experiment, future research studying RuBisCo activity, and other enzymes involved, would provide more information.

Citrus are subtropical species that tolerate temperature rises without showing damage to the photosystem or limiting growth (Pereira et al., 2017; Vincent et al., 2020). In this work, individual HS increased A, affected neither φPSII nor photosynthetic protein content, and did not cause important leaf damage in plants of the two studied genotypes (Figures 1–3). HL applied individually reduced

φPSII in plants of both genotypes but only decreased A in Carrizo. On the contrary, Cleopatra plants showed increased levels of gs and E, suggesting a differential physiological response between genotypes that may affect A under HL (Figure 2). Previous experiments in *Vitis vinifera* demonstrated that varieties with higher stomatal aperture under high light intensity (1000–2000 μmol m^{-2} s^{-1} irradiances) showed higher photosynthetic rates, even under moderate water scarcity conditions (Costa et al., 2012). Results obtained in our work suggest that gs and E increases were positive responses to HL in citrus, although further research would be necessary to confirm this. Under HL + HS, gs and E increased significantly in both genotypes (Figure 2), which is the prevailing plant response to HS in *A. thaliana* (Balfagón et al., 2022). Thus, increasing transpiration to reduce the impact of high temperatures on leaves appears to be a key physiological response to this stress combination. The inability of Cleopatra to maintain low leaf temperatures, even at 23°C (CT), suggests that an increase in ambient temperature or a reduction in transpiration will result in higher increases in leaf temperature than that observed in Carrizo (Figure 2). The stress pressure caused by higher leaf temperatures may be the cause of the greatest loss of PSII photochemical efficiency observed in the photosynthetic apparatus of Cleopatra (Mathur et al., 2014; Figure 3).

Photosynthetic apparatus functioning is highly dependent on PSII steady state (Järvi et al., 2015; Tikkanen et al., 2014). Transcriptomic data showed that, under stress combinations, Carrizo was more efficient than Cleopatra in inducing gene expression to face photodamage and degradation of the photosynthetic apparatus, especially that related to the PSII complex (Figures 4 and 5). Downregulation of transcripts of the PETC and the PSII-repairing system was more marked in Cleopatra than in Carrizo under all stress combinations (Figures 4 and 6A). In addition, Carrizo was able to enhance the expression of these transcripts under HL + HS and WS + HL + HS, conditions that strongly affected PSII integrity and efficiency in this genotype (Figures 4 and 6A). Under stress conditions, chloroplast retrograde signals play a role in the activation of genes related to the photosynthetic apparatus (Crawford et al., 2017). However, under the conditions reported in this work, there was not a strong activation of different TFs involved in this pathway in any genotype (Yurina & Odintsova, 2019; Table S9). These results suggest that the differences in the activation of genes of the photosynthetic apparatus between genotypes may be controlled by other pathways or signals (i.e., metabolic signals; Crawford et al., 2017; Yurina & Odintsova, 2019). The enhanced transcriptomic response in Carrizo leaves was translated into a lower downregulation of proteins of the PSII repairing system (Figure 6B and Table S6). The content of proteins involved in PSII repair significantly declined in Cleopatra plants under two and three stress factor combinations, and Ftsh1 and Ftsh5 (two key proteins directly linked to D1 turnover; Nath et al., 2013) content was more reduced in Cleopatra than in Carrizo (Figure 6B,C). It has been reported that the induction of the PSII repair system, the increase of D1 turnover and a high PSII reassembly rate reduce PSII damages and enhance tolerance to high irradiance and heat stress conditions (Balfagón et al., 2019; Nath et al., 2013). In addition, under adverse environmental conditions, Cyt-b_6f and F-type ATP synthase accumulation is strongly repressed (Kohzuma et al., 2009). In Cleopatra plants, a significant decrease of proteins from these complexes was observed when plants were subjected to triple stress combination (Figures S4 and S5), which could indicate a higher stress pressure on the photosynthetic apparatus. Damage or malfunctioning of these complexes would disrupt photosynthetic flux and increase the photoxidative damage, especially on PSI proteins (Schöttler et al., 2015; Suorsa et al., 2012). The higher modulation of ROS-responsive transcripts in Cleopatra than in Carrizo under triple stress conditions could indicate a higher accumulation of ROS in Cleopatra leaves (Figure S6 and Table S7). In addition, the activation of ROS-responsive pathways in Cleopatra was not translated into a higher modulation of ROS-responsive protein levels than Carrizo (Figure S6 and Table S8). In this sense, previous studies showed that, under oxidative stress situations, the accumulation and activity of key proteins involved in ROS response were more efficient in Carrizo than in Cleopatra (Balfagón et al., 2018; Zandalinas et al., 2017). Indeed, Cleopatra plants showed damage on PSI, mostly on the light harvest complexes, which would correspond to a greater oxidative damage (Figure 7). On the other hand, photodamage in Carrizo was limited to PSII, with a strong recovery capacity (Tikkanen et al., 2014), and PSI, Cyt-b_6f and F-type ATP were not significantly affected by the different stress combinations (Figure 7; Figures S4 and S5). It has been reported that, under stress conditions, the restriction of photodamage to PSII increases the tolerance to high irradiance by protecting PSI, which importantly supports photosynthesis (Lima-Melo et al., 2019). Therefore, our results indicate that the Carrizo photosynthetic apparatus was not as compromised as the Cleopatra one under abiotic stress combinations, resulting in less foliar damage (Figure 1 and Figure S1) and a greater recovery of PSII efficiency after stress (Figure 3).

In summary, together with the physiological responses that may alleviate heat stress on PSII, the transcriptomic responses aiming to increase the protein levels of the photosynthetic apparatus and the PSII repair system play a key role on the tolerance of citrus plants to multiple abiotic stress combinations. Therefore, the photosynthetic apparatus of Carrizo showed better performance under WS, HL and HS factorial stress combination than that of Cleopatra due to physiological responses that alleviated heat impact on PETC complexes (e.g., leaf temperature) and transcriptomic response that contributed to maintaining the integrity of photosynthetic proteins. This work reveals important information about the effects of multiple abiotic stress conditions on the photosynthetic system of citrus plants, raising the importance of studying complex stress conditions on crops (Rivero et al., 2022). Those results could be also the bases for genetic improvements aiming to obtain tolerant varieties to adverse environmental conditions resulting from climate change.

AUTHOR CONTRIBUTIONS

Damián Balfagón and Aurelio Gómez-Cadenas designed the experiments. Damián Balfagón performed the experiment, harvest plant material and analyzed samples. Tadeu dos Reis de Oliveira, Claudete Santa-Catarina and Damián Balfagón performed the proteomic analysis. Aurelio Gómez-Cadenas supervised the project and provided

funding. Damián Balfagón, Sara I. Zandalinas and Aurelio Gómez-Cadenas wrote the manuscript and prepared figures. All authors have read and approved the final version.

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DATA AVAILABILITY STATEMENT

RNA-Seq data files were deposited in Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) under the accession no. GSE203331. The mass spectrometry proteomic data have been deposited with ProteomeXchange consortium via the PRIDE (https://www.ebi.ac.uk/pride/) partner repository with the dataset identifier PXD034419.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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