Plant strategies to deal with a combination of drought and high temperatures

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TESIS DOCTORAL

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__ Noviembre 2016 __

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Castellón de la Plana, Noviembre 2016

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ABBREVIATIONS

A - Net photosynthetic rate

ABA - Abscisic acid

ABAGE - ABA-glycosyl ester

ABRE - ABA-responsive element

AOG - ABA O-glycosyl transferase

AOX - Alternative oxidase

APX - Ascorbate peroxidase

AREB/ABF - ABA-responsive element-binding protein/ABA-binding factor

CAT - Catalase

CBF/DREB - Cold-binding factor/dehydratation responsive element binding

C_i/C_a - Ratio of substomatal to ambient CO_2 concentration

CO_2 - Carbon dioxide

Cu/Zn-SOD - Copper/zinc superoxide dismutase

CYP707A - ABA 8'-hydroxylase

DPA - Dehydrophaseic acid

DRE - Dehydration-responsive element

DW - Dry weight

E - Transpiration

ERTCA - Ethylene response transcriptional co-activator

F_v/F_m - Maximum efficiency of photosystem II

FW - Fresh weight

GC - Gas chromatography

GPX - Glutathione peroxidase

GR - Glutathione reductase

g_s - Stomatal conductance

GST - Glutathione S transferase

HCA - Hierarchical cluster analysis

HSF - Heat shock factor

HSP - Heat shock protein

ICS - Isochorismate synthase

IPCC - Intergovernmental Panel on Climate Change

IS - Internal standard

JA - Jasmonic acid

MBF1c - Multiprotein bridging factor 1c

M_d - Dry mass

MDA - Malondialdehyde

M_f - Fresh mass

MS - Mass spectrometry

M_t - Turgid mass

NCED - 9-neoxanthin-*cis*-epoxicarotenoid dioxygenase

NO - Nitric oxide

PA - Phaseic acid

PAL - Phenylalanine ammonia lyase

PLS-DA - Partial least squares coupled to discriminant analysis

PR - Pathogenesis-related

PSII - Photosystem II

PP2C - Protein phosphatase 2C

PYR/PYL/RCAR - Pyrabactin resistance1/PYR-like/regulatory components of ABA receptor

Φ_PSII - Quantum efficiency of PSII photochemistry

QTOF - Quadrupole time-of-flight mass spectrometer

RAB18 - Responsive to ABA-related gene 18

RBOH - Respiratory burst oxidase homologue

RI - Retention index

ROS - Reactive oxygen species

RWC - Relative water content

SA - Salicylic acid

SnRK - Sucrose non-fermenting 1-related protein kinase

TF - Transcription factor

TPX - Thioredoxin peroxidase

TW - Turgid weight

UPLC - Ultra-performance liquid chromatography

WUE - Water use efficiency

SUMMARY

Adverse conditions occur simultaneously in natural environments, constituting a new stress that cannot be predicted from the study of the individual stresses. Among them, drought and heat are one the most frequent abiotic stress combinations affecting global crop productivity. Plant responses to these unfavorable conditions are specially mediated by phytohormones, among which abscisic acid (ABA) constitutes an important stress-signaling hormone implicated in the regulation of stomatal closure and up-regulation of genes leading to adaptive responses during abiotic stress conditions.

The present work is divided into three chapters. In Chapter 1, it is demonstrated the different impact of combined drought and high temperatures on citrus plants. Our study reveals that Carrizo citrange represents a more tolerant genotype under combined stress conditions than Cleopatra mandarin, likely as a result of a higher transpiration rate that could allow a more efficient cooling of leaf surface, along with a decreased impact of oxidative stress. The study of the hormonal profiling shows that salicylic acid levels increase in an additive manner in response to combined stress conditions. Interestingly, ABA content is modulated depending on the particular stress via the activation of hormone biosynthesis, catabolism and/or conjugation. Apart from changes in plant physiology, metabolism reconfiguration is also involved in the adaptation of plants to adverse environmental conditions. For this reason, Chapter 2 is focused on the study of primary and secondary metabolism of these citrus genotypes under individual or combined stress situations. Our results show different patterns in metabolite accumulation between plants of both citrus genotypes subjected to drought, heat or a combination of these stresses. In general, Cleopatra plants suffer deep alterations in metabolism oriented to mitigate stress damage whereas the higher ability of Carrizo to tolerate adverse situations prevents further modifications of metabolism. Once the different responses of citrus plants to combined drought and heat stresses are demonstrated, Chapter 3 aims to further investigate the role of ABA during stress combination. For this purpose, *Arabidopsis thaliana* plants are used due to the availability of ABA-deficient (*aba1-1*) and ABA-insensitive (*abi1-1*) mutants. Our data demonstrate that ABA is necessary for plant tolerance to this combined situation as well as for the accumulation of key proteins (APX1, MBF1c and HSP101) required for plant acclimation to environmental adverse conditions. Additionally, based on our results of

Arabidopsis abi1-1 mutant, ABI1 is not required for stomatal closure during the stress combination and H_2O_2 can signal stomatal reduction independently of ABA signaling.

On the whole, this work provides novel information about citrus mechanisms of tolerance to a combination of drought and heat stress in terms of physiological, hormonal and metabolic responses. In addition, data presented here suggest that ABA is an essential hormone during the plant acclimation to combined drought and heat stress.

RESUMEN

En condiciones naturales es frecuente que varios estreses abióticos ocurran de forma simultánea, generando así un estrés nuevo cuyos efectos no se pueden predecir a partir del estudio de los estreses individuales. Entre ellos, la sequía y las altas temperaturas representan una combinación de estreses que se da frecuentemente en campo afectando el rendimiento de los cultivos. La respuesta de las plantas a estas condiciones desfavorables está mediada principalmente por fitohormonas, entre las que destaca el ácido abscísico (ABA). El ABA está implicado en la regulación estomática y la expresión de genes relacionados con las respuestas adaptativas de las plantas frente a condiciones de estrés abiótico.

Esta tesis doctoral está dividida en tres capítulos. En el Capítulo 1 se demuestra que la combinación de sequía y altas temperaturas tiene un impacto diferente en plantas de cítricos. Nuestro estudio indica que el genotipo citrange Carrizo es más tolerante a la combinación de estreses que el mandarino Cleopatra como resultado de una mayor transpiración dirigida a enfriar la superficie foliar, unida a un menor impacto de estrés oxidativo. El perfil hormonal de las hojas indica que el ácido salicílico se acumula de forma aditiva en respuesta a la combinación de estreses. Curiosamente, el contenido de ABA se modula en función del tipo de estrés mediante la activación de su biosíntesis, catabolismo y/o conjugación. Además de cambios en la fisiología, la reconfiguración del metabolismo es otra respuesta de adaptación de las plantas a las condiciones ambientales adversas. Por ello, el Capítulo 2 se centra en el estudio del metabolismo primario y secundario de ambos genotipos de cítricos en condiciones de sequía, altas temperaturas y la combinación de ambos estreses. Nuestros resultados muestran que existen distintos patrones de acumulación de metabolitos entre plantas de ambos genotipos en respuesta a los distintos estreses. En general, Cleopatra altera más marcadamente su metabolismo con el fin de mitigar el daño causado por los estreses. Sin embargo, la mayor tolerancia de Carrizo a estos factores ambientales da lugar a una menor alteración del metabolismo. Una vez demostradas las distintas respuestas de las plantas de cítricos a la combinación de sequía y altas temperaturas, en el Capítulo 3 se ahonda en el papel del ABA en la respuesta de las plantas a esta combinación de estreses. Con tal objetivo, se hace uso de plantas de *Arabidopsis thaliana* debido a la disponibilidad de mutantes deficientes e insensibles a ABA (*aba1-1* y *abi1-1*, respectivamente). Nuestro trabajo demuestra que esta hormona es necesaria para la

tolerancia de las plantas a esta combinación de estreses así como para la acumulación de proteínas clave (APX1, MBF1c y HSP101) implicadas en la aclimatación de las plantas a condiciones ambientales adversas. Además, según los resultados obtenidos en los mutantes de *Arabidopsis abi1-1*, ABI1 no determina el cierre estomático durante la combinación de sequía y altas temperaturas, siendo el H_2O_2 un posible inductor de la disminución de la apertura estomática, independiente de la señalización mediada por ABA.

En conclusión, este trabajo ofrece información novedosa sobre las respuestas fisiológicas, hormonales y metabólicas de los cítricos frente a situaciones de combinación de sequía y altas temperaturas. Finalmente, nuestros datos sugieren que el ABA es una hormona esencial para la aclimatación de las plantas a esta combinación de estreses.

RESUM

En condicions naturals és freqüent que diversos estressos abiòtics tinguen lloc de forma simultània, generant així un estrès nou els efectes del qual no es poden predir a partir de l'estudi dels estressos individuals. Entre ells, la sequera i les altes temperatures representen una combinació d'estressos que es dóna en camp freqüentment afectant el rendiment dels cultius. La resposta de les plantes a aquestes condicions desfavorables està regulada principalment per les fitohormones, entre les que destaca l'àcid abscísic (ABA). L'ABA està implicat en la regulació estomàtica i l'expressió de gens relacionats amb les respostes adaptatives de les plantes durant condicions d'estrès abiòtic.

Aquesta tesi doctoral està dividida en tres capítols. En el Capítol 1 es demostra que la combinació de sequera i altes temperatures té un impacte diferent en distints genotips de cítrics. El nostre estudi indica que el genotip citrange Carrizo és més tolerant a la combinació d'estressos que el mandarí Cleopatra com a resultat d'una major transpiració dirigida a disminuir la temperatura foliar, unida a un menor impacte de l'estrès oxidatiu. El perfil hormonal de les fulles indica que l'àcid salicílic s'acumula de forma additiva en resposta a la combinació d'estressos. Curiosament, el contingut d'ABA es modula en funció de l'estrès mitjançant l'activació de la seua biosíntesi, catabolisme i/o conjugació. A més a més, la reconfiguració del metabolisme és una altra resposta d'adaptació de les plantes a les condicions ambientals adverses. Per la qual cosa, el Capítol 2 es centra en l'estudi del metabolisme primari i secundari d'ambdós genotips de cítrics en condicions de sequera, altes temperatures i la combinació d'aquests estressos. Els nostres resultats indiquen que existeixen distints patrons d'acumulació de metabolits entre plantes dels dos genotips en resposta als diferents estressos. En general, Cleopatra altera més marcadament el seu metabolisme amb la finalitat de mitigar el dany causat pels estressos. Contràriament, la major tolerància de Carrizo a aquests factors ambientals dóna lloc a una menor alteració del metabolisme. Una volta demostrades les diferents respostes de les plantes de cítrics a la combinació de sequera i calor, en el Capítol 3 s'aprofundeix en la funció de l'ABA en la resposta de les plantes a aquesta combinació d'estressos. Amb aquest objectiu, es fa ús de plantes d'*Arabidopsis thaliana* degut a la disponibilitat de mutants deficients i insensibles a ABA (*aba1-1* i *abi1-1,* respectivament). El nostre treball demostra que aquesta hormona és necessària per a la tolerància de les plantes a aquesta combinació d'estressos així com per a l'acumulació de proteïnes clau (APX1, MBF1c i HSP101) implicades en

l'aclimatació de les plantes a condicions ambientals adverses. A més a més, els resultats obtinguts en els mutants d'*Arabidopsis abi1-1* suggereixen que ABI1 no determina el tancament estomàtic durant la combinació de sequera i altes temperatures, sent el H_2O_2 un possible inductor de la disminució de l'obertura estomàtica, independent de la senyalització de l'ABA.

En conclusió, aquest treball ofereix informació nova sobre les respostes fisiològiques, hormonals i metabòliques dels cítrics sotmesos a situacions de combinació de sequera i altes temperatures. Finalment, les nostres dades suggereixen que l'ABA és una hormona essencial per a l'aclimatació de les plantes a aquesta combinació d'estressos.

Introduction

The interactions of water deficit with other abiotic stress conditions.

Abstract

Under field conditions, crops are routinely subjected to a number of different abiotic stress factors simultaneously. Recent studies revealed that the response of plants to a combination of different abiotic stresses is unique and cannot be directly extrapolated from simply studying each of the different stresses applied individually. These studies have also identified specific regulatory transcripts, combinations of metabolites and proteins and physiological responses that are unique to specific stress combinations, highlighting the importance of studying abiotic stress combination in plants. Here we describe the interactions between water deficit and other abiotic stresses with emphasis on drought and heat stress. We review the different molecular, physiological and metabolic adaptations of different plants and crops to this stress combination and we highlight the importance of reactive oxygen species (ROS) metabolism and stomatal responses for plant acclimation to water deficit and heat stress combination. We further emphasize the need for developing crops with enhanced tolerance to water deficit and heat stress combination in order to mitigate the negative impacts of predicted global climatic changes on agricultural production worldwide.

Introduction

The impact of global climatic changes resulting from the constant elevation in atmospheric carbon dioxide (CO_2) levels is exacerbated by the constant decline in agricultural land availability and quality (Peters et al. 2011). According to the Intergovernmental Panel on Climate Change 2014 (IPCC 2014), the number of warm days and nights and the frequency of heat waves have increased in large parts of the world. Relative to 1850–1900, the global surface temperature change is projected to exceed 2 °C by the end of the 21st century. Furthermore, changes in precipitation will become more erratic, making high latitudes and the equatorial Pacific likely to experience an increase in annual mean precipitation in the next years. In contrast, mean precipitation is expected to decrease in many mid-latitude and subtropical dry regions. Finally, mean world ocean temperatures will continue to increase throughout the 21st century, with the strongest warming trend projected for tropical and subtropical regions in the Northern Hemisphere (IPCC 2014, **Figure 1)**. This scenario will subject crops to a greater range and number of environmental stresses that could occur simultaneously with severe consequences (Mittler and Blumwald 2010). As predicted by the IPCC 2014 report, decreases in food production and quality, mainly resulting from heat and drought stresses, are considered a major future risk in many areas.

Abiotic stress conditions, including heat, cold, drought, salinity, oxidative stress and nutrient deficiency, are the primary cause of crop loss worldwide, reducing average yields and quality (Carmo-Silva et al. 2012, Awasthi et al. 2014). These environmental challenges lead to a wide range of responses in plants, including morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity. Plant cells perceive stress stimuli via various sensors that in turn activate signaling pathways involving secondary messengers, plant hormones, signal transducers and transcriptional regulators (Cvikrová et al. 2013, Danquah et al. 2014, Gilroy et al. 2014). Multiple signals, therefore, converge to regulate stress-inducible genes that encode proteins and enzymes directly involved in stress metabolism, contributing to the specificity of the acclimation response to a given stress stimuli (Casaretto et al. 2016).

Abiotic stress conditions could potentially cause the accumulation of reactive oxygen species (ROS), including H_2O_2 that function as signal transduction molecules, but can

also cause extensive cellular damage and inhibition of photosynthesis. To prevent damage, ROS are normally removed by antioxidant machinery, which can be, in turn, impaired by the effect of stresses themselves (Baxter et al. 2014).

(A)

(B)
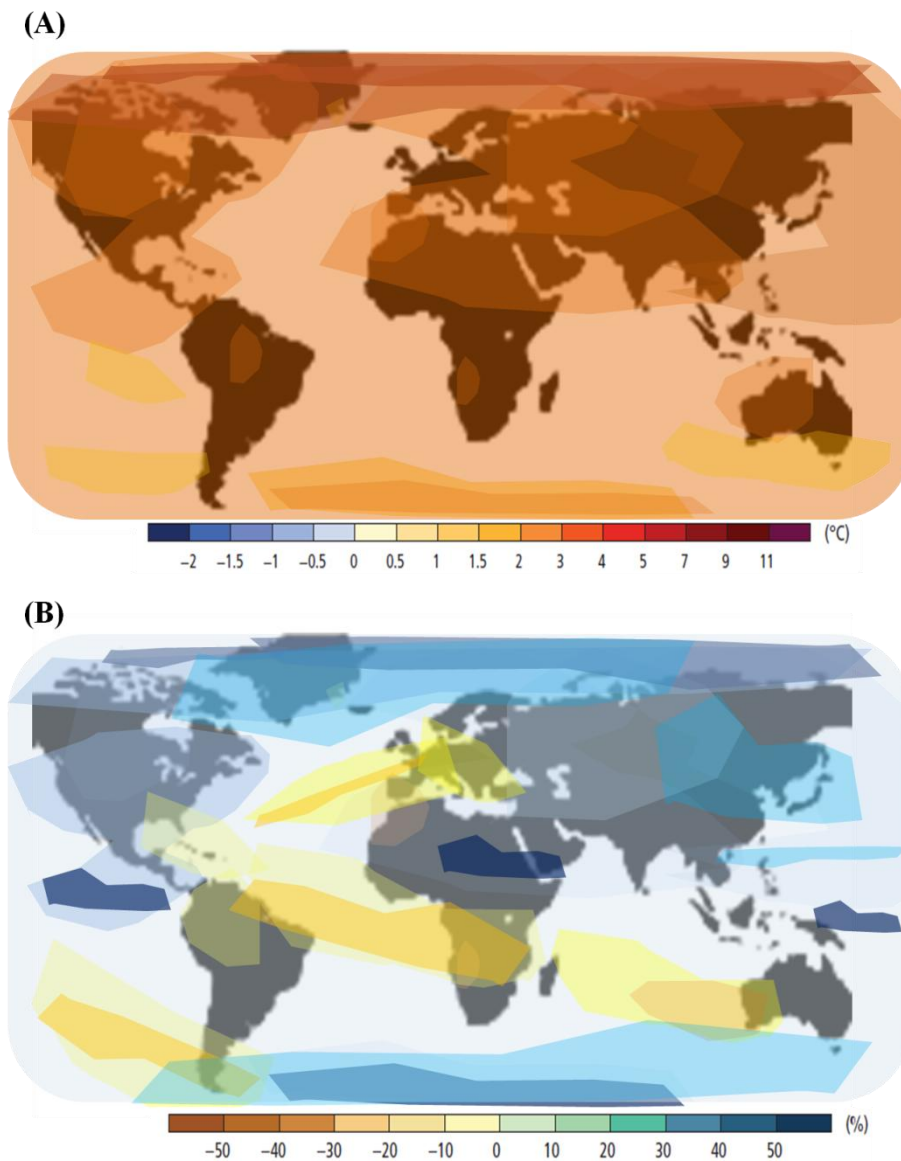
Figure 1. Predicted changes in average surface temperature (A) and in average precipitation (B) by 2100. Color scales indicate the decrease or the increase in Celsius degrees (A) and the % of change in precipitation (B) (IPCC 2014).

In addition to ROS, plant responses to abiotic stress are mediated by phytohormones, which coordinate complex stress-adaptive signaling cascades (Muñoz-Espinoza et al. 2015). Specifically, the plant hormone abscisic acid (ABA) functions as a key regulator in the activation of plant cellular adaptation to drought and salinity (Danquah et al. 2014). Among the main cellular mechanisms orchestrating plant acclimation to

environmental cues are stress-response transcription factors (TFs). Studies on the transcriptional regulation of drought and salinity have uncovered both ABA-dependent and ABA-independent pathways in which ABA-dependent responses are mediated via TFs such as AREB/ABF (ABA-responsive element-binding protein/ABA-binding factor) family, whereas ABA-independent responses are mediated via TFs such as CBF/DREB (cold-binding factor/dehydratation responsive element binding) TFs (Yoshida et al. 2014).

The importance of investigating abiotic stress combination

Based on the different models for climate change, the probability that plants will encounter new or more severe combinations of abiotic stresses in the near future is likely to be higher than previously anticipated (Rizhsky et al. 2004, Mittler 2006, Suzuki et al. 2014). Although past and current research on plants subjected to a single abiotic stress condition such as water deficit or heat, have provided important information over the last decades, many of these results cannot be used to infer the effects of a combination of two or more different stresses on plants (Mittler 2006, Suzuki et al. 2014). Recently, significant progress has been made in understanding the physiological, metabolic and molecular responses of several plant species to a combination of different abiotic stress conditions (Rizhsky et al. 2002, Rizhsky et al. 2004, Barnabás et al. 2008, Rasmussen et al. 2013, Ahmed et al. 2013, Rivero et al. 2013, Cvikrová et al. 2013, Zinta et al. 2014, Ahmed et al. 2014, Perdomo et al. 2014, Hu et al. 2015, Zandalinas et al. 2016a, Zandalinas et al. 2016b). These studies have concluded that each combination of two or more stress conditions imposes a specific set of requirements on the plant. Therefore, different stress combinations required the tailoring of unique metabolic and signaling responses including TFs, photosynthesis, antioxidant mechanisms, hormone signaling and osmolyte synthesis (Rizhsky et al. 2004, Koussevitzky et al. 2008, Rasmussen et al. 2013, Iyer et al. 2013, Atkinson et al. 2013, Prasch and Sonnewald 2013, Pandey et al. 2015). These specific responses, with emphasis on stress combinations that include water deficit as one of the stress components involved, are described below.

Specificity in plant responses to environmental stress combination

Many studies have reported that stress interactions, including drought and heat, salinity and heat, ozone and salinity, ozone and heat, nutrient stress and drought, nutrient stress

and salinity, UV and heat, UV and drought and high light intensity combined with heat, drought or chilling have a significantly higher negative impact on crop productivity than each of the different stress components applied individually (Mittler and Blumwald 2010, Suzuki et al. 2014). Nonetheless, other studies have reported beneficial effects as a result of the interaction of two different stresses applied simultaneously (reviewed in Suzuki et al. 2014). **Figure 2** shows "the Stress Matrix" created by Mittler (2006) and updated in Mittler and Blumwald (2010), Suzuki et al. (2014) and in this review. This matrix depicts different combinations of abiotic factors along with their potential positive or negative effects on plant productivity and growth.
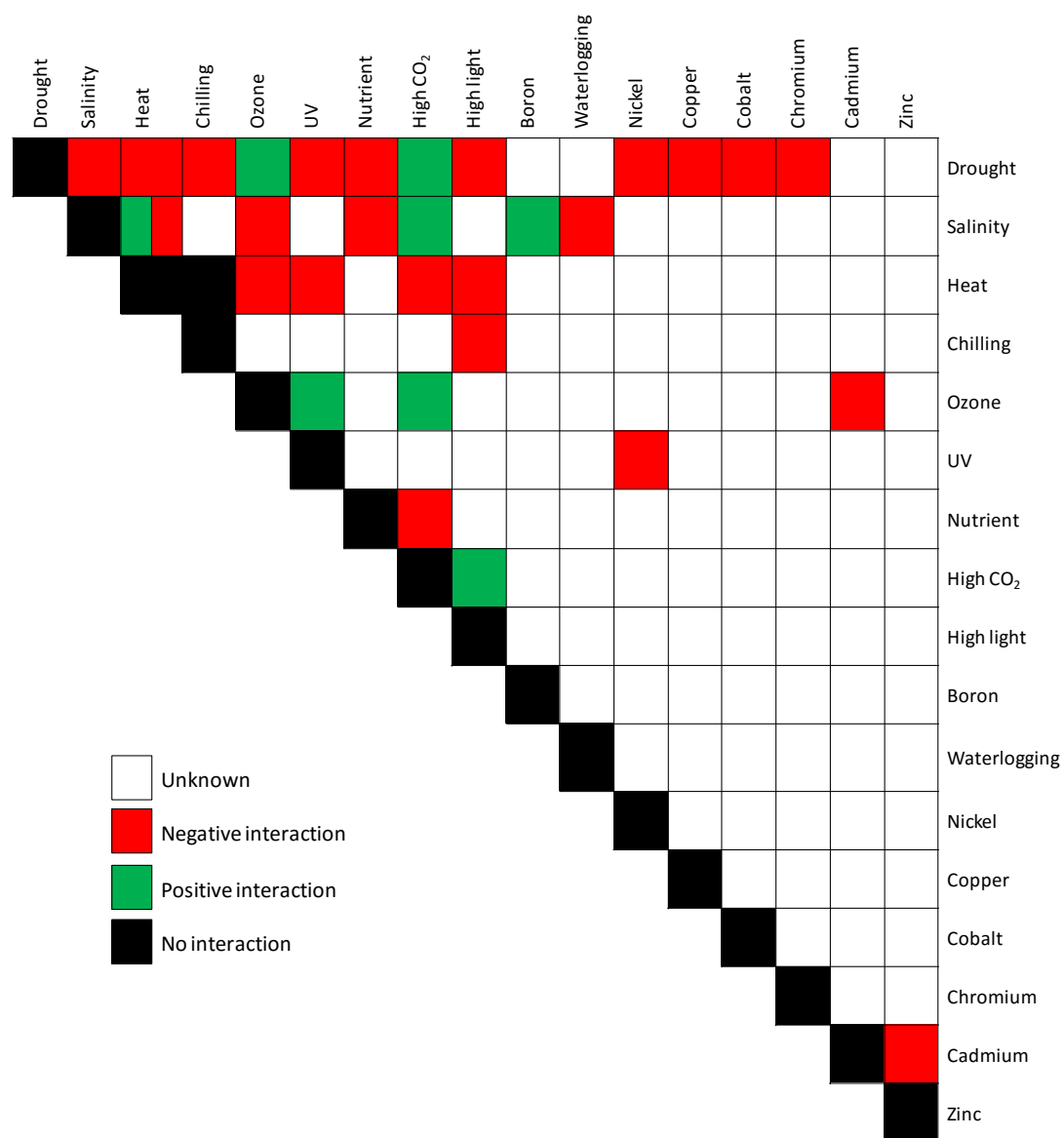
Figure 2. "The Stress Matrix", adapted from Mittler (2006) and modified in Mittler and Blumwald (2010), Suzuki et al. (2014) and in this review.

The combination of salinity and heat stress in wheat can, for example, exacerbate the damaging effects of both stresses when applied individually (Keles and Oncel 2002). In cultivated barley, either salinity or drought stress decreased growth, chlorophyll content, photosynthetic rate, maximal photochemical efficiency of photosystem II (PSII) (F_v/F_m), water potential and osmotic potential, but the stress combination had a higher negative effect than each of the individual conditions (Ahmed et al. 2013). Negative interactions have also been demonstrated in plants subjected to high light and drought (Giraud et al. 2008) or cold stress (Haghjou et al. 2009). In these stress combinations, high light intensities resulted in high photosynthetic energy absorbed by plants that in turn enhanced ROS accumulation. Nutrient deficiency can aggravate the impact of other abiotic stress conditions when combined because energy and mineral resources are important for the acclimation of plants to stress and micronutrients are required for the function of many ROS scavenging enzymes. Other negative interactions between two different abiotic factors have been reported in other studies; as examples, drought combined with cold stress (Sales et al. 2013), heavy metals combined with UV (Srivastava et al. 2012), heat stress and high CO_2 (Wang et al. 2016), zinc and cadmium combination (Cherif et al. 2011), waterlogging combined with salinity (Alhdad et al. 2013) or ozone interactions with heat stress (Mittler and Blumwald 2010) or with cadmium (Castagna et al. 2015) were reported.

In contrast, some stress combinations can have a favorable effect on plants compared to each of the stresses applied individually. Examples include combinations of ozone and drought, in which the reduction in stomatal conductance due to the effect of drought stress could decrease ozone intake through stomata (Iyer et al. 2013). In addition, elevated CO_2 levels have been reported to be advantageous when combined with other stresses such as ozone (Ainsworth et al. 2008), salt or high light (Pérez-López et al. 2013). In tomato plants, a combination of salinity and heat stress enhances the protection against the damaging effects of salinity, suggesting that the accumulation of osmoprotectants such as glycine betaine and trehalose could play an important role protecting plants against this stress combination (Rivero et al. 2013). In contrast, Suzuki et al. (2016) found that this stress combination had a significant negative impact on *Arabidopsis* (**Figure 2**).

Transcriptomic and proteomic studies in different plants have identified several putative genes involved in the regulation and acclimation of plants to multiple abiotic stresses,

suggesting potential targets for the improvement of crop stress tolerance (e.g., *Apx1*, *Mbf1c*, At1g26580; Rizhsky et al. 2002, 2004; Priyanka et al. 2010; Wang et al. 2010, Kumar et al. 2015; Suzuki et al. 2016; Koussevitzky et al. 2008). According to these studies, a combination of abiotic stresses activated specific sets of transcripts that represented new programs of gene expression, providing a molecular explanation for the unique response of plants to simultaneous stresses. Transcript expression data in rockcress (*Boechera spp.*) plants under combined heat and high light stress revealed for example two important marker genes for these stress responses: *APX2* (Ascorbate peroxidase 2) and *HSFA2* (Heat Shock Factor A2) (Gallas and Waters 2015). Transcriptome analyses in *Arabidopsis thaliana* under different combinations of stresses concluded that 61% of the transcripts altered in plants subjected to stress combination could not be predicted from the responses to the single stress factors when applied individually (Rasmussen et al. 2013). In addition, Rasmussen et al. (2013) showed that plants prioritized between potentially antagonistic responses for only 5% to 10% of the responding transcripts, suggesting that plants have evolved to cope with a combination of different stresses.

A limited number of studies conducted proteomic analysis of plants subjected to combined stresses including drought and ozone in poplar (Bohler et al. 2013), drought and heat in *Arabidopsis thaliana*, barley and *Carissa spinarum* (Koussevitzky et al. 2008, Zhang et al. 2010, Rollins et al. 2013), heavy metals as mercury and salinity in *Suaeda salsa* (Liu et al. 2013) and high temperature and humidity in *Portulaca oleracea* (Yang et al. 2012). Importantly, all transcriptomic and proteomic analyses, performed in several plant species subjected to different stress combinations, have highlighted the importance of the antioxidant defense machinery. Plants with higher antioxidant capacity or lower ROS accumulation have generally showed increased tolerance to stress combination (Koussevitzky et al. 2008, Suzuki et al. 2014).

Water deficit and heat stress as a model for abiotic stress combination

The incidences of drought combined with a heat wave are expected to increase in the near future (IPCC 2014), thus, underscoring an excellent case for studying this stress combination for the purpose of improving the tolerance of future crops. Several reports have investigated the effects of drought and heat stress combination on plant growth and productivity, concluding that this stress combination causes a disproportionate damage

compared to each of its individual stress components (Keles and Oncel 2002, Rizhsky et al. 2002, Barnabás et al. 2008, Boeck et al. 2015, Zandalinas et al. 2016a, Zandalinas et al. 2016b). In general, the negative impacts of drought and heat stress are considered not to be additive, pointing to a certain degree of independence between the mechanisms involved in the responses of plants to drought or heat stress (Suzuki et al. 2014). Studies in *Arabidopsis thaliana* concluded for example that plant growth was more severely reduced under a combination of drought and heat stress than when each of the stresses was applied individually (Rizhsky et al. 2004, Vile et al. 2012). In addition, crop yield, grain number, spikelet fertility, chlorophyll content and harvest index were severely affected by the combined effect of drought and heat stress in spring wheat (Prasad et al. 2011). In barley, drought caused reductions in biomass, height and spike numbers whereas heat stress did not alter these traits but increased aborted spikes and decreased kernel weight. Interestingly, the strongest phenotypic changes were observed under the stress combination treatment (Rollins et al. 2013).

Plants display different responses to different stresses depending on their developmental stage. It has been demonstrated for example that reproductive tissues are more sensitive than vegetative tissues to drought, heat stress and their combination and that both individual stresses influenced reproductive processes differently (Barnabás et al. 2008). In spring wheat for example drought can inhibit grain weight, pistillate flower development and ovule functions while heat stress affects pollen fertility and grain number (Prasad et al. 2011). Importantly, the combined effects of these stresses are greater than the effects of drought or heat stress alone on reproductive tissues, causing a higher damaging effect of this stress combination on crop yield.

Physiological responses to a combination of drought and heat stress

Alterations in physiological responses under drought, heat and their combination have been studied in several species. In general, photosynthetic activity is repressed when plants are subjected to abiotic stresses via destabilization of Rubisco and damage to PSII (Nishiyama and Murata 2014). However, the effects of drought, heat stress and their combination on photosynthesis could be different depending on plant species. According to Wang et al. (2010), a combination of drought and heat stress negatively affected wheat photosynthetic rates to a more severe level than each of the different stresses applied individually. *Nicotiana tabacum* plants subjected to drought and heat

combination displayed suppressed photosynthesis, enhanced respiration, closure of stomata and increased leaf temperature (Rizhsky et al. 2002). This stress combination also led to higher reduction in photosynthetic activity and enhanced production of ROS in *Populus yunnanensis* (Li et al. 2014) and a severe reduction in photochemical efficiency of PSII in *Festuca arundinacea* and *Lolium perenne* (Jiang and Huang 2001). Sainz et al. (2010) reported a significant disruption in PSII function in *Lotus japonicus* plants under combined drought and heat stress. In leaves of *Cicer arietinum*, Rubisco activity increased in response to heat stress whereas it decreased in response to water deficit or drought and heat combination (Awasthi et al. 2014). In drought-tolerant and drought-sensitive cotton cultivars, heat stress alone or in combination with drought increased leaf temperature and decreased net photosynthetic rate and stomatal conductance, resulting in a more damaging impact under the combined stress conditions. Drought-sensitive cultivars displayed greater changes in these features, showing lower activity of Rubisco compared to drought-tolerant cultivars under the stress combination (Carmo-Silva et al. 2012). These results suggest that maintenance of photosynthetic activity is important for the acclimation of plants to a combination of drought and heat stress, and that both drought- and heat-induced limitations act simultaneously to inhibit photosynthesis under combined stress conditions in the field.

Changes in stomatal aperture are a primary and rapidly-occurring response to environmental stresses aimed at regulating the flow of CO_2, leaf temperature (via transpiration) and water loss. Stomatal responses to drought and heat combination represent a challenging situation in which plants must balance between preventing water loss and protecting from over-heating. Whereas heat causes increases in stomatal conductance as the plant attempts to cool down its leaves by transpiration, drought has an opposite effect to prevent water loss (Mittler and Blumwald 2010). Several reports have highlighted the importance of maintaining leaf temperature under control for the tolerance of plants to a combination of drought and heat. Tobacco plants subjected to this stress combination exhibited a higher leaf temperature than plants subjected only to heat stress, probably as a result of the inability of plants under the stress combination to increase transpiration because their stomata are closed (Rizhsky et al. 2002). Similarly, stomatal conductance of *Arabidopsis thaliana* and citrus plants subjected to drought or to a combination of drought and heat stress decreased (Rizhsky et al. 2004, Zandalinas et al. 2016a, Zandalinas et al. 2016b), suggesting that the effect of drought on stomatal

aperture, aimed to prevent water loss, could prevail over the effect of heat to cool leaf surface. The response to a combination of drought and heat stress can vary among different genotypes of a specific plant species as reported by Aprile et al. (2013) and Zandalinas et al. (2016a). In the first work, combined drought and heat stress conditions led to differences in leaf temperature between two durum wheat cultivars, Ofanto and Cappelli, which differ in water use efficiency (WUE). Cappelli, characterized by higher WUE and lower stomatal conductance, showed higher leaf temperature in response to drought and heat stress combination compared to Ofanto (Aprile et al. 2013). In the second study, two closely-related citrus genotypes, Carrizo citrange and Cleopatra mandarin, showed contrasting abilities to tolerate the combination of drought and heat due to different transpiration rates along with different incidence of oxidative stress. Therefore, Carrizo, that is more tolerant to the stress combination than Cleopatra, showed higher transpiration rate, pointing to a lower leaf temperature (Zandalinas et al. 2016a). Stomatal density is also affected by a combination of drought and heat stress. Vile et al. (2012) reported that *Arabidopsis* plants subjected to drought increased stomatal density whereas heat had the opposite effect. In addition, under combined stresses, stomatal density decreased, suggesting that plant developmental processes are apparently determined by the more severe stress, in this case, drought.

Molecular responses to a combination of drought and heat stress

In addition to physiological studies, transcriptomic analysis of plants subjected to simultaneous drought and heat combination demonstrated that this stress combination resulted in a new profile of transcript expression that could not be predicted by the study of each of the different stresses applied individually (Mittler and Blumwald 2010, Pandey et al. 2015). *Arabidopsis thaliana* (Rizhsky et al. 2004), tobacco (Rizhsky et al. 2002), durum wheat (Rampino et al. 2012) and sorghum (Johnson et al. 2014) for example displayed unique transcriptomic changes under the stress combination which depended on the plant species, duration and stress severity (Pandey et al. 2015). Transcriptomic studies in *Arabidopsis thaliana* plants under drought, heat and their combination indicated that this stress combination altered the expression of more than 770 unique transcripts, not altered by drought or heat stress applied individually, including those encoding different heat shock proteins (HSPs), MYB TFs, several protein kinases, proteins involved in ROS detoxification, proteases and enzymes involved in lipid biosynthesis and starch degradation (Rizhsky et al. 2004). **Figure 3**

shows gene ontologies of transcripts specifically up-regulated in response to the combination of drought and heat and their relative abundance in *Arabidopsis thaliana* (Rizhsky et al. 2004). Tobacco plants subjected to a combination of drought and heat stress specifically up-regulated HSP coding transcripts, including small HSPs, HSP70, HSP90 and HSP100, as well as transcripts encoding pathogenesis related (PR) and phenylalanine ammonia lyase (PAL) proteins, WRKY TFs and ethylene response transcriptional co-activator (MBF1c) (Rizhsky et al. 2002). In this study, differences were also observed in the type of ROS detoxification genes induced under individual and combined stresses, reflecting stress-dependent ROS-detoxification mechanisms. Therefore, while drought resulted in the induction of catalase (CAT) and glutathione peroxidase (GPX), heat stress induced cytosolic APX and thioredoxin peroxidase (TPX). In contrast, the stress combination specifically induced transcripts encoding alternative oxidase (AOX), GPX, glutathione reductase (GR), copper/zinc superoxide dismutase (Cu/Zn-SOD), and glutathione S transferase (GST). Furthermore, Koussevitzky et al. (2008) found that the *Apx1* gene was specifically required for the tolerance of *Arabidopsis thaliana* plants to drought and heat stress combination. When exposed to heat stress combined with drought, an APX1-deficient mutant (*apx1*) was significantly more sensitive to the stress combination than wild type, suggesting that cytosolic APX1 could play a key role in the acclimation of plants to a combination of drought and heat stress. Interestingly, a mutant deficient in thylakoid APX did not display similar sensitivity to the stress combination, suggesting that ROS accumulation at the cytosol plays a key regulatory role in this response (Koussevitzky et al. 2008). In addition, the sensitivity of *Festuca arundinacea* plants to combined drought and heat was recently associated with lower SOD activity and higher H_2O_2 accumulation (Bi et al. 2016). Furthermore, Zandalinas et al. (2016b) demonstrated that the accumulation of MBF1c during a combination of drought and heat stress depended on ABA function through ABI1.

In general, induction of ROS detoxification enzymes was found to be a common response among plant species to drought and heat stress combination, suggesting that a higher antioxidant capacity is associated with the tolerance of plants to stress combination. Additionally, TFs such as MYB (Rizhsky et al. 2004, Casaretto et al. 2016), HSPs as well as transcripts involved in photosynthesis and glycolysis are also specifically accumulated in response to combined drought and heat, indicating that

plants must maintain a balance between energy and resource allocation in order to maintain growth and undergo stress acclimation.
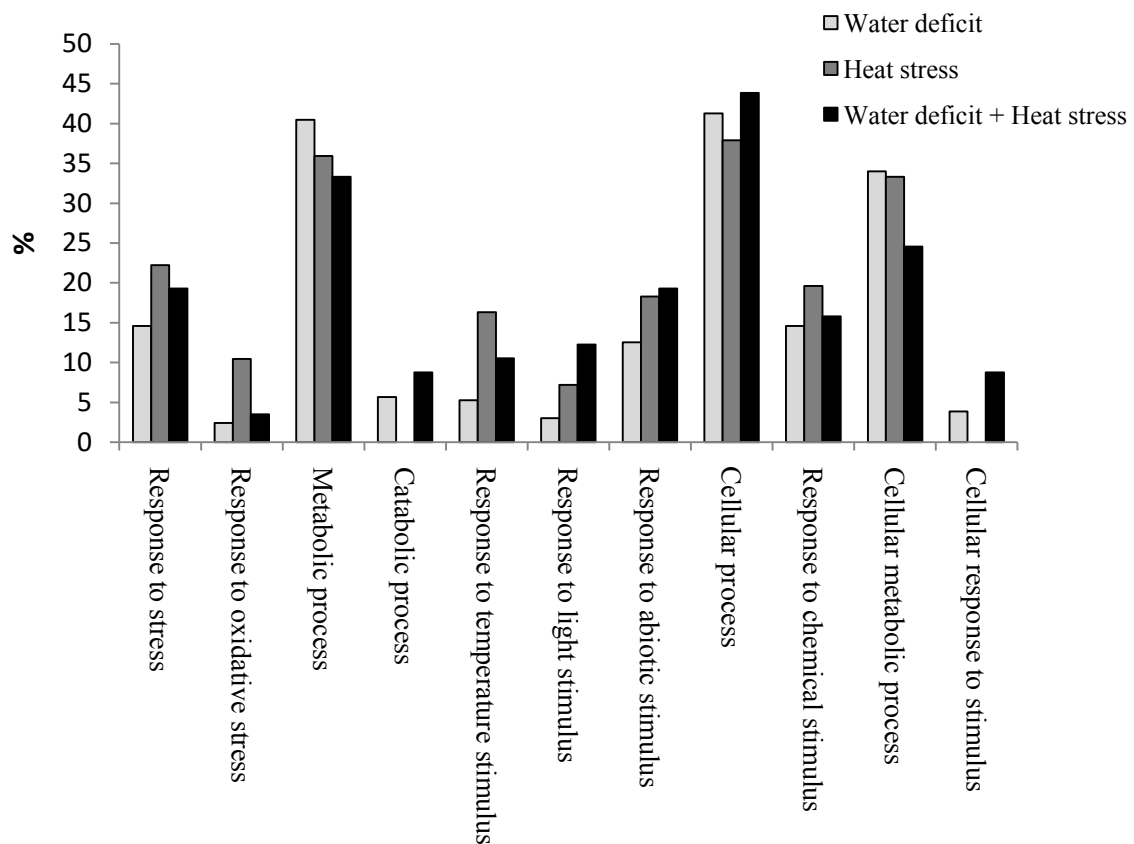
Figure 3. Different gene ontologies of transcripts specifically accumulated in response to drought and heat combination and their relative abundance (%). Data was obtained from Rizhsky et al. (2004).

