



## Final Report

Identification and genetic modification of ERF transcription factors for development of waterlogging-tolerant jatropha

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Identification and genetic modification of ERF transcription factors for  
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## Abstract

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**Project Code :** MRG5680032

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### Abstract:

Waterlogging usually creates low oxygen environment, which can adversely affect plant growth and production. This project concentrates on a family of ERF (Ethylene Responsive Factor) DNA binding transcription factors, which have been identified as key-regulators for low oxygen and submergence survival in *Arabidopsis* (*Arabidopsis thaliana*) and rice (*Oryza sativa*). Recent publications show that the stability of ERFs is regulated by N-end rule targeted proteolysis in an oxygen- dependent manner. The N-end rule regulation involves a conserved N-terminal amino acid sequence and mutation of this sequence resulted in improved submergence and hypoxia tolerance. Intriguingly, the genetic modification of ERF targets of oxygen-regulated N-end rule pathway turnover might be key factors to improve survival of crop plants under low oxygen condition.

The continuous depletion of petroleum resources stimulates a search for alternative energy. *Jatropha* (*Jatropha curcus*) is a drought-tolerant, non-food, oil-rich crop listed as a potential candidate for alternative fuel sources. However, *Jatropha* is known to awfully perform when grown on poorly-drained soil or frequently-flooded area. This project focuses on utilizing molecular analysis and transcriptome profiling approach to examine the mechanism underlying waterlogging response in *Jatropha*. The molecular objective is to identify waterlogging-regulated ERFs by gene expression analysis. These experiments will provide a foundation for genetic engineering of *Jatropha* to improve waterlogging tolerance.

**Keywords :** Waterlogging, Ethylene responsive factors (ERFs), *Jatropha curcas*

## Final report content:

### 1. Abstract

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The continuous depletion of petroleum resources stimulates a search for alternative energy. *Jatropha* (*Jatropha curcus*) is a drought-tolerant, non-food, oil-rich crop listed as a potential candidate for alternative fuel sources. However, *Jatropha* is known to awfully perform when grown on poorly-drained soil or frequently-flooded area. This project focuses on utilizing molecular analysis and transcriptome profiling approach to examine the mechanism underlying waterlogging response in *Jatropha*. The molecular objective is to identify waterlogging-regulated ERFs by gene expression analysis. These experiments will provide a foundation for genetic engineering of *Jatropha* to improve waterlogging tolerance.

### 2. Executive summary

Plant biodiesel is proposed to deliver a solution for alternative fuel sources. *Jatropha* is a drought tolerant, non-food, oil-rich crop that can be used as a potential material for biodiesel production. However, *Jatropha* is highly susceptible to waterlogging. In Thailand the monsoon season can last approximately 6 months from May to October. The heavy rains make some parts of the country suffered from waterlogging creating uncultivable area and drastically reducing the land productivity. As a result, it is impossible to grow *Jatropha* in flood-prone areas of Thailand.

Our goal is to evaluate functional roles of ethylene responsive transcription factors, candidates of low oxygen response regulator, in response to waterlogging and profile the transcrional changes in roots of *Jatropha* subjected to waterlogging treatment. This research will provide a foundation for genetic engineering of *Jatropha* to improve waterlogging tolerance. In the long run, this may improve *Jatropha* yields and productivity.

### 3. Objectives

- To identify waterlogging-responsive JcERFs by gene expression analysis
- To profile transcriptome adjustment of *Jatropha* roots upon waterlogging stress

## 4. Methodology

### Aim 1: Expression analysis of JcERFs in response to waterlogging.

Rationale: Our preliminary analysis discovered three putative group VII-ERFs within *Jatropha* genome and show that there is a conserved N-terminal motif among the group VII-JcERFs. In *Arabidopsis*, this motif is shown to be involved in oxygen dependent N-end rule regulation. In addition, overexpression of *Arabidopsis* group VII-ERFs (HRE1, HRE2, RAP2.2, and RAP2.12) enhances hypoxia and/or submergence tolerant in *Arabidopsis*. To begin assigning function to these ERFs, we will analyze their transcript accumulation in response to waterlogging.

