Running head: Transcriptomics of drought response in *J. curcas*

Corresponding Author:

M. Margarida Oliveira, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Genomics of Plant Stress (GPlantS), Av. da República, 2780-157 Oeiras, Portugal

Phone: +351 214469647; Fax: +351 214411277; E-mail: mmolive@itqb.unl.pt

Research Area: Ecophysiology and Sustainability

Integrating transcriptomics and physiology to assess the drought stress and recovery behaviour of the biodiesel plant *Jatropha curcas*

Helena Sapeta[1], Tiago Lourenço[1], Stefan Lorenz[3], Christian Grumaz[3], Joaquim Miguel Costa[2], Kai Sohn[3] and M. Margarida Oliveira[1]

[1] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Genomics of Plant Stress, Av. da República, 2780-157 Oeiras, Portugal and iBET, Apartado 12, 2781-901 Oeiras, Portugal
[2] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Plant Molecular Ecophysiology Lab, Av. da República, 2780-157 Oeiras, Portugal and iBET, Apartado 12, 2781-901 Oeiras, Portugal
[3] Functional Genomics Lab, Fraunhofer IGB, Nobelstr. 12, 70569, Stuttgart, Germany

One-sentence Summary:

Transcriptomics and morpho-physiology data demonstrated *Jatropha curcas* capacity to cope with drought stress by rapidly adjusting, withstanding severe stress, and rapidly recovering after re-watering.

Footnotes:

Financial source: The present study was funded by Fundação para a Ciência e a Tecnologia (FCT) (project PTDC/AGR-GPL/101435/2008). H Sapeta, JM Costa and T Lourenço were also supported by FCT through grants SFRH/BD/89781/2012 (HS), SFRH/BPD/34429/2006 (JMC) and SFRH/BPD/34943/2007 (TL).

Corresponding author: M. Margarida Oliveira (mmolive@itqb.unl.pt)

Abstract

Jatropha curcas is a multipurpose plant attracting much attention due to its high oil content and quality for biofuel. Moreover it can grow in semi-arid climates. Since water scarcity is an increasing problem, our study aimed to contribute to understand this ability at the transcriptomic and morpho-physiological levels. We used two accessions from different climate origins (wet tropical and semi-arid). One month-old potted plants were subjected to continuous well-watered conditions, or water withholding followed by re-watering. Soil and plant water status, growth and biomass partitioning, leaf gas exchange and chlorophyll *a* fluorescence were assessed. RNA was collected at several time points along drought (49-days) and re-watering (7-days) for leaf and root tissues. We sequenced 22 cDNA libraries using Next Generation Sequencing (RNA-Seq). Sequences were annotated to the publicly available Jatropha genome database (http://www.kazusa.or.jp/jatropha/). Both accessions were found to behave similarly under stress and control conditions, both at transcriptome level (R=0.913-0.996), and morpho-physiology. A hierarchical clustering analysis of the transcriptomic data grouped all samples under 3 major clades, leaf samples, root samples and a third cluster joining root and leaf tissues under maximum stress (day 49). At maximum stress, we found ~4000 drought-responsive genes (2000 in roots and 2000 in leaves). Gene ontology enrichment analysis revealed pathways altered by drought, such as chlorophyll biosynthesis/breakdown. Further functional validation (RT-qPCR, HPLC) confirmed expression of genes putatively involved in chlorophyll biosynthesis/breakdown, and the expression was correlated with chlorophyll metabolites. *J. curcas* responds rapidly to drought, withstanding severe water stress and recovering fast after rehydration.

1. Introduction

Drought is a major environmental factor restricting plant productivity and distribution (Bartels and Sunkar, 2005). Reduced soil water potential increases the difficulty in water uptake by the plant, thus leading to quick reduction in the rate of cell expansion in growing tissues reduced photosynthesis and overall reduced yield (Chaves et al., 2011).

Climate models predict more frequent 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 reduced water and plants able to maximize water use efficiency for the scenarios of increasing water scarcity.

Jatropha curcas (purging nut) is a soft-woody oil-seed bearing plant of the Euphorbiaceae family that is attracting much attention due to its high seed oil content and lipid composition similar to that of fossil oil resources, making it a promising sustainable source of biodiesel (Fairless, 2007). This species has been described as drought tolerant and capable of growing in marginal and poor soils without competing with food production for land use (Fairless, 2007; Divakara et al., 2010). However, the strategies used by Jatropha to deal with water scarcity are still poorly elucidated.

Recently, the genomic sequence of *Jatropha curcas* has become publically available (Sato et al., 2011) which is a major help for breeding purposes and molecular studies aimed to uncover metabolic pathways. Regarding transcriptomic information, several expressed sequence tags (ESTs) databases have been produced for *Jatropha curcas* using various organs, such as mature leaves, flowers, mature embryo, developing and germinating seeds (Costa et al., 2010; Natarajan et al., 2010; King et al., 2011; Natarajan and Parani, 2011). Eswaran and co-workers (2010, 2012) addressed *J. curcas* salt stress resistance using GAL1 functional genetic screens with salt stressed root cDNA expression libraries. Moreover the authors have examined the expression for some of the isolated genes. Other studies have focus on isolating specific stress responsive genes of *J. curcas* to various abiotic stress conditions, including salt, drought, heat and cold (Tang et al., 2007; Zhang et al., 2007; Zhang et al., 2008; Liu et al., 2010; Wang et al., 2011; Jang et al., 2013). The drought stress conditions applied, however, were only simulated using Polyethylene glycol (PEG). Still the authors were able to identify and characterized genes responding to drought (PEG), such as a betaine aldehyde dehydrogenase gene (Zhang et al., 2008), an AP2/EREBP domain-containing transcription

factor (Tang et al., 2007), a Phospholipase D (Liu et al., 2010), a D-myo-inositol-3- phosphate synthase (Wang et al., 2011) and two aquaporins (Zhang et al., 2007; Jang et al., 2013).

Other authors have studied the physiological responses of *J. curcas* under drought stress, assessing biomass production and partitioning, plant-water relationships, leaf gas exchange and osmotic adjustment (Maes et al., 2009; Achten et al., 2010; Pompelli et al., 2010; Silva et al., 2010a,b,c; Diaz-Lopez et al., 2012; Fini et al., 2013). However, the water stress treatments are very diverse in these studies. Water stress was induced either by water withhold (Pompelli et al., 2010; Silva et al., 2010a); deficit irrigation (Maes et al., 2009; Achten et al., 2010; Silva et al., 2010b; Diaz-Lopez et al., 2012; Fini et al., 2013), or by osmotic stress (PEG) (Silva et al., 2010c). These different approaches result in different stresses intensities, and this is further accentuated by the fact that stress was imposed at different plant developmental stages. In spite of the information already available, both at the physiological and molecular level, the knowledge for this species performance under water limiting conditions is fragmented. In this context, the knowledge of this plant specific genes/pathways regulating abiotic stress tolerance is a fundamental tool not only to develop *J. curcas* lines with enhanced stress tolerance without compromising oil yields, but also to help improving drought sensitive crops. We have previously studied *J. curcas* morpho-physiological behavior under drought stress by gradual reduction of soil water availability along a 28 days period followed by one week recovery (Sapeta et al., 2013). We have found that this species improves its water use efficiency along mild to moderate stress keeping a reasonable growth under soil water conditions up to 30% of soil water content, moreover *J. curcas* showed a good recovery capacity after re-watering, We have also reported the drought stress did not reduce chlorophyll contents but led to decreased chlorophyll *a* to *b* ratio.

To contribute to uncover the molecular basis of *Jatropha curcas* efficiency in coping with drought stress, and to understand the metabolic changes of adaptation, in this study we have applied an integrative strategy combining whole trascriptome changes and morpho-physiology analysis. Furthermore, we have design an experiment to extend the drought treatment to extreme conditions and therefore monitor the water levels in the soil. After identifying the time points for analysis we performed a transcriptomic analysis by Next Generation Sequencing (RNA-Seq), which providing a whole transcriptome view. To further understand the impact of the extensive changes occurring in *J. curcas* transcriptome, we have also performed a morpho-physiology study in parallel and further confirmed relevant

expression patterns of genes involved in chlorophyll metabolism by reverse transcription quantitative PCR (RT-qPCR). This study provides in-depth characterization of Jatropha drought response.

2. Material and methods

2.1. Plant material and growth conditions

We tested two accessions of *Jatropha curcas* from two distinct climate regions, one from a wet tropical region (Indonesia-Ind) and the other from an arid climate (Cape Verde islands – CVi). Seeds were obtained from a single mother plant from either Cape Verde or Indonesia, a more detailed description of the accessions provenances can be found in (Sapeta et al., 2013). Homogeneous seeds were germinated in clean sand, and 10-day-old homogeneous seedlings were transplanted to 7.5 L pots containing a soil mixture of sand, peat and soil (3:1:1) and supplemented with a commercial fertilizer (Osmocote, Scotts, Netherlands) (5 g/pot) (N:P:K:Mg, 16:9:12:2.5). Plants were daily irrigated until the beginning of the treatments. Experiments were carried out in a greenhouse under natural photoperiod (29 Jun. - 24 Aug. 2011, Oeiras, Portugal) with a day/night temperature of 29±3 to 20±2ºC, and day/night relative humidity of 39±8 to 69±4%. Pots were randomly moved every week to minimize position effects.

2.2. Drought stress conditions

Potted 36-day-old seedlings (at the five-leaf stage) from both accessions were subjected to drought stress (Stress) or continuously grown under well-watered conditions (Control). Drought stress was imposed by water withhold for a maximum period of 49 days, followed by a 7-day re-watering period.

Soil water content

Soil water content (SWC) was calculated as SWC= [(Pot weight-Minimum Pot Weight) / (Maximum Pot Weight – Minimum Pot Weight)] x 100, as described in (Sapeta et al., 2013).

Sampling

Six to twelve plants (half/half from each accession) were allocated per treatment for each sampling point. Sampling was performed from different sets of plants to avoid wounding effect (for more details see Fig. S1).

2.3. High-throughput sequencing (RNA-seq)

RNA extraction

Root tips and young leaves (<2cm in length) were collected along the drought assay and re-watering period (from a group of tree to six plants per treatment and accession) being immediately frozen in liquid nitrogen and stored at -80 ºC until further use. The samples were collected from a pool of six plants for days 0 and 49 or 3 plants for days 13 and 52. Total RNA was extracted using the RNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's instructions, however for leaf samples the lysis buffer was supplemented with 0.2% of a PEG 20000, as suggested by Gehrig et al. (2000). After the extraction, samples were treated with DNAse (Turbo DNA-free Kit, AM1907, Ambion, USA) according to the manufacturer's instructions to minimize genomic contaminants. RNA quality and quantity was evaluated using an Agilent 2100 Bioanalyzer with RNA 6000 Nano Chips, following manufacturer's protocol; RNA integrity numbers (RIN) ranged from 8.3 - 9.9.

cDNA library preparation for high-throughput sequencing

cDNA libraries were prepared using an initial amount of 400 ng of total RNA, and the Illumina's TruSeq^{TM} RNA Sample Preparation v2 protocol. Indexed barcodes were used for pooling, and sequencing was performed on an Illumina HiSeq^{TM} 2000. To assess concentration and ensure an appropriate size distribution (between 200-570 bp) the cDNA libraries were checked using Bioanalyzer DNA 1000 chips. The sequencing run was carried out on a fully loaded flow cell with single-end 50 bp reads following manufacturer's instructions with a loading amount of 9 pmol cDNA per lane. On average, each sample was covered by about 82 million reads.

Mapping and quantification of RNA-Seq data

Mapping was performed using TopHat v1.4.1 with standard settings. We used sequences and annotations from *Jatropha curcas* genome version 3.0 as reference (Sato et al., 2011) (ftp://ftp.kazusa.or.jp/pub/jatropha/JAT_r3.0/). Quantification and differential gene expression profiling was carried out according to Grumaz et al. (2013). Gene expression levels were calculated according to Mortazavi et al. (2008) and transcript abundance was calculated as RPKM (=reads per kilobase of exon model per million mapped reads).

For normalization the standard quantile normalization was performed using R with the limma package and adding 1 RPKM to each expression value due to statistical reasons. Testing for differential expression was conducted with the DEGSeq package developed for RNA-Seq data (Wang et al., 2010). Here, we considered genes with an adjusted p-value ≤ 0.001 (Storey and Tibshirani, 2003) and a fold change ≤ -4 or ≥ 4 as being significantly differentially regulated between two conditions.

Z-score calculation

Calculation of the Z-score was determined based on the RPKM-normalized log2-transformed transcript count data as follows: $Z = (X-\mu)/s$, where X is the transcript count of a gene for a specific organ/time point, μ is the mean transcript count of a gene across all organs/time points and s is the transcript count standard deviation of a gene across all organs/time points. All calculations were performed using the Mayday software (Battke et al., 2010).

Functional categorization

In order to determine the biological function of genes differentially expressed between control and drought stress treatments, we have performed functional annotation based on Gene Ontology (GO) enrichment analysis. The most significantly differentially expressed genes of leaves and roots were tested for enrichment according to Conesa and Gotz (2008) with the criterion FDR (false discovery rate) ≤ 0.05. The characterization was based on BLAST results (BLASTx, nucleotide sequence of transcripts against NCBI's nr database) linked to functional information stored in the Gene Ontology database and a motif/domain search using InterProScan. Blast2GO was used to perform these analyses and enabled associating GO-Terms to transcripts.

2.4. Morpho-physiology

To validate the relevance of the RNA-Seq data and to understand the impact of the changes identified, several morpho-physiology studies were performed in parallel.

Growth and morphology

Stem growth characteristics were weekly monitored by measuring length (from substrate surface to the apical meristem) and diameter (at the base) and by counting the number of leaves longer than 2cm.

Leaf gas exchange and Chl$_a$ fluorescence

Leaf gas exchange and chlorophyll *a* fluorescence were assessed with a portable infrared gas analyzer (LI-6400; LI-COR Inc., Lincoln, USA) equipped with a fluorometer (LI-6400-40, LI-COR Inc., Lincoln, USA). For these analyses we used a block temperature of 28 ºC, a CO_2 concentration of 400 µL L^{-1}, 300 mol photons m^{-2} s^{-1} of light intensity and an air flow rate of 500 µmol s^{-1} and monitored net photosynthesis (A_n, µmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapour (g_s, mol H_2O m^{-2} s^{-1}) and internal CO_2 concentration (C_i, µL L^{-1}). Data was regularly collected at 11 a.m, never extending over a total period of 90 minutes. By simultaneously measuring Chl$_a$ fluorescence we could also estimate the photosystem II operating efficiency (ΦPSII), which translates photochemistry efficiency (Genty et al., 1989; Maxwell and Johnson, 2000). ΦPSII was calculated as (Fm'- Fs)/F'm (Genty et al., 1989). Fs and Fm' represent the steady state and maximum fluorescence measured under light adapted conditions. We estimated the electron transport rate (ETR = ΦPSII x PPFD x 0.5 x 0.84, µmol electron m^{-2} s^{-1}) (Bjorkman and Demmig, 1987), where the PPFD is the photon flux density of photosynthetically active radiation (400-700nm), 0.84 is the assumed light absorbance of the sample, and 0.5 is the fraction of excitation energy diverted to the photosystem II. For all parameters, the youngest fully expanded leaf of at least 12 plants was measured.