The number of transcripts altered by each individual or combined stress condition could reflect the complexity of plant molecular responses to adverse situations. For example, transcriptomic analysis in sorghum plants under individual and combined stress conditions revealed that 448, 1554 and 2043 transcripts were specifically modulated under drought, heat and their combination, respectively (Johnson et al. 2014), indicating that the number of transcripts specifically altered during a combination of drought and heat stress tends to be higher than that altered by each of the individual stresses, suggesting thereby that the molecular response of plants to the stress combination requires a higher complexity.

Changes in plant metabolism in response to a combination of drought and heat stress

The acclimation mechanisms of plants to a combination of drought and heat are coordinated with the activation of specific physiological and molecular responses as mentioned above. These responses, in turn, lead to changes in plant metabolism that mitigate the damaging effects of the stress combination. Metabolite profiling of different plant species subjected to drought, heat stress and their combination has unraveled a wide range of metabolites specifically altered during stress combination, including osmoprotectants, carbohydrates, polyols, aminoacids or Krebs cycle intermediates (Suzuki et al. 2014).

Under abiotic stresses, the accumulation of osmoprotectants (compatible solutes) is a common plant response aimed to stabilize proteins and membranes and to contribute to the cell osmotic pressure. In particular, proline accumulates in many plant species in response to environmental stress. However, its role during combined stress conditions remains unclear. It has been reported that proline accumulated in *Arabidopsis thaliana* plants in response to drought but not in response to heat stress, or the combination of drought and heat. In contrast, in plants subjected to a combined heat and drought stress treatment, sucrose accumulated, potentially to protect mitochondria and other cellular components from the adverse effects of water deficit (Rizhsky et al. 2004). These results suggest that sucrose could replace proline in *Arabidopsis thaliana* plants under a combination of drought and heat stress, functioning as a major osmoprotectant. Similar results were reported in purslane plants (Jin et al. 2016), where proline was slightly accumulated under individual drought and heat conditions, but not by combined stress. In contrast, Cvikrová et al. (2013) demonstrated the possible involvement of proline in the protection of tobacco plants from drought and heat stress combination, possibly by modulating polyamine biosynthesis.

Other specific metabolic changes in plants subjected to drought and heat stress combination include the accumulation of sugars, polyols or Krebs cycle intermediates. In response to a combination of drought and heat stress, *Arabidopsis thaliana* plants specifically accumulated sucrose, probably derived from starch breakdown (**Figure 4**; Rizhsky et al., 2004). Therefore, this process coupled with energy production in the mitochondria could play an important role in plant metabolism during a combination of

drought and heat stress (Rizhsky et al. 2002, Rizhsky et al. 2004). Accumulation of amino acids could also be associated with storage of available substrates for protein synthesis that would promote rapid recovery of plant metabolism from stress, as well as an osmotic adjustment. *Arabidopsis* (**Figure 4**; Rizhsky et al. 2004) and purslane plants (Jin et al. 2016) specifically accumulated certain amino acids during a combination of drought and heat stress, including glutamine, ornithine, tyrosine, valine and tryptophan, suggesting a cellular osmotic adjustment aimed to keep the leaf turgor during this stress condition.
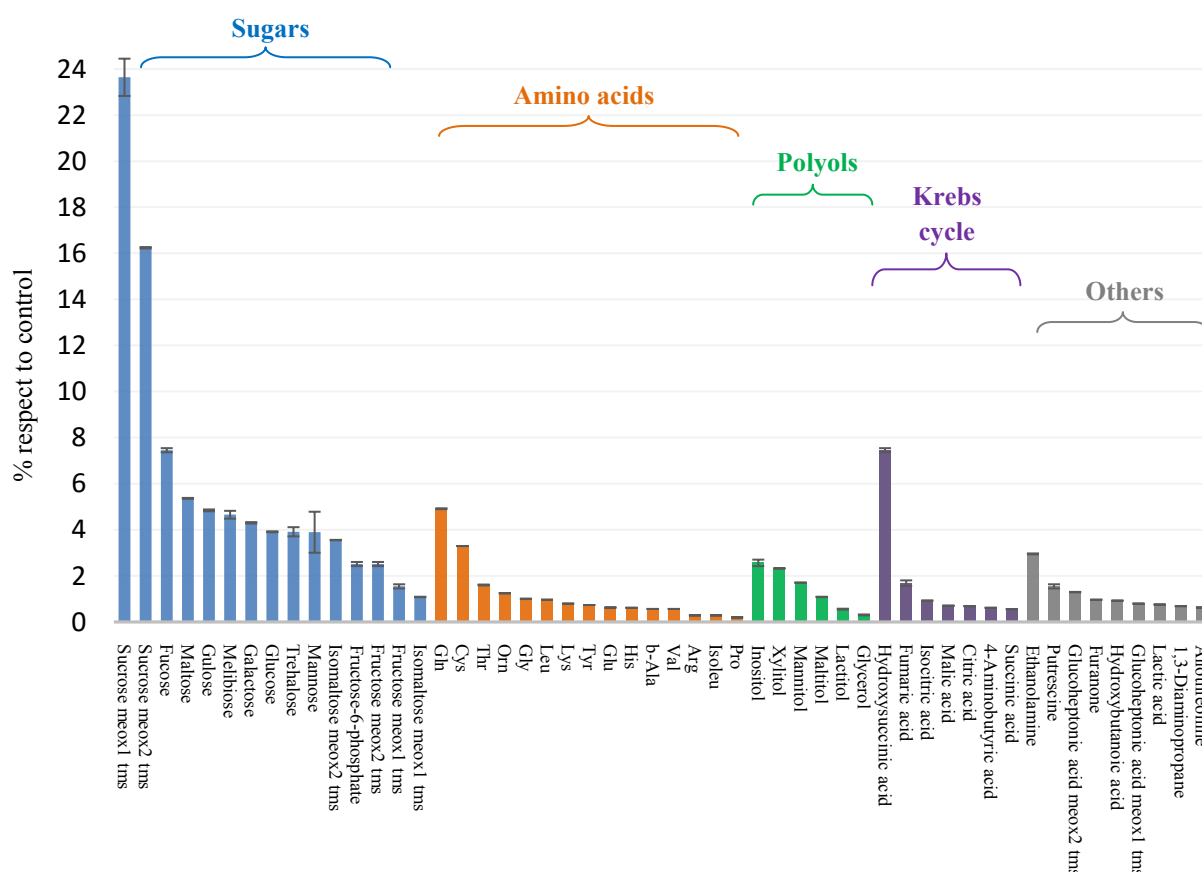
Figure 4. Polar metabolites detected by GC-MS specifically accumulated during a combination of drought and heat stress in *Arabidopsis* plants. Data was obtained from Rizhsky et al. (2004).

Krebs cycle intermediates were also implicated in the response of plants to combined stresses (**Figure 4**; Rizhsky et al. 2004; Koussevitzky et al. 2008). Based on proteins specifically accumulated during the stress combination, Koussevitzky et al. (2008) demonstrated that malate metabolism could be involved in the response of *Arabidopsis thaliana* to a combination of drought and high temperatures. In this study, the

accumulation of malic enzyme during the combined stress treatment correlated with its enhanced activity and the decrease in malate and oxaloacetate levels.

The studies described above highlight the idea that a combination of drought and heat stress imposes on plants a specific metabolic demand compared to drought or heat stress, demonstrating the ability of plants to respond to complex environmental conditions that naturally occur in the field. However, a recent study of metabolite profiles of maize leaves under drought, heat and their combination demonstrated that only a few metabolites specifically responded to the stress combination (Obata et al. 2015). Additionally, most of the metabolic changes were predictable from the sum of responses to each single stress, suggesting that metabolic pathways were regulated to meet the metabolic demands under each stress condition, resulting thus in an additive metabolite profile under stress combination (Obata et al. 2015).

Strategies for improving tolerance to abiotic stress combination in crop plants

New approaches to investigate the response of plants to a combination of different abiotic stresses are urgently needed to develop plants tolerant to a wide range of stresses. These studies could significantly improve our chances of developing crops with enhanced tolerance to field conditions by unraveling the complex networks of molecular interactions controlling plant acclimation to field conditions (Mittler and Blumwald 2010, Suzuki et al. 2014). Some success has already been achieved in the development of crops tolerant to a particular abiotic stress condition by genetic manipulation of stress-inducible genes such as TFs, LEA proteins and antioxidant proteins (Mittler and Blumwald 2010, Lawlor 2013, Shaik and Ramakrishna 2014). Further improvement could, however, be obtained by studying the tolerance of plants to a wide range of stress combinations that are likely to co-occur in the field. In addition, studies could be conducted on field-grown elite cultivars subjected to different stress combinations. Dissecting the mechanisms that regulate plant responses to adverse factors that simultaneously impact the plant under laboratory or field conditions could, therefore, be key for the development of broad-spectrum stress-tolerant crops. The meta-analysis of whole transcriptome expression patterns in response to several abiotic stresses can be accessed using bioinformatics tools. More recently, computational tools such as co-expression modules provided additional means for identifying candidate genes for engineering broad-spectrum resistance based on the availability of large

volumes of genome-scale gene expression data. Transcriptomic data from several plant species subjected to different stress combinations certainly provided a snap shot of the changes in the steady-state mRNA levels (Rizhsky et al. 2002, Rizhsky et al. 2004, Priyanka et al. 2010, Rasmussen et al. 2013, Johnson et al. 2014, Suzuki et al. 2016). In addition, this type of data can be integrated into interaction networks between genes and/or proteins based on their degree of co-expression, helping to visualize and interpret data in a systems biology context. This type of approach allows the identification of functional modules (e.g. highly coordinated gene or protein networks or protein-protein interactions), pointing to highly interconnected genes or proteins as potentially involved in stress tolerance. Additional omics studies conducted on plants and crops grown in the lab and/or the field, coupled with the use of bioinformatics tools could yield a more defined list of target genes for biotechnological applications. These genes could then be tested in large-scale lab and field studies and introduced into elite cultivars. Although the study of stress combination is just beginning, the urgent need to develop plants and crops that would be able to withstand the predicted climatic changes in years to come (**Figure 1**), highlight the importance of studying this particular aspect of plant acclimation. In a future of rapidly changing environment we would desperately need crops that could tolerate multiple stresses with a minimal effect on their yield.

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Hypothesis and objectives

Previous studies focused on combining abiotic stresses in plants have unraveled physiological and molecular responses that are unique and cannot be extrapolated from the study of the impact of each individual stress. Specifically, drought and high temperatures represent a frequent stress combination that take place during summers in Mediterranean areas. This stress combination constitutes an agricultural problem for citriculture and will be aggravated in future years due to the current climate change. Nowadays, different citrus rootstocks are adapted to specific climate conditions, determining the crop yield and quality. However, limited information regarding citrus responses to a combination of drought and heat are currently available, highlighting the importance of better understanding plant behavior to this stress combination to enhance citrus yield in a future of rapidly changing environment.

Therefore, the present work hypothesizes that the combination of drought and high temperatures represents a unique and more severe condition than individual stresses and that particularly alters citrus physiology and hormonal profiles.

Among hormones, ABA plays a central role during plant responses to abiotic stresses, suggesting its relevant involvement in plant tolerance to combined drought and heat. To analyze the ABA role during drought and heat stress combination, the use of *Arabidopsis thaliana* plants is needed due to the availability of mutants, specifically ABA-deficient (*aba1-1*) and ABA-insensitive (*abi1-1*) mutants.

Our hypothesis points to ABA as a key hormone to tolerate the combination of drought and high temperatures in plants.

The main objective of this work is to study the impact of the combination of drought and high temperatures on plant physiology.

To achieve this aim, we have planned partial objectives that are described below:

1. Determine the physiological responses to drought, high temperatures and their combination in two citrus genotypes (Carrizo citrange and Cleopatra mandarin).

2. Link citrus tolerance responses to changes in hormonal profiles, especially with differential SA and ABA accumulation and signaling.

3. Analyze the impact of drought, high temperatures and their combination in the primary and secondary metabolism of Carrizo citrange and Cleopatra mandarin and to correlate the differential metabolite accumulation with the contrasting tolerance of these genotypes to adverse situations.

4. Evaluate ABA involvement in the tolerance of plants to combined drought and high temperatures.

Results

Chapter 1

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.

Zandalinas *et al*. (2016) BMC Plant Biology 16:105

Abstract

Background: In natural environments, several adverse environmental conditions occur simultaneously constituting a unique stress factor. In this work, physiological parameters and the hormonal regulation of Carrizo citrange and Cleopatra mandarin, two citrus genotypes, in response to the combined action of high temperatures and water deprivation were studied. The objective was to characterize particular responses to the stress combination.

Results: Experiments indicated that Carrizo citrange is more tolerant to the stress combination than Cleopatra mandarin. Furthermore, an experimental design spanning 24 h stress duration, heat stress applied alone induced higher stomatal conductance and transpiration in both genotypes whereas combined water deprivation partially counteracted this response. Comparing both genotypes, Carrizo citrange showed higher phostosystem-II (PSII) efficiency and lower oxidative damage than Cleopatra mandarin. Hormonal profiling in leaves revealed that salicylic acid (SA) accumulated in response to individual stresses but to a higher extent in samples subjected to the combination of heat and drought (showing an additive response). SA accumulation correlated with the up-regulation of pathogenesis-related gene 2 (*CsPR2*), as a downstream response. On the contrary, abscisic acid (ABA) accumulation was higher in water-stressed plants followed by that observed in plants under stress combination. ABA signaling in these plants was confirmed by the expression of responsive to ABA-related gene 18 (*CsRAB18*). Modulation of ABA levels was likely carried out by the induction of 9-neoxanthin cis-epoxicarotenoid dioxygenase (*CsNCED*) and ABA 8'-hydroxylase (*CsCYP707A*) while conversion to ABA-glycosyl ester (ABAGE) was a less prominent process despite the strong induction of ABA O-glycosyl transferase (*CsAOG*).

Conclusions: Cleopatra mandarin is more susceptible to the combination of high temperatures and water deprivation than Carrizo citrange. This is likely a result of a higher transpiration rate in Carrizo that could allow a more efficient cooling of leaf surface ensuring optimal CO_2 intake. Hence, SA induction in Cleopatra was not sufficient to protect PSII from photoinhibition, resulting in higher malondialdehyde (MDA) build-up. Inhibition of ABA accumulation during heat stress and combined stresses was achieved primarily through the up-regulation of *CsCYP707A* leading to phaseic acid (PA) and dehydrophaseic acid (DPA) production. To sum up, data indicate

that specific physiological responses to the combination of heat and drought exist in citrus. In addition, these responses are differently modulated depending on the particular stress tolerance of citrus genotypes.

Key words: Carrizo citrange, Cleopatra mandarin, combined stress conditions, heat, hormone regulation, salicylic acid.

Background

Plants respond to adverse environmental challenges by activating specific molecular and physiological changes to minimize damage. The great majority of studies focusing on plant stress tolerance have considered a single stress condition. However, under field conditions, several abiotic stress situations are most likely to occur simultaneously constituting a unique new stress condition and not a mere additive combination of the effects of the individual stress factors [1, 2]. Therefore, the future development of broad-spectrum stress-tolerant plants will require the understanding of the responses to multiple abiotic threats and, hence, new experimental approaches have to be developed in order to mimic stress combinations [2]. Particularly, drought and elevated temperatures represent the most frequent abiotic stress combination occurring in natural environments [1]. This situation has important detrimental effects on plant growth and productivity [3–5]. Additionally, plant responses to a combination of drought and high temperatures have been suggested to be exclusive and different from plant responses to drought or heat stress applied individually [6–8].

Plant responses to external stimuli are mainly mediated by phytohormones, whose involvement in abiotic stress responses has been deeply studied [9–12]. Under drought or high salinity, abscisic acid (ABA) seems to be an important stress-signaling hormone [13, 14], involved in the regulation of stomatal closure, synthesis of compatible osmolytes and up-regulation of genes leading to adaptive responses. Increase of ABA levels is accompanied by the up-regulation of 9-neoxanthin cis-epoxicarotenoid dioxygenase (NCED) that converts 9-neoxanthin to xanthoxin and is considered the bottleneck in ABA biosynthesis. Inactivation of ABA is achieved by its cleavage to 8'-OH-ABA catalyzed by an ABA 8'-hydroxylase (CYP707A) and this compound is converted spontaneously to phaseic acid (PA) and subsequently to dehydrophaseic acid (DPA) as main degradation products. Additionally, another pathway for removing active ABA pools is the conjugation to hexoses by an ABA O-glycosyl transferase (AOG) yielding ABA-glycosyl ester (ABAGE) [15]. Finally, active ABA can be released after cleavage of ABAGE by an ABAGE β-glycosidase (BG18) [16 and Additional file 1A].

Salicylic acid (SA) has been associated to defense responses against biotrophic pathogens [17]. However, recent studies have suggested that SA also plays an important

role in abiotic stress-induced signaling and tolerance [11, 18]. Particularly, it has been proposed that SA may induce thermotolerance in several plant species [19–22]. Studies in *Arabidopsis* mutants suggest that SA-signaling pathways involved in the response to biotic stresses overlap with those promoting basal thermotolerance. In this sense, pathogenesis-related (*PR*) genes are not only induced by biotic stresses but also in response to high temperatures [21]. This plant hormone is synthesized from chorismate in a reaction catalyzed by isochorismate synthase (ICS) and subsequently by isochorismate pyruvate lyase. In addition, SA is also synthesized from phenylalanine and the key enzyme catalyzing this reaction is phenylalanine ammonia lyase (PAL) [23 and Additional file 1B]. SA accumulation induced by stress, exogenous application or genetic manipulation has been associated to positive responses against high temperature stress in different plant species such as poplar [24], *Agrostis stolonifera* [25], *Avena sativa* [26] and grapevine [27]. The benefits of SA accumulation seem to be associated to an improvement in antioxidant activity and the protection of the photosynthetic machinery avoiding electron leakage [28]. In addition, an improvement in the responses to other abiotic stress conditions such as salinity, drought or chilling have been reported [11].

Despite these advances in hormonal physiology, it is still unclear how different signaling pathways with such clear roles interact to induce defense responses in plants when several stress conditions concur. For instance, stomatal responses, which are essential in acclimation to abiotic stress conditions, have been recently associated to the interaction of reactive oxygen species (ROS), ABA and Ca^{2+} waves [29]. Briefly, upon ABA sensing, mediated by pyrabactin resistance1/PYR-like/regulatory components of ABA receptors (PYR/PYL/RCAR) and protein phosphatases 2C (PP2Cs), sucrose non-fermenting 1-related protein kinases (SnRKs) 2.3 is released and phosphorylates slow anion channel-associated 1 (SLAC1), a membrane ion channel that mediates anion release from guard cells promoting stomatal closure. In addition, SnRKs2.3 also phosphorylates and activates a plasma-bound NADPH oxidase (RBOH) involved in $O_2^{\bullet-}$ production that is dismutated into H_2O_2 by apoplastic superoxide dismutases. The elevated ROS levels enhance ABA signaling through inhibition of PP2Cs and activate influx Ca^{2+} channels, increasing its cytosolic concentration. Subsequently, this Ca^{2+} accumulation contributes to inhibit ion influx into guard cells and maintain stomatal closure. This mechanism is in line with apoplastic ROS modulating the responsiveness

of guard cells to ABA [30]. Moreover, ROS have been shown to promote ABA biosynthesis and inhibit its degradation, resulting in an increase of endogenous ABA levels [29].

In this work, we aimed to study the physiological and hormonal responses to drought, heat and their combination in two citrus genotypes with contrasting stress tolerance, Carrizo citrange and Cleopatra mandarin, and link tolerance responses to a differential SA and ABA accumulation and signaling.

Methods

Plant material and growth conditions

True-to-type Carrizo citrange (*Poncirus trifoliata* L. Raf. x *Citrus sinensis* L. Osb.) and Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) plants were purchased from an authorized commercial nursery (Beniplant S.L., Penyíscola, Spain). One-year-old seedlings of both citrus genotypes were placed in 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 ºC and 18.0 ± 3.0 ºC, respectively). Later, plants of both genotypes were maintained for 2 weeks in growth chambers to acclimate to a 16-h photoperiod at 300 μmol m^{-2} s^{-1} at 25º C and relative moisture at approximately 80%. Temperature and relative moisture were recorded regularly with a portable USB datalogger (OM-EL-WIN-USB, Omega, New Jersey, USA).

Stress treatments and experimental designs

To evaluate heat stress tolerance, Carrizo citrange and Cleopatra mandarin seedlings were subjected to 40 ºC for 10 days and the number of intact sprouts (sprouts with no visual symptoms of damage: wilting, bronzing and/or abscission at gentle touch) was recorded regularly. Similarly, citrus plants were maintained at 40 ºC while imposing water withdrawal to investigate the effects of the stress combination. Percentage of intact sprouts was calculated at 0, 2, 4, 6, 8 and 10 days after imposing stress treatments.

Additionally, we designed a 24-h experiment in which severe drought, imposed by transplanting plants to dry perlite, was applied alone or in combination with high temperatures (40ºC). Prior to imposition of drought regime, heat stress was applied for 7

days to a group of well-watered Carrizo and Cleopatra plants whereas another group was maintained at 25 ºC. Thereby, we established four experimental groups of each genotype: well-watered plants at 25 ºC (CT) and 40 ºC (HS) and plants subjected to drought at 25 ºC (WS) and at 40 ºC (WS+HS). Leaf tissue was sampled at 24 hours after subjecting plants to both stresses.

Physiological parameters

Gas exchange and chlorophyll fluorescence parameters were measured in parallel on plants of each treatment between 9:00 and 11:00 h. Leaf gas exchange parameters were measured with a LCpro+ portable infrared gas analyzer (ADC bioscientific Ltd., Hoddesdon, UK) under ambient CO_2 and moisture. Supplemental light was provided by a PAR lamp at 1000 µmol m^{-2} s^{-1} photon flux density and air flow was set at 150 µmol mol^{-1}. After instrument stabilization, ten measurements were taken on three mature leaves (from an intermediate position on the stem) in three replicate plants from each genotype and treatment. Quantum yield (Φ_{PSII}) and maximum efficiency of photosystem II (PSII) photochemistry, as F_v/F_m ratio, were analyzed on the same leaves and plants using a portable fluorometer (FluorPen FP-MAX 100, Photon Systems Instruments, Czech Republic).

Proline analysis

0.05 g ground, frozen leaf tissue was extracted in 5 ml of 3% sulfosalicylic acid (Panreac, Barcelona, Spain) by sonication for 30 min. After centrifugation at 4000 g for 20 min at 4 ºC, extracts were assayed for proline as described by Bates and others [31] with slight modifications. Briefly, 1 ml of the supernatant was mixed with 1 ml of glacial acetic acid and ninhydrin reagent (Panreac) in a 1:1 (v:v) ratio. The reaction mixture was incubated in a water bath at 100 ºC for 1 h. After centrifuging at 2000 g for 5 min at 4 ºC, absorbance was read at 520 nm. A standard curve was performed with standard proline (Sigma-Aldrich, St. Louis, MO, USA).

Leaf water status

Leaf relative water content (RWC) was measured using adjacent leaves, which were immediately weighed to obtain a leaf fresh mass (M_f). Then, leaves were placed in a beaker of water overnight in the dark, so leaves could become fully hydrated. Leaves were reweighed to obtain turgid mass (M_t) and dried at 80 ºC for 48 h to obtain dry

mass (M_d). Finally, RWC was calculated as $[(M_f - M_d) \times (M_t - M_d)^{-1}] \times 100$ according to [32].

Malondialdehyde analysis

Malondialdehyde (MDA) content was measured following the procedure of [33] with some modifications. Ground frozen leaf tissue (0.2 g approximately) were homogenized in 2 mL 80% cold ethanol by sonication for 30 min. Homogenates were centrifuged 12000 g for 10 min and different aliquots of the supernatant were mixed either with 20% trichloroacetic acid or with a mixture of 20% trichloroacetic acid and 0.5% thiobarbituric acid. Both mixtures were incubated in a water bath at 90 ºC for 1 h. After that, samples were cooled in an ice bath and centrifuged at 2000 g for 5 min at 4 ºC. The absorbance at 440, 534 and 600 nm of the supernatant was read against a blank.

Plant hormonal analysis

Hormone extraction and analysis were carried out as described in [34] with few modifications. Shortly, for ABA, PA, DPA and SA extractions, 0.3 g of ground frozen leaf tissue was extracted in 2 mL of ultrapure water after spiking with 50 ng of $[^2H_6]$-ABA, $[^{13}C_6]$-SA and $[^2H_3]$-PA in a ball mill (MillMix20, Domel, Železniki, Slovenija). After centrifugation at 4000 g at 4 ºC for 10 mins, supernatants were recovered and pH adjusted to 3 with 30% acetic acid. For ABAGE extraction, the aqueous layer was recovered and after adding 0.1 M sodium hydroxide, was incubated in a water bath at 60 ºC for 30 min. Then, samples were cooled in an ice bath and 50 ng of $[^2H_6]$-ABA was added. pH was adjusted to 3 with 0.5% chlorhydric acid. All water extracts were partitioned twice against 2 mL of diethyl-ether and then the organic layer was recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). Once dried, the residue was resuspended in a 10:90 methanol:water solution by gentle sonication. The resulting solution was filtered through 0.22 μm polytetrafluoroethylene membrane syringe filters (Albet S.A., Barcelona, Spain) and directly injected into an ultra performance liquid chromatography system (Acquity SDS, Waters Corp., Milford, MA, USA). Chromatographic separations were carried out on a reversed-phase C18 column (Gravity, 50 × 2.1 mm 1.8-μm particle size, Macherey-Nagel GmbH, Germany) using a methanol:water (both supplemented with 0.1% acetic acid) gradient at a flow rate of 300 μL min^{-1}. Hormones were quantified with a triple quadrupole mass spectrometer (Micromass, Manchester,

UK) connected online to the output of the column though an orthogonal Z-spray electrospray ion source.

Total RNA isolation and cDNA synthesis

About 100 mg of ground Carrizo and Cleopatra leaf tissue was used to isolate total RNA by RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Then, 5 µg RNA was treated with RNase-free DNase (Promega Biotech Ibérica, SL. Madrid, Spain) according to the manufacturer in order to remove genomic DNA contamination. The integrity of the RNA was assessed by agarose gel electrophoresis and ethidium bromide staining. Total RNA concentration was determined using spectrophotometric analysis (NanoDrop, Thermo Scientific, Wilmington, DE, USA), and the purity was assessed from the ratio of absorbance readings at 260 and 280 nm. Reverse transcription was carried out from 1 µg of total RNA using Primescript RT reagent with oligo(dT) primer (Takara Bio, Inc. Japan).

qRT-PCR analyses

Gene-specific primers were designed with primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) using orthologous sequences retrieved from *Citrus sinensis* genome (http:\\www.phytozome.org) (Additional table 1). Designed primers were then evaluated with IDT-oligoanalyzer tools (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/) following parameters: Tm around 60 ºC, amplicon length of 125 to 200 bp, primer length of 18 to 22 nucleotides with an optimum at 20 nucleotides and, finally, a GC content of 45 to 55%. Amplicon specificity was evaluated by agarose gel electrophoresis and by melting-curve analyses. The expression of all genes was normalized against the expression of two endogenous control genes (tubulin and actin). Relative expression levels were calculated by using REST software [35], comparing the expression of the gene at a particular time point to a common reference sample from the tissue at the first time point and then expression values were expressed as fold change of control values for each stress conditions. qRT-PCR analyses were performed in a StepOne Real-Time PCR system (Applied Biosystems, CA, USA). The reaction mixture contained 1 µL of cDNA, 5 µL of SYBR Green (Applied Biosystems) and 1 µM of each gene-specific primer pair in a final volume of 10 µL. The following thermal profile was set for all

amplifications: 95 ºC for 30 s followed by 40 cycles of 95 ºC for 5 s and 60 ºC for 30 s. Three technical replicates were analyzed on each biological replicate.

Statistical analyses

Statistics were evaluated with the Statgraphics Plus v.5.1. software (Statistical Graphics Corp., Herndon, VA, United States). Data are means of three independent determinations and were subjected to one-way analysis of variance (ANOVA) followed by Tukey posthoc test ($p<0.05$) when a significant difference was detected. In addition, data were also subjected to analysis of variance using a two-way ANOVA with two citrus genotypes x four stress treatments followed by Tukey posthoc test ($p<0.05$) when a significant difference was detected.

Results

Tolerance of Carrizo and Cleopatra plants to high temperatures and combined drought and heat

The citrus genotypes used in this study, Carrizo citrange and Cleopatra mandarin, were chosen due to their differences in tolerance to different abiotic stress conditions [36]. However, little is known about their ability to tolerate high temperatures. Hence, the relative tolerance to high temperature of the two genotypes employed in this study was firstly investigated. To accomplish this, both genotypes were subjected to continuous heat stress (40 ºC) for 10 days. The ability to produce new flushes and maintain sprouts healthy throughout the experimental period was taken as a tolerance trait. All seedlings growing at 40 ºC showed an intense flushing of new sprouts compared to those grown at normal temperature (25 ºC) (Additional file 2A and 2B, 2D and 2E). However, as the experiment progressed, new sprouts in Cleopatra started browning and withering (Additional file 2E-F), affecting more than 70% of the new flushes after 6 days of treatment (Additional file 2G). On the contrary, new sprouts appearing on Carrizo did not show any damage symptom throughout the experimental period (Additional file 2B-C). Only at the end of the experimental process, 20% of the new flushes in Carrizo showed symptoms of damage (Additional file 2G). These results clearly evidenced the higher tolerance of Carrizo to high temperatures compared to Cleopatra. Moreover, we also recorded the number of intact sprouts in Carrizo and Cleopatra seedlings subjected to a combination of heat (40 ºC) and water deprivation for 10 days (Figure 1). After 4

days of treatment, only 50% of new sprouts in Cleopatra plants remained unaffected whereas all sprouts on Carrizo looked healthy. At 8 days of treatment, Carrizo sprouts started showing symptoms of damage, but a 75% still remained intact. At this point, however, only 15% of Cleopatra sprouts showed no apparent damage. At the end of the experiment (10 days), 60% of Carrizo sprouts still remained unaffected by stress treatment, while all Cleopatra sprouts were severely damaged, thus evidencing a higher ability of Carrizo to tolerate drought and heat applied in combination. To this respect, tolerance to high temperatures of both genotypes greatly mirrored tolerance to heat and water stress combination.
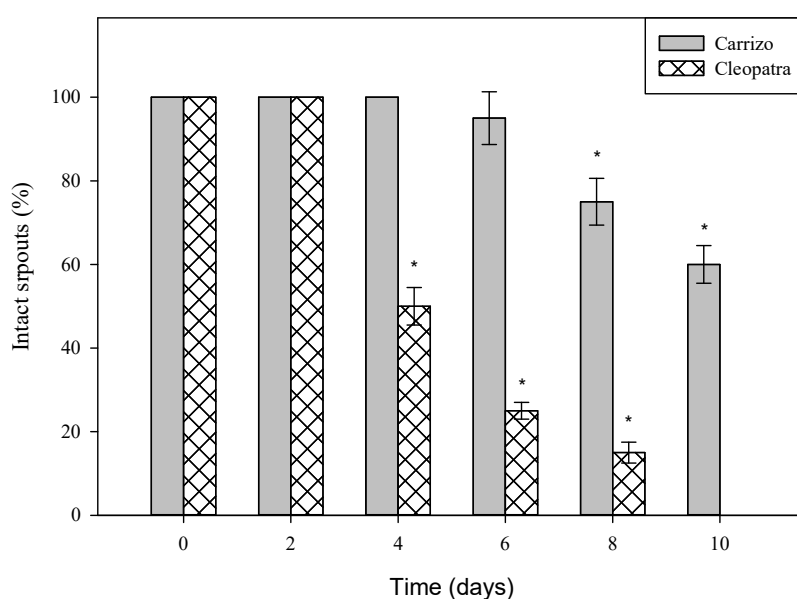
Figure 1. Phenotypic traits of citrus plants in response to a combination of drought and heat stress. Intact sprouts (%) of Carrizo and Cleopatra seedlings subjected to drought and heat stress (40 ºC) in combination for 10 days. For each genotype, asterisks denote statistical significance with respect to initial values at $p \leq 0.05$. Values are mean $\pm$ SD.

Effects on osmotic status under drought, heat and combined stresses

Leaf RWC was measured for each genotype and stress treatment (Figure 2a). In the conditions assayed in this work, abiotic stress conditions induced similar significant decreases in RWC in both genotypes. When applied individually, water stress and heat stress induced similar decreases in leaf RWC in plants of Carrizo and Cleopatra (60-70% of control values). Interestingly, stress combination had an additive effect on this parameter. Therefore, WS+HS plants exhibited the most dramatic reduction in leaf

RWC showing levels that were 48.4% and 34.3% of control values in Carrizo and Cleopatra, respectively.

In line with the observed variations in RWC, endogenous proline levels, as a compatible osmolyte, were inspected (Figure 2b). In response to WS, proline levels increased by 1.4-fold and 1.3-fold, respect to control values in Carrizo and Cleopatra, respectively. Moreover, HS induced an accumulation of proline in leaves of Carrizo whereas in Cleopatra, it had no significant effect. As for RWC, the stress combination had an additive effect on proline levels, inducing the highest leaf proline accumulation of all treatments, an average of 52.7 nmol g^{-1} fresh weigh (FW) in both genotypes (Figure 2b). Interestingly, proline levels in leaves of non-stressed Cleopatra seedlings were higher than in Carrizo (37.5 nmol g^{-1} FW versus 21.0 nmol g^{-1} FW, respectively). A correlation analysis between RWC and proline was performed, showing R values of 0.8065 and 0.6504 in Carrizo and Cleopatra, respectively, and p-values of <0.01 in both citrus genotypes.
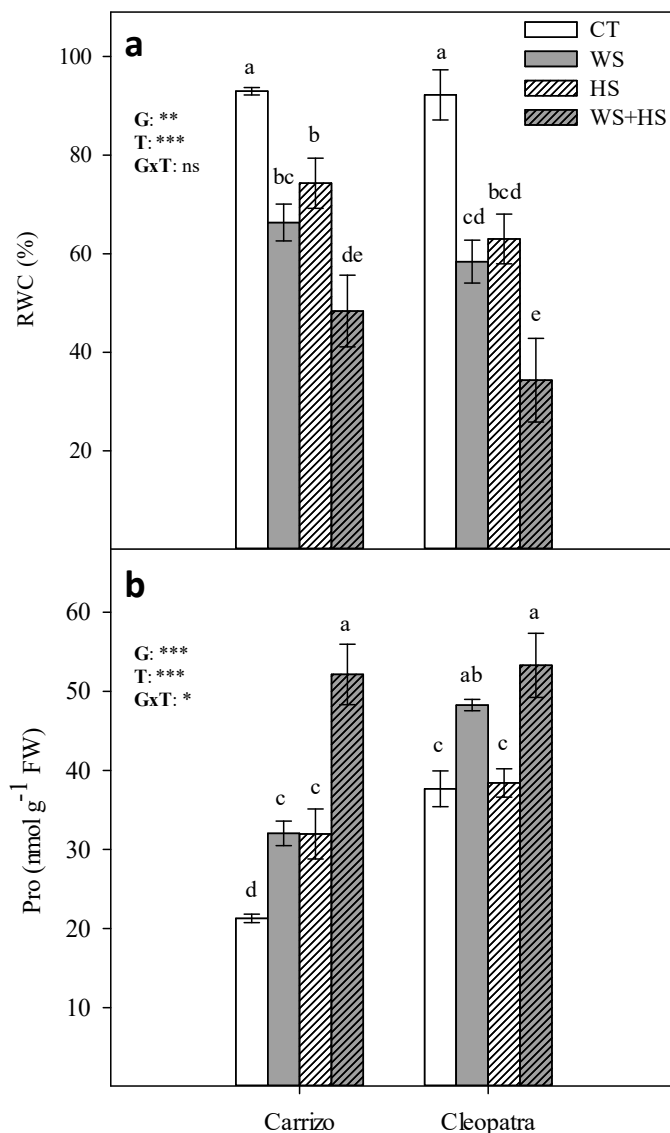
Figure 2. Relative water content (RWC) (a) and proline concentration (b) in Carrizo and Cleopatra plants subjected to drought (WS), heat (HS) and their combination (WS+HS). Different letters denote statistical significance at p≤0.05. G: genotypes; T: stress treatment; GxT: interaction genotype x stress treatment. *P<0.05; **P<0.01; ***P<0.001; ns: no statistical differences. Values are mean ± SD.

Leaf gas exchange and fluorescence parameters under drought, heat and combined stresses

Leaf photosynthetic rate (A), transpiration (E), carboxylative efficiency (in terms of substomatal-to-ambient CO_2, C_i/C_a ratio) and stomatal conductance (g_s) were measured in both genotypes (Figure 3). In general, WS and WS+HS reduced A, E and g_s parameters compared to unstressed plants mainly in Cleopatra. On the other hand, HS increased these parameters, especially in Carrizo, almost doubling Cleopatra levels in some cases (Figures 3a, b and d).
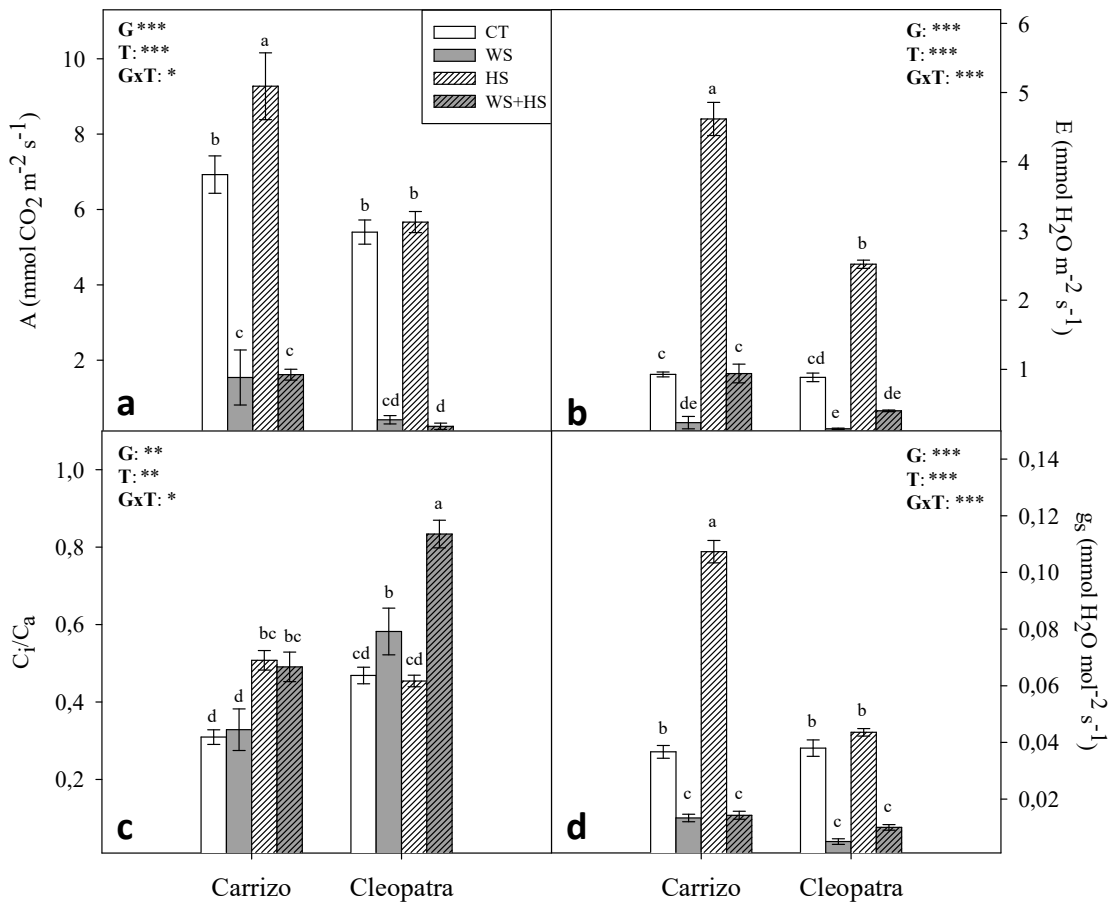
Figure 3. Gas exchange parameters in citrus plants subjected to different stress treatments. Leaf photosynthetic rate, A (a), transpiration, E (b), ratio of substomatal-to-ambient CO_2, C_i/C_a (c), stomatal conductance, g_s (d) in Carrizo and Cleopatra plants subjected to drought (WS), heat (HS) and their combination (WS+HS). Different letters denote statistical significance at $p \leq 0.05$. G: genotypes; T: stress treatment; GxT: interaction genotype x stress treatment. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ns: no statistical differences. Values are mean ± SD.

However, this effect of HS was counteracted by WS under WS+HS conditions. Plants subjected to stress combination showed similar gas exchange values to those obtained for WS plants in both genotypes. Additionally, carboxylative efficiency was affected by

HS and stress combination in Carrizo. In Cleopatra, C_i/C_a ratio increased slightly in response to WS. However, stress combination had a pronounced effect on carboxylative efficiency in this genotype (Figure 3c).

In addition to this, we measured the quantum efficiency of PSII photochemistry (Φ_{PSII}) and the maximum efficiency of PSII photochemistry (F_v/F_m ratio) that correlated with gas exchange parameters (Figure 4). In Carrizo, WS had a predominant effect over HS on electron transport between photosystems (Φ_{PSII}) whereas WS, HS or their combination was detrimental for this parameter in Cleopatra, having HS a more pronounced effect than WS alone. Moreover, F_v/F_m measurements mostly mirrored results obtained for Φ_{PSII} showing a negative effect of HS applied alone only in Cleopatra whereas stress combination affected both genotypes similarly.
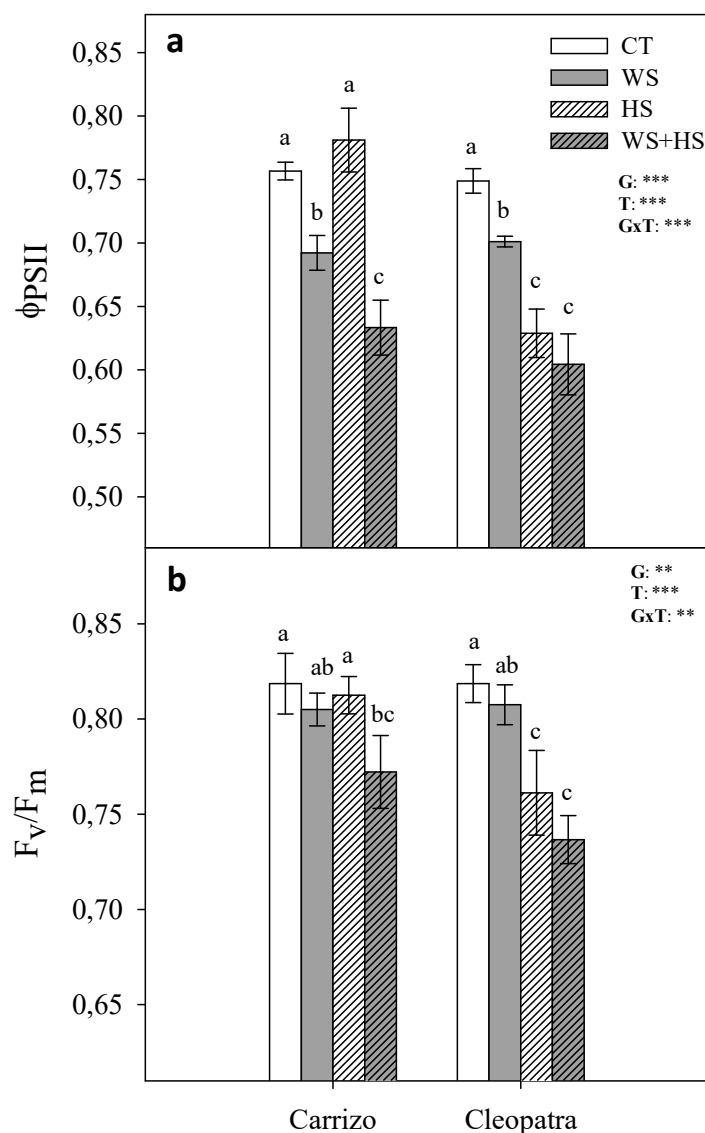
Figure 4. Chlorophyll fluorescence parameters in citrus plants subjected to different stress treatments. Quantum efficiency (Φ_{PSII}) (a) and maximum efficiency of PSII photochemistry (F_v/F_m ratio) (b) in Carrizo and Cleopatra plants subjected to drought (WS), heat (HS) and their combination (WS+HS). Different letters denote statistical significance at p≤0.05. G: genotypes; T: stress treatment; GxT: interaction genotype x stress treatment. *P<0.05; **P<0.01; ***P<0.001; ns: no statistical differences. Values are mean ± SD.

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MDA accumulation

Lipid peroxidation was measured in terms of MDA content. According to data (Table 1), MDA accumulated significantly in Carrizo leaves only in response to WS+HS. In Cleopatra leaves, MDA content increased in the three experimental conditions but higher levels were found under the combined effect of WS+HS, reaching values of 234.2 nmol g^{-1} FW, representing three times the MDA content of control plants (85.1 nmol g^{-1} FW).

Table 1. Malondialdehyde (MDA) concentration in citrus plants subjected to stress treatments: drought (WS), heat (HS) and their combination (WS+HS). Different letters denote statistical significance at p≤0.05. Values are mean ± SD. G: genotypes; T: stress treatment; GxT: interaction genotype x stress treatment. *P<0.05; **P<0.01; ***P<0.001; ns: no statistical differences.

MDA content (nmol g^{-1} FW)		
Stress condition	**Carrizo**	**Cleopatra**
CT	112.83^{cd} ± 3.46	85.11^{d} ± 7.15
WS	120.89^{c} ± 1.30	118.16^{cd} ± 5.95
HS	106.82^{cd} ± 1.15	116.15^{cd} ± 3.26
WS+HS	160.02^{b} ± 0.48	234.21^{a} ± 16.55
G	*	
T	***	
GxT	***	

SA metabolism and signaling under drought, heat and combined stresses

We measured SA levels in citrus leaves subjected to drought, heat stress and the combination of both stresses (Figure 5c). WS and HS and the combination of stresses increased SA levels in leaves of both genotypes respect to CT values, but higher levels were always observed in WS+HS plants. Interestingly, Cleopatra plants under WS+HS and WS showed SA levels 2.2-fold and 3.0-fold respectively higher than Carrizo. Moreover, we analyzed the relative expression of *CsPAL* and *CsICS*, two genes involved in SA biosynthetic pathways [23] in response to WS, HS and WS+HS. No statistical differences were found between genotypes or stress treatments in *CsPAL* transcript levels (Figure 5a) whereas *CsICS* expression was significantly altered during HS and WS+HS in Carrizo leaves and during WS+HS in Cleopatra (Figure 5b), showing the highest expression levels, respectively.
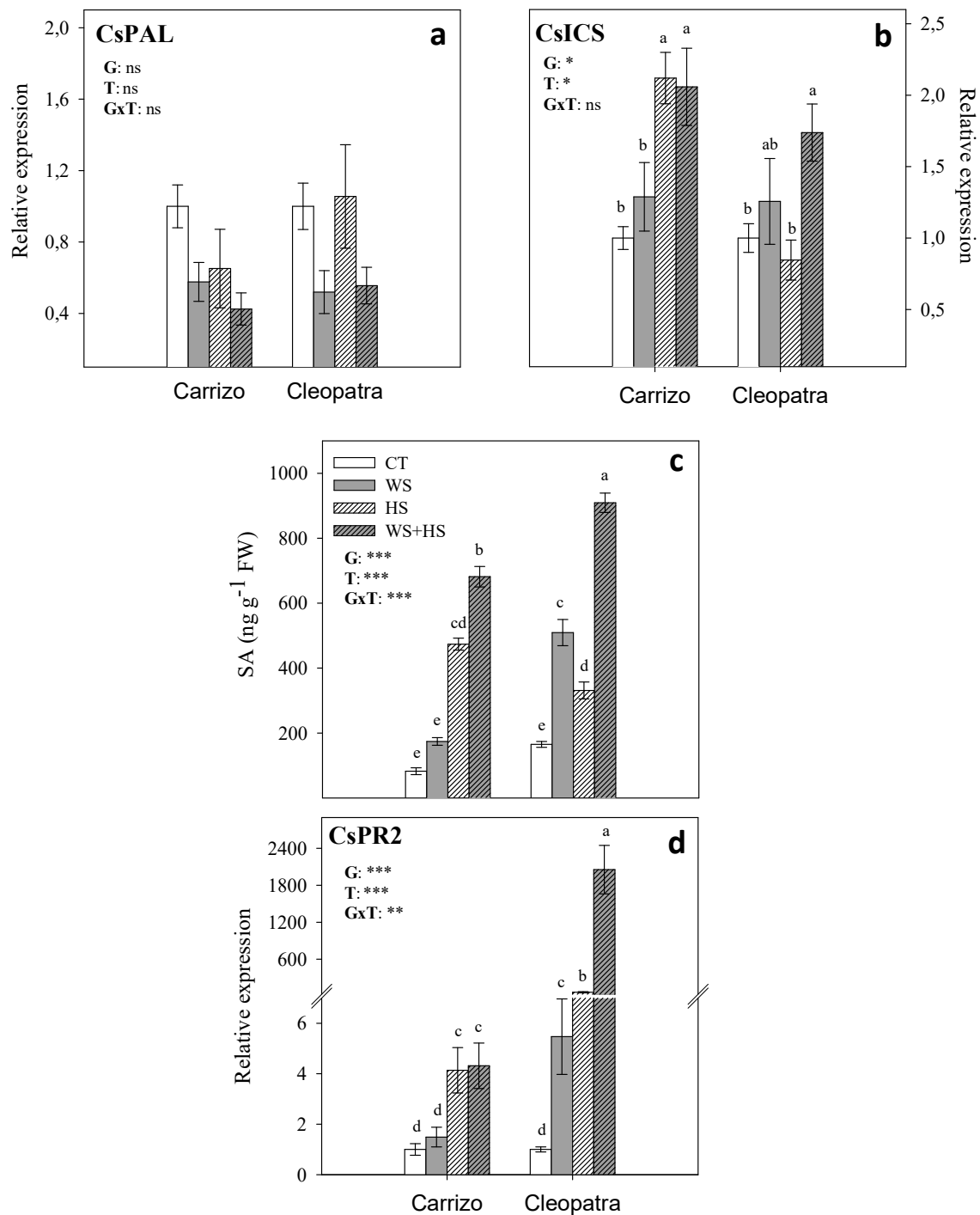
Figure 5. Effect of the different stress treatments on metabolism and signaling of SA. *CsPAL* (a) and *CsICS* (b) relative expressions, SA concentration (c) and *CsPR2* relative expression (d) in Carrizo and Cleopatra plants subjected to drought (WS), heat (HS) and their combination (WS+HS). Different letters denote statistical significance at p≤0.05. Values are mean ± SD. G: genotypes; T: stress treatment; GxT: interaction genotype x stress treatment. *P<0.05; **P<0.01; ***P<0.001; ns: no statistical differences.

To confirm SA signaling, we also analyzed the expression of *CsPR2*, a protein functioning as β-1,3-glucanase activity involved in defense against biotrophic pathogens that is induced by SA [37]. *CsPR2* transcript abundance correlated with SA accumulation in leaves of citrus, being strongly induced in leaves of WS+HS Cleopatra plants, showing the greatest SA levels. In general, abiotic stress induced higher SA build-up in Cleopatra than in Carrizo and hence a stronger *CsPR2* expression. Moreover, in Carrizo, only treatments involving heat (HS and WS+HS) resulted in a significant increase in *CsPR2* transcript levels whereas all abiotic stress treatments induced expression of this gene in Cleopatra plants (Figure 5d).

ABA metabolism under drought, heat and combined stresses

Analysis of ABA showed that WS and, to a much lower extent, WS+HS combination increased ABA levels in both citrus genotypes, reaching about 831.9 and 1340.1, and 290.9 and 225.7 ng g^{-1} FW, respectively (Figure 6a). Conversely, HS did not have any significant influence on ABA concentration in any of the two genotypes. To further investigate ABA metabolism in these stress conditions, concentration of PA and DPA as main ABA degradation products (Figure 6b-d) as well as the accumulation of ABAGE (Figure 6c) were measured. WS increased PA and DPA levels in leaves of both citrus genotypes but only Cleopatra exhibited a significant increment of ABAGE. In addition, HS induced the accumulation of DPA and reduced ABAGE content below control levels in both genotypes. During heat stress treatment, only Carrizo showed a significant PA accumulation (Figure 6b). Finally, WS+HS combination resulted in a strong accumulation of PA and DPA in both citrus genotypes that was significantly higher than in WS treatment. Stress combination slightly induced ABAGE accumulation only in Carrizo.
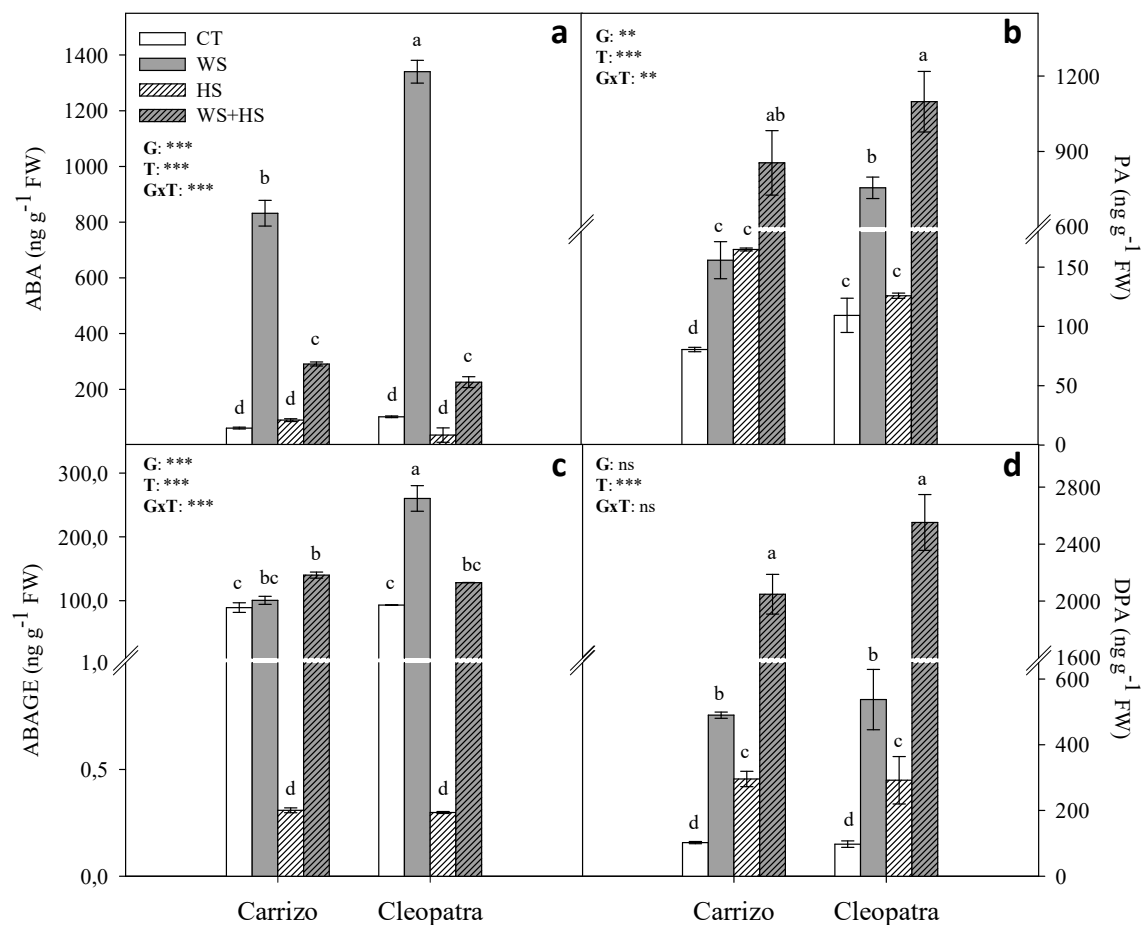
Figure 6. ABA, ABAGE, PA and DPA levels in citrus plants subjected to different stress treatments. ABA (a), ABAGE (c), PA (b) and DPA (d) levels in Carrizo and Cleopatra plants subjected to drought (WS), heat (HS) and their combination (WS+HS). Different letters denote statistical significance at p≤0.05. Values are mean ± SD. G: genotypes; T: stress treatment; GxT: interaction genotype x stress treatment. *P<0.05; **P<0.01; ***P<0.001; ns: no statistical differences.

Genes involved in ABA metabolism and signal transduction

To understand how ABA metabolism is modulated under the stress conditions assayed, the relative expression of genes encoding proteins involved in ABA biosynthesis, catabolism and conjugation were analyzed. In addition, responsive to ABA-related gene 18 (*CsRAB18*) expression was measured to confirm the occurrence of ABA signal transduction (Figure 7).

When WS was applied alone or in combination, the expression of *CsNCED1* was induced in leaves of Carrizo and, to a lower extent, in Cleopatra. But, on the contrary,

HS did not change the expression of this gene in any of the genotypes studied (Figure 7a). Hence, in stress combination, HS always counteracted the WS-dependent induction of *CsNCED1*. Additionally, *CsCYP707A1* expression was up-regulated in all stress treatments but showed different induction profiles depending on the genotype. Overall, expression was higher in Carrizo than in Cleopatra but, conversely to *CsNCED1*, stress treatments involving heat (HS and WS+HS) also induced *CsCYP707A1* expression (Figure 7b). No differences were recorded for *CsCYP707A1* expression values among stress treatments in Carrizo. In Cleopatra, WS increased *CsCYP707A1* expression up to 6-fold while HS had a more moderate impact and WS+HS combination induced its expression up to 50-fold. In the ABA conjugation pathway, *CsAOG* expression pattern was similar to that of *CsNCED1* but showing a more intense up-regulation in Cleopatra than in Carrizo upon WS+HS imposition and a significant slight induction by HS alone (Figure 7c). Although *CsAOG* gene was primarily induced by WS, stress combination had an additive effect on its expression showing values of 90.2 and 1704.9 in Carrizo and Cleopatra, respectively (Figure 7c). Moreover, *CsBG18* expression was up-regulated primarily in response to WS in both genotypes and in response to HS and WS+HS only in Carrizo (Figure 7d); in Cleopatra HS induced a significant down-regulation whereas stress combination had no significant effect.

Additionally, stress signal transduction mediated by ABA was assessed by studying the expression of *CsRAB18*, encoding a dehydrin protein, as an ABA-responsive gene. The expression pattern of this gene followed greatly that shown by *CsNCED1* (Figure 7a) and also that exhibited by ABA levels (Figure 6a). Accumulation of *CsRAB18* transcripts in leaves of both genotypes was observed mainly in response to WS and WS+HS and it was more pronounced in Carrizo. In this genotype, HS induced a slight increment in *CsRAB18* expression, while no changes were observed in Cleopatra (Figure 7e).
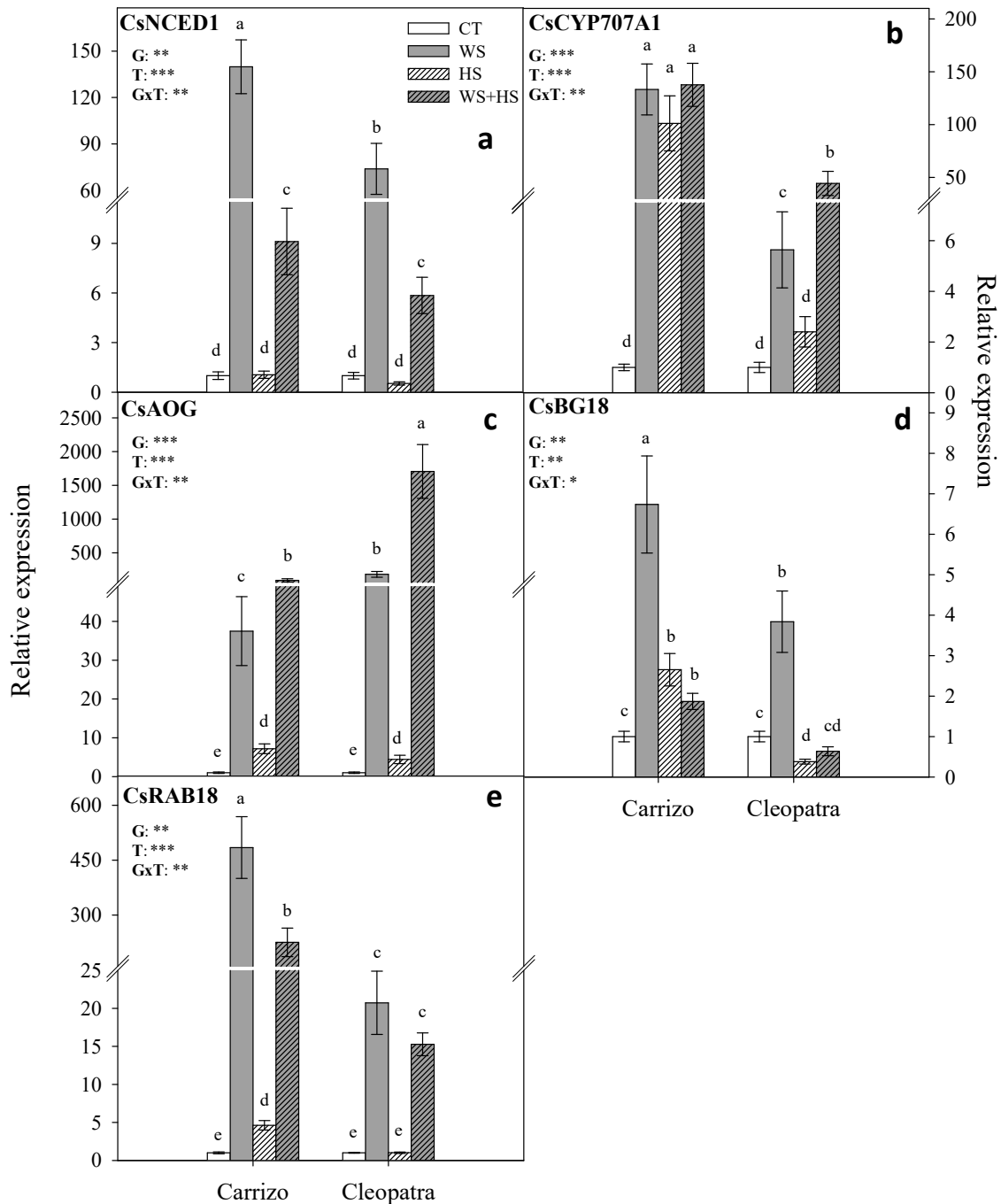
Figure 7. Expression of genes involved in ABA biosynthesis, catabolism, conjugation and signaling in citrus plants subjected to different stress treatments. Relative expression of ABA-biosynthetic gene *CsNCED1* (a), ABA-related catabolism gene *CsCYP707A1* (b), ABA-related conjugation genes *CsAOG* and *CsBG18* (c-d) and ABA-signaling gene *CsRAB18* (e) in leaves of Carrizo and Cleopatra plants in response to drought (WS), heat (HS) and their combination (WS+HS). Different letters denote statistical significance at $p \leq 0.05$. Values are mean ± SD. G: genotypes; T: stress treatment; GxT: interaction genotype x stress treatment. *P<0.05; **P<0.01; ***P<0.001; ns: no statistical differences.

Discussion

In the field, plants are often subjected to a combination of different abiotic stress conditions. Most research projects have focused on plant responses to a single stress factor under controlled environment. However, it is predicted that responses of plants to a combination of stress conditions could not be inferred simply from the study of each individual stress [1, 7]. For this reason, there is a need to understand the nature of responses to multiple stresses in order to develop plants more tolerant to environmental cues in a climate change scenario. In this context, drought and heat represent two stress conditions that are expected to increase their incidence in the next 50-100 years, drastically affecting global agricultural systems (IPCC, 2007). In the Mediterranean climate, summer drought is accompanied by high temperatures that limit crop plant growth, development and production. In the present research, we studied the relative tolerance and the physiological and molecular responses to heat, drought and a combination of both stress conditions of two citrus genotypes: Carrizo citrange and Cleopatra mandarin. These two citrus species show contrasting ability to tolerate different abiotic stress conditions. It has been reported that Cleopatra is more tolerant to drought and salinity than Carrizo, whereas the latter is more tolerant to soil flooding conditions [36]. However, information on citrus responses to heat stress is scarce. For this reason, in a preliminary study, we assessed heat susceptibility of both citrus genotypes by analyzing sprout emission and survival of plants subjected to a 10-day period of high temperatures (40 ºC) alone and combined with water withdrawal (Additional file 2 and Figure 1). Heat stress had a detrimental effect on Cleopatra sprout survival whereas Carrizo sprouts remained visibly healthy until the end of the experiment (Additional file 2), indicating that Carrizo is more tolerant to heat stress than Cleopatra. Similarly, Carrizo showed higher ability to tolerate heat stress combined with drought since 60% of sprouts remained intact after 10 days of stress combination. On the other hand, all sprouts were damaged in Cleopatra by the end of the experiment, showing only 50% of intact sprouts after 4 days of WS+HS (Figure 1). Nevertheless, it is worthwhile noting that at day 8 all sprouts in Cleopatra plants were damaged in response to HS (Additional file 2), whereas in response to WS+HS, 12.5% of sprouts still remained healthy on the same date (Figure 1). This apparent inconsistency could be explained by the effect of water stress and high temperature combination on stomatal closure. Cleopatra plants have been previously reported to be tolerant to salt stress due

to a fast decrease in transpiration rate during the osmotic phase of salinity that prevents build-up of chloride ions [38]. In this sense, the similar effect caused by WS would lead to a sharp decrease in transpiration rate during WS+HS conditions respect to HS that would prevent further desiccation. Hence, WS would act buffering the damaging effects of HS subsequently yielding a significantly higher percentage of intact sprouts in WS+HS plants.

Physiological responses of citrus to WS, HS and their combination

Water deprivation induced similar decreases of RWC in plants of both genotypes, indicating that the impact of WS was identical to both genotypes. On the other hand, HS incremented plant transpiration in both citrus genotypes. The combination of both stress conditions resulted in drastic decreases in leaf RWC, probably due to the additive effects of the individual stresses (drought induced water loss and high temperatures increased transpiration). In this sense, the accumulation of the compatible osmolyte proline was also highest in WS+HS treatments. Proline is an osmotically active molecule [39–43] although it is also accumulated in response to other types of stresses. Therefore, besides its known role as a compatible osmolyte, proline exhibits many other protective effects, including maintenance of redox balance and radical scavenging, maintenance of protein native structure acting as a molecular chaperonin enhancing the activities of different enzymes and contributing to lessen cell membrane damage [40, 44]. Under our conditions, proline accumulation was associated to water loss induced by soil drought, the elevated transpiration rates associated to the high temperatures or both. To show this association, we have performed a correlation analysis between RWC and proline, obtaining p-values <0.01 and R values of 0.8065, 0.6504 for Carrizo and Cleopatra, respectively. As previously shown [36 and references therein], different basal levels of proline between genotypes, as well as other protective and regulatory mechanisms, could be behind the higher tolerance of Cleopatra plants to drought. In well-watered plants of this genotype, proline levels are higher than in those of Carrizo. This seems to protect cells against stress for an extended period without significant additional increases in proline concentration [36]. Hence, a stronger stress pressure or longer treatment periods are required to cause further proline accumulation. Therefore, the high constitutive levels of proline prevent subsequent osmolyte biosynthesis until more severe stress conditions are reached, hence altering the linear relationship between the phenotypic trait (RWC) and the biochemical response.

To further characterize the responses to stress combination, gas exchange and chlorophyll fluorescence parameters were analyzed. As expected, WS alone induced stomatal closure, reducing transpiration and net photosynthetic rate in both citrus genotypes. On the contrary, HS induced an increase of transpiration probably oriented to decrease leaf surface temperature via evaporative cooling. However, HS had a different impact on transpiration in Carrizo and Cleopatra. In this latter genotype, high temperature treatment resulted in a lower increase of transpiration that did not have a concomitant impact on A or g_s. These different gas exchange responses could constitute a physiological advantage of Carrizo over Cleopatra since, as previously reported [8], higher transpiration rate could be linked to a lower leaf temperature. Moreover, stress also had an effect on net CO_2 assimilation, which was more pronounced in Cleopatra than in Carrizo, as evidenced by C_i/C_a ratio. In this sense, the capability of plants to modulate leaf gas exchange parameters and maintain optimal CO_2 assimilation rates under heat stress is directly associated to high temperature stress tolerance [45]. Moreover, under stress combination (WS+HS), the effect of WS on gas exchange parameters predominated over HS indicating that induction of stomatal closure to minimize water loss prevailed over responses that could lead to a reduction of leaf surface temperature (Figure 3). Chlorophyll fluorescence data further evidenced the detrimental influence of HS on the ability of Cleopatra plants to photosynthesize. In this genotype, PSII performance (F_v/F_m) and photosynthetic electron flow (Φ_{PSII}) significantly decreased in both HS treatments (HS and WS+HS) while PSII values in Carrizo plants were affected only by WS+HS combination (Figure 4). PSII, and especially the oxygen-evolving complex, is the most heat-sensitive component of the photosynthetic system [27, 45, 46]. These results are coherent with gas exchange data and further support the higher tolerance of Carrizo citrange to increased temperatures. WS+HS combination had the most detrimental effect on both genotypes evidenced by a reduction in F_v/F_m. In addition, the concomitant Φ_{PSII} reduction could be attributed to the lower PSII efficiency and the increased stomatal closure induced by water stress. As indicated by the parallel response of gas exchange and chlorophyll fluorescence parameters, the reduction in the ability to fix CO_2 could be associated to impairment in the performance of PSII, linked to the decrease in photosynthetic electron flow and, to a lesser extent, to the drought-induced stomatal closure. In other plant systems, similar results have been reported. In *Arabidopsis thaliana*, the combination of heat and drought resulted in the simultaneous enhancement of respiration and suppression of

photosynthesis. Heat stress induced stomatal opening and enhanced photorespiration whereas drought caused a suppression of photosynthesis linked to stomatal closure [7]. Similarly, tobacco plants subjected to drought exhibited a severe reduction in net photosynthetic rate while application of heat shock resulted in stomatal opening and an increase in transpiration and photorespiration but without alteration of net photosynthetic rate. Overall, the combination of drought and heat suppressed transpiration and photosynthesis but induced an increase in photorespiration rate [8]. Our data are in agreement with these reports, since combination of drought and heat affected plants in a different manner, suggesting that plants under combined stresses that could not cool their leaves by increasing transpiration as in heat stress conditions, faced a more damaging situation.

The effect on photochemistry is often linked to electron leakage and induction of oxidative damage [47]. The degree of lipid peroxidation (determined by monitoring changes in the levels of MDA) can be related to the balance between ROS production and antioxidant activity within a given cell or tissue. WS+HS combination increased MDA levels in leaves of both citrus genotypes although a higher accumulation was observed in Cleopatra respect to Carrizo seedlings (Table 1). The higher levels of MDA observed in Cleopatra leaves under the combination of WS and HS indicate a stronger incidence of oxidative damage associated to a higher ROS production and also to a less efficient ROS detoxification system, as previously shown [48]. Interestingly, these results also correlate to net photosynthetic rate and C_i/C_a ratio (Figure 3a-c) since during WS+HS, Cleopatra displayed a more pronounced reduction in net CO_2 assimilation than Carrizo. It has been shown that ROS have an influence on ABA biosynthesis and signaling that alters Ca^{2+} influx to stomata guard cells and modulates stomatal opening [29, 30]. Hence, this higher ROS production in combination with an active ABA signaling could be behind the stomatal closure observed under WS+HS combination [29]. On the other hand, the lower MDA accumulation observed in Carrizo is compatible with a lower ROS production possibly associated to the more efficient antioxidant system of this genotype [48] and a less sensitive photosynthetic system to abiotic stressors that is able to modulate excess photosynthetic electron input even under adverse environmental conditions [49, 50].

Involvement of ABA and SA to the response to drought, heat and combined stresses

To our knowledge, little is known on hormonal responses of citrus plants to heat or its combination with other stress conditions. In this work, the hormonal profile revealed significant changes in ABA and SA, hormones that have been involved in abiotic stress [12, 51] and plant thermotolerance [19–22, 52], respectively.

The role of SA in plant–pathogen interactions has been extensively investigated, being involved in systemic acquired resistance (SAR), a stronger defense response mediated by the PR proteins [17, 23, 53]. In addition to defense responses, SA plays an important role in the response to abiotic stresses [11] and especially in the tolerance to high temperatures [19–22, 52]. Moreover, previous studies indicate that SA-signaling pathways involved in SAR overlap with those promoting basal thermotolerance since mutations known to affect SA-signaling in pathogen defenses also affect heat tolerance [21]. In the present study, SA levels increased in response to stresses applied individually and more prominently in both citrus genotypes subjected to WS+HS combination showing an additive output. This major SA accumulation observed during WS+HS was accompanied by a significant up-regulation of *CsICS*, whereas the involvement of the PAL pathway in the active SA biosynthesis could be considered marginal. This data would reinforce the idea that stress combination has a higher impact to physiology than individual stresses applied alone. Additionally, the relative expression of *CsPR2*, a gene induced by SA in citrus plants [37], remarkably increased in response to heat treatments (HS and WS+HS) mainly in Cleopatra leaves. This confirmed the stronger response of Cleopatra probably intended to mitigate the detrimental effects of heat stress. In previous reports, SA has been proposed to protect PSII complex [27, 54] and could be involved in the maintenance of membrane integrity during heat stress [21]. Data presented here are in agreement with these proposals as the most affected genotype, Cleopatra, also showed the strongest SA build-up and *CsPR2* transcript accumulation. This genotype exhibited a stronger accumulation of MDA, therefore, requiring a higher accumulation of protective SA. Nevertheless, the higher accumulation of SA found in Cleopatra leaves under WS+HS conditions was not sufficient to prevent heat-induced membrane damage. In general, data in this study indicate that SA, as well as *CsPR2* transcripts, are predominantly accumulated in response to WS+HS and could be related to the higher damage induced by the two

stress situation acting together. Therefore, stress combination would represent a more damaging situation for plants than disconnected stresses.

The role of ABA on plant responses to water stress is well-known but there is not much information on its involvement in heat stress. Different stress treatments induced similar ABA accumulation patterns in both genotypes. An interesting finding is the fact that while WS induced the typical ABA accumulation in citrus tissues, HS (alone or applied in combination with WS) greatly inhibited this response, suggesting that during different abiotic stress conditions citrus leaves undergo substantially different programs regulating ABA homeostasis. Endogenous ABA levels are regulated through the coordinated action of biosynthesis, catabolism and conjugation yielding ABAGE [15, 55–57]. In our study, WS induced a strong accumulation of ABA in plants of both genotypes, coincident with stomatal closure and accompanied by a concerted up-regulation of *CsNCED1*, *CsCYP707A1*, *CsAOG* and *CsBG18* gene expression leading to significant amounts of PA, DPA and ABAGE. On the contrary, HS did not vary either ABA levels or *CsNCED1* expression in leaves of both genotypes. However, PA and DPA accumulation along with the up-regulation of *CsCYP707A1* indicated an induction of ABA catabolism under high temperature conditions. Apart from catabolism, conjugation to hexoses was also activated since a significant accumulation of *CsAOG* transcripts was observed. Despite this up-regulation, an increment of ABAGE content during HS could not be observed. This could be associated to the fact that HS induced the ABA catabolic pathway without a concomitant induction of *CsNCED1*. Therefore, although *CsAOG* gene expression was up-regulated, there was not enough ABA to be conjugated.

Strikingly, under combination of drought and heat, ABA levels moderately increased in parallel with a moderate *CsNCED1* up-regulation, much lower than that observed under WS. In this sense, HS could prevent the huge accumulation of ABA through the partial down-regulation of *CsNCED1* and up-regulation of genes encoding for ABA 8'-hydroxylase and ABA glycosyl transferase. Additionally, analytical and gene expression data indicated that ABA catabolism actively participates in the reduction of hormone levels under HS and WS+HS conditions whereas conjugation to hexoses had a marginal role in the conditions assayed, despite the strong *CsAOG* transcript accumulation observed. Nevertheless, the role of hexose conjugation as an additional mechanism to precisely modulate active ABA levels under particular stress conditions

cannot be ruled out. Despite the strong reduction in ABA levels during WS+HS respect to WS conditions, data indicate that either the remaining amount of hormone found was enough to close stomata and reduce photosynthetic rate or, as suggested previously [29], an interaction between ROS and ABA signaling cooperatively contributed to close stomata. In this sense, it has been previously discussed that treatments with H_2O_2 induce a reduction in stomatal aperture when ABA signaling is repressed [58]. In this sense, the increased incidence of oxidative damage observed in WS+HS conditions is an indication of an excess H_2O_2 production that probably participated in the reduction of gas exchange parameters as observed under WS. Moreover, *CsRAB18* expression confirmed ABA signaling, which correlated with ABA content under all stress conditions. Overall, our data indicate that while WS increases ABA contents via *de novo* biosynthesis, HS would activate pathways involved in removing active hormone pools mainly through catabolism. At the physiological level, the observed inhibition of WS-induced ABA accumulation associated to HS could be a specific response aimed to down-regulate ABA signaling and increase stomatal opening and transpiration, allowing an adequate refrigeration of leaves. In line with these data, a recent report in *Arabidopsis thaliana* revealed a decrease of leaf ABA in response to HS correlated with a down-regulation of *AtNCED3* expression and an up-regulation *AtCYP707A3* [59], supporting the idea that ABA reduction in photosynthetic organs is a necessary response to induce stomatal opening and subsequently to enhance transpiration as a direct response to the heat stimulus.

Our results indicate that Cleopatra is more sensitive to heat stress than Carrizo, especially when combined with drought. This higher tolerance of Carrizo could be associated to an improved leaf cooling via enhanced transpiration along with the ability to modulate photosynthetic electron flow, resulting in a lower incidence of oxidative damage. In both citrus genotypes, stress combination represents a more damaging situation than the individual stresses, inducing a higher damage to PSII leading to the accumulation of MDA. In addition, SA accumulation and signaling paralleled stress sensitivity and correlated with a higher requirement of SA-mediated protective responses, being both greater in Cleopatra mandarin than in Carrizo citrange. ABA levels increased in response to WS but not during HS and, interestingly, WS+HS resulted in lower hormone levels than drought applied alone. Furthermore, the transcriptional regulation of ABA metabolism during drought and heat applied alone

and in combination pointed to a unique mechanism of response for each stress condition as reported in previous studies [1, 60]. Although it is widely accepted that NCED has a central role in the regulation of ABA levels under stress conditions, as shown in different plant species [57, 61, 62], the up-regulation of *CsCYP707A1*, *CsAOG* and *CsBG18* could act cooperatively to modulate active ABA pools during WS when NCED activity is elevated. Therefore, these results indicate that the activation of ABA degradation and conjugation could contribute to fine-tune hormone levels during WS and HS.

Conclusions

At present, information on the combined effect of heat and drought stress in citrus is rather limited. In this work, we have demonstrated the different ability of two citrus genotypes, Carrizo citrange and Cleopatra mandarin, to tolerate drought and heat applied alone or in combination. In this sense, physiological responses in terms of gas exchange parameters and chlorophyll fluorescence, along with MDA accumulation as an estimation of oxidative damage, evidenced the susceptibility of Cleopatra mandarin to combined heat and drought conditions. The different pattern of ABA accumulation (along with specific transcriptional regulation of genes involved in ABA metabolism) in response to each individual stress situation and their combination pointed to a unique mechanism of response to each stress condition. Additionally, SA levels and associated signaling positively correlated with stress sensitivity, being more pronounced in Cleopatra mandarin. Tolerance to a combination of different stress factors mimicking field conditions should be the focus of future research programs aimed to develop genetically-engineered plants with enhanced tolerance to several environmental conditions. Additionally, study of hormone crosstalk already observed in citrus and other species [63] could be also relevant under combined abiotic stress conditions leading to plant tolerance. The understanding of the underlying mechanisms of response and the existing interactions among abiotic stressors will provide valuable information for crop improvement.

Declarations

List of abbreviations

A: Net photosynthetic rate; ABA: Abscisic acid; ABAGE: ABA-glycosyl ester; AOG: ABA O-glycosyl transferase; C_i/C_a: ratio of substomatal to ambient CO_2 concentration;

CYP707A: ABA 8'-hydroxylase; DPA: Dehydrophaseic acid; E: Transpiration; F_v/F_m: Maximum efficiency of photosystem II; g_s: Stomatal conductance; ICS: Isochorismate synthase; MDA: Malondialdehyde; NCED: 9-neoxanthin *cis*-epoxicarotenoid dioxygenase; PA: Phaseic acid; PAL: Phenylalanine ammonia lyase; PR2: pathogenesis-related gene 2; PSII: Photosystem II; PP2C: Protein phosphatase 2C; PYR/PYL/RCAR: Pyrabactin resistance1/PYR-like/regulatory components of ABA receptor; Φ_{PSII}: Quantum efficiency of PSII photochemistry; RAB18: Responsive to ABA-related gene 18; ROS: Reactive oxygen species; RWC: Relative water content; SA: Salicylic acid; SnRK: Sucrose non-fermenting 1-related protein kinase.

Consent to publish

Not applicable

Competing interests

Authors declare that they have no competing interests

Authors' contributions

SIZ, AGC and VA planned and designed the experiments. RMV and VM provided laboratory infrastructure and greenhouse space and aided in the interpretation of results. SIZ performed greenhouse experiments, harvesting of plant material and analyzed samples. SIZ and VA wrote the first draft of the manuscript and prepared figures. SIZ, AGC and VA revised subsequent versions of the manuscript and prepared the final version. All authors have read and approved the final version of the manuscript.

Availability of data and materials

Raw data could be obtained by request to the corresponding author. The datasets supporting the conclusions of this article are included within the article and its additional files. Gene sequences are available in the Phytozome database (see Additional Table 1 and https://phytozome.jgi.doe.gov/pz/portal.html).

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Additional files

a

PLASTID

zeaxanthin

9-cis-violaxanthin —NCED1→ xanthoxin

xanthoxin

BIOSYNTHESIS

ABA

CYP707A1

PA

DPA

signaling

AOG
BG18

ABAGE

CATABOLISM

CONJUGATION

RAB18

b

chorismate

phenylalanine   isochorismate

PAL   ICS

BIOSYNTHESIS

SA

signaling

PR2
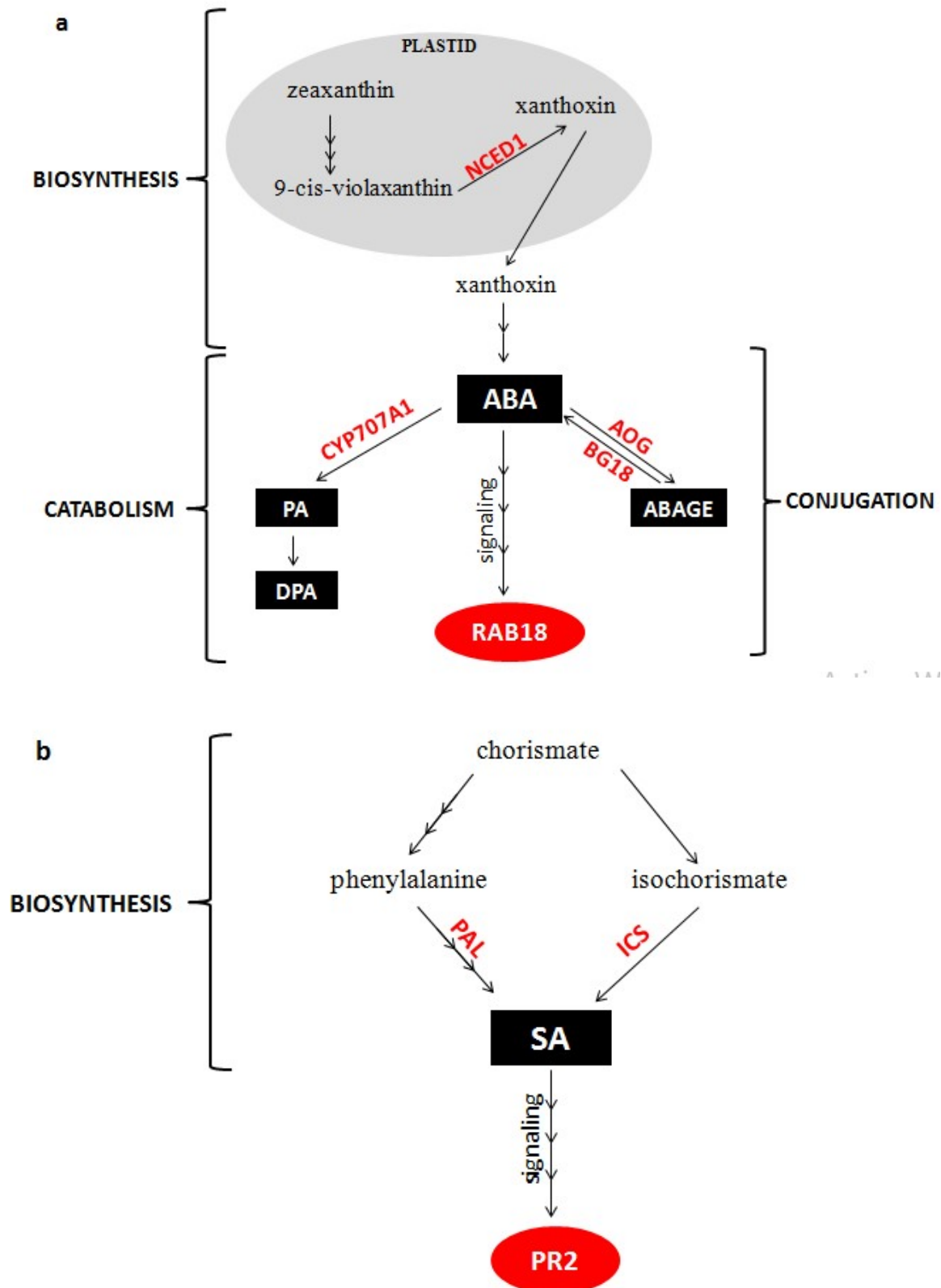
Additional file 1. Schematic diagram showing the biosynthetic and signaling pathways of ABA (a) and SA (b). Names in red are the genes analyzed in this work and different metabolites studied are presented in black squares.
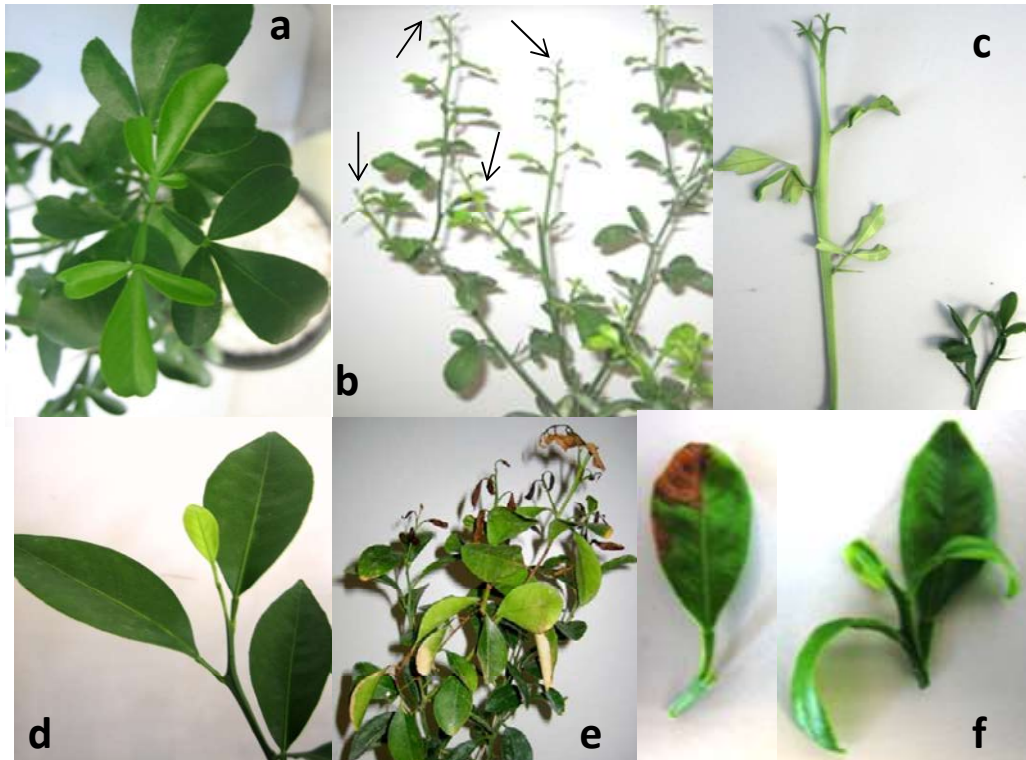
Additional file 2. Effects of heat stress treatment on citrus sprouts. Carrizo control sprouts (25 ºC) (a), Carrizo plants subjected to heat stress for 10 days (b), sprouts on control (right) and heat-stressed (left) Carrizo plants (c), Cleopatra control sprouts (d), Cleopatra plants subjected to heat stress for 10 days (e), sprouts on control (right) and heat-stressed (left) Cleopatra plants (f), integral sprouts (%) of Carrizo and Cleopatra seedlings subjected to heat stress for 10 days (g). For each genotype, asterisks denote statistical significance at $p \leq 0.05$ respect to initial values. Values are mean ± SD.

Additional Table 1. Designed primers for gene expression analyses by quantitative RT-PCR.

Citrus gene	Locus	Forward / Reverse	Sequence (5'→3')	Amplicon size (bp)
CsNCED1	orange1.1g007379m	F	AATGCTTGGGAAGAGCCTGAG	147
		R	AGTGGACTCGCCGGTCTTTAG	
CsCYP707A1	orange1.1g038621m	F	TCAATGTTGCACTGCTCTCC	246
		R	CTTTGGCACCCATGAAAGAT	
CsAOG	orange1.1g022744m	F	CGGGTTCACTGTTTGGTCTT	176
		R	GCCTCGAGAGAAATGGATGT	
CsBG18	orange1.1g010588m	F	CAAGGCAAAACAGGGTGGAT	212
		R	CAGCCTCAGAGCTGGTGAAT	
CsRAB18	orange1.1g028210m	F	CTGAAGCTGAACGGGAGATT	195
		R	TTGTGGTGGTAGAGGTGGTG	
CsICS	orange1.1g044177m.g	F	GTTGAATGTGGTGCGTCATC	150
		R	CCATGTGGACATTGGTGTGT	
CsPAL	orange1.1g004955m.g	F	GAAACGGATGATTGACGAGT	113
		R	GCTCCACCTTGACTCCAGAG	
CsACT	orange1.1g037845m	F	CCCTTCCTCATGCCATTCTTC	105
		R	CGGCTGTGGTGGTAAACATG	
CsTUB	orange1.1g013335m	F	GGGGCAAAATGAGCACTAAA	187
		R	CGCCTGAACATCTCCTGAAT	

Results

Chapter 2

Increased activation of metabolism in citrus plants is related to sensitivity to combined drought and high temperatures.

Zandalinas *et al*. (2016) Physiologia Plantarum. Submitted.

Abstract

Simultaneous drought and heat are one the most frequent abiotic stress combinations constraining global crop productivity. Metabolism reconfiguration is often behind the adaptation of plants to adverse environmental conditions. Carrizo citrange and Cleopatra mandarin, two citrus genotypes with contrasting ability to tolerate combined heat and drought conditions, showed different metabolite patterns: a strong induction of glycolysis and TCA and glyoxylate/dicarboxylate cycles in the stress-sensitive Cleopatra suggested a higher cellular energy demand and a requirement for NADPH and ATP recycling in this genotype, both produced during photosynthetic light reactions. The phenylpropanoid pathway was also activated in Cleopatra in response to stress in order to accumulate defensive metabolite scopolin whereas tolerant Carrizo induced the accumulation of sinapic acid and sinapoyl aldehyde, direct precursors of lignins. Finally, Cleopatra showed an accumulation of flavonols and glycosylated and polymethoxylated flavones. The activation of flavonoid biosynthesis in Cleopatra could be aimed to mitigate the higher oxidative damage observed in this genotype. In addition, limonoids were, in general, more deeply altered in Cleopatra than in Carrizo in response to stress imposition. To conclude, all metabolite changes observed in Cleopatra appear to be a response oriented to mitigate the damaging effects of stress: induction of photoprotective and antioxidant compounds, and metabolic pathways involved in ATP and NADPH recycling. Conversely, the higher ability of Carrizo to maintain the photosynthetic activity and to cope with oxidative stress prevented further modifications of metabolism.

Introduction

The acclimation and adaptation mechanisms of plants to challenging environmental conditions are based on the activation of specific physiological and molecular responses. These responses, in turn, lead to changes in plant metabolism to minimize stress-induced damage. Studies focusing on abiotic factors in isolation do not represent the particular response of plants to a combination of different stresses likely affecting to crops growing in the field (Boeck et al. 2015; Hu et al. 2015; Liu et al. 2015; Mittler and Blumwald 2010; Suzuki et al. 2014; Zhang et al. 2015). Specifically, combination of drought and heat stresses are considered one of the most recurrent conditions that take place in natural environments, affecting plant growth and productivity (Boeck et al. 2015; Craufurd et al. 2008; Jiang and Huang 2001; Mittler 2006; Savin and Nicolas 1996; Zandalinas et al. 2016). In order to face combinations of drought and heat stresses, plants specifically alter gene expression in a very different way than changes occurring in plants grown under isolated conditions of drought or heat (Mittler 2006; Rizhsky et al. 2002, 2004). These alteration in gene expression leads to specific regulation of metabolome, depending on the stress and the plant species (Rizhsky et al. 2004; Sun et al. 2016).

Plant metabolome comprises a huge diversity of compounds, including carbohydrates, acids, amino acids, phenols, polyols, polyamines, lipids and others, with many different biological functions. This wide variety of compounds makes difficult the selection of a single exhaustive analytical technology to attain metabolome analysis. Most popular analytical platforms are based on mass spectrometry (MS) coupled to a separation technique such as ultra-performance liquid chromatography (UPLC), gas chromatography (GC) or capillary electrophoresis. However, GC-MS has been established as a gold-standard procedure for the analysis of polar primary metabolites, providing highly efficient metabolite separation, detection and quantification of metabolites with a high reproducibility (Jorge et al. 2015). Using GC-MS, many compounds with different chemical composition including organic acids, sugars, amino acids, sugar alcohols, aromatic amines, tricarboxylic acid (TCA) cycle intermediates and fatty acids can be profiled and identified by matching spectra with available spectral libraries (Shulaev et al. 2008). These compounds, known as primary metabolites, are essential to plant cellular life, among which carbohydrates as direct products of photosynthetic performance and different effectors of osmotic readjustment (e.g.

proline) are found. Different studies comparing primary metabolites of plants subjected to abiotic stresses such as drought, salinity, excess light or low and high temperatures, have identified metabolites involved in plant acclimation to abiotic stress but also compounds that respond to each stress condition (Caldana et al. 2011; Cramer et al. 2007; Kaplan et al. 2004; Maruyama et al. 2009). For instance, energy metabolism, particularly TCA cycle, gluconeogenesis and photorespiration are activated under osmotic stress, therefore leading to increased glucose, malate and proline levels to adjust osmotically and to cope with ROS production and photoinhibition (Cramer et al. 2007). In addition, monosaccharides, disaccharides, trisaccharides and sugar alcohols accumulate in *Arabidopsis* under cold and dehydration conditions. Interestingly, a comparative metabolomic study in *Arabidopsis* plants determined that the great majority of primary metabolites responding to heat shock overlapped with those produced in response to cold stress, suggesting that a metabolic network of primary metabolites such as compatible solutes, monosaccharides, galactinol and raffinose, could have an important role in the tolerance to temperature stress (Kaplan et al. 2004). Moreover, although phenylpropanoids and their derivatives are sometimes classified as secondary metabolites, these compounds constitute an important source of plant metabolites required for plant survival and establish a starting point for the production of many other important compounds, such as flavonoids, coumarins and lignans. Therefore, phenylpropanoid metabolism represents a link between primary and secondary metabolism (Fraser and Chapple 2011). Phenylpropanoids are involved in the production of the hydroxycinnamyl alcohols, also known as monolignols: building blocks of lignin which confer structural support, vascular integrity and pathogen resistance to plants (Boerjan et al. 2003).

Apart from GC-MS, one of the most useful metabolite profiling techniques is UPLC coupled to hybrid quadrupole time-of-flight mass spectrometers (QTOF) since UPLC is the most suitable technique for analyzing biomolecules. In addition, the accurate mass measurement, true isotopic pattern recognition and high sensitivity of QTOF instruments are appropriate for calculations of elemental composition of mass signals. By using reversed phase LC-MS, it is possible to analyze semi-polar to non-polar secondary metabolites (Roepenack-Lahaye et al. 2004). These metabolites perform a variety of physiological roles, including antioxidants, ROS scavengers, coenzymes, photoprotection and also as regulatory molecules (Zhao et al. 2005). The variety of

secondary metabolites is specific to plant species and their biosynthesis is highly regulated by the developmental stage, tissue or cell group and stress situations (Ahmed et al. 2014; Arbona et al. 2013; Rizhsky et al. 2004). One of the most important and diverse group of secondary metabolites involved in plant responses to adverse environmental conditions are phenolic compounds. These metabolites include derivatives of phenylpropanoids such as flavonoids, anthocyanins and tannins (Arbona et al. 2013; Dixon and Paiva 1995; Naoumkina et al. 2010). In addition to this, limonoids are naturally occurring compounds derived from isoprenoids found in plant species of the Rutaceae and Meliaceae families whose antioxidant activity has been demonstrated (Perez et al. 2009; Yu et al. 2005).

Our previous work (Zandalinas et al. 2016) focused on the particular physiological and hormonal responses of citrus genotypes Carrizo citrange and Cleopatra mandarin to the combined action of high temperatures and water deficit. In that work, Carrizo was found to be more tolerant to high temperatures applied alone or in combination with water deprivation. Higher transpiration rate along with reduced oxidative damage were identified as the key traits behind increased tolerance. However, metabolic changes of these citrus plants could be also involved in this contrasting ability to tolerate different abiotic stresses applied individually or in combination.

Therefore, the aim of this study was to analyze the impact of drought and heat, two major abiotic stresses, and their combination, in the primary and secondary metabolism of Carrizo citrange and Cleopatra mandarin and to correlate the differential metabolite accumulation with the contrasting tolerance to adverse situations.

Results

Analysis of polar metabolites

Non-targeted GC-MS analysis was performed to determine different patterns of polar metabolite accumulation in leaves of citrus plants subjected to WS, HS and WS+HS. Results revealed different metabolite profiles in both citrus genotypes under individual and combined stress conditions **(Figures 1 and 2, and Supplementary Figures S1 and S2)**. In Carrizo, stress was the main source of variability (21.4%) allowing the differentiation of a "control" cluster of samples from the rest of treated samples. Within this "stressed" cluster, all three treatments (WS, HS and WS+HS) could be easily

differentiated **(Figure 1A)**. Conversely, the main source of variability (17.1%) of polar metabolites in Cleopatra was the imposition of combined stress conditions (WS+HS) with a minor contribution of WS (11.5%). Interestingly, control samples could not be clearly differentiated from HS samples **(Figure 1B)**. Analysis of variance revealed a total of 94 polar metabolites differentially altered (56 up-regulated and 38 down-regulated, out of 1143 detected) in Carrizo plants, of which 14 were up-regulated only during WS **(Figure 2A)**, including the aminoacids tryptophan, glutamate and acetylated aspartate and long chain hydroxylated fatty acids **(Supplementary Figure S1)**. Only 8 compounds were found specifically up-regulated in Carrizo during WS+HS **(Figure 2A)** among which dimethoxylated cinnamate were found **(Supplementary Figure S1)**. Heat stress alone induced the accumulation of 12 compounds including ascorbate, glycerol 3-P and panthenol **(Figure 2A and Supplementary S1)**. Moreover, concentration of several metabolites increased in response to more than one stress condition. For instance, aminoacids such as glycine and tyrosine, along with mannitol and coumarine were up-regulated by WS and WS+HS; both HS and WS induced the accumulation of 3-oxoglutarate and 3-dehydroshikimate whereas HS and stress combination slightly induced the increased in levels of four compounds including succinate and inositol **(Supplementary Figure S1)**. Stress imposition down-regulated a total of 20 metabolites comprising different metabolic pathways **(Figure 2A)**: the auxin conjugate indolyl-3-acetyl aspartate, ornithine, cinnamoyl aldehyde or malate **(Supplementary Figure S1)**. The impact of individual stresses depressing metabolite levels was more restricted **(Figure 2A)**.

In Cleopatra, stress imposition significantly altered a total of 87 polar metabolites out of 1113 detected in GC-MS analyses, of which 77 were up-regulated and 10 down-regulated **(Figure 2B)**. Stress combination induced the accumulation of 49 metabolites including the carbohydrates isomaltose, fucose, sucrose, raffinose, lactose, fructose and glucose disaccharides and trehalose; aminoacids such as tryptophan, leucine and isoleucine, homoglutamine and acetylated phenylalanine; some dipeptides and the IAA-Asp conjugate; also the polyamines agmatine, putrescine and spermidine were significantly up-regulated. The glycolytic end-product pyruvate as well as the TCA intermediate succinate were also up-regulated upon imposition of stress combination **(Supplementary Figure S2)**. HS and WS applied alone induced the accumulation of only 8 and 1 polar metabolites in Cleopatra, respectively. Both HS and WS+HS caused

the increase in levels of 15 metabolites including the aminoacid tyrosine, the carbohydrate sorbose and 3-dehydro shikimate, an intermediate in phenylpropanoid biosynthesis **(Figure 2B and Supplementary Figure S2)**. As mentioned above, stress imposition did not have such a strong impact on down-regulation of polar metabolites in Cleopatra compared to Carrizo (4 metabolites were down-regulated by stress imposition, 5 compounds shared by HS and WS and only one was significantly down-regulated by HS applied alone) **(Figure 2B)**.
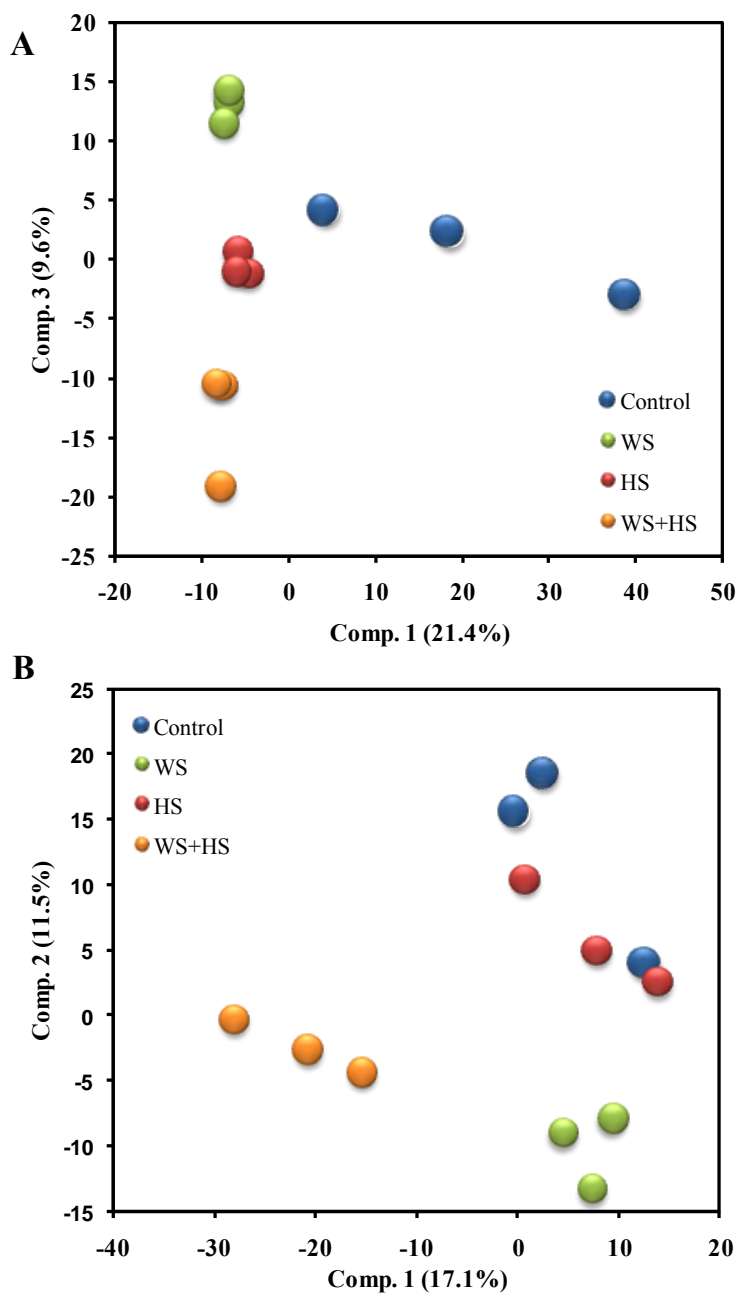
Figure 1. Partial Least Squares-Discriminant Analysis (PLS-DA) of polar metabolite profiles in Carrizo (A) and Cleopatra (B).

A

B
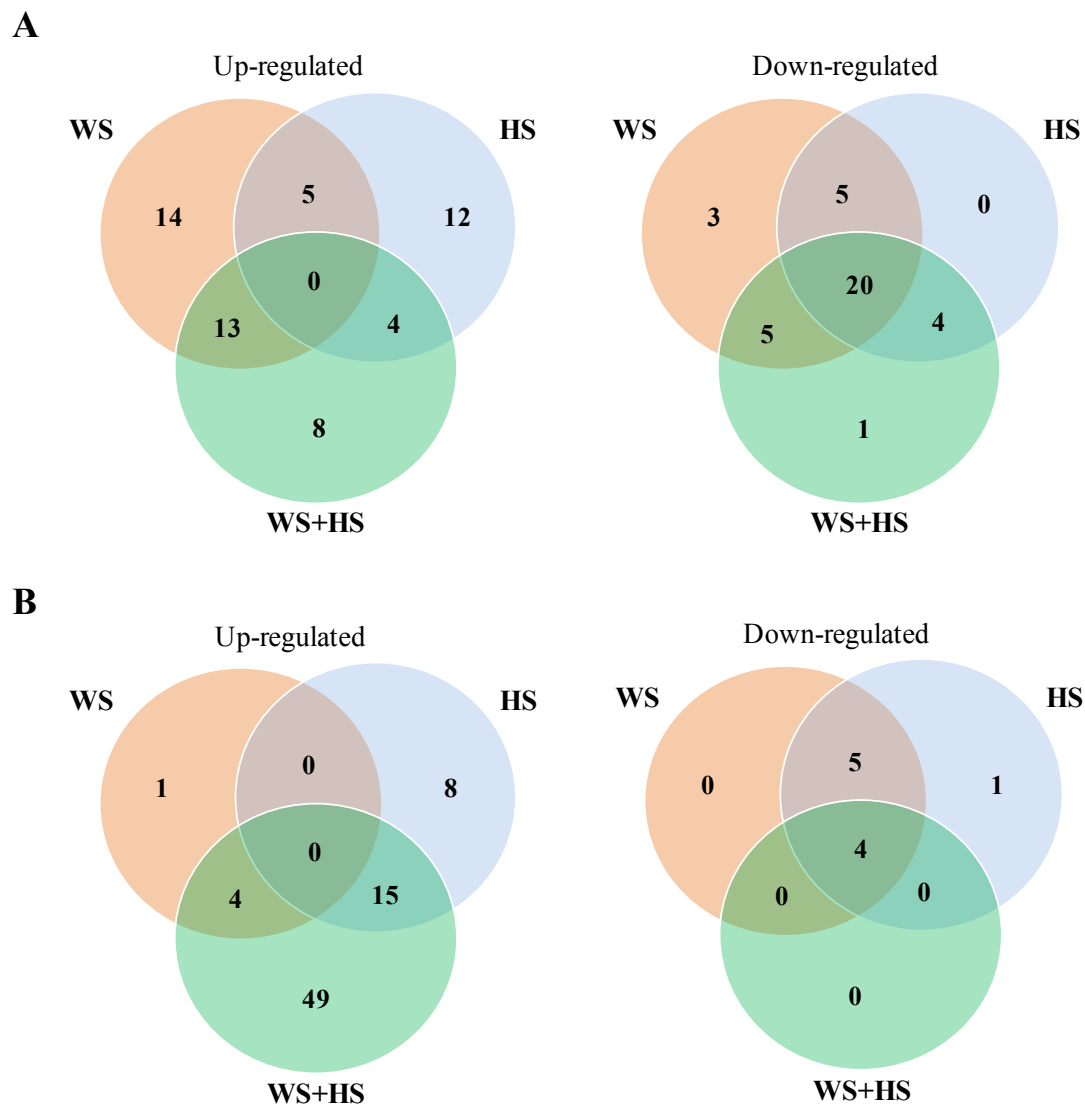
Figure 2. Venn diagrams depicting overlapping polar metabolites significantly altered during WS, HS and WS+HS in Carrizo (A) and Cleopatra (B).

Pathway analysis of polar metabolites: glycolysis-TCA cycle

To investigate differences between genotypes in the metabolic fluxes throughout different pathways, metabolite levels were represented within their respective pathways. Carrizo and Cleopatra displayed different metabolite flows through glycolysis and TCA cycle depending on stress type. As a general trend, stress imposition induced fructose accumulation, although WS had a less pronounced effect in Cleopatra on this metabolite **(Figure 3)**. As a clear difference between both citrus genotypes, abiotic stress induced glucose-6-P accumulation in Cleopatra but not in Carrizo, where it only showed a significant accumulation in response to WS but a depletion in response to HS and

WS+HS. However, fructose-6-P levels were dramatically depleted in Cleopatra plants subjected to WS or stress combination, in clear contrast to Carrizo response **(Figure 3)**.
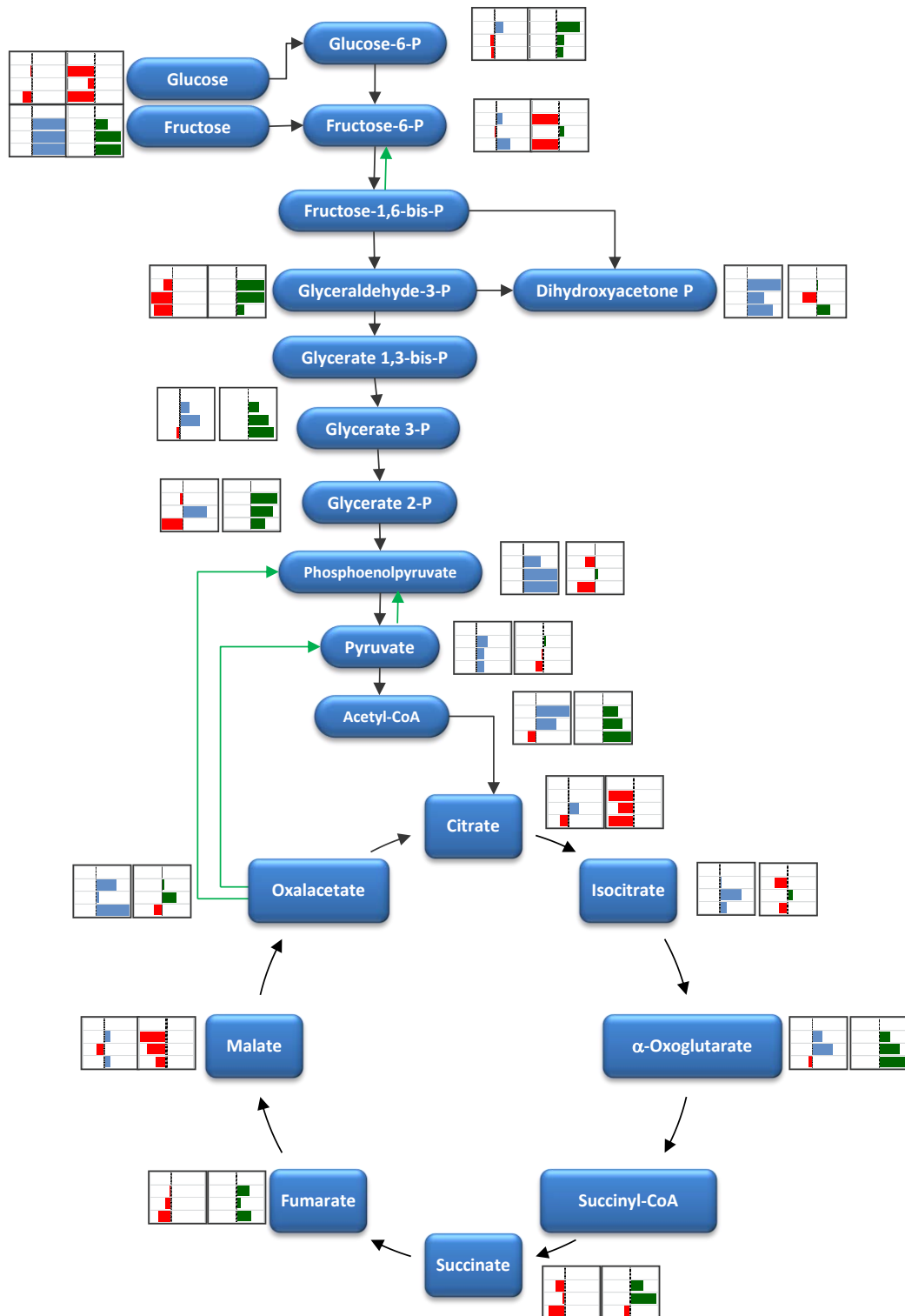
Figure 3. Pathway analysis of metabolites involved in glycolysis and TCA cycle. Relative levels (expressed as log₂[stress/control], control values on first row of graphs were always 0, followed by WS, HS and WS+HS, in descending order) are given besides each detected metabolite as a bar graph: Carrizo (left graph, positive values in blue, negative in red) and Cleopatra (right graph, positive values in green, negative in red).

Glyoxylate/dicarboxylate pathway

In general, stress induced the accumulation of different intermediates of the chloroplastic step of the pathway in Cleopatra. The accumulation of glycolate from ribulose-1,5-bis-P was impaired in both genotypes under WS+HS conditions. Nevertheless, glycolate was significantly higher in WS and HS samples in Carrizo and Cleopatra, respectively, showing a clear correlation with glycine levels. Detected Calvin-cycle intermediates were primarily accumulated in Cleopatra under all stress conditions with specific differences: glycerate-3-P levels were higher in this genotype under WS+HS combination whereas the precursors glyceraldehydes-3-P showed strongly reduced levels in these conditions. These metabolites showed a different trend in Carrizo, since glyceraldehydes-3-P showed strongly reduced levels under all stress conditions, specially under those involving HS. On the contrary, glycerate-3-P accumulated under WS and to a higher extent in HS, but stress combination had the opposite effect, inducing a strong reduction in its levels **(Figure 4)**. On the contrary, ribulose-5-P showed strikingly lower levels in Cleopatra under all stress conditions and in Carrizo under WS and HS, showing a slightly high accumulation under stress combination. Sucrose synthesis, the output of the pathway, was significantly increased in Carrizo, showing a strong accumulation under HS conditions, followed by stress combination and WS; whereas in Cleopatra, stress combination had a detrimental effect on sucrose build-up, while a moderate reduction was observed under HS. Only WS slightly induced sucrose accumulation in this genotype **(Figure 4)**.
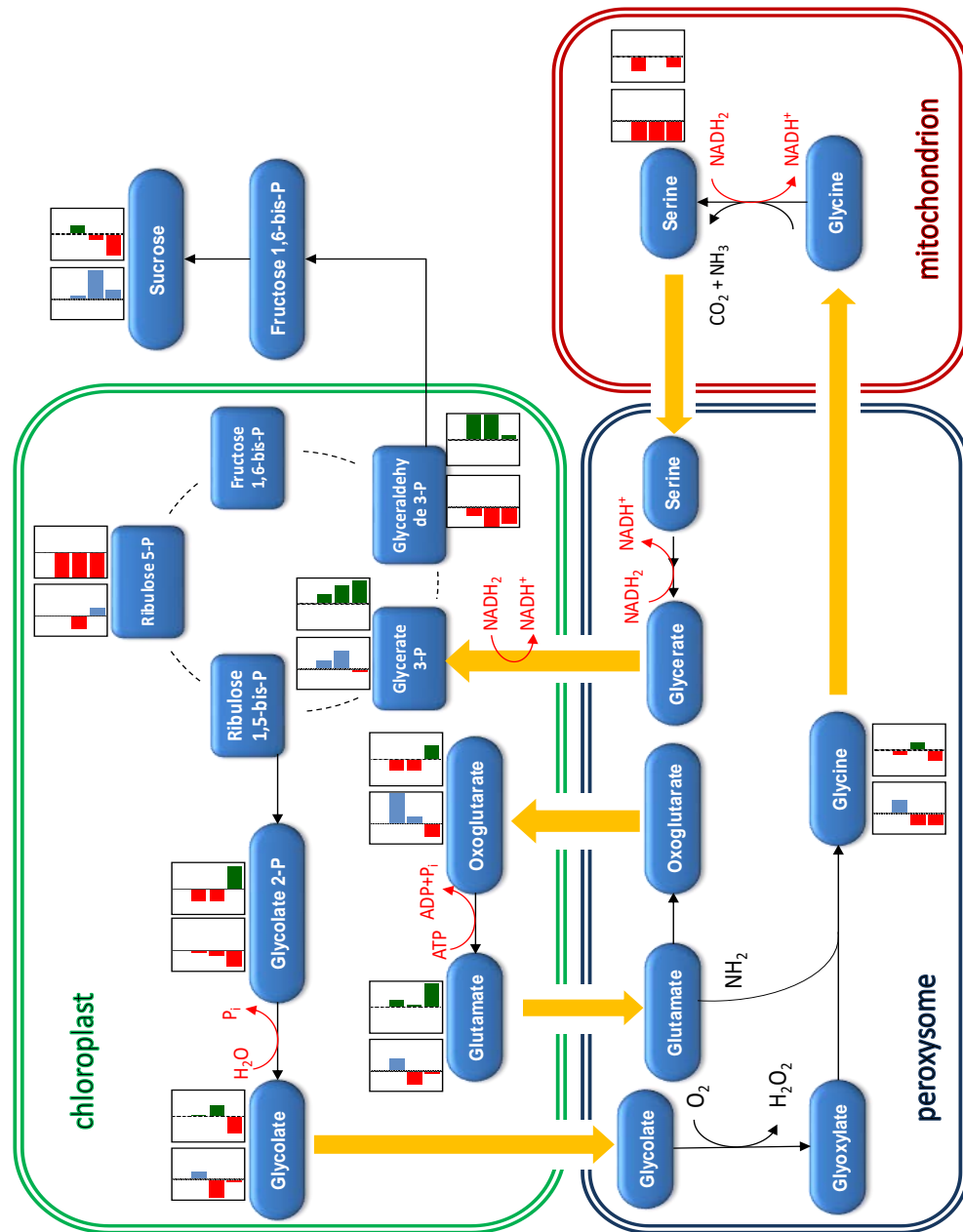
Figure 4. Pathway analysis of metabolites involved in the glyoxylate/dicarboxylate pathway. Relative levels (expressed as log₂[stress/control], control values on first row of graphs were always 0, followed by WS, HS and WS+HS, in descending order) are given besides each detected metabolite as a bar graph: Carrizo (left graph, positive values in red) and Cleopatra (right graph, positive values in green, negative in red).

Phenylpropanoid pathway

Another enriched metabolic pathway in polar metabolite profiles was the phenylpropanoid pathway arising from the aminoacid phenylalanine **(Figure 5)**. The first phenolic acid product of the reaction catalyzed by phenylalanine ammonia lyase enzyme is cinnamic acid whose levels were severely reduced after stress imposition, as well as the product of cinnamate hydroxylation, coumaric acid, in both citrus plants. On

the contrary, caffeic acid levels increased in both genotypes in response to stress following different profiles. In this sense, its levels were higher in Carrizo plants subjected to WS whereas a minor accumulation could be observed upon WS+HS imposition in this genotype. Contrastingly, all stress conditions induced significant amounts of caffeic acid in Cleopatra, although highest levels were observed in plants subjected to stress combination. Ferulic acid levels increased only in Carrizo plants subjected to HS, whereas the rest of treatments reduced its concentration. In addition, scopoletin, derived from hydroxylation of feruloyl CoA (Kai et al. 2008), was up-regulated in response to WS and HS and down-regulated under WS+HS in Carrizo while it was repressed under stress imposition in Cleopatra. However, scopolin, a glucoside of scopoletin, accumulated in response to all stress treatments in both genotypes, especially in Cleopatra plants subjected to WS+HS. Moreover, sinapic acid levels also responded positively to stress imposition in Carrizo, showing enhanced levels in response to WS or HS and, to a lesser extent, to WS+HS. In Cleopatra, however, levels of this metabolite increased significantly only in response to HS, whereas stress combination reduced its levels.
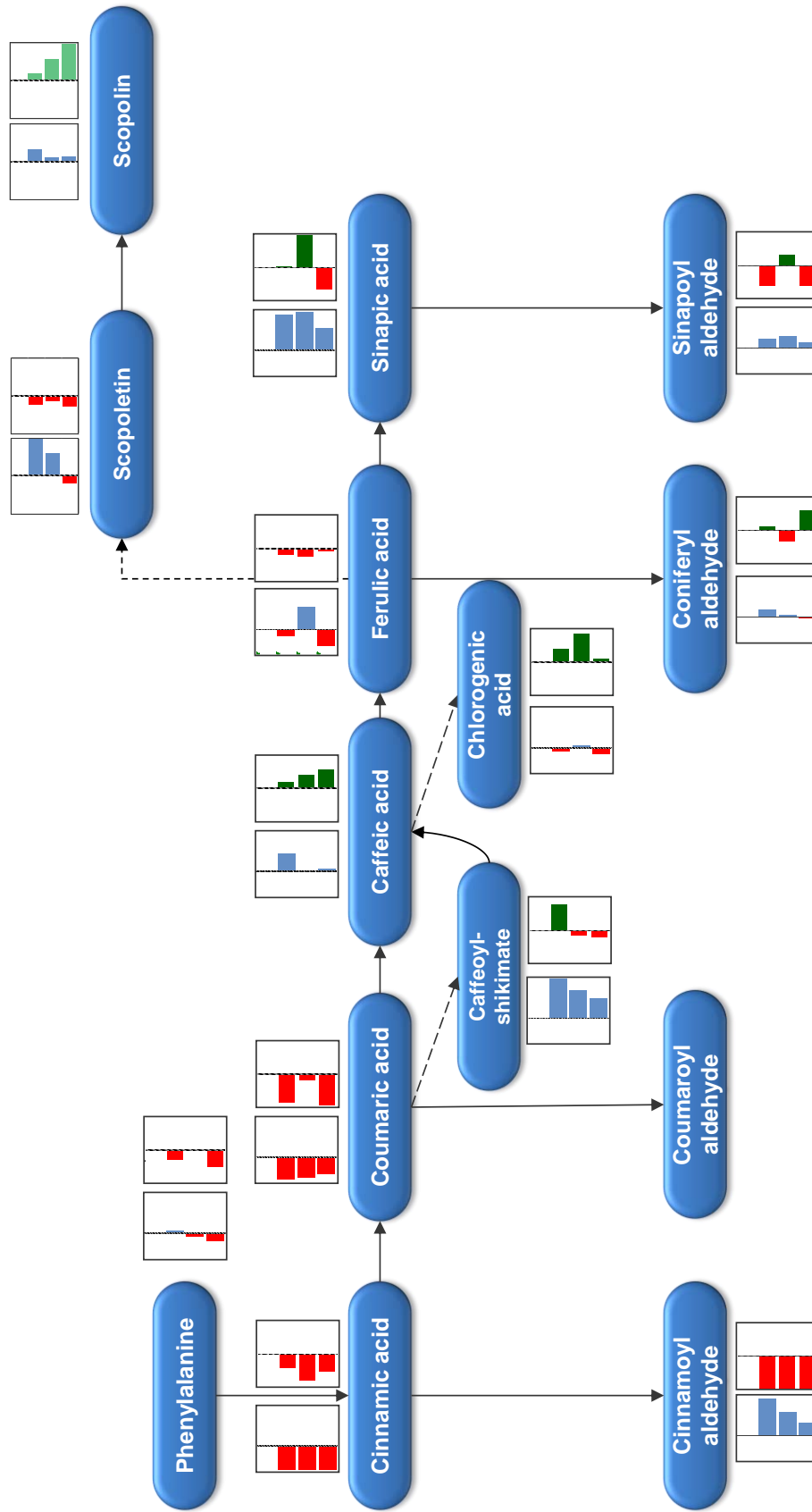
Figure 5. Pathway analysis of metabolites involved in the phenylpropanoid pathway. Relative levels (expressed as log$_2$[stress/control], control values on first row of graphs were always 0, followed by WS, HS and WS+HS, in descending order) are given besides each detected metabolite as a bar graph: Carrizo (left graph, positive values in blue, negative in red) and Cleopatra (right graph, positive values in green, negative in red).

Phenolic acids are usually converted into the respective aldehydes via conjugation with coenzyme A and subsequently reduced to alcohols constituting the building blocks for lignins (Boerjan et al. 2003). Sinapoyl aldehyde exhibited a general induction in response to all stress conditions in Carrizo, being higher upon WS, whereas it was reduced below control levels in Cleopatra. This metabolite, on the contrary, was accumulated in Cleopatra only in plants subjected to HS but to a limited extent. Coniferyl aldehyde (derived from feruloyl-CoA) accumulated in Cleopatra in response to WS and WS+HS and reduced in response to HS. In Carrizo, this metabolite accumulated in response to WS and HS only, and showed no differences respect to controls under WS+HS combination. Unfortunately, coumaroyl aldehyde could not be detected in GC-MS profiles but, on the contrary, significant amounts of caffeoyl shikimate were detected. This metabolite derives from coumaric acid through conjugation with coenzime A, shikimic acid moiety transfer and reduction of the phenolic acid constituent (Humphreys and Chapple 2002). Caffeoyl shikimate acts as a precursor either of caffeoyl CoA, and subsequently caffeoyl aldehyde, or caffeic acid itself. In turn, caffeic acid can also divert into the production of chlorogenic acid or caffeoyl quinic acid (Humphreys and Chapple 2002), that was also detected in polar metabolite profiles. This latter showed significantly increased level in Cleopatra in response to all stress conditions, especially HS. However, Carrizo plants showed a reduction in its levels upon WS and WS+HS and a slight increase in response to HS. Caffeoyl shikimate, on the contrary, showed high levels in Carrizo in response to all stress conditions and in Cleopatra in response to WS, whereas the rest of treatments caused a reduction **(Figure 5)**.

Analysis of secondary metabolites by LC-MS

To determine the accumulation of secondary metabolites in leaves of citrus plants subjected to WS, HS and their combination, we performed a LC-MS analysis of compounds extracted from leaves of both citrus plants subjected to the different stresses. Xcms processing of LC-MS metabolite profiles rendered a total of 7195 and 8453 peaks in Carrizo and Cleopatra, respectively, of which 1001 and 2730 had significant variations in area values in the different treatments, as revealed by ANOVA analysis. Partial Least Squares coupled to Discriminant Analysis (PLS-DA) showed a clear impact of heat stress on secondary metabolite composition in Carrizo (26.3% of explained variability) whereas water stress had a lower contribution (18.8%) **(Figure**

6A). In Cleopatra, however, both heat and water stress had an influence on secondary metabolite composition (21.1%) although the stress combination had a stronger contribution (26.2%) **(Figure 6B)**.
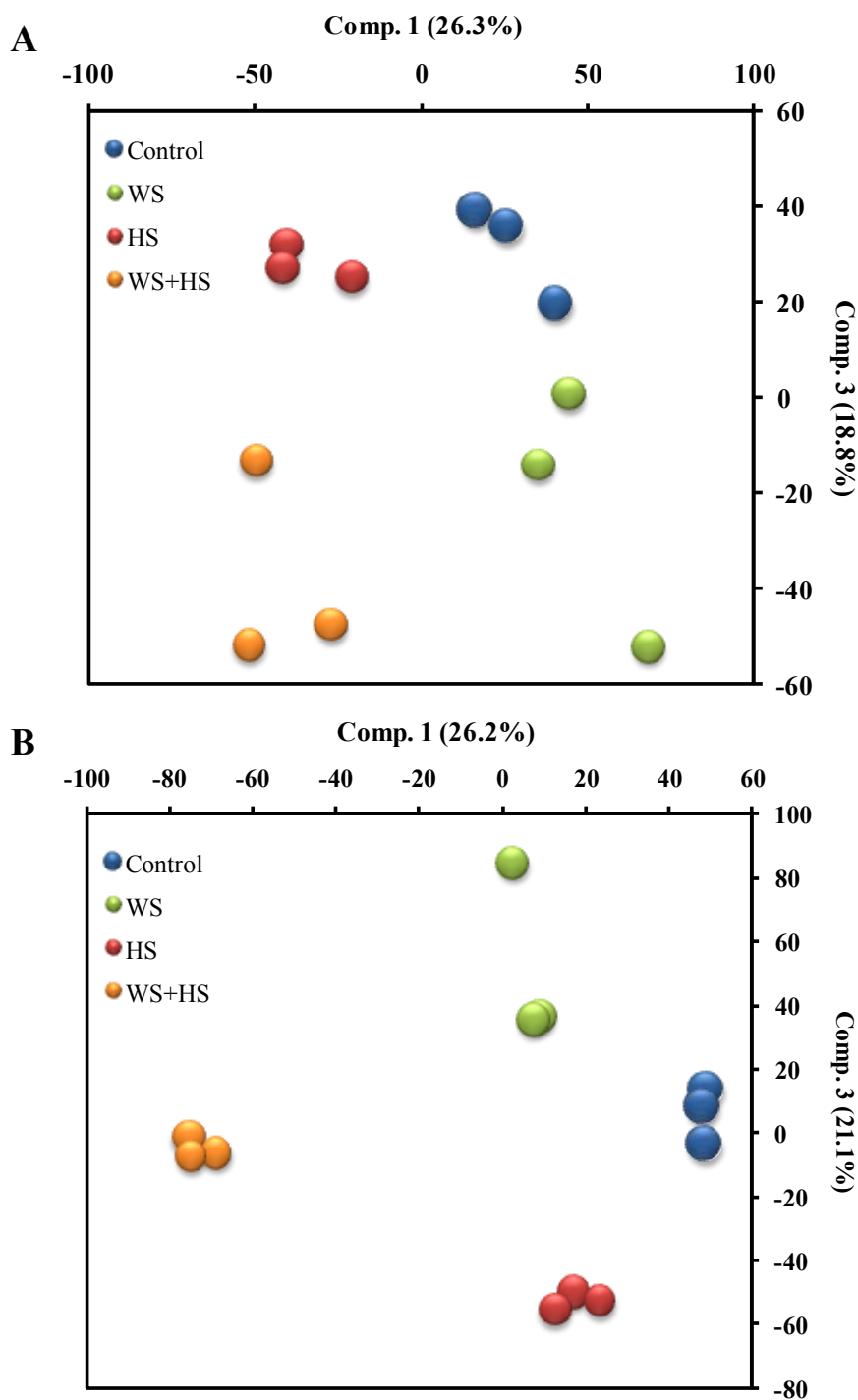
Figure 6. Partial Least Squares-Discriminant Analysis (PLS-DA) of secondary metabolite profiles in Carrizo (A) and Cleopatra (B).

The specific profile for each metabolite as well as the metabolite overlapping among treatments was studied and results are depicted as Venn diagrams in **Figure 7.** Carrizo leaves displayed a clearly response to HS applied individually or in combination with WS. In this sense, 87 and 66 up- and down-regulated compounds, respectively, were significantly altered during HS and WS+HS, likely as a result of the incidence of HS **(Figure 7A)**. Identification of metabolites is shown in **Table 1**.
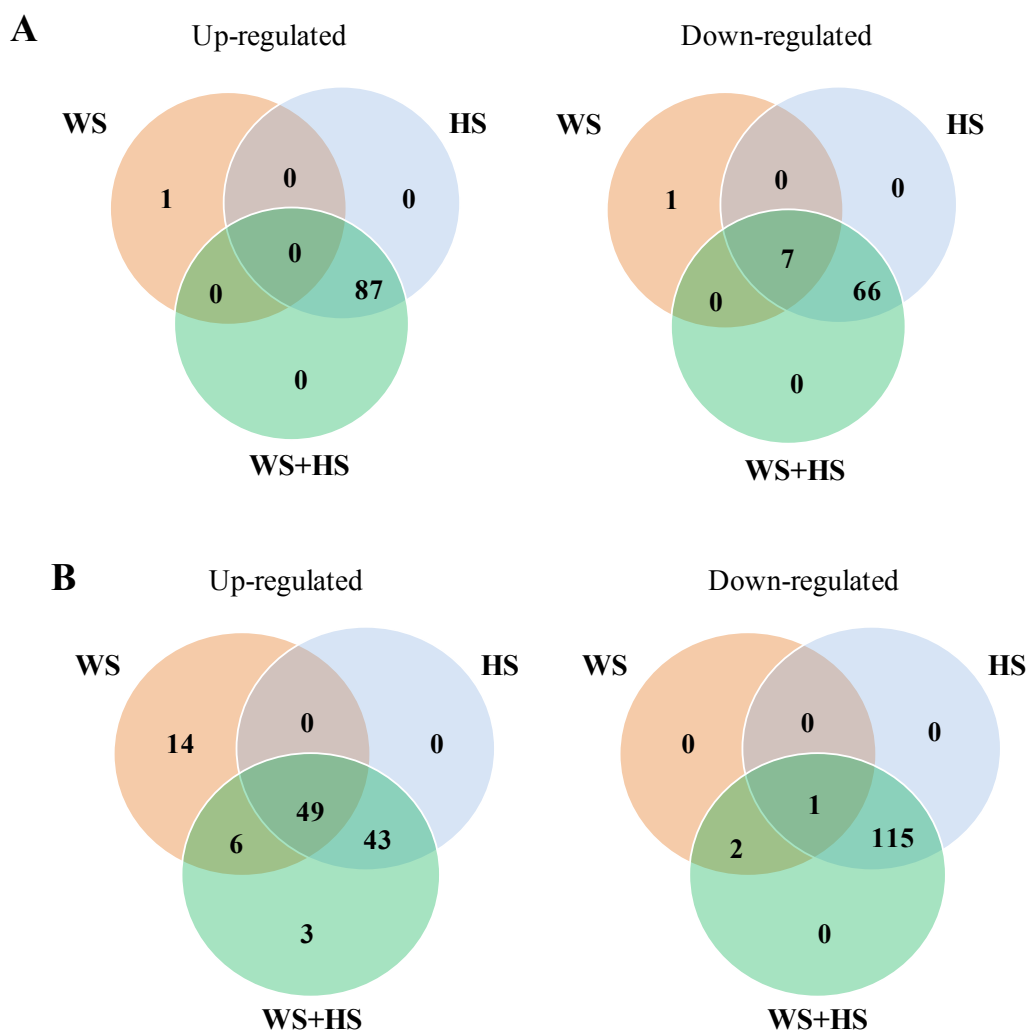
Figure 7. Venn diagrams depicting overlapping secondary metabolites significantly altered during WS, HS and WS+HS in Carrizo (A) and Cleopatra (B).

Among them, we found up-regulated flavonoids such as isorhamnetin hexoside desoxyhexoside, limonoids (nomilin deoxyhexoside) or the phospholipid derivative lysophosphatidyl choline. Furthermore, we also found several flavonoids like kaempferol derivatives or apigenin deoxyhexose hexose, that were down-regulated in

response to HS and WS+HS in Carrizo leaves **(Figure 8)**. On the other hand, as indicated also by PLS-DA **(Figure 6B)**, we observed a significant response of Cleopatra to WS, finding 14 secondary metabolites up-regulated during WS applied individually, including flavonoids (quercetin hexoside deoxyhexoside and hesperetin hexoside deoxyhexoside), limonoids (nomilin deoxyhexoside) or lipid derivatives such as linolenic acid hexoside hexoside **(Figure 8)**. Additionally, 49 compounds were up-regulated in response to all stress treatments **(Figure 7B)**, including lysophophatidyl choline, flavonoids such as kaempferol and quercetin derivates or limonoids as obacunone **(Figure 8)**, and 43 secondary metabolites specifically accumulated in response to HS and WS+HS in Cleopatra leaves. Moreover, 6 secondary metabolites were up-regulated in Cleopatra plants in response to WS and WS+HS and only 3 compounds were found to be significantly accumulated in response to WS+HS. Moreover, a total of 115 metabolites were specifically down-regulated in Cleopatra leaves subjected to HS and WS+HS. Only one compound was down-regulated in response to all stress treatments and 2 metabolites were also down-regulated in Cleopatra plants in response to WS and WS+HS **(Figure 7B)**.
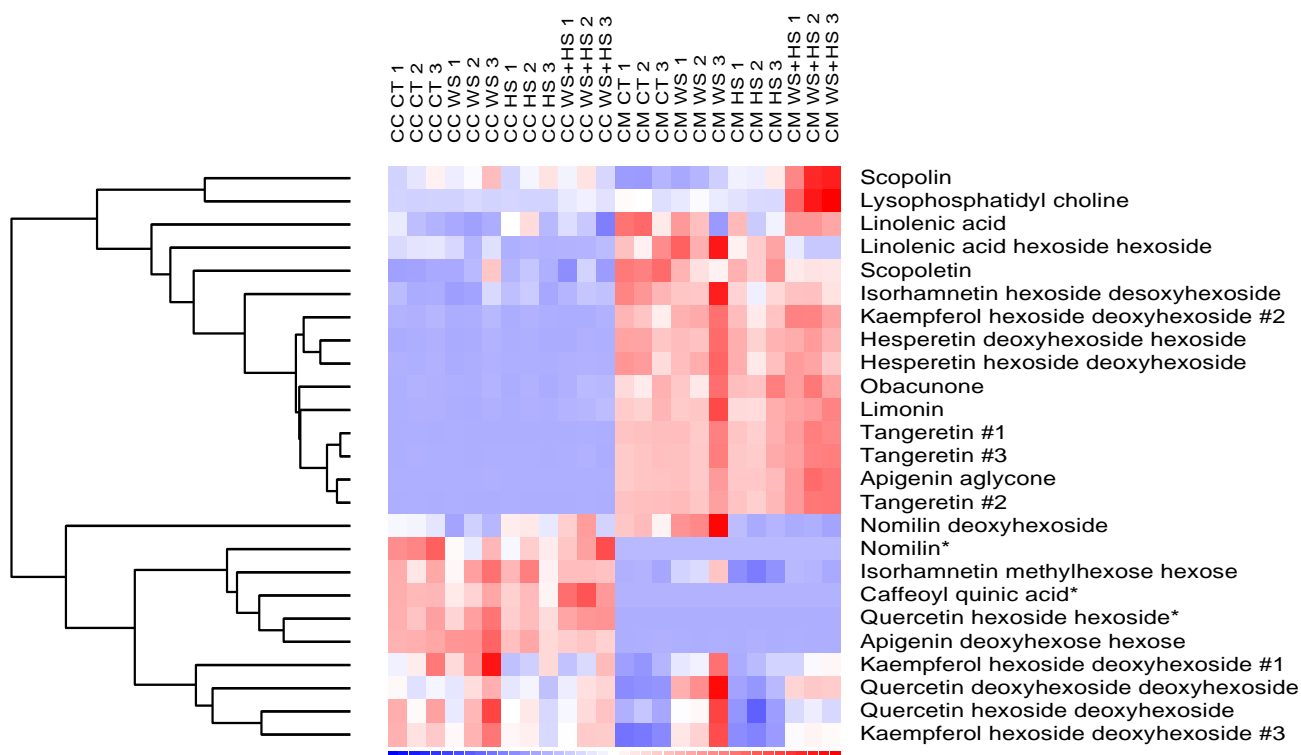
Figure 8. Heatmap displaying identified secondary metabolites significantly altered in leaves of Carrizo (CC) or Cleopatra (CM) plants in response to stress. (*, metabolites not detected in Cleopatra).

Table 1. Identification of compounds analyzed by LC-MS.

Flavonoids	ESI+		ESI-		Rt (min)	Rt (s)
Quercetin hexoside hexoside	627.16	[M+H]+	625.14	[M-H]-	5.50	330
	465.11	[M-hexose]+				
	303.05	[M-2hexose]+				
Quercetin hexoside deoxyhexoside	609.15	[M+H]+	607.14	[M-H]-	6.00	360
	303.06	[M-hexose-deoxyhexose]+				
	449.10	[M-hexose]+				
Quercetin deoxyhexoside deoxyhexoside	595.17	[M+H]+	593.16	[M-H]-	6.34	380.4
	449.12	[M-deoxyhexose]+				
	303.07	[M-deoxyhexose]+				
Hesperetin deoxyhexoside hexoside	303.09	[M-deoxyhexose]+	609.18	[M-H]-	6.60	396
	611.19	[M+H]+	301.08	[M-deoxyhexose-hexose]-		
	449.15	[M-hexose]+				
Hesperetin hexoside deoxyhexoside	611.20	[M+H]+	609.18	[M-H]-	6.77	406.2
	449.15	[M-hexose]+	301.08	[M-hexose-deoxyhexose]-		
	303.09	[M-deoxyhexose]+	447.14	[M-hexose]-		
Kaempferol hexoside deoxyhexoside #1	287.07	[M-deoxyhexose-hexose]+	285.06	[M-deoxyhexose-hexose]-	5.99	359.4
	595.16		593.15	[M-H]-		
	449.10		447.09	[M-deoxyhexose]-		
Kaempferol hexoside deoxyhexoside #2	595.16	[M+H]+	593.16	[M-H]-	6.13	367.8
	287.07	[M-hexose-deoxyhexose]+	285.06	[M-hexose-deoxyhexose]-		
			447.11	[M-deoxyhexose]-		
Kaempferol hexoside deoxyhexoside #3	595.16	[M+H]+	593.16	[M-H]-	6.35	381
	449.12	[M-hexose]+	285.06	[M-hexose-deoxyhexose]-		
	287.07	[M-hexose-deoxyhexose]+				
Isorhamnetin hexoside desoxyhexoside	625.18	[M+H]+	623.17	[M-H]-	6.40	384
	317.07	[M-deoxyhexose-hexose]+	315.07	[M-deoxyhexose-hexose]-		
	479.13	[M-deoxyhexose]+				
Isorhamnetin methylhexose hexose	623.16	[M+H]+	621.15	[M+H]-	6.90	414
	317.07	[M-methylhexose-hexose]+	315.06	[M-methylhexose-hexose]-		
Apigenin deoxyhexose hexose	271.06	[M-deoxyhexose-hexose]+	269.05	[M-deoxyhexose-hexose]-	6.45	387
	579.17	[M+H]+	577.16	[M-H]-		
	433.11	[M-deoxyhexose]+				
Apigenin aglycone	271.06	[M+H]+	269.06	[M-H]-	8.66	519.6
Tangeretin #1	373.14	[M+H]+	-		9.40	564
Tangeretin #2	373.14	[M+H]+	-		9.90	594
Tangeretin #3	373.14	[M+H]+	-		11.20	672
Phenylpropanoids						
Scopolin	193.07	[M-hexose]+	-		4.90	294
	355.11	[M+H]+				
Scopoletin	193.07	[M+H]+	-		5.80	348

Caffeoyl quinic acid	135.13	[M-H$_2$O]$^+$	-		11.90	714
	377.14	[M+Na]$^+$				
	337.15	[M-H$_2$O]$^+$				
	393.11	[M+K]$^+$				
	355.16	[M+H]$^+$				
	163.04	[M-C$_{12}$H$_{16}$O$_2$]$^+$				
Triterpenoids						
Nomilin deoxyhexoside	515.22	[M-deoxyhexose]$^+$	531.22	[M-deoxyhexose+H$_2$O]$^-$	7.90	474
	487.23	[M-CO]$^+$				
	469.22	[M-CH$_2$O$_2$]$^+$				
	455.21	[M-C$_2$H$_4$O$_2$]$^+$				
	413.20	[M-C$_4$H$_6$O$_3$]$^+$				
	661.27	[M+H]$^+$				
Nomilin	515.22	[M+H]$^+$	453.19	[M-C$_2$H$_3$O$_2$]$^-$	10.50	630
	487.23	[M-CO]$^+$	513.22	[M-H]$^-$		
			425.20	[M-CO]$^-$		
Obacunone	455.21	[M+H]$^+$	453.20	[M-H]$^-$	11.10	666
	411.17	[M-C$_2$H$_4$O]$^+$				
Limonin	471.20	[M+H]$^+$	469.19	[M-H]$^-$	9.90	594
	443.21	[M-CO]$^+$				
	425.20	[M-H$_2$O]$^+$				
Lipids and derivatives						
Lysophosphatidyl choline	496.34	[M-C$_{18}$H$_{32}$O]$^+$	-		13.80	828
	263.24	[M-C$_{24}$H$_{49}$NO$_7$P]$^+$				
Linolenic acid	279.23	[M+H]$^+$	277.22	[M-H]$^-$	12.30	738
Linolenic acid hexoside hexoside	353.27	[M-hexose+H$_2$O]$^+$	277.22	[M-hexose-hexose]$^+$	11.57	694.2
	497.31	[M-hexose]$^+$	657.35	[M-H]$^-$		
	261.22	[M-hexose-hexose-H$_2$O]$^+$				
	659.36	[M+H]$^+$				
	335.26	[M-hexose]$^+$				

Flavonoid-related metabolites

A group of secondary metabolites that play important roles in the responses of plants to abiotic stresses is flavonoids. In this study we analyzed the accumulation of different compounds involved in the biosynthetic pathway of flavonoids in Carrizo and Cleopatra leaves subjected to WS, HS and WS+HS. As shown in **Figure 9**, different pattern of flavonoid accumulation was observed between citrus genotypes and also among stress treatments. Therefore, levels of flavones such as tangeritin significantly increased in response to WS and WS+HS in Carrizo, whereas in Cleopatra, only WS+HS induced significant accumulation of these compounds. Other flavones, apigenin derivatives, were down-regulated in response to WS+HS in Carrizo whereas individual stresses had different effects: apigenin deoxyhexose hexose levels were reduced under HS but increased under WS and apigenin aglycone accumulation was strongly repressed in response to WS but slightly induced under HS. In general, stress imposition down-regulated apigenin deoxyhexose hexose but induced the accumulation of apigenin aglycone in Cleopatra plants. Stress treatments had a little impact in hesperetin derivative accumulation in both citrus genotypes. Furthermore, almost opposite pattern of flavonol accumulation was observed between both citrus genotypes. Whereas HS and WS+HS down-regulated kaempferol-related compounds in Carrizo leaves, all stress treatments induced a remarkable up-regulation of these metabolites in Cleopatra leaves, especially under WS and WS+HS conditions. Similarly, the effect of stress treatments in quercetin derivatives (quercetin hexoside deoxyhexoside and quercetin deoxyhexoside hexoside) was more prominent in Cleopatra, mainly in response to WS and WS+HS. In addition, poor impact in the accumulation of isorhamnetin-related compounds was shown in Carrizo leaves under stress imposition. On the other hand, in Cleopatra, isorhamnetin hexoside desoxyhexoside was down-regulated in response to HS and WS+HS whereas isorhamnetin methylhexose hexose was up-regulated under these conditions but down-regulated in response to HS.
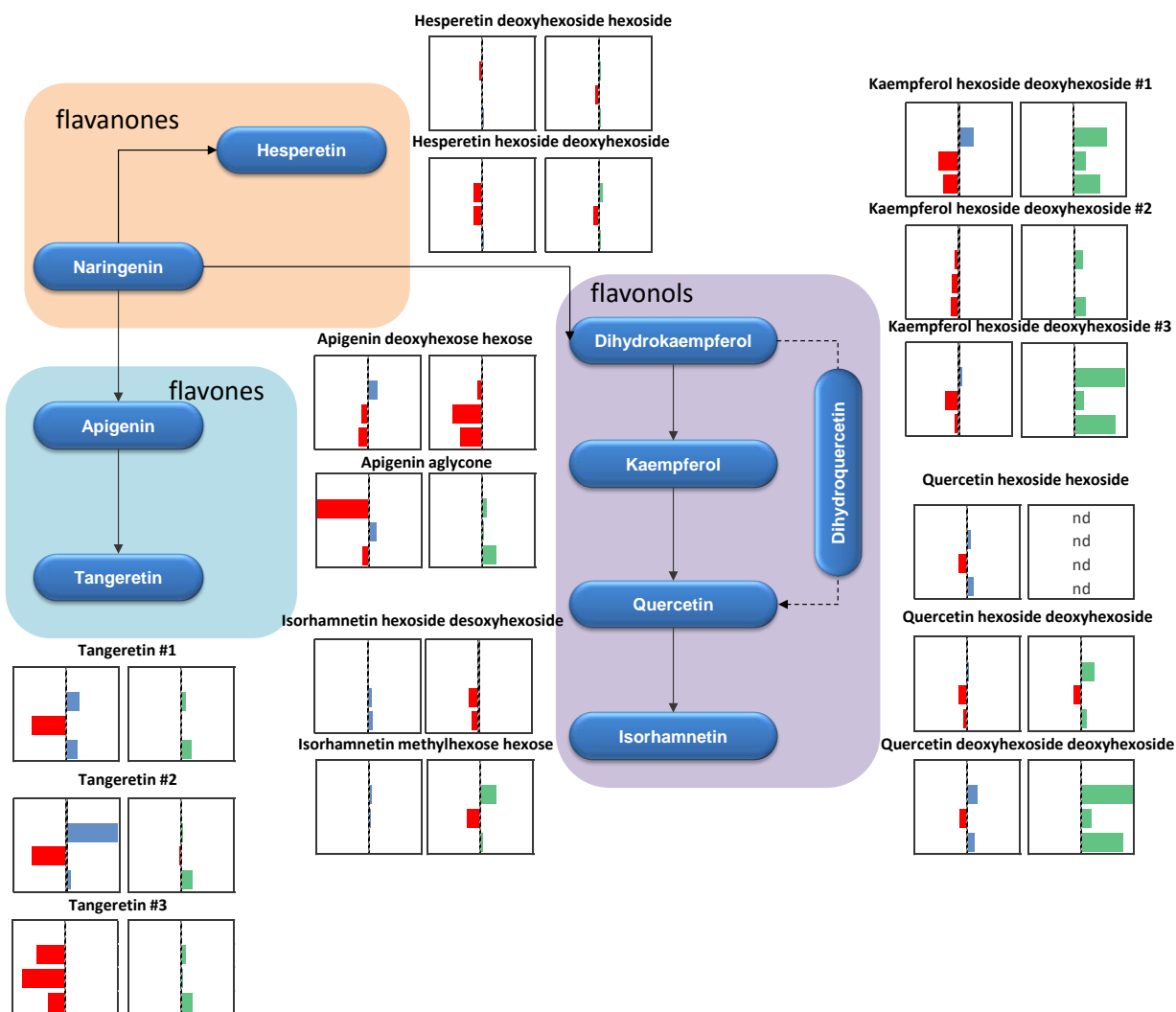
Figure 9. Pathway analysis of metabolites involved in the flavonoid pathway. Relative levels (expressed as log$_2$[stress/control], control values on first row of graphs were always 0, followed by WS, HS and WS+HS, in descending order) are given besides each detected metabolite as a bar graph: Carrizo (left graph, positive values in blue, negative in red) and Cleopatra (right graph, positive values in green, negative in red).

Limonoid-related metabolites

To further study the involvement of limonoids in the response of citrus plants to WS, HS and WS+HS, we analyzed the accumulation of metabolites involved in the biosynthetic pathway of limonin, considered as a terpenoid with functions in the interaction with the surrounding environment, arising from nomilin **(Figure 10)**. Carrizo leaves under stress imposition repressed the accumulation of nomilin whereas this compound could not be detected in Cleopatra leaves. The accumulation of nomilin deoxyhexoside followed opposite patterns in both citrus genotypes: while Carrizo

down-regulated this metabolite under WS but up-regulated it under HS and WS+HS, in Cleopatra leaves, it was significantly accumulated under WS but repressed under HS and WS+HS. Poor impact had stress treatments in obacunone regulation in Carrizo, whereas in Cleopatra we observed an additive accumulation of this metabolite. Finally, limonin was down-regulated in response to individual stresses but up-regulated under stress combination in Carrizo. On the other hand, limonin accumulation in Cleopatra leaves showed differential response under the distinct adverse conditions: its levels decreased in response to HS and increased under WS or WS+HS conditions.
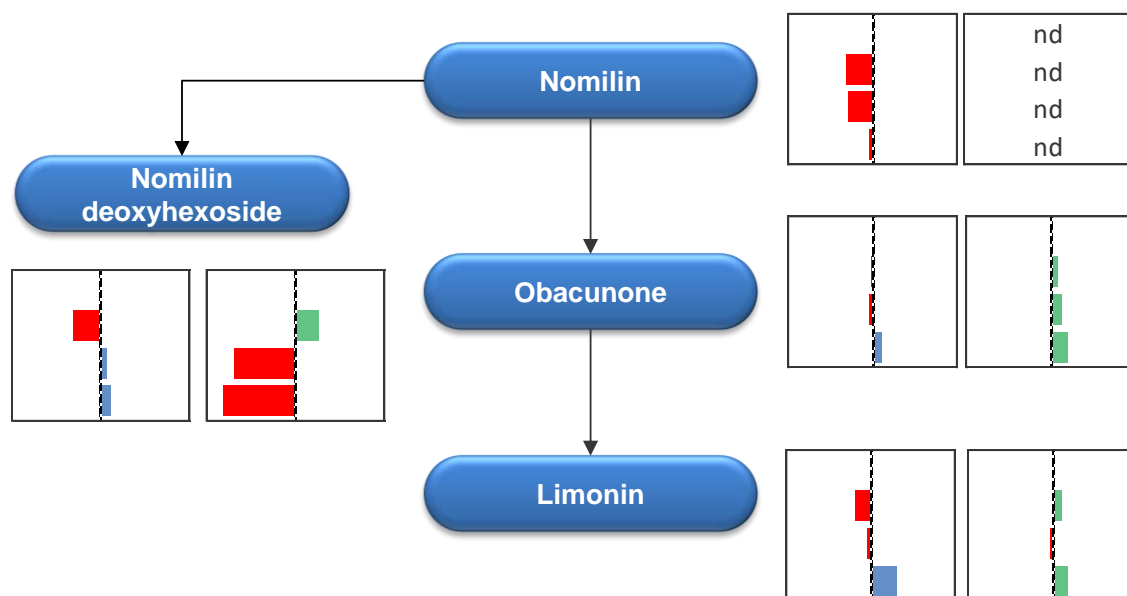
Figure 10. Pathway analysis of metabolites involved in the limonoid pathway. Relative levels (expressed as $\log_2$[stress/control], control values on first row of graphs were always 0, followed by WS, HS and WS+HS, in descending order) are given besides each detected metabolite as a bar graph: Carrizo (left graph, positive values in blue, negative in red) and Cleopatra (right graph, positive values in green, negative in red).

Discussion

In natural environments, plants have to face different combinations of abiotic stresses, tailoring specific physiological and molecular responses to face a new stress situation different from the individual stresses (Boeck et al. 2015; Hu et al. 2015; Liu et al. 2015; Mittler and Blumwald 2010; Suzuki et al. 2014; Zhang et al. 2015).

We recently conducted a study focusing on the physiological and hormonal responses of two citrus genotypes Carrizo citrange and Cleopatra mandarin to a combination of WS

and HS. Data demonstrated that Carrizo is more tolerant than Cleopatra to HS alone or in combination with WS, concluding that higher transpiration rate along with a stronger antioxidant defense is crucial for citrus tolerance to a combination of drought and heat stress (Zandalinas et al. 2016). However, it is also relevant to investigate metabolic changes caused by each stress condition in order to identify additional mechanisms underlying the superior tolerance of Carrizo to HS and WS+HS. In the present work, primary and secondary metabolites differentially altered during each stress condition were identified. Cleopatra and Carrizo leaves showed a similar number of altered polar primary compounds spanning different metabolic pathways. However, in Cleopatra, a large number of polar metabolites were up-regulated in response to WS+HS **(Figure 2)**. Therefore, a total of 77 primary compounds increased in leaves of Cleopatra seedlings subjected to stress, of which 49 responded to WS+HS, 8 to HS and only 1 to WS. Only 8 polar metabolites were up-regulated in Carrizo leaves in response to stress combination. These data suggest that under the same stress conditions, both citrus genotypes alter their metabolism in different manners showing diverse polar metabolite profiles.

The TCA cycle is a central metabolic pathway for aerobic processes and is responsible for a major portion of carbohydrate, fatty acid and amino acid oxidation leading to the production of energy and reducing power (Fernie et al. 2004). In our work, some metabolites involved in TCA cycle increased in Cleopatra in response to stress, including α-oxoglutarate and fumarate **(Figure 3)**. In addition, Cleopatra leaves seemed to notably activate glycolysis since some intermediates such as glyceraldehyde-3-P, glycerate-3-P or glycerate-2-P were accumulated upon stress imposition. Some of these intermediates involved in glycolysis and TCA cycle (glycerate-3-P, acetyl-CoA, α-oxoglutarate) were accumulated in an additive manner in Cleopatra, suggesting that the combination of WS and HS could have a higher impact on glycolysis and TCA cycle in Cleopatra plants compared to Carrizo, paralleling the higher sensitivity of Cleopatra to this stress combination (Zandalinas et al. 2016) and suggesting a major requirement of energy to cope with combined stress conditions.

Glyoxylate/dicarboxylate pathway is importantly modulated in response to stress in green tissues to dissipate excess reducing equivalents as well as energy. Therefore, ROS accumulation is prevented, by mitigating thus oxidative stress under abiotic stress conditions (Voss et al. 2013).

Increasing temperatures with closed stomata, as observed in Cleopatra, induce CO_2 limitation for the Calvin cycle, resulting in an excess of reducing power (NADPH) and energy (ATP). To prevent oxidative stress under these conditions, the glyoxylate/dicarboxylate cycle could assume a more prominent role in consuming reducing equivalents and energy to prevent ROS accumulation (Voss et al. 2013). In Carrizo plants, we observed an increased accumulation of sucrose via gluconeogenesis in response to HS and WS+HS in contrast to Cleopatra. This *de novo* carbohydrate biosynthesis could be related to the higher photosynthetic rate observed under combined stress conditions in Carrizo (Zandalinas et al. 2016). Hence, carbohydrate biosynthesis, transport and, probably, accumulation appears to be an advantage under stressful conditions in citrus. On the other hand, Cleopatra down-regulated sucrose and consumed ATP (synthesizing glutamate from oxoglutarate) and reducing power during the conversion of oxoglutarate to glutamate and during the production of glycerate-3-P from glycerate, in response to WS+HS **(Figure 4)**. This result is in accordance with our previous work (Zandalinas et al. 2016), in which Cleopatra suffered higher oxidative damage associated to a higher ROS production during combined WS and HS. In this sense, the consumption of reducing power and energy could contribute to mitigate increased ROS production found in this genotype under WS+HS.

Phenylpropanoid pathway intermediates represent a major flow of carbon from primary metabolism into secondary metabolism. Among these metabolites, coumarin scopoletin, derived from feruloyl-CoA, is converted into scopolin by glycosylation, and both metabolites are likely to be involved in plant responses to stress (Kai et al. 2008). Our results suggest that Cleopatra plants activate the phenylpropanoid pathway in order to accumulate scopolin under individual stresses and especially under combined conditions. On the other hand, in response to stress imposition, Carrizo induced the accumulation of sinapic acid and its derivative compound sinapoyl as well as cinnamoyl aldehydes **(Figure 5)**, direct precursors of lignins, suggesting that Carrizo plants could accumulate lignin to protect plants from heat and drought.

On the whole, our data indicate that Cleopatra, as a susceptible citrus genotype under WS+HS situations (Zandalinas et al. 2016), deeply alters its primary metabolism in order to face the damaging effects caused by stress combination by activating glycolysis and TCA cycle to support cellular energy requirements as well as phenylpropanoid-

derived scopolin. On the other hand, Carrizo shows reduced modifications in primary metabolism, associated to a lower incidence of stress-induced damage.

Plant secondary metabolites are often referred to as compounds that have no fundamental role in the maintenance of life processes in plants, but they are important in the interaction with the surrounding environment. In this sense, different stresses regulate the production of secondary metabolites, which are most often involved in plant defense and stress acclimation (Zhao et al. 2005). After studying different pathways of plant primary metabolism, our next aim was to examine whether secondary metabolites could play an important role in the tolerance of citrus plants to WS, HS and WS+HS. In general terms, alteration in levels of secondary metabolites overlapped between HS and WS+HS (**Figure 7)**. Among them, some flavonoids and limonoids were differently altered during WS and HS applied alone or in combination in both citrus genotypes **(Figures 9 and 10)**.

Although the specific physiological role of flavonoids in citrus remains largely unknown, recent studies in citrus juices have identified the main flavonoids and derivatives of chemotaxonomic significance (Arbona et al. 2015) and with high radical scavenging activity (Patil et al. 2009). In general, flavanones and flavones are poorly represented in plants, except in citrus. Actually, flavanones, along with flavones and flavonols, are one of the main secondary metabolites found in citrus juices (Arbona et al. 2015; Djoukeng et al. 2008). In addition, naringenin and hesperetin are considered the most abundant flavanones mainly occurring as glycoside derivatives. In citrus tissues, flavones are presented as glycoside derivatives, and flavonols, synthesized from flavanones by hydroxylation, include kaempferol, quercetin and isorhamnetin (Arbona et al. 2015; Carisiti et al. 2006; Djoukeng et al. 2008). In our work, we could identify several compounds in the flavonoid biosynthetic pathway starting from naringenin **(Table 1 and Figure 9)**, finding important differences between both citrus genotypes subjected to individual and combined WS and HS. Stress imposition had a poor impact in flavanone accumulation but flavonols were greatly induced in Cleopatra seedlings. In this sense, kaempferol-derived compounds (#1, #2 and #3) were remarkably accumulated in response to WS and WS+HS in Cleopatra leaves, whereas in Carrizo, levels of these metabolites generally decreased, except for kaempferol hexoside deoxyhexoside during WS, which was slightly up-regulated. Similarly, quercetin hexoside deoxyhexoside and quercetin deoxyhexoside deoxyhexoside levels increased

in response to WS and WS+HS in Cleopatra. In contrast, quercetin-related metabolites were minimally up-regulated in Carrizo in response to WS and WS+HS, except for quercetin hexoside deoxyhexoside levels that were slightly reduced in response to HS and WS+HS. In general, stress had a significant impact on flavonol accumulation in Cleopatra leaves. In line with our previous report (Zandalinas et al. 2016), the stronger flavonol accumulation observed in Cleopatra could be related to its higher sensitivity to stress. Hence, activation of the biosynthesis of these flavonoids could constitute a photoprotective response in leaves of sensitive Cleopatra. Moreover, flavones derived from naringenin were accumulated differently in both genotypes in response to stress treatments. Therefore, while apigenin deoxyhexose hexose was down-regulated in both citrus genotypes in response to HS and WS+HS, apigenin aglycone was significantly accumulated in Cleopatra mainly in response to WS+HS. In Carrizo, however, this compound was repressed during WS and WS+HS and slightly induced under HS. Finally, different tangeretin isomers (#1, #2 and #3) were up-regulated in response to WS+HS in Cleopatra whereas Carrizo showed different patterns: tangeretin #1 and #2 accumulated during WS and WS+HS but down-regulated in response to HS, and tangeretin #3 levels decreased under all stress treatments. These polymethoxylated flavones could be involved in an enzymatic mechanism aimed to maintain a high antioxidant activity under stress as previously shown (Yu et al. 2005). These differences in flavone content are consistent with previous data (Zandalinas et al. 2016), in which higher oxidative damage was observed in Cleopatra during WS+HS, requiring thus an increased antioxidant response respect to Carrizo.

Limonoids are highly oxygenated triterpenes present in Rutaceae and Meliaceae (Hasegawa et al. 1980) that have been reported to exhibit a role in ROS detoxification through the induction of glutathione S-transferase (GST) activity (Perez et al. 2009; Yu et al. 2005). Our results indicate that all nomilin equivalents diverted into biosynthesis of obacunone and subsequently limonin in Cleopatra in response to stress treatments and, hence, it could not be detected in this genotype **(Figure 10)**, suggesting an active nomilin metabolism. On the contrary, in Carrizo nomilin was partly directed to the synthesis of its glycoside in response to HS and WS+HS but a larger portion was directed to the synthesis of obacunone and limonin under WS+HS conditions.

In general, different patterns in the accumulation of secondary metabolites were observed in Carrizo and Cleopatra plants in response to stress combination. It has been

previously reported that *Arabidopsis thaliana* and *Thellungiella halophila* exhibited different regulation of secondary metabolism with a low degree of overlap among them when subjected to identical stress conditions (Arbona et al. 2010). In addition, Morsy et al. (2007) found that two rice genotypes with contrasting abiotic stress tolerance, showed different patterns of metabolite accumulation under low temperature, salt and osmotic stress related to their respective ability to tolerate abiotic stress. Results presented here expand these previous findings, since different profiles of secondary metabolite variations were observed in the two citrus genotypes under each stress condition applied individually or in combination. As previously suggested, this different metabolism in citrus under adverse environmental conditions could be a result of the specificity of the secondary metabolism and also the different stress tolerance of Carrizo and Cleopatra plants (Zandalinas et al. 2016).

On the whole, particular basal metabolism as well as different regulation of metabolism, could explain these differential responses in citrus plants. In this sense, it has previously reported that different metabolic response to a combination of drought and heat in two durum wheat cultivars leads to opposite stress-responsive strategies (Aprile et al. 2013). Furthermore, the higher capability to prevent stress-induced oxidative damage of Carrizo (Arbona et al. 2008) and the particular adjustment of transpiration rate during WS+HS (Zandalinas et al. 2016) also lead to an increased tolerance of Carrizo plants under WS+HS respect to Cleopatra that, in turn, could prevent further modification of metabolism. In this sense, due to its high sensitivity, Cleopatra, requires a deep alteration of its primary and secondary metabolism in order to prevent further stress-induced damages.

At present, information on the combined effect of heat and drought stress on citrus metabolism is scarce. It is a fact that abiotic stress factors influence changes in plant metabolism (Akula and Ravishankar 2011; Bolton 2009) and the characterization of the metabolome is a key aspect in basic research and plant breeding. In this work, we have studied the changes in primary and secondary metabolism that two closely-related citrus genotypes, Carrizo citrange and Cleopatra mandarin, display in response to drought and heat, applied individually or in combination. Data suggest that different metabolic patterns of two closely related citrus genotypes in response to the same stress conditions exist and that the different regulation of primary and secondary metabolic pathways

could be also behind the different tolerance of citrus subjected to individual or combined abiotic stress conditions.

Materials and methods

Plant material and growth conditions

True-to-type Carrizo citrange (*Poncirus trifoliata* L. Raf. x *Citrus sinensis* L. Osb.) and Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) plants were purchased from an authorized commercial nursery (Beniplant S.L., Penyíscola, Spain). One-year-old seedlings of both citrus genotypes were placed in 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 ºC and 18.0 ± 3.0 ºC, respectively). After that, plants of both genotypes were maintained for 2 weeks in growth chambers to acclimate to a 16-h photoperiod at 500 μmol m^{-2} s^{-1} at 25º C and relative moisture at approximately 80%. Temperature and relative moisture were recorded regularly with a portable USB datalogger (OM-EL-WIN-USB, Omega, New Jersey, USA).

Stress treatments and experimental designs

A 24-h experiment was performed in which severe drought, imposed by transplanting plants to dry perlite, was applied alone or in combination with high temperatures (40ºC). Prior to imposition of drought, heat stress was applied for 7 days to a group of well-watered Carrizo and Cleopatra plants whereas another group was maintained at 25 ºC. Thereby, we established four experimental groups of each genotype: well-watered plants grown at 25 ºC (CT) and 40 ºC (HS) and plants subjected to drought at 25 ºC (WS) and at 40 ºC (WS+HS). Leaf tissue was sampled at 24 hours after subjecting plants to the adverse conditions.

GC-MS extraction and derivatization

For primary metabolite analysis by GC-MS, 50 mg of fresh leaf tissue was extracted in 300 μL of pure methanol (LC-MS grade, Panreac, Barcelona, Spain) spiked to 0.2 mg ml^{-1} with ribitol as internal standard (IS), as described previously in Roessner et al. (2001). Extractions were performed by ultra-sonication for 10 min at room temperature. After centrifugation, supernatants were recovery and mixed with 200 μL of chloroform

and 400 µL of water followed by centrifugation at 10000 rpm and 4 °C for 10 min. The upper water layer was then recovered and evaporated to dryness using a SpeedVac (Jouan, Saint HerblainCedex, France). Dry residues were re-dissolved with 50 µL of 20 mg mL^{-1} methoxyamine in pyridine followed by incubation at 30 ºC in a water bath for 90 min. Later, 70 µL of methylsilyltrifluoroacetamide (Macherey-Nagel, Germany) were added and subsequently incubated at 37 ºC for 30 min. Finally, the solution was mixed with 10 µL of a commercial mixture of fatty acid methyl esters (C8-C24 FAME mix, Sigma-Aldrich, Madrid, Spain) as retention index (RI) markers.

Instrumental conditions for GC-MS analysis

Derivatized extracts were independently injected into a GC-MS system composed of a gas chromatograph equipped with an autosampler and a quadrupole mass analyzer (GCMS-QP2010 Ultra, Shimadzu Corporation, Japan) equipped with an electron impact ion source. The GC separation was performed using a fused silica BPX5 capillary column with a length of 30 m x 0.25 mm ID and a film thickness of 0.25 µm (SGE Analytical Science, Victoria, Australia). The oven program was set as follows: 80 ºC (2 min); 10 ºC min^{-1} to 325 ºC (3.5 min) for a total runtime of 30 min. Injections of 1 µL of sample extracts were performed in split mode (1:200) at a temperature of 230 ºC. Helium (99.999%; Praxair, Valencia, Spain) was used as the carrier gas at a constant flow rate of 2 mL min^{-1}. The interface and source temperatures were set to 325 ºC and 230 ºC respectively. Scan rate was set at 5 scan s^{-1} within 50 to 700 amu mass range. After acquisition, chromatogram files were converted to NetCDF for further processing.

GC-MS data processing

Mass chromatographic features were extracted with xcms (Smith et al. 2006) and subsequently processed with TargetSearch software (Cuadros-Inostroza et al. 2009) using Gölm Metabolite Database (available from http://www.mpimp-golm.mpg.de/). Briefly, this software calculates RI for all compounds in chromatograms based on tabulated RI values for the constituents of the FAME mix and then matches RI and mass fragments of compounds in samples with those found in databases. Identified metabolites were cross-referenced in chromatograms using GCMS-solution software (Shimadzu Corp.). Peak areas of identified metabolites were normalized to the IS (ribitol) area before statistical analyses.

LC-MS extraction

Extraction was performed essentially as previously described in Zandalinas et al. (2012) with slight modifications. Briefly, 500 μL of 70% methanol supplemented with biochanin A at 1 mg L^{-1} (IS) was added to 0.1 g of frozen leaf powder. After 10 min of sonication, extracts were heated at 80 ºC in a water bath for 15 min and allowed to cool down at room temperature. Then, samples were centrifuged at 10000 g for 10 min at 4 °C and supernatants filtered through 0.22 μm PTFE syringe filters (Whatman International Inc., Kent, United Kingdom), prior to UPLC-QTOF-MS analysis.

LC-MS instrumental conditions

Chromatographic separations were performed on an Acquity SDS system (Waters Corp. Ltd., Milford, MA) interfaced to a QTOF Premier from Micromass Ltd. through an ESI source. A reversed-phase column was used as follows: 100 mm × 2.1 mm, i.d. 1.8 μm (ProntoSil, Bischoff, Leonberg, Germany). Sample aliquots (10 μL) were injected onto the UPLC system using the partial loop-filling option. Separations were carried out using a flow rate of 300 μL min^{-1} for 30 min as follows: 0−2 min, isocratic 95% A [water:formic acid, 99.9:0.1 (v/v)] and 5% B [acetonitrile: formic acid, 99.9:0.1 (v/v)]; 2−17 min, gradient 5−95% B; 17−20 min, return to initial conditions; 20−25 min, re-equilibration period. During analyses, the column temperature was maintained at 40 °C and samples were maintained at 10 °C to slow down degradation. Samples were analyzed in both negative and positive ionization modes. Two functions were set in the instrument: in function 1, data were acquired in profile mode from 50 to 1000 Da using a scan time of 0.2 s and a collision energy of 2 eV; in function 2, the scan range was the same, but a collision ramp between 4 and 65 eV was set. During all measurements, the electrospray capillary voltage was set to 4 kV and the cone voltage was set to 25 V. The source temperature was maintained at 120 °C and the desolvation gas temperature was set at 350 °C. Argon was used as the collision gas and nitrogen was used as the nebulizer as well as desolvation gas set at 60 and 800 L h^{-1}, respectively. Exact mass measurements were provided by monitoring the reference compound lockmass leucine-enkephalin.

LC-MS data processing

Data were processed using Masslynx v.4.1. Raw data files were converted to netCDF format using the application databridge from Masslynx and processed using the xcms package as previously described (Smith et al. 2006; Zandalinas et al. 2012). Chromatographic peak detection was performed using the matchedFilter algorithm, applying the following parameter settings: snr = 3, fwhm = 15 s, step = 0.01 D, mzdiff = 0.1 D, and profmethod = bin. Retention time correction was achieved in three iterations applying the parameter settings minfrac = 1, bw = 30 s, mzwid = 0.05 D, span = 1 and missing = extra = 1 for the first iteration; minfrac = 1, bw = 10 s, mzwid = 0.05 D, span = 0.6 and missing = extra = 0 for the second iteration; and minfrac = 1, bw = 5 s, mzwid = 0.05 D, span = 0.5 and missing = extra = 0 for the third iteration. After final peak grouping (minfrac = 1, bw = 5 s) and filling in of missing features using the fillPeaks routine of the xcms package, a data matrix consisting on feature × sample was obtained.

Statistical analyses

Significantly altered metabolites were extracted from datasets following ANOVA analysis at p≤0.05 using sample treatment as factor. To investigate metabolite profiles over samples, genotypes and treatments, peak areas of significantly altered metabolites were represented as a heatmap to facilitate visualization. Both heatmaps and hierarchical cluster analysis (HCA) on heatmaps was performed using Dchip software (http://www.hsph.harvard.edu/cli/complab/dchip/). PLS-DA was performed with Simca-P+ 11.0 (Umetrics AB, Umeá, Sweden).

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Supplementary Figures

Column labels (left to right): CC CT 1, CC CT 2, CC CT 3, CC WS 1, CC WS 2, CC WS 3, CC HS 1, CC HS 2, CC HS 3, CC WS+HS 1, CC WS+HS 2, CC WS+HS 3

Row labels (top to bottom):
*Indole-3-lactic acid (2TMS)
*Artemisinic acid (1TMS)
*beta-D-Fructofuranosyl-(2,1)-beta-D-Fructofurano
*Propane, 1,2-diamino- (4TMS)
*Cytosine (2TMS)
Isoascorbate*Isoascorbic acid (4TMS)
*Tyramine, 3-methoxy- (3TMS)
*Imidazole-4-acetic acid (2TMS)
Ascorbate*Ascorbic acid (4TMS)
Calystegine B2*Calystegine B2 (1MEOX) (3TMS) MP
*Panthenol (4TMS)
*Glucaric acid-1,4-lactone (4TMS)
*D305620
*Acetamide, 2-phenyl- (2TMS)
Glycerol-3-P*Glycerol-3-phosphate (4TMS)
Indole-3-acetyl-aspartate*Aspartic acid, N-(3-ind
*Hydrocortisone (2MEOX) (3TMS) MP
monomethylphosphate*Phosphoric acid monomethyl es
*Acetic acid, 4-hydroxyphenyl- (2TMS)
*Azelaic Acid (2TMS)
Tryptamine*Tryptamine (2TMS)
Adenine*Adenine (2TMS)
GABA*Butanoic acid, 4-amino- (2TMS)
*D208496
*D285215
*Trans-1,2-0-benzylidene-D-glycerol (1TMS)
*Methionine, N-acetyl- (2TMS)
*Malic acid, 3-oxalo- (TMS)
*Neuraminic acid, N-acetyl- (1MEOX) (7TMS) MP
Adenosine*Adenosine, alpha- (4TMS) BP
*Inosine-5'-monophosphate (5TMS)
*Maleamic acid, N-ethyl- (2TMS)
*Norbornane-2-carboxylic acid, 2-amino- (2TMS) BP
*Phenylpyruvic acid (1MEOX) (1TMS) BP
*Lysine, N-epsilon-acetyl- (3TMS)
Ornithine*Ornithine (3TMS)
*Mannosamine, N-acetyl- (1MEOX) (5TMS) BP
*NA 645810.81
*Ribose-5-phosphate (1 MEOX) (5TMS) MP
*Cinnamaldehyde, trans- (1MEOX) MP
Calystegine B3*Calystegine B3 (1MEOX) (4TMS) MP
*NA135011 (classified unknown
*Nonanoic acid (1TMS)
*Phenylpyruvic acid (2TMS)
N-Heptanoyl-homoserine lactone*Homoserine lactone
*Butanoic acid, 4-amino-3-hydroxy- (2TMS)
*Phenoxyacetic acid, 4-hydroxymethyl-3-methoxy- (
*Cinnamic acid, 3,4-dimethoxy-, cis- (1TMS)
*Kestose, 1- (11TMS)
*Propionic acid, 3-ureido- (3TMS)
Nicotinamide*Nicotinamide (2TMS)
ABA*Abscisic acid (1MEOX) (1TMS) BP
*Synephrine (3TMS)
*beta-D-Allose (5TMS)
*Gluconic acid-1,4-lactone (4TMS)
*Sinapic acid, cis- (2TMS)
*Pyruvic acid, 4-hydroxyphenyl- (1MEOX) (2TMS) MP
tyrosine*Tyrosine (2TMS)
Coumarine*Coumarine
mannitol*Mannitol (6TMS)
glycine*Glycine (2TMS)
isoleucine*Isoleucine (1TMS)
asparagine*Asparagine [-H2O (2TMS)
glucose*Glucopyranose [-H20 (4TMS)
*Erythronic acid (4TMS)
*Serine, cyclo- (2TMS)
pyroglutamate*Pyroglutamic acid (2TMS)
Norleucine*Norleucine (1TMS)
*Threonine, O-methyl-, L- (2TMS)
*Diethanolamine (2TMS)
N-Heptanoyl-homoserine lactone*Homoserine lactone
succinate*Succinic acid (2TMS)
*Propanoic acid, 2-amino-3-ureido- (3TMS)
*Inositol, allo- (6TMS)
*D286510
*Glycyl-phenylalanine (4TMS)
*D222462
Vitamin K1*Phylloquinone
aconitate*Aconitic acid, cis- (3TMS)
*Acrylic acid, 4-imidazole- (2TMS)
Tryptophan*Tryptophan (2TMS)
*Butanoic acid, 2,4-diamino- (4TMS)
*Aspartic acid, N-acetyl- (3TMS)
*Estrone, 2-methoxy- (1MEOX) (1TMS) MP
Tryptophan*Tryptophan (3TMS)
*Fluorescein, 5(6)-carboxy- (4TMS)
*NA145015
*NA 659384.31
glutamate*Glutamic acid (2TMS)
*Hypotaurine (3TMS)
6-Benzylaminopurine-9-(beta-glucoside)*Purine, 6-
*3-Hydroxyundecan-4-one (1MEOX) (1TMS)
*Butanoic acid, 4-amino-3-hydroxy- (3TMS)
*3-Hydroxydodecan-4-one (1MEOX) (1TMS)
*Biotin, dethio- (3TMS)
*Taurine, N-tri(hydroxymethyl)methyl- (4TMS)
*Kynurenine (2TMS)
3-Oxoglutarate*Glutaric acid, 3-oxo- (1MEOX) (3TM
*Shikimic acid, 3-dehydro- (1MEOX) (3TMS) BP

Supplemental Figure S1. Hierarchical cluster analysis of differentially accumulated polar metabolites in Carrizo.

Supplemental Figure S2. Hierarchical cluster analysis of differentially accumulated polar metabolites in Cleopatra.

Chapter 3

ABA is required for the accumulation of APX1 and MBF1c during a combination of water deficit and heat stress.

Abstract

Abscisic acid (ABA) plays a key role in plant acclimation to abiotic stress. Although recent studies suggested that ABA could also be important for plant acclimation to a combination of abiotic stresses, its role in this response is currently unknown. Here we studied the response of mutants impaired in ABA signaling (*abi1-1*) and biosynthesis (*aba1-1*) to a combination of water deficit and heat stress. Both mutants displayed reduced growth, biomass and survival when subjected to stress combination. Focusing on *abi1-1*, we found that although its stomata were impaired in their response to water deficit, remaining significantly more open than wild type, when subjected to the stress combination its stomata aperture was surprisingly reduced. Stomatal closure during stress combination in *abi1-1* was accompanied by higher levels of H_2O_2 in leaves, suggesting that H_2O_2 might play a role in this response. In contrast to the almost 'wild type'-stomatal closure phenotype of *abi1-1* during stress combination, the accumulation of ascorbate peroxidase 1 and multiprotein bridging factor 1c proteins, required for acclimation to a combination of water deficit and heat stress, was significantly reduced in *abi1-1*. Our findings reveal a key function for ABA in regulating the accumulation of essential proteins during a combination of water deficit and heat stress.

Introduction

Under natural conditions, or when grown in the field, plants are subjected to a combination of different abiotic stresses (Mittler, 2006; Mittler and Blumwald, 2010; Suzuki *et al.*, 2014). Recent studies identified specific physiological and molecular responses of plants to a combination of different abiotic stresses, demonstrating the importance of studying stress combination (Mittler and Blumwald, 2010; Suzuki *et al.*, 2014; Boeck *et al.*, 2015; Hu *et al.*, 2015; Liu *et al.*, 2015; Zhang *et al.*, 2015). Water deficit and high temperature represent one of the most frequent abiotic stress combinations occurring under natural conditions (Savin and Nicolas, 1996; Jiang and Huang, 2001; Mittler, 2006; Craufurd *et al.*, 2008; Boeck *et al.*, 2015). Previous studies have shown that the transcriptome of plants subjected to a combination of water deficit and heat stress is different from that of plants subjected to water deficit or heat stress (Rizhsky *et al.*, 2002, 2004; Mittler, 2006), suggesting that the development of broad-spectrum abiotic stress-tolerant crops will require a more detailed study of the impact of multiple environmental conditions on plants and crops (Mittler and Blumwald, 2010).

Several different phytohormones play a pivotal role in the response of plants to abiotic stress (Peleg and Blumwald, 2011; De Ollas *et al.*, 2013; Miura and Tada, 2014; Yoshida *et al.*, 2014). Abscisic acid (ABA), for example, plays a key role in the response of plants to water deficit, salinity and heat, as it regulates stomatal closure and the expression of different acclimation proteins (Bartels and Sunkar, 2005; Finkelstein, 2013). Guard cell ABA signaling has been extensively studied using the ABA-insensitive dominant mutant allele *abi1-1* which severely reduces the catalytic activity of the ABI1 type 2C protein phosphatase (PP2C; Koornneef *et al.*, 1984; Merlot *et al.*, 2001), and characterization of the redox sensitivity of ABI1 revealed strong enzymatic inactivation by H_2O_2 (Meinhard and Grill, 2001). Production of reactive oxygen species (ROS) by respiratory burst oxidase homologue proteins (RBOH), activation of Ca^{2+} channels at the plasma membrane and activation of SLAC1, required to drive stomatal closure, were all found to be impaired in the *abi1-1* mutant (Wu *et al.*, 2003; Nilson and Assmann, 2006). Jasmonic acid (JA) is also involved in stomatal responses during abiotic stresses (Munemasa *et al.*, 2011; Daszkowska-Golec and Szarejko, 2013; Murata *et al.*, 2015), and ROS production in guard cells is also dependent on jasmonates, which interact with the ABA pathway by increasing the influx of Ca^{2+} (Munemasa *et al.*, 2011; Daszkowska-Golec and Szarejko, 2013). It has been reported that the stomata of the *abi1-1* mutant are insensitive to jasmonates and that jasmonates might affect regulation of the ABA

receptor complexes in guard cells (Murata *et al.*, 2015). In addition to JA, salicylic acid (SA) can also induce stomatal closure that is accompanied by extracellular and intracellular ROS accumulation and inward-rectifying K^+ channel inactivation in guard cells (Khokon *et al.*, 2011).

Aside from regulating stomatal responses, ABA is involved in transcriptional regulation, for example via the ABRE (ABA-responsive element) or DRE (dehydration-responsive element) elements (Shinozaki and Yamaguchi-Shinozaki, 2007; Umezawa *et al.*, 2010; Nakashima and Yamaguchi-Shinozaki, 2013). A recent genome-wide search for ABRE and DRE cis-motifs in *Arabidopsis* identified 2052 genes containing these elements (Mishra *et al.*, 2014). In addition, about 1354 genes had impaired transcript accumulation in the *abi1-1* mutant (Hoth *et al.*, 2002).

Mutants impaired in ABA biosynthesis (*aba1-1*) or ABA signaling (*abi1-1*) were recently reported to be impaired in their acclimation to a combination of salt and heat stress (Suzuki *et al.*, 2016), suggesting an important role for ABA in plant acclimation to abiotic stress combination. Nevertheless, it was not known whether this impairment is due to ABA's role in regulating stomatal responses, transcript expression, or both (Suzuki *et al.*, 2016).

Here we report that *abi1-1* and *aba1-1* plants are impaired in their acclimation to a combination of water deficit and heat stresses. Focusing on the *abi1-1* mutant, we found that although the stomata of *abi1-1* plants displayed an impaired response to water deficit stress, remaining significantly more open than wild type, when subjected to a combination of water deficit and heat stresses they surprisingly remained closed, similar to wild type, demonstrating a potentially unique stomatal regulation mechanism during the stress combination. Interestingly, stomatal closure in the *abi1-1* mutant during stress combination was accompanied by higher levels of H_2O_2 in leaves, suggesting that H_2O_2 might play a role in this response. In contrast to the almost 'wild type-response' of stomatal closure displayed by *abi1-1* plants during the stress combination, the accumulation of ascorbate peroxidase 1 (APX1) and multiprotein bridging factor 1c (MBF1c), two proteins required for plant survival during a combination of water deficit and heat stress (Suzuki *et al.*, 2005; Koussevitzky *et al.*, 2008), was significantly reduced in the *abi1-1* mutant, compared to wild type. Our findings reveal a potential role for H_2O_2 in regulating stomatal responses during stress combination and point to a key role for ABA in regulating the accumulation of APX1 and MBF1c during a combination of water deficit and heat stresses.

Materials and Methods

Plant material, growth conditions and stress treatments

Arabidopsis thaliana Ler (cv Landsberg erecta), *aba1-1* and *abi1-1* (Assmann *et al.*, 2000) plants were grown in 240-cm^2 inserts on soil mixture (MetroMix 200, SUN GRO) under controlled conditions: 21 ^{o}C, 10-h light cycle, 100 μmol m^{-2}s^{-1}, and relative humidity of 70% (AR-66, Percival Scientific) as described in Suzuki *et al.* (2016). All stress treatments were performed in parallel as described in Rizhsky *et al.* (2004) with the following modifications: water deficit was applied by withdrawing water from 10-d-old plants until reaching 40% of control soil weight, typically within 20-25 days. Heat stress was imposed by transferring 30-d-old plants to 38 ^{o}C for 8 hours as follows: 06:00-08:00, 21 °C; 08:00-16:00, 38 °C. Water deficit and heat stress combination was performed by applying heat stress to 30-d-old plants under water deficit (Supplementary Fig. S1). Rosettes from Ler and *abi1-1* plants were sampled at the same time and all measurements were performed in parallel after each stress condition (Supplementary Fig. S1). Following the stress treatments, plants were recovered under controlled conditions for 5 days and scored for survival. Temperature and relative humidity were recorded regularly with a portable USB datalogger (OM-EL-USB-2-LCD-PLUS, OMEGA Engineering, INC., Stamford, Connecticut, USA; Supplementary Fig. S1B). All experiments were repeated at least three times.

Growth characteristics

Fresh weight, dry weight and plant diameter were measured as described in Suzuki *et al.* (2005, 2016). Relative water content (RWC) was measured using rosettes, which were immediately weighed after stress treatments to obtain a fresh weight (FW). Rosettes were then placed in a beaker of water overnight in the dark, allowing them to become fully hydrated, reweighed to obtain turgid weight (TW) and dried at 40 ºC for 4 days to obtain dry weight (DW). Finally, RWC (%) was calculated as [(FW - DW) × (TW- DW)$^{-1}$] × 100. For plant survival, plants were recovered for 5 days under controlled conditions (well-watered and 21 °C) and % survival of plants was scored and calculated as described in Koussevitzky *et al.* (2008) and Suzuki *et al.* (2016).

Stomatal conductance

Stomatal conductance (gs) was measured in parallel on *aba1-1* and *abi1-1* plants of each treatment by using a LCpro+ portable infrared gas analyzer (ADC bioscientific Ltd., Hoddesdon, UK). After instrument stabilization, at least ten measurements were taken on three leaves in three replicate plants from each mutant and stress treatment.

Plant hormone analysis

Hormone extraction and analysis were carried out as described in Durgbanshi *et al.* (2005) with few modifications. Shortly, 0.1 g of dry tissue was extracted in 2 mL of ultrapure water after spiking with 50 ng of [^{2}H$_6$]-abscisic acid, [C$_{13}$]-salicylic acid and dihydrojasmonic acid in a ball mill (MillMix20, Domel, Železniki, Slovenija). After centrifugation at 4000 g at 4 ºC for 10 mins, supernatants were recovered and pH adjusted to 3 with 30% 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). Once dried, the residue was resuspended in a 10:90 MeOH:H$_2$O solution by gentle sonication. The resulting solution was filtered through 0.22 µm PTFE membrane syringe filters (Albet S.A., Barcelona, Spain) and directly injected into a UPLC system (Acquity SDS, Waters Corp., Milford, MA, USA). Chromatographic separations were carried out on a reversed-phase C18 column (Gravity, 50 × 2.1 mm 1.8-µm particle size, Macherey-Nagel GmbH, Germany) using a MeOH:H$_2$O (both supplemented with 0.1% acetic acid) gradient at a flow rate of 300 µL min^{-1}. Hormones were quantified with a TQS triple quadrupole mass spectrometer (Micromass, Manchester, UK) connected online to the output of the column though an orthogonal Z-spray electrospray ion source.

Stomatal aperture

Stomatal aperture analysis was performed as described in Morillon and Chrispeels (2001). Briefly, three leaves of Ler and *abi1-1* from each plant were cut and the lower surface was immediately stuck to a microspore slide with a medical adhesive (Hollister, Libertyville, IL). After 1-2 mins, the leaf was peeled away under distilled water. The lower epidermis stuck to the glass was visualized under the microscope and stomatal images were recorded. Measurements of stomatal aperture were performed using the imaging software Image J, version 6.

H₂O₂ measurement

H$_2$O$_2$ accumulation in rosettes tissues was measured using the Amplex Red Hydrogen Peroxide-Peroxidase Assay kit (Molecular Probes, Invitrogen, Carlsbad, CA) as described in Suzuki *et al.* (2015). In short, 500 µL of 50-mM sodium phosphate buffer (pH 7.4) containing 50 µM Amplex Red and 0.05 U mL^{-1} horseradish peroxidase was added to ground, frozen tissues. Samples were centrifuged at 12,000 g for 12 min at 4 °C and after that, 450 µL of supernatant was transferred into fresh tubes and incubated for 30 min at room temperature in the dark. Absorbance at 560 nm was measured using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The concentration of H$_2$O$_2$ in each sample was determined from a standard curve consisting of 0, 0.5, 1, 3, 6 and 9 µM of H$_2$O$_2$. Following the measurement of absorbance, tissue samples were completely dried using a speed vacuum concentrator for 90 min and H$_2$O$_2$ accumulation per g dry weight was calculated.

H₂O₂ and ABA treatments

Hydrogen peroxide and ABA treatments were conducted by spraying 1 mM H$_2$O$_2$ or 30 µM ABA on 30-d-old Ler and *abi1-1* plants. Control plants were simultaneously sprayed with distilled water. Stomatal aperture for control and treated leaves was measured after 30 and 60 mins of each treatment in plants kept in the light or in the dark.

Protein blot analysis

Protein was isolated quantified and analyzed by protein blot as previously described (Miller *et al.*, 2007). Coomassie Blue staining of protein gels was used to control for protein loading.

Statistical analysis

Genotypic differences were discriminated by one-tailed Student's *t-test*. Results are presented as the Mean ± SD (* $P<0.05$). Stress acclimation data was also subjected to analysis of variance using a two-way ANOVA with the interaction genotype x stress followed by Tukey posthoc test ($P<0.05$) when a significant difference was detected (Supplementary Table S1).

Results

Growth of wild type (Ler), aba1-1 *and* abi1-1 *plants subjected to a combination of water deficit and heat stresses*

Rosette fresh and dry weight, RWC, plant diameter, survival and gs of Ler, *aba1-1* and *abi1-1* plants subjected to water deficit, heat stress and a combination of water deficit and heat stresses were characterized (Figs. 1, 2). Compared to wild type Ler plants, *aba1-1* plants showed decreased fresh and dry weight, as well as rosette diameter, in response to all stress treatments. In addition, water deficit and a combination of water deficit and heat stress significantly reduced RWC whereas gs increased in *aba1-1* in response to all stress treatments, compared to wild type. Plant survival under combined stress conditions was 90% and 50% in Ler and *aba1-1* plants, respectively (Fig. 1A). Compared to wild type, *abi1-1* plants showed a significant reduction in fresh and dry weight in response to all stress treatments.

(A)
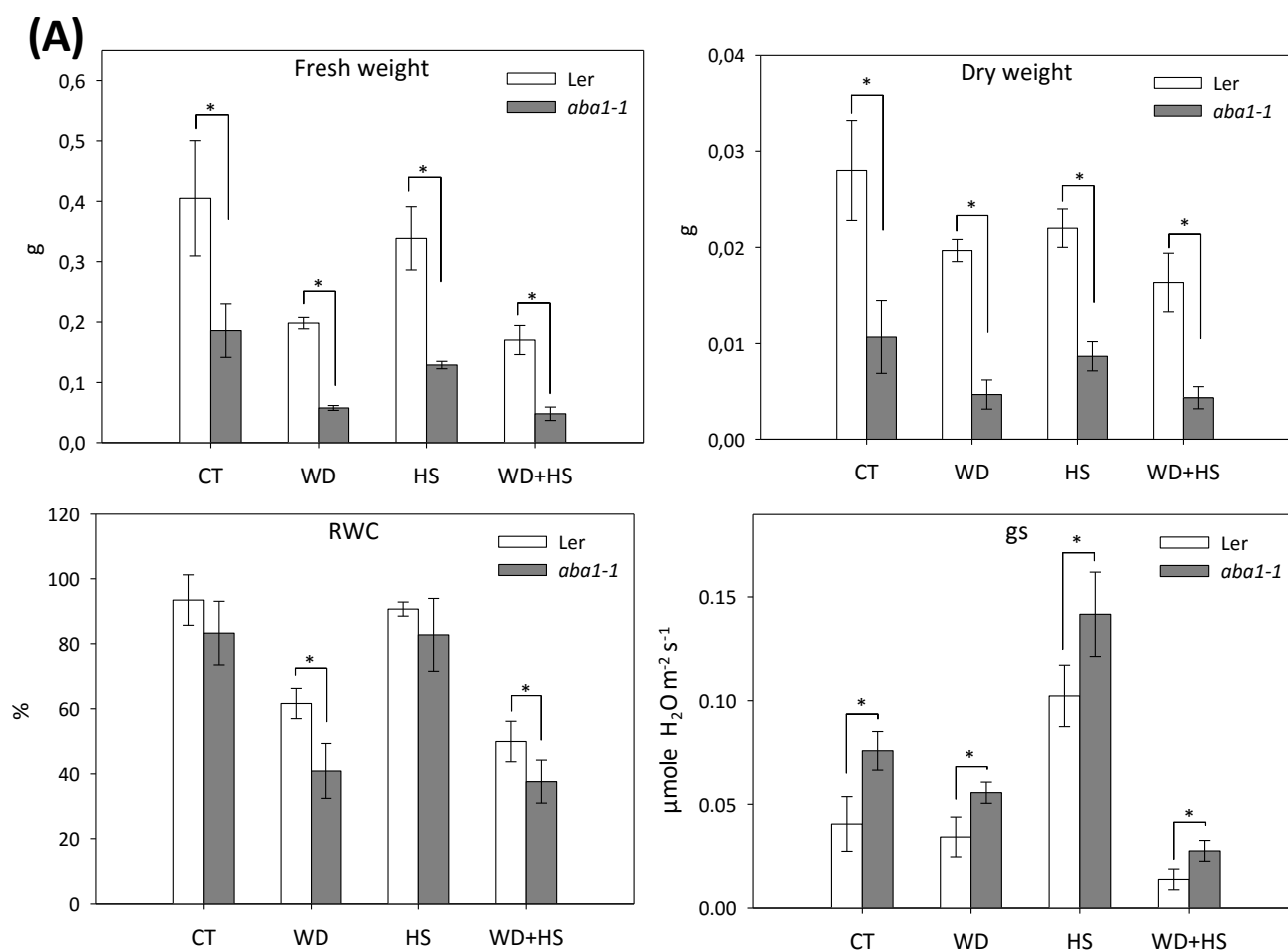
Figure 1. Growth, biomass, survival, RWC and stomatal conductance of wild type and *aba1-1* plants subjected to a combination of water deficit and heat stresses. (A) Shoot fresh and dry weight (g; average of five individual rosettes), RWC, rosette diameter, survival and stomatal conductance (gs) of plants subjected to water deficit (WD), heat stress (HS) and a combination of water deficit and heat stresses (WD+HS). **(B)** Representative images of wild type and *aba1-1* plants subjected to the different stresses. *, Student's test significant at $P < 0.05$. Error bars represent SD.

In contrast, decreased diameter and RWC were only observed in *abi1-1* plants in response to water deficit or water deficit combined with heat stress (Fig. 2A). Interestingly, whereas 95% of Ler plants survived the stress combination, only about 40% of *abi1-1* survived the exposure to a combination of water deficit and heat stress (Fig. 2A). Increased gs was observed in *abi1-1* plants compared to Ler in control conditions as well as in response to water deficit (Fig. 2A). The results shown in Figs. 1 and 2 demonstrate that although the growth of *abi1-1* and *aba1-1* plants is negatively impacted by water deficit or heat stress, compared to wild type, these treatments had no adverse effect on survival. In contrast, the combination of water deficit and heat stress significantly impacted the survival of *abi1-1* and *aba1-1* plants, demonstrating that mutants impaired in ABA biosynthesis or signaling are impaired in their acclimation to this stress combination. A *genotype x stress* interaction analysis further confirmed the dependency of plant survival on ABA function with a *p* value that is equal to or less than 0.001 (Supplementary Table S1).

To further study the role of ABA in plant acclimation to a combination of water deficit and heat stress, we focused our studies on the *abi1-1* mutant that is impaired in ABA signaling (Assmann *et al.*, 2000; Wu *et al.*, 2003; Nilson and Assmann, 2006).
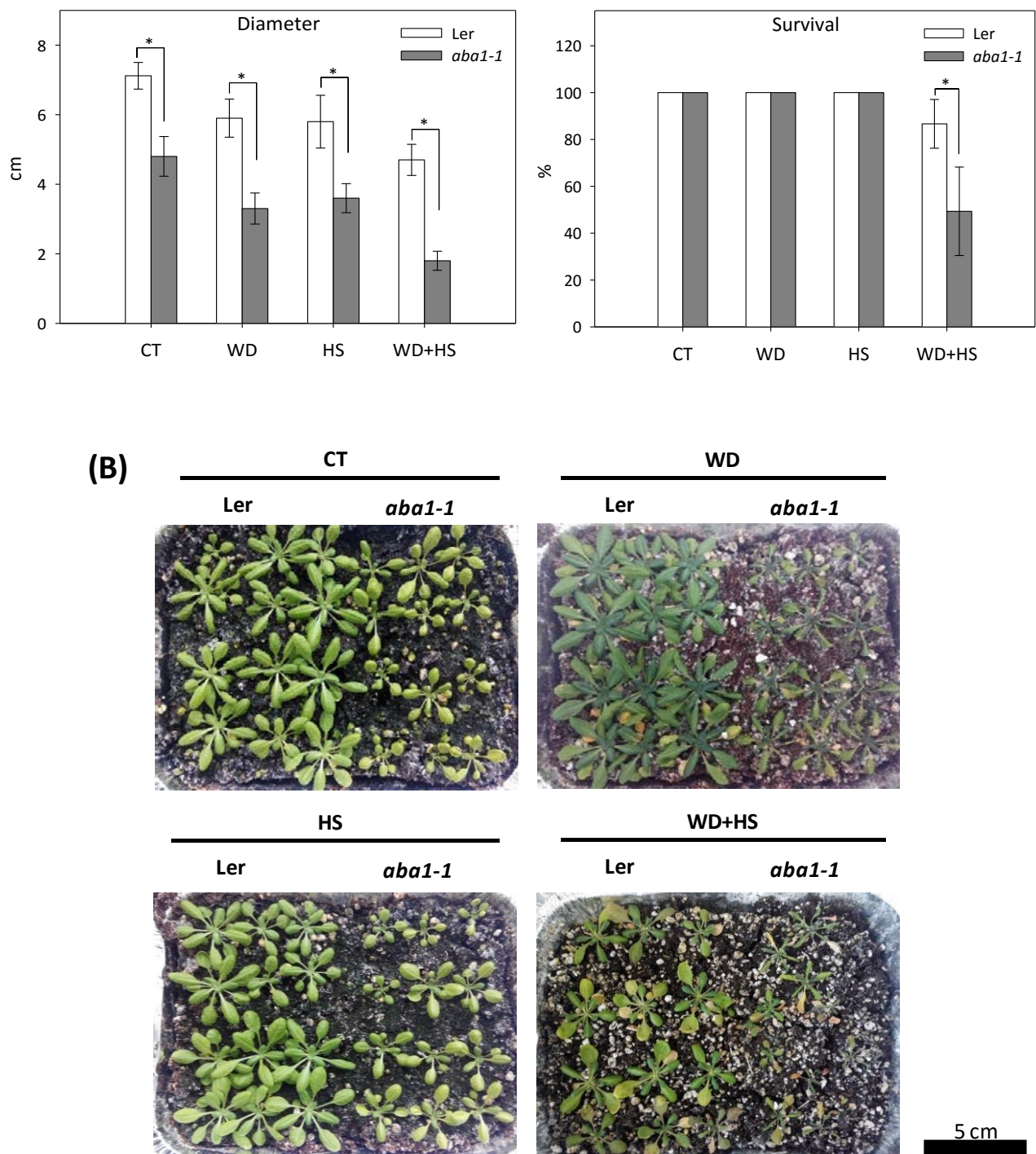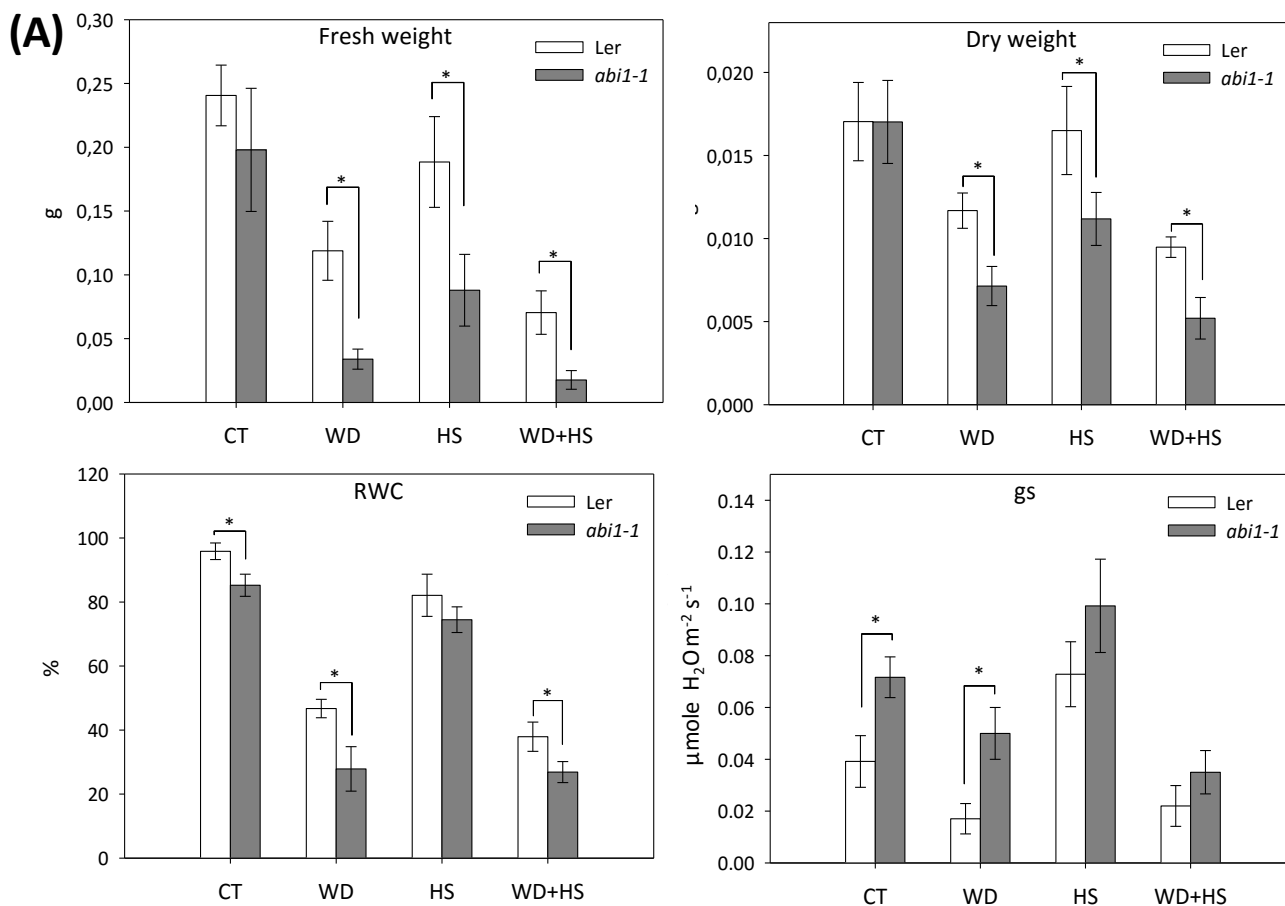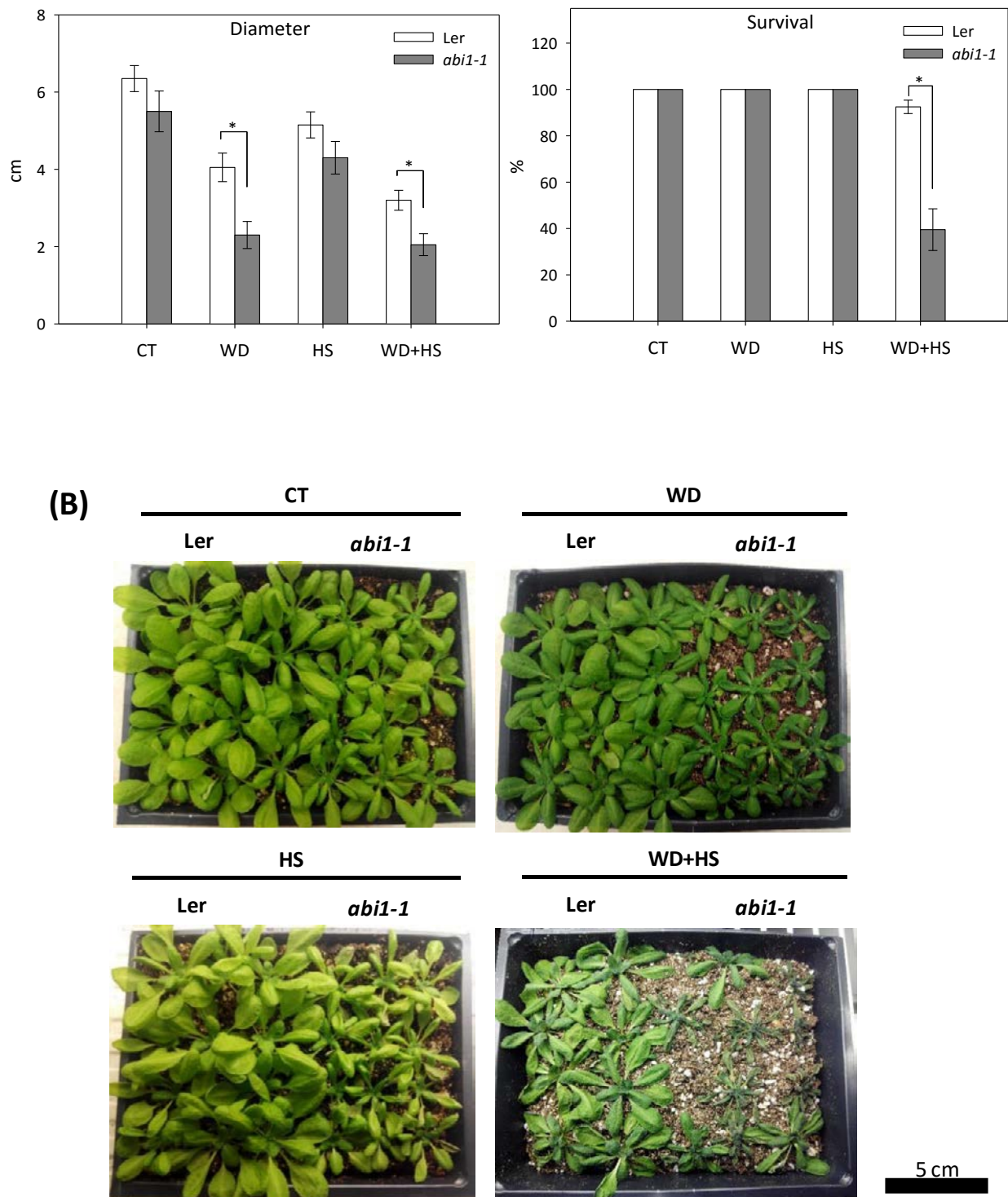
Figure 2. Growth, biomass, survival, RWC and stomatal conductance of wild type and *abi1-1* plants subjected to a combination of water deficit and heat stresses. (A) Shoot fresh and dry weight (g; average of five individual rosettes), RWC, rosette diameter, survival and stomatal conductance (gs) of plants subjected to water deficit (WD), heat stress (HS) and a combination of water deficit and heat stresses (WD+HS). **(B)** Representative images of wild type and *abi1-1* plants subjected to the different stresses. *, Student's test significant at P < 0.05. Error bars represent SD.

Stomatal aperture of Ler and abi1-1 *plants subjected to a combination of water deficit and heat stresses*

To determine whether the differences observed between the survival of *abi1-1* and wild type plants in response to the stress combination (Fig. 2) were related to the impaired stomatal responses of *abi1-1* (Wu *et al.*, 2003; Nilson and Assmann, 2006), the stomatal aperture of Ler and *abi1-1* plants was measured under water deficit, heat stress and a combination of water deficit and heat stresses (Fig. 3A, B). Under controlled conditions, the stomatal aperture of *abi1-1* plants was larger than that of wild type Ler (Fig. 3A, B). The stomatal aperture of *abi1-1* plants did not decrease in response to water deficit reflecting the impairment of *abi1-1* in stomatal responses (Fig. 3A, B; Wu *et al.*, 2003; Nilson and Assmann, 2006). In contrast, the stomatal aperture of *abi1-1* increased in response to heat stress and was larger than that of Ler showing that the stomata of *abi1-1* could open in response to heat stress (Fig. 3A, B). Surprisingly, and in contrast to the differences in stomatal responses and stomatal aperture observed between wild type and *abi1-1* under controlled conditions, heat stress or water deficit (Fig. 3A, B), in response to the stress combination the stomatal aperture of *abi1-1* was reduced to levels similar to that of wild type plants (Fig. 3A, B). The measurements of stomatal aperture in control and *abi1-1* plants during stress combination (Fig. 3A, 3B) were in agreement with the stomatal conductance measurements of control and *abi1-1* subjected to the stress combination (Fig. 2A), providing further confidence to this finding. To determine whether the density of stomata in the *abi1-1* mutant would be a factor in affecting overall transpiration during the different stresses we also measured the stomatal density of wild type and *abi1-1* plants. As shown in Fig. 3C, no significant difference was observed between wild type and *abi1-1* plants, both harboring about 200 stomata per mm^{-2}. The results shown in Fig. 3 could explain the differences between reduced growth, biomass and RWC of *abi1-1* and wild type plants shown in Fig. 2, but not likely the differences in *abi1-1* survival in response to the stress combination (Fig. 2).
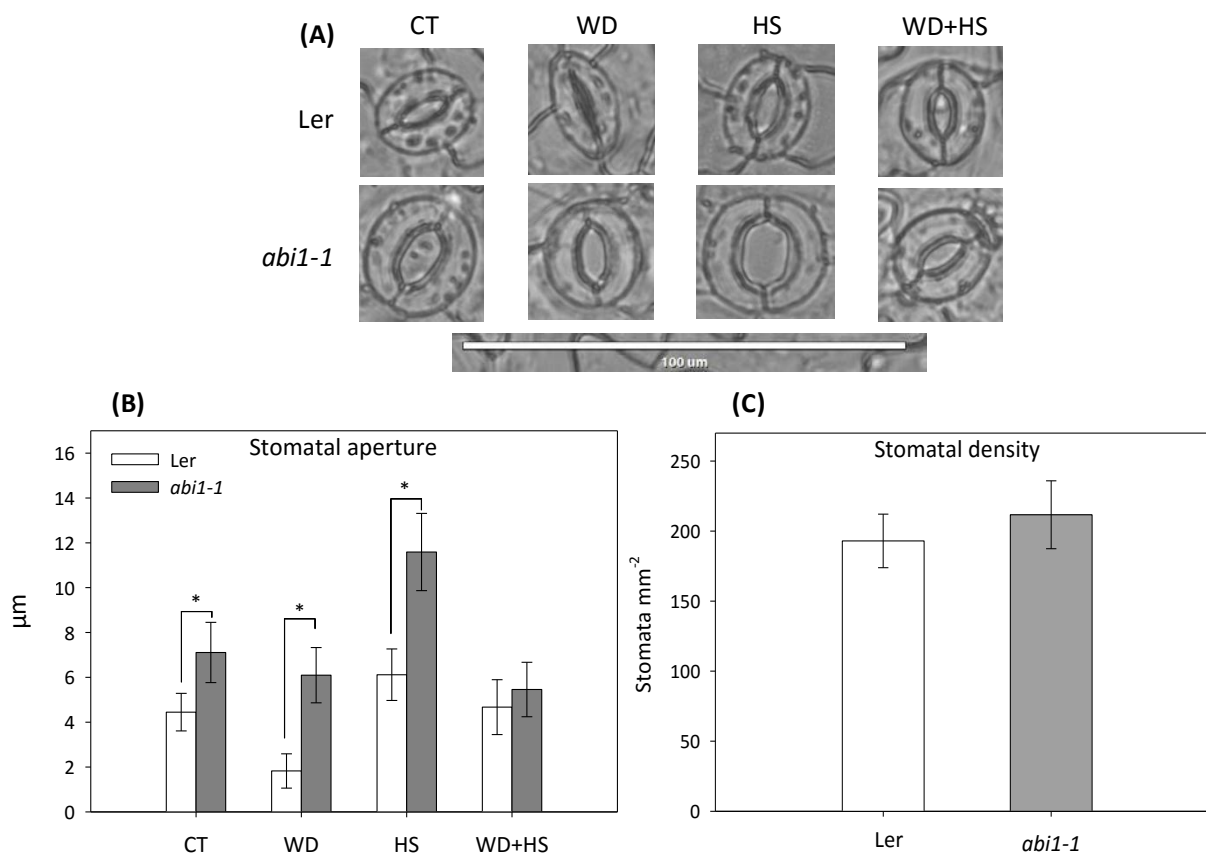
Figure 3. Stomatal aperture of wild type and *abi1-1* plants subjected to a combination of water deficit and heat stresses. (A) Representative images of stomata from wild type and *abi1-1* plants subjected to water deficit (WD), heat stress (HS) and a combination of water deficit and heat stresses (WD+HS). **(B)** Measurements of stomatal aperture of wild type and *abi1-1* plants subjected to the different stresses. **(C)** Stomatal density of wild type and *abi1-1* plants. *, Student's test significant at P < 0.05. Error bars represent SD.

Accumulation of ABA, JA and SA in wild type and abi1-1 *plants subjected to a combination of water deficit and heat stresses*

To examine whether the differences in stomatal responses and plant survival observed between wild type and *abi1-1* plants in response to the stress combination (Figs. 2, 3) were related to the accumulation of ABA, JA and/or SA in leaves, we measured the levels of these hormones in wild type and *abi1-1* plants subjected to the different stresses (Fig. 4). As shown in Fig. 4A, water deficit and a combination of water deficit and heat stress was accompanied by ABA, but not SA or JA accumulation in wild type plants. In addition, JA and SA did not accumulate in wild type leaves in response to water deficit, heat stress, or their combination (Fig. 4B, C). In contrast, *abi1-1* plants accumulated high levels of ABA in response to all stress treatments (Fig. 4A; this observation was further supported by correlation analysis between RWC and ABA in plants subjected to the different stresses; Supplementary Fig. S2),

high levels of SA under control conditions and in response to heat stress (Fig. 4B), and high levels of JA in response to heat stress and a combination of water deficit and heat stress (Fig. 4C). The combination of water deficit and heat stress resulted in the highest accumulation of ABA in both *abi1-1* and wild type plants, suggesting that ABA is important for plant acclimation to this stress combination. The results shown in Fig. 4 could implicate JA-related pathways in the stomatal reduction aperture response of *abi1-1* plants during the stress combination (Fig. 3). In addition they reflect the deficiency in ABA signaling in *abi1-1* plants that result in higher accumulation of ABA in response to stress (Fig. 4A).
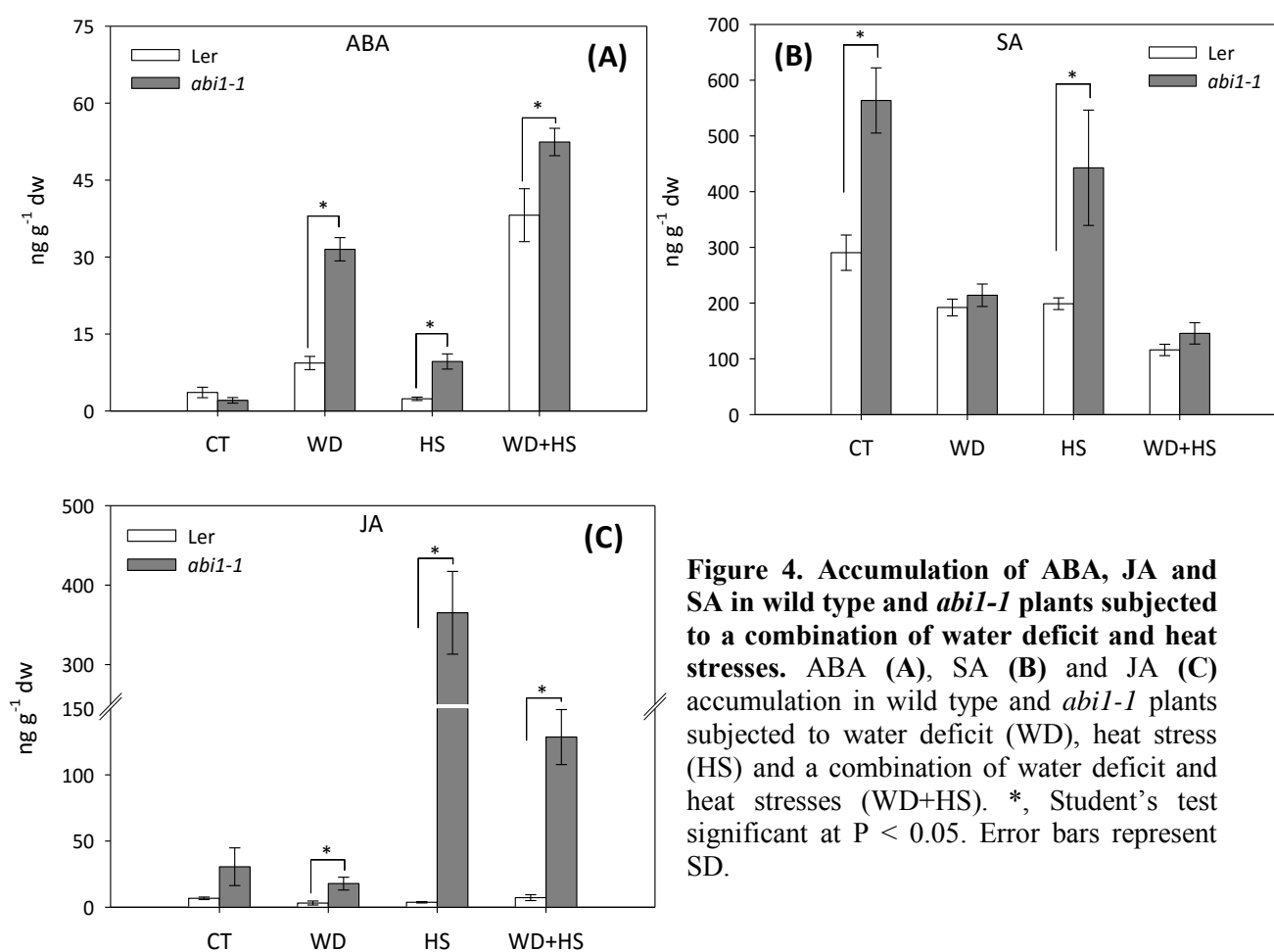
Figure 4. Accumulation of ABA, JA and SA in wild type and *abi1-1* plants subjected to a combination of water deficit and heat stresses. ABA **(A)**, SA **(B)** and JA **(C)** accumulation in wild type and *abi1-1* plants subjected to water deficit (WD), heat stress (HS) and a combination of water deficit and heat stresses (WD+HS). *, Student's test significant at P < 0.05. Error bars represent SD.

H₂O₂ accumulation in wild type and abi1-1 *plants in response to a combination of water deficit and heat stress*

Hydrogen peroxide plays an important role in abiotic stress and stomatal responses (Song *et al.*, 2014; Qiao *et al.*, 2014). To determine whether H_2O_2 plays a role in the regulation of stomatal aperture during a combination of water deficit and heat stress, we measured the levels of H_2O_2 in leaves from plants subjected to the different stresses. As shown in Fig. 5A, H_2O_2 accumulated in wild type plants in response to water deficit and a combination of water deficit and heat stress. In contrast, H_2O_2 accumulated in response to all stress treatments in the *abi1-1* mutant, with the highest levels obtained in *abi1-1* plants subjected to a combination of water deficit and heat stress (Fig. 5A). The high levels of H_2O_2 measured in the leaves of the *abi1-1* mutant in response to the stress combination (Fig. 5A) could explain the closure of stomata in the *abi1-1* mutant during a combination of water deficit and heat stress (Fig. 3). We therefore examined how H_2O_2 or ABA application would affect the stomatal aperture of wild type and *abi1-1* plants. As shown in Fig. 5B, D, application of H_2O_2 (1 mM), but not ABA (30 µM) resulted in a significant reduction of stomatal aperture in the *abi1-1* mutant. The results presented in Fig. 5 could implicate H_2O_2 as an important signaling molecule that promotes stomatal closure in wild type and the *abi1-1* mutant during abiotic stress combination.
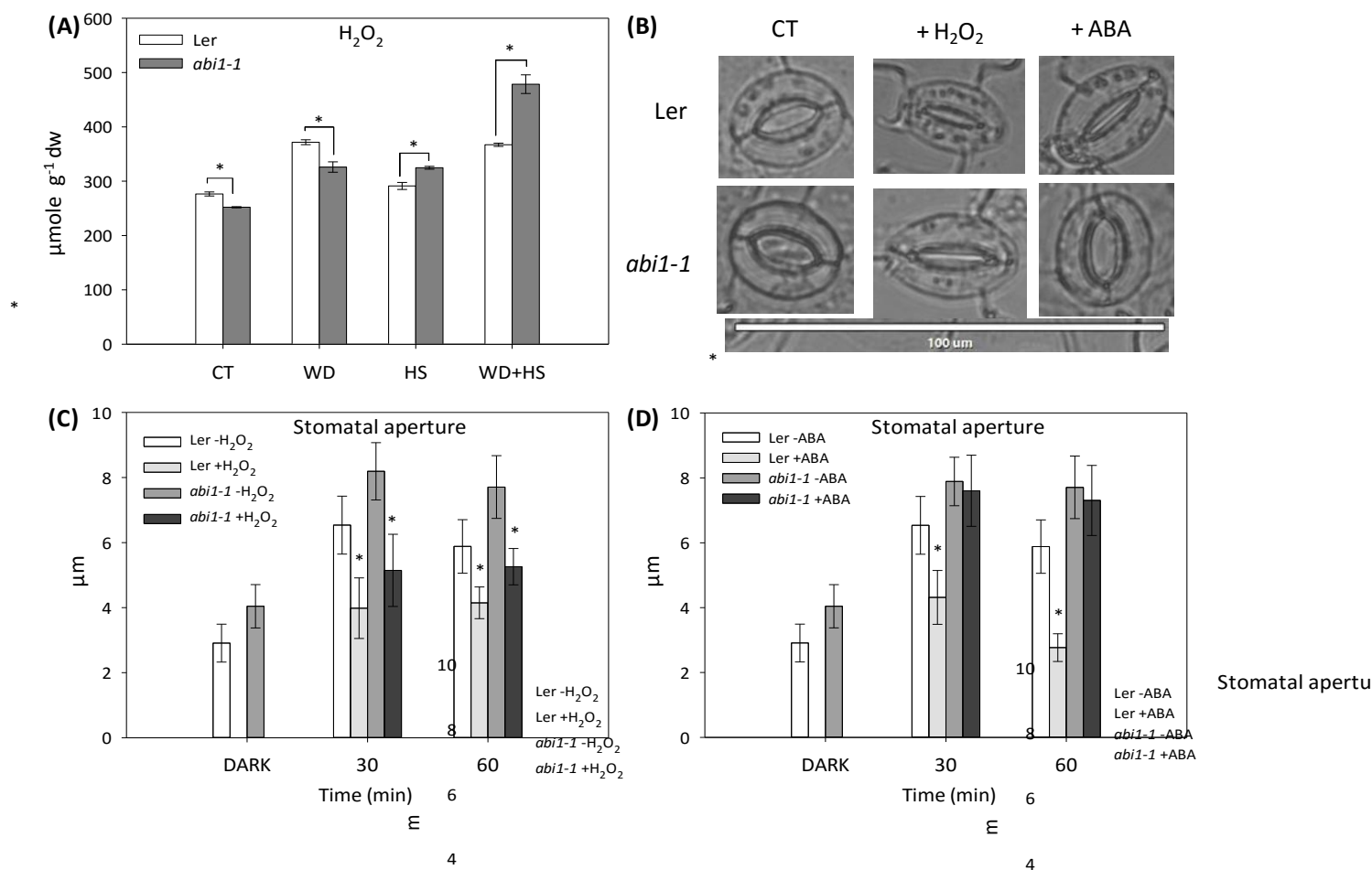
Figure 5. H$_2$O$_2$ accumulation in wild type and *abi1-1* plants in response to a combination of water deficit and heat stress. **(A)** H$_2$O$_2$ accumulation in wild type and *abi1-1* plants subjected to water deficit (WD), heat stress (HS) and a combination of water deficit and heat stresses (WD+HS). **(B)** Representative images of stomata of *Arabidopsis* plants 60 min following the application of H$_2$O$_2$ or ABA. **(C)** Measurements of stomatal aperture of wild type and *abi1-1* plants following application of 1 mM H$_2$O$_2$. **(D)** Measurements of stomatal aperture of wild type and *abi1-1* plants following application of 30 μM ABA. *, Student's test significant at $P < 0.05$. Error bars represent SD.

Accumulation of key acclimation proteins involved in the response of plants to a combination of water deficit and heat stresses in wild type and abi1-1 *plants*

Although the *abi1-1* mutant was more susceptible to a combination of water deficit and heat stress than wild type (Fig. 2), this susceptibility was not reflected in its stomatal responses to the stress combination (Fig. 3), and may not be explained therefore by excessive water loss of the *abi1-1* mutant during the stress combination.

Figure 6. Accumulation of key acclimation proteins involved in the response of plants to a combination of water deficit and heat stresses in wild type and *abi1-1* plants. (A) Protein blot analysis of MBF1c, **(B)** HSP101 and **(C)** APX1 accumulation in leaves of wild type and *abi1-1* plants subjected to water deficit (WD), heat stress (HS) and a combination of water deficit and heat stresses (WD+HS). Top: Quantification bar graphs for fold change in protein accumulation. Bottom: Protein blots and Coomassie-stained gels of total protein. Quantification of protein expression was performed per total protein for 3 different experiments. *, Student's test significant at P < 0.05. Error bars represent SD.

Because ABA plays a dual role during the response of plants to abiotic stress, controlling stomatal responses as well as stress-response transcript and protein expression, we measured the accumulation of 3 key proteins important for plant acclimation to osmotic, heat or oxidative stress (HSP101 and MBF1c; Queitsch *et al.*, 2000; Arce *et al.*, 2010; Suzuki *et al.*, 2011), or a combination of water deficit and heat stress (MBF1c and APX1; Suzuki *et al.*, 2005; Koussevitzky *et al.*, 2008), in wild type and *abi1-1* mutants subjected to water deficit, heat stress and their combination. As shown in Fig. 6, compared to wild type, the accumulation of all three proteins was suppressed in the *abi1-1* mutant in response to the stress combination. Because the expression of APX1 and MBF1c is required for plant acclimation to a combination of water deficit and heat stress (Suzuki *et al.*, 2005; Koussevitzky *et al.*, 2008), the results shown in Fig. 6 could explain the higher susceptibility of the *abi1-1* mutant to the stress combination (Fig. 2), as well as highlight the important role ABA could play in the regulation of acclimation mechanisms during abiotic stress combination.

Meta-analysis of transcriptomic data from ABA-treated abi1-1 *and wild type plants, and wild type plants subjected to a combination of water deficit and heat stress*

The results presented in Fig. 6 strongly support a role for ABA in the accumulation of different acclimation proteins during water deficit and heat stress combination. To examine how broad this role might be we compared the transcriptome of wild type plants (Col) subjected to a combination of water deficit and heat stress (Rizhsky *et al.*, 2004) with that of wild type plants (Ler) treated with 50 μM ABA (Hoth *et al.*, 2002). As shown in Fig. 7A, 106 transcripts were common between the 1187 up-regulated transcripts during water deficit and heat stress combination (Rizhsky *et al.*, 2004) and the 659 transcripts up-regulated following ABA treatment of unstressed wild type plants (Hoth *et al.*, 2002). A similar overlap of 55 transcripts was found between the 791 down-regulated transcripts during water deficit and heat stress combination and the 694 transcripts down-regulated following ABA treatment of unstressed wild type plants (Fig. 7B). When the same overlap was tested between transcripts up- or down-regulated in wild type (Col) plants in response to a combination of water deficit and heat stress (Rizhsky *et al.*, 2004), and transcripts up- or down-regulated in the *abi1-1* mutant (Ler) in response to ABA treatment of unstressed plants (50 μM; Hoth *et al.*, 2002) (Fig. 7C, D), it was found that the number of overlapped transcripts between the two groups decreased by about 75% (up-regulated) and 50% (down-regulated) respectively. This finding could suggest that in addition to HSP101, MBF1c and APX1 (Fig. 6), *abi1-1* could be

involved in the regulation of several other acclimation pathways in plants in response to a combination of water deficit and heat stress. A list of the transcripts that could be under the control of *abi1-1* in response to the stress combination is included in Supplementary Table S2.
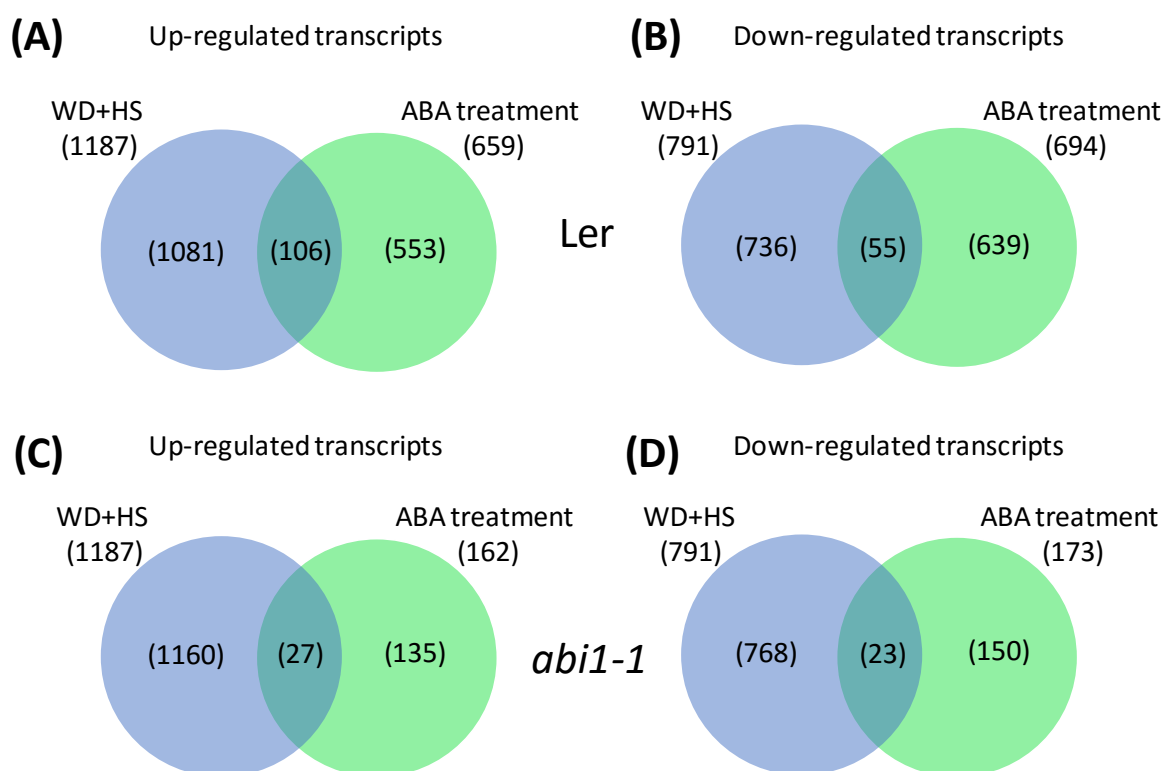
Figure 7. Meta-analysis of transcriptomics data from ABA-treated *abi1-1* and wild type plants, and wild type plants subjected to a combination of water deficit and heat stress. Top: Venn diagrams showing the overlap between transcripts specifically up- **(A)** or down- **(B)** regulated in response to a combination of water deficit and heat stresses, or ABA treatment, of wild type plants. Bottom: Venn diagrams showing the overlap between transcripts specifically up- **(C)** or down- **(D)** regulated in response to a combination of water deficit and heat stresses of wild type plants, or ABA treatment in *abi1-1* plants. References used for the meta-analysis are Hoth *et al.* (2002) and Rizhsky *et al.* (2004).

Discussion

We recently conducted transcriptomic analysis of *Arabidopsis* plants subjected to a combination of salinity and heat stress and identified many ABA-response transcripts within the group of transcripts that were specifically expressed in response to the stress combination (Suzuki *et al.*, 2016). We subsequently determined that mutants impaired in ABA synthesis (*aba1-1)*, or signaling (*abi1-1*), were more susceptible than wild type plants to a combination of salinity and heat stress (Suzuki *et al.*, 2016). Nonetheless, whether this enhanced susceptibility to the stress combination was due to impaired stomatal responses, deficiency in the expression of different acclimation transcripts and proteins, or both, was unclear.

To deepen our understanding of ABA's role in the response of plants to a combination of different abiotic stresses, we focused in the current study on the acclimation of plants to a combination of water deficit and heat stress. Our findings that the *abi1-1* and *aba1-1* mutants are susceptible to this stress combination (Figs. 1, 2; Supplementary Table S1), that the stress combination was accompanied by elevated accumulation of ABA in wild type and *abi1-1* plants (Fig. 4), and that many transcripts involved in the response of plants to a combination of water deficit and heat stress are ABA-response transcripts (Fig. 7), demonstrated that ABA is required for the acclimation of plants to yet another type of stress combination, i.e., water deficit and heat stress. These findings underscored a possible general role for ABA in the acclimation of plants to abiotic stress combinations. Further studies focused on the acclimation of mutants deficient in ABA metabolism and signaling to additional abiotic stress combinations would reveal how broad spectrum the role of ABA is in the response of plants to stress combination.

We further focused on the *abi1-1* mutant and examined whether ABA is required for stomatal responses, expression of different acclimation transcripts and proteins, or both (Figs. 3, 6), and surprisingly found that the stomata of *abi1-1* plants, although impaired in their responses to water deficit (Figs. 2, 3), reduced their aperture to levels that are similar to that of wild type plants in response to the combination of heat stress and water deficit (Fig. 3). This finding suggested that ABI1 might not be required for stomatal closure during the stress combination, and that the decreased survival of *abi1-1* plants subjected to the stress combination (Fig. 2), could not be simply explained by enhanced water loss during the stress combination due to impaired stomatal responses (although some loss of RWC was observed in *abi1-1* plants during the stress combination; Fig. 2A). In contrast to the almost wild type

stomatal phenotype of *abi1-1* plants under the stress combination (Fig. 3), the accumulation of three proteins important for plant acclimation to heat stress (HSP101 and MBF1c) and a combination of water deficit and heat stress (MBF1c and APX1) was attenuated in *abi1-1* plants during the stress combination (Fig. 6). Taken together, the findings shown in Figs. 3 and 6 suggest that the cause of the enhanced susceptibility of *abi1-1* plants to the stress combination (Fig. 2) could be an outcome of the inability of these plants to mount an acclimation response involving the accumulation of MBF1c and APX1 (Fig. 6), as opposed to their inability to close their stomata (Fig. 3). ABA may therefore be required for the accumulation of key proteins required for the acclimation of plants to a combination of different stresses (Fig. 6).

In an attempt to address the question of how the stomata of *abi1-1* had a reduced aperture during the stress combination, we measured the levels of ABA, JA, SA and H_2O_2 in plants subjected to the stress combination. As shown in Figs. 4 and 5, the stress combination was accompanied by a unique combination of high H_2O_2, high JA and low SA in leaves of *abi1-1* plants. Under this combination, H_2O_2 and JA can signal stomatal closure, independent of ABA signaling, by enhancing nitric oxide (NO) levels and triggering Ca^{2+} and SLAC1 function (Fig. 8; Daszkowska-Golec and Szarejko, 2013; Murata *et al.*, 2015). In contrast, during heat stress when both SA and JA are enhanced (Fig. 4), SA could antagonize JA function and stomata will open in *abi1-1* (Figs. 3, 8; Thaler *et al.*, 2012; Caarls *et al.*, 2015). In support of the proposed role of H_2O_2 in mediating stomatal closer in *abi1-1* plants, the application of H_2O_2, but not ABA, was able to induce stomatal closer in unstressed *abi1-1* plants (Fig. 5B, D). Our findings point to an alternative pathway that may be involved in the stomatal responses of plants subjected to stress combination, involving JA and/or H_2O_2 (Fig. 8).
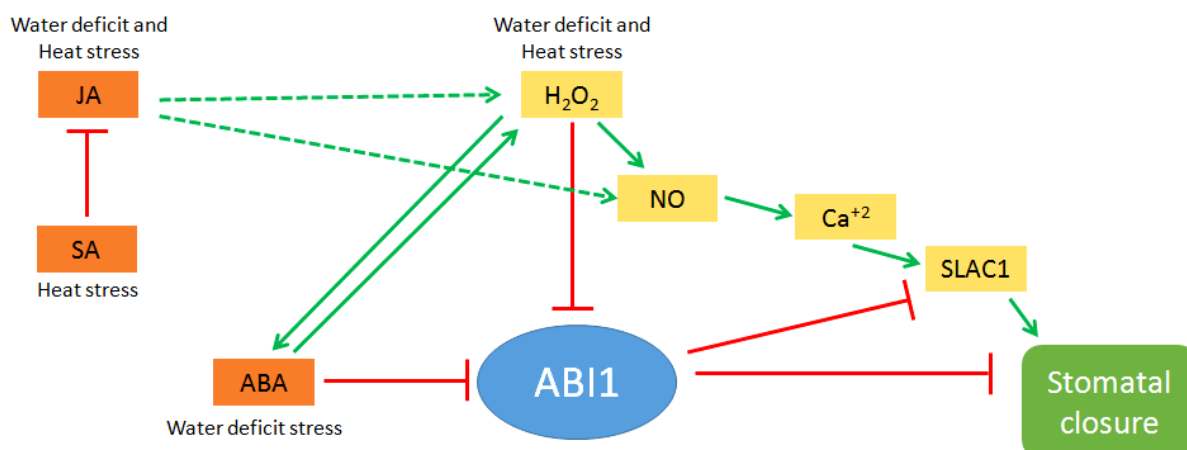
Figure 8. A hypothetical model for the signaling role of JA, SA, ABA and H₂O₂ in the regulation of stomatal aperture in *abi1-1* during water deficit, heat stress and a combination of water deficit and heat stress. Dotted lines indicate hypothetical interactions. Solid lines and arrows indicate positive and negative regulation based on published literature, respectively. Abbreviations: JA, Jasmonic acid; SA, Salicylic acid; ABA, Abscisic acid; NO, Nitric oxide; SLAC1, SLOW ANION CHANNEL-ASSOCIATED 1.

Further studies are of course needed to address this possibility, including the analysis of additional mutants in ABA, JA and ROS signaling and direct measurements of H_2O_2, JA, SA, NO and ABA in the stomata of plants subjected to stress combination. Because ABA is required for different physiological acclimation responses, as well as for the regulation of protein and transcript accumulation during different stresses and their combination, it could function as an overall regulator that tailors the plant response to the different environmental conditions.

Acknowledgements

Conflicts of Interest

Authors declare that there are no conflicts of interest.

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Author contribution

SIZ and RM designed and supervised the research. SIZ, DB, MAI, VA and AGC performed the research. SIZ and RM wrote the manuscript.

Supplementary Material
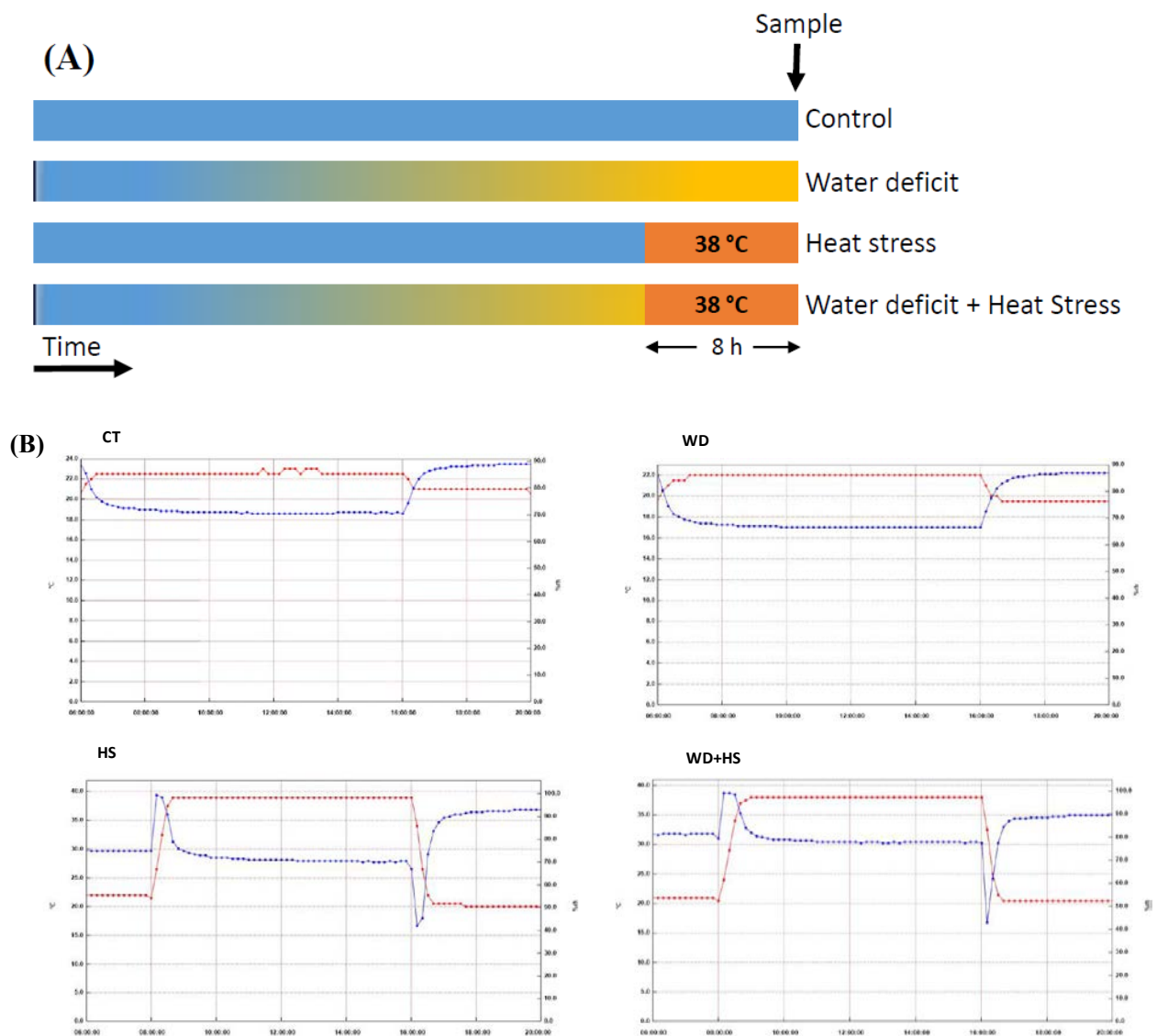
Figure S1. (A) The experimental design used to subject wild type, *aba1-1* and *abi1-1* plants to water deficit (yellow), heat stress (red) and a combination of water deficit and heat stress (orange). **(B)** Temperature and humidity measurements for control (CT), water deficit (WD), heat stress (HS) and a combination of water deficit and heat stresses (WD+HS), recorded inside the growth chamber with a datalogger.
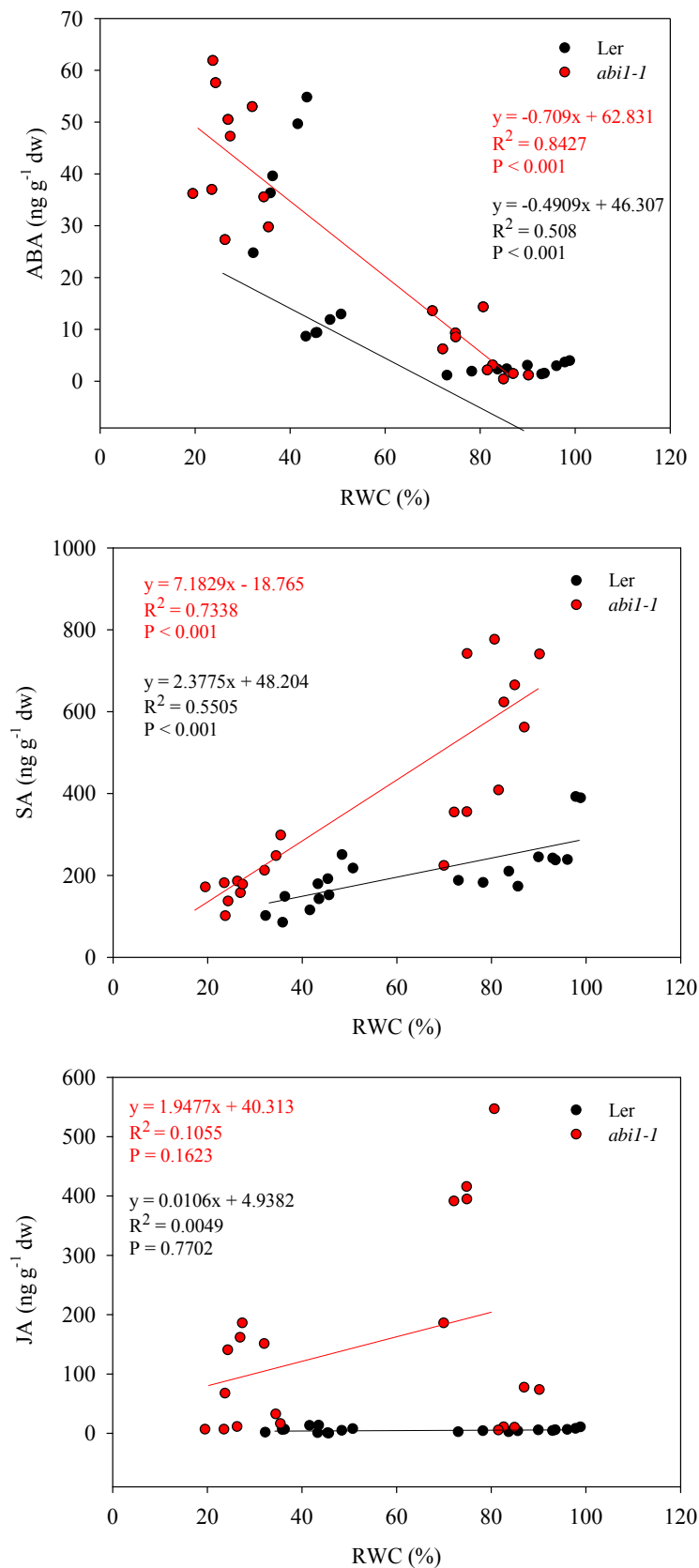
Figure S2. Correlation analysis between RWC and hormonal concentrations (ABA, SA and JA) obtained for Ler and *abi1-1* plants under water deficit, heat stress and a combination of water deficit and heat stress.

Table S1. Analysis of variance of growth characteristic parameters and hormonal concentrations for *aba1-1* and *abi1-1* plants. P<0.05 denotes statistical significance.

		P-value		
	Parameter	Genotype	Stress	Interaction *genotype x stress*
aba1-1	FW	<0.0001	<0.0001	0.1648
	DW	<0.0001	0.0002	0.4085
	RWC	0.0008	<0.0001	0.5047
	Diameter	<0.0001	<0.0001	0.4163
	Survival	0.0085	<0.0001	0.0010
	gs	<0.0001	<0.0001	0.0007
abi1-1	FW	<0.0001	<0.0001	0.0782
	DW	<0.0001	<0.0001	0.0105
	RWC	<0.0001	<0.0001	0.0575
	Diameter	<0.0001	<0.0001	0.0005
	Survival	<0.0001	<0.0001	<0.0001
	gs	<0.0001	<0.0001	0.0250
	ABA	<0.0001	<0.0001	0.0001
	JA	<0.0001	<0.0001	<0.0001
	SA	0.0001	<0.0001	0,0086

Table S2. List of the transcripts that could be under the control of *abi1-1* in response to the stress combination.

| Upregulated: 27	
AT number	**Short description**
At1g52690	Late embryogenesis abundant protein (LEA) family protein; BEST Arabidopsis thaliana protein match is: Late embryogenesis abundant protein (LEA) family protein (TAIR:AT3G15670.1)
At5g06760	Late embryogenesis abundant protein LEA like (D113), encodes LEA4-5, a member of the Late Embryogenesis Abundant (LEA) proteins which typically accumulate in response to low water availability conditions imposed during development or by the environment.
At5g66400	Dehydrin RAB18-like protein, belongs to the dehydrin protein family. ABA- and drought-induced glycine-rice dehydrin protein.
At2g37870	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein.
At3g48530	SNF1-related protein kinase regulatory subunit gamma 1 (KING1); FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: plant-type cell wall; EXPRESSED IN: 23 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: Cystathionine beta-synthase, core (InterPro:IPR000644)
At4g27410	Encodes a NAC transcription factor induced in response to dessication. It is localized to the nucleus and acts as a transcriptional activator in ABA-mediated dehydration response.
At1g48970	NagB/RpiA/CoA transferase-like superfamily protein; FUNCTIONS IN: GTP binding, translation initiation factor activity; INVOLVED IN: translational initiation, cellular metabolic process; LOCATED IN: eukaryotic translation initiation factor 2B complex; EXPRESSED IN: 23 plant structures; EXPRESSED DURING: 13 growth stages.
At2g39800	AtP5C1, encodes a delta1-pyrroline-5-carboxylate synthase that catalyzes the rate-limiting enzyme in the biosynthesis of proline. Expression is induced by abscisic acid and salt stress in a light-dependent manner.
At2g33380	AtRD20, encodes a calcium binding protein whose mRNA is induced upon treatment with NaCl, ABA and in response to dessication. mRNA expression under drought conditions is apparent particularly in leaves and flowers. Isoform of caleosin with a role as a peroxygenase involved in oxylipin metabolism during biotic and abiotic stress.
At4g26080	AtABI1 Involved in abscisic acid (ABA) signal transduction. Negative regulator of ABA promotion of stomatal closure.
At5g12030	Heat shock protein 17.6A, encodes a cytosolic small heat shock protein with chaperone activity that is induced by heat and osmotic stress and is also expressed late in seed development.
At4g34000	Abscisic acid responsive elements-binding factor (ABRE/ABF3). Encodes an ABA-responsive element-binding protein with similarity to transcription factors that is expressed in response to stress and abscisic acid.
At5g11110	Encodes a protein with putative sucrose-phosphate synthase activity.Involved in pollen exine formation.
At4g30460	Glycine-rich protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: endomembrane system; EXPRESSED IN: 20 plant structures; EXPRESSED DURING: 12 growth stages
At3g04000	ChlADR is an aldehyde reductase that catalyzes the reduction of the aldehyde carbonyl groups on saturated and alpha,beta-unsaturated aldehydes with more than 5 carbons in vitro. The N-terminal region of this protein directs GFP to the chloroplast where where ChlADR likely helps to maintain the photosynthetic process by detoxifying reactive carbonyls formed during lipid peroxidation.
At2g42540	A cold-regulated gene whose product is targeted to the chloroplast. Cor15am protects stromal proteins from aggregation under various stress conditions. Constitutive expression increases freezing tolerance in protoplasts in vitro and chloroplasts in vivo. NMR and x-ray diffraction studies suggest that COR15a alters the intrinsic curvature of the inner membrane of chloroplast envelope. Late Embryogenesis abundant protein (LEA).

At3g08860	Encodes a protein that is predicted to have beta-alanine aminotransferase activity.
At3g57540	Remorin family protein; CONTAINS InterPro DOMAIN/s: Remorin, C-terminal (InterPro:IPR005516); BEST Arabidopsis thaliana protein match is: Remorin family protein (TAIR:AT2G41870.1)
At2g47470	Encodes a protein disulfide isomerase-like (PDIL) protein, a member of a multigene family within the thioredoxin (TRX) superfamily. Transcript levels for this gene are up-regulated in response to three different chemical inducers of ER stress (dithiothreitol, beta-mercaptoethanol, and tunicamycin). AtIRE1-2 does not appear to be required for this response, but the atbzip60 mutant has a diminished response.
At3g53980	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein; FUNCTIONS IN: lipid binding; INVOLVED IN: lipid transport; LOCATED IN: endomembrane system; EXPRESSED IN: 10 plant structures; EXPRESSED DURING: 7 growth stages; CONTAINS InterPro DOMAIN/s: Bifunctional inhibitor/plant lipid transfer protein/seed storage (InterPro:IPR016140)
At5g61820	FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: vacuole; EXPRESSED IN: 24 plant structures; EXPRESSED DURING: 15 growth stages; CONTAINS InterPro DOMAIN/s: Stress up-regulated Nod 19 (InterPro:IPR011692)
At1g34630	BEST Arabidopsis thaliana protein match is: Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein (TAIR:AT5G51150.1)
At1g79520	Cation efflux family protein; FUNCTIONS IN: cation transmembrane transporter activity; INVOLVED IN: cation transport, transmembrane transport; LOCATED IN: membrane; EXPRESSED IN: 18 plant structures; EXPRESSED DURING: 10 growth stages; CONTAINS InterPro DOMAIN/s: Cation efflux protein (InterPro:IPR002524)
At3g60350	ARABIDILLO-2 and its homolog, ARABIDILLO -1, are unique among Arabidopsis Arm-repeat proteins in having an F-box motif and fall into a phylogenetically distinct subgroup from other plant Arm-repeat proteins Similar to arm repeat protein in rice and armadillo/beta-catenin repeat family protein / F-box family protein in Dictyostelium.
At2g43570	Chitinase, putative (CHI); FUNCTIONS IN: chitin binding, chitinase activity; INVOLVED IN: carbohydrate metabolic process, cell wall macromolecule catabolic process; LOCATED IN: apoplast, plant-type cell wall; EXPRESSED IN: 15 plant structures; EXPRESSED DURING: 10 growth stages.
At4g16190	Papain family cysteine protease; FUNCTIONS IN: cysteine-type peptidase activity, cysteine-type endopeptidase activity; INVOLVED IN: proteolysis; LOCATED IN: vacuole; EXPRESSED IN: 23 plant structures;
At1g74310	Heat shock protein 101, encodes ClpB1, which belongs to the Casein lytic proteinase/heat shock protein 100 (Clp/Hsp100) family. Involved in refolding of proteins which form aggregates under heat stress. Also known as AtHsp101. AtHsp101 is a cytosolic heat shock protein required for acclimation to high temperature.

Downregulated 23	
AT number	**Short description**
At5g10390	Histone superfamily protein; FUNCTIONS IN: DNA binding; INVOLVED IN: nucleosome assembly; LOCATED IN: chloroplast, nucleosome; EXPRESSED IN: 23 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: Histone H3 (InterPro:IPR000164), Histone-fold (InterPro:IPR009072), Histone core (InterPro:IPR007125)
At4g16500	Cystatin/monellin superfamily protein; FUNCTIONS IN: enzyme regulator activity, cysteine-type endopeptidase inhibitor activity; INVOLVED IN: biological_process unknown; LOCATED IN: cell wall, vacuole; EXPRESSED IN: 24 plant structures; EXPRESSED DURING: 15 growth stages.
At5g59870	Encodes HTA6, a histone H2A protein.
At2g38310	Encodes a member of the PYR (pyrabactin resistance)/PYL(PYR1-like)/RCAR (regulatory components of ABA receptor) family proteins with 14 members. PYR/PYL/RCAR family proteins function as abscisic acid sensors. Mediate ABA-dependent regulation of protein phosphatase 2Cs ABI1 and ABI2.
At4g20780	Calcium sensor involved in trichome branching.
At5g05440	Encodes a member of the PYR (pyrabactin resistance)/PYL(PYR1-like)/RCAR (regulatory components of ABA receptor) family proteins with 14 members. PYR/PYL/RCAR family proteins function as abscisic acid sensors. Mediate ABA-dependent regulation of protein phosphatase 2Cs ABI1 and ABI2.
At4g11290	Peroxidase ATP19a, peroxidase superfamily protein; FUNCTIONS IN: peroxidase activity, heme binding; INVOLVED IN: response to oxidative stress, oxidation reduction; LOCATED IN: endomembrane system; EXPRESSED IN: 11 plant structures; EXPRESSED DURING: 7 growth stages.
At2g06850	Endoxyloglucan transferase (EXGT-A1) gene.
At2g10940	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein; FUNCTIONS IN: lipid binding; INVOLVED IN: lipid transport; LOCATED IN: chloroplast thylakoid membrane, apoplast, chloroplast, membrane; EXPRESSED IN: 21 plant structures; EXPRESSED DURING: 13 growth stages.
At2g22170	Lipase/lipooxygenase, PLAT/LH2 family protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: thylakoid, chloroplast, membrane; EXPRESSED IN: 19 plant structures; EXPRESSED DURING: 13 growth stages.
At1g56430	Encodes a protein with nicotianamine synthase activity.
At2g14560	Encodes LURP1, a member of the LURP cluster (late upregulated in response to Hyaloperonospora parasitica) which exhibits a pronounced upregulation after recognition of the pathogenic oomycte H. parasitica. LURP1 is required for full basal defense to H. parasitica and resistance to this pathogen mediated by the R-proteins RPP4 and RPP5.
At3g05730	Encodes a defensin-like (DEFL) family protein.
At5g64290	2-oxoglutarate/malate translocator, dicarboxylate transport 2.1 (DIT2.1); FUNCTIONS IN: oxoglutarate:malate antiporter activity; INVOLVED IN: malate transport, response to nematode; LOCATED IN: chloroplast, membrane, chloroplast envelope; EXPRESSED IN: 23 plant structures; EXPRESSED DURING: 13 growth stages.
At1g67330	Protein of unknown function (DUF579); FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: endomembrane system; EXPRESSED IN: 8 plant structures; EXPRESSED DURING: LP.06 six leaves visible, LP.04 four leaves visible, LP.10 ten leaves visible, 4 leaf senescence stage.
At3g06750	hydroxyproline-rich glycoprotein family protein; Has 6317 Blast hits to 3436 proteins in 319 species: Archae - 0; Bacteria - 429; Metazoa - 2195; Fungi - 739; Plants - 2175; Viruses - 83; Other Eukaryotes - 696 (source: NCBI BLink).
At3g12710	DNA glycosylase superfamily protein; FUNCTIONS IN: DNA-3-methyladenine glycosylase I activity, catalytic activity;

At3g00750	hydroxyproline-rich glycoprotein family protein; Has 6517 Blast hits to 5450 proteins in 519 species: Archae - 0; Bacteria - 429; Metazoa - 2195; Fungi - 739; Plants - 2175; Viruses - 83; Other Eukaryotes - 696 (source: NCBI BLink).
At3g12710	DNA glycosylase superfamily protein; FUNCTIONS IN: DNA-3-methyladenine glycosylase I activity, catalytic activity; INVOLVED IN: DNA repair, base-excision repair; EXPRESSED IN: 20 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro.
At5g65360	Histone superfamily protein; FUNCTIONS IN: DNA binding; INVOLVED IN: nucleosome assembly; LOCATED IN: chloroplast, nucleosome; EXPRESSED IN: 23 plant structures; EXPRESSED DURING: 14 growth stages; CONTAINS InterPro
At5g62920	Encodes a Type-A response regulator that is responsive to cytokinin treatment. Its C-ter domain is very short in comparison to other Arabidopsis ARRs (17 total). Arr6 protein is stabilized by cytokinin.

General discussion

General discussion

Plants must deal with a wide range of environmental challenges which often occur simultaneously, imposing severe consequences on plants. The responses to these stress combinations are based on the activation of specific changes in plant physiology, transcriptome and metabolism, that allow plants to avoid damages (Mittler 2006, Mittler and Blumwald 2010). These specific responses cannot be predicted from the study of the effects of individual stress conditions and, therefore, new experimental approaches need to be developed in order to model broad-spectrum stress-tolerant plants in the future (Rizhsky et al. 2004, Mittler 2006). Among the stress combinations, drought and high temperatures are the most frequent abiotic stress interaction occurring in natural environments (Mittler 2006). This situation has been previously suggested to have important damaging effects on plant growth and productivity (Savin and Nicolas 1996, Jiang and Huang 2001, Craufurd et al. 2008). In addition, according to different studies in several plant species, the response of plants to a combination of drought and high temperatures is exclusive and different of that of drought or heat applied individually (Rizhsky et al. 2002, Rizhsky et al. 2004, Koussevitzky et al. 2008, Prasad et al. 2011, Vile et al. 2012, Rollins et al. 2013). This stress combination is especially important in the Mediterranean area, where summer drought accompanied by high temperatures is relatively frequent, limiting crop growth, development and productivity. For this reason, in the first chapter of this work, we aimed to study the physiological and molecular responses and the relative tolerance of two citrus genotypes (Carrizo citrange and Cleopatra mandarin) subjected to drought, heat or to a combination of both stress factors. These two citrus rootstocks, widely used in citriculture, have been previously reported to show different tolerance to abiotic stress conditions: whereas Cleopatra is more tolerant to drought and salinity, Carrizo is more tolerant to soil flooding (Argamasilla et al. 2013). On the contrary, limited information of the responses of citrus plants to combined stress conditions is currently available. According to our phenotypical, physiological and biochemical data, Cleopatra is more susceptible than Carrizo to heat stress, applied individually or in combination with water deficit. In this sense, new sprouts on Carrizo remained visibly healthy during a longer period of time than those on Cleopatra when plants were subjected to heat stress alone or in combination with drought. Hence, this result reflects the higher ability of Carrizo

seedlings to tolerate high temperatures. In addition, the analysis of physiological responses in terms of gas exchange and chlorophyll fluorescence parameters supports this conclusion. Heat stress increased transpiration in both citrus genotypes, probably aimed to decrease leaf surface temperature via evaporative cooling. However, a lower increase in transpiration rate was observed in Cleopatra and this increment did not have a parallel effect on A or g_s. Therefore, the higher transpiration rate observed in Carrizo plants under high temperatures could be involved in a lower leaf temperature as previously reported (Rizhsky et al. 2002), constituting an advantage against heat. Moreover, inhibition of net CO_2 assimilation was more pronounced in Cleopatra than in Carrizo during high temperatures, as evidenced by C_i/C_a ratio. Consequently, the capacity of plants to regulate leaf gas exchange maintaining optimal CO_2 assimilation is directly associated to high temperature stress tolerance (Mathur et al. 2014). On the other hand, during the combination of drought and high temperatures, the effect of drought on gas exchange parameters predominated over heat stress, suggesting that stomatal closure to minimize water loss prevailed over responses that could lead to a reduction of leaf surface temperature. Despite this, transpiration rate remained significantly higher in Carrizo than in Cleopatra, pointing again to an improved mechanism to refrigerate leaf surface. Studies in *Arabidopsis thaliana* (Rizhsky et al. 2004) and tobacco plants (Rizhsky et al. 2002) subjected to drought, heat and their combination have obtained similar results, suggesting that, overall, combination of drought and heat affects plants in a different manner and that this stress combination does not allow plants to cool their leaves by increasing transpiration as in heat stress, facing thus a more damaging situation.

The stronger negative effect of heat stress on Cleopatra, especially when combined with drought, was confirmed by chlorophyll fluorescence data, since PSII, and particularly the oxygen-evolving complex, has been reported to be one of the most heat-sensitive component of the photosynthetic system (Lu and Zhang 2000, Wang et al. 2010, Mathur et al. 2014). Therefore, while PSII performance (F_v/F_m) and photosynthetic electron flow (Φ_{PSII}) significantly decreased during heat and a combination of drought and heat in Cleopatra plants, PSII values in Carrizo were only affected by the stress combination. In addition, combined heat and drought showed the most detrimental effect in both citrus genotypes, resulting in a strong reduction in maximum PSII efficiency (F_v/F_m). In line with the impairment of photochemistry, induction of oxidative damage due to ROS

production was higher in citrus plants subjected to drought and heat combination than to individual stresses, as indicated by MDA accumulation. Although both citrus genotypes showed increased MDA levels under stress combination, a higher accumulation was observed in Cleopatra respect to Carrizo, indicating a stronger incidence of oxidative damage associated to a higher ROS production and also to a less efficient antioxidant system, as previously shown (Arbona et al. 2008).

In this first chapter, we also analyzed hormonal responses of both citrus plants under individual and combined drought and heat stresses to provide a more comprehensive view of the responses of citrus plants to the combination of abiotic stress factors. Our results showed that ABA and SA levels were significantly altered during our stress conditions. While ABA is a key hormone involved in tolerance to abiotic stress (Danquah et al. 2014, Yoshida et al. 2014), SA has been involved in biotic responses (Vlot et al. 2009, Dempsey et al. 2011, Boatwright and Pajerowska-Mukhtar 2013) as well as in induction of plant thermotolerance (Dat et al. 2000, Clarke et al. 2004, Larkindale and Huang 2005, Wang and Li 2006, Clarke et al. 2009). In response to stress treatments, SA levels increased in both citrus genotypes, especially during a combination of drought and heat, showing an additive accumulation and reinforcing the higher impact of combined stresses on plant physiology. In addition, *CsPR2* transcripts, a gene induced by SA in citrus plants (Coqueiro et al. 2015), accumulated in response to heat stress applied individually or in combination with drought, mainly in Cleopatra seedlings. This result suggests that Cleopatra could boost up this response in order to alleviate the damaging effects of heat stress on the photosynthetic system, since SA has been reported to be involved in protecting PSII complex (Wang et al. 2010, Wang et al. 2014) and in the maintenance of membrane integrity during heat stress (Clarke et al. 2004). Whereas SA levels followed an additive accumulation pattern in response to individual and combined stress conditions, ABA content showed opposite trends in response to heat or drought. Therefore, the combination of the two stress conditions induced a nearly intermediate response. Drought induced a strong ABA accumulation, coincident with stomatal closure, whereas high temperatures greatly inhibited this response. On the other hand, combined drought and heat induced a more moderate ABA accumulation with respect to drought. However, these remaining ABA levels, along with the increased incidence of oxidative damage (indicating an excess H_2O_2 production) participated in the reduction of gas exchange parameters as observed under

drought (Murata et al. 2001, Mittler and Blumwald 2015). The regulation of ABA content is mediated by a coordinated action of biosynthesis, catabolism and conjugation yielding ABAGE (Nambara and Marion-Poll 2005, Priest et al. 2006, Lee et al. 2006, Umezawa et al. 2010). Our results indicate that the strong accumulation of ABA in citrus plants during drought was accompanied by an up-regulation of *CsNCED1*, *CsCYP707A1*, *CsAOG* and *CsBG18* leading to significant amounts of PA, DPA and ABAGE. On the other hand, heat stress induced ABA degradation mainly through catabolism, probably aimed to increase stomatal opening and transpiration, allowing an adequate cooling of leaves, as recently shown in *Arabidopsis thaliana* (Dobrá et al. 2015). The combination of drought and heat moderately increased ABA levels in parallel with a moderate *CsNCED1* up-regulation, and activated ABA catabolism, preventing a strong accumulation of ABA as during drought.

Overall, in this first chapter it is demonstrated the different ability of two citrus genotypes, Carrizo citrange and Cleopatra mandarin, to tolerate drought and heat applied alone or in combination. Results indicate that Carrizo is more tolerant to heat applied alone or in combination with drought than Cleopatra due to an improved leaf cooling via enhanced transpiration along with the ability to modulate photosynthetic electron flow and a lower incidence of oxidative damage. In both citrus genotypes, stress combination represents a more damaging situation than the individual stresses, inducing a higher damage to PSII leading to the accumulation of MDA. In addition, SA levels and associated signaling positively correlate with stress sensitivity, being more pronounced in Cleopatra. Although *de novo* biosynthesis has a key role regulating ABA levels under stress as shown in different plant species (Nambara and Marion-Poll 2005, Han et al. 2009, Woo et al. 2011), the activation of ABA degradation and conjugation could contribute to fine-tune hormone levels under different stress conditions. In this sense, the different pattern of ABA accumulation and the specific transcriptional regulation of genes involved in ABA metabolism suggest a unique mechanism of response to each stress condition applied individually or in combination.

The response and acclimation mechanisms of plants to abiotic stress not only include the activation of specific physiological, hormonal and molecular responses, but also changes in plant metabolism. In the second chapter, therefore, we intended to study changes in primary and secondary metabolism of Carrizo and Cleopatra in response to individual drought and heat, and combined stresses. Primary and secondary metabolites

were differentially altered during each stress condition in both citrus genotypes. In Cleopatra, a total of 77 polar compounds increased in response to stress, of which 49 responded to combined stress factors. On the other hand, only 8 polar metabolites were up-regulated in Carrizo leaves in response to this stress combination. Among polar metabolites studied in this second chapter, some compounds involved in TCA cycle and glycolysis were identified. In Cleopatra, these primary pathways were activated in an additive manner, suggesting that the combination of drought and heat stress could have a higher impact in Cleopatra compared to Carrizo, paralleling with the higher sensitivity to this stress combination. Since TCA cycle represents a key metabolic pathway for aerobic processes to produce energy and reducing power (Fernie et al. 2004), our data suggest a higher requirement of energy in Cleopatra with respect to Carrizo to face a the stress combination. Apart from TCA cycle, we also analyzed the glyoxylate/dicarboxylate cycle as a pathway involved in dissipating excess of reducing equivalents and energy to prevent ROS accumulation, mitigating thus oxidative stress (Voss et al. 2013). In this sense, Cleopatra consumed more ATP and reducing power and down-regulated sucrose in response to combined stresses, suggesting a higher impact of oxidative stress associated to a higher ROS production as shown in the first chapter. On the other hand, Carrizo accumulated sucrose via gluconeogenesis in response to heat stress and the combination of drought and heat stress, probably related to higher photosynthetic rate observed previously and constituting hence an advantage under these conditions. In addition, phenylpropanoid pathway intermediates are reported to be involved in plant responses to stress (Kai et al. 2008). In this sense, whereas Cleopatra leaves accumulated scopolin especially under combined conditions, Carrizo induced the accumulation of sinapic acid and derivatives, direct precursors of lignins. In short, we demonstrated that Cleopatra, as a susceptible citrus genotype to combined drought and heat, deeply alters its primary metabolism in order to activate glycolysis and TCA cycle to support cellular energy requirements as well as phenylpropanoid-derived scopolin. On the other contrary, Carrizo shows reduced modifications in primary metabolism, associated to a lower incidence of stress-induced damage.

Apart from primary metabolism, in this second chapter we analyzed plant secondary metabolism to determine its possible involvement in the acclimation of citrus plants to individual and combined stress (Zhao et al. 2005). Among them, we found some

flavonoids and limonoids, metabolites with ROS scavenging activities (Yu et al. 2005, Perez et al. 2009, Patil et al. 2009) differently altered during stress imposition in both citrus genotypes. Flavanones (including naringenin and hesperetin), flavones (including kaempferol, quercetin and isorhamnetin) and flavonols, are especially found in citrus juices (Djoukeng et al. 2008, Arbona et al. 2015). In this part, we identified several differences between both citrus genotypes subjected to individual and combined drought and heat stress regarding flavonoid accumulation. Therefore, flavonols were greatly accumulated in Cleopatra with respect to Carrizo, probably due its higher sensitivity to stress. Hence, activation of the biosynthesis of these flavonoids could be related to a photoprotective response in Cleopatra. In addition, flavones derived from naringenin were also differently accumulated in both genotypes in response to stress treatments, being, in general, accumulated to a greater extent in Cleopatra than in Carrizo. Consequently, Cleopatra, which presents higher oxidative damage during combined stresses, would induce an increased antioxidant response to cope with ROS produced under stress conditions, whereas in Carrizo this response seems to be constitutive. Finally, whereas Cleopatra activated nomilin metabolism during stress, nomilin equivalents were directed to the synthesis of obacunone and limonin in Carrizo under the combined stress conditions.

As reported previously in rice (Morsy et al. 2007) and in durum wheat (Aprile et al. 2013), we observed different patterns in the accumulation of secondary metabolites in response to stress in two close-related citrus plants, Carrizo and Cleopatra. Due to its higher sensitivity, Cleopatra would require a deep alteration of its metabolism as a consequence of its inability to mitigate the damaging effects of adverse environmental conditions. In general, our results suggest that the contrasting ability to tolerate abiotic stress of Carrizo and Cleopatra could be a result of the specificity of metabolism activation along with the higher capability to prevent stress-induced oxidative damage of Carrizo (Arbona et al. 2008) and the particular adjustment of transpiration rate during the combination of drought and heat stress found in the first chapter.

Further investigations in citrus varieties as well as in root tissue will expand knowledge on the response of citrus plants to combined drought and heat stress. In addition, it is worth keeping studying the response of citrus plants to other combinations of stresses, including salinity and heat or salinity and drought in order to develop, in the future, tolerant plants to a wide range of combined stresses. Finally, field studies are necessary

to evaluate constant interactions with weeds or pest and to assess the real natural effect of the heterogeneous environmental conditions on plants.

In the first chapter, we demonstrated that individual and combined drought and heat had a different impact on ABA accumulation. To further understand the role of ABA in abiotic stress combination, we conducted a study of the tolerance of plants to a combination of water deficit and heat stress, focusing on the responses of wild type *Arabidopsis* plants (Ler), an ABA-insensitive mutant impaired in stomatal responses (*abi1-1*) and an ABA-deficient mutant (*aba1-1*). Both mutants were susceptible to this stress combination as well as during a combination of heat and salinity as recently reported (Suzuki et al. 2016), suggesting a key role of ABA in the acclimation of plants to different combined abiotic conditions. Therefore, in the third chapter we aimed to elucidate if the higher susceptibility of *Arabidopsis* mutants to stress combination could be due to impaired stomatal responses or is related to a deficiency in the expression of transcripts and proteins involved in the acclimation process.

Our findings show that the stress combination induced an increased accumulation of ABA in Ler and *abi1-1* plants. In addition, many transcripts involved in the response of plants to a combination of drought and heat stress were ABA-response transcripts, suggesting a requirement of ABA for the acclimation of plants to combined stress conditions. Focusing on the *abi1-1* mutant, we found that the stomatal aperture of this mutant under combined stress conditions were similar to that of Ler plants, indicating that ABI1 could not be involved in the reduction of stomatal opening during the combination of water deficit and heat. To understand how the stomata of *abi1-1* reduced its aperture during the stress combination, we measured the levels of ABA, JA, SA and H_2O_2 in plants subjected to the stress combination. Therefore, our results indicate that H_2O_2 and JA can induce stomatal closure, independently of ABA signaling, probably by increasing nitric oxide (NO) levels and triggering Ca^{2+} and SLAC1 function (Daszkowska-Golec and Szarejko 2013, Murata et al. 2015). Supporting this proposal, H_2O_2 treatment, but not ABA, induced a reduction in the stomatal aperture in *abi1-1* plants under control conditions. These findings point to an alternative pathway involved in stomatal closure in plants subjected to stress combination involving JA and/or H_2O_2. In addition, the accumulation of three important proteins for plant acclimation to heat stress (HSP101 and MBF1c) and a combination of water deficit and heat stress (MBF1c and APX1) was attenuated in *abi1-1* plants during the stress combination. These results

suggest that the higher susceptibility of *abi1-1* plants to the stress combination could be related to their inability to accumulate MBF1c and APX1 that might be ABA-dependent. Overall, our findings indicate that ABA, required for physiological responses and regulation of gene expression and protein accumulation, could act as a regulator of plant responses to different stresses and their combination. Nevertheless, the role of other hormones such as SA or JA in the tolerance of plants to combined abiotic stresses cannot be ruled out. Therefore, further studies using *Arabidopsis* plants (including JA- and SA-deficient mutants) are, of course, needed to understand hormonal crosstalks that contribute to the whole response of plants to combined stresses.

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Conclusions

Conclusions

1. Carrizo citrange is more tolerant than Cleopatra mandarin to heat stress applied individually or in combination with drought. This is likely as a result of an improved leaf cooling via enhanced transpiration along with the ability to modulate photosynthetic electron flow, resulting in a lower incidence of oxidative damage.

2. The combination of drought and high temperatures represents a more damaging situation than the individual stresses to citrus plants, inducing a higher impairment to PSII leading to an enhanced oxidative stress.

3. Hormonal profiling in leaves reveals different accumulation pattern in response to individual and combined stress. Salicylic acid accumulation and signaling parallel the impact of individual and combined stresses in leaves of citrus plants, being more pronounced in Cleopatra mandarin. ABA accumulation and metabolism has a central role for the acclimation of plants to a combination of water deficit and heat stress. The pattern of ABA variation is specific for each stressful situation and, in particular, during combined stress, ABA catabolism along with partial down-regulation of biosynthesis participates in the reduction of ABA levels with respect to those obtained during drought.

4. Cleopatra mandarin requires a deep adjustment of its primary and secondary metabolism in order to prevent further stress-induced damages: a strong induction of glycolysis and TCA and glyoxylate/dicarboxylate cycles to supply a higher demand of cellular energy, activation of phenylpropanoid pathway to accumulate defensive metabolites and enhanced flavonoid biosynthesis to mitigate the higher oxidative damage. On the contrary, the higher ability of Carrizo to maintain the photosynthetic activity and to cope with oxidative stress during combined stresses prevents further modifications of metabolism.

5. In the *Arabidopsis* system, though ABI1 is involved in stomatal closure during stress responses, it is not required for stomatal closure during the stress

combination, and H_2O_2 can induce stomatal reduction, independently of ABA signaling.

6. ABA is required for the accumulation of three important proteins (MBF1c, HSP101 and APX1) for the acclimation of *Arabidopsis* plants to a combination of water deficit and heat stress.

Conclusiones

1. El genotipo de cítricos citrange Carrizo es más tolerante que el mandarino Cleopatra a las altas temperaturas aplicadas de forma individual o combinada con sequía. La mayor transpiración que permite el enfriamiento de las hojas y la óptima modulación del flujo fotosintético de electrones que resulta en una menor incidencia de estrés oxidativo parecen ser los factores determinantes de esta mayor tolerancia.

2. La combinación de sequía y altas temperaturas representa una situación más perjudicial para las plantas de cítricos que los estreses individuales, induciendo un mayor daño en el PSII y consiguientemente un mayor estrés oxidativo.

3. El perfil hormonal de las hojas de cítricos revela un patrón diferente de acumulación en respuesta a los estreses individuales y combinados. La acumulación de ácido salicílico y su señalización evidencian el impacto de los distintos estreses, siendo más pronunciados estos procesos en el mandarino Cleopatra. La acumulación y metabolismo del ABA tienen un papel importante en la aclimatación de las plantas a la combinación de sequía y altas temperaturas. El patrón de acumulación del ABA es específico para cada situación de estrés. Así pues, durante la combinación de ambas situaciones adversas, el catabolismo del ABA junto con la disminución parcial de su biosíntesis, contribuyen a la reducción de los niveles de ABA respecto de los obtenidos durante la sequía.

4. El mandarino Cleopatra activa el metabolismo primario y secundario para prevenir daños producidos por los estreses: la mayor inducción de la glicólisis y del TCA y del ciclo del glioxilato/dicarboxilato tienen como objetivo suplir la demanda de energía celular. Por otro lado, la activación de la ruta de los fenilpropanoides contribuye a la acumulación de metabolitos de defensa tales como los flavonoides que ayudan a mitigar el daño oxidativo. Por el contrario, la mayor capacidad del citrange Carrizo para mantener la actividad fotosintética y prevenir el estrés oxidativo durante la combinación de sequía y altas temperaturas previene mayores modificaciones en el metabolismo.

5. En plantas de *Arabidopsis thaliana*, a pesar de que la proteína fosfatasa 2C ABI1 está implicada en el cierre estomático durante la respuesta a estrés, durante la combinación de sequía y altas temperaturas no participa en el cierre de estomas, siendo el H_2O_2 un promotor del cierre estomático en estas condiciones de forma independiente al ABA.

6. El ABA está implicado en la acumulación de tres proteínas importantes (MBF1c, HSP101 and APX1) para la aclimatación de plantas de *Arabidopsis* a la combinación de sequía y altas temperaturas.

Conclusions

1. El genotip de cítrics citrange Carrizo és més tolerant que el mandarí Cleopatra a les altes temperatures i a la combinació de sequera i altes temperatures. Aquest fenomen és resultat d'una major transpiració que permet un refredament més eficient de les fulles i de l'òptima modulació del flux fotosintètic d'electrons que resulta en una menor incidència d'estrès oxidatiu.

2. La combinació de sequera i altes temperatures representa una situació més perjudicial per a les plantes de cítrics que els estressos individuals, induint un major dany en el PSII i conseqüentment un major estrès oxidatiu.

3. El perfil hormonal de les fulles dels cítrics revela un patró diferent d'acumulació en resposta als estressos aplicats de forma individual i combinada. L'acumulació de l'àcid salicílic i la seua senyalització evidencien l'impacte dels diferents estressos, sent més pronunciats aquests processos en el mandarí Cleopatra. L'acumulació i metabolisme de l'ABA tenen un paper important en l'aclimatació de les plantes a la combinació de sequera i altes temperatures. El patró d'acumulació de l'ABA és específic per a cada situació d'estrès. Així doncs, durant la combinació d'ambdues situacions adverses, el catabolisme de l'ABA, unit a la disminució parcial de la seua biosíntesi, contribueixen a la reducció dels nivells d'ABA respecte als obtinguts durant la sequera.

4. El mandarí Cleopatra activa el seu metabolisme primari i secundari per a prevenir danys produïts pels estressos: la inducció de la glicòlisi, del TCA i del cicle del glioxilat/dicarboxilat contribueixen a suplir la major demanda d'energia cel·lular. Per altra banda, l'activació de la ruta dels fenilpropanoides contribueix l'acumulació de metabòlits de defensa com ara els flavonoides que participen en mitigar el dany oxidatiu. Pel contrari, la major capacitat del citrange Carrizo per a mantenir l'activitat fotosintètica i prevenir l'estrès oxidatiu durant la combinació de sequera i altes temperatures evita majors modificacions en el metabolisme.

5. En plantes d'*Arabidopsis thaliana*, malgrat la funció de la proteïna fosfatasa 2C ABI1 en el tancament estomàtic durant la resposta a estrès, durant la combinació de sequera i calor no participa en el tancament d'estomes, sent el H_2O_2 un promotor del tancament estomàtic en aquestes condicions de forma independent a l'ABA.

6. L'ABA està implicada en l'acumulació de tres proteïnes importants (MBF1c, HSP101 and APX1) per a l'aclimatació de plantes d'*Arabidopsis* a la combinació de sequera i altes temperatures.