Transcriptomics and physiological analyses reveal co-ordinated alteration of metabolic pathways in *Jatropha curcas* drought tolerance

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Abstract

*Jatropha curcas*, a multipurpose plant attracting a great deal of attention due to its high oil content and quality for biofuel, is recognized as a drought-tolerant species. However, this drought tolerance is still poorly characterized. This study aims to contribute to uncover the molecular background of this tolerance, using a combined approach of transcriptional profiling and morphophysiological characterization during a period of water-withholding (49 d) followed by rewatering (7 d). Morphophysiological measurements showed that *J. curcas* plants present different adaptation strategies to withstand moderate and severe drought. Therefore, RNA sequencing was performed for samples collected under moderate and severe stress followed by rewatering, for both roots and leaves. *Jatropha curcas* transcriptomic analysis revealed shoot- and root-specific adaptations across all investigated conditions, except under severe stress, when the dramatic transcriptomic reorganization at the root and shoot level surpassed organ specificity. These changes in gene expression were clearly shown by the down-regulation of genes involved in growth and water uptake, and up-regulation of genes related to osmotic adjustments and cellular homeostasis. However, organ-specific gene variations were also detected, such as strong up-regulation of abscisic acid synthesis in roots under moderate stress and of chlorophyll metabolism in leaves under severe stress. Functional validation further corroborated the differential expression of genes coding for enzymes involved in chlorophyll metabolism, which correlates with the metabolite content of this pathway.

Key words: Chlorophylls, gene profiling, *Jatropha curcas*, leaf and root, leaf gas exchange, morphology, progressive drought, purging nut, RNA-Seq, water stress–recovery cycle.

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; A<sub>n</sub>, net photosynthesis; CAO, chlorophyll a oxygenase; CAT, catalase; Chalase, chlorophyllase; CPM, counts per million; CS, chlorophyll synthase; DEG, differentially expressed gene; F<sub>D</sub>/F<sub>m</sub>, maximum quantum yield of photosystem II; GO, gene ontology; g<sub>s</sub>, stomatal conductance to water vapor; NCED, 9-cis-epoxycarotenoid dioxygenase; NPO, non-photochemical quenching; PAO, pheophorbide a oxygenase; PPH, pheophytinase; ProDH, proline dehydrogenase; PSII, photosystem II; PSOS, 1-pyrroline-5-carboxylate synthetase; RNA-Seq, RNA sequencing; RS, raffinose synthase; RT-qPCR, reverse transcription–quantitative PCR; RWC, relative water content; SWC, soil water content; TLA, total leaf area; TW, turgid weight; Φ<sub>PSII</sub>, PSII operating efficiency.

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Introduction

Drought is a major environmental constraint, limiting plant productivity and distribution (Bartels and Sunkar, 2005). Current climate models predict increased drought episodes due to the long-term effects of global warming (Dai, 2013). This emphasizes the urgent need to invest in agricultural strategies more appropriate to deal with water scarcity and breeding of plants for higher water use efficiency. Drought response mechanisms have been investigated at the molecular level in a variety of species, but mostly in model species such as Arabidopsis (Matsui et al., 2008; Harb et al., 2010), poplar (Cohen et al., 2010; Chen et al., 2013), and rice (Lenka et al., 2011; Moumeni et al., 2011). However, model plants such as Arabidopsis and rice are very sensitive to drought, and some authors have pointed out that their responses may reflect senescence or death, rather than adaptations to drought (Deyholos, 2010).

Jatropha curcas (purging nut) is a soft woody oil-seed-bearing plant of the Euphorbiaceae family that is attracting a great deal of attention as a promising sustainable source of biodiesel (Fairless, 2007). This species has the advantage of showing high drought tolerance and the capacity to grow in marginal and poor soils without competing with food production for land use (Divakara et al., 2010). Therefore, the transcript profiling of J. curcas, as of other plants adapted to arid and semi-arid climates, can be a valuable tool to characterize resistance mechanisms and screen for drought tolerance candidate genes.

We previously studied J. curcas morphophysiological behavior under drought by gradually reducing soil water availability during a 28 d period followed by 1 week recovery (Sapeta et al., 2013). Jatropha curcas drought tolerance can be classified as a drought avoidance mechanism, presenting a typical water-saving strategy (Diaz-Lopez et al., 2012; Sapeta et al., 2013). Physiological adjustments under drought include a tight stomatal regulation in response to soil water availability, preventing water loss, arresting growth, and, in extreme drought, shedding older leaves (Achten et al., 2010; Pompelli et al., 2010; Silva et al., 2010a, b; Diaz-Lopez et al., 2012; Sapeta et al., 2013).

Transcriptomic approaches to investigate J. curcas drought adaptation have been performed mainly targeting single genes. To that extent, J. curcas genes responding to drought have been identified and cloned, such as a betaine aldehyde dehydrogenase gene (Zhang et al., 2008), an AP2/EREBP domain-containing transcription factor gene (Tang et al., 2007), a phospholipase D gene (Liu et al., 2010), a n-myo-inositol-3-phosphate synthase gene (Wang et al., 2011), and two aquaporin genes (Jang et al., 2013). Recently, studies aiming to provide a broader view of transcriptome changes under drought have been performed. Cartagena et al. (2014) have developed a J. curcas oligomicroarray that allowed the identification of 332 genes as drought responsive and 374 genes involved in drought/recovery. Zhang et al. (2015) have analyzed genome-wide transcriptional profiles of J. curcas seedlings after 1, 4, and 7 d withholding water. The authors found enrichment in abscisic acid (ABA) synthesis and signal transduction in roots, and enrichment in ABA and ethylene signal transduction pathways in leaves.