Waterlogging assay was performed using one-month old *Jatropha* seedlings grown in pot soil. Seedlings will be subjected to soil waterlogging (3 cm above soil surface). Total RNAs were extracted from waterlogged and non-treated root and leaf tissues by use of TRIzol RNA extraction reagents. Total RNA samples were treated with RNase-free DNase to get rid of contaminated genomic DNA. cDNA (complementary DNA) libraries were synthesized. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and quantitative realtime PCR (qRT-PCR) performed by use of primers specific to JcERFs coding sequences (CDS). The primers were designed based on *Jatropha* genome information (<http://www.kazusa.or.jp/jatropha/>)

### Aim 2: Transcriptomic analysis of waterlogged *Jatropha*

Rationale: In recent years, studies in *Arabidopsis* (*Arabidopsis thaliana*), gray poplar (*Populus × canescens*), cotton (*Gossypium hirsutum*), and rice (*Oryza sativa*) have revealed that low oxygen can cause drastic changes in transcription, translation, and metabolite levels (Branco-Price et al., 2008; Kreuzwieser et al., 2009; Mustroph et al., 2009; Narsai et al., 2009; Christianson et al., 2010; Lee et al., 2011; Juntawong et al., 2014). While several published studies of model plants have provided some fundamental clues, quite a few studies were performed in hypoxic chambers, in which the entire plants were subjected to low oxygen conditions, grown on artificial media, or subjected to complete darkness. These conditions would not imitate the impact observed in plants exposed to soil waterlogging. Recently, high-throughput sequencing technology has been used as a powerful tool for genomic analysis in both model and non-model species. This method can provide quantitative descriptions of gene expression at the genome-scale level, accompanying high accuracy, low background noise, and large dynamic ranges. High-throughput transcriptome analysis could help provide a basic understanding of the molecular responses to waterlogging in *Jatropha*.

#### Aim 2.1 RNA extraction, library preparation, and sequencing

Total RNA was extracted with TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Total RNA samples were subjected to DNase treatment and RNA cleanup by a GF-1 RNA extraction kit (Vivantis). Poly(A)<sup>+</sup> mRNAs were isolated from the total RNAs by using an Absolutely mRNA purification kit (Agilent). *Jatropha* mRNA (200 ng) was used to construct a sequencing library, by using a Ion Total RNA seq kit (Life Technologies). For each treatment, two independent

biological replicates were sequenced on a Ion Proton sequencer (Life Technologies). FASTQ files were obtained with the base caller provided by the instrument and subjected to quality control filtering and 3' end trimming using the FASTX-toolkit ([http://hannonlab.cshl.edu/fastx\\_toolkit/index.html](http://hannonlab.cshl.edu/fastx_toolkit/index.html)) included in the Torrent Suite™ Software (Life Technologies). The preprocessed reads with a minimum of 35 nt and a quality value of 17, were deposited in the NCBI GEO database under the accession number GSE57428.

### **Aim 2.2 Transcriptome analysis**

FASTQ reads were mapped to the *J. curcas* genome release 4.5, downloaded from <http://www.kazusa.or.jp/jatropha/> (Sato et al., 2011; Hirakawa et al., 2012) with TMAP mapping program (<https://github.com/iontorrent/TMAP>), using the default settings and “mapall” command. A binary format of sequence alignment files (BAM) was generated and subsequently used to create read count tables using HTseq library (Anders et al., 2014) for differential gene expression analysis. Statistical analysis of DEGs was calculated in the R environment (R Development Core Team, 2005), by using a generalized linear model (GLM) approach from the edgeR package (Robinson et al., 2010). Genes with the count per million (CPM) values  $\geq 1$  in at least two library samples were included in this analysis. In brief, the calcNormFactors function was applied for data normalization using the trimmed mean of M-values (TMM) method. Subsequently, the GLMTagwiseDisp function was used to generate the estimated tagwise dispersion. Next, the glmFit function was applied to fit the negative binomial GLM for each tag. Finally, the glmLTR function was applied to carry out the likelihood ratio test. Significant DEGs were filtered, based on false discovery values (FDR < 0.05).

Assignment of the DEGs into functional bins and the creation of a mapping file from the *Jatropha* genome were performed using the Mercator pipeline for automated sequence annotation (Lohse et al., 2013). The inputs for the Mercator were *Jatropha* protein sequences supplied by <http://www.kazusa.or.jp/jatropha/>. This analysis was performed with a blast score cutoff > 80. For visualization, the MapMan (Thimm et al., 2004) and the PageMan (Usadel et al., 2006) programs were used.

Gene ontology (GO) enrichment analysis was performed in the R environment. Firstly, the GO terms for each gene were derived from homology searching of plant protein databases using GOANNA with an e-value cutoff < E-10 (McCarthy et al., 2006). Subsequently, a gene annotation file was generated. GO term enrichment analysis in up or down regulated DEGs was performed by the GOHyperGALL function (Horan et al., 2008). Significant GO terms were filtered based on the adjusted p-value < 0.05.

### **Aim 2.3 Analysis of publicly available microarray data**

Publicly available microarrays were downloaded from the Gene Expression Omnibus (GEO) database. Microarray data were normalized using the Robust Multi-chip Average (RMA) method. Differential expression analysis was performed on RMA-normalized data. False discovery

rates (FDR) for significant differences between genes in each comparison were generated using p-value distributions (Smyth, 2004). For each comparison, DEGs were selected based on criteria of  $FDR < 0.05$  and the absolute values of  $\log_2$  fold change ( $\log_2FC$ )  $\geq 1$ .

#### Aim 2.4 Ortholog identification

Orthologs among *Jatropha*, *Arabidopsis*, rice, and poplar were identified based on protein sequence homology using ORTHOMCL (OMCL) version 1.4 (Li et al., 2003). The inflate number used to perform this analysis was set to 1.2. Protein sequences used in this step were downloaded from *Jatropha*, *Arabidopsis*, rice, and poplar genome databases (ftp://ftp.kazusa.or.jp/pub/jatropha/JAT\_r4.5.aa.gz, ftp://ftp.arabidopsis.org/home/tair/Sequences/blast\_datasets/TAIR10\_blastsets/TAIR10\_pep\_20110103\_representative\_gene\_model\_updated, ftp://ftp.jgi-psf.org/pub/compngen/phytozome/v9.0/Osativa/annotation/Osativa\_204\_protein\_primaryTranscriptOnly.fa.gz, and ftp://ftp.jgi-psf.org/pub/compngen/phytozome/v9.0/Ptrichocarpa/annotation/Ptrichocarpa\_210\_protein\_primaryTranscriptOnly.fa.gz, respectively)

## 5. Results

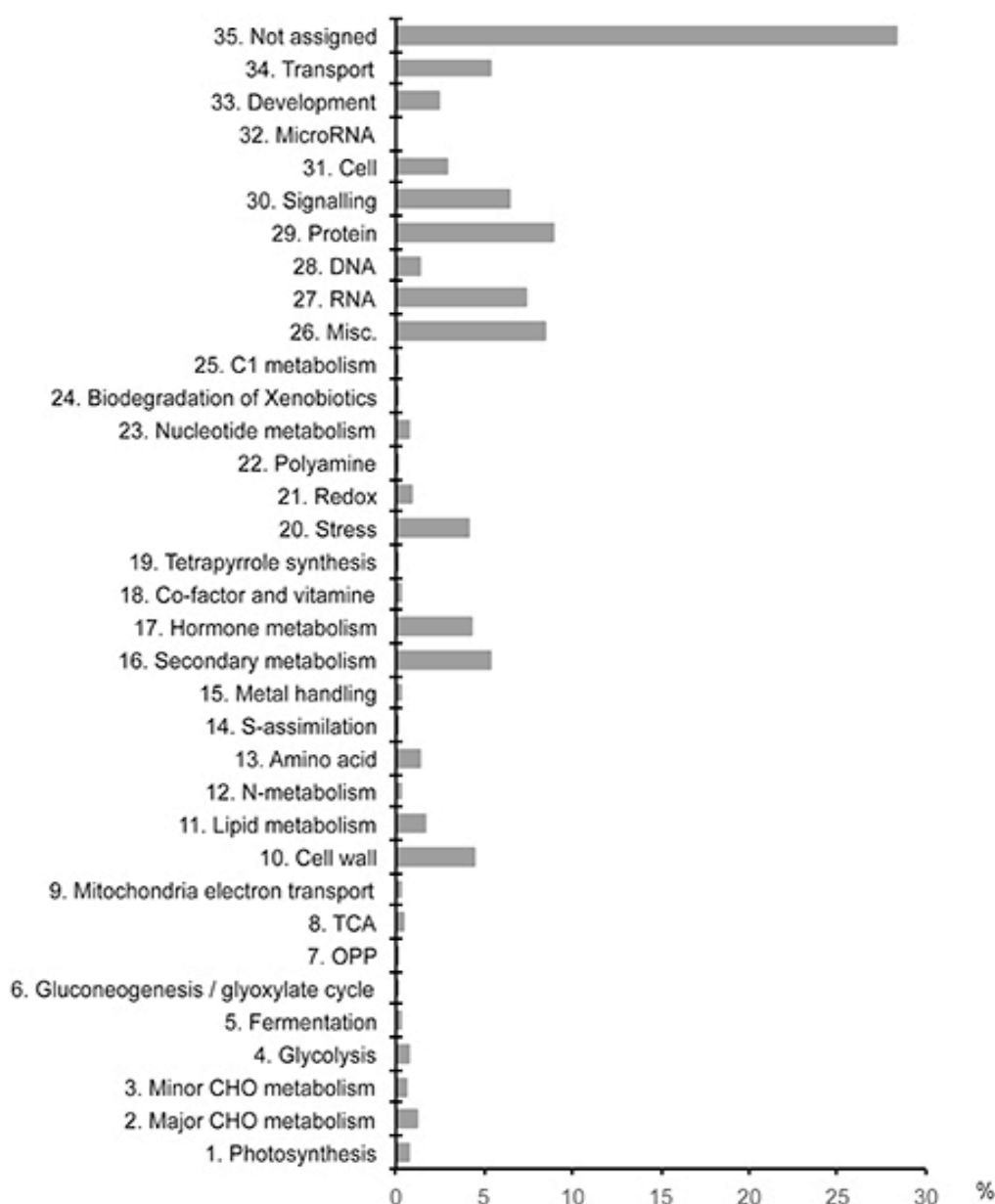
### 5.1 Waterlogging stress triggered transcriptome re-adjustment in *jatropha* roots

To compare the effects of waterlogging on the *Jatropha* roots, we quantitatively profiled transcriptome from waterlogging roots (WS) and non-stress root (NS) samples. Over 4 million reads were obtained for each of the two independent biological replicates. The majority of reads (93–96%) mapped to the *Jatropha* genome (Figure 1)

Sample	Total Read	Mapped read	Reads mapped to reference genome
Non-stress rep#1	13,037,445	12,552,917	96.28%
Non-stress rep#2	4,091,321	3,846,314	94.01%
Waterlogging rep#1	9,656,225	9,262,378	95.92%
Waterlogging rep#2	7,908,492	7,371,466	93.21%

**Figure 1.** Statistic and quality control of RNA-seq data.

Transcriptome analysis identified 1968 differentially expressed genes (DEGs) with significant changes in expression evaluated by the false discovery rate ( $FDR < 0.05$ ). Of these, 931 genes (47%) were up-regulated and 1037 genes (53%) were down-regulated. Assignment of the DEGs to functional bins, using a well-established Mercator pipeline, categorized the DEGs into 35 functional bins (Figure 2). The genes that related to cell wall, secondary metabolism, hormone metabolism, stress, RNA, protein, signaling, and transport were abundant in the DEG dataset.



**Figure 2.** Functional classification of waterlogging-responsive DEGs. DEGs (1968 genes) were assigned into 35 functional bins using the Mercator annotation pipeline.

To further evaluate the response to waterlogging, we considered the biological function of the DEGs that were co-regulated in response to waterlogging. GO enrichment analysis was performed with the up-regulated or down-regulated DEGs. Co-regulation of DEGs with similar functions was observed (Figure 3A). The up-regulated DEGs were involved in the response to stress (adjusted p-value:  $1.45\text{E-}08$ ), response to hypoxia (adjusted p-value:  $2.37\text{E-}03$ ), and response to ethylene (adjusted p-value:  $7.34\text{E-}03$ ). In addition, we also observed enrichment of genes encoding transcription factors (adjusted p-value:  $4.08\text{E-}02$ ) following WS. The down-regulated DEGs were enriched for cell wall organization or biogenesis (adjusted p-value:  $9.03\text{E-}10$ ), cellular carbohydrate biosynthetic process (adjusted p-value:  $6.21\text{E-}04$ ), secondary metabolite biosynthetic process (adjusted p-value:  $1.20\text{E-}03$ ), and growth (adjusted p-value:  $1.96\text{E-}03$ ).



To confirm the results from the GO enrichment analysis, we performed functional enrichment analysis using PAGEMAN tools (Figure 3B). The PAGEMAN results were highly correlated with those derived from the GO enrichment analysis. In fact, results from PAGEMAN further demonstrated that DNA synthesis/chromatin structure related genes were down-regulated (p-value:  $3.15\text{E-}03$ ). On the other hand, the expression of the genes involved in protein degradation, calcium signaling, and sugar transport were enhanced (p-value:  $4.68\text{E-}02$ ,  $9.59\text{E-}03$ , and  $4.07\text{E-}03$ , respectively). Altogether, the GO enrichment and PAGEMAN results confirmed that waterlogging caused global gene expression reprogramming in *Jatropha* roots.

A

Up	BP:GO:0006950	response to stress (1.45E-08)
	BP:GO:0001666	response to hypoxia (2.37E-03)
	BP:GO:0009723	response to ethylene (7.34E-03)
	MF:GO:0003700	sequence specific DNA binding transcription factor activity (4.08E-02)
	BP:GO:0009061	anaerobic respiration (4.22E-02)
Down	BP:GO:0071554	cell wall organization or biogenesis (9.03E-10)
	BP:GO:0034637	cellular carbohydrate biosