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New insights into short-term water stress tolerance through transcriptomic and metabolomic analyses on pepper roots

Yaiza Gara Padilla [a], Ramón Gisbert-Mullor [b], Eduardo Bueso [c], Leilei Zhang [d], Javier Forment [c], Luigi Lucini [d], Salvador López-Galarza [b], Ángeles Calatayud [a,*]

[a] Centro de Citricultura y Producción Vegetal, Instituto Valenciano de Investigaciones Agrarias, CV-315, Km 10,7, Moncada, 46113 Valencia, Spain
[b] Departamento de Producción Vegetal, CVER, Universitat Politècnica de València, Camí de Vera s/n, 46022 Valencia, Spain
[c] Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universitat Politècnica de València-C.S.I.C., Valencia, Spain
[d] Department for Sustainable Food Process, Research Centre for Nutrigenomics and Proteomics, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy

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ABSTRACT

In the current climate change scenario, water stress is a serious threat to limit crop growth and yields. It is necessary to develop tolerant plants that cope with water stress and, for this purpose, tolerance mechanisms should be studied. NIBER® is a proven water stress- and salt-tolerant pepper hybrid rootstock (Gisbert-Mullor et al., 2020; López-Serrano et al., 2020), but tolerance mechanisms remain unclear. In this experiment, NIBER® and A10 (a sensitive pepper accession (Penella et al., 2014)) response to short-term water stress at 5 h and 24 h was studied in terms of gene expression and metabolites content in roots. GO terms and gene expression analyses evidenced constitutive differences in the transcriptomic profile of NIBER® and A10, associated with detoxification systems of reactive oxygen species (ROS). Upon water stress, transcription factors like *DREB*s and *MYC* are upregulated and the levels of auxins, abscisic acid and jasmonic acid are increased in NIBER®. NIBER® tolerance mechanisms involve an increase in osmoprotectant sugars (*i.e.*, trehalose, raffinose) and in antioxidants (spermidine), but lower contents of oxidized glutathione compared to A10, which indicates less oxidative damage. Moreover, the gene expression for aquaporins and chaperones is enhanced. These results show the main NIBER® strategies to overcome water stress.

1. Introduction

Crop yields are being limited by climate change *via* many direct and not so direct effectors, such as droughts, fluctuating rainfall, floods and the dissemination of diseases and pests to new locations (FAO, 2022). Of abiotic stressors, water stress is one of the main factors to affect vegetative growth, successful reproduction and, in the end, plant survival (Arbona et al., 2013). According to the FAO (2022), water stress has become the severest natural hazard for the population all over the world in the last 40 years, but has also to the increased affected land. Hence, the need to act and improve plant tolerance to the severe water stress, that takes part in the global warming scenario (Ozturk et al., 2021).

Water stress is a multidimensional stress that provokes several diverse plant responses by affecting physiological, morphological and molecular levels (Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Among others, water stress impacts major physiological processes, such as photosynthesis, respiration and stomatal movement, which lead to plant growth restrictions (Yang et al., 2021). In addition, leaf size becomes smaller, which appear cutinized, have a thicker surface and premature induced senescence. This comes with decreased total biomass, which extends to lower plant development (Anjum et al., 2011). The onset of all plant processes affected by water stress involves multiple signals, which are transmitted through signaling pathways to drive the expression of water stress-responsive genes such as transcription factors, heat shock proteins, dehydrins, aquaporins, late embryogenesis abundant proteins, among others (Kaur and Asthir, 2017).

To overcome water stress effects, plants have developed different strategies that range from improving water uptake in roots to minimizing transpiration water loss on leaves (Farooq et al., 2009). Roots are the first organs to sense the presence of water stress and preserving root growth is an indicator of water stress tolerance (Mia et al., 2020). Furthermore, there are more specific mechanisms available to cope with water stress, for instance synthesis of organic compatible solutes and antioxidants and antioxidant enzymes, hormonal regulation, leaf

* Corresponding author.
E-mail address: calatayud_ang@gva.es (Á. Calatayud).

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rolling, epicuticular wax accumulation and a higher root/shoot ratio (Athar and Ashraf, 2009; Seleiman et al., 2021). These strategies are part of a reprogramming process at the whole plant level, triggered by gene expression changes that enable plants to survive under water stress conditions. Thus understanding plants modulation at the 'omics' level when they face water stress could help to achieve water stress-resilient plants.

The increasing number of molecular biotechnology studies is helping to untangle the molecular mechanisms involved in water stress response by gene expression approaches (You et al., 2019). Plant gene expression strategies have been conducted to study water stress tolerance on several crops, such as maize (Song et al., 2017; Zenda et al., 2019; Zheng et al., 2020; Li et al., 2021) sorghum (Fracasso et al., 2016; Varoquaux et al., 2019; Zhang et al., 2019; Kumari et al., 2021) and soybean (Xu et al., 2018; Tamang et al., 2021; Wang et al., 2022). With Solanaceae plants, the transcriptomics approach has been mostly applied to tobacco and tomato as model plants. In tobacco, Yang et al. (2017) showed upregulation of genes related to alpha-linolenic acid, arginine and proline metabolisms when stress was applied, while Khan et al. (2019) observed an enhanced expression for the genes involved in plant hormone signal transduction, starch and sucrose metabolism, and arginine and proline metabolism in the water stress-tolerant tobacco cultivar. In tomato, Lee et al. (2018) reported the down-regulation of the gene expression for the signaling pathways associated with abiotic stress responses, lignification, cell wall development and hormones in the sensitive genotype. In tomato, Iovieno et al. (2016) observed the down-regulation of the genes implicated in photosynthesis, cell proliferation and cell cycle after two water stress cycles and final rewatering. However, there have been very few studies to date on pepper plants transcriptomic responses under water stress. Borràs et al. (2021) described the complex role of *NAC* transcription factors when facing water stress on *Capsicum annuum*, because one *NAC* gene usually responds to the different stress agents that participate in multiple signaling pathways as a positive or negative regulator. Kang et al. (2020) provided the transcriptomic data of pepper exposed to different abiotic stresses to be used as information in other studies, including the expression pattern of top differentially expressed genes (DEGs) (*i.e.*, *ABI2*, *PP2CA*, *GLP3*) and representative stress-related GO terms.

In the present study, a transcriptomic experiment was performed to broaden knowledge about water stress response mechanisms on *Capsicum annuum* and understand how tolerance could be achieved through the analysis of gene expression changes. To do so, we used two *C. annuum* genotypes: NIBER® and A10. A10 is a pepper accession that has been characterized as sensitive to water stress (Penella et al., 2014). NIBER® is a hybrid pepper rootstock that has been proven tolerant to water stress (Gisbert-Mullor et al., 2020) and salt (López-Serrano et al., 2020). It was obtained and has been patented by our research team. NIBER® was able to confer the grafted variety tolerance to deficit irrigation when used as a rootstock (Gisbert-Mullor et al., 2020) due to sustained photosynthesis and lesser reduction in both biomass and fruit yield. However, there is no information to date on the short-term molecular mechanisms involved in NIBER® performance under water stress conditions that lead to its resilience. The transcriptome profile of NIBER® roots under water stress conditions could provide useful information about the hybrid's prompt responses that constitute master regulators in tolerance achievement. For this purpose, changes in gene expression and primary and secondary metabolisms were studied for NIBER® and A10 in a short-term water stress experiment.

2. Material and methods

2.1. Plant material and greenhouse conditions

Two *C. annuum* genotypes were selected as plant material according to previous studies: A10 (Habanero accession) for being sensitive to water stress (Penella et al., 2014) and the NIBER® hybrid for being tolerant to water stress conditions (Gisbert-Mullor et al., 2020). Seeds were sown in 104-hole seed trays filled with enriched substrate for germination. When plants had 6–8 real leaves, roots were cleaned to remove substrate and plants were placed in 2 L polyethylene pots covered with aluminium to block light. Pots were filled with a nutrient solution containing (mmol L^{-1}): 12.3 NO^{3-}, 1.02 H_2PO_4, 2.45 SO_4^{2-}, 3.24 Cl^-, 5.05 K^+, 0.6 NH_4^+, 4.23 Ca^{2+}, 2.2 Na^+, 2.55 Mg^{2+}; micronutrients (μmol L^{-1}): 15.8 Fe^{2+}, 10.3 Mn^{2+}, 4.2 Zn^{2+}, 43.5 B^+ and 2.14 Cu^{2+} with artificial aeration. The electrical conductivity (EC) and pH of this nutrient solution were 2.15 dS m^{-1} and 6.6, respectively. The nutrient solution was refilled daily to compensate for absorption. After 14 days of plant acclimation, water stress was induced by replacing nutrient solution with 4% polyethylene glycol (PEG) nutrient solution in the pots corresponding to the water stress treatment, whereas the pots for the control conditions were refilled with nutrient solution. Osmotic potential was − 0.55 MPa for the water stress pots (4% PEG) and − 0.05 MPa for the control pots (0% PEG), measured by a vapor osmometer (Digital osmometer, Wescor, Logan, USA). The layout design was completely randomized with 20 plants per genotype and treatment.

During the culture and experiment, plants were grown in a greenhouse at the Polytechnic University of Valencia (UPV, Valencia, Spain) under natural light conditions (800–1000 μmol m^{-2} s^{-1}), with a temperature range of 21–25 °C and 50–70% relative humidity (RH). Root samples were taken immediately before stress induction (T0), 5 h after stress induction (T1) and 24 h after stress induction (T2), and were immediately frozen in liquid nitrogen to be stored at − 80 °C.

2.2. RNA sequencing and data processing for the gene expression analysis

Four root samples per treatment, genotype and sampling time (T0, T1 and T2) were ground in liquid nitrogen with a mortar for RNA extraction. Total RNA was extracted using the Rneasy Plant Mini Kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions and treated to remove the remaining genomic DNA with the Rnase-Free Dnase Set (Qiagen, USA). RNA concentration and purity were measured by a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) to select the samples with a proper RNA concentration and appropriate A260/A280 and A260/A230 absorption ratios.

Twenty-million 100 nt reads (pair ends) per library were sequenced by DNBseq™ eukaryotic transcriptome resequencing by BGI Genomics (Cambridge, MA, USA). Three replicates were used per treatment and genotype. After adapter removal and the quality trimming of raw reads with cutadapt (Martin, 2011), a quality analysis of clean reads was done with FastQC (Andrews, 2010). Clean read pairs longer than 20 nt were then mapped to the *Capsicum annuum* genome assembly ASM51225v2 from EnsemblPlants (Capsicum_annuum - Ensembl Genomes (n.d.); Yates et al., 2022) using HISAT2 (Kim et al., 2019) with default parameters. The number of read counts mapped to one and only one of the annotated genes of the genome (uniquely mapped) were obtained with htseq-count (Anders et al., 2015). A differential expression analysis was done with DESeq2 (Love et al., 2014). The differential expression analysis was performed between both genotypes under the control conditions (NIBER®/A10) to study the constitutive differences. Another differential expression analysis was performed between water stress and the control conditions in each genotype to study the water stress-associated differences.

2.3. Gene Ontology analysis and functional annotation of DEGs

The Protein Analysis Through Evolutionary Relationships (PANTHER) classification system (Mi et al., 2019) was employed to perform a Statistical Overrepresentation Test against the *Capsicum annuum* genome to find statistically over- and under-represented GO terms among the differentially expressed genes. GO terms belong to the Gene Ontology Consortium and include the "Biological Process",

"Molecular Function" and "Cellular Component" categories. GO terms are included in a hierarchical classification, where an *Ancestor* term occupies a higher position and is followed by *Child* terms. The functional annotation of DEGs was performed with the UniProt Consortium (Bateman et al., 2021) and The Arabidopsis Information Resource (TAIR) (Berardini et al., 2015).

2.4. Metabolites quantification

Four root samples per treatment and genotype were used for metabolites quantification.

For hormones quantification, ground root samples from sampling times T1 and T2 (5 h and 24 h) were suspended in 80% methanol and 1% acetic acid containing internal standards and mixed by shaking for 1 h at 4 °C. The extract was kept at − 20 °C overnight and then centrifuged. The supernatant was dried in a vacuum evaporator. The dry residue was dissolved in 1% acetic acid and passed through an Oasis HLB (reverse-phase) column, as described in (Seo et al., 2011). The dried eluate was dissolved in 5% acetonitrile-1% acetic acid, and hormones were separated using an autosampler and reverse phase UPHL chromatography (2.6 μm Accucore RP-MS column, 100-mm length 2.1-mm inner diameter; ThermoFisher Scientific) with a 5–50% acetonitrile gradient containing 0.05% acetic acid at 400 μL min^{-1} for 21 min. Hormones were analyzed in a Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific) by targeted Selected Ion Monitoring (SIM). The concentrations of hormones in extracts were determined using embedded calibration curves and XCALIBUR 2.2 SP1 build 48 and TRACEFINDER. The internal standards for the quantification of each different plant hormones were the deuterium-labeled hormones.

For the other metabolites, the samples from sampling time T2 (24 h) were lyophilized in a vacuum evaporator (Labconco Corporation, Kansas City, USA) and extracted by the ultrasound method in a mixture of 20% (v/v) methanol + 2 mM EDTA and acidified with 1% (v/v) formic acid at a ratio of 1:40. Extracts were than centrifuged at 8000 g for 10 min at 4 °C (Eppendorf 5810R, Hamburg, Germany). Next, supernatants were collected, syringe-filtered (0.22 μm pore size), and transferred to vials for the subsequent analysis.

The trehalose, galactinol, raffinose, and stachyose oligosaccharides quantification on root samples was performed by a high-performance anion exchange chromatography coupled with a pulsed amperometric detection approach (HPAEC-PAD). Equipment consisted in a Dionex ICS-5000 + (Thermo Fisher Scientific, Waltham, USA), containing an electrochemical cell with a gold working electrode combined with a pH-Ag/AgCl reference electrode as the detection system. The separation method applied for the HPAEC-PAD analysis was optimized for all four oligosaccharides through a Dionex CarboPac PA200 column (3 x 250 mm) coupled to a guard column (3 x 50 mm) as the stationary phase (both purchased from Thermo Scientific), which confers high-resolution separation of monosaccharides and linear oligosaccharides (Rocchetti et al., 2022). Specifically for trehalose and raffinose, the mobile phase was a binary solvent system composed by H_2O milliQ (eluent A) and 30 mM NaOH (eluent B). An isocratic separation method was applied with a total run time of 30 min, the flow rate was adjusted at 0.4 mL/min and the temperatures for both the column and detector compartments were set at 300 °C. Second, the stachyose oligosaccharide was separated using a binary solvent system composed of H_2O milliQ (eluent A) and 25 mM NaOH (eluent B). An isocratic separation method was applied with a total run time of 30 min, the flow rate was adjusted at 0.25 mL/min and the temperatures for both the column and detector compartments were set at 30 °C. Finally, the galactinol oligosaccharide was separated by a binary solvent system comprising H_2O milliQ (eluent A) and 500 mM NaOH (eluent B). An isocratic separation method was applied with a total run time of 30 min, the flow rate was adjusted at 0.4 mL/min and the temperatures for both the column and detector compartments were set at 30 °C. Quantification of oligosaccharides was achieved according to the standard curves of trehalose dihydrate, galactinol dihydrate, raffinose pentahydrate (Merck KGaA, Darmstadt, Germany), and stachyose hydrate (Cayman Chemical, Michigan, USA), designed with five concentration points.

The target analysis for polyamines and oxidized glutathione was performed using a Q Exactive Focus Hybrid Ultra-High Pressure Liquid Chromatography Quadrupole-Orbitrap Mass Spectrometer (UHPLC-HRMS; Thermo Scientific) coupled to a Vanquish UHPLC pump and equipped with a heated electrospray ionization-II probe (Thermo Scientific; (Rocchetti et al., 2021)). Chromatographic separation was based on a water-acetonitrile (LC-MS grade, Sigma-Aldrich) gradient elution (6–94% acetonitrile in 35 min), employing 0.1% formic acid as the phase modifier and an Agilent Zorbax Eclipse Plus C18 column (50 × 2.1 mm, 1.8 μm) at a flow rate of 200 μL/min and an injection volume of 6 μL.

Data acquisition was performed in the data-dependent (Top N = 3) MS/MS mode, in a full scan mass resolution of 17500 at m/z 200 using positive ionization for spermine, spermidine and putrescine, and negative ionization for oxidized L-glutathione. Identification was performed with an AGC target value of 1×10^5, a maximum injection time of 100 ms and an isolation window of 1.0 m/z. The Top N ions were fragmented employing a stepped Normalized Collisional Energy (*i.e.*, 10, 20, 40 eV). The heated electrospray ionization parameters were the following: sheath gas flow 40 arbitrary units, auxiliary gas flow 20 arbitrary units, spray voltage 3.5 kV, capillary temperature 320 °C. Specifically, quantification of polyamines and glutathione was performed with the standard calibration curves of spermine, spermidine, putrescine and oxidized L-glutathione (Merck KGaA, Darmstadt, Germany).

2.5. Statistical analysis

As mentioned in 2.1., the layout design of the experiment was completely randomiszd and included 20 plants per treatment and genotype combination. The samples for metabolites quantification (2.4.) were tested by a one-way ANalysis Of VAriance (ANOVA) with Statgraphics Centurion XVIII (Statgraphics Technologies, Inc., The Plains, Virginia, USA). Fisher's Least Significant Difference test (LSD) was performed at $p < 0.05$. For oligosaccharides, data were expressed as the difference between the oligosaccharide content under the water stress and control conditions for each genotype.

3. Results

3.1. Gene expression constitutive differences between NIBER® and A10 roots

The transcriptome comparison between genotypes NIBER® and A10 under the control conditions showed 1597 up-regulated and 1430 down-regulated DEGs. The Gene Ontology Overrepresentation test on DEGs was performed for Biological Process (Fig. 1), Molecular Function (Fig. S1) and Cellular Component (Fig. S2).

The GO test on Biological Process showed statistically over-represented categories (Fig. 1) for NIBER® *versus* A10, classified by an increasing Gene Ratio to include "response to stimulus" and "response to chemical" with more genes (222 and 115 genes, respectively) and a lower p-value ($p < 0.01$), followed by "response to oxidative stress", "response to inorganic substance" and "photosynthesis, light harvesting in photosystem I" with the highest Gene Ratio and fewer genes (32, 25 and 10 genes, respectively). All the over-represented categories were statistically significant ($p < 0.05$).

The GO test on Biological Process also showed statistically under-represented categories (Fig. 1) for NIBER® *versus* A10, classified by a lowering Gene Ratio, including "primary metabolic process", "protein metabolic process", "nitrogen compound metabolic process" and "macromolecule metabolic process" with more genes (393, 198, 326 and 288 genes, respectively), followed by "nucleobase-containing compound metabolic process", "RNA metabolic process", "gene expression"

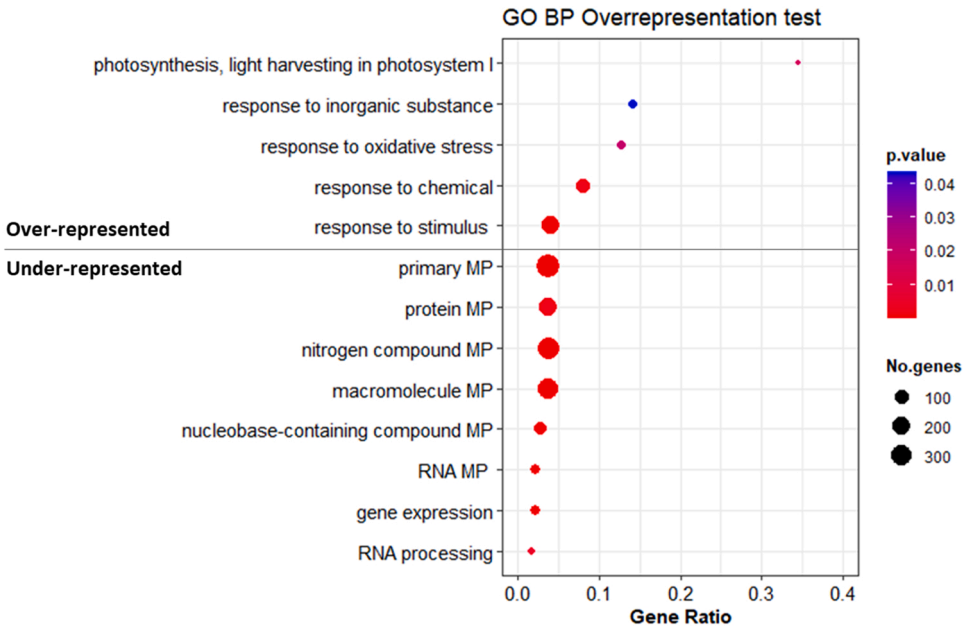

Fig. 1. GO Overrepresentation test for the DEGs resulting from NIBER® in relation to A10 comparison under the control conditions showing the statistically over- and under-represented categories for Biological Process. The Gene Ratio is the percentage of DEGs in relation to the total number of genes associated with the GO term in the *Capsicum annuum* genome. MP means Metabolic Process.

and "RNA processing" with the lowest Gene Ratio and fewer genes (82, 39, 46 and 16 genes, respectively). All the under-represented categories were statistically significant ($p < 0.01$).

The GO test on Molecular Function (Fig. S1) showed statistically over- and under-represented categories with $p < 0.01$. The over-represented categories included "oxidoreductase activity" (188 genes), "heme binding" (68 genes) and "terrapyrrole binding" (79 genes), classified by an increasing Gene Ratio. The under-represented categories included "RNA binding" (32 genes) and "cysteine-type peptidase activity" (7 genes), classified by a lowering Gene Ratio.

The GO test on Cellular Component (Fig. S2) showed statistically over- and under-represented categories with $p < 0.05$. The over-represented categories included "extracellular region" (69 genes), "chloroplast thylakoid" (25 genes) and "photosystem I" (16 genes), classified by an increasing Gene Ratio. The under-represented categories included "intracellular organelle" (396 genes), "protein-containing complex" (113 genes), "intracellular non-membrane-bounded organelle" (43 genes) and "ribonucleoprotein complex" (13 genes), classified by a lowering Gene Ratio.

The DEGs under the control conditions suggested constitutive differences in the stress-related processes between NIBER® and A10 (Table S1). NIBER® is a hybrid pepper rootstock with the feature that overcomes water stress situations (Gisbert-Mullor et al., 2020). However, the gene signaling network involved in its tolerance remains unclear.

In this analysis we were able to find DEGs related to **osmoprotection**, specifically for trehalose synthesis. Genes such as *TPS1* (trehalose-phosphate synthase 1, T459_35236, (Fichtner et al., 2020)) and *TPP* (trehalose 6-phosphate phosphatase, T459_05222, (Vogel et al., 1998)) were down-regulated in NIBER®. In addition, sugar transporters like *SWEET* (T459_11884) and other genes related to glucose transport (T459_19291 and T459_08278; Dyson et al., 2014) were also down-regulated in NIBER®. This up-regulation of the sugar-related genes in A10 is in accordance with the *SS1* (soluble starch synthase 1, T459_09622) down-regulation in A10.

Regarding **hormones**, auxin synthesis and signaling are key for explaining differences in plant growth. We confirmed that two important genes, *i.e.*, *TIR1* (auxin receptor, T459_15901; Dharmasiri et al., 2005) and *ILR1* (an amidohydrolase that releases active indole-3-acetic acid from conjugates, T459_25791; Bartel and Fink, 1995) were up-regulated in NIBER®. Similarly, four genes encoding putative indole-3-acetic acid-amido synthetase *GH3* (T459_33260, T459_24904, T459_20103, T459_33259; Staswick et al., 2005) to, thus favour indole-3-acetic acid (IAA) inactivation, were down-regulated in NIBER®. Abscisic acid (ABA) is also crucial for overcoming abiotic stress. *NCED1* (a main enzyme involved in ABA synthesis, T459_20461; Schwartz et al., 2001) was up-regulated in NIBER®, but *PYL1*, one of the receptors of this hormone, was down-regulated (T459_17022; Hao et al., 2011). Finally, we found five putative *ACO* genes (1-amino-cyclopropane-1-carboxylate oxidase, T459_24052, T459_14026, T459_07834, T459_22421, T459_07844) coding for a key enzyme in the ethylene synthesis (Jafari et al., 2013) and down-regulated in NIBER®.

Photosynthetic performance is one of the main processes with a differential response to stress when comparing tolerant and sensitive plants (Padilla et al., 2021). In agreement with this, we were able to find that *APO1* (T459_00386) was up-regulated in NIBER®, a protein required for the accumulation of Photosystem I and NADH dehydrogenase complexes in the chloroplast (Amann et al., 2004). *CYP89A9* (T459_09773) is a cytochrome involved in the formation of major chlorophyll catabolites during leaf senescence in Arabidopsis (Christ et al., 2013) and was up-regulated in NIBER®, together with *TGD1*, a permease involved in lipid transfer from the endoplasmic reticulum (ER) to the chloroplast (T459_33667; Xu et al., 2003). However, we found down-regulated genes coding for Photosystem II subunit P-2 (T459_26094; Kochhar et al., 1996), two subunits of the cytochrome b6-f complex (T459_23921 and T459_31770; Munekage et al., 2001), two genes coding for protein gradient regulation (*PGR5*, T459_04004 and *PGRL1A*, T459_07652; DalCorso et al., 2008), subunit K of the Photosystem I reaction center (T459_03540; Varotto et al., 2002) and a protein required for anchoring the FNR flavoenzyme to the thylakoid membrane (T459_21968; Jurić et al., 2009).

We also observed changes in important genes for **membrane homeostasis**. Calcium is one of the most significant cations for stress signaling (Edel et al., 2017). Two calcineurin-binding proteins, *i.e.*, *CBL7* and *CBL9* (T459_08611, T459_16980; Batistič et al., 2009), and *CIPK20* (T459_05348, a CBL-interacting protein kinase) were up-regulated in

NIBER®. Nevertheless, calcium-dependent protein kinase *CPK4* (T459_17188; Zhu et al., 2007) and calmodulin *CML11* (T459_07898; McCormack and Braam, 2003) were down-regulated. Other down-regulated genes in this rootstock were aquaporin *PIP2–4* (T459_26048; Quigley et al., 2001) and vacuolar proton pump *AVP1* (T459_34590; Gaxiola et al., 2001).

Reactive oxygen species (ROS) formation is also a consequence of stress exposure in plants (Gill and Tuteja, 2010). Constitutive differences in **detoxification systems** are key for overcoming future stress (Landi et al., 2017). Based on this, the analysis showed the DEGs involved in the scavenging of these toxic compounds. Formate and malate dehydrogenase (T459_06869, T459_31834) are enzymes involved in coping with oxidative stress, which were up-regulated in NIBER® (Heyno et al., 2014; Thomas et al., 2016). *HIRD11* was also up-regulated in NIBER® and is a dehydrin that can reduce ROS formation (T459_15197; Hara et al., 2013). In contrast, we found up-regulation in A10 of two glutathione transferases, enzymes that are involved in detoxification (T459_27445, T459_31012), and *RbohB* was up-regulated, which is one of the main isozymes responsible for superoxide anion production (T459_34097; Sagi and Fluhr, 2006). It has been demonstrated that polyamines function in stress tolerance by modulating ROS homeostasis (Alcázar et al., 2006). We found that *PAO4* was up-regulated in NIBER®, which is a flavoprotein that catalyzes the oxidative conversion of spermine into spermidine in *Arabidopsis thaliana* (T459_24048; Kamada--Nobusada et al., 2008), as well as *PUT4*, a polyamine uptake transporter (T459_06454; Mulangi et al., 2012). Finally, T459_03483, coding for spermidine synthase, was up-regulated in the sensitive rootstock (Hanzawa et al., 2002).

Chaperones are crucial in preventing protein denaturation (Wang et al., 2004). This analysis displayed *BAG4* as being up-regulated in NIBER® (T459_16041; Yan et al., 2003), but also the downregulation of one isoform of a heat shock protein (HSP) 90 co-chaperone (T459_26371; D'Alessandro et al., 2015) and the small HSP (sHSP) 18.2KDa (T459_25314).

3.2. Water stress impact on gene expression and associated GO categories

The comparison of the response under water stress (simulated by PEG addition) and the control conditions 5 h after treatment (T1) resulted in 437 specific DEGs for A10, 168 DEGs in NIBER®, and 222 DEGs were shared by both genotypes (Fig. 2A, Table S1). However, at 24 h after treatment (T2), the number of DEGs of NIBER® and A10 dropped in relation to T1, and only 68 DEGs were specific for A10, 108 DEGs for NIBER®, and 22 DEGs in both genotypes (Fig. 2B, Table S1).

The GO Overrepresentation test for the aforementioned DEGs showed that several BP categories were shared by both genotypes involved in the response to water stress at T1 and T2 (Fig. 3), but there were also some categories only present in either A10 (Fig. 4) or NIBER® (Fig. 5).

The BP shared by both genotypes (Fig. 3) belong to the *ancestor* categories "response to stimulus", "biological regulation", "primary metabolism", "other metabolic processes" and "CC organization or biogenesis" (the last one for T2). For "response to stimulus" the *child* terms were "response to oxygen-containing compound", "oxidant detoxification" and "auxin-activated signaling pathway" for T1 and T2; "hydrotropism" and "response to abscisic acid" for T1; "response to oxidative stress" and "response to auxin" for T2. The "Biological regulation" *child* terms were "cellular ion and lipid homeostasis" and "regulation of stomatal closure" for T1, and "negative regulation of proteolysis" for T2. "Cellular component organization or biogenesis", which appeared at T2, was associated with cell wall. The "Primary metabolism" at T1 included the carbohydrate metabolic processes for trehalose biosynthesis, L-arabinose and galacturonan; the lipid processes for steroids, fatty acids and lipid oxidation; several protein processes related to ubiquitination, retrograde transport and catabolism of misfolded proteins. However, the primary metabolism in T2 could be summarized in lipid catabolism. Lastly, "other metabolic processes" comprised glutathione biosynthesis and hydrogen peroxide catabolism for T1 and T2; the polyamine biosynthesis and photosynthesis-related processes involving the electron transport chain and PSI for T1.

The BP exclusive for A10 under water stress in relation to control conditions (Fig. 4) belong to the *ancestor* categories "response to stimulus", "transport", "biological regulation", "primary metabolism", "organelle organization" (for T1) and "other metabolic processes" (for T2). The "Response to stimulus" *child* terms were "abscisic acid-activated signaling pathway" for T1 and T2; "response to water deprivation" for T1; response to abscisic acid and ethylene, and "ethylene-activated signaling pathway" for T2. The "Transport" associated categories were "phospholipid translocation" and vesicle-mediated transport including docking, budding from membrane and fusion for T1; "protein import into nucleus" for T2. "Organelle organization", which appeared at T1, was related to the vacuole and chloroplast accumulation and avoidance movements. For "biological regulation" the *child* terms were auxin homeostasis and catabolism, "regulation of brassinosteroid-mediated signaling pathway" and "protein stabilization" for T1; "regulation of protein dephosphorylation" for T2. The "Primary metabolism" at T1 comprised the carbohydrate biosynthesis processes for glucose, sucrose and cell wall cellulose; lipid processes, such as abscisic and jasmonic acid metabolism and phospholipid biosynthesis; protein metabolism, with some catabolism processes involving ubiquitination and/or the proteasome, but also proline catabolism and arabinogalactan protein metabolism. At T2, the "primary metabolism" included

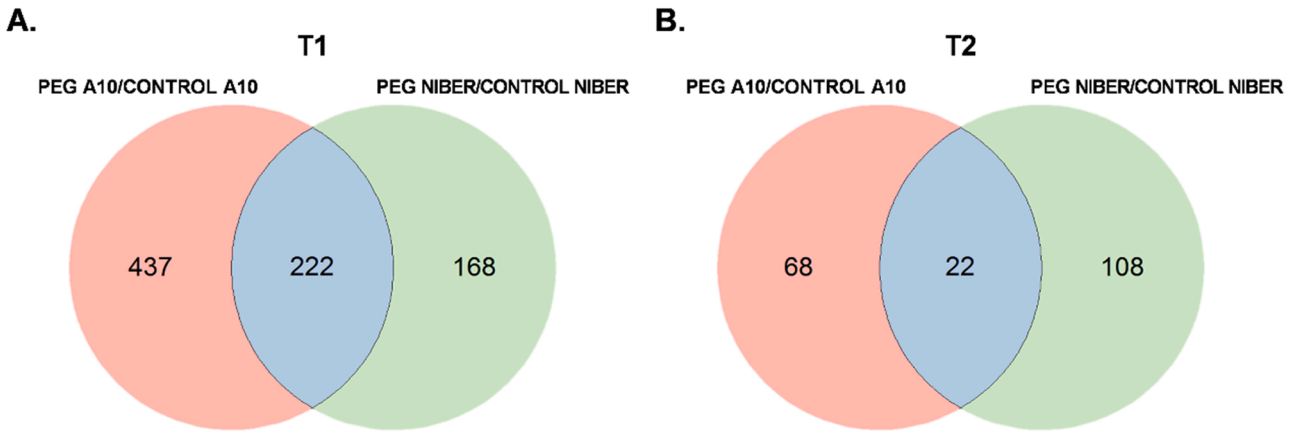

Fig. 2. Venn diagram for the DEGs under water stress/control conditions at T1 (A) and T2 (B) for each genotype. Red depicts the DEGs exclusive for A10, green the DEGs specific for NIBER® and blue the DEGs shared by both genotypes.

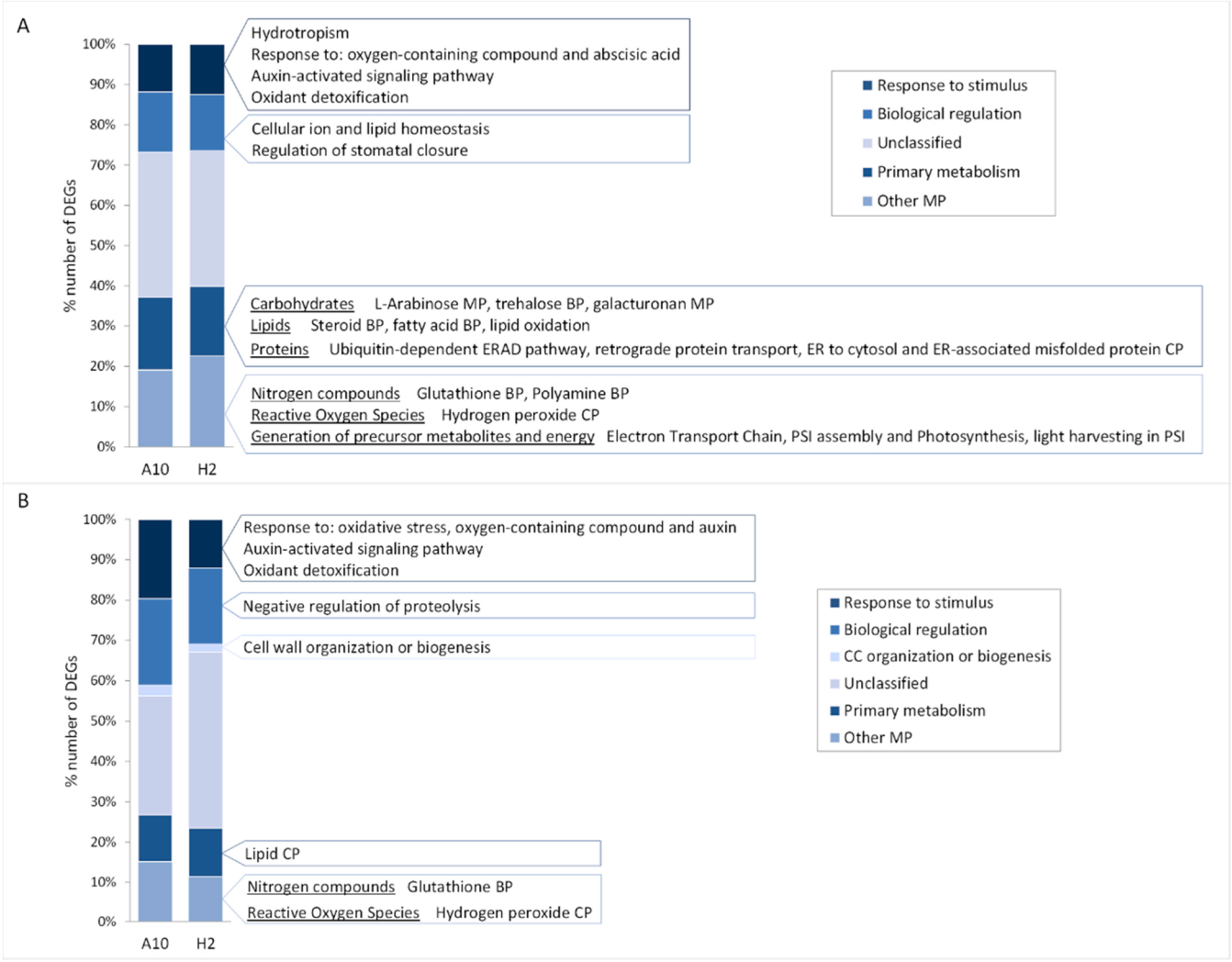

Fig. 3. *Ancestor* and *Child* BP categories shared by NIBER® and A10 for the water stress/control conditions at T1 (A) and T2 (B). MP is Metabolic Process, BP is Biological Process, ER is Endoplasmic Reticulum, CP is Catabolic Process, PSI is Photosystem I and CC is Cellular Component.

galacturonan metabolism for carbohydrates and protein autophosphorylation, but also a common process at T1 and T2, which was the "SFC-dependent proteasomal ubiquitin-dependent protein catabolic process". Lastly, "other metabolic processes" was present at T2 and was associated with oxoacid metabolism.

The specific BP in NIBER® under water stress in relation to the control conditions (Fig. 5) belong to the *ancestor* categories "response to stimulus", "transport", "biological regulation", "protein folding", "primary metabolism" and "other metabolic processes". The "Response to stimulus" *child* terms were "response to hydrogen peroxide" for T1 and T2, response to salicylic acid and unfolded protein for T1, response to osmotic stress and ER-unfolded protein for T2. "Transport" at T1 was related to "plasmodesmata-mediated intercellular transport", while T2 included lipid, water and vesicle-mediated transport. For "biological regulation" the *child* terms were "brassinosteroid homeostasis", "regulation of jasmonic acid-mediated signaling pathway", "regulation of proteolysis" and "regulation of photosynthesis, light reaction" for T1; hormone biosynthesis and regulation of hormone levels and cellular response to hypoxia for T2. "Protein folding" can be summarized in "chaperone cofactor-dependent protein folding" for T1 and T2. The "Primary metabolism" common process at T1 and T2 was cellulose catabolism; at T1 there were lipid processes, such as "fatty acid alpha-oxidation" and biosynthesis of brassinosteroid and unsaturated fatty acid. At T2 there was hemicellulose metabolism for carbohydrate-related processes and ubiquitination and dephosphorylation for protein-related processes. Lastly, "other metabolic processes" was associated with polyamine biosynthesis, which was a common *child* term at T1 and T2, but could be identified as spermine and spermidine biosynthesis at T1.

3.3. Outstanding genes with differential expression under water stress

NIBER® and A10 have a common response after water stress, but also a specific one for each genotype (Table S1).

Both genotypes respond to water stress with the deregulation of the genes involved in **carbohydrates** synthesis. For instance, the genes involved in raffinose synthesis (Nishizawa et al., 2008) were upregulated as T459_10139 in NIBER® and T459_06047 in A10. We specifically found *GolS2* (galactinol synthase 2, T459_06371; Taji et al., 2002) was up-regulated in NIBER® and a trehalose-phosphate synthase (T459_18743) in A10. However, the analysis also displayed down-regulated genes in both genotypes; for instance T459_16904 (a trehalose-phosphate phosphatase).

We also detected the deregulation of other **osmoprotectant**-related genes in A10. By way of example, the genes involved in proline catabolism (T459_06801; Funck et al., 2010) and transport (T459_23231; Rentsch et al., 1996) were up-regulated, while a glycine betaine biosynthesis gene (T459_17755; Missihoun et al., 2015) was

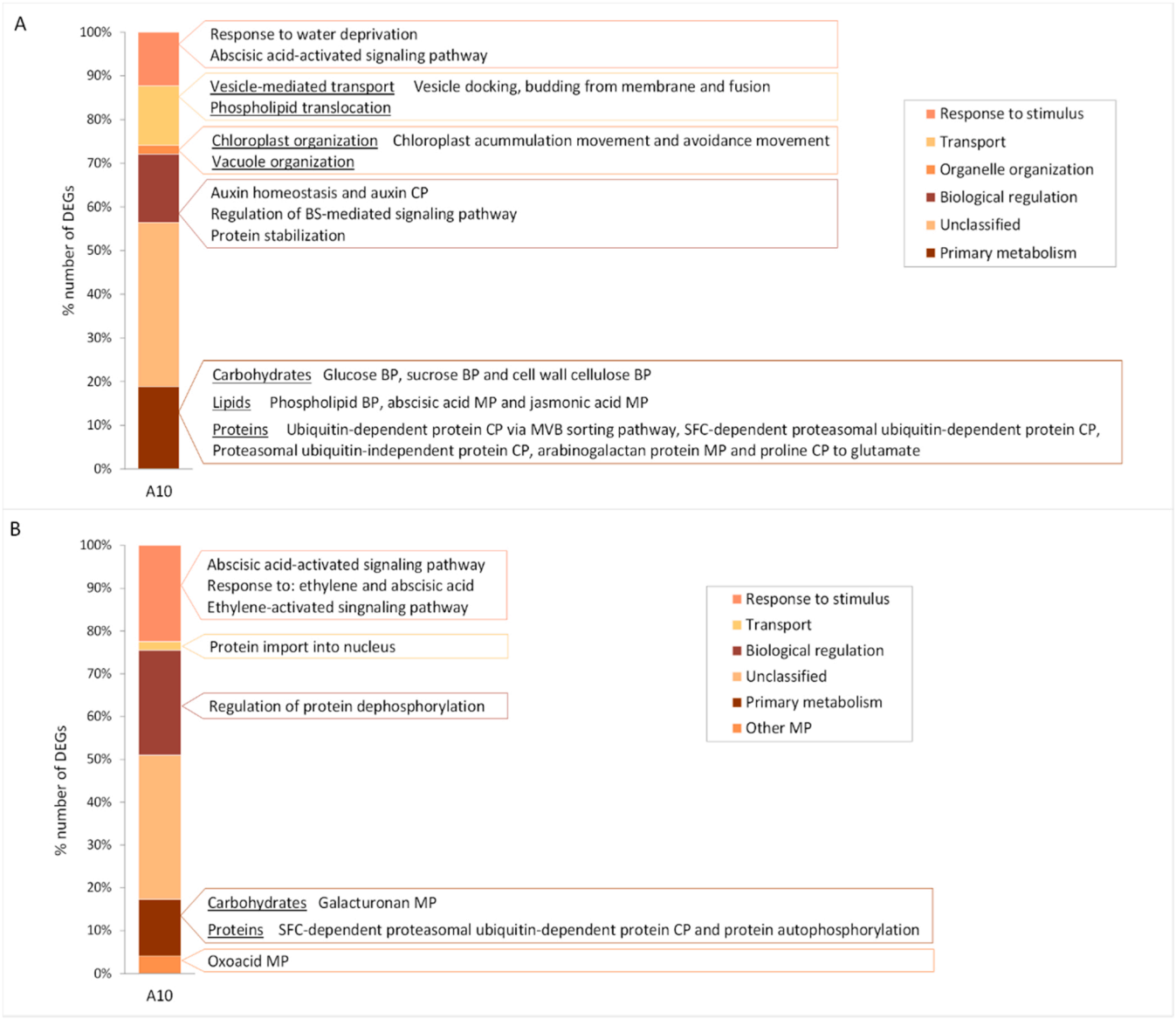

Fig. 4. *Ancestor* and *Child* BP specific categories in A10 for the water stress/control conditions at T1 (A) and T2 (B). CP is Catabolic Process, BS is brassinosteroid, BP is Biological Process, MP is Metabolic Process and MVB is multivesicular body.

down-regulated.

The genes coding for **hormone** signaling or synthesis are commonly deregulated after stress. Lower synthesis, inactivation or degradation of **auxins** usually happen in all plant species after stress exposure. We found two auxin-related genes that are down-regulated in both genotypes, *i.e.*, *MIZ1* (T459_12756, plays a role in maintaining auxin levels; Moriwaki et al., 2011) and *ABCG37* (T459_33922, transporter of indole-3-butyric acid and probably other auxin metabolites; Růžička et al., 2010). In A10 we found that the genes involved in the synthesis of IAA amino-acid conjugates (putative indole-3-acetic acid-amido synthetase *GH3.8*, T459_06630) were up-regulated and a gene homolog, which irreversibly inactivates this hormone in rice (2-oxoglutarate-dependent dioxygenase *DAO*, T459_04872: Zhao et al., 2013)).

Cell division in plants typically slows down after stress. **Cytokinin** (CK) synthesis is blocked, and hormone degradation starts. In this experiment in A10 there were several genes related to CK biosynthesis that were down-regulated (*IPT* genes, T459_23598 and T459_26831; Takei et al., 2001), but only the last one was also down-regulated in NIBER®. Besides, in A10, we also found the up-regulation of a gene that regulates the degradation of this hormone (cytokinin dehydrogenase, T459_24347; Werner et al., 2003). Curiously, a gene involved in CK activation (*LOG1*, T459_13377; Kuroha et al., 2009) was up-regulated in both genotypes.

Regarding **ABA**-related gene expression, we noted differences between the two rootstocks. For instance, a protein phosphatase 2 C (T459_14080; Schweighofer et al., 2004) was down-regulated in A10, but up-regulated in NIBER®. In A10, we also observed the down-regulation of a gene involved in ABA catabolism (abscisic acid 8'-hydroxylase 1, T459_03838; Kushiro et al., 2004) and the up-regulation of another gene involved in protein trafficking and sorting (*NHL6*, T459_04332; Bao et al., 2016).

Ethylene is another hormone involved in stress. We found the deregulation of several genes related to ethylene biosynthesis (1-aminocyclopropane-1-carboxylate oxidase genes). However, there was no clear regulation trend in this case because genes indistinctively appeared up- and down-regulated.

Photosynthesis, as a general indicator of plant performance under stress, can provide information about tolerance mechanisms through the deregulation of associated genes. In this experiment, several photosynthetic chain components were down-regulated for A10, *i.e.*, NAD(P)H-

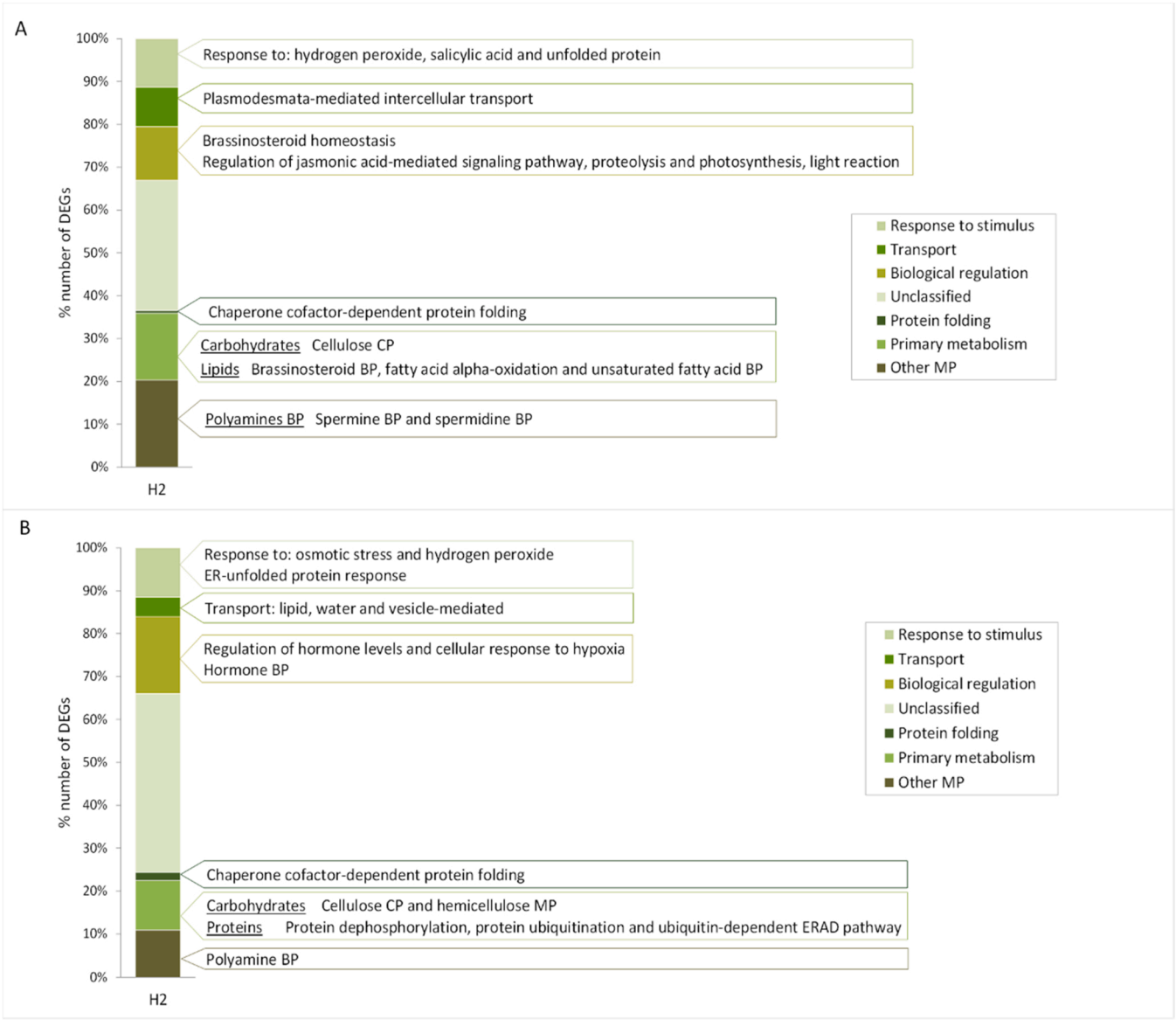

Fig. 5. *Ancestor* and *Child* BP categories found only in NIBER® for the water stress/control conditions at T1 (A) and T2 (B). CP is Catabolic Process, BP is Biological Process, ER is Endoplasmic Reticulum and MP is Metabolic Process.

quinone oxidoreductase subunit T (it acts as an electron shuttle in the photosynthetic chain and probably in the chloroplast respiratory chain, T459_08381; Yamamoto et al., 2011) and chlorophyll a-b binding protein (Light Harvesting Complex, T459_05321). However, some chlorophyll synthesis genes were up-regulated for NIBER®, *i.e.*, magnesium-chelatase subunit *ChlH* (plastid-to-nucleus retrograde signaling and ABA perception, T459_13633; Mochizuki et al., 2001; Tsuzuki et al., 2011) and geranylgeranyl diphosphate reductase (it provides phytol for both tocopherol and chlorophyll synthesis, T459_10457; Keller et al., 1998), but also an early light-induced protein that modulates chlorophyll synthesis to prevent photooxidative stress (T459_07967; Tzvetkova-Chevolleau et al., 2007).

Chaperones' transcription deregulation after water stress treatment took place because chaperone gene dnaJ 20 was down-regulated for both genotypes (T459_20966; Pulido et al., 2013). Besides, we observed many different chaperone genes up-regulated in NIBER®, such as heat shock cognate 70 kDa (T459_18252; Zhang et al., 2018), dnaJ 8 (T459_17550; Chen et al., 2011) and sHSP 22.0 kDa class IV (T459_13892; Li et al., 2018).

Calcium signaling was also affected after water stress. So, calcium-binding protein *CP1* (T459_22923; Jang et al., 1998) was up-regulated, while calcineurin T459_14199 was down-regulated for both genotypes. Specifically, calcineurin *CBL4* was down-regulated in A10 (T459_09604; Halfter et al., 2000).

Some gene **transporters** were also regulated. Aquaporin *TIP1–3* (T459_28400) was down-regulated for both genotypes, while putative aquaporin *TIP-2-like* (T459_16939) was down-regulated in A10 and up-regulated in NIBER®. Other aquaporins were down-regulated and specific for A10, such as aquaporin *PIP1–5* (T459_03898) and aquaporin *TIP1-like* (T459_26097). Curiously, the genes coding for vacuolar iron transporters (T459_14318, T459_32063, T459_32062, T459_32064 and T459_12384; Kim et al., 2006; Gollhofer et al., 2014) were down-regulated for both genotypes. For A10 S-type anion channel *SLAH1* (involved in anion homeostasis maintenance, T459_22888; Negi et al., 2008) was down-regulated. For NIBER®, vacuolar proton pump *AVP1* (T459_34590) was also down-regulated.

Transcription factors regulate gene expression and constitute the starting point in several signaling pathways in plants exposed to biotic and abiotic stresses. The regulation of transcription factors after water stress differed depending on the genotype. In NIBER®, the response was

more robust because *WRKY70* (T459_25223, involved in preventing stomatal closure; Li et al., 2013), *MYC2* (T459_04029, a master regulator in jasmonic acid (JA) signaling; Fernández-Calvo et al., 2011) and many *DREBs* (T459_09275, T459_09274, T459_15138; Sakuma et al., 2002) were up-regulated. In A10, the response was weaker because only *JUB1* (T459_21054; Wu et al., 2012) was specifically down-regulated.

As previously mentioned, plants under stress produce **ROS** and its ability to eliminate these compounds is related to plant tolerance. Glutathione is an important player in oxidative stress metabolism. Indeed, both genotypes show up-regulated glutathione-related genes, like the glutathione biosynthesis gene (glutamate-cysteine ligase, T459_00299; Vernoux et al., 2000), glutathione peroxidase (T459_00315), glutaredoxin C9 (T459_24935; Huang et al., 2016) and many glutathione transferase genes. However, the glutaredoxin C6 gene (T459_21376) was down-regulated in both genotypes.

In this experiment, the glycolate oxidase 2 gene, which encodes an enzyme that produces H_2O_2 from glycolate oxidation (T459_24757; Dellero et al., 2015) was specifically down-regulated in NIBER® and, consequently, the catalase gene (T459_31096) was also down-regulated. Nevertheless, we were unable to find any marked trend in peroxidase genes because there were more than 20 differentially expressed genes, and some were up- and some were down-regulated in both genotypes. However in A10, we noted two up-regulated genes that code for suberization associated anionic peroxidases (T459_31642 and T459_05761), and root suberization a common response to oxidative stress, together with lignification. Accordingly in A10, the up-regulation of four laccase genes (T459_19889, T459_15127, T459_16504, T459_19890; Cai et al., 2006) involved in lignin formation was observed.

Other genes involved in the **oxidative stress response** were up-regulated in A10, such as formate dehydrogenase (T459_06869; Olson et al., 2000) and mitochondrial uncoupling protein 6 (T459_29341), the latter of which functions in decreasing oxidative phosphorylation and increasing heat production (Borecký et al., 2006). Conversely in A10, we detected the down-regulation of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (T459_18879), which generates NADPH that is essential for antioxidant systems (Kirch et al., 2004). Lastly for polyamines, we observed the up-regulation in NIBER® for the spermidine synthase-coding gene (T459_03483).

3.4. Water stress-related metabolite responses

The targeted metabolomics performed in the root samples for various metabolic families showed differential regulation when plants were exposed to water stress in relation to the control conditions.

The **oligosaccharides** quantification at T2 (24 h) (Fig. 6) revealed an increase in trehalose content after stress for both genotypes, but with a greater increase in NIBER® than in A10. Galactinol was also affected by stress because both genotypes significantly decreased its content compared to the control conditions, but there was no significant difference between genotypes. With raffinose, A10 and NIBER® showed an opposite effect for its content. The A10 raffinose content dropped and NIBER® raffinose content rose after 24 h under water stress. Stachyose content lowered for both genotypes under water stress compared to the control conditions, and was significantly higher in A10.

The **hormones** quantification in roots at T1 (5 h) and T2 (24 h) is shown in Fig. 7. IAA increased significantly at T1 for both genotypes under stress *versus* the control conditions (Fig. 7A). At T2, IAA decreased for A10 in relation to the control, while there were no significant differences for IAA content in NIBER® under the stress conditions (Fig. 7A). ABA content significantly decreased at T1, but only for A10 under the stress *vs*. The control conditions (Fig. 7B). At T2, significant differences were noted for ABA content under the stress *vs*. the control conditions, where ABA increased for both genotypes (Fig. 7B). There was a difference for JA at both T1 and T2 because NIBER® JA content significantly increased, and A10 JA content was not affected by stress compared to the control conditions during both events (Fig. 7C).

The **polyamines** quantification in roots at T2 (24 h) (Fig. 8) exhibited significant differences for spermine content in both genotypes because it decreased under the stress conditions, but this decrease was greater in NIBER®. Spermidine content increased significantly under stress *vs*. the control conditions for both genotypes, and the A10 increase was greater. However, there were no significant differences in putrescine content between genotypes and when comparing the stress and control conditions.

The **glutathione** quantification for the oxidized form in roots at T2 (Fig. 9) revealed a significant increase in GSSG content for both genotypes when stress was applied, but the increase in GSSG content in A10 was greater (68%) than in NIBER® (23%) *versus* the control conditions.

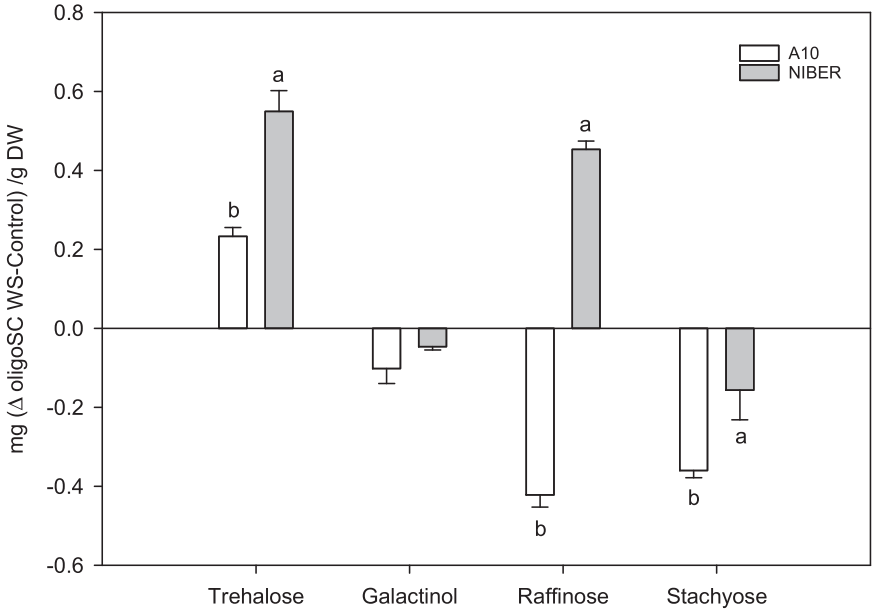

Fig. 6. Oligosaccharides quantification (trehalose, galactinol, raffinose, stachyose) as a difference between water stress and the control conditions (mg)/g DW (dry weight) in the A10 and NIBER® roots. Different letters indicate statistical differences for the LSD test with a p-value of < 0.05 for $n = 4$. OligoSC means oligosaccharide.

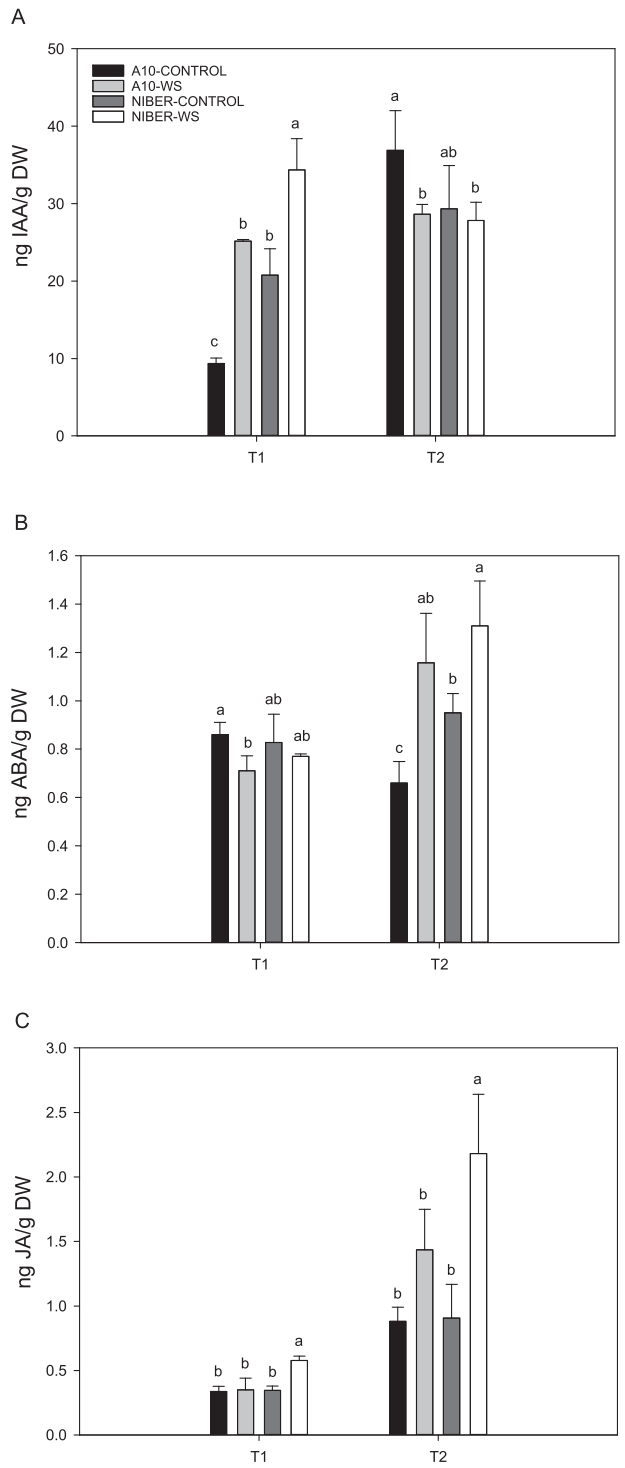

Fig. 7. Hormones quantification for IAA (A), ABA (B) and JA (C) as ng of hormone/g DW (dry weight) in the A10 and NIBER® roots. Different letters indicate statistical differences for the LSD test with a p-value of < 0.05 for $n = 4$. IAA is indoleacetic acid, ABA is abscisic acid and JA is jasmonic acid.

4. Discussion

In this experiment, a water stress-tolerant hybrid rootstock (NIBER®; Gisbert-Mullor et al., 2020) was studied under water stress conditions together with the A10 accession, which is considered sensitive to this stress (Penella et al., 2014). Both genotypes were analyzed for changes in transcriptomic and different metabolites to address the differential patterns related to the short-term water stress response. The results uncovered some essential gene signaling pathways and metabolites to cope water stress. Some of these mechanisms are genotype-constitutive, while others are regulated after stress exposure.

4.1. Constitutive differences in gene expression

Under the non-water stress conditions, we observed differences between genotypes in gene expression terms, which could influence plant performance once stress has been applied. Up to 3000 genes were differentially expressed between NIBER® and A10 when water stress was absent. This constitutive difference between accessions has been observed by Fracasso et al. (2016) in sorghum genotypes with contrasting water use efficiency, where the tolerant genotype showed an up-regulation in the genes associated with the "secondary metabolic process" and "glutathione transferase activity" GO terms under well-watered conditions. Similarly, López-Serrano et al. (2021) stated advantageous characteristics in salt-tolerant pepper accession A25 under control conditions compared to sensitive accession A6, with a marked abundance of genes related to GO terms such as "response to stress", "response to abiotic or biotic stimulus", "transport", among others. Similarly, in this study, NIBER® showed an over-representation of the "response to stimulus", "response to chemical", "response to oxidative stress" and "response to inorganic substance" GO terms compared to A10 under the control conditions (Fig. 1).

The genes related to these over-represented GO terms in NIBER® (Table S1) could be associated with the constitutive tolerance that this rootstock manifests under water stress. Hence, several of the genes involved in ROS detoxification were up-regulated in NIBER®/A10 under the control conditions (formate and malate dehydrogenases), while superoxide anion production gene *RbohB* was down-regulated. ROS production could be promoted in a hormonal-dependent way (Mori and Schroeder, 2004), and frequently by the activation of NADPH oxidases, which are encoded by *Rboh* genes (Sagi and Fluhr, 2006). Accordingly, ethylene has been proven to manage ROS production by regulating *Rboh* genes transcription through MAPK cascades and by triggering cell death under stress conditions (Xia et al., 2015). We found five down-regulated putative *ACO* genes in NIBER®/A10 under the control conditions. The ACO enzyme catalyses the final step in the ethylene biosynthesis pathway (Houben and van de Poel, 2019) and is encoded by *ACO* genes. Ethylene has been described to act in an antagonistic manner to ABA, because the presence of high ABA and ethylene levels results in the inhibition of the production of both hormones (Müller and Hasanuzzaman, 2021). The main enzyme responsible for ABA synthesis, *NCED1*, was up-regulated in NIBER®/A10 under the control conditions, but ABA receptor *PYL1* was down-regulated. High ABA levels cause the down-regulation of PYR/PYL receptors to prevent detrimental ABA accumulation effects (Ruiz-partida et al., (2021)). Moreover, PYL receptors have been linked with ABA-induced stomatal closure regulation, and also under optimal conditions (Gonzalez-Guzman et al., 2012). *PYL1* down-regulation in NIBER® could avoid ABA-dependent stomatal closure and reduce undesirable growth effects from enhanced ABA synthesis (*NCED1*). Besides the role of these stress-related hormones, oscillations in the levels of other plant hormones take place in the initial stress response phases by bringing about several metabolic changes that lead to modifications in the growth pattern to help to adapt to stress conditions (Verma et al., 2016). For instance, auxin homeostasis is achieved by several strategies, one of which is conjugation and de-conjugation (Hayashi et al., 2021). In this experiment, auxin receptor *TIR1* and amidohydrolase *ILR1* that releases free IAA from conjugates were up-regulated in NIBER®/A10, while four putative *GH3* genes that inactivate IAA by forming conjugates were down-regulated under the control conditions. Therefore, NIBER® constitutively showed an active IAA regulation pattern in relation to A10 that could help to achieve its better performance when the stress comes into play.

An increase in IAA levels has been related to improved survival to water stress because of the auxin-dependent protective effect on

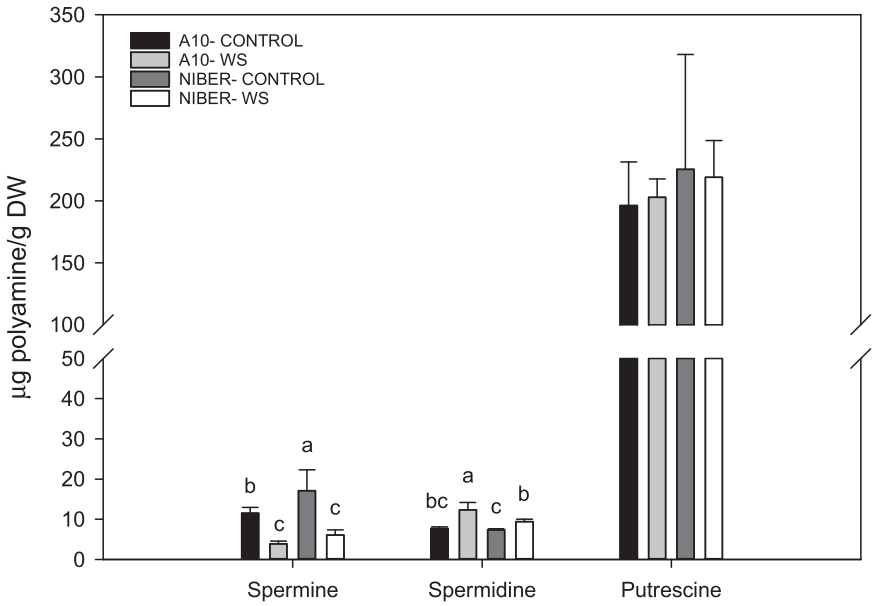

Fig. 8. Polyamines quantification (spermine, spermidine, putrescine) as µg of polyamine/g of DW (dry weight) in the A10 and NIBER® roots. Different letters indicate statistical differences for the LSD test with a p-value of < 0.05 for $n = 4$.

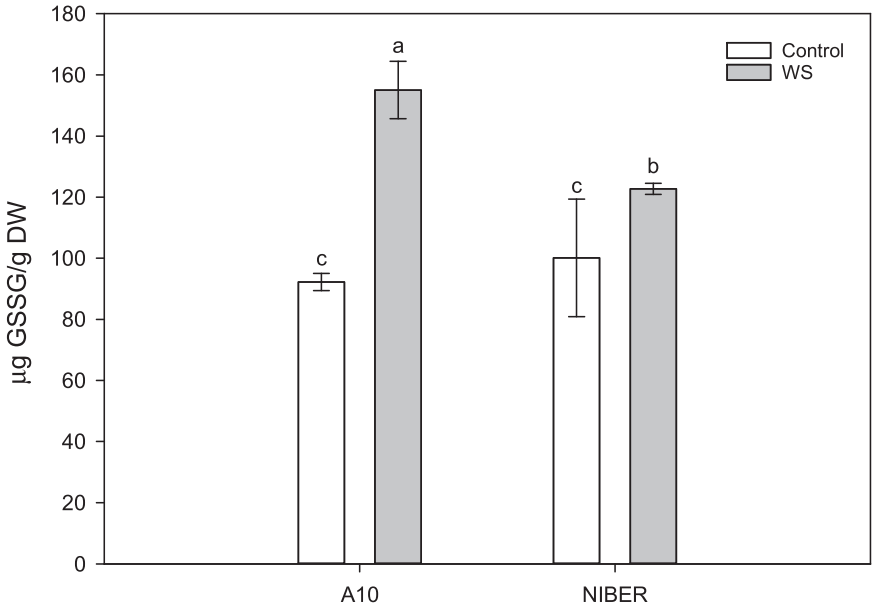

Fig. 9. Oxidized glutathione quantification as µg of GSSG/g of DW (dry weight) in A10 and NIBER®. Different letters indicate statistical differences for the LSD test with a p-value of < 0.05.

photosynthesis (Tognetti et al., 2010). It has been suggested that IAA protection to photooxidative inhibition exists by altering the chloroplast structure and pigments composition given that photosynthesis is one of the earliest affected processes when abiotic stress like water stress is present (Tognetti et al., 2012). Accordingly, we found several DEGs that encode for photosynthetic components and pigments, which were up-regulated (*APO1*, *CYP89A9*, *TGD1*) and down-regulated (*PSBP1*, cytochrome b6-f subunits, *PSI-K*, *PGRL1A, PGR5*, among others) when comparing NIBER® and A10 under the control conditions. However, the NIBER® down-regulated genes *PGRL1A* and *PGR5* have been associated with cyclic electron flow (CEF) around Photosystem I, which constitutes an alternative electron transfer pathway to achieve photoprotection under stress conditions (Nawrocki et al., 2019). These results suggest that A10 may already be performing CEF under the control conditions, and NIBER® mainly performs the linear electron flow (LEF).

Calcium is involved in the regulation of the genes related to chloroplast components and it is known as a major signaling cation when plants sense an external stimulus (Wang et al., 2019). Several calcium-associated genes, such as calcineurin-binding proteins (*CBL7* and *CBL9*) and CBL-interacting kinase *CIPK20,* were up-regulated in NIBER®/A10, but calcium-dependent protein kinase *CPK4* and calmodulin *CML11* were down-regulated in NIBER®/A10 under the control conditions. Unlike CDPKs, CBLs and CIPKs interact in a very specific way by conducting calcium signaling in certain locations, *i.e.*, vacuole membrane, where calcium can be sensed only by the CBL-CIPK interaction (Edel et al., 2017). Nevertheless, calcium is a wide-range and complex component of many signaling transduction pathways, and it is present as a ubiquitous second messenger in many biological processes

(Batistič et al., 2009), including the response to environmental stresses and developmental processes (Pirayesh et al., 2021).

Calcium signaling, together with phytohormones and MAP kinases, regulates the expression of the genes included in osmolytes synthesis pathways in the presence of abiotic stress (Jogawat, 2019). Osmolytes synthesis and accumulation are key for plant survival under stress conditions because osmolytes diminish the detrimental effects of water starvation by preserving the water potential (Ozturk et al., 2021). However, several genes related to osmoprotectant sugars synthesis and transport were downregulated in NIBER®/A10 under the control conditions, such as trehalose synthase genes (*TPS1* and *TPP*) and a *SWEET* transporter. Notwithstanding, some authors have reported negative trehalose overproduction effects under control conditions that have resulted in penalty growth and, thus, suggest employing tissue-specific promoters or those induced by stress conditions (Iqbal and Nazar, 2016).

Polyamines are molecules involved in osmoprotection, but are also considered for their ability to detoxify excess ROS and to avoid oxidative damage by acting as antioxidants themselves or by promoting the action of other antioxidant enzymes and molecules (Zulfiqar et al., 2019). Two genes related to polyamines were up-regulated in NIBER®/A10 under the control conditions (*PAO,* that catalyzes spermine to spermidine conversion, and *PUT4*, a polyamine transporter), and one spermidine synthase gene was down-regulated. Additionally, dehydrins are soluble proteins whose expression is modified in the presence of abiotic stress, and are associated with the protection of other proteins, but also with osmotic adjustment with water stress (Ozturk et al., 2021). Thus, dehydrin *HIRD11* was up-regulated in NIBER®/A10 under the control conditions. Furthermore, this dehydrin has been proven to reduce ROS formation, including H_2O_2 and hydroxyl radicals (Hara et al., 2013).

Lastly, molecular chaperones usually present an enhanced expression under stress conditions, and they are in charge of rearranging protein conformation when it has been altered due to stress (Wang et al., 2004). Despite the role of chaperones being essential in protein homeostasis, an HSP90 co-chaperone and *sHSP 18.2KDa* genes were down-regulated in NIBER®/A10 under the control conditions. These results could be supported by the minimal expression of sHSPs, which is typically found when abiotic stress is absent, except for specific reproductive developmental stages, such as embryogenesis, germination, among others (Sun et al., 2002). Besides, *BAG4* was up-regulated in NIBER®/A10 under the control conditions, and this chaperone has been linked with stomatal movement regulation through the interaction with potassium channels (Locascio et al., 2019).

4.2. Transcriptomic and metabolomic changes under water stress conditions

Under water stress conditions, the number of DEGs was 4-fold bigger at 5 h (T1) than at 24 h (T2) (Fig. 2), which indicates that short-term gene transcription is crucial for water stress responses. Similarly to the control conditions, the water stress that impacted both genotypes was different in gene expression regulation terms because A10 showed more than double the amount of specifically DEGs compared to NIBER® at 5 h (T1) (Fig. 2A). Nevertheless, there are common pathways in the stress response for both genotypes, notably mechanisms that counteract oxidant compounds effects, such as peroxidases, calcium signaling and glutathione regulation (glutathione synthase, peroxidases, transferases and glutaredoxins). Regarding glutathione, it is well-known that GSH is oxidized to GSSG under stress conditions for redox homeostasis (Dorion et al., 2021), and lower GSSG content has been associated with water stress tolerance (Hasanuzzaman and Fujita, 2011). In this experiment, the GSSG content under stress was higher in A10 than in NIBER® (Fig. 9). Moreover, in A10 the regulation of the genes related to oxidants detoxification (formate dehydrogenase, suberization associated anionic peroxidases, *JUG1*) occurred, while the down-regulation of H_2O_2-related genes (*CAT*, glicolate oxidase) took place in NIBER®, which suggests a stronger impact of stress in A10. Fracasso et al. (2016) reported a significantly larger amount of DEGs, especially ROS detoxification genes, in the sensitive genotype in relation to the tolerant genotype, for sorghum water-stressed plants.

Photosynthetic activity triggers oxidants production in the chloroplast, and ROS should be scavenged to avoid oxidative damage. A10 showed the down-regulation of chlorophyll a-b binding protein (*LHC*) and NAD(P)H-quinone oxidoreductase subunit T (*ndhT*), both of which are photosynthetic chain elements, as well as the up-regulation of mitochondrial uncoupling protein 6 (*PUMP6*), which helps to avoid oxidative damage by increasing heat production (Borecký et al., 2006). Rivero et al. (2010) associated photosynthetic apparatus degradation in sensitive plants under water stress with increased non-photochemical quenching, plus a diminished electron transfer, while the photosynthesis of tolerant plants was not affected. NIBER® showed the up-regulation of chlorophyll synthesis genes and early light-induced protein (*ELIP*) (which prevents free chlorophyll accumulation to protect from photooxidative stress) and showed no inhibition of photorespiration in gene expression terms.

Furthermore, a common response to abiotic stress in plants is higher polyamines content (Hussain et al., 2011), which was higher for spermidine in both genotypes when stress was applied (Fig. 8). Several reports suggest an antagonistic role for polyamines and ethylene because they have a common precursor (S-adenosylmethione, SAM) (Imai et al., 2004). In this experiment, the down-regulation of *SAMDC* was noted in NIBER® at 5 h after water stress, as was the up-regulation of *SPDS1* at 24 h, which may counteract and result in the above-mentioned increased spermidine after the stress treatment. In addition, the changes in gene expression for the *ACO* genes did not follow a specific trend, but there were several ethylene-related GO terms in A10, and ethylene contribution should be further studied (Fig. 4). Polyamines have also been linked with ABA and *DREB*s because the promoters of polyamines synthesis genes, such as *SPDS1,* contain DRE and ABA-responsive elements (Alcázar et al., 2006). Accordingly, the up-regulation of three *DREB*s occurred in NIBER® with water stress compared to the control conditions, which showed the ability to develop a quick response to water stress because increases in *DREB*s expression have been observed at 5 h from water and osmotic stress exposure (Sakuma et al., 2002). *MYC2* is also a transcription regulator which has been associated with the ABA-dependent signaling pathway for water stress tolerance (Abe et al., 2003) and, to a greater extent, with dehydration resistance through JA signaling (Li et al., 2019). NIBER® JA content increased after 5 h of water stress and doubled after 24 h compared to the control conditions, while A10 JA content did not change (Fig. 7C). *MYC2* was upregulated in NIBER® 24 h after water stress treatment and Li et al. (2019) linked the accumulation of the JA that derived from water stress exposure with the activation of *MYC2*, which promotes the gene expression of the downstream genes associated with dehydration resistance.

ABA content increased under water stress in both A10 and NIBER® at 24 h (Fig. 7B). However, we were able to find find different transcriptomic response in the genes related to the signaling and synthesis of this hormone. In NIBER®, the up-regulation of *CHLH* took place after 5 h of water stress, which is involved in both chlorophyll synthesis and the ABA signaling pathway for stomatal movements (Tsuzuki et al., 2011), together with the up-regulation of *WRKY70*, which is a negative regulator of stomatal closure (Li et al., 2013). In A10, the down-regulation of ABA catabolism gene (ABA 8-hydroxylase 1) at 5 h after water stress and up-regulation of an ABA signaling and synthesis gene (*NHL6*) occurred at 24 h, *NHL6* overexpression has been linked with sensitivity to salt and osmotic stress due to excess ABA and hypersensitivity (Bao et al., 2016). These results suggest that in early water stress exposure stages, NIBER® could avoid stomatal closure, whereas A10 could promote its closure, which agrees with *PP2C72* differential expression under water stress (down-regulated in A10 at 5 h and up-regulated in NIBER® at 24 h). PP2C-type protein phosphatases are negative regulators of ABA signaling that prevent stomatal closure (Lee et al., 2009). Besides, A10

showed related GO terms, such as "chloroplast accumulation and avoidance movements", "ABA metabolic process", "ABA-activated signaling pathway" and "response to water deprivation" for stress compared to the control conditions (Fig. 4), that were not found in NIBER®. Accordingly, many of the genes coding for aquaporins (*PIP1–2, TIP1–1, TIP1–2, TIP-type RB7–5A*) were down-regulated in A10, which could result in weaker water transport and slight uncharged molecules transport (Takano et al., 2017). NIBER® showed GO terms such as "water transport" and "plasmodesmata-mediated intercellular transport" (Fig. 5), together with the up-regulation of aquaporin *TIP-type RB7–5A*. Moreover, stomatal closure as a water-saving strategy leads to decreased photosynthetic activity from CO_2 unavailability, and aquaporins function to achieve CO_2 homeostasis (Afzal et al., 2016). Sade et al. (2009) observed more growth and bigger yields in tomato plants with the constitutive expression of *SlTIP2–2* that derived from the maintenance of transpiration and CO_2 uptake under water stress conditions. In previous experiments, the photosynthetic balance in plants grafted onto NIBER® under high salt stress conditions was better, compared to self-grated and ungrafted plants (López-Serrano et al., 2020). This improvement was attributed to the sustained stomatal opening that resulted in more growth.

Changes in the shoot/root growth ratio under water stress are regulated by auxins and cytokinins because auxin promotes root growth and inhibits shoot growth, whereas cytokinin could act as antagonist (Kurepa and Smalle, 2022). A10 and NIBER® showed increased IAA 5 h after the stress treatment (Fig. 7A), along with a down-regulation of an auxin polar transporter (*ABCG37*) and an auxin homeostasis regulator (*MIZ1*). These results are related to the "Hydrotropism" GO term, which is common for both genotypes (Fig. 3) because it is defined as growth or movement toward or away from water. For CKs, the up-regulation of CKs activation gene (*LOG1*) and the down-regulation of CKs synthesis (*IPT1*) occurred in both NIBER® and A10 at 5 h after water stress treatment. The transcriptomic results suggested that both genotypes avoid negative feedback and auxin export in roots, while stimulating CKs activation but inhibiting CKs synthesis. Furthermore, A10 showed the down-regulation of a second *IPT* gene and the up-regulation of cytokinine dehydrogenase gene at 5 h after water stress, which suggest a decrease in CKs to promote root growth over shoot growth. Despite this, A10 displayed the up-regulation of an auxin inactivation gene (*DAO*) and an auxin conjugation gene (*GH3.8*) at 5 h after water stress, which is consistent with the "auxin homeostasis" and "auxin catabolic process" GO terms (Fig. 4). These results agree with the drop in the IAA content observed in A10 at 24 h under water stress compared to the control conditions, which was not observed in NIBER® (Fig. 7A). While A10 seems to downregulate auxins activation and CKs synthesis, NIBER® apparently maintains the levels for both phytohormones.

Osmolytes accumulate in plants to protect them from oxidative damage, but also counteract existing harm, and include sugars (trehalose, RFOs), amino acids (proline), quaternary ammonium compounds (glycine betaine) and sugar alcohols (Jogawat, 2019; Ejaz et al., 2020).

Trehalose is speculated to be an osmoprotectant sugar given the tolerant genotype observed in transgenic plants for trehalose biosynthesis genes (van Houtte et al., 2013). In this experiment, trehalose content increased for both genotypes under stress *vs*. the control conditions (Fig. 6). Notwithstanding, the *TPP* gene was down-regulated for both genotypes 5 h after water stress, and it is involved in the conversion of trehalose-6-phosphate into free trehalose, which should end with lower trehalose content. Moreover, *TPS* gene expression in A10 under water stress increased at 24 h, and TPS is the enzyme responsible for trehalose-6-phosphate synthesis. These results suggest that A10 promoted trehalose-6-phosphate synthesis, probably due to its role as a signaling molecule in regulating photosynthesis and starch synthesis in plastids (Iturriaga et al., 2009). For NIBER® there were no expression changes in *TPS*, but trehalose content was higher compared to the control conditions.

Nishizawa et. al (2008) proposed an additional role for galactinol and raffinose sugars as direct ROS scavengers, apart from osmoprotectants and membrane stabilizers, by ensuring photosynthesis protection under adverse conditions like water stress. A10 and NIBER® showed higher galactinol-sucrose galactosyltransferase gene expression at 5 h after water stress, the enzyme responsible for raffinose synthesis from galactinol. This is consistent with the drop in galactinol content under stress compared to the control conditions in both genotypes (Fig. 6). However, the matching increase in raffinose content was observed only for NIBER® (Fig. 6), which also showed the up-regulation of the galactinol synthase gene (*GolS2*) at 24 h after the stress treatment. It would seem that NIBER® could provide galactinol for raffinose synthesis, as reflected in oligosaccharides quantification. Conversely, raffinose content in A10 could not be associated with the observed gene expression changes, regulation mechanisms could act at the post-translational level, and/or the processes affecting the metabolome could be delayed in relation to gene expression changes (Feussner and Polle, 2015). Moreover, oligosaccharides quantification was performed in roots and sugars could accumulate in leaves because they would be required for ROS scavenging and osmoprotection, as it has been previously mentioned.

Accumulation of glycine betaine (GB) and proline has been widely reported in multiple plant species under abiotic stresses, and these compounds are considered main organic osmolytes (Ashraf and Foolad, 2007). Proline functions like osmolyte include cell turgor maintenance and cellular homeostasis, but it also functions like an antioxidant in scavenging ROS (Hayat et al., 2012). In the A10 genotype, the up-regulation of the proline dehydrogenase gene, together with the up-regulation of a proline transporter and the GO term "proline catabolism", occurred at 5 h after water stress. Proline degradation has been observed as a strategy to favor polyamine synthesis because they have a common precursor (Ejaz et al., 2020). However, no marked increase in polyamines content in A10 other than that observed in NIBER® was noted. Thus, we cannot confirm this strategy, plus proline content should also be studied. Regarding GB, the betaine aldehyde dehydrogenase gene in A10 was down-regulated, which is responsible for the last GB synthesis step in chloroplasts. GB accumulates mainly under water stress conditions to protect chloroplasts through osmoregulation by avoiding damage to thylakoid membranes and preserving the photosynthetic yield (Dikilitas et al., 2020). Both proline and GB are capable of stabilizing membranes and proteins, and the high concentrations of these and/or other osmolytes could be an effective strategy to recover from the protein unfolding that derives from stress exposure (Ortbauer, 2013).

Protein stability and conformations are strongly affected by internal and external stimuli, including unexpected environmental changes that lead to protein unfolding and misfolding (Ha and Loh, 2012). HSPs are chaperones that assist in protein folding, and in the refolding of unfolded or misfolded proteins under abiotic stress conditions (Wang et al., 2004). With water stress *versus* the control conditions, NIBER® showed GO terms such as "response to unfolded protein", "endoplasmic reticulum unfolded protein response" and "chaperone cofactor-dependent protein folding". Accordingly, chaperone dnaJ 8 was upregulated in NIBER®, as were heat shock cognate 70 kDa and *22 kDa sHSP*. As described for the control conditions, the expression of HSPs typically increases when plants are exposed to stress conditions, which is consistent with the results obtained for NIBER®, which seems to promote chaperones as a tolerance strategy that was not observed in A10.

5. Conclusions

Briefly, NIBER® displays multiple mechanisms to cope with water stress (Fig. 10). The transcriptomic results suggest constitutive mechanisms, mainly involved in detoxification, which are amplified under stress conditions. These results also evidence the important role of transcription factors like *DREB*s and *MYC* at the beginning of the stress response. Hormones regulation under water stress in NIBER® comprises

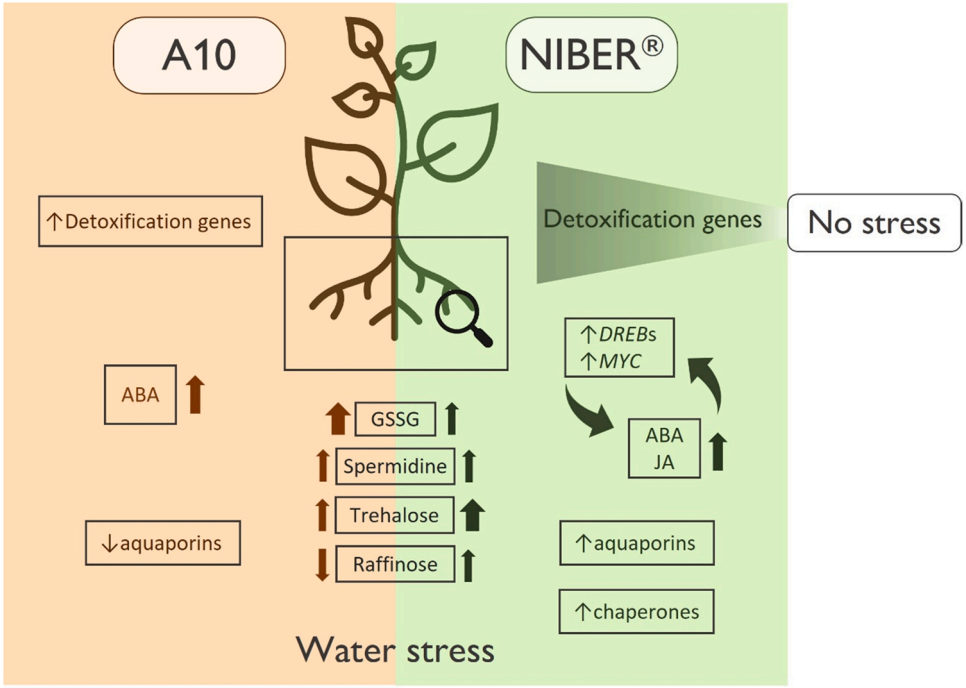

Fig. 10. Water stress response mechanisms in the NIBER® and A10 roots. Framed arrows depict a higher or lower expression level for the corresponding genes. Nonframed arrows denote higher or lower metabolite content. The wider the arrow, the higher the content.

the up-regulation of auxins, ABA and JA, which might benefit the rootstock in growth and development terms. These changes are accompanied by the regulation of osmoprotectants in terms of gene expression and metabolites content. The gene expression of raffinose, galactinol and spermidine synthesis genes increases under water stress, together with enhanced trehalose, raffinose and spermidine content. For glutathione, its synthesis gene has a higher expression under water stress, and NIBER® has lower oxidised glutathione content compared to A10. All these osmoprotectant-antioxidant components contribute to NIBER® tolerance under water stress, together with aquaporins and chaperones, which display an increased gene expression for NIBER®. This study provides useful insight into rootstock (NIBER®) tolerance mechanisms, but further studies should be performed to move forward.

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CRediT authorship contribution statement

YGP: Conducted Methodology, Investigation, Data curation, Writing – review & editing. **RGM:** conducted experiments, Methodology, Investigation. **EB:** Supervision, Conceptualization, Investigation, Data curation, Writing – review & editing. **LZ:** Methodology, Data curation, writing. **JF:** Methodology, Data curation. **LL:** Supervision. **SLG:** Supervision, Conceptualization, Investigation. **ÁC:** Supervision, Conceptualization, Investigation, Writing – review & editing. All authors have read and have approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All data is included in the manuscript and supplementary information.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111731.

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