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Additional Information

Title

Depletion of abscisic acid levels in roots of flooded Carrizo citrange (*Poncirus trifoliata* L. Raf. ×
Citrus sinensis L. Osb.) plants is a stress-specific response associated to the differential
expression of PYL/PYR/RCAR receptors.

Running title

ABA metabolism and signaling in waterlogged roots

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Acknowledgements

Abstract

Soil flooding reduces root abscisic acid (ABA) levels in citrus, conversely to what happens under drought. Despite this reduction, microarray analyses suggested the existence of a residual ABA signaling in roots of flooded Carrizo citrange seedlings. The comparison of ABA metabolism and signaling in roots of flooded and water stressed plants of Carrizo citrange revealed that the hormone depletion was linked to the upregulation of *CsAOG*, involved in ABA glycosyl ester (ABAGE) synthesis, and to a moderate induction of catabolism (*CsCYP707A,* an ABA 8'-hydroxylase) and buildup of dehydrophaseic acid (DPA). Drought strongly induced both ABA biosynthesis and catabolism (*CsNCED1,* 9-*cis*-neoxanthin epoxycarotenoid dioxygenase 1, and *CsCYP707A*) rendering a significant hormone accumulation. In roots of flooded plants, restoration of control ABA levels after stress release was associated to the upregulation of *CsBGLU18* (an ABA β-glycosidase) that cleaves ABAGE. Transcriptional profile of ABA receptor genes revealed a different induction in response to soil flooding (*CsPYL5)* or drought (*CsPYL8).* These two receptor genes along with *CsPYL1* were cloned and expressed in a heterologous system. Recombinant *CsPYL5* inhibited ΔNHAB1 activity *in vitro* at lower ABA concentrations than *CsPYL8* or *CsPYL1*, suggesting its better performance under soil flooding conditions. Both stress conditions induced ABA-responsive genes *CsABI5* and *CsDREB2A* similarly, suggesting the occurrence of ABA signaling in roots of flooded citrus seedlings. The impact of reduced ABA levels in flooded roots on *CsPYL5* expression along with its higher hormone affinity reinforce the role of this ABA receptor under soil-flooding conditions and explain the expression of certain ABA-responsive genes.

Key words: abiotic stress, drought, flooding, hormones, photosynthesis, signaling

Introduction

Plants are continuously exposed to challenging environmental conditions some of which affect growth, development or even compromise survival. Drought is one of the most damaging situations since it limits water supply to the canopy (Lawlor 2013). Plants respond to water limitation by closing stomata and reducing leaf water potential to maintain water availability to photosynthetic organs. However, if the adverse condition persists, progressive leaf wilting, photosynthetic inhibition and other leaf injuries appear (Arbona et al. 2009; Lawlor 2013). All these defense responses are regulated by abscisic acid (ABA), the key regulator of abiotic stress adaptations. In citrus, drought induces accumulation of ABA in roots and leaves, triggering several adaptive responses such as leaf abscission (De Ollas et al. 2013). Soil waterlogging also induces ABA accumulation in leaves but, on the contrary, causes a severe reduction of the hormone levels in roots in clear contrast to water deficit (Arbona and Gómez-Cadenas 2008; Argamasilla et al. 2013).

Water stress-induced increase of ABA concentration is accompanied by the upregulation of 9-*cis*-epoxicarotenoid dioxygenase (*CsNCED1*) that converts 9-neoxanthin to xanthoxin and appears to be regulated by a previous JA accumulation (De Ollas et al. 2013). Xanthoxin is further metabolized to ABA aldehyde by xanthoxin dehydrogenase (encoded by *AtABA2* in *Arabidopsis thaliana*) and then oxidized to ABA by an ABA aldehyde oxidase (González-Guzmán et al. 2002; Nambara and Marion-Poll 2005). The main catabolic pathway of ABA involves the hydroxylation at C-8' to generate 8'-OH-ABA, which is converted spontaneously to phaseic acid (PA) and subsequently to dihydrophaseic acid (DPA) in a reaction catalyzed by the PA reductase ABH2 (Krochko et al. 1998; Weng et al. 2016). Another pathway to remove active ABA pools is conjugation to hexoses by an ABA *O*-glycosyl transferase (AOG), yielding inactive ABA-glycosyl ester (ABAGE; Priest *et al.*, 2006). ABA can be released after cleavage of this metabolite by an ABAGE β-glycosidase (BGLU18, Schroeder and Nambara 2006).

Active ABA binds to PYR/PYL/RCAR cytosolic receptors that sequester different protein phosphatases 2C (PP2Cs) such as ABI2, ABI1, HAB1, or PP2CA/AHG3 (Santiago et al. 2012). The formation of a ternary complex ABA-receptor-PP2C inactivates the phosphatase activity, releasing Sucrose non-fermenting-1-Related Kinases 2 (SnRK2) proteins, which become active (Szostkiewicz et al. 2010; Santiago et al. 2012). These kinases phosphorylate different transcription factors from the ABRE/ABF/ABI5 family that bind to particular *cis*- elements in promoters of ABA-responsive genes, inducing their expression.

The physiological processes regulated by ABA need to be modulated according to the pressure of the stress exerted and the pre-existing physiological status of the plant (Arbona and Gómez-Cadenas 2008; Romero et al. 2012b). This implies a fine adjustment of the magnitude of the physiological response and requires a more complex regulatory system than the sole presence/absence of ABA. Indeed, there are 14 loci encoding for ABA receptors in *Arabidopsis*,

each of them having different intrinsic affinities for the hormone and could also bind to specific PP2Cs (Dupeux et al. 2011; Santiago et al. 2012; Antoni et al. 2013; Fuchs et al. 2014). Besides the fine-tuning of the ABA signal, modulation of the endogenous hormone levels is associated to the specific environmental situation and the stress pressure, which adds further complexity to the model (Seiler et al. 2011; Romero et al. 2012b; Baron et al. 2012; De Ollas et al. 2013). Therefore, increases or decreases in ABA levels respect to non-stressed values could be considered as a signal, multiplying the number of combinations to fine-tune the physiological response. To this respect, soil waterlogging reduces root ABA levels below control values shortly after stress imposition in citrus (Arbona and Gómez-Cadenas 2008) and Arabidopsis (Hsu et al. 2011). Although ABA is primarily known for its role in seed germination and regulation of adaptive responses to water deprivation (Finkelstein and Rock 2002), it is also involved in plant responses to submergence or soil waterlogging. In deep water rice or *Rumex palustris*, reduction of ABA levels is associated to internode and shoot elongation (Benschop et al. 2007; Saika et al. 2007). Moreover, in non-adapted species such as soybean, secondary aerenchyma formation in response to soil flooding seems associated to ABA signaling (Shimamura et al. 2014). In citrus, soil flooding reduced root hydraulic conductance associated to the downregulation of aquaporin expression (Rodríguez-Gamir et al. 2011). In addition, ABA metabolism in leaves and roots of soil-flooded plants differs greatly (Arbona and Gómez-Cadenas 2008; Hsu et al. 2011) surely leading to tissue-specific responses.

In this work it is hypothesized that the reduction in ABA levels in roots of flooded citrus plants is an orchestrated mechanism leading to particular physiological responses. Experiments consisting of short-term stress exposure followed by alleviation were carried out in citrus seedlings to investigate stress-specific responses previous to any visible symptom of damage. Water stress experiments were also performed to compare hormonal and molecular responses to those of soil-flooded plants.

Materials and Methods

Plant materials and treatments

One-year-old true-to-type seedlings of Carrizo citrange (*Citrus sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.) were acclimated for 2 months in a greenhouse under natural photoperiod and temperature (averaging 16:8 L:D and 25.0 ± 3.0 ºC, respectively). Plants were grown in 2.5-L plastic pots filled with a mixture of sterile peat moss, perlite and vermiculite (80:10:10) and watered three times a week with 0.5 L of a half-strength Hoagland solution. Only uniform plants were used for the assays. Harvesting of plant material was performed in the morning (9:00 a.m.) and only fibrous roots were collected, washed with distilled water to remove substrate, blotted dry and immediately frozen in liquid nitrogen.

After the two-months acclimation period, two experimental systems were set up:

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Flooding and recovery

Flooding conditions were imposed by submerging the entire root system in tap water reaching c.a. 2 cm above the soil surface. Based on previous data (Arbona et al. 2009), no visible symptoms of damage could be observed when the soil flooding conditions were maintained for 7 days. After this period, water excess was drained and plants allowed to recover. An additional set of plants was regularly watered as above and set as control.

Water deficit and recovery

Plants were transplanted to dry and wet perlite to establish water deficit treatment and control conditions, respectively. The stress condition was maintained for 24 hours and subsequently all groups of plants were fully watered (as control plants) to allow recovery.

Physiological parameters

Flooding and water deficit experiments were carried out in parallel and physiological parameters were measured between 9:00 and 11:00 a.m. on randomly chosen plants to avoid bias between treatments. The physiological descriptors considered for this study were leaf gas exchange parameters that were measured with an LCpro+ portable infrared gas analyzer (ADC Bioscientific Ltd., Hoddesdon, UK) under ambient CO_2 and humidity. Supplemental light was provided by a LED array providing PAR at 1000 µmol m^{-2} s^{-1} photon flux density and air flow was set at 150 µmol mol^{-1}. After instrument stabilization, measurements were taken on three mature leaves (from an intermediate position on the stem) in 3 replicate plants from both experimental designs. In addition, quantum yield [Φ_{PSII}= (Fm´−Fs)/Fm´] was analyzed in parallel to gas exchange and on the same leaves using a portable fluorometer (FluorPen FP-MAX 100, Photon Systems Instruments, Czech Republic) after actinic light adaptation (Arbona et al. 2009).

Hormone analyses

Analyses of ABA and catabolites were carried out by UPLC coupled to tandem mass spectrometry as described in Argamasilla *et al.* (2013). Briefly, 0.2 g of powdered frozen root tissue was spiked with 50 ng of [$^2H_6$]-ABA and 50 ng of [$^2H_3$]-PA as internal standards before extraction in ultrapure water using a tissue homogenizer (Ultra-Turrax, Ika-Werke, Staufen, Germany). Samples were centrifuged at 4700$\times g$ and 4ºC, supernatants recovered and pH adjusted to 3.0 with a 30% acetic acid solution. Subsequently, supernatants were partitioned twice against di-ethyl-ether (Panreac, Barcelona, Spain) and the organic layers combined and evaporated in a centrifuge vacuum evaporator (Jouan, Saint-Herblain, France). The dry residue was thereafter resuspended in a water:methanol (9:1) solution, filtered, and injected onto the UPLC system (Acquity SDS, Waters Corp. Milford. USA). ABAGE content was estimated as free ABA released after alkaline hydrolysis. Following partition, 2 mL of 0.1 M NaOH were added to

the aqueous fractions, mixed and the resulting solution incubated at 60ºC for 30 min. Afterwards, extracts were acidified, partitioned and processed as above. All samples were separated by reversed-phase chromatography on a Gravity C18 column (50 × 2.1 mm 1.8-µm particle size, Macherey-Nagel GmbH, Germany) using methanol and ultrapure water both supplemented with glacial acetic acid to a concentration of 0.1%. The mass spectrometer, a Waters TQD triple quadrupole was operated in negative ionization electrospray mode and plant hormones were detected according to their specific transitions using a multiresidue mass spectrometric method.

Microarray hybridization and analysis

A Citrus genome-wide cDNA microarray was used including 21,081 putative unigenes. Three independent microarray hybridization experiments were carried out with cDNA isolated from control and flooded roots of Carrizo citrange. Microarray hybridizations were performed essentially as described in (Agustí et al. 2008). In each experiment, equal amounts of Cy5-labeled cDNA from stressed samples and Cy3-labeled cDNA from control samples (40–60 pmol of each dye) were mixed in 55 µL hybridization solution containing 3x SSC, 0.1% SDS and 0.1 mg mL^{-1} of salmon sperm DNA. The hybridization mix was denatured at 95°C for 60 s and applied to a previously pre-hybridized microarray slide (incubation at 50 °C in 3x SSC, 0.1% SDS and 0.1 mg mL^{-1} BSA for 60 min, followed by two washes with SSC (0.1x) and once with distilled water, and finally drying by centrifugation at 1000 rpm for 5 min. The slide was placed in a hybridization chamber (Olympus, Tokyo, Japan) and incubated overnight (14–16 h) at 50 °C. After hybridization, slides were washed by consecutive incubations in 2x SSC/0.1% SDS buffer at 42 °C for 5 min, two times in 0.1x SSC/0.1% SDS buffer at 28 °C for 5 min, and five times in 0.1x SSC at 28 °C for 1 min. Finally, samples were washed in 0.01x SSC buffer at 28 °C for a few seconds. Slide surface was dried by centrifugation at 1000 rpm for 5 min. Microarray readings were carried out with a GenePix 4000B microarray scanner (Axon Instruments, Inc., Union City, CA, USA) operated with GenePix Pro 4.1 image acquisition software at 5–10 µm resolution. High-resolution *.tiff images were generated and used for quantification of gene expression data. Spot positions were identified on the color images and quality flags were automatically assigned (and manually corrected, if required) to individual spots.

Raw data were imported into the R-computing environment for preprocessing, visualization and statistical analysis. To identify probes showing significant differential gene expression between samples, the LIMMA package was used (Ritchie et al. 2015). Preprocessing and normalization of two-color microarray data including signal intensity, background correction, uniformity of the expression ratio over the microarray surface (within-array normalization), and normality of M-value [defined as log$_2$(stressed/control)] distributions were also evaluated. *P*-values associated to the statistical analysis of differential expression obtained from LIMMA analysis were corrected for multiple comparisons using the B&H false discovery rate (FDR) procedure and differences in gene expression were considered to be significant when M-value was ≥/0.7/ and the FDR-

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adjusted *p*-value was smaller than 0.05. GO-enrichment was carried out by pasting the closest Arabidopsis orthologue onto the AgriGO Analysis Toolkit online (http://bioinfo.cau.edu.cn/agriGO/index.php). Results are shown as GO-enriched categories (Supporting Figure S1) and individual annotated orthologues (Supporting Tables S1 and S2).

Total RNA isolation, cDNA synthesis and qRT-PCR analyses

About 100 mg of ground Carrizo root tissue was used to isolate total RNA by EZNA Plant RNA Purification Kit (Omega Bio-Tek, Inc. Norcross, GA, USA) following the manufacturer's instructions. Then, the RNA extract underwent a treatment with RNase-free DNase (Promega Biotech Ibérica, SL. Madrid, Spain) in order to remove any possible genomic DNA contamination. The integrity of the RNA was assessed by agarose gel electrophoresis and ethidium bromide staining. The total RNA concentration was determined using a spectrophotometric analysis (NanoDrop, Thermo Scientific, Wilmington, DE, USA), and the purity assessed using the 260/280 nm absorbance ratio. cDNA was synthesized from 1 µg of total RNA using Primescript RT (Takara Bio, Inc. Japan) with oligo(dT) primers.

Gene-specific primers were designed with primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi, see Supporting Table S3) using orthologous sequences retrieved from the *Citrus sinensis* genome (http:\\www.phytozome.org, see Supporting Table S4 for protein homology of retrieved sequences). Designed primers were then evaluated with IDT-oligoanalyzer tools (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/) following parameters: Tm around 60ºC, 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. Forward (F) and reverse (R) sequences of each primer, as well as the amplicon sizes are shown in Supporting Table S3. qRT-PCR analyses were performed in a SmartCycler™ (Cepheid, Sunnyvale, CA, USA). The reaction mixture contained 2 µL of cDNA, 0.5 µM of each gene-specific primer pair and 10 µL of SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA) including dye, uracil-DNA glycosylase and AmpliTaq® DNA polymerase to a final volume of 20 µL. The following thermal profile was set for all amplifications: 95ºC for 30 s followed by 40 cycles of 95ºC for 5 s and 60ºC for 30 s. Two technical replicates were analyzed for each biological replicate. The expression of genes encoding for ABA receptors was normalized against the expression of the endogenous control gene (Tubulin, *CsTUB*, see Supporting Table S3). Relative expression for the rest of genes was calculated using $\Delta\Delta Ct$ method. Subsequently, expression values were expressed as $\log_2$ of ΔCt values to facilitate interpretation of data.

Purification of recombinant proteins

Sequences orange1.1g046151m, orange1.1g038201m and orange1.1g028067m coding for putative ABA receptors were cloned into a modified pCR8/GW/TOPO using NcoI/BamHI

restriction enzymes (Gonzalez-Guzman et al. 2014). These sequences were checked by sequencing and then subcloned into pETM11 after NcoI/BamHI double digestion. *Escherichia coli* BL21 (DE3) cells were transformed with the corresponding pETM11 construct and allowed to grow for 3 h at 37ºC in 50 mL of Luria–Bertani medium supplemented with 50 µg mL^{-1} kanamycin (OD$_{600}$ between 0.6 and 0.8). To induce the expression of recombinant proteins, a solution containing isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After a 5 h-incubation period at 28ºC, cell suspensions were centrifuged for 30 min at 4ºC and cell pellets collected. Pellets were resuspended in 6 ml of HIS buffer (50 mM TRIS-HCl, pH 7.6, 250 mM KCl, 10% glycerol, 0.1% Tween-20, and 10 mM mercaptoethanol) and cells were then disrupted by ultrasonication (Branson sonifier) until complete clarification of cell suspension. Cell lysates were afterwards centrifuged at 14,000 rpm and 4ºC for 15 min and supernatants were loaded onto a 0.5 ml nickel–nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen). The column was washed with 10 mL of HIS buffer supplemented with 20% glycerol and 30 mM imidazole. Retained protein was then eluted with HIS buffer supplemented with 20% glycerol and 250 mM imidazole in successive 200 µL aliquots. Protein concentration was determined using Bradford protein dye method (Bio-Rad) and aliquots containing significant amounts of recombinant protein stored at -80ºC for subsequent analyses.

Phosphatase activity inhibition assays

Phosphatase activity was measured using the RRA(phosphoT)VA peptide as substrate (Gonzalez-Guzman et al. 2014). Assays were performed in a 100 µl reaction volume containing 25 mM TRIS-HCl, pH 7.5, 10 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 25 µM peptide substrate, and 0.5 µM of recombinant ΔNHAB1 from *Arabidopsis thaliana*. Blank controls without recombinant ABA receptors or without ABA (HIS buffer) or ABA agonists (DMSO) were included as a 100% phosphatase activity reference. For ABA response curves, concentrations of the hormone ranging between 0 and 10 µM were used. ABA agonists quinabactin (Life Chemicals, Kyiv, Ukraine) and pyrabactin (Sigma Aldrich) were applied at 1 and 10 µM concentrations. The different ABA receptors were added to a final concentration of 2 µM (ratio receptor:ΔNHAB1 was 4:1). Reaction mixtures were incubated for 60 min at 30 ºC and then the reaction was stopped by the addition of 30 µL of molybdate dye. The absorbance was read at 630 nm with a 96-well microplate reader.

Statistical analyses

Statistics were evaluated with the Statgraphics Plus v.5.1. software (Statistical Graphics Corp., Herndon,VA. United States). Student's t-test between controls and stressed samples was used to assess differences at $p \leq 0.05$ considering n=3.

Results

Genome-wide microarray expression analyses

To provide a global view of the soil flooding-induced responses, a genome-wide microarray analysis was carried out comparing control with root samples from flooded citrange Carrizo seedlings (Supporting Figure S1 and Table S1). Flooding resulted in changes in gene expression involving 159 upregulated genes and 469 downregulated genes (showing at least a $\log_2$ value of 1 or -1, respectively, and a p-value below 0.05). Among the upregulated genes, 'Cellular metabolic process', 'Response to stress', and 'Response to abiotic stimulus' were the most overrepresented Gene Ontology (GO) categories, followed by 'Hormone signaling and metabolism', including abscisic acid, gibberellin and auxin. Within the 'Cellular metabolic process' category, we found genes involved in oxidative reactions such as alcohol dehydrogenase, monooxygenase or cytochrome P450 79A1 and 79A2; genes involved in carbohydrate metabolism such as glucose-6-phosphate isomerase, phosphofructokinase, fructose bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, enolase 1, ribose 5-phosphate isomerase, etc... suggesting an activation of the oxidative metabolism, possibly fermentation. However, orthologues of PDCs 1 and 2 (involved in conversion of pyruvate into acetyl-CoA in the mitochondrial matrix) and several gene orthologues encoding different hemoglobins were included; hence, it is likely that certain oxidative phosphorylation takes place allowing the production of significant amounts of ATP. Other genes included in the list were peroxydase, hydroperoxyde lyase, glutathione S-transferase and catalase, involved in the detoxification of reactive oxygen species. In addition, genes encoding proteins that are involved in the synthesis of indolics (such as anthranilate synthase and anthranilate N-benzoyltransferase), phenylpropanoids (hydroxycinnamoyl CoA quinate transferase and cinnamate 4-hydroxylase CYP73), isoprenoids (homogentisate geranylgeranyl transferase) and flavonoids (chalcone synthase). Moreover, ABA catabolism was represented by the induction of ABA 8'-hydroxylase CYP707A1, which catalyzes the production of PA. In the 'Responses to abscisic acid stimulus' genes involved in ABA signal transduction such as several NAC-containing domain proteins (NAC1, NAC2, and ATAF1) and the regulatory β subunit of SnRK1. Also, genes encoding calcium-binding proteins involved in intracellular signal amplification (EF hand motif-contaning proteins, CPK1 and CPK5). Moreover, auxin metabolism was also represented in a putative indole-3-acetic acid-amido synthetase GH3.1, as well as signaling as ARF2 or ARF-GAP. Finally, gibberellin metabolism and signaling pathways were also activated in reponse to soil flooding and represented by the activation of GA-2 oxidase and a scarecrow-3-like protein involved in distal specification of the root quiescent center, and stem cell fate determination.

Among the downregulated genes (Supporting Figure S1 and Table S2), 'Cell metabolism', 'Response to stimulus' and 'Response to oxidative stress' were the most represented categories. The GO term 'Cell metabolism' included genes involved in secondary metabolism such as anthocyanidin and flavonoid biosynthesis: Leucoanthocyanidin dioxygenase-like protein

and Putative chalcone isomerase, phenylpropanoid biosynthesis: cinnamyl-alcohol dehydrogenase, eugenol O-methyltransferase, hydroxycinnamoyl transferase and caffeic acid 3-O-methyltransferase, diterpenoid and gibberellin biosynthesis: ent-kaurenoic acid oxidase; energy and carbohydrate metabolism: Phosphoenolpyruvate carboxylase, Malate dehydrogenase, Putative 6-phosphogluconolactonase, Raffinose synthase, Putative cellulose synthase, Aldose 1-epimerase or the starch branching enzyme. Within the 'Response to stimulus' category, several dehydrin genes (ERD14 and ERD7), the cold regulated 15, COR15 LEA protein and aquaporins were included. In addition, two lipoxygenase genes potentially involved in jasmonic acid biosynthesis along with allene oxide synthase were included in this group. Other genes involved in plant hormone biosynthesis included ACC oxidase, responsible for the oxidization of 1-aminoacyclopropane 1-carboxylic acid (ACC) to ethylene. As mentioned above, another important enriched category was 'Response to oxidative stress' including several genes encoding catalase, ascorbate peroxidase, monodehydro and dehydroascorbate reductase (involved in reduction of oxidized ascorbate), several peroxidases and glutathione S-transferases and a Fe-superoxide dismutase.

Although important responses to soil flooding were associated to changes in primary,and secondary metabolism, and antioxidant activity, genes involved in ABA metabolism and signal transduction were upregulated under these conditions.

Physiological and biochemical characterization of Carrizo citrange seedlings subjected to soil flooding and water deficit

Soil flooding and water deficit reduced stomatal conductance in Carrizo citrange seedlings after a short period of stress but it rapidly recovered to control values after draining water excess and rewatering, respectively (Figures 1a and 1b). Indeed, stomatal conductance in plants flooded for seven days was 63% of that in control plants. Although stomatal aperture was reduced in response to both stress conditions, only drought affected carboxylative efficiency (as shown by the increase in Ci/Ca) and electron transport between photosystems (depicted by a decrease in Φ_{PSII}). This concomitant opposite variation of Φ_{PSII} and Ci/Ca indicated that the reduction in the ability to fix CO_2 was not only associated to stomatal factors but to a reduction in the photosynthetic electron flow. Moreover, the compatible osmolyte proline accumulated in roots of citrus to almost identical maximum values (two-fold higher than controls) in response to soil flooding or drought. Stress release restored control values for all parameters in both stress conditions (Figures 1a through 1h).

Root hormonal profiles

In response to soil flooding, root ABA concentration decreased to very low levels (88% reduction respect to controls after seven days of stress, Figure 2a). On the contrary, 24 h of severe water deprivation induced a rise in the levels of this hormone to reach 47.1-fold above control levels. As expected, stress release in both cases induced the recovery of control root ABA levels

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(Figures 2a and 2b). Levels of PA also decreased after soil flooding (Figure 2c). Conversely, DPA levels followed an opposite tendency to that of its precursor PA after seven days of treatment (Figure 2e). Moreover, waterlogging caused ABAGE levels to decrease 20% respect to controls; however, levels of the conjugated hormone exceeded 4 times the concentration of free ABA (Figure 2g). In water deficit experiments, ABA, PA, DPA and ABAGE levels increased simultaneously and returned to control levels after rewatering (Figures 2b, 2d, 2f and 2h). As for the physiological parameters, stress release induced the recovery of hormone, catabolite and ABAGE levels to control values (Figures 2a through 2h).

Root gene expression profiles

qRT-PCR analysis of genes involved in ABA biosynthesis, catabolism and conjugation

The preliminary microarray analysis showed that ABA metabolism and ABA-dependent signaling were overrepresented GO terms in the upregulated dataset (see Supporting Table S1). Therefore, to get a deeper insight of the transcriptional responses of genes involved in ABA metabolism, the expression of genes encoding enzymes involved in ABA biosynthesis, catabolism and conjugation: *CsNCED1*, *CsCYP707A*, *CsAOG* and *CsBGLU18* was analyzed by qRT-PCR (Figure 3). In citrus roots, *CsNCED1* expression was not affected by soil flooding whereas water deficit upregulated this gene up to a $\log_2$ value of 3.2. Release of water deficit caused *CsNCED1* expression to return to control values (Figures 3a and 3b). Soil flooding induced a 2.5-fold increase in *CsCYP707A* expression (Figure 3c), whereas drought repressed it 11.1 times respect to control values (Figure 3d). Stress release in soil-flooded seedlings reduced *CsCYP707A* expression to control values but rewatering induced its expression up to 4.2-fold. Moreover, waterlogging upregulated *CsAOG* 7.6 times respect to controls (Figure 3e) and water drainage returned its transcript levels to non-stressed values. A similar response was observed in response to water deficit, although rewatering did not completely recover control values. The expression of the ABA β-glycosidase encoding gene, *CsBGLU18*, decreased in roots upon imposition of soil flooding (showing values 50% lower than controls). After drainage, *CsBGLU18* expression increased to reach levels above control values (8.6-fold respect to control values, Figure 3g). Similarly, drought also repressed *CsBGLU18* expression in roots of Carrizo seedlings up to 86% respect to control values and rewatering recovered non-stressed transcript levels (Figure 3h).

Transcriptional analysis of ABA receptors

Analysis of the annotated genome of *Citrus sinensis* rendered 11 putative ABA receptors of which six were considered for expression analysis following previous work (Romero et al. 2012a). With the exception of *Cs31700*, annotated citrus ABA receptors were distributed in three different subfamilies (clades) as described for the *Arabidopsis* PYR/PYL/RCAR ABA receptors (Figure 4). The subfamily I included *Arabidopsis* dimeric receptors (*AtPYR1* and *AtPYL1* to *AtPYL3*) and contained four citrus genes of which *Cs46151* and *Cs46697* were

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annotated as *CsPYL1* and *CsPYL2*, respectively (Figure 4a). Subfamily II of ABA receptors is formed by *AtPYL4* to *AtPYL6* and *AtPYL11* to *AtPYL13*. In this case only two genes, *Cs38201* and *Cs26007* named as *CsPYL5* and *CsPYL4* after Romero *et al.*(2012*a*), were identified. In subfamily III, including ABA receptor genes *AtPYL7* to *AtPYL10*, five genes from citrus were found of which two were annotated as *CsPYL8* and *CsPYL9* according to Romero *et al.* (2012*a*). No close relatives for the Arabidopsis *AtPYL11* to *AtPYL13* were found in the citrus genome. All identified putative ABA receptors in the citrus genome contain the β3/β4 and β5/β6 loops, known as gating loops or gate and latch domains (Figure 4b), that control access to the ABA-binding pocket (Dupeux et al. 2011).

The expression profile of the six annotated ABA receptors, including representative members of the three subfamilies described in Figure 4a, was analyzed in root of citrus plants under stress and control conditions and after the recovery period (Figure 5). Interestingly, *CsPYL2*, *CsPYL4* and *CsPYL5* showed enhanced expression under soil waterlogging compared to control roots, despite the fact that ABA levels were notably reduced in waterlogged roots. In particular, the expression of *CsPYL5* in roots was induced more than 20-fold after waterlogging. The expression of *CsPYL4* was also induced under these conditions but only 1.6 fold respect to non-stressed values; nevertheless, its transcript levels under waterlogged conditions were 2.5 times lower than *CsPYL5* (Figure 5a). These results suggest that, at least, a subset of ABA receptors is induced when ABA levels decay, which might represent a feedback mechanism to maintain a certain level of ABA signaling under soil waterlogging conditions. In contrast, the expression of *CsPYL4* and *CsPYL5* in roots was downregulated upon drought imposition (reductions of 65% and 90% respect to non-stressed values), when ABA levels were notably increased. These results suggest that a negative feedback regulatory mechanism operates in citrus roots to reduce the expression of some ABA receptors when ABA levels are high or to enhance their expression when ABA levels are low.

Several receptor proteins are often co-expressed in the same tissue and cell type probably each of them carrying specialized functions, although this extent has not been sufficiently studied (Dupeux et al. 2011). In this sense, basal transcript levels were compared for all putative ABA receptors to assess their relative contribution to signaling in roots of citrus (Figure 5, white bars). Under non-stressful conditions, the ABA receptor showing the highest expression levels was *CsPYL9* followed by *CsPYL4* and *CsPYL8*. However, soil flooding donwregulated *CsPYL9* whereas drought did not modify its expression respect to basal values. Control transcription levels of *CsPYL5* were lower than those measured for *CsPYL4* (Figure 5a). However, soil flooding upregulated it to 21.8-fold (versus 1.6-fold for *CsPYL4* under the same conditions). Unlike other ABA receptors analyzed in this work, *CsPYL8* showed a 1.5-fold upregulation in response to drought. Based on transcriptional data, this ABA receptor could have a strong contribution to ABA signaling under water deprivation in citrus (Figure 5b). It is worth noting that

the stress release period was sufficient to recover basal transcript values for all ABA receptor-encoding genes.

Inhibition assay of Arabidopsis ΔNHAB1 by putative Citrus ABA receptors

Biochemical characterization of *Citrus* ABA receptors is required to validate annotation studies. To accomplish this, *CsPYL5* and *CsPYL8* were chosen due to their particular transcriptional profile whereas the putative dimeric ABA receptor *CsPYL1* was included as a reference (Figure 4a). Their respective ORFs were cloned and expressed in *E.coli* to obtain purified recombinant proteins. An *in vitro* phosphatase assay was performed using the *Citrus* ABA receptor proteins and the catalytic core of *Arabidopsis* HAB1 (ΔNHAB1, aminoacids 170-511) from *Arabidopsis*. PYR/PYL/RCAR receptors have been reported to inhibit clade A PP2Cs from different plant species in the presence of ABA (Gonzalez-Guzman et al. 2014). Likewise, *Citrus* ABA receptors inhibited ΔNHAB1 phosphatase activity in an ABA concentration-dependent manner (Figure 6a). Data indicated that the putative ABA receptors are functional and able to inhibit PP2C activity in the presence of hormone. Although all recombinant receptors responded to ABA, differences in the IC_{50} were found among them (Figure 6a). For instance, *CsPYL1*, a putative dimeric ABA receptor, showed an IC_{50} of 0.82 µM ABA whereas for *CsPYL8*, a putative monomeric ABA receptor, this value was lower, 0.51 µM. Therefore, the amount of ABA required to inhibit 50% of ΔNHAB1 phosphatase activity was significantly lower in monomeric *CsPYL8* than in dimeric *CsPYL1*, as expected. Moreover, *CsPYL5*, another monomeric ABA receptor, showed an IC_{50} value of 0.22 µM ABA, four and two times lower than the amount required for *CsPYL1* and *CsPYL8*, respectively. These results suggest the existence of notable differences among ABA receptors in the concentration of hormone required to trigger ABA signaling.

To further characterize these receptors, the ability of recently discovered ABA agonists, such as pyrabactin and quinabactin, to bind *CsPYL1*, *CsPYL5* and *CsPYL8* and induce ABA-dependent ΔHAB1 inhibition was tested in this work. Each recombinant ABA receptor was incubated with either 1 or 10 µM pyrabactin or quinabactin, respectively. *CsPYL8* did not respond to quinabactin or pyrabactin, whereas *CsPYL1* responded to both, showing similar ΔNHAB1 activity inhibition irrespective of the concentration. Finally, *CsPYL5* was insensitive to pyrabactin but was able to inhibit phosphatase activity upon incubation with quinabactin (Figure 6b).

Transcriptional analysis of stress-responsive genes

In this work, the expression of several stress- and ABA-inducible genes in roots subjected to waterlogging or drought was investigated. Thus, we analyzed expression of *CsABI5*, encoding a leucine zipper transcription factor family member involved in ABA-dependent gene expression (Tanaka et al. 2012; Chen et al. 2012); *CsDREB2A* that encodes an AP2/ERF transcription factor that specifically binds to DRE/CRT *cis-* elements of drought-responsive genes (Naika et al. 2013); *CsRD29*, an orthologous of drought and ABA-responsive *AtRD29A* and *AtRD29B*

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from Arabidopsis; *CsRD22,* which does not contain any typical ABRE consensus sequence in its promoter and is dependent on a MYC transcription factor for stress-inducible expression (Abe et al., 2003) and *CsRAB18*, that encodes an ABA-responsive dehydrin protein family member (Agarwal and Jha 2010). In response to soil flooding, expression of both genes, *CsABI5* and *CsDREB2A*, increased: *CsABI5* expression was induced 5.2-fold in response to soil flooding whereas *CsDREB2A* induction was more moderate (Figures 7a and 7c). Water drainage reduced expression of *CsDREB2A* and *CsABI5* to control values. In response to water deprivation, the expression of both genes was also upregulated. Transcript levels of *CsABI5* and *CsDREB2A* were 4.8 and 2.6 times higher than controls, respectively (Figures 7b and 7d).

CsRD29 showed a strong upregulation (a log_2 value of 4.6) in response to drought, whereas soil flooding only increased its expression 2.0-fold (Figures 7e and 7f). On the other hand, *CsRD22* expression in citrus roots increased after soil flooding showing a log_2 value of 1.5 (3.0-fold respect to controls, Figure 7g) whereas 24 h of dehydration had only a marginal effect, showing a log_2 value of 0.9 (Figure 7h). Finally, *CsRAB18* expression showed a moderate 2.0-fold expression in response to drought, despite the important ABA accumulation observed, whereas soil flooding did not have any significant effect on the expression of this gene (Figures 7i and 7j). Interestingly, after releasing soil-flooded plants from stress, *CsRD29* expression downregulated, showing log_2 of -1.8 (a 34% reduction respect to control values) whereas for the rest of genes, stress release restored control values.

Discussion

Water stress is the most important constraint to worldwide crop production that can be manifested either as water deficit (drought) or as water excess (waterlogging). Both environmental conditions limit the availability of capillary water that can be efficiently absorbed by plant roots (Yamaguchi and Sharp 2010). In citrus, drought causes a sharp and transient accumulation of JA followed by a progressive ABA buildup whereas soil waterlogging reduces root ABA and JA levels drastically (Arbona and Gómez-Cadenas 2008; Argamasilla et al. 2013). This puzzling observation led to hypothesize that ABA depletion is a highly regulated process and not a metabolic outcome resulting from oxygen deprivation in waterlogged conditions.

A microarray analysis carried out on roots of control and waterlogged Carrizo citrange seedlings showed that 'Response to stress', and 'Response to abiotic stimulus' were over-represented terms among the differentially expressed genes. Among these, several genes involved in ABA metabolism (namely ABA 8′-hydroxylases) and in ABA signal transduction such as several NAC domain-containing proteins (NAC1, NAC2, and ATAF1) and the regulatory β subunit of SnRK1 were found. This particular enrichment in ABA metabolism and signal transduction of upregulated genes in flooded roots of Carrizo citrange suggested that ABA mediates root responses to soil waterlogging. Therefore, a more focused study was conducted including analysis of ABA its catabolites and ABAGE, expression of genes involved in ABA metabolism

and signal perception (PYR/PYL/RCAR ABA receptors, not included in the microarray analysis) and genes encoding for ABA-dependent transcription factors and effector proteins. In two parallel experiments, Carrizo citrange seedlings were subjected to a short period of soil flooding and water deprivation followed by alleviation to compare ABA accumulation and signaling in roots of waterlogged plants with a well-known stress model. Stress release and subsequent recovery, allowed the association of the responses observed with the presence of the stress factor. Both soil flooding and drought affected gas exchange, chlorophyll fluorescence and root proline accumulation similarly. After stress release, all physiological and biochemical parameters returned to pre-stress values, indicating that the metabolic and physiological changes observed were adaptive plant stress responses and the ability of plants to recover after stress was not affected.

The two experimental conditions assayed induced opposite changes in ABA concentration (Figs. 2a and 2b) but only drought caused a parallel effect in *CsNCED1* expression (Figs. 3a and 3b). In addition, changes in PA levels always paralleled those of ABA concentration which is in line with the proposal that active ABA levels are not only regulated by its biosynthesis but also by its catabolism (Nambara and Marion-Poll 2005). To this respect, *CsCYP707A1* expression slightly increased in roots of citrus subjected to soil flooding whereas it strongly decreased in response to water deficit (Figs. 3c and 3d), suggesting that modulation of ABA levels in water stressed citrus roots is carried out partially through degradation to PA and subsequently to DPA although other mechanisms should contribute to the rapid and drastic ABA decrease in roots of soil flooded citrus (Figs. 2c, 2d, 2e and 2f). After stress release, *CsCYP707A* expression in roots returned to basal levels. In certain plant organs, such as imbibed seeds, degradation is the predominant mechanism controlling the active ABA pool (Okamoto et al. 2006). Indeed, perturbation of any of the CYP707A-encoding alleles in *Arabidopsis* led to a delay in germination (Okamoto et al. 2006).

Conjugation was investigated as an alternative mechanism to reduce ABA levels. Waterlogging and drought induced *CsAOG* expression but only roots of plants subjected to water deprivation significantly accumulated ABAGE. This is probably a result of the strong ABA accumulation in response to drought; therefore, *CsCYP707A* and *CsAOG* upregulation and accumulation of PA, DPA and ABAGE could act coordinately to fine-tune ABA levels when NCED activity is elevated, as in response to severe water deprivation. Soil waterlogging did not downregulate *CsNCED1* but, despite this, a significant reduction in ABA levels was observed. This observation cannot be explained solely by the moderate increase in DPA and the slight upregulation of *CsCYP707A* observed in roots of flooded citrus seedlings (Figs. 2g and 3c). Moreover, under these conditions, a strong induction of *CsAOG* was recorded (Fig. 3e). These observations along with the slight reduction in ABAGE concentration (only a 20% reduction respect to control values) and the 80% reduction in ABA levels (respect to control values), indicate that the decrease in ABA concentration induced by soil flooding results from a combination of degradation and

conjugation. After water drainage, hydrolysis of ABAGE to ABA by *CsBGLU18* contributes to restore basal hormone levels in the absence of *CsNCED1* upregulation (Figs. 2a, 3a, 3c, 3e and 3g). On the other hand, the increase of ABA levels in roots of citrus plants subjected to water deficit required the induction of *CsNCED1* expression along with the repression of *CsCYP707A*. Conversion of ABA to ABAGE by induction of *CsAOG* and repression of *CsBGLU18* could then contribute to fine-tune ABA levels (Fig. 3f). After re-watering, reduction of ABA to control levels is achieved at expenses of catabolism activation and return of *CsNCED1* expression to control values (Figs. 3b and 3d). Taken together, results indicate the existence of different synchronized mechanisms aimed to precisely regulate active ABA levels under stress conditions by simultaneously controlling ABA biosynthesis, degradation and conjugation.

In this and previous works (Arbona and Gómez-Cadenas 2008; Argamasilla et al. 2013), ABA depletion induced by soil waterlogging was proposed to be a specific signal to this environmental cue. In this work, some putative ABA receptors and some genes involved in ABA-dependent signaling showed an upregulation in response to soil flooding (Figs. 5a and 7). This transcriptional response, in an environmental situation where ABA levels are extremely low, might reflect an attempt of plants to maintain the basal ABA signaling necessary to develop adaptive responses to stress. Additionally, ABA-independent stress response pathways could be activated in the absence of huge amounts of the hormone. In response to drought, ABA receptors showed trancriptional downregulation or no change but, interestingly, *CsPYL8* expression followed an opposite pattern (Fig. 5b). In *Arabidopsis*, *AtPYL8* plays an important role in ABA signaling interacting with at least five PP2Cs in *Arabidopsis*, accounting for a certain specificity in the ternary complex formation (Antoni et al. 2012; Antoni et al. 2013). The different expression patterns shown by *CsPYL5* and *CsPYL8* suggest a specialization of ABA receptors in response to varying hormone concentrations that could be linked to a different affinity for their hormone ligand.

To investigate this essential aspect in determining potential role of the putative ABA receptors, these were cloned. The resulting recombinant proteins were assayed *in vitro* as functional ABA receptors measuring *Arabidopsis* ∆NHAB1 phosphatase activity in the presence of ABA or ABA agonists (Fig. 6). Data showed that the amount of ABA required to induce 50% ∆NHAB1 phosphatase activity inhibition was much higher in dimeric (*CsPYL1*) than in monomeric ABA receptors and two-fold higher in *CsPYL8* than in *CsPYL5*. This is line with previous findings in tomato where ABA IC_{50} for *PYR1* and *PYL1*-like was between 1 and 10 µM whereas for Sl6g050500 (similar to *Arabidopsis PYL5* within subfamily II) ranged between 0.1 and 0.5 µM (Gonzalez-Guzman et al. 2014). Moreover, Szostkiewicz *et al.* (2010) showed that different receptor complexes exhibited varying sensitivity to ABA as well as selectivity for different ABA isomers. In addition, phosphatase assays performed with different combinations of ABA receptors and PP2Cs showed that specific interactions between receptors and phosphatases occur (Antoni et al., 2012). This evidence allows proposing other possibilities to fine-tune ABA

responses by channeling hormone signal through specific receptor-PP2C partners which probably act modifying particular transcription factors and other target molecules (Zhao et al., 2014). In this sense, the recombinant citrus receptors showed varying responses to the ABA agonists pyrabactin and quinabactin (Fig. 6b) following the same trend described for *Arabidopsis* PYR/PYL/RCAR receptors in Okamoto et al. (2013).

The canonical model for ABA perception and signal transduction postulates that when no hormone is present, PP2Cs act as negative regulators of ABA signaling. In the preliminary microarray analysis, several PP2Cs-encoding genes were downregulated upon imposition of soil flooding (Supporting Table S2). Moreover, qRT-PCR analysis of several citrus orthologues of PP2Cs (ABI2, AHG3, HAB1 and HAI1) in flooded roots confirmed this downregulation (data not shown) whereas water deprivation upregulated these genes, following a similar pattern to that of endogenous ABA. This response has been proposed to act as a negative feedback regulation on ABA signaling (Valdés et al. 2012). In response to drought, this negative feedback mechanism is required to mitigate the strong ABA input signal that is not required under soil flooding conditions when hormone levels are low.

Analysis of stress-responsive genes revealed the upregulation of both *CsABI5* and *CsDREB2A* in roots of citrus exposed to both stress conditions (Figs. 7a through 7d). In *Arabidopsis*, *ABI5* is a member of the AREB1/ABF2 transcription factor family that is phosphorylated on Ser/Thr residues by SnRK2s. Indeed, the SnRK2-AREB/ABF interaction governs the major ABA-mediated ABRE-dependent gene expression in response to stress (Lee and Luan 2012). In cotton, overexpression of *AtABI5* induced drought tolerance (Mittal et al. 2014) and in rice *OsABI5* seems to be also involved in stress tolerance (Zou et al. 2008). Therefore, it is quite likely that this gene also plays a role in ABA-dependent stress responses in citrus. Interestingly, *CsDREB1A* expression in roots of water-deprived plants with increased ABA levels was lower than in soil-flooded ones where ABA depletion was accompanied by a slight upregulation of this gene (data not shown). This is in line with findings in rice where expression of *OsDREB1A* increased upon imposition of drought but decreased after application of ABA (Fukao et al. 2011). Moreover, *CsDREB2A* was reversibly upregulated under the two stress conditions assayed. In *Arabidopsis*, *DREB2A* has been reported to be ABA-responsive further supporting the activation of ABA signaling in roots of waterlogged citrus seedlings. In addition, under these circumstances, the induction of stress responses, such as *CsRD22* upregulation and root proline accumulation, was also evidenced.

In roots of plants subjected to abiotic stress, active ABA homeostasis is achieved through coordination of biosynthesis, degradation and conjugation. Hence, conjugation to hexoses appears as a plausible mechanism to reversibly regulate endogenous root ABA levels rather than diminishing precursor input or accelerating degradation. Conversely to what happens under drought, where NCED and ABA hydroxylases are key enzymes in controlling hormone

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levels, conjugation is complementary to degradation to achieve ABA homeostasis in response to soil waterlogging. Moreover, this mechanism allows a metabolically-inexpensive restoration of control hormone levels by *BGLU18*-catalyzed hydrolysis of ABAGE. Expression of ABA receptors and ABA-dependent genes suggest that reduction of endogenous ABA levels far below controls could constitute a specific signal to soil flooding. Moreover, *in vitro* phosphatase assays showed that *CsPYL5* was able to inhibit ΔNHAB1 activity at low ABA concentrations. Taken together, these results suggest that certain members of the ABA signaling pathway could operate at low hormone concentrations to trigger specific adaptive responses to soil flooding in coordination with ABA-independent responses.

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Figure legends

Figure 1. Physiological responses of Carrizo citrange seedlings subjected to soil flooding and drought: stomatal conductance (gs; a and b), substomatal to ambient CO_2 ratio (Ci/Ca; c and d), PSII quantum efficiency (ΦPSII; e and f) and root proline accumulation (g and h). Asterisks denote statistical significance at $p≤0.05$.

Figure 2. Hormone levels in roots of Carrizo citrange seedlings subjected to soil flooding and water deficit. Abscisic acid (ABA; a and b), phaseic acid (PA; c and d), dehydrophaseic acid (DPA; e and f) and abscisic acid glycosyl ester (ABAGE; g and h). Asterisks denote statistical significance at $p≤0.05$.

Figure 3. Relative expression of genes involved in ABA biosynthesis and catabolism in roots of Carrizo citrange seedlings subjected to soil flooding and water deficit: 9-neoxanthin carotenoid epoxydase (*CsNCED1*; a and b), ABA 8'-hydroxylase (*CsCYP707A*; c and d), ABA *O*-glycosyl transferase (*CsAOG*; e and f) and ABA β-glycosidase (*CsBGLU18*; g and h).

Figure 4. Cladogram and amino acid sequence alignment of citrus PYR/PYL ABA receptors. a) Cladogram of the multiple sequence alignment of citrus and *Arabidopsis* PYR/PYL receptors (receptors chosen for subsequent cloning and recombinant expression are marked in bold). b) Sequence and secondary structure alignment of tomato PYR/PYL ABA receptors and *Arabidopsis* PYR1 protein. The predicted secondary structure of the citrus proteins was indicated, taking as a model the crystallographic structure of PYR1 (Protein DataBank Code 3K90) and using the Espript interface (http://espript.ibcp.fr/). Boxes indicate the position of the gate and latch loops. Black asterisks mark residues K59, A89, E94, R116, Y120, S122, and E141 of PYR1 involved in ABA binding.

Figure 5. Relative expression of genes encoding for ABA receptors in roots of Carrizo citrange subjected to soil flooding (a) or drought (b). On white bars, different letters denote statistical significance at p≤0.05 following ANOVA and LSD posthoc test.

Figure 6. ABA-dependent ΔNHAB1 inhibition mediated by citrus ABA receptors (a). PP2C activity was measured *in vitro* using a phosphopeptide substrate in the absence or presence of ABA at a 1:4 ratio of phosphatase:receptor (0.5:2 µM stoichiometry). Data are mean values ± standard error for three independent experiments Different letters denote statistical significance at $p≤0.05$ following ANOVA and LSD posthoc test. ΔNHAB1 phosphatase assay test performed with the citrus ABA receptors *CsPYL1*, *CsPYL8* and *CsPYL5* using the ABA agonists quinabactin and pyrabactin at 1 and 10 µM concentrations (b). Note: Green color denotes presence of Pi in the medium and high phosphatase activity and yellow denotes no Pi in the medium and no phosphatase activity.

Figure 7. Relative expression of genes involved in ABA-dependent gene expression in roots of Carrizo citrange seedlings subjected to soil flooding and water deficit: Abscisic acid Insensitive

5 (*CsABI5*; a, b), Dehydration Responsive Element 2A (*CsDREB2A*; c, d), Responsive to Dehydration 29 and 22 (*CsRD29* and *CsRD22*; e, f and g, h) and Responsive to ABA 18 (*CsRAB18*; i, j).

Supporting information

Supporting Figure S1. Enriched GO terms among upregulated and downregulated genes.

Supporting Table S1. Upregulated genes and enriched categories.

Supporting Table S2. Downregulated genes and enriched categories.

Supporting Table S3. Primers designed for gene expression analyses by qRT-PCR.

Supporting Table S4. Comparison of protein sequences between *Arabidopsis thaliana* and *Citrus sinensis*.

Title

Depletion of abscisic acid levels in roots of flooded Carrizo citrange (*Poncirus trifoliata* L. Raf. × *Citrus sinensis* L. Osb.) plants is a stress-specific response associated to the differential expression of PYL/PYR/RCAR receptors.

Key messsage:

In this work, microarray data also suggested the involvement of ABA in the responses of citrus to soil flooding. In a series of experiments conducted the reduction of root ABA levels in response to soil waterlogging is primarily a result of conjugation (production of ABAGE) and degradation (production of PA and DPA) without significant reduction in biosynthesis; subsequently, after stress release restoration of control hormone levels is achieved at the expenses of ABAGE hydrolysis. The reduction of root ABA levels is accompanied by the upregulation of ABA-responsive genes (such as the citrus orthologues of ABI5 or DREB2A) In addition, CsPYL5 (the citrus orthologue of the Arabidopsis PYL5) showed a positive transcriptional response to soil flooding. In addition, purified recombinant CsPYL5 was able to inhibit HAB1 phosphatase *in vitro* at lower ABA concentrations than other receptors. These results indicate that reduction of root ABA levels in response to soil flooding could constitute a specific signal to this stress condition.

Title

Depletion of abscisic acid levels in roots of flooded Carrizo citrange (*Poncirus trifoliata* L. Raf. ×
Citrus sinensis L. Osb.) plants is a stress-specific response associated to the differential
expression of PYL/PYR/RCAR receptors.

Author contributions statements:

V.A. and A.G.C. planned and designed the experiments and analyzed microarray data, M.M.
and S.I.Z. performed experiments and analyzed citrus samples, P.R. and M.G.G. planned and
designed PYR/PYL receptor cloning, V.A. and M.G.G. performed cloning of selected genes and
in vitro analyses, V.A., M.G.G., M.M. and S.I.Z. analyzed data and prepared figures and tables
and V.A. wrote first draft of the manuscript. V.A., A.G.C., P.R., M.G.G., M.M. and S.I.Z.
participated in the discussion of results and manuscript writing. All authors have read and
approved the final version of the manuscript and declare that they have no conflicts of interest.

Figure 1

Figure 2

Figure 3

Figure 4

(a)

(b)

Figure 5

(a)

(b)

Supplementary figure 1
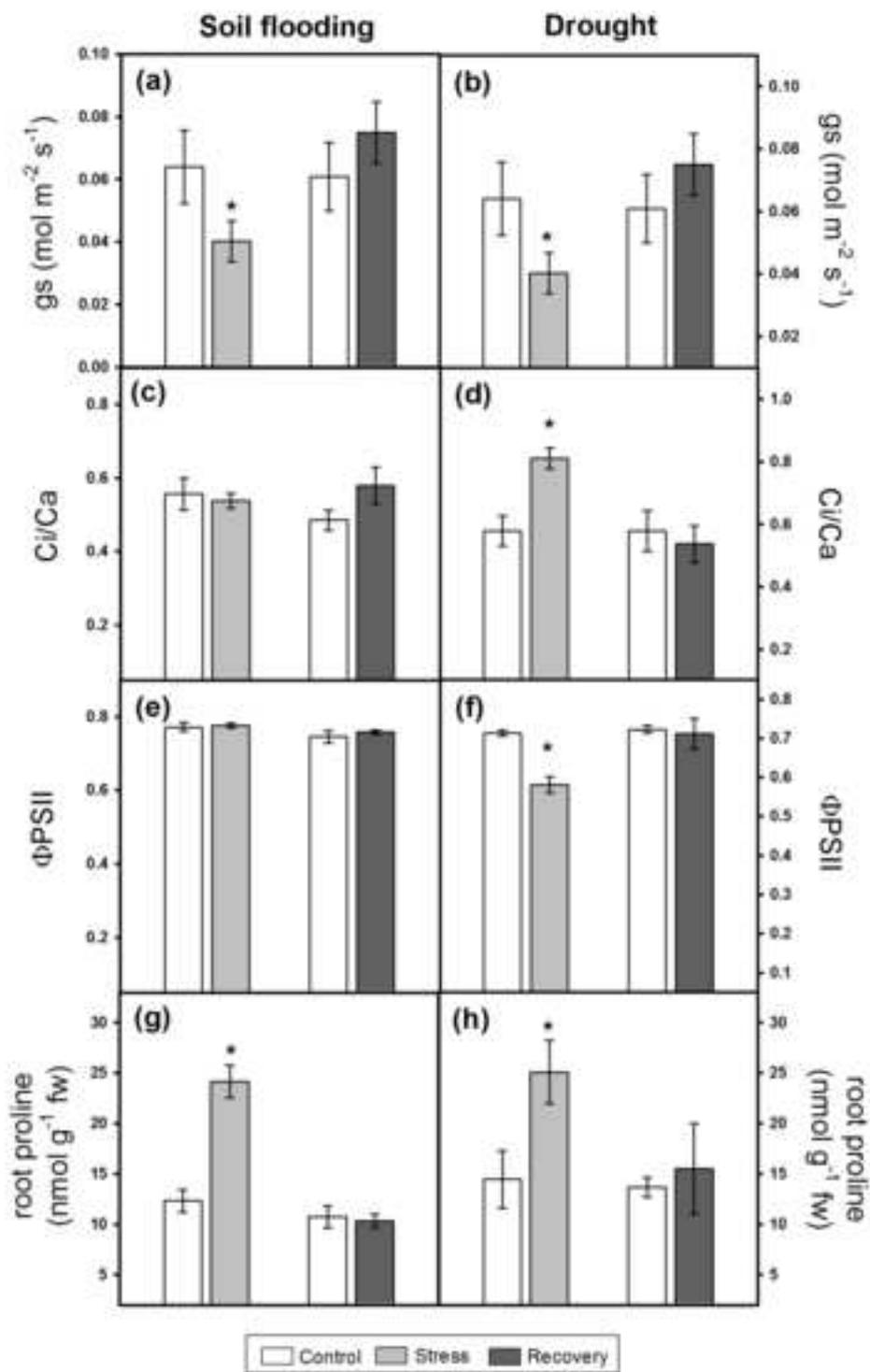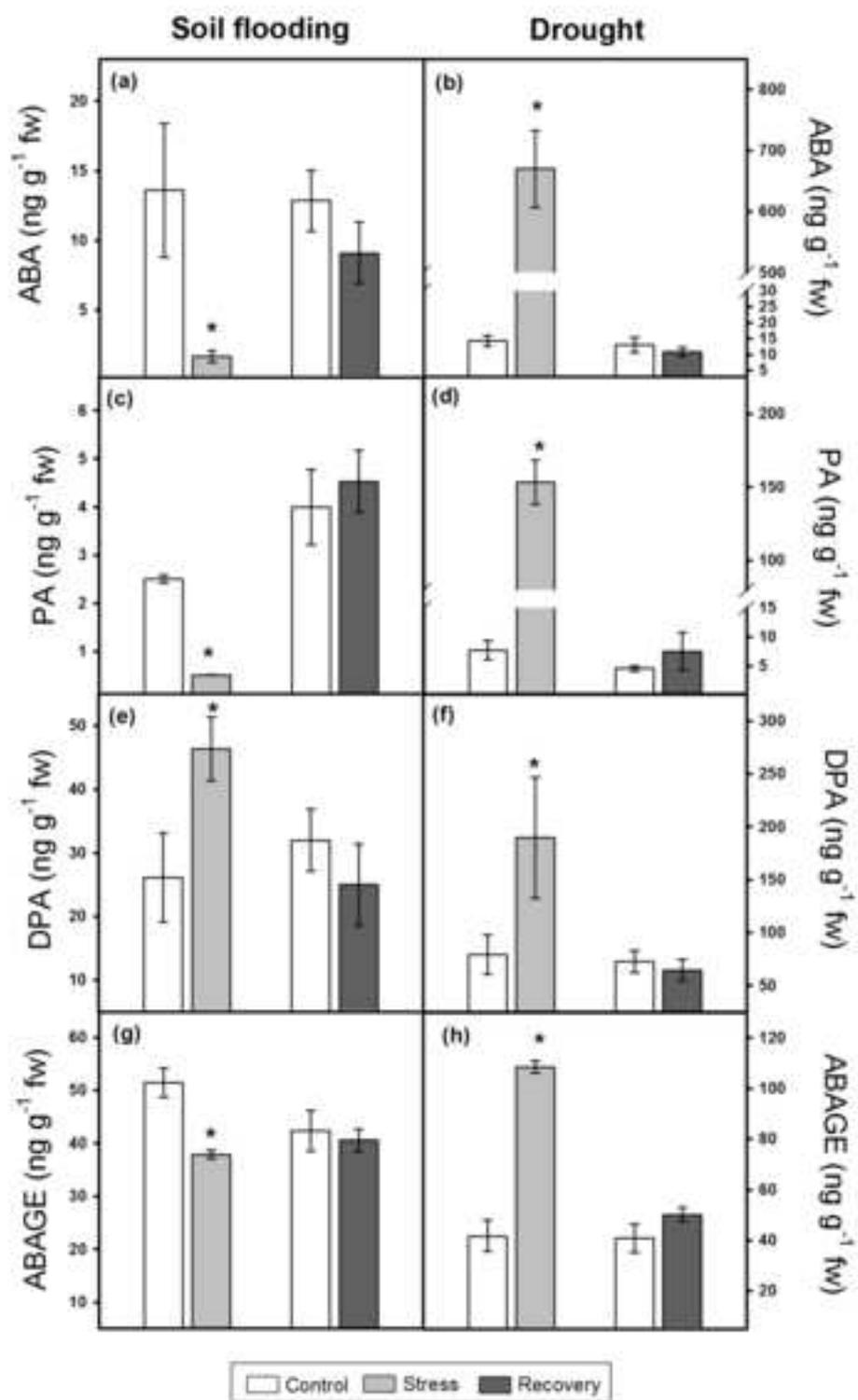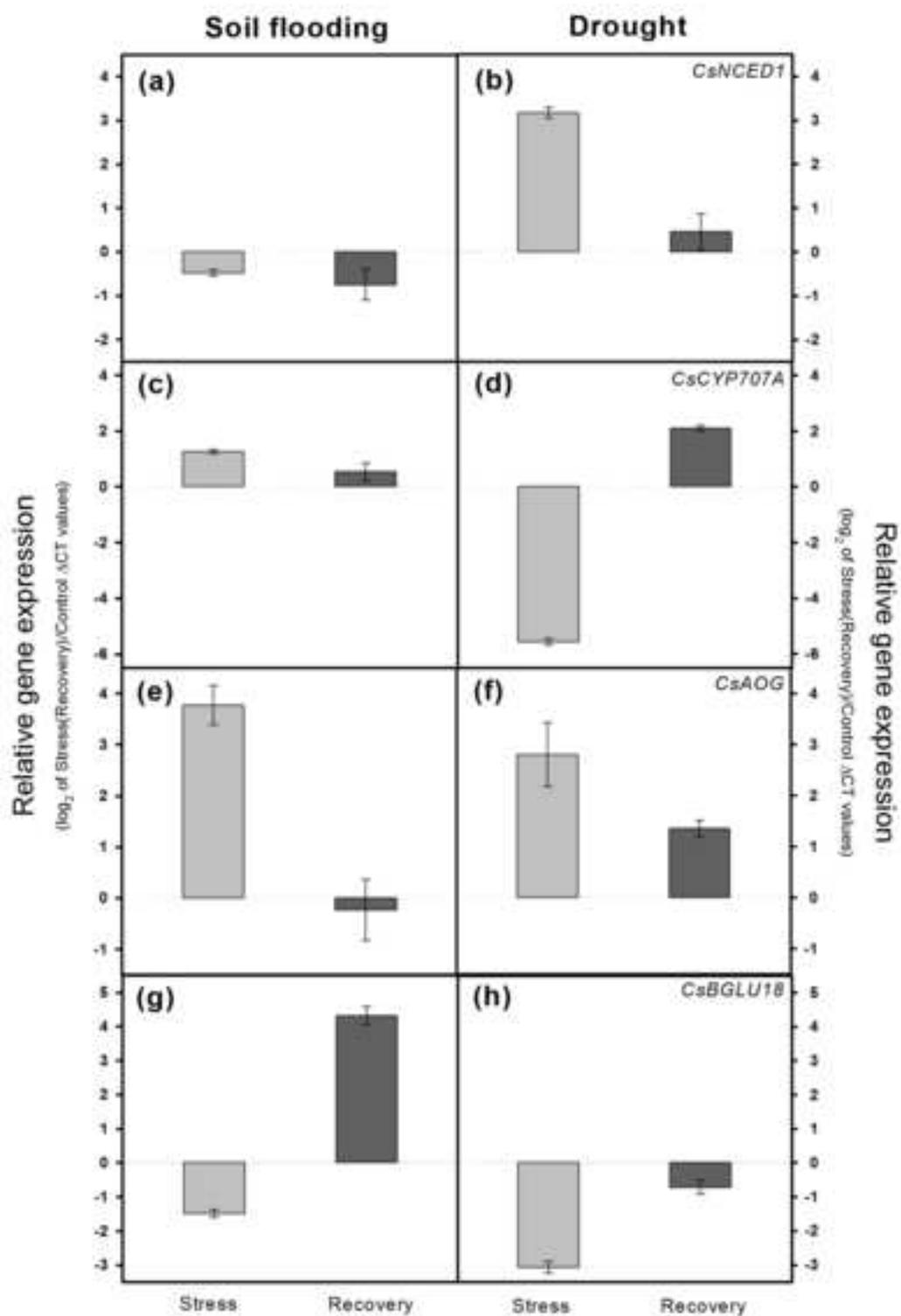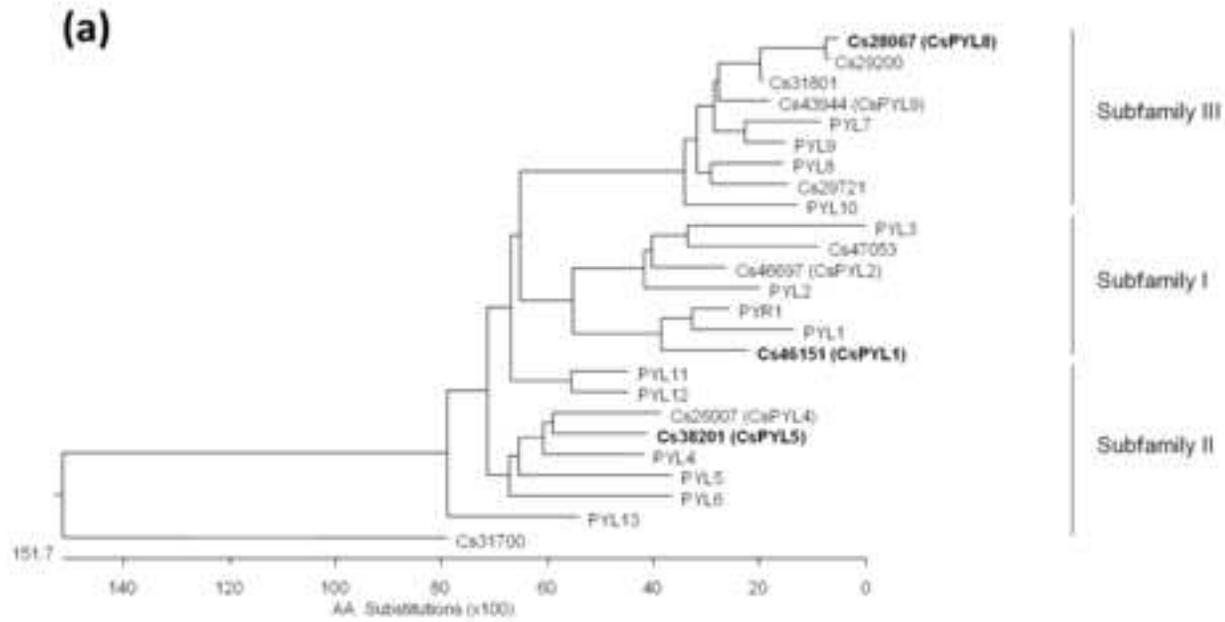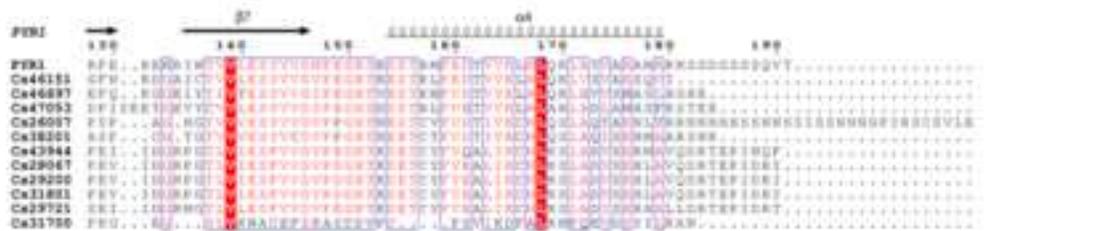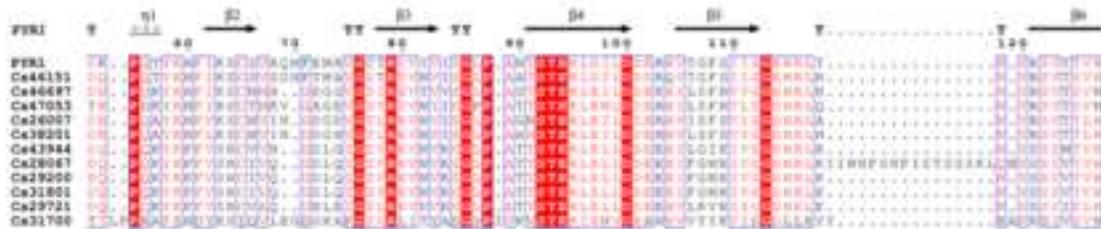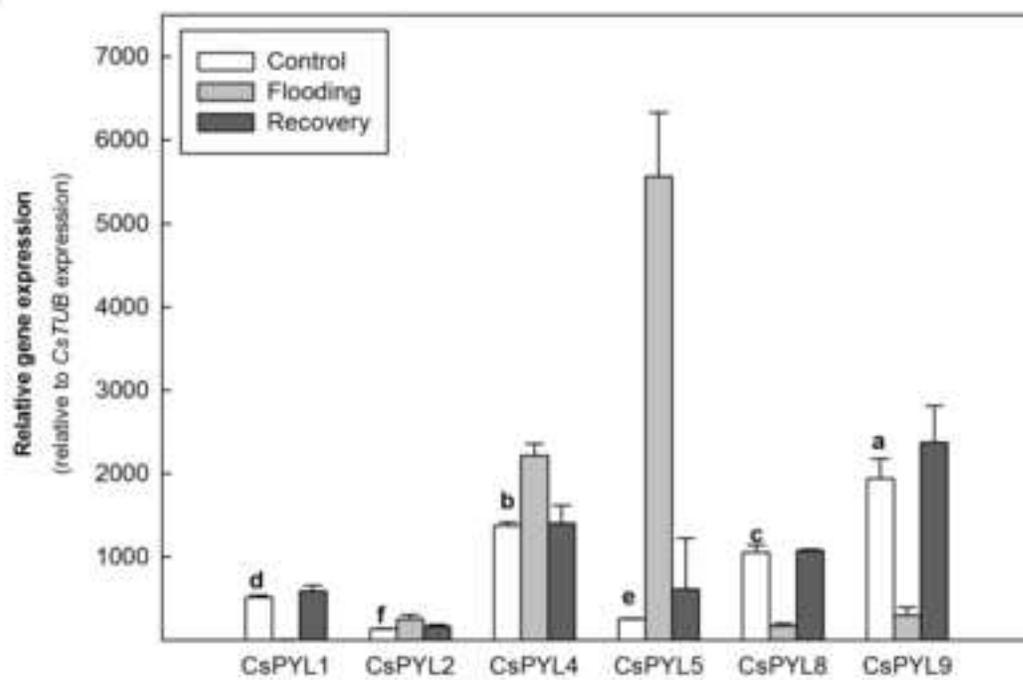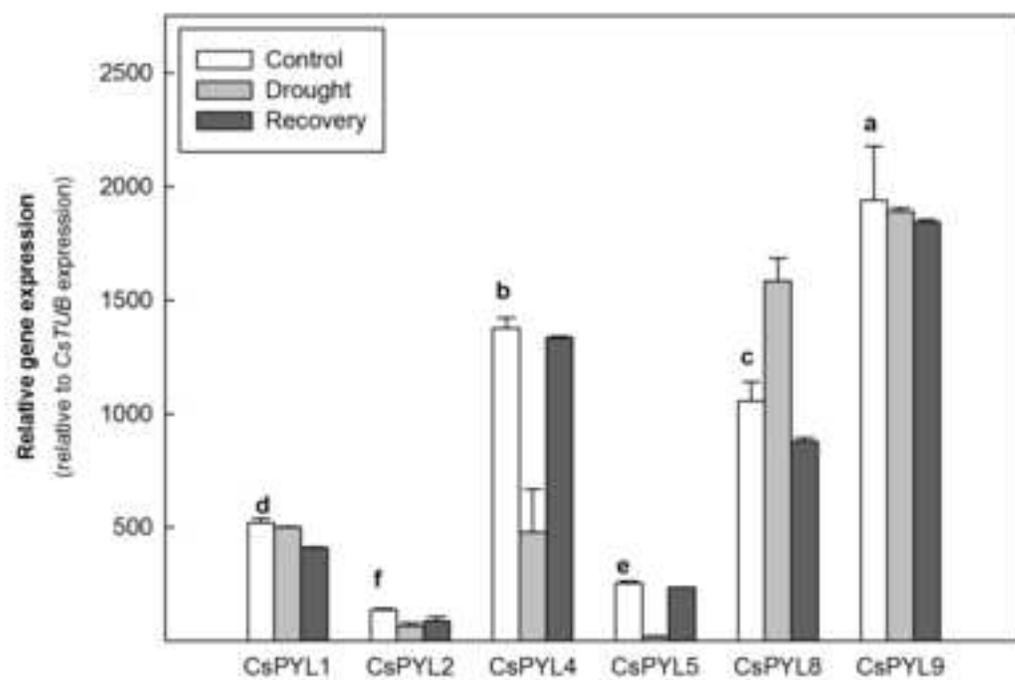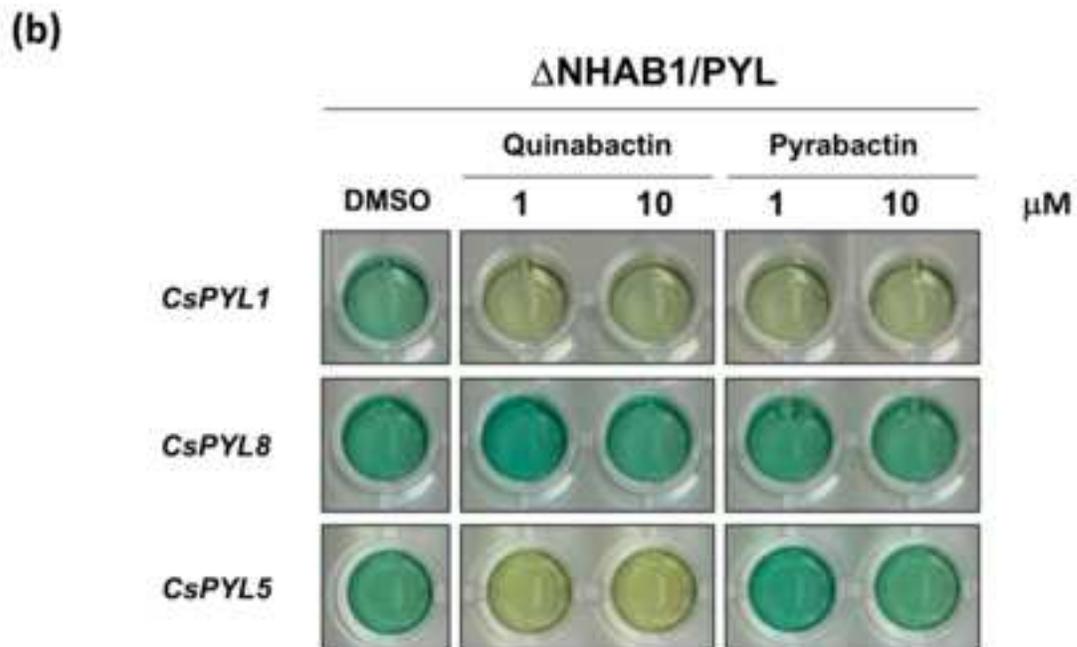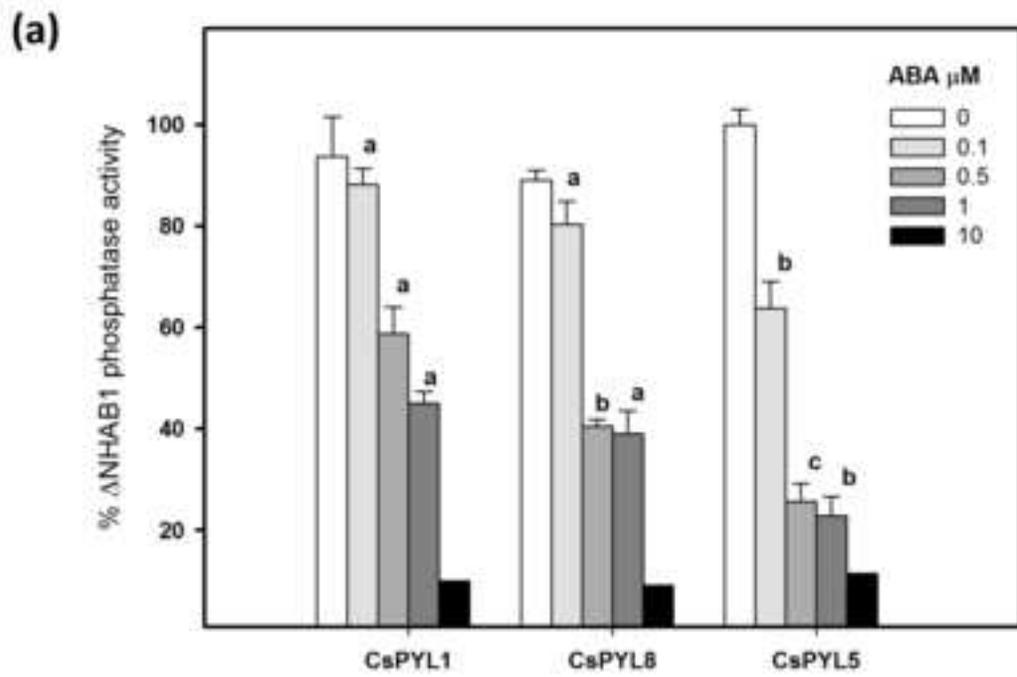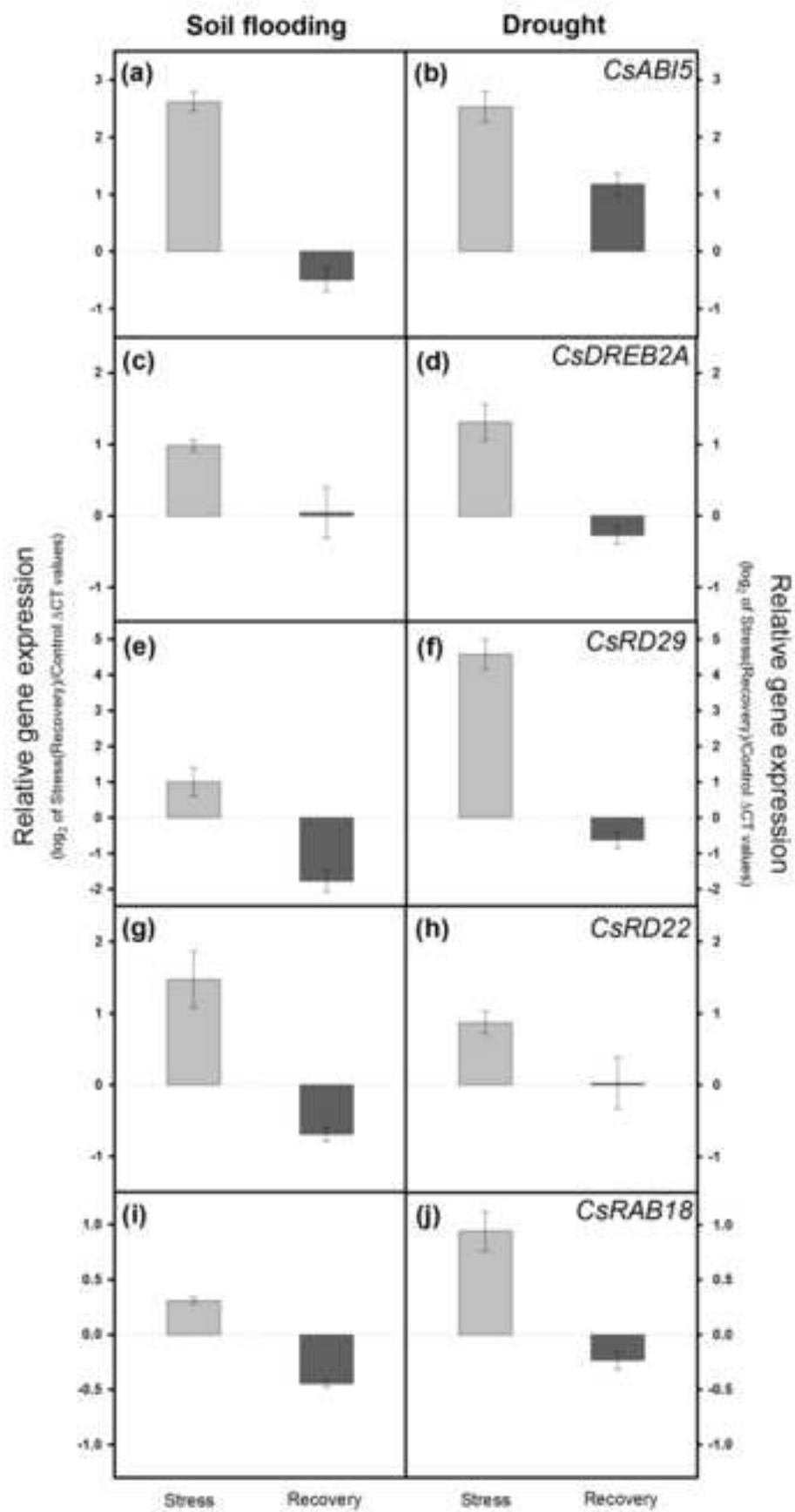
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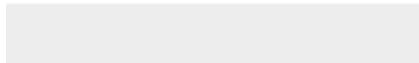
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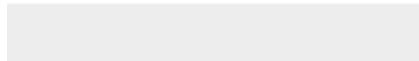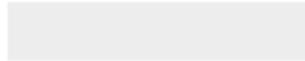
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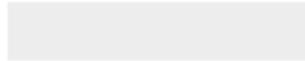
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