Chl$_a$, Chl$_b$ and pheophorbide a determination

The contents (mg g^{-1} DW) of chlorophyll *a* (Chl$_a$) and chlorophyll *b* (Chl$_b$) were determined according to Lichtenthaler (1987) along the drought stress period (day 0; 13 and 49) and at the end of the experiment (day 56). Three leaf discs (Ø = 19mm) were collected for each plant from the three youngest fully expanded leaves (1 disc per leaf). Six to twelve plants were analyzed per condition (stress *vs.* control), six in day 13 and twelve in the other sampling points. Absorbance was measured with a spectrophotometer (DU-70 Spectrophotometer, Beckman, USA) at 663.2 and 646.8 nm. The Chl *a* to *b* ratio (Chl$_{a/b}$) was also calculated.

This study was repeated using a different analytical method and further including pheophorbide *a* quantification. Samples were collected from the three youngest fully expanded leaves of plants subjected to 49 days of drought (maximum stress) or control conditions. The contents (mg g^{-1} DW) of Chl$_a$, Chl$_b$ and of pheophorbide *a* were assessed by high performance liquid chromatography (HPLC) was described by Hwang et al. (2005) with some alterations. Fine grounded samples (~0.005g DW) were homogenized in 1.5 ml HPLC

grade acetone and stored at -20ºC over night. The homogenate was ultrasonicated for 2 min and centrifuged at 8,000xg for 20 min at 4ºC. The supernatant was filtered through a 0.22µm nylon membrane, and analyzed by HPLC for Chl_a and Chl_b determinations. For pheophorbide *a* detection, the filtered supernatants were first concentrated 2.5 and 5 x. For this, complete dehydration was obtained under vacuum (90 min), and the pellet resuspended in the appropriate acetone volume.

HPLC was performed in a Hitachi model equipped with 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 LiChrospher® RP-18, 5 µm column (200x4mm) and a 100 RP-18, 5 µm guard column.

The injection volume was 20µL and the flow rate 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-methanol-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% - 8 min, 70% - 1 min, 50% - 2 min, 20% - 1 min, 10% - 6min and 0% - 4 min). The column was equilibrated with 20% A - 2 min and 100% A -8 min. Calibration curves for quantitative determinations were prepared using standards for Chl_a, Chl_b and pheophorbide *a* diluted in acetone in the range of 5 to 40 mg mL^{-1} (5, 10, 15, 20, 25, 30 and 40), 5 to 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

Morpho-physiological data was subjected to t-test for single comparisons and analysis of variance (ANOVA) with the General Linear Model procedure for multiple comparisons. The statistical software package SIGMAPLOT 11.0 (Systat Software Inc., Chicago, USA) was used and differences were considered significant for $P \leq 0.05$.

2.5. Reverse transcriptional quantitative PCR (RT-qPCR) analysis

Based on the expression levels observed from the RNA-Seq and on the physiology indications that pointed for a modified chlorophyll metabolism (Sapeta et al., 2013 and this work), the genes selected for the RT-qPCR reactions were: *Chlorophyll a oxygenase* (JcCA0070011.10); *Chlorophyllase-1* (JcCD0049300.10); *Chlorophyll synthase* (JcCB0002271.10); *Pheophorbide a oxygenase* (JcCA0287191.10) and *Pheophytinase* (JcCB0033751.10).

Quantitative PCR was performed using the LightCycler 480 system (Roche, Basel, Switzerland) and the SYBR Green I Master mix (Roche, Basel, Switzerland). The PCR running conditions were as follows: one cycle at 95ºC for 5 min and 45 cycles of amplification at 95ºC for 10 s, 59ºC for 10 s and 72ºC for 10 s. The CT values were calculated from means of three technical replicates and the relative expression of transcripts (to the internal control) was calculated using the method of relative quantification with kinetic PCR efficiency correction (Pfaffl, 2001). The *ARF GTPase-activating protein* gene (JcCA0131111.10) was selected from the RNA-Seq data (based on the lowest coefficient of variance method, Severin et al., 2010) to be used as internal control.

The primers selected for all PCR amplifications are described in Supplementary Table S1. The cDNA for the RT-qPCR validation was prepared with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland) from 1 µg total RNA isolated as previously described.

3. Results

Defining the stress scale for transcriptomics

Experiments were designed to assess the behaviour of two *J. curcas* accessions in conditions of drought stress (water withhold) for 49 days followed by one week of recovery (re-watering), as compared to plants maintained in well-watered conditions (control). Soil and plant (leaf) water content (SWC and RWC, respectively) assessed during the experiment showed that SWC decreased rapidly along the imposed drought period until day 15 (when it reached 30% of field capacity - FC) and continued to slowly decline until it reached 10% at day 49 (maximum stress) (Fig. 1). However, RWC was found to be stable (with only minor differences with no statistical significance) throughout the whole stress and re-watering periods and in both accessions, even at maximum stress (10% SWC) (Table S2).

Since drought stress did not affect leaf water status, we have defined the stress levels based on soil water content (% of FC) (Fig. 1) and on previous physiology studies we have conducted (Sapeta et al., 2013). Moderate stress was defined as corresponding to 30% FC and severe stress to 10% FC. Based on the stress scale defined, we have collected samples for RNA isolation and transcriptomic analyses, at 4 time points: beginning of the experiment (day 0), moderate stress (day 13), severe stress (day 49) and after 3 days of re-watering (day 52).

Transcriptome deep sequencing and mapping to the reference Jatropha curcas genome

Twenty-two *Jatropha curcas* drought stress and control cDNA samples (from leaves and roots) were used for deep sequencing by the Illumina platform, providing roughly 1.8 billion reads, each with 50 nucleotides. The number of reads per sample ranged from 59 to 130 million, with an average number of approximately 82 million reads.

Reads generated from all the libraries were mapped using TopHat v1.4.1 to *Jatropha curcas* reference genome version 3.0 (Sato et al., 2010) and categorized into three classes: uniquely mapped reads (1.4 billion corresponding to 76% of the total), unmapped reads (305 million, 17%) and multiple mapped reads (117 million, 6.52%) (Table S3).

Out of the 59,269 genes annotated in the *Jatropha curcas* database genome version 3.0 (Sato et al., 2011) we found 34,969 expressed genes, corresponding to 59% (Table S3).

Transcriptomic differences are higher at maximum stress, clustering together leaves and roots

Before testing for differential expression, the consistency of the data between the 22 samples was tested. Read counts mapping to the annotated genome were normalized by reads per kilobase of exon model per million mapped reads (RPKM).

A high correlation was observed in gene expression between the two accessions, thus the Pearson's correlation coefficient between the normalized expression values (RPKM), for the two accessions, ranged from 0.913 to 0.996 (Table S4). At the maximum stress tested (day 49) the Pearson's correlation between accessions was 0.988 for leaves and 0.982 for root samples subjected to drought stress (Fig. 2). Considering the high degree of similarity between the accessions in all tested conditions, they were used as biological replicates. Moreover, since each RNA sample was isolated from a pool of 3 to 6 plants, each replicate was considered as an average sample.

To gain insight on the transcriptional changes occurring during drought stress and re-watering, we performed hierarchical clustering analysis (Fig. 3). The high correlation already observed between accessions was further reflected in the clustering. Root samples from both accessions under control, re-watering and moderate stress conditions clustered together in the first clade, as did the leaf samples in a second one. However, both leaf and root samples clustered together at maximum stress, reflecting a specific effect on transcriptional regulation. It was also observed that at the same conditions the two accessions tended to cluster as nearest neighbors. Overall, this analysis showed that major differences in gene expression occur at maximum stress, justifying further characterization of drought mechanisms at the molecular level.

Main expression differences in moderate stress and recovery occur in roots

To identify drought responding genes, samples from control (well-watered) and drought stress (water withholding) after 13 and 49 days were analyzed, as well as after 3 days of subsequent re-watering (day 52). The transcript abundance of each gene (in RPKM) for the two biological replicates was used for testing differential expression using the DEGSeq package.

Analysis of differential gene expression revealed a higher number of differentially expressed genes in roots as compared with leaves of plants subjected to drought stress (Fig. 4). At

moderate drought (day 13), only 26 differentially expressed genes were found in leaves, while 445 were found in roots. Similarly, after 3 days of re-watering (day 52) we found 84 differentially expressed genes for leaves and 1103 for roots. However, under maximum stress (day 49) the number of differentially expressed genes for both organs was similar, 1897 for leaves and 1990 for roots. It was interesting to find that 258 differentially expressed (DE) genes were common in both leaves and roots at day 49 (Table S5). Thus, 1639 and 1732 DE genes were found specifically in leaves and roots at day 49, respectively. Among the common 258 DE genes, 30 were classified as conserved hypothetical protein or had no hits. Moreover, we found enrichment in sequences coding for putative heat shock proteins, late embryogenesis abundant proteins, and leucine-rich repeat containing proteins.

Considering only leaf samples, genes specifically differentially expressed in leaves at day 13 showed high homology to heat-shock proteins (JcCA0239021.10; JcCB0143541.10; JcCB0002131.20), lipoxygenases (JcCB0620091.10; JcCB0760501.10; JcCC0606721.10) and proteinase inhibitors (JcCB0390721.10; JcCD0002166.10), among others. Furthermore, six gene sequences were found to be specifically expressed in leaves at both moderate and maximum stress (days 13 and 49). Among these, some presented high homology to *Ricinus communis* sequences encoding gibberellin-regulated protein 3 (JcCA0285481.10), GA-like protein (JcCB0068571.20) and light-regulated protein (JcCB0368851.10). Another sequence with high homology to a *Ricinus communis* putative vacuolar cation/proton exchanger 1a (JcCB0051311.10) was differentially expressed in all analyzed conditions (in leaves), as compared to controls.

For the re-watering time point (day 52) we found genes coding for germin-like protein (JcCB0055961.20); elongation factor 1-alpha (JcCA0075581.10) and sugar transporter (JcCB0249171.10), in leaves.

Identities of the top 40 leaf genes with the highest up- and down-regulation at maximum stress are presented in table I. We found up-regulation of genes known to be stress-responsive, such as Cytochrome P450 and LEA 6, as well as transcription factors (NF-YA1 and NAC domain-containing protein). As down-regulated genes, we found several encoding histones (Histone H2A and H3) and defense proteins (Chitinases).

Functional categorization of the drought-regulated genes

Gene Ontology (GO) enrichment analysis reveled 347 and 830 enriched GO terms for up-regulated and down-regulated genes, respectively, for leaves under severe stress (day 49) (Table S6). Most enriched GO terms from up-regulated genes were associated with response to stress or water loss such as response to: stimulus (GO:0050896); stress (GO:0006950); abiotic stimulus (GO:0009628); osmotic stress (GO:0006970); water deprivation (GO:0009414) and water stimulus (GO:0009415), among others. Response to hormones was also highly represented, such as response to: hormone stimulus (GO:0009725); abscisic acid stimulus (GO:0009737); oxylipin biosynthetic and oxylipin metabolic process (GO:0031408 and GO:0031407); hormone-mediated signaling pathway (GO:0009755); abscisic acid transport and abscisic acid mediated signaling pathway (GO:0080168 and GO:0009738), and salicylic acid stimulus (GO:0009751). Additionally, several GO terms associated with wax production and cell wall thickening were also found, namely wax metabolic process (GO:0010166); wax biosynthetic process (GO:0010025); cell wall modification (GO:0042545), defense response by cell wall thickening (GO:0052482). Leaf senescence (GO:0010150) associated genes were also enriched, as well as genes involved in antioxidant activity (GO:0016209) and osmoregulation such as trehalose biosynthetic process (GO:0005992) and trehalose-phosphatase activity (GO:0004805).

Interestingly, the categories chlorophyll catabolic and metabolic process (GO:0015996 and GO:0015994) were enriched with up-regulated genes. On the other hand, growth associated GO terms were found to be strongly down-regulated in leaves of *J. curcas* plants subjected to 49 days of water withholding, such as cell proliferation (GO:0008283); cell cycle (GO:0007049); cell cycle process (GO:0022402); cell division (GO:0051301); meristem growth (GO:0035266); growth (GO:0040007) and developmental growth (GO:0048589), among others. Other down-regulated GO terms were related to plant hormones such as auxin polar transport (GO:0009926) and response to cytokinin stimulus (GO:0009735). Moreover, GO terms related to water stress were also found, namely cellular response to stress (GO:0033554); superoxide dismutase activity (GO:0004784) and water transport (GO:0006833), among others.

GO enrichment analysis for leaves under moderate stress and re-watering (days 13 and 52, respectively) can also be found in supplementary Table S6. GO enrichment analysis for roots

under moderate, severe and re-watering (days 13, 49 and 52, respectively) can be found in Table S7.

Growth reduces at moderate water deficit and older leaves are lost at severe stress

Water deficit caused a significant reduction in growth ($p<0.001$). Stem length and diameter (Fig. 5 A and B) were the first parameters affected, showing growth reduction by day 14 onwards (65% SWC). By day 49 (10% SWC) stressed plants had a 39% reduction in stem diameter, 22% reduction in stem length and 46% reduction in leaf number, as compared to control plants. The most affected parameter was the total number of leaves derived from the apical meristem, due to the arrest of leaf emergence and to leaf shed. Flushing of older leaves was observed from day 35 onwards (15% SWC) being more accentuated from day 42 to 49 (Fig. 5 C). Despite the severe growth arrest observed along the drought period stem elongation, stem diameter enlargement and emergence of new leaves was rapidly resumed after re-watering (Fig. 5, from day 49 to 56). Roots were also quickly rehydrated, and new roots were observed already by day 52 (the third day after re-watering) (data not shown). Additionally, drought stress significantly (p-value=0.001) reduced biomass production and total leaf area (Fig. S2).

At the morphological level we have observed that the petioles curved down in drought stressed plants from day 7 onwards (Fig. 6 A). This alteration resulted in more vertically positioned leaf blades throughout the remaining drought period. However after one night of re-watering, the petiole curvature reduced, returning to a more straight shape (Fig. 6B). The morphological appearance of the stress plants at the beginning of stress imposition, through stress and re-watering can be found in Fig. S3.

Reduction of stomatal conductance precedes photosynthesis shut-down

Water stress significantly reduced stomatal conductance, photosynthesis, photosystem II efficiency and the electron transport rate ($p<0.001$) (Fig. 7). A reduction in net photosynthesis (Fig. 7 A) of 37% (control versus drought stressed plants) was observed after 12 days of water withhold (40% SWC). Net photosynthesis continued to decrease in stressed plants as drought stress continued. In fact, at maximum stress (10% SWC), a 95% reduction was observed in stressed plants as compared to control.

There was also a strict stomatal control in response to drought stress (Fig. 7 B). Stomatal conductance reduced by 20%, as compared with the control, after one week of water withhold (60% SWC), while after 12 days (SWC of 40%), an 88% reduction was observed (Fig. 7 B). When minimum soil water availability was reached (10%), stomatal conductance reached values close to zero. The reduction of stomatal conductance occurred before the reduction of net photosynthesis, resulting in an increase of instantaneous water use efficiency (data not shown).

The ΦPSII of stressed plants remained at control levels until day 16 after the beginning of water withhold (25% SWC). By day 20, ΦPSII started to decline, being reduced by 75% on day 23 (20% SWC) for the stress treated plants as compared with the control. These values were maintained throughout the drought stress period until maximum stress. A complete recovery was observed for all measured parameters within 5 days of re-watering (day 54). Maximum quantum yield of PSII (Fv/Fm) was also significantly affected by drought stress (Fig. S4). After 49 days of drought (maximum stress) the Fv/Fm was reduced from 0.83 to 0.78 (p-value <0.001). Nevertheless, after 1 day of re-watering (day 50), the stressed plants already showed some recovery of Fv/Fm, which reached control levels at the end of the re-watering period (day 56).

Chlorophylls a and b increase at maximum drought stress while $Chl_{a/b}$ ratio decreases

Chlorophyll *a* (Chl_a) content was similar for both stress and control plants at days 0 and 13 (roughly 6 mg g^{-1} DW) (Fig. 8 A). However, at maximum stress (day 49) Chl_a content was significantly higher in stressed plants than in controls (3.2 versus 4.5 mg g^{-1} DW in control versus drought stress plants, p-value=0.011). The same pattern was observed for the chlorophyll *b* (Chl_b) content (Fig. 8 B, day 49, 1.1 versus 2.0 mg g^{-1} DW in control versus drought stress plants, p-value<0.001). 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 chlorophyll *a* to *b* ratio (Fig. 8 C, day 49) due to drought. This ratio was quickly resumed to control values (~3) after 7 days of re-watering (day 56), supporting that its reduction was a consequence of the drought treatment.

Real-time PCR and HPLC validate the modified Chlorophyll metabolism

Among 110 Chl associated genes, seven showed significant up-regulation in leaves of plants at maximum drought stress. Genes encoding putative Pheophorbide a oxygenase and Chlorophyll *a* oxygenase (Table S8) were among those seven.

To confirm the accuracy and reproducibility of the Illumina RNA-Seq results, and to further investigate chlorophyll biosynthesis and breakdown, genes involved in these pathways and showing differential and non-differential expression under drought, were selected for reverse transcription quantitative PCR (RT-qPCR). Quantitative expression analysis also validated as a good housekeeping gene for drought stress assays in *Jatropha curcas* plants, the RNA-Seq-selected gene encoding the putative Arf GTPase-activating protein (JcCA0131111.10).

Correlation between RNA-Seq and RT-qPCR using fold change measurements revealed that the expression trends of these genes showed significant similarity (r = 0.9077, Fig. S5). Moreover, RT-qPCR showed a significant increase of transcripts abundance of *Chlorophyll a oxygenase* (*CAO*, JcCA0070011.10) in leaves of drought stressed *J. curcas* plants (Fig. 9 A), and no significant differences after re-watering (3 days after maximum stress), further supporting the RNA-Seq data (Fig. S6). A significant increase in transcripts abundance was also observed at maximum stress for the chlorophyll breakdown pathway genes *Pheophorbide a oxygenase* (*PAO*, JcCA0287191.10) and *Pheophytinase* (*PPH*, JcCA0287191.10), however *Chlorophyllase-1* (*Chlase*, JcCD0049300.10) expression was similar in control and drought stressed plants (Fig. 9 B). Similarly, the *Chlorophyll synthase gene* (*CS*, JcCB0002271.10) showed no significant expression changes (Fig. 9 B).

The HPLC analyses performed to quantify the changes occurring in chlorophyll content after 49 days of drought stress, further supported the reduction in chlorophyll *a to b* ratio (Fig. 10A, p-value=0.006) first assessed spectrophotometrically. Pheophorbide *a* quantifications showed significant lower levels in drought-stressed plants as compared to controls (Fig. 10B, p-value=0.016).

4. Discussion

A better understanding of plant stress physiology can help designing improved and more targeted breeding strategies directed to improve plant performance in a context of water scarcity. Moreover, the integration of the recently available transcriptomics tools (RNA-Seq) may additionally provide relevant indications about genes and pathways altered by the stress.

Drought-response mechanisms have been investigated at the molecular level in a variety of species, but mostly in models 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, models like Arabidopsis and rice are very sensitive to drought, and some authors have pointed out that their responses may reflect senescence or death, rather than useful adaptations (Munns and Tester, 2008 *in* Deyholos, 2010). In this context, transcript profiling of plants adapted to arid and semi-arid climates can be a valuable tool to characterize resistance mechanisms and screen for drought tolerance candidate genes.

Transcriptomic studies designed to uncover drought tolerance mechanisms have been conducted in several legume species such as common bean (Recchia et al., 2013), soybean (Fan et al., 2013) and chickpea (Wang et al., 2012). Plants adapted to extreme environments such as, Agave sp. (Gross et al., 2013) or resistant to extreme desiccation like the species *Craterostigma plantagineum* (Rodriguez et al., 2010) were also studied at transcriptomic level.

In previous studies (Sapeta et al., 2013) it was observed that, under severe drought stress (20% SWC), the emerging biodiesel plant *Jatropha curcas* responds by shutting down all systems (stomatal exchanges first and then photosynthesis). This was most remarkable and was the drive for the present study in which we combined transcriptomics with morpho-physiology to investigate the mechanisms behind the high drought-tolerance of this species.

Based on our previous work (Sapeta et al., 2013) we have defined three main stages for characterization of the transcriptome, (1) moderate stress; (2) severe stress and (3) recovery, which in the present study, corresponded to Days 13, 49 and 52, as assessed from SWC. Transcriptomic and morpho-physiological changes occurring in these periods were correlated aiming to understand the plant adjustments to the environment. We used RNA sequencing (RNA-Seq) to assess both leaf and root transcriptomes under moderate stress (to identify

sensing and signaling events), prolonged drought (to uncover the molecular controls of plant performance and protection), and finally three days after stress alleviation (re-watering), to assess recovery efficiency. Differently from our previous work (Sapeta et al., 2013), here drought stress was imposed by water withhold for 49 days (instead of progressive reduction of irrigation along 28 days), thus resulting in more defined stages of moderate and severe stress.

In this study we aimed to compare two *J. curcas* accessions, original from two contrasting climates, semi-arid and wet tropical, in extended drought conditions. Genotypes differing only in their tolerance to one stress or adapted to different climatic origins can be good materials for the identification of genes conferring stress tolerance (Deyholos, 2010). This strategy has been used with Eucalyptus (Villar et al., 2011; Thumma et al., 2012), horse gram (Bhardwaj et al., 2013) and rice (Lenka et al., 2011), among other species. In this work with Jatropha, the transcriptomics comparisons between, control, two stress intensities and recovery showed a high similarity between both accessions. Moreover, this result could be correlated with the morphophysiology data. We have thus used both accessions as biological replicates in order to increase the statistical value of the transcriptomics data. Thumma et al. (2012) have performed RNA-Seq to compare drought response of *Eucalyptus camaldulensis* populations originating from contrasting climates (humid, dry and semi-arid) and also found very similar transcriptome and morphophysiological profiles for the three populations tested. As in our study, these authors used the three populations as biological replicates for differential expression analysis (Thumma et al., 2012). Interestingly, other studies comparing various *J. curcas* accessions revealed little variation among them (Maes et al., 2009; Achten et al., 2010), most probably due to the reduced genetic diversity that *J. curcas* has low outside its centre of origin (Kumar et al., 2008; Kumar et al., 2009; Pamidimarri et al., 2009; Pamidimarri et al., 2009; Popluechai et al., 2009).

In this study all screened collection points showed, in comparison with the control, transcriptomic alterations in response to drought. However severe stress (49 days) resulted in the most drastic changes, as shown by hierarchical cluster analysis. Shoots and roots were separated in two clusters each joining the transcriptomes expressed in control conditions, at moderate stress (day 13) and at re-watering (day 52), while under severe stress, the transcriptomes expressed by roots and leaves clustered together in the same group. This suggests that the severity of the stress reduced the functional differences between the two organ types inducing a common response in drought.

Changes in transcriptomic profiles occurred earlier in roots than in leaves, with a higher number of differentially expressed genes at lower stress intensity (day 13, moderate drought). Furthermore, as compared with leaves, roots also showed a larger number of differentially expressed genes in severe stress and re-watering. Also in soybean, chickpea and horse gram, other authors have found that roots respond faster and more strongly to drought stress than leaves (Wang et al., 2012; Bhardwaj et al., 2013; Fan et al., 2013).

It was most surprising to find that, by day 13 (moderate stress), only 26 genes were found differentially expressed in leaves (12 up- and 14 down-regulated), while by then stomatal conductance was already drastically reduced, net photosynthetic rate had decreased by more than 50% and stem growth rate was also reduced. Among the genes up-regulated in this condition in leaves we found the putative *Lipoxygenase 2* and *3* (*LOX2* and *3*). *LOX* genes have been well studied in model plants like *A. thaliana*. *AtLOX 2* and *3* were proposed to be located in plastids and be responsible for the first step in jasmonate biosynthesis (Bell and Mullet, 1993; Seltmann et al., 2010), thus being putatively involved in the biosynthesis of oxylipins of the jasmonate family (Wasternack, 2007). Furthermore, the GO enrichment analysis showed the up-representation of linoleate 13S-lipoxygenase activity and oxylipin biosynthetic/metabolic process. Moreover these GO categories were still over represented in leaves under severe stress (day 49). Oxylipins are known to accumulate in leaves in response to both biotic and abiotic stresses (Wasternack, 2007; Grebner et al., 2013) and they were described as playing a role in stress-associated stomata closure and senescence (Suhita et al., 2004; Montillet et al., 2013; Savchenko et al., 2014). The oxylipin metabolism under drought stress may thus deserve further investigation in *J. curcas*.

At severe stress (day 49) we found several genes up- or down-regulated (≥4 fold change). Many of the induced genes were classified as putatively involved in stress responses, such as cytochrome P450 enzymes, UDP-glucuronyl-glycosyl-transferases, ABC transporters, and heat shock proteins, among others. As expected, the GO Terms categories related to drought stress response (e.g: response to stimulus/ stress/ abiotic stimulus/ osmotic stress, etc) were enriched. Identical enrichment in these GO Terms were also reported for some 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 drought stress genes were induced by the stress treatment, a higher number was down-regulated (1181 down-regulated against 716 up-regulated). The genes repressed included those involved in cell cycle regulation and cell

growth (e.g.: putative Histone and Chitinase coding genes). Gene ontology indicated that down-regulated genes are mainly associated with growth, development, cell cycle and cell replication (e.g.: cell cycle; cell cycle process; cell division; meristem growth; growth and developmental growth). These changes correlate with the growth arrest observed from moderate to severe stress and agree with published data (Thumma et al., 2012). In addition to genes with high homology to genes previously characterized, we also found several novel and/or unknown ones differentially expressed. Functional analysis of these genes may provide novel insights into the genetic control of drought tolerance in *Jatropha curcas*.

During drought stress recovery (Day 52) several genes involved in protein synthesis (e.g.: Elongation factors) were found. This was also observed in the enrichment analysis of the up-regulated genes (e.g.: translation elongation factor activity; translational elongation; translation factor activity; nucleic acid binding) concordant with the re-growth phase observed after stress alleviation.

The transcriptomic data generated in the present work also provides an opportunity to identify those genes with a more stable expression during stress and recovery periods, and thus more appropriate to use as housekeeping (HKG) in expression studies. Therefore, we have also performed a preliminary study to identify the most appropriate HKG for these studies. Among the candidate genes one putatively encoding an ARF GTPase-activating protein was selected due to its stable expression in both roots and leaves in all tested conditions. ARF GTPase-activating proteins belong to the ARF-family GTPases and were described as involved in multiple membrane trafficking processes, as well as in cytoskeletal organization (Vernoud et al., 2003).

Morpho-physiological adaptations to drought

The morpho-physiological data gathered here support our previous findings and the idea that the main strategy of *J. curcas* to endure drought stress is via a strict stomatal control and reduction of transpiration area by leaf growth arrest. In fact, *Jatropha curcas* plants succeeded to maintain reasonably constant relative water content during the whole stress and re-watering period. Stem length and diameter were the first to suffer from drought, followed by leaf number (leaves produced by the apical meristem). Biomass and total leaf area were particularly reduced at maximum stress, and started recovering after re-watering. A progressive shut-down was observed in leaves on a weekly basis during drought stress,

stomatal conductance was the first parameter to show a strong decline (by day 7), followed by net photosynthesis (by day 14), although leaf number only reduced from the second to the third week of drought. Still, by day 20, when SWC was close to 25% field capacity, the efficiency of photosystem II and the electron transfer rate still showed normal values, falling abruptly by day 23, and reaching the minimum values at maximum stress (day 49). The maximum quantum yield of PSII (Fv/Fm) showed the maximum reduction at maximum stress, increasing thereafter and approaching control values along the rehydration week. Considering that these observations were obtained for the younger fully expanded leaves, it is important to stress that other leaves may have different behaviour.

Additionally to the reduction of total leaf area by abscission and initiation arrest, we also observed, from day 7 onwards, leaf inflection bringing the leaf blade towards a more vertical orientation (with the tip pointing down, Fig. 6A). This was also reported by Maes et al. (2011) for *Jatropha curcas* under drought stress. Although these authors have not analyzed RWC in their plants, in our experiments we found that leaf inflection did not correlate with a reduction in RWC, which was maintained at 70% throughout the whole experiment. Maes et al. (2011) reported that smaller and more vertically oriented leaves reflect a *J. curcas* adaptation to drought stress to reduce leaf temperature. Our results (RWC and morphology) seem to agree with this conclusion of Maes et al. (2011), especially because leaf re-orientation was only observed after complete stomatal closure, reverting to normal position upon rehydration.

Chl a to b ratio adjustment under drought stress

In previous work we found that under drought stress *J. curcas* shows a reduction of Chl *a* to *b* ratio (Sapeta et al., 2013). The transcriptomic and physiological data presented here further validated that work. As above described, this is a consequence of an increase in chlorophyll content relative to dry weight, which is stronger in the case of chlorophyll *b*.

The plasticity of $Chl_{a/b}$ in response to light variations is well known, mainly due to the enzyme Chlorophyllide *a* oxygenase (CAO) that plays a predominant role in determining the Chl *a* to *b* ratios (Tanaka et al., 1998; Espineda et al., 1999; Pattanayak et al., 2005; Tanaka and Tanaka, 2005). Under severe stress (day 49 of water withholding) a significant increase in transcripts was observed for the gene putatively encoding *J. curcas* CAO (for review of the Chl synthesis, cycle and breakdown enzymes and metabolites see Fig. 11). The up-regulation of CAO was expected from the observed $Chl_{a/b}$ reduction from 2.85 to 2.19, respectively in

control and stressed plants. Like previously described we also observed a full recovery after 7 days of rehydration where $Chl_{a/b}$ was 2.96 and 2.98, respectively in control and drought stressed plants. Moreover, CAO transcripts were found to be already normal after 3 days of re-watering (day 52). Thus, the results strongly suggest that the decrease observed in $Chl_{a/b}$ is a consequence of increased CAO activity. The CAO pathway was already identified as the sole pathway for Chl_b biosynthesis in *A. thaliana* (Espineda et al., 1999; Oster et al., 2000). Moreover, Arabidopsis full-length CAO overexpressed in tobacco plants resulted in increased Chl_b and therefore reduced $Chl_{a/b}$ (Pattanayak et al., 2005). Recently, Biswal et al. (2012) using the same overexpressing line described in Pattanayak et al. (2005) showed that CAO overexpressing tobacco plants had more light-harvesting chlorophyll proteins, as well as electron transport chain proteins, leading to an increase in light capture and electron transport rates in photosystems I and II. Moreover light saturated photosynthetic carbon assimilation, starch content, and dry matter accumulation were increased (Biswal et al., 2012). Sakuraba et al., (2012) have further demonstrated that increased Chl_b synthesis through overexpression of the catalytic domain of *Prochlorococcus hollandica* CAO in *Arabidopsis* plants delayed senescence. In addition, the authors have performed a large-scale real-time PCR analyzing several transcription factors. In their study, they found several differentially expressed senescence associated TFs. Furthermore, genes encoding Chl degradation enzymes were down-regulated and genes involved in photosynthesis were up-regulated. These results highlighted the role of CAO and, together with our data, we may hypothesize that the up-regulation of CAO is involved in delaying drought-induced leaf senescence.

Additionally, we found in RNA-Seq analyses, a significant increase in transcripts of *J. curcas* putative PAO, which was further confirmed by real time PCR analysis. Moreover, pheophorbide *a*, described by Hortensteiner et al. (1995) and Pruzinska et al. (2003) as the preferential substrate for PAO, was found to significantly decrease during severe stress in our experiments. PAO activity has mainly been associated with senescence and its up-regulation can also be found in response to various environmental challenges, such as osmotic stress and pathogen infections, conditions in which chlorophyll breakdown is also observed (Thomas et al., 2001). In our case, we only observed leaf senescence of older leaves but not of the young ones that were sampled for transcriptomic study. We hypothesize that the increase of PAO is most likely for leaf protection rather than senescence, since accumulation of pheophorbide *a* leads to O_2^- and is involved in the signaling pathway of programmed cell death (Hirashima et

al., 2009). To summarize, the results here reported, support the relevance of chlorophyll synthesis and breakdown genes such as *CAO* and *PAO* in *J. curcas* drought adaptations, in a process that deserves further investigation.

5. Conclusions

The capacity of a plant to withstand adverse condition largely depends on the capacity to anticipate those adverse conditions. Jatropha presents this capacity for drought stress with its sensitive stomatal control that is followed, if the adverse conditions persist, by a gradual shut down of photosynthesis and growth. The success of this strategy is evidenced in its recovery capacity after stress. Only three days after re-watering, growth and photosynthesis were resumed, highlighting this species capacity to withstand severe drought. In this study we have combined transcriptomics with morpho-physiology to unveil the molecular basis of *J. curcas* drought responses and physiologically relevant processes. This powerful strategy allowed us to identify over 4000 drought-responsive genes. Functional validation of these drought responsive genes will help to understand drought tolerance at molecular level.

Acknowledgments

José de Vega-Bartol, from the Forest Biotech Lab (ITQB, Oeiras, Portugal) is acknowledged for support in the GO enrichment analysis. Prof**.** Baishnab Tripathy **(**Jawaharlal Nehru University, New Delhi, India) is acknowledged for helpful discussions. Maria Cristina Leitão (ITQB, Oeiras, Portugal) is acknowledged for HPLC determinations support. The authors would like to acknowledge Maria da Graça Palha (Instituto Nacional de Investigação Agrária e Veterinária, I.P., Oeiras, Portugal) for making available the colour image analysis system.

Table I. Gene identities of the transcripts most differentially expressed in leaves after 49 days of water withhold.

Sequence ID[a]	Description[a]	Log_2 FC		
		Day 13	Day 49	Day 52
Up-regulated				
JcCB0111631.20	STS14 protein precursor, putative OS=*Ricinus communis*	-0.2	**7.6**	-0.2
JcCA0045481.10	Ferritin OS=*Ricinus communis*	2.4	**7.4**	0.6
JcCB0119141.10	Major allergen Pru ar, putative OS=*Ricinus communis*	-0.7	**7.3**	-0.2
JcCA0131871.10	Cytochrome P450, putative OS= *Ricinus communis*	0.3	**7.2**	0.4
JcCC0144682.10	Major allergen Pru ar, putative OS=*Ricinus communis*	-0.6	**7.0**	-0.1
JcCA0216451.10	Major allergen Pru ar, putative OS=*Ricinus communis*	-0.7	**7.0**	0.0
JcCB0638961.10	Putative uncharacterized protein OS=*Ricinus communis*	0.5	**6.9**	-0.2
JcCB0931941.10	STS14 protein precursor, putative OS=*Ricinus communis*	-0.3	**6.9**	-0.1
JcCD0145641.10	Cytochrome P450, putative OS= *Ricinus communis*	0.5	**6.6**	0.4
JcCA0019971.10	Putative uncharacterized protein OS=*Ricinus communis*	1.7	**6.4**	-0.4
JcCB0465511.10	Stem-specific protein tsjt1, putative OS=*Jatropha curcas*	0.2	**6.2**	0.9
JcCB0116051.10	NF-YA1 (Nuclear factor Y, Subunit A1), TF, OS= *A. thaliana*	0.8	**6.1**	0.0
JcCA0300321.10	STS14 protein precursor, putative OS=Ricinus communis	0.6	**6.1**	-0.2
JcCB0149421.10	Casbene synthase, chloroplast precursor, putative OS=R. communis	0.0	**6.0**	0.0
JcCA0270891.20	Conserved hypothetical protein, OS=*Ricinus communis*	-0.3	**6.0**	0.0
JcCB0200081.10	Late embryogenesis abundant 6, OS= *A. thaliana*	0.5	**6.0**	-0.2
JcCA0152811.10	Cytochrome P450, putative, OS= *Ricinus communis*	-0.3	**5.9**	0.0
JcCB0037771.10	UDP-glucosyltransferase, putative, OS=Ricinus communis	0.3	**5.9**	-0.3
JcCA0305001.10	Conserved hypothetical protein, OS=*Ricinus communis*	0.2	**5.8**	0.6
JcCB0053051.10	NAC domain-containing protein, putative, OS= *Ricinus communis*	1.2	**5.8**	-0.4
Down-regulated				
JcCB0172181.10	Histone H2A, OS= *Glycine max*	-0.2	**-6.7**	-0.2
JcCB0614261.10	Histone H3, OS= *Picea sitchensis*	-0.5	**-6.5**	-0.6
JcCA0140591.10	Early nodulin 55-2 precursor, putative, OS=Ricinus	-0.4	**-6.4**	-0.2

	communis			
JcCB0009711.20	Histone H2A, OS=*Ricinus communis*	-0.4	**-6.2**	-0.5
JcCA0314821.10	Class IV chitinase, putative, OS= *Ricinus communis*	0.2	**-6.2**	-4.9
JcCD0157886.10	Conserved hypothetical protein, OS= *Ricinus communis*	-0.2	**-6.1**	-0.1
JcCB0190791.20	Histone H3, OS= *Arabidopsis thaliana*	-0.6	**-6.0**	-0.6
JcCB0417591.10	Hydroxyproline-rich glycoprotein family protein, OS=*Arabidopsis thaliana*	-0.6	**-6.0**	-0.3
JcCB0065801.20	Histone H3, OS= *Arabidopsis thaliana*	-0.6	**-5.9**	-0.3
JcCA0106661.10	Class IV chitinase, putative, OS=*Ricinus Communis*	0.2	**-5.9**	-4.7
JcCA0317561.20	Patellin-4, putative, OS=*Ricinus communis*	-0.5	**-5.8**	-0.3
JcCA0150061.20	Histone h3, putative, OS= *Ricinus communis*	-0.4	**-5.8**	-0.5
JcCB0079621.10	CDK (CYCLIN-DEPENDENT KINASE), putative, OS= *Ricinus communis*	-0.3	**-5.8**	0.2
JcCA0301181.10	Putative uncharacterized protein, OS=*Ricinus communis*	-0.7	**-5.7**	0.5
JcCB0689751.10	Putative proline-rich cell wall protein, OS=*Arabidopsis thaliana*	-0.2	**-5.7**	1.4
JcCA0135621.20	Replication factor A 1, rfa1, putative, OS=*Ricinus communis*	-0.4	**-5.7**	-0.4
JcCA0313351.20	Histone H3, OS=*Arabidopsis thaliana*	-0.3	**-5.6**	-0.7
JcCA0313581.30	Rhicadhesin receptor precursor, putative, OS=*Ricinus communis*	0.2	**-5.6**	0.2
JcCB0008031.30	Syntaxin, putative, OS=*Ricinus communis*	-0.6	**-5.6**	0.0
JcCB0531671.10	Chitinase, putative, OS=*Ricinus communis*	0.0	**-5.6**	-3.3

[a] Gene ID and annotations from *Jatropha curcas* genome version 3.0 database (Sato et al., 2011) (ftp://ftp.kazusa.or.jp/pub/jatropha/JAT_r3.0/)

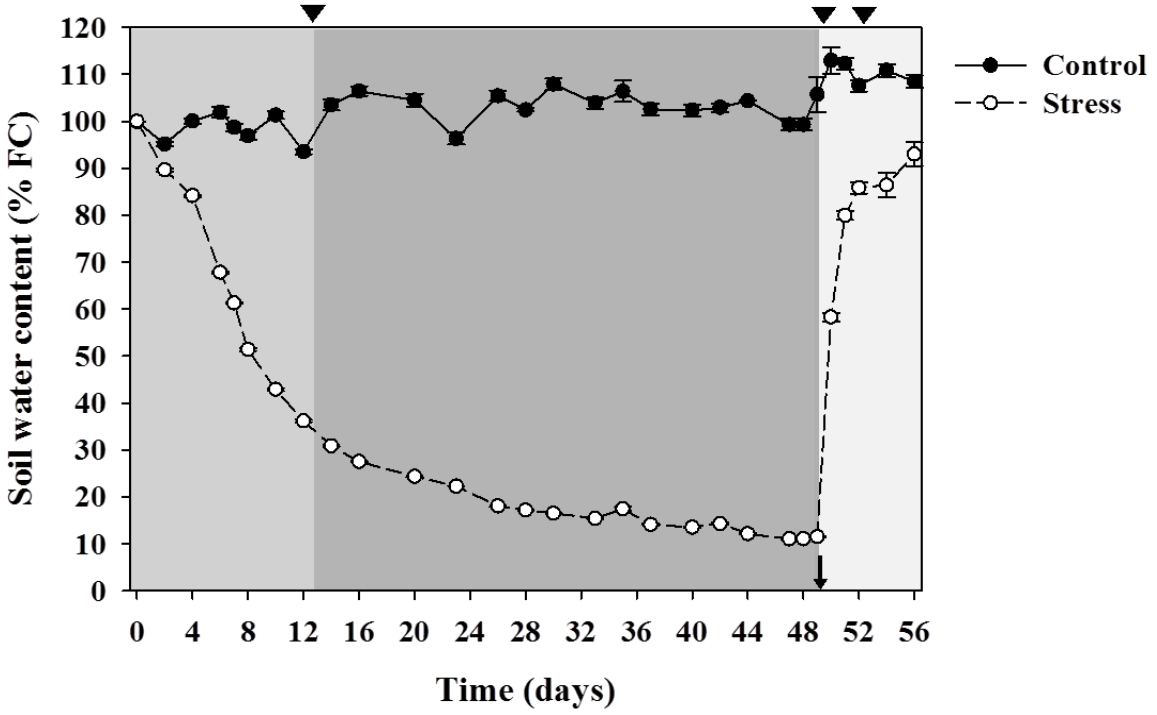

Figure 1. Soil water content along the experiment for 36-days-old *Jatropha curcas* plants continuously grown under well-watered conditions (Control) or subjected to drought stress (Stress) for 49 days followed by a 7-day re-watering period. Background colour indicates: non to moderate stress (100 – 30% FC) (grey); moderate to severe stress (30-10% FC) (dark grey) and re-watering (light grey). The arrow at day 49 indicates re-watering start. Triangles show time points for RNA-Seq analysis. Values are means. Bars represent ± SE (n=12).

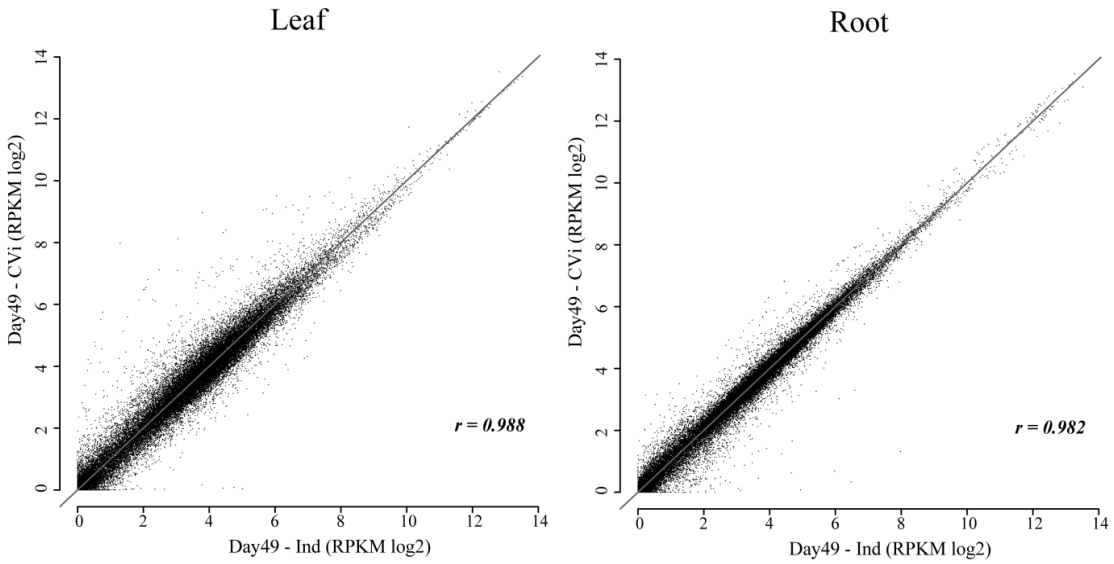

Figure 2. Scatter plots comparing gene expression in leaves and roots of *Jatropha curcas* plants from Indonesia (Ind) versus Cape Verde islands (CVi) subjected to 49 days of drought stress by water withholding.

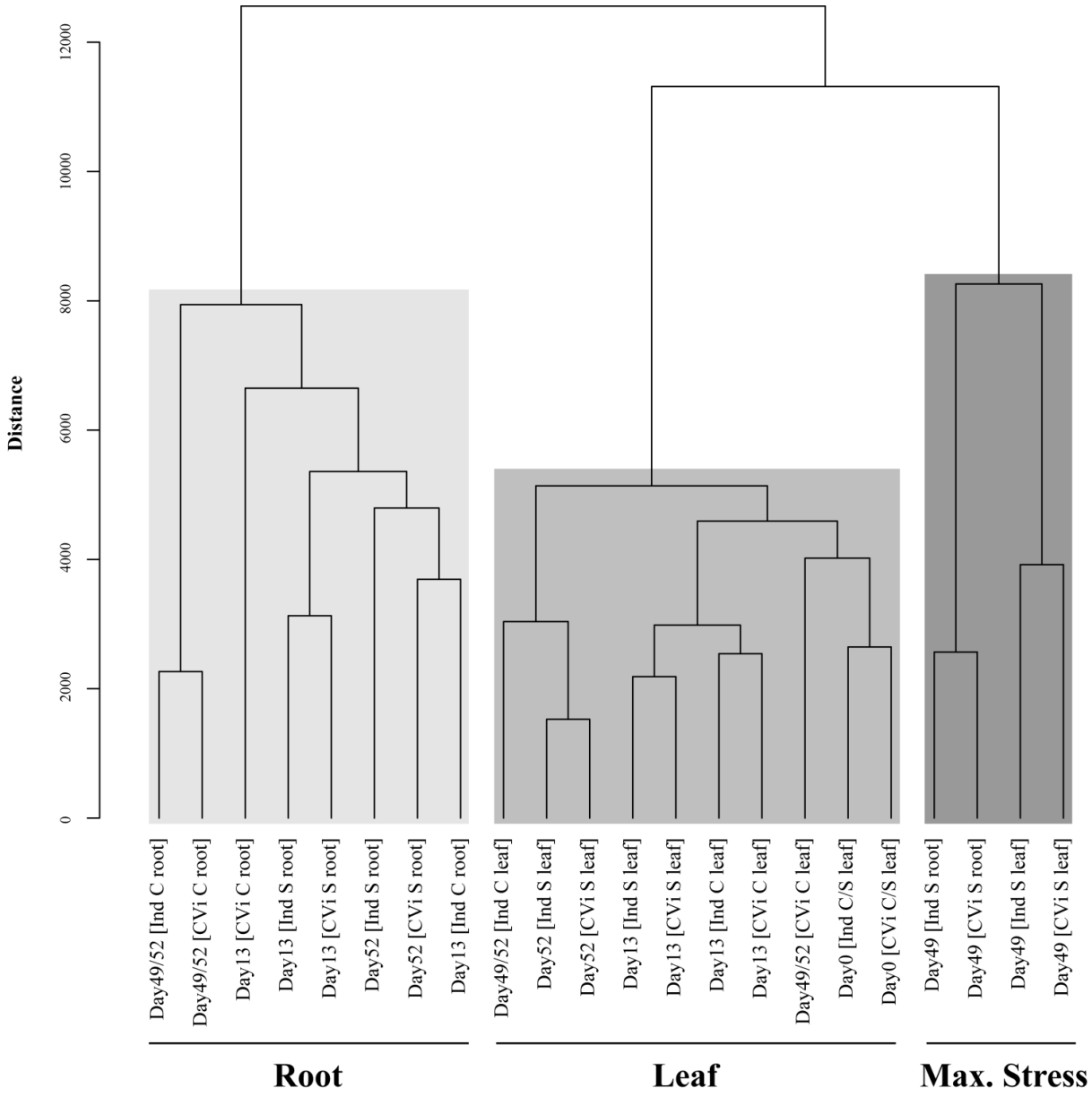

Figure 3. Hierarchical clustering of transcriptional profiles of leaves and roots of *Jatropha curcas* plants from Indonesia (Ind) and Cape Verde islands (CVi), continuously grown under well-watered control conditions (C) or subjected to drought stress (S) for 13 days (day 13, moderate stress) or 49 (day 49, maximum stress) followed by a 3-day re-watering period (day 52, re-watering). RNA of control samples collected at days 49 and 52 was pooled for sequencing, thus referred as Day 49/52. Hierarchical clustering analysis of the transcriptional profiles was performed using the hclust command in R plotted as a dendogram (R Development Core Team, 2009).

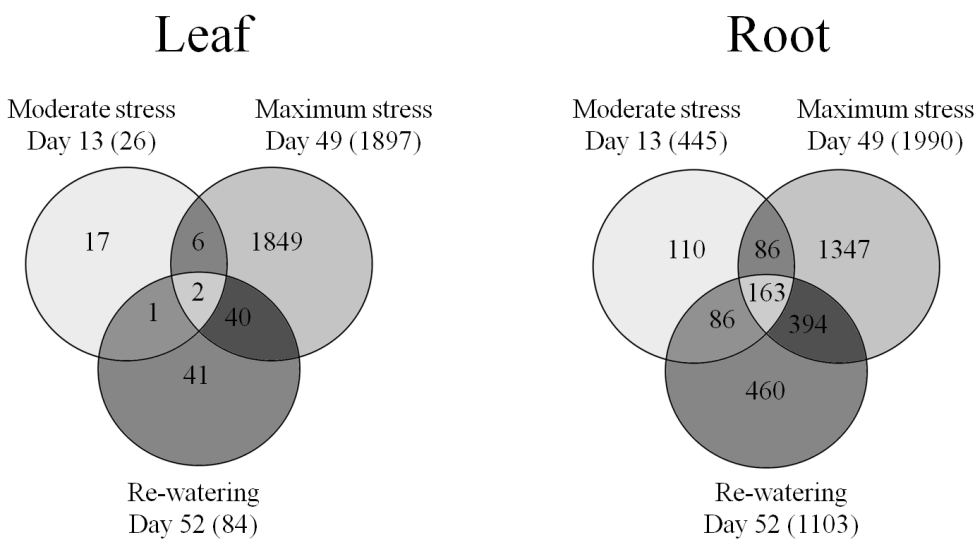

Figure 4. Venn diagrams showing the number of genes differentially expressed in drought stress. Differentially expressed genes were considered if the adjusted p-value was ≤ 0.001 and the fold change ≤-4 or ≥4 (stress versus respective control$_{).}$). The plants used in the experiment were grown for 36 days and subjected to drought stress for 13 days (day 13, moderate stress) up to 49 (day 49, maximum stress) followed by a 3-day re-watering period (day 52, re-watering). Leaves (A) and roots (B) were collected for analyses. The Venn diagram was built using the VENNY tool (Oliveros, 2007).

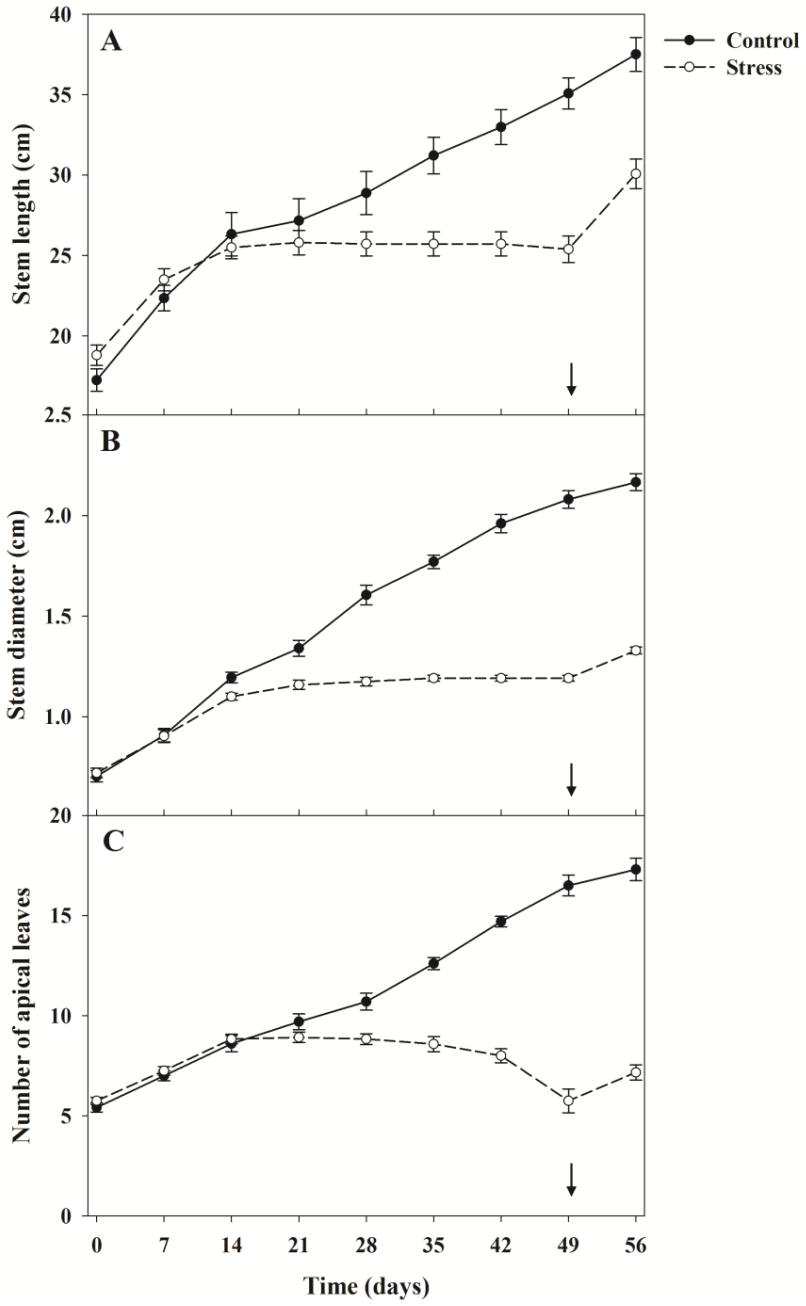

Figure 5. Effect of drought stress and re-watering on (**A**) stem length, (**B**) stem diameter and (**C**) number of apical leaves measured for 36-days-old *Jatropha curcas* plants continuously grown under well-watered control conditions (Control) or subjected to drought stress (Stress) for 49 days followed by a 7-day re-watering period. Arrows at day 49 indicate re-watering start. Values are means. Bars represent ± SE (n=12).

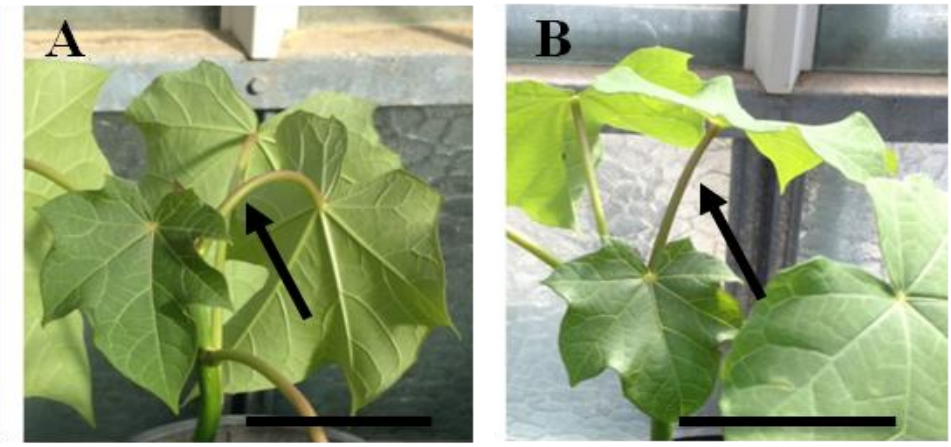

Figure 6. Effect of drought stress and re-watering on leaf petiole angle. Images were collected for 36-days-old *Jatropha curcas* plants subjected to drought stress for 49 days (**A**) followed by a 1-day re-watering (**B**). Arrows highlight the difference in the petiole and leaf angle, scale bar stands for 10cm.

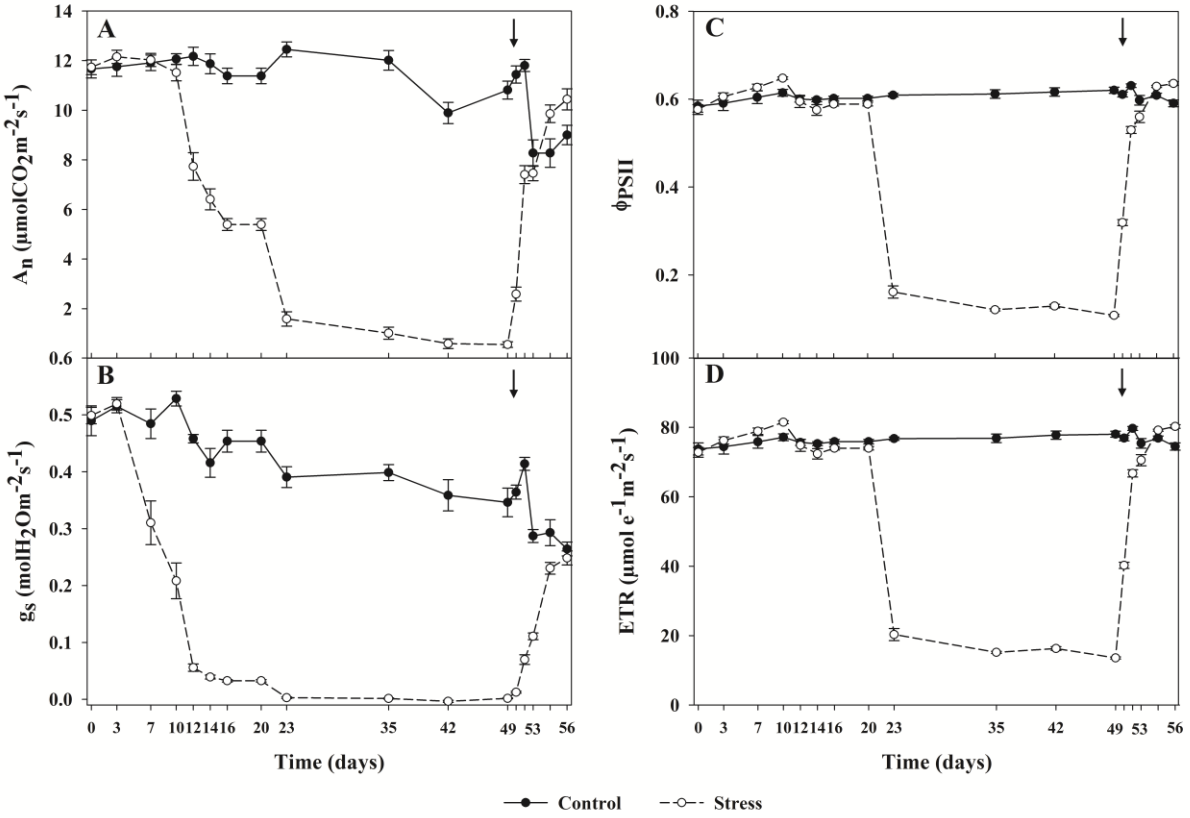

Figure 7. Effect of drought stress and re-watering on (**A**) net photosynthesis (A_n), (**B**) stomatal conductance to water vapour (g_s), (**C**) photosystem II operating efficiency (ΦPSII), (**D**) electron transport rate (ETR), measured for *Jatropha curcas* plants (36-days-old at the start of the experiment) continuously grown under well-watered conditions (Control) or subjected to drought stress (Stress) for 49 days followed by a 7-day re-watering period. Arrows at day 49 indicate re-watering start for stressed plants. Values are means. Bars represent ± SE (n=12).

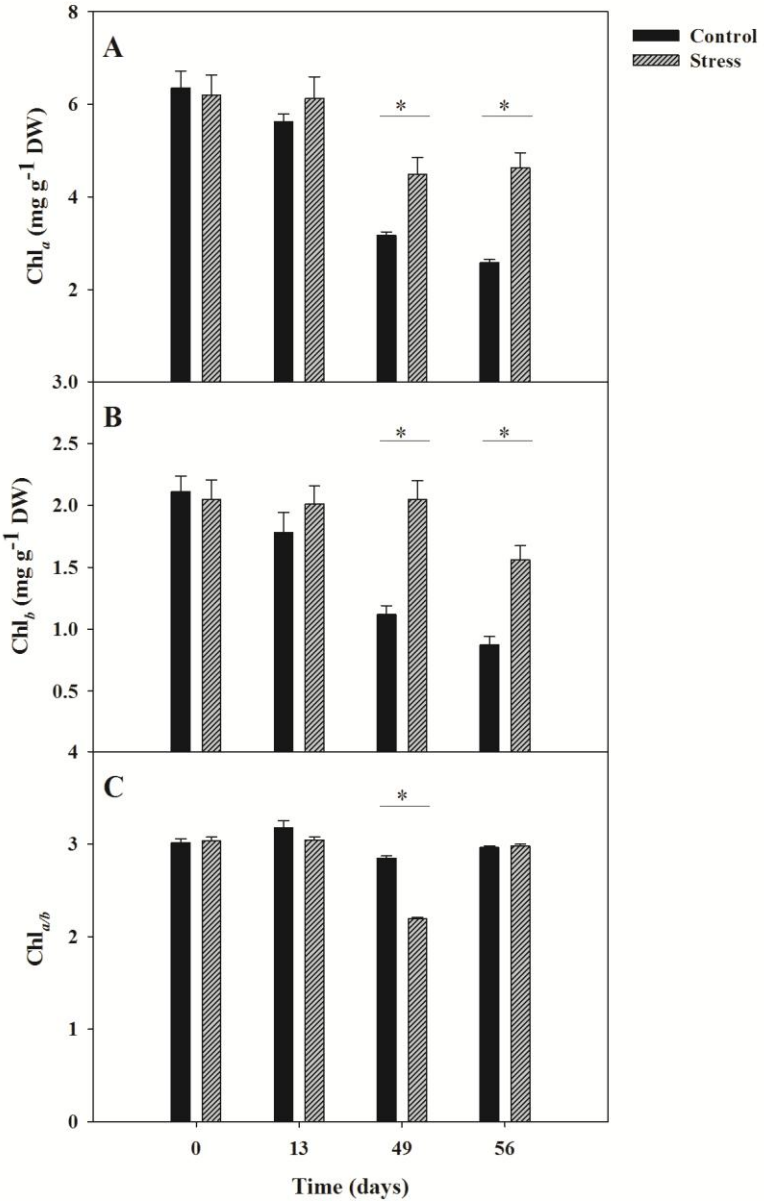

Figure 8. Effect of drought stress and re-watering on chlorophyll (Chl) content assessed spectrophotometrically (**A**) Chl$_a$ content, (**B**) Chl$_b$ content and (**C**) Chl$_{a/b}$ ratio measured for leaves of *Jatropha curcas* plants (36-days-old at the start of the experiment) continuously grown under well-watered conditions (Control) or subjected to drought stress (Stress) for 49 days followed by a 7-day re-watering period. Values are means. Bars represent ± SE. Samples were collected from 12 plants, except for day 13 in which only six plants were used. The symbol * in each time point indicates significant differences between stress and control according to t-test (p<0.05)

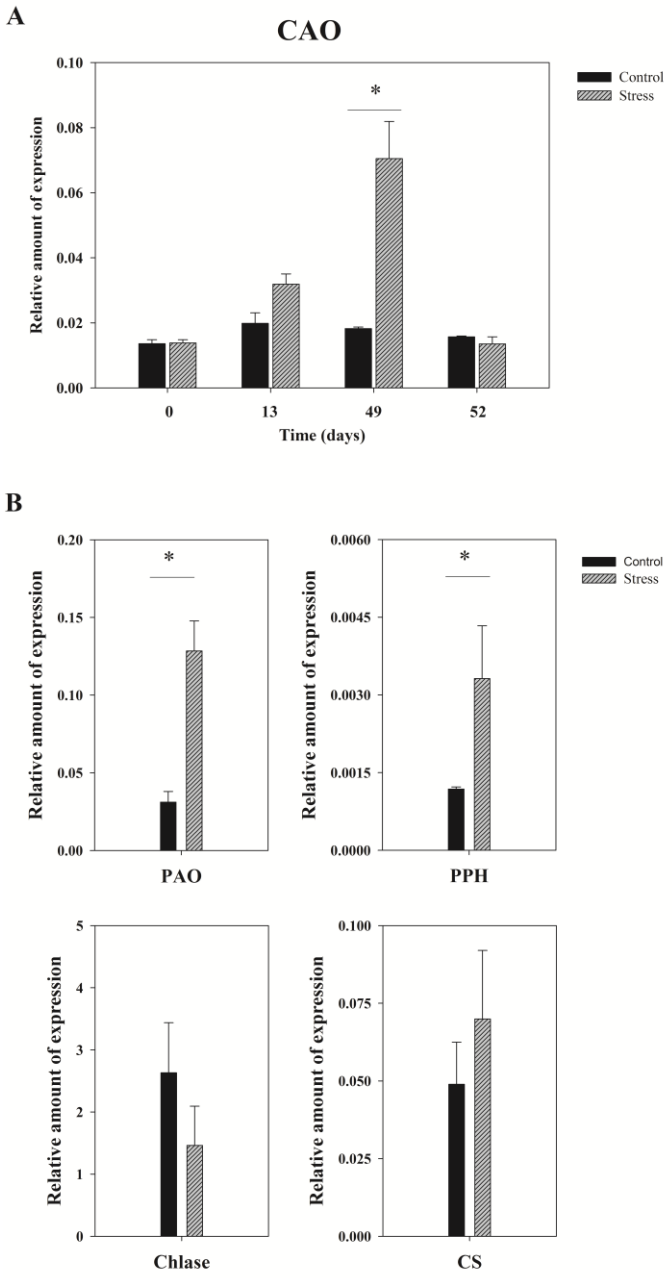

Figure 9. RT-qPCR expression analysis of *Chlorophyll a oxygenase* (*CAO*, JcCA0070011.10); *Pheophorbide a oxygenase* (PAO, JcCA0287191.10); *Pheophytinase* (*PPH*, JcCA0287191.10); *Chlorophyllase-1* (*Chlase*, JcCD0049300.10) and *Chlorophyll synthase* (*CS*, JcCB0002271.10) in *Jatropha curcas* leaves. 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 days after re-watering) or for 49 days of drought stress (B). All values are relative to the expression of the selected housekeeping gene (JcCA0131111.10). Error bars represent SE (n=2).

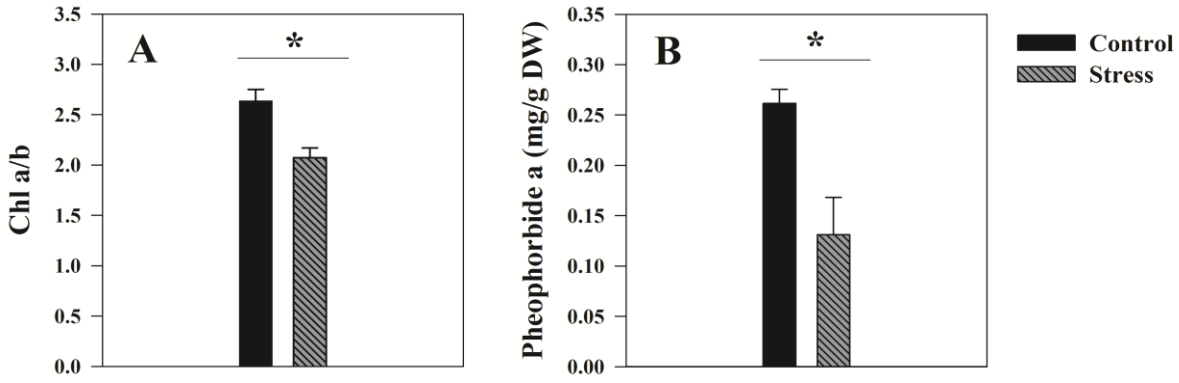

Figure 10. Effect of drought stress in chlorophyll (Chl) and pheophorbide *a* contents assessed by HPLC. (**A**) $Chl_{a/b}$ ratio and (**B**) pheophorbide *a* content measured for leaves of *Jatropha curcas* plants (36-days-old at the start of the experiment) continuously grown for 49 days under well-watered control conditions (Control) or subjected to drought stress (Stress) for 49 days. Values are means. Bars represent ± SE. Samples were collected from 5 plants, except for pheophorbide *a* which was only detected in 4 out of the 5 plant samples used. The symbol * indicates significant differences between stress and control according to t-test ($p<0.05$).

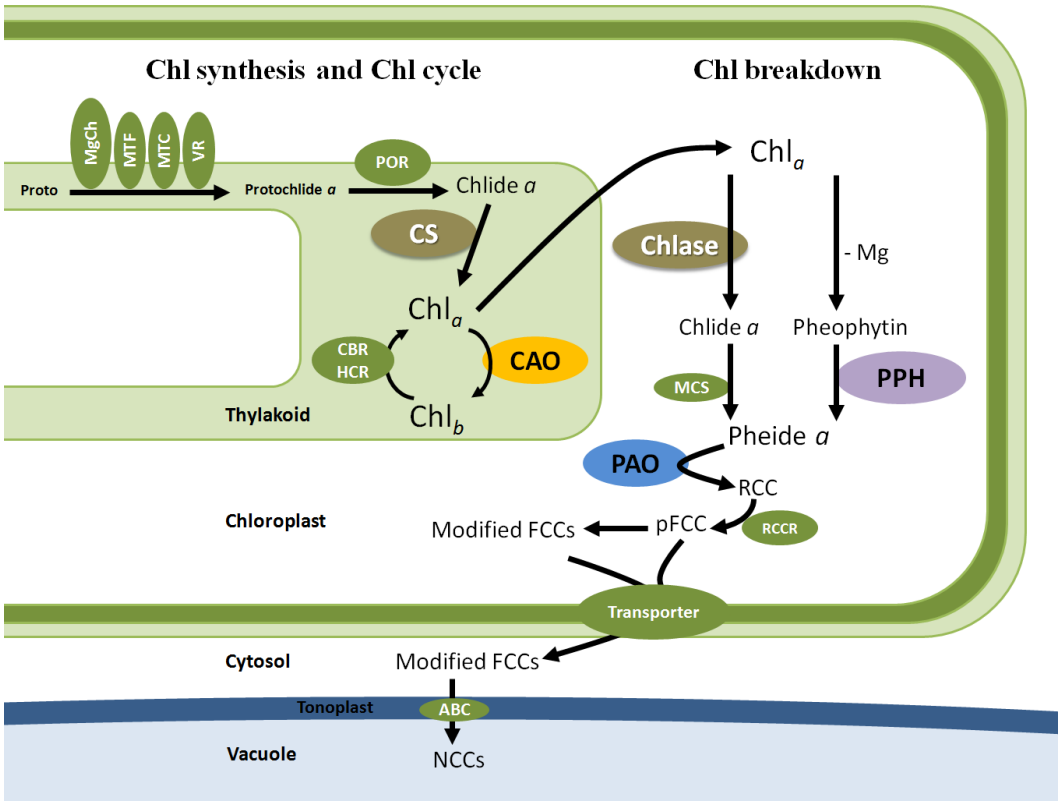

Figure 11. Schematic representation of the chlorophyll synthesis, cycle and breakdown metabolism. The chlorophyll (Chl) synthesis pathway involves several enzymes, in the thylakoids Proto is converted into ProtoChlide by the enzymes MgCh; MTF; MTC and VR. Afterwards, POR catalysis the reduction of one of the double bonds in ring D, using NADPH. The final step in the Chl biosynthetic pathway is the attachment of the phytol tail, which is catalyzed by CS. In the Chl cycle, Chl_b is synthesized from Chl_a and after it is reconverted to Chl_a. This interconversion between Chl_a and Chl_b is maintained by CAO an enzyme that converts Chl_a in to Chl_b, on the other hand the enzymes CBR and HCR convert Chl_b back in to Chl_a. During Chl breakdown, Chl_a is converted trough a multistep pathway to colorless breakdown products that are stored in the vacuole. Chl_a can be converted into Pheide *a* via the enzyme Chlase that converts Chl_a through hydrolysis into Chlide *a*, followed by MSC that converts Chlide *a* into Pheide *a*, or Mg-free Chl_a (pheophytin) can be catalyzed by PPH forming Pheide *a*. After Pheide *a* is formed, the poryphin ring is cleaved by PAO forming RCC causing the plant to lose its green color. RCC is then broken down into pFCC by RCCR which is translocated to the cytoplasm originating NCCs that are stored in the vacule. Not all enzymes and metabolites from the entire pathway are represented. Abbreviations of enzymes and metabolites in the chlorophyll synthesis: Chlorophyllide (Chlide); Chlorophyll (Chl); protoporphyrin IX (Proto); Mg-chelatase (MgCh); Mg-protoporrphytin IX Methyltransferase (MTF); Mg-protoporrphytin IX monomethylester cyclase (MTC); 8- vinyl reductase (VR), NADPH-ProtoChlide oxireductase (POR); Chl synthase (CS); chlorophyll cycle: Chl *a* oxygenase (CAO), Chl *b* reductase (CBR), hydroxychlorophyll *a* reductase (HCR), and on the Chl breakdown: Chlorophyllase (Chlase); Mg-dechelatase (MSC); Pheophytinase (PPH); Pheophorbide (Pheide); Pheide *a* oxygenase (PAO), Red Chl catabolite (RCC); Red Chl catabolite reductase (RCCR); Fluorescent Chl catabolite (FCC); Primary FCC (pFCC); Nonfluorescent Chl catabolite (NCC). Adapted from Eckhardt et al. (2004) and Schelbert et al. (2009).

SUPPLEMENTAL MATERIAL

List of supplementary tables (available in a Microsoft Excel file)

Table S1. Primer list used for the confirmation of gene expression (RT-qPCR validation). Primers were designed with the primer design software Primer 3 (Koressaar and Remm, 2007; Untergrasser et al., 2012).

Table S2. Effect of drought stress and re-watering in leaf relative water content (RWC). Plants from two *Jatropha curcas* accessions original from Indonesia (Ind) and Cape Verde islands (CVi) were continuously grown under well-watered conditions (C) or drought stress (S) for 49 days followed by 7 days of re-watering. Sampling points were collected at the beginning of the stress period (day 0), moderate drought (day 13), maximum stress (day 49) and during re-watering (day 50, 52 and 56). Values are means ± SE. Six leaf discs (Ø = 19mm) were collected per plant selecting the three youngest expanded leaves to isolate 2 discs per leaf. Six plants per treatment and accession were analyzed in days 0, 49 and 56 (grey background) while for days 13, 50 and 52, only 3 were used. RWC was calculated as previously described in Sapeta et al. (2013). Different letters within the same day differ significantly according to Tukey's test ($p<0.05$).

Table S3. Summary for Illumina HiSeqTM 2000 sequencing libraries (RNA-Seq).

Table S4. Pearson's correlation between the normalized transcript expression values (RPKM) registered for both *Jatropha curcas* accessions along the drought stress and recovery period, versus control. Samples included leaves and roots after 0, 13 or 49 days of drought stress (S) followed by 3 days of re-watering (day 52). Control plants (C) were maintained at field capacity. RNA of control samples collected at days 49 and 52 was pooled for sequencing, thus referred as day 49/52.

Table S5. Differentially expressed genes found in *Jatropha curcas* leaves and roots under drought stress and re-watering. Differentially expressed genes were found in leaves and roots of *J. curcas* plants subjected to 13 or 49 days of drought stress, followed by 3 days of re-watering (day 52). Differentially expressed genes were considered if the adjusted p-value was ≤ 0.001 and the fold change $\leq$-4 or $\geq$4 (stress versus respective control). Magenta: up regulation, Green: down-regulation, White: not differentially expressed. OS stands for organism species.

Table S6. Gene Ontology (GO) enrichment analysis of differentially expressed genes found in *Jatropha curcas* leaves under drought stress and re-watering. GO enrichment analysis was performed with the Blast2GO tool. Differentially expressed genes were found in leaves of *J. curcas* plants subjected to 13 or 49 days of drought stress, followed by 3 days of re-watering (day 52). Differentially expressed genes were considered if the adjusted p-value was ≤ 0.001 and the fold change $\leq$-4 or $\geq$4 (stress versus respective control). Enrichment was determined with Fishers exact's test against all coding sequences available at the Jatropha genome database version 3.0 (ftp://ftp.kazusa.or.jp/pub/jatropha/JAT_r3.0/). Sequences were

annotated based on blast results (BLASTx, nucleotide sequence of transcripts against NCBI's nr database) linked to functional information stored in the GO database and a motif/domain search using InterProScan. GO terms were considered to be enriched if the FDR (false discovery rate) ≤ 0.05. Analyses were performed for all collection time points, up and down regulated genes were analyzed independently. Categories letters stand for C - cellular component, P - biological process or F - molecular function.

Table S7. Gene Ontology (GO) enrichment analysis of differentially expressed genes found in *Jatropha curcas* roots under drought stress and re-watering. GO enrichment analysis was performed with the Blast2GO tool. Differentially expressed genes were found in roots of *J. curcas* plants subjected to 13 or 49 days of drought stress, followed by 3 days of re-watering (day 52). Differentially expressed genes were considered if the adjusted p-value was ≤ 0.001 and the fold change ≤-4 or ≥4 (stress versus respective control). Enrichment was determined with Fishers exact's test against all coding sequences available at the Jatropha genome database version 3.0 (ftp://ftp.kazusa.or.jp/pub/jatropha/JAT_r3.0/). Sequences were annotated based on blast results (BLASTx, nucleotide sequence of transcripts against NCBI's nr database) linked to functional information stored in the GO database and a motif/domain search using InterProScan. GO terms were considered to be enriched if the FDR (false discovery rate) ≤ 0.05. Analyses were performed for all collection time points, up and down regulated genes were analyzed independently. Categories letters stand for C - cellular component, P - biological process or F - molecular function.

Table S8. Z-score normalized expression for the seven most enriched chlorophyll associated genes. Z-score was calculated using RPKM normalized Log_2-transformed transcription count for expression data of leaves from *Jatropha curcas* plants from Indonesia (Ind) and Cape Verde islands (CVi) subjected to 0, 13 or 49 days of drought stress, followed by 3 days of re-watering (day 52), or continuously grown under control conditions. RNA of control samples collected at days 49 and 52 was pooled for sequencing, thus referred as day 49/52.

List of supplementary figures (available in a pdf file)

Figure S1. Schematic representation of the experimental design. After germination homogeneous seedlings were grown in the green house under well watered conditions. *Jatropha curcas* plants (36-days-old) were randomly divided into two groups, control and drought stress (groups contained equal amount of plants from both accessions). Control plants were continuously grown under well-watered conditions while, drought stress plants were subjected to 49 days of water withhold followed by 7 days of re-watering. Sampling points were collected at the beginning of the stress period (day 0), moderate drought (day 13), maximum stress (day 49) and during re-watering (day 50, 52 and 56). The amount of plants per treatment used in each collection time can be found in the figure. Arrows indicate harvest days, in such days both control and drought stress plants would be destroyed to allow root and aerial part collection to be used for RNA extractions, chlorophyll content (Chl), relative water content (RWC), biomass and total leaf area (TLA) determinations. Plants used for leaf gas exchange and morphological measurements (stem diameter, length and number of apical leaves) were kept throughout the experiment and used for destructive measurements on day 56 (last day of the experiment).

Figure S2. Biomass and total leaf area of *Jatropha curcas* plants submitted to drought stress. *Jatropha curcas* plants (36-days-old) were submitted to 49 days water withhold and 7 days re-watering (Stress) or continuously grown under well-watered conditions (Control). Analyses were performed for biomass (A) and total leaf area (TLA) (B) in stress versus control conditions. Sampling points were taken at the beginning of the stress period (day 0), moderate drought (day 13); maximum stress (day 49) and during re-watering (days 50, 52 and 56). Biomass and total TLA were determined as described in Sapeta et al. (2013). Values are means. Bars represent ± SE. Samples were collected from 12 plants, except for day 13 in which only six plants were used.

Figure S3. Morphological appearance of *Jatropha curcas* plants under drought stress. Morphological appearance of 36-days-old *Jatropha curcas* plants (A, day 0) subjected to 35 (B) or 49 (C) days of drought stress by water withhold followed by one day of re-watering (D). Scale bar stands for 20cm.

Figure S4. Effect of drought stress and re-watering on maximum quantum yield of PSII (Fv/Fm). Fv/Fm was determined for *Jatropha curcas* plants (36-days-old at the start of the experiment) continuously grown under well-watered conditions (Control) or subjected to drought stress (Stress) for 49 days followed by a 7-day re-watering period. Fv/Fm was measured in dark adapted leaves at predawn (4-5 a.m.). Measurements were performed for the youngest fully expanded leaf. Values are means. Bars represent ± SE (n=18).

Figure S5. Validation of RNA-Seq expression values using RT-qPCR. Correlation of RNA-Seq (y axis) and RT-qPCR (x axis) expression (fold change) of chlorophyll associated genes showing up, down or no differentially regulation in *Jatropha curcas* subjected to drought stress versus respective control.

Figure S6. RNA-Seq expression values for chlorophyll associated genes. Normalized expression values (RPKM) for *Chlorophyll a oxygenase* (*CAO*, JcCA0070011.10), *Pheophorbide a oxygenase* (*PAO*, JcCA0287191.10); *Pheophytinase* (*PPH*, JcCA0287191.10); *Chlorophyllase-1* (*Chlase*, JcCD0049300.10) and *Chlorophyll synthase* (*CS*, JcCB0002271.10) in leaves of *Jatropha curcas* plants subjected to 0, 13 or 49 days of drought stress followed by 3 days of re-watering (day 52).

Literature Cited

Achten WMJ, Maes WH, Reubens B, Mathijs E, Singh VP, Verchot L, Muys B (2010) Biomass production and allocation in *Jatropha curcas* L. seedlings under different levels of drought stress. Biomass & Bioenergy **34:** 667-676

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. Critical Reviews in Plant Sciences **24:** 23-58

Battke F, Symons S, Nieselt K (2010) Mayday - integrative analytics for expression data. BMC Bioinformatics **11:** 121

Bell E, Mullet JE (1993) Characterization of an Arabidopsis lipoxygenase gene responsive to methyl jasmonate and wounding. Plant Physiology **103:** 1133-1137

Bhardwaj J, Chauhan R, Swarnkar MK, Chahota RK, Singh AK, Shankar R, Yadav SK (2013) Comprehensive transcriptomic study on horse gram (*Macrotyloma uniflorum*): *De novo* assembly, functional characterization and comparative analysis in relation to drought stress. BMC Genomics **14:** 647

Biswal AK, Pattanayak GK, Pandey SS, Leelavathi S, Reddy VS, Govindjee, Tripathy BC (2012) Light intensity-dependent modulation of chlorophyll *b* biosynthesis and photosynthesis by overexpression of chlorophyllide *a* oxygenase in Tobacco. Plant Physiology **159:** 433-449

Bjorkman O, Demmig B (1987) Photon yield of O2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. Planta **170:** 489-504

Chaves MM, Costa JM, Saibo NJM (2011) Recent advances in photosynthesis under drought and salinity. In I Turkan, ed, Plant responses to drought and salinity stress: developments in a post-genomic era, Ed 1 Vol 57, Academic Press Ltd - Elsevier science Ldt, Lodon, pp 14-19

Chen JH, Song YP, Zhang H, Zhang DQ (2013) Genome-wide analysis of gene expression in response to drought stress in *Populus simonii*. Plant Molecular Biology Reporter **31:** 946-962

Cohen D, Bogeat-Triboulot MB, Tisserant E, Balzergue S, Martin-Magniette ML, Lelandais G, Ningre N, Renou JP, Tamby JP, Le Thiec D, Hummel I (2010) Comparative transcriptomics of drought responses in Populus: a meta-analysis of genome-wide expression profiling in mature leaves and root apices across two genotypes. BMC Genomics **11:** 630

Conesa A, Gotz S (2008) Blast2GO: A comprehensive suite for functional analysis in plant genomics. Int J Plant Genomics **2008:** 619832

Costa GGL, Cardoso KC, Del Bem LEV, Lima AC, Cunha MAS, de Campos-Leite L, Vicentini R, Papes F, Moreira RC, Yunes JA, Campos FAP, Da Silva MJ (2010) Transcriptome analysis of the oil-rich seed of the bioenergy crop *Jatropha curcas* L. BMC Genomics **11:** 462

Dai AG (2013) Increasing drought under global warming in observations and models. Nature Climate Change **3:** 52-58

Deyholos MK (2010) Making the most of drought and salinity transcriptomics. Plant Cell and Environment **33:** 648-654

Diaz-Lopez L, Gimeno V, Simon I, Martinez V, Rodriguez-Ortega WM, Garcia-Sanchez F (2012) *Jatropha curcas* seedlings show a water conservation strategy under drought conditions based on decreasing leaf growth and stomatal conductance. Agricultural Water Management **105:** 48-56

Divakara BN, Upadhyaya HD, Wani SP, Gowda CLL (2010) Biology and genetic improvement of *Jatropha curcas* L.: a review. Applied Energy **87:** 732-742

Eckhardt U, Grimm B, Hortensteiner S (2004) Recent advances in chlorophyll biosynthesis and breakdown in higher plants. Plant Molecular Biology **56:** 1-14

Espineda CE, Linford AS, Devine D, Brusslan JA (1999) The *AtCAO* gene, encoding chlorophyll *a* oxygenase, is required for chlorophyll *b* synthesis in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences of the United States of America **96:** 10507-10511

Eswaran N, Parameswaran S, Anantharaman B, Kumar GRK, Sathram B, Johnson TS (2012) Generation of an expressed sequence tag (EST) library from salt-stressed roots of *Jatropha curcas* for identification of abiotic stress-responsive genes. Plant Biology **14:** 428-437

Eswaran N, Parameswaran S, Sathram B, Anantharaman B, Kumar GRK, Tangirala SJ (2010) Yeast functional screen to identify genetic determinants capable of conferring abiotic stress tolerance in *Jatropha curcas*. BMC Biotechnology **10:** 23

Fairless D (2007) Biofuel: The little shrub that could - maybe. Nature **449:** 652-655

Fan XD, Wang JQ, Yang N, Dong YY, Liu L, Wang FW, Wang N, Chen H, Liu WC, Sun YP, Wu JY, Li HY (2013) Gene expression profiling of soybean leaves and roots under salt, saline-alkali and drought stress by high-throughput Illumina sequencing. Gene **512:** 392-402

Fini A, Bellasio C, Pollastri S, Tattini M, Ferrini F (2013) Water relations, growth, and leaf gas exchange as affected by water stress in *Jatropha curcas*. Journal of Arid Environments **89:** 21-29

Gehrig HH, Winter K, Cushman J, Borland A, Taybi T (2000) An improved RNA isolation method for succulent plant species rich in polyphenols and polysaccharides. Plant Molecular Biology Reporter **18:** 369-376

Genty B, Briantais J-M, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta - General Subjects **990:** 87-92

Grebner W, Stingl NE, Oenel A, Mueller MJ, Berger S (2013) Lipoxygenase6-dependent oxylipin synthesis in roots is required for abiotic and biotic stress resistance of arabidopsis. Plant Physiology **161:** 2159-2170

Gross SM, Martin JA, Simpson J, Abraham-Juarez MJ, Wang Z, Visel A (2013) *De novo* transcriptome assembly of drought tolerant CAM plants, *Agave deserti* and *Agave tequilana*. BMC Genomics **14:** 563

Grumaz C, Lorenz S, Stevens P, Lindemann E, Schock U, Retey J, Rupp S, Sohn K (2013) Species and condition specific adaptation of the transcriptional landscapes in *Candida albicans* and *Candida dubliniensis*. BMC Genomics **14:** 212

Harb A, Krishnan A, Ambavaram MMR, Pereira A (2010) Molecular and physiological analysis of drought stress in arabidopsis reveals early responses leading to acclimation in plant growth. Plant Physiology **154:** 1254-1271

Hirashima M, Tanaka R, Tanaka A (2009) Light-independent cell death induced by accumulation of pheophorbide *a* in *Arabidopsis thaliana*. Plant and Cell Physiology **50:** 719-729

Hortensteiner S, Vicentini F, Matile P (1995) Chlorophyll breakdown in senescent cotyledons of rape, *Brassica-Napus* L.: enzymatic cleavage of pheophorbide *a in vitro*. New Phytologist **129:** 237-246

Hwang DF, Tsai YS, Chou SS, Liu SM, Wu JT, Lin SJ, Tu WC (2005) HPLC determination of pheophorbide *a* and pyropheophorbide *a* in dried laver product implicated in food poisoning. Shokuhin Eiseigaku Zasshi **46:** 45-48

Jang HY, Yang SW, Carlson JE, Ku YG, Ahn SJ (2013) Two aquaporins of Jatropha are regulated differentially during drought stress and subsequent recovery. Journal of Plant Physiology **170:** 1028-1038

King AJ, Li Y, Graham IA (2011) Profiling the developing *Jatropha curcas* L. seed transcriptome by pyrosequencing. Bioenergy Research **4:** 211-221

Kumar RS, Parthiban KT, Govinda Rao M (2009) Molecular characterization of Jatropha genetic resources through inter-simple sequence repeat (ISSR) markers. Molecular Biology Reports **36:** 1951-1956

Kumar RV, Dar SH, Yadav VP, Tripathi YK, Ahlawat SP (2008) Genetic variability in *Jatropha curcas* accessions. Range Management and Agroforestry **29:** 10-12

Lenka SK, Katiyar A, Chinnusamy V, Bansal KC (2011) Comparative analysis of drought-responsive transcriptome in Indica rice genotypes with contrasting drought tolerance. Plant Biotechnology Journal **9:** 315-327

Lichtenthaler HK (1987) Chlorophylls and carotenoids - pigments of photosynthetic biomembranes. Methods in Enzymology **148:** 350-382

Liu B, Yao L, Wang WG, Gao JH, Chen F, Wang SH, Xu Y, Tang L, Jia YJ (2010) Molecular cloning and characterization of phospholipase D from *Jatropha curcas*. Molecular Biology Reports **37:** 939-946

Maes WH, Achten WMJ, Reubens B, Muys B (2011) Monitoring stomatal conductance of *Jatropha curcas* seedlings under different levels of water shortage with infrared thermography. Agricultural and Forest Meteorology **151:** 554-564

Maes WH, Achten WMJ, Reubens B, Raes D, Samson R, Muys B (2009) Plant-water relationships and growth strategies of *Jatropha curcas* L. seedlings under different levels of drought stress. Journal of Arid Environments **73:** 877-884

Matsui A, Ishida J, Morosawa T, Mochizuki Y, Kaminuma E, Endo TA, Okamoto M, Nambara E, Nakajima M, Kawashima M, Satou M, Kim JM, Kobayashi N, Toyoda T, Shinozaki K, Seki M (2008) Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. Plant and Cell Physiology **49:** 1135-1149

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence - a practical guide. Journal of Experimental Botany **51:** 659-668

Minh-Thu PT, Hwang DJ, Jeon JS, Nahm BH, Kim YK (2013) Transcriptome analysis of leaf and root of rice seedling to acute dehydration. Rice **6**

Montillet JL, Leonhardt N, Mondy S, Tranchimand S, Rumeau D, Boudsocq M, Garcia AV, Douki T, Bigeard J, Lauriere C, Chevalier A, Castresana C, Hirt H (2013) An abscisic acid-independent oxylipin pathway controls stomatal closure and immune defense in Arabidopsis. Plos Biology **11:** e1001513

Mortazavi A, Williams BA, Mccue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nature Methods **5:** 621-628

Moumeni A, Satoh K, Kondoh H, Asano T, Hosaka A, Venuprasad R, Serraj R, Kumar A, Leung H, Kikuchi S (2011) Comparative analysis of root transcriptome profiles of two pairs of drought-tolerant and susceptible rice near-isogenic lines under different drought stress. BMC Plant Biology **11:** 174

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annual Review of Plant Biology **59:** 651-681

Natarajan P, Kanagasabapathy D, Gunadayalan G, Panchalingam J, Shree N, Sugantham PA, Singh KK, Madasamy P (2010) Gene discovery from *Jatropha curcas* by sequencing of ESTs from normalized and full-length enriched cDNA library from developing seeds. BMC Genomics **11:** 606

Natarajan P, Parani M (2011) *De novo* assembly and transcriptome analysis of five major tissues of *Jatropha curcas* L. using GS FLX titanium platform of 454 pyrosequencing. BMC Genomics **12:** 191

Oliveros JC (2007) VENNY. An interactive tool for comparing lists with Venn Diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html.

Oster U, Tanaka R, Tanaka A, Rudiger W (2000) Cloning and functional expression of the gene encoding the key enzyme for chlorophyll *b* biosynthesis (CAO) from *Arabidopsis thaliana*. Plant Journal **21:** 305-310

Pamidimarri DVNS, Chattopadhyay B, Reddy MP (2009) Genetic divergence and phylogenetic analysis of genus Jatropha based on nuclear ribosomal DNA ITS sequence. Mol Biol Rep **36:** 1929-1935

Pamidimarri DVNS, Mastan SG, Patel J, Reddy MP (2009) Molecular characterization and identification of markers for toxic and non-toxic varieties of *Jatropha curcas* L. using RAPD, AFLP and SSR markers. Mol Biol Rep **36:** 1357-1364

Pattanayak GK, Biswal AK, Reddy VS, Tripathy BC (2005) Light-dependent regulation of chlorophyll *b* biosynthesis in chlorophyllide *a* oxygenase overexpressing tobacco plants. Biochemical and Biophysical Research Communications **326:** 466-471

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research **29:** e45

Pompelli MF, Barata-Luis R, Vitorino HS, Goncalves ER, Rolim EV, Santos MG, Almeida-Cortez JS, Ferreira VM, Lemos EE, Endres L (2010) Photosynthesis, photoprotection and antioxidant activity of purging nut under drought deficit and recovery. Biomass & Bioenergy **34:** 1207-1215

Popluechai S, Breviario D, Mulpuri S, Makkar HPS, Raorane M, Reddy AR, Palchetti E, Gatehouse AMR, Syers JK, O'Donnell AG, Kohli A. (2009) Narrow genetic and apparent phenetic diversity in *Jatropha curcas*: initial success with generating low phorbol ester interspecific hybrids. Nature Precedings: hdl:10101/npre.2009.2782.1.

Pruzinska A, Tanner G, Anders I, Roca M, Hortensteiner S (2003) Chlorophyll breakdown: Pheophorbide *a* oxygenase is a Rieske-type iron-sulfur protein, encoded by the accelerated cell death 1 gene. Proceedings of the National Academy of Sciences of the United States of America **100:** 15259-15264

R Development Core Team (2009) R: A Language and Environment for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, http://www.R-project.org

Recchia GH, Caldas DGG, Beraldo ALA, da Silva MJ, Tsai SM (2013) Transcriptional analysis of drought-induced genes in the roots of a tolerant genotype of the common bean (*Phaseolus vulgaris* L.). International Journal of Molecular Sciences **14:** 7155-7179

Rodriguez MCS, Edsgard D, Hussain SS, Alquezar D, Rasmussen M, Gilbert T, Nielsen BH, Bartels D, Mundy J (2010) Transcriptomes of the desiccation-tolerant resurrection plant *Craterostigma plantagineum*. Plant Journal **63:** 212-228

Sakuraba Y, Balazadeh S, Tanaka R, Mueller-Roeber B, Tanaka A (2012) Overproduction of Chl *b* retards senescence through transcriptional reprogramming in Arabidopsis. Plant and Cell Physiology **53:** 505-517

Sapeta H, Costa JM, Lourenço T, Maroco J, van der Linde P, Oliveira MM (2013) Drought stress response in *Jatropha curcas*: Growth and physiology. Environmental and Experimental Botany **85:** 76-84

Sato S, Hirakawa H, Isobe S, Fukai E, Watanabe A, Kato M, Kawashima K, Minami C, Muraki A, Nakazaki N, Takahashi C, Nakayama S, Kishida Y, Kohara M, Yamada M, Tsuruoka H, Sasamoto S, Tabata S, Aizu T, Toyoda A, Shin-i T, Minakuchi Y, Kohara Y, Fujiyama A, Tsuchimoto S, Kajiyama S, Makigano E, Ohmido N, Shibagaki N, Cartagena JA, Wada N, Kohinata T, Atefeh A, Yuasa S, Matsunaga S, Fukui K (2011) Sequence Analysis of the Genome of an Oil-Bearing Tree, *Jatropha curcas* L. DNA Research **18:** 65-76

Savchenko T, Kolla VA, Wang CQ, Nasafi Z, Hicks DR, Phadungchob B, Chehab WE, Brandizzi F, Froehlich J, Dehesh K (2014) Functional convergence of oxylipin and abscisic acid pathways controls stomatal closure in response to drought. Plant Physiolgy **164:** 1151-1160

Schelbert S, Aubry S, Burla B, Agne B, Kessler F, Krupinska K, Hortensteiner S (2009) Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in Arabidopsis. Plant Cell **21:** 767-785

Seltmann MA, Stingl NE, Lautenschlaeger JK, Krischke M, Mueller MJ, Berger S (2010) Differential impact of lipoxygenase 2 and jasmonates on natural and stress-induced senescence in Arabidopsis. Plant Physiology **152:** 1940-1950

Severin AJ, Woody JL, Bolon YT, Joseph B, Diers BW, Farmer AD, Muehlbauer GJ, Nelson RT, Grant D, Specht JE, Graham MA, Cannon SB, May GD, Vance CP, Shoemaker RC (2010) RNA-Seq atlas of *Glycine max*: a guide to the soybean transcriptome. BMC Plant Biology **10:** 160

Silva EN, Ferreira-Silva SL, Fontenele AD, Ribeiro RV, Viegas RA, Silveira JAG (2010a) Photosynthetic changes and protective mechanisms against oxidative damage subjected to isolated and combined drought and heat stresses in *Jatropha curcas* plants. Journal of Plant Physiology **167:** 1157-1164

Silva EN, Ferreira-Silva SL, Viegas RA, Silveira JAG (2010b) The role of organic and inorganic solutes in the osmotic adjustment of drought-stressed *Jatropha curcas* plants. Environmental and Experimental Botany **69:** 279-285

Silva EN, Ribeiro RV, Ferreira-Silva SL, Viegas RA, Silveira JAG (2010c) Comparative effects of salinity and water stress on photosynthesis, water relations and growth of *Jatropha curcas* plants. Journal of Arid Environments **74:** 1130-1137

Storey JD, Tibshirani R (2003) Statistical significance for genomewide studies. Proceedings of the National Academy of Sciences of the United States of America **100:** 9440-9445

Suhita D, Raghavendra AS, Kwak JM, Vavasseur A (2004) Cytoplasmic alkalization precedes reactive oxygen species production during methyl jasmonate- and abscisic acid-induced stomatal closure. Plant Physiology **134:** 1536-1545

Tanaka A, Ito H, Tanaka R, Tanaka NK, Yoshida K, Okada K (1998) Chlorophyll *a* oxygenase (CAO) is involved in chlorophyll *b* formation from chlorophyll *a*. Proceedings of the National Academy of Sciences of the United States of America **95:** 12719-12723

Tanaka R, Tanaka A (2005) Effects of chlorophyllide *a* oxygenase overexpression on light acclimation in *Arabidopsis thaliana*. Photosynthesis Research **85:** 327-340

Tang MJ, Sun JW, Liu Y, Chen F, Shen SH (2007) Isolation and functional characterization of the *JcERF* gene, a putative AP2/EREBP domain-containing transcription factor, in the woody oil plant *Jatropha curcas*. Plant Molecular Biology **63:** 419-428

Thomas H, Ougham H, Hortensteiner S (2001) Recent advances in the cell biology of chlorophyll catabolism. Advances in Botanical Research, **35:** 1-52

Thumma BR, Sharma N, Southerton SG (2012) Transcriptome sequencing of *Eucalyptus camaldulensis* seedlings subjected to water stress reveals functional single nucleotide polymorphisms and genes under selection. BMC Genomics **13:** 364

Vernoud V, Horton A, Yang Z, Nielsen E (2003) Analysis of the small GTPase gene superfamily of Arabidopsis. Plant Physiology **131:** 1191–1208

Villar E, Klopp C, Noirot C, Novaes E, Kirst M, Plomion C, Gion JM (2011) RNA-Seq reveals genotype-specific molecular responses to water deficit in eucalyptus. BMC Genomics **12**: 538

Wang LK, Feng ZX, Wang X, Wang XW, Zhang XG (2010) DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics **26:** 136-138

Wang XS, Liu Y, Jia YY, Gu HY, Ma HY, Yu T, Zhang H, Chen QJ, Ma L, Gu AX, Zhang JS, Shi SB, Ma H (2012) Transcriptional responses to drought stress in root and leaf of chickpea seedling. Molecular Biology Reports **39:** 8147-8158

Wang Y, Huang J, Gou C, Dai X, Chen F, Wei W (2011) Cloning and characterization of a differentially expressed cDNA encoding myo-inositol-1-phosphate synthase involved in response to abiotic stress in *Jatropha curcas*. Plant Cell, Tissue and Organ Culture **106:** 269-277

Wasternack C (2007) Jasmonates: An update on biosynthesis, signal transduction and action in plant stress response, growth and development. Annals of Botany **100:** 681-697

Zhang FL, Niu B, Wang YC, Chen F, Wang SH, Xu Y, Jiang LD, Gao S, Wu J, Tang L, Jia YR (2008) A novel betaine aldehyde dehydrogenase gene from *Jatropha curcas*, encoding an enzyme implicated in adaptation to environmental stress. Plant Science **174:** 510-518

Zhang LD, Yu SW, Zuo KJ, Luo LJ, Tang KX (2012) Identification of gene modules associated with drought response in rice by network-based analysis. Plos One **7:** e33748

Zhang Y, Wang YX, Jiang LD, Xu Y, Wang YC, Lu DH, Chen F (2007) Aquaporin JcPIP2 is involved in drought responses in *Jatropha curcas*. Acta Biochimica et Biophysica Sinica **39:** 787-794