Despite the information already available, at both the physiological and the molecular levels, the knowledge regarding the response/performance of this species under different water-limiting conditions after short- and long-term drought imposition is still scarce. To understand which genes play a role in moderate and/or severe drought responses, and which physiological parameters/metabolic pathways are controlled by such genes, is of major importance to assess the relationship between gene expression and drought adaptation. In this context, an integrative view of physiological responses and genes/pathways regulating abiotic stress tolerance constitutes a fundamental tool not only to develop J. curcas lines with enhanced stress tolerance minimizing reductions in oil yields, but also to explore the translation of the tolerance mechanism to drought-sensitive crops. Therefore, in this study we used an integrative strategy combining whole transcriptomic and morphophysiological analysis. We have defined three main stages to characterize the transcriptome, two during the stress imposition and one at the recovery stage. RNA sequencing (RNA-Seq) was used to assess both leaf and root transcriptomes at the selected time points to identify global alterations after short- and long-term drought exposure followed by recovery. We present a comprehensive and detailed overview of the changes occurring during the experiment, integrating the transcriptomics with the morphophysiological data. Our aim was to identify candidate genes potentially relevant to improve drought tolerance in crops.

Materials and methods

Plant material and growth conditions

Seeds from two batches of Jatropha curcas grown in Cape Verde (described in Sapeta et al., 2013) were germinated in clean sand, and 10-day-old uniform size seedlings were transplanted to 7.5 liter pots containing a mixture of sand, peat, and soil (3:1:1) and supplemented with a commercial fertilizer (Osmocote, Scotts, The Netherlands) (5 g per pot) (N:P:K: Mg, 16: 9:12: 2.5). Plants were irrigated daily until the beginning of the treatments. Experiments were carried out in a greenhouse under natural photoperiod (29 June–24 August 2011, Oeiras, Portugal) with a day/night temperature of 29±3 °C to 20±2 °C, day/night relative humidity of 39±8% to 69±4%, and an average light intensity at plant level of 411±226 µmol photon m−2 s−1 (11:00–12:00 h). Additionally, a second independent experiment was conducted in the same greenhouse from 2 August to 27 September 2012, under natural photoperiod with a day/night temperature of 27±2 °C to 22±1 °C, day/night relative humidity of 56±3% to 78±3%, and an average light intensity at plant level of 316±170 µmol photon m−2 s−1 (11:00–12:00 h). In both experiments, pots were randomly moved every week to minimize position effects.

Drought conditions

Potted 36-day-old seedlings (at the five-leaf stage) were subjected to drought (Stress) or continuously grown under well-watered conditions (Control). Drought was imposed by withholding water for a maximum period of 49 d, followed by a 7 d rewatering period, and soil water content (SWC) was monitored (as described in Sapeta et al., 2013). Leaf relative water content (RWC) was also determined, for six leaf discs (Ø=19 mm)
collected for each plant from the three youngest expanded leaves (two discs per leaf). Calculations were according to Barrs and Weatherley (1962), using the formula: \[ RWC \% = \frac{(FW - DW)}{(TW - DW)} \times 100, \] where FW, DW, and TW are fresh, dry, and turgid weight, respectively. For all measurements, 6–12 plants were allocated per treatment for each sampling point. Sampling was performed from different sets of plants to avoid wronging effects (see Fig. 1A for further details). Details of sampling for the additional experiment can be found in Supplementary Fig. S1 available at JXB online.

Morphophysiology

Growth and morphology

Stem growth characteristics were monitored weekly by measuring length (from the substrate surface to the apical meristem) and diameter (at the base) and by counting the number of leaves longer than 2 cm (main vein). DW (g) was determined after drying plant material at 70 °C (until a constant weight was achieved). Total leaf area (TLA) was determined with a color image analysis system (WinDIAS 2, Delta-T Devices, Cambridge, UK).

Leaf gas exchange and Chl a fluorescence

Leaf gas exchange and Chl a fluorescence were assessed with a portable infrared gas analyzer (LI-6400: LI-COR Inc., Lincoln, NE, USA) equipped with a fluorometer (LI-6400-40, LI-COR Inc.). We used a block temperature of 28 °C, a CO2 concentration of 400 µL L−1, 300 µmol photons m−2 s−1 of light intensity (10% blue light), and an air flow rate of 500 µmol s−1 to monitor net photosynthesis (A), µmol CO2 m−2 s−1) and stomatal conductance to water vapor (g, µmol H2O m−2 s−1). Data were collected at around 11:00 h within a 90 min period. By simultaneously measuring Chl fluorescence, we could also estimate the photosystem II operation efficiency (Fv/Fm), which translates photochemistry efficiency (Genty et al., 1989). Fv/Fm was calculated as (Fv/Fm′) (Genty et al., 1989). The ratio of variable to maximum fluorescence (Fv/Fm′) was calculated as (Fv/Fm′)(Fv/Fm′) (Genty et al., 1989), and the non-photochemical quenching (NPQ) was calculated as (Fv/Fm′)(Fv/Fm′) (Roháček, 2002). Fv and Fm represent the minimum and maximum fluorescence yield, respectively, measured in dark-adapted leaves (between 04:00 h and 05:00 h), whereas Fv′ and Fm′ represent the steady-state and maximum fluorescence yield, respectively, measured under light-adapted conditions. For all parameters, the youngest fully expanded leaf of at least 10 plants was measured.

Chl a, Chl b, and pheophorbid a determination

The contents (mg g−1 DW) of Chl a and Chl b were determined according to Lichtenthaler (1987) during the drought period (day 0, 13, and 49) and after the recovery period (day 56). Three leaf discs (Ø=19 mm) were collected per plant from the three youngest fully expanded leaves (one disc per leaf). Six to 12 plants were analyzed per condition (stress versus control), six on day 13, and 12 at the other sampling points. Absorbance was measured with a spectrophotometer (DU-70 Spectrophotometer, Beckman, USA) at 663.2 nm and 646.8 nm. The Chl a to b ratio (Chl/a/b) was also calculated.

This study was repeated using HPLC for higher accuracy and further including pheophorbid a quantification. Samples were collected from the three youngest fully expanded leaves of plants subjected to 49 d of drought (maximum stress) or control conditions. The contents (mg g−1 DW) of Chl a, Chl b, and of pheophorbid a were assessed as described by Hwang et al. (2005) with some alterations. Finely ground samples (~0.005 g DW) were homogenized in 1.5 ml of HPLC grade acetone and stored at −20 °C overnight. The homogenate was ultrasonicated for 2 min and centrifuged at 8000 g for 20 min at 4 °C. The supernatant was filtered through a 0.22 µm nylon membrane, and analyzed by HPLC for Chl a and Chl b determination. For pheophorbid a detection, the filtered supernatants were first concentrated 2.5- and 5-fold. For this, complete dehydration was obtained under vacuum (90 min), and the pellet was resuspended in the appropriate acetone volume.

For HPLC, a Hitachi model was used, equipped with an L-2130 pump, L-2200 sample injector, L-2300 column oven, and L-2455 diode array detector (operating in the range of 400–700 nm), and with a LiChroPrep® RP-18, 5 µm column (200×4 mm) and a 100 µL RP-18, 5 µm guard column.

The injection volume was 20 µl and the flow rate was 0.6 ml min−1. For the mobile phase, solutions A, ethyl acetate–methanol–water (15:65:20, v/v/v); and B, ethyl acetate–methylamine–water (60:30:10, v/v/v); were used, and the elution was carried out using a graded descending series of A in B (100% for 8 min, 70% for 1 min, 50% for 2 min, 20% for 1 min, 10% for 6 min, and 0% for 4 min). The column was equilibrated with 20% A for 2 min and 100% A for 8 min. Calibration curves for quantitative determinations were performed by linear regression of standard peak area versus the respective concentration. Standards of Chl a, Chl b, and pheophorbid a (Sigma-Aldrich) were diluted in acetone in the range of 5–40 mg mL−1 (5, 10, 15, 20, 25, and 40), 5–25 mg mL−1 (5, 10, 15, 20, and 25), and 1.25 to 25 mg mL−1 (1.25, 2.5, 5, 10, 15, 20, and 25), respectively.

Statistics

Morphophysiological data were subjected to t-test using the statistical software package SIGMAPLOT 11.0 (Systat Software Inc., Chicago, IL, USA). Differences were considered significant for P≤0.05.

High-throughput sequencing (RNA-Seq)

RNA extraction

Root tips and young leaves were collected (between 14:00 h and 16:00 h) during the drought assay and subsequent rewetting period. In detail, one leaf (≤2 cm in length) from the apical shoot was excised per plant and immediately frozen in liquid nitrogen. For root collection, plants were gently removed from the pot within 30 s; 1 cm long root ends (10–15) were excised, carefully brushed to remove attached soil, and immediately flash-frozen in liquid nitrogen. A pool of six plants for days 0 and 49, and three plants for days 13 and 52 were sampled per replicate and treatment. Samples were stored at −80 °C until further use. Before extraction, the frozen material was ground in liquid nitrogen with a mortar and pestle into a fine powder. Total RNA was extracted using the RNasy plant mini kit (Qiagen, Germany) according to the manufacturer’s instructions; however, for leaf samples, the lysis buffer was supplemented with 0.2% polyethylene glycol (PEG) 20 000 as suggested by Gehrig et al. (2000). After the extraction, samples were treated with DNase (Turbo DNA-free Kit, AM1907, Ambion, CA, USA) according to the manufacturer’s instructions to minimize contamination. RNA quality and quantity were evaluated using an Agilent 2100 Bioanalyzer with RNA 6000 Nano Chips, following the manufacturer’s protocol. RNA integrity numbers ranged from 8.3 to 9.9.

cDNA library preparation for high-throughput sequencing

cDNA libraries were prepared using an initial amount of 400 ng of total RNA, and Illumina’s TrueSeq™ RNA Sample Preparation v2 protocol. Indexed barcodes were used for pooling, and sequencing was performed using an Illumina HiSeq™ 2000. To assess concentration and ensure an appropriate size distribution (200–500 bp) the cDNA libraries were checked with Bioanalyzer DNA 1000 chips. The sequencing run was carried out on a fully loaded flow cell with single-end 50 bp reads following the manufacturer’s instructions with a loading amount of 9 pmol cDNA per lane. On average, each sample was covered by ~ 82 million reads.

Mapping and quantification of RNA-Seq data

Mapping was performed with NextGenMap v0.4.12 using default settings (Sedlacek et al., 2013). We used sequences and annotations from J. curcas genome version 4.5 as reference (http://www.
Pathway analysis and functional categorization

In order to determine the broad biological function of genes differentially expressed between control and drought treatments, we have performed functional annotation based on gene ontology (GO) enrichment analysis. DEGs of leaves and roots were tested for enrichment according to Conesa and Gotz (2008) with the criterion FDR ≤0.05. The characterization was based on BLASTx results against NCBI’s nr database (from June 2014 linked to functional information stored in the GO database and a motif/domin search using InterProScan). Blast2GO was used to perform these analyses and enabled the association of GO terms and enzyme codes with transcripts. Metabolic pathway construction was based on the attributed enzyme and KEGG pathways (Kanehisa and Goto, 2000).

Reverse transcription–quantitative PCR (RT–qPCR) analysis

RT–qPCRs were performed for selected genes involved in Chl metabolism (chlorophyll a oxygenase, Jcr4S00496.20; chlorophyllase-1, Jcr4S01992.60; chlorophyll synthase, Jcr4S03698.40; pheophorbide a oxygenase, Jcr4S01668.30; and pheophytinase, Jcr4S06489.10); reactive oxygen species (ROS) detoxification (catalase, Jcr4S01023.80; and ascorbate peroxidase, Jcr4S00717.110); osmoprotection (raffinose synthase, Jcr4S00099.110; proline dehydrogenase, Jcr4S00221.50; and 1-pyrroline-5-carboxylate synthetase, Jcr4S04565.30); and ABA metabolism (9-cis-epoxycarotenoid dioxygenase, Jcr4S03683.20). Quantitative PCR was performed using the LightCycler 480 system (Roche, Basel, Switzerland) and the SYBR Green I Master mix (Roche). The PCR running conditions were as follows: one cycle at 95 °C for 5 min and 45 cycles of amplification at 95 °C for 10 s, 59 °C for 10 s, and 72 °C for 10 s. The CT values were calculated from means of three technical replicates, and the relative (to the internal control) expression of transcripts was calculated using the method of relative quantification with kinetic PCR efficiency correction (Pfaffl, 2001). The AFR GPTase-activating protein gene (Jcr4S05054.10) was selected from RNA-Seq data based on the lowest coefficient of variance method, to be used as internal control.

Primers selected for all PCR amplifications are described in Supplementary Table S1 at JXB online. The cDNA for RT–qPCR validation was prepared with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) from 1 µg of total RNA isolated as previously described.

Results

Defining the stress scale for transcriptomics

Experiments were designed to assess the physiological response of J. curcas in conditions of drought (imposed by withholding water) for 49 d followed by 1 week of recovery (by rewatering), as compared with plants maintained in well-watered conditions (Fig. 1A). SWC showed a fast reduction during the imposed drought period until day 15 (when it reached 30% of SWC) and continued to decline slowly until it reached 10% by day 49 (maximum stress) (Fig. 1B). A similar reduction in SWC was found in an additional experiment performed 1 year later, and described in Supplementary Fig. S2 at JXB online. Leaf RWC was not affected by water deficit (see Supplementary Tables S2, S3). During the drought/recovery experiment, when looking at the water content in different organs (leaf, stem, and root), we only found a reduction in stressed roots (day 13 and 49), with full recovery to control levels 3 d after rewatering (Supplementary Tables S2, S3).

Since drought did not affect the leaf hydration status, we have defined two major stress stages based on SWC (Fig. 1B). Stress was defined as moderate corresponding to 30% SWC and severe corresponding to 10% SWC, and samples were collected for RNA isolation and transcriptomic analyses at four stages: the beginning of the experiment (day 0), moderate stress (day 13), severe stress (day 49), and after 3 d of rewatering (day 52). Shortly after withholding water (by day 7), J. curcas leaves were found to reposition, with an obvious leaf inflection that positioned the leaf blade towards a more vertical orientation (with the tip pointing downwards, Fig. 1C). These changes, however, were not related to any reduction in RWC, as shown in Supplementary Tables S2 and S3 at JXB online.

J. curcas displays a drought avoidance strategy drastically restricting its growth, shedding older leaves under severe stress, and exhibiting a rapid recovery after rehydration

Water deficit caused a significant reduction in growth (P<0.001). Stem length and diameter (Fig. 2A, B) were the first parameters affected, showing growth reduction by day 7 onwards (65% SWC). By day 49 (10% SWC), stressed plants had a 39% reduction in stem diameter, a 22% reduction in stem length, and a 46% reduction in leaf number, as compared with control plants. Therefore, the total number of leaves derived from the apical meristem was the most affected parameter, due to the arrest of leaf primordia initiation from day 14 onwards (30% SWC) and leaf shed from day 35 onwards (15% SWC) (Fig. 2C). Despite the severe growth arrest observed during the drought period, growth was resumed rapidly after rewatering (Fig. 3, day 49–56). Roots were also quickly rehydrated (Supplementary Table S2, S3 at JXB online), and new root growth was observed already by day 52 (3 d after rewatering) (data not shown). Additionally, drought reduced biomass production and total leaf area (P<0.001) (Supplementary Fig. S3). All the tendencies observed were further confirmed in the additional experiment described in Supplementary Figs S4 and S5). The morphological aspect of the stressed plants at the beginning of stress imposition, maximum stress, and after rewatering is shown in Supplementary Fig. S6.
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Drought-stressed *J. curcas* shows high stomatal control and protection of photosystem II

Water stress significantly reduced \( P<0.001 \) stomatal conductance to water vapor \( (g_s) \), photosynthesis \( (A_n) \), and photosystem II efficiency \( (\Phi_{PSII}) \) (Fig. 3). A reduction in \( A_n \) (Fig. 3A) of 37\% (control versus drought) was observed after 12 d withholding water (40\% SWC), continuing to decrease as drought stress persisted, and reaching a 95\% reduction at maximum stress (10\% SWC).

A strict stomatal control in response to drought was observed (Fig. 3B). The \( g_s \) was reduced by 20\%, as compared with control, after 7 d withholding water (60\% SWC), while after 12 d (40\% SWC) there was an 88\% reduction. At maximum stress (10\% SWC), \( g_s \) reached values close to zero.

The \( \Phi_{PSII} \) (Fig. 3C) only started to decline by day 23 (75\%, stress versus control). Despite the severe reductions, a complete recovery was observed for all measured parameters within 5 d of rewatering (day 54). All tendencies observed were further confirmed in the additional experiment performed (see Supplementary Fig. S7 at *JXB* online).

Maximum quantum yield of PSII \( (F_v/F_m) \) was also significantly affected by drought (Supplementary Fig. S8A at *JXB* online). However, it should be emphasized that despite the severity of the stress imposed, the reduction of the \( F_v/F_m \) was rather small (from 0.83 in controls to 0.78 under severe drought). Moreover, after 1 d of rewatering (day 50), the stressed plants already showed some recovery of \( F_v/F_m \) with full recovery by the end of the rewatering period (day 56). NPQ (see Supplementary Fig. S8B) was significantly increased under severe stress, and the values decreased after rewatering until reaching control levels by day 56 (7 d after rewatering).

**Chla to b ratio decreases under stress**

The Chla content was similar for both drought-stressed and control plants at days 0 and 13 (Fig. 4A). However, at maximum stress, the Chla content was significantly higher in stressed plants than in the control (4.5 mg g\(^{-1}\) DW versus...
3.2 mg g⁻¹ DW, respectively); a similar trend was observed for the Chl b content (Fig. 4B, 2.0 mg g⁻¹ DW versus 1.1 mg g⁻¹ DW, respectively). Moreover, Chl b content relative to total chlorophylls also increased at maximum stress, shifting from 25% in control to 32% in stressed plants. This resulted in a decrease in the Chla/b (Fig. 4C). This ratio was quickly reverted to control values after 7 d of rewatering. A similar decrease of the Chla/b was observed in an additional drought experiment (see Supplementary Fig. S9 at JXB online).

RNA-Seq performance in J. curcas

Twenty-two cDNA samples (four conditions/two replicates/leaves and roots/control and stress, pooling some of the controls) (see Fig. 5 for further details) were used for deep sequencing by the Illumina platform, providing ~1.8 billion reads, each with 50 nucleotides. The number of reads per sample ranged from 59 million to 130 million, with an average number of ~82 million reads. Mapping efficiency, with a total of 1.5 billion uniquely assigned reads, was very high (84%, Supplementary Table S4 at JXB online). Thus, gene expression of the 57,437 annotated genes could be quantified on a robust basis, revealing ~27,000 expressed above threshold (Supplementary Table S4). The high reproducibility of the overall experimental design and the applied technical methods for transcriptional profiling can be observed between the two replicates, with stable Pearson correlation coefficients ranging from 0.935 to 0.994 (see Supplementary Table...
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S5), confirming a good analytical quality performance. Also, comparison between RNA-Seq and RT–qPCR fold change ratios revealed highly similar expression trends of selected genes with a Pearson correlation coefficient of \( R^2 = 0.96 \) (see Supplementary Fig. S10).

To test further the consistency of the data across all samples, a hierarchical clustering of the transcriptional profiles was performed (Fig. 5). Root samples under control, rewatering, and moderate stress conditions clustered together in the first clade, as did the leaf samples in the second clade. However, leaf and root samples clustered together at maximum stress, reflecting the common transcriptional regulation of both organs under this severe stress condition.

To identify drought-responsive genes, samples from control and drought treatments were compared after 13 d and 49 d, as well as after 3 d of subsequent rewatering (day 52). The pairwise testing of differential gene expression showed that roots respond rapidly to drought, with 409 DEGs already under moderate stress (Fig. 6A, C). In leaves, however, impressive changes in expression patterns were only observed under maximum stress, indicating that J. curcas leaves do not respond to drought as rapidly as roots. However, the number of DEGs for both leaves and roots under maximum stress was nearly the same, each with ~2000 candidates. Beyond that, both DEGs groups showed an altered expression pattern compared with all other conditions, as revealed by organ-specific hierarchical clustering (Fig. 6B). After rewatering, the leaf transcriptome returned to control faster than the root transcriptome (Fig. 6A, C).

Taking a closer look at DEGs in leaves, we found 13 genes differentially regulated only by day 13 (Fig. 6C), showing high homology to tonoplast dicarboxylate transporter-like (Jcr4S01423.50 and Jcr4S09289.30), lipoxigenases (Jcr4S06599.10 and Jcr4S14032.10), and proteinase inhibitors (Jcr4S03455.30), among others (Supplementary Table S6 at JXB online). Furthermore, 11 gene sequences were found to be expressed in leaves under both moderate and maximum stress, including a gene with high homology to a Ricinus communis sequence encoding gibberellin-regulated protein 3 (Jcr4S05849.30), a putative zinc finger protein (Jcr4S00350.30), and a heat-shock protein (Jcr4S04325.10). Another sequence with high homology to a putative zinc finger protein was also found to be differentially expressed in leaves in all stress phases, as compared with controls. In the recovery treatment, we found genes coding for putative germin-like protein (Jcr4S20460.10), proteinase inhibitor (Jcr4S06006.10, Jcr4S12693.10, and Jcr4S14888.10), and a sugar transporter (Jcr4S02245.90).

Comparing the expression profiles in roots at days 13, 49, and 52 (Fig. 6C; Supplementary Table S6 at JXB online), we found 99 genes exclusively expressed under moderate stress, and among those we found up-regulation of sequences putatively coding for leucine-rich repeat-containing proteins (Jcr4S00370.20, Jcr4S02192.10, and Jcr4S0493.20), several zinc finger transcription factors (Jcr4S06510.20 and Jcr4S00242.60), and a putative beta-carotene hydroxylase (Jcr4S01869.30), among others. Under severe stress (day 49), 1644 genes were found specifically in this stage, while only 512 genes were found specifically after rewatering. Moreover, 150 genes were differentially expressed between roots of control and drought-treated plants under moderate and severe stress, and on subsequent rewatering.

Drought-responsive metabolic pathways

Under severe stress (day 49), 447 common DEGs were found between leaves and roots (Fig. 6D; Supplementary Table S6 at JXB online).
A comprehensive pathway analysis of the DEGs found in both roots and leaves at maximum stress revealed enrichments in lipids (e.g. fatty acid degradation), carbohydrates (e.g. starch and sucrose metabolism), and secondary metabolism (e.g. flavonoid biosynthesis).

Genes involved in ROS detoxification were also found (see Supplementary Fig. S11A at JXB online): sequences coding for ascorbate peroxidase (APX) and glutathione S-transferase were among the common DEGs. Moreover, a strong up-regulation of a sequence coding for catalase (CAT) was found in leaves. Both CAT and APX up-regulation were validated by RT–qPCR (see Supplementary Fig. S11B, C).

ABA metabolism-associated genes were also among the common leaf and root DEGs. Taking a closer look at the RNA-Seq expression profiles of all ABA metabolism-associated genes (see Supplementary Fig. S12A at JXB online), we found an up-regulation of the ABA biosynthetic pathway under moderate stress. Under severe stress (day 49), the up-regulation of ABA metabolism was observed in both roots and leaves. Common up-regulated DEGs at day 49 included sequences putatively encoding zeaxanthin epoxidase, 9-cis-epoxycarotenoid dioxygenase (NCED), and (+)-ABA 8′-hydroxylase (see Supplementary Fig. S12B). To validate the up-regulation of the pathway, we analyzed the expression of a sequence putatively coding for the rate-limiting enzyme NCED by RT–qPCR, confirming its up-regulation (see Supplementary Fig. S12C).

Moreover, in both leaves and roots under maximum stress there was an induction of sequences coding for the putative l-pyrroline-5-carboxylate synthetase (P5CS), while a sequence coding for proline dehydrogenase (ProDH) was up-regulated in leaves and down-regulated in roots. These two genes are described as involved in proline metabolism (P5CS in biosynthesis and ProDH in degradation) (reviewed in Verbruggen and Hermans, 2008). Furthermore, the expression of both genes was validated by RT–qPCR (see Supplementary Fig. S13A, B).

Since starch, sucrose, and galactose pathways were found to have the highest number of enzymes coded by DEGs shared in both organs (Fig. 6D; Supplementary Table S7 at JXB online), further investigation of alterations in these pathways at maximum stress was performed (Fig. 7). A conserved down-regulation in both roots and leaves of genes coding for enzymes involved in cellulose and fructose biosynthesis was observed. In contrast, sequences coding for putative enzymes involved in galactinol, raffinose, and trehalose synthesis were up-regulated in both organs under maximum stress. The expression pattern of the sequence coding for raffinose synthase (RS) was further validated by RT–qPCR (see Supplementary Fig. S13C).
Functional categorization of the drought-regulated genes

When looking at the RNA-Seq data using GO enrichment analysis, we found that most enriched GO terms from up-regulated genes in both leaves and roots were associated with response to stress, such as response to: stimulus, stress, heat, abiotic stimulus, temperature stimulus. GO terms associated with regulation of transcription were also found to be up-regulated in both leaf and root, such as sequence-specific DNA-binding transcription factor activity, transcription, DNA-dependent and nucleic acid-binding transcription factor activity. Genes associated with the production of osmolytes such as proline were also enriched. Moreover, growth-associated GO terms were found to be down-regulated in leaves and roots of J. curcas plants subjected to severe drought, such as cell wall organization or biogenesis, cellulose biosynthetic and metabolic process, and cellulose synthase activity. GO enrichment analysis for leaves and roots under moderate and severe stress followed by rewatering (days 13, 49, and 52) can be found in Supplementary Tables S8 and S9 at JXB online.

RT–qPCR and HPLC validate the modified chlorophyll metabolism

Among 110 chlorophyll-associated genes, seven displayed significant up-regulation in leaves of plants subjected to severe drought. Genes encoding putative pheophorbide a...
Discussion

A better understanding of plant water stress physiology can help to design improved and more targeted breeding strategies for improved plant/crop performance in a context of water scarcity. In previous studies, we observed that, under drought, *J. curcas* plants respond first by reducing gₛ, followed by Aᵣ, while maintaining a high water content under drought (Sapeta et al., 2013). These results were the driver for the present study in which we applied a much more severe stress (higher intensity and duration) and combined transcriptomics with morphophysiology to investigate further the mechanisms underlying the high drought endurance of this species.

In our transcriptomic analysis, we found that roots are the first plant organ to perceive stress, showing an earlier transcription reprogramming (Fig. 6) that can be correlated with a decrease in root water content (see Supplementary Table S2 at JXB online). This seems to be a conserved mechanism, since a faster and stronger transcriptomic response was also observed in soybean, chickpea, rice, and horse gram roots as compared with leaves (Wang et al., 2012; Bhardwaj et al., 2013; Fan et al., 2013; Minh-Thu et al., 2013). Still, it was surprising that, by day 13 (moderate stress), although gₛ was drastically reduced, Aᵣ had decreased by >50% and the stem growth rate was also reduced; only 25 genes were found to be differentially expressed in leaves (15 up- and 10 down-regulated). Despite the major physiological changes at this stress point, no differential regulation of genes involved in growth, cell expansion, or photosynthesis was found for leaves. In fact, up-regulated genes under this condition were involved in oxylipin biosyntheses/metabolism, such as putative *Lipoxygenases* 2 and 3 (*LOX2* and 3). *LOX* genes have been well studied in model plants such as *Arabidopsis thaliana*. At*LOX2* and 3 were proposed to be located in plastids.
and to be responsible for the first step in the biosynthesis of oxylipins, such as jasmonate (Bell and Mullet, 1993; Seltmann et al., 2010). Oxylipins are known to accumulate in leaves in response to both biotic and abiotic stresses (Wasternack, 2007; Grebner et al., 2013) and they have been described as playing a role in stress-associated stomatal closure and senescence (Suhita et al., 2004; Montillet et al., 2013; Savchenko et al., 2014). LOX genes were also found to be down-regulated in roots under severe stress. Oxylipins in roots were also associated with lateral root development (Vellosoillo et al., 2007). Down-regulation of the oxylipin pathway in J. curcas roots under severe stress may be related to growth arrest.

As expected, under severe stress, the transcriptomes of both leaves and roots show large changes that mostly relate to drought response (e.g. response to stress). This response was previously observed for model plants when submitted to drought, such as rice (Zhang et al., 2012; Minh-Thu et al., 2013) and Arabidopsis (Harb et al., 2010). While several genes were induced by the stress treatment, a higher number were down-regulated (1345 out of 2157 in leaves, and 1657 out of 2343 in roots). GO analysis indicated that down-regulated genes are mainly associated with growth and development. These changes correlate with the growth arrest observed from moderate to severe stress and agree with previous data for *Eucalyptus camaldulensis* (Thumma et al., 2012).

Even after 49 d withholding water, the leaf RWC of *J. curcas* was maintained at control levels, indicating an efficient adjustment to drought of physiological and biochemical parameters. Under mild stress (day 7), the water status was maintained by a strict stomatal regulation, reducing transpiration water loss while maintaining *A*n, inducing higher *A*n/*g*s ratios favorable to improved water use efficiency (Silva et al., 2010a; Diaz-Lopez et al., 2012; Sapeta et al., 2013). However, with the progression of the stress, the transcriptomic data indicate that the accumulation of osmoprotectors is essential to avoid dehydration and injury. Expression induction of putative RS and P5CS transcripts suggests an increase in the production of compatible solutes (raffinose and proline). Moreover, Silva et al. (2010b) showed an increase in proline contents in leaves and roots of drought-treated *J. curcas* plants. Furthermore, sequences coding for putative galactinol synthases (also involved in raffinose biosynthesis) also showed up-regulation under severe drought in both roots and leaves. Recently, Cartagena et al. (2014) also found increased transcription of the same genes in *J. curcas* leaves under drought. In *Medicago truncatula*, Zhang et al. (2014) found a positive correlation between up-regulation of genes coding for P5CS, proline content, and stress tolerance. Sequences coding for both trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase were also found to be up-regulated in drought-treated leaves and roots. The increase in the ABA biosynthesis transcripts also supports an increase in ABA hormone and thus a reduced transpiration due to stomatal closure (under mild stress) and growth arrest (under moderate stress). The arrested leaf emergence, increased leaf shed, and leaf repositioning, led to a reduction in light capture and photooxidation. Leaf repositioning was also reported by Maes et al. (2011) in *J. curcas*. Since we observed no correlation of leaf repositioning with leaf RWC, this mechanism does not

![Fig. 8. Validation of drought-induced alterations in gene expression and metabolites of chlorophyll metabolism. RT-qPCR expression analysis of (A) chlorophyll a oxygenase (CAO; Jcr4S00496.20) and (B) pheophorbide a oxygenase (PAO; Jcr4S01668.30), pheophytinase (PPH; Jcr4S06489.10); chlorophyllase-1 (Chlase; Jcr4S01992.60), and chlorophyll synthase (CS; Jcr4S03698.40) in *J. curcas* leaves. Two pools of leaves were collected from 12 plants and used as biological replicates, except for days 13 and 52 in which only six plants were used per pool. Analyses were performed after various periods of stress and recovery (A: day 13, moderate stress; day 49, maximum stress; day 52, 3 d after rewatering) or for 49 d of drought (B). All values are relative to the expression of the selected housekeeping gene (Jcr4S05054.10). Values are means ±SE (n=2). (C) Effect of maximum stress on the Chla and pheophorbide a contents assessed by HPLC. Values are means ±SE (n=5). An asterisk indicates significant differences between stress and control according to t-test (P<0.05).](https://academic.oup.com/jxb/article-abstract/67/3/845/2893349)
seem to be related to wilting but instead is an adaptation to avoid excess light as well as temperature increase (leaf re-orientation was only observed after complete stomatal closure). Moreover, the reduction of Fv/Fm only occurred after a drastic reduction of A\textsubscript{ie}, indicating that electron transport from PSII was redirected for alternative sinks different from the Calvin cycle. Protection of PSII could be further observed by the determination of Chl\textsubscript{a}/b, due to the enzyme CAO that plays a predominant role in the photosynthetic carbon assimilation, starch content, and dry matter accumulation were increased. Further studies have demonstrated that increased Chl\textsubscript{b} synthesis through overexpression of the catalytic domain of Prochlorococcus hollandica CAO in Arabidopsis plants delayed senescence (Sakuraba et al., 2012). These authors have performed a large-scale RT–qPCR analysis where they found several differentially expressed genes.

\textbf{Fig. 9.} Overview of the drought-induced alterations in Jatropha curcas gene expression and metabolites of chlorophyll synthesis, cycle, and breakdown. Drought reduced Chl\textsubscript{a}/b, as a result of increased Chl\textsubscript{b} content. Expression of the sequences putatively coding for CAO and PAO was up-regulated under stress, correlating with an increase of Chl\textsubscript{a}/b and decrease of Pheide \textalpha{} contents. Enzymes and metabolites altered by drought are represented in bold. The CAO pathway has already been identified as the sole pathway for Chl\textsubscript{b} biosynthesis in \textit{A. thaliana} (Espineda et al., 1999; Oster et al., 2000). Moreover, Arabidopsis full-length CAO overexpressed in tobacco plants resulted in increased Chl\textsubscript{b} and therefore reduced Chl\textsubscript{a}/b (Pattanayak et al., 2005). Biswal et al. (2012) showed that CAO-overexpressing tobacco plants had more light-harvesting Chl proteins and higher electron transport rates. Moreover, light-saturated photosynthetic carbon assimilation, starch content, and dry matter accumulation were increased. Further studies have demonstrated that increased Chl\textsubscript{b} synthesis through overexpression of the catalytic domain of \textit{Prochlorococcus hollandica} CAO in Arabidopsis plants delayed senescence (Sakuraba et al., 2012). These authors have performed a large-scale RT–qPCR analysis where they found several differentially expressed genes.
Fig. 10. Summary of drought-induced changes in *Jatropha curcas* seedlings. For illustration, several changes under moderate and severe stress, and rewatering are highlighted, and some representative genes are given in italic. Gene regulation is represented by arrows pointing up or down, representing up- or down-regulation, respectively. Circles represent the plant material collected for RNA-Seq. The dotted hexagon contains alterations common for both leaves and roots under severe stress. Abbreviations: αMY, α-amylase; βMY, β-amylase; ABA, abscisic acid; APX, ascorbate peroxidase; Chl, chlorophyll; CAO, Chl a oxygenase; CAT, catalase; LEA, late embryogenesis abundant; LOX, lipoxygenase; NCED, 9-cis-epoxy-carotenoid dioxygenase; NPQ, non-photochemical quenching; PAO, phosphoribid a oxygenase; PIP, plasma membrane intrinsic protein; PSCS, 1-pyruvyl-5-carboxylate synthase; ROS, reactive oxygen species; RS, raffinose synthase; SPP, sucrose-6-phosphate phosphatase; SPS, sucrose-6-phosphate synthase; TPP, trehalose-6-phosphate phosphatase; TPS, trehalose-6-phosphate synthase; ZE, zeaxanthin epoxidase. (This figure is available in colour at JXB online.)
senescence-associated transcription factors. Furthermore, Sakuraba et al. (2012) found that genes encoding Chl degradation enzymes were down-regulated while those involved in photosynthesis were up-regulated. Taking this into account, we hypothesize that the up-regulation of CAO may be involved in delaying drought-induced leaf senescence and fast recovery of the photosynthetic apparatus after rewatering.

Additionally, an increase in transcripts of the gene putatively encoding PAO was observed. The substrate of PAO, pheophorbide a (Pruzynska et al., 2003), was found to decrease during severe stress in our experiments. PAO activity has been mainly associated with senescence, and its up-regulation can also be found in response to various environmental challenges, such as osmotic stress and pathogen infections, or under conditions in which chlorophyll breakdown is also observed (Thomas et al., 2001). We observed leaf senescence of older leaves but not of the young ones that were sampled for the transcriptomic study. Therefore, we hypothesize that the increase in PAO transcript levels is more likely to act in leaf protection rather than in senescence, since accumulation of pheophorbide a leads to O₂⁻ formation and is involved in programmed cell death signaling (Hirashima et al., 2009).

A fast and efficient recovery after stress is a key factor for survival and adaptation. In our study, after 3 d of recovery, we found up-regulation of several genes involved in protein synthesis, translation, sugar metabolism, and transport in leaves. These transcription data agree with the re-growth phase observed after stress alleviation and with the transcriptomic data reported by Cartagena et al. (2014). Similarly, genes involved in the same pathways were also found in grapevine petioles after recovery from drought (Perrone et al., 2012).

A concise summary of the major changes in J. curcas drought response and recovery is presented in Fig. 10, highlighting the major changes observed in genes and metabolic pathways, at the leaf and root level during the stress and recovery. Taken together, our data suggest that drought tolerance in J. curcas is associated with a favorable and co-ordinated expression of several genes involved in protection, especially hormones and compatible solutes. In addition, this species shows a very efficient stomatal apparatus, and thus allowing a rapid recovery after rehydration.

### Supplementary data

Supplementary data are available at JXB online.

- **Table S1.** Primer list for RT–qPCR.
- **Table S2.** Effect of drought and rewatering on RWC and WC.
- **Table S3.** Effect of drought and rewatering on RWC and WC (additional experiment).
- **Table S4.** Summary of Illumina HiSeqTM 2000 sequencing libraries.
- **Table S5.** Pearson's correlation for the normalized transcript expression values for both replicates.
- **Table S6.** DEGs found in J. curcas leaves and roots under drought and rewatering.

### References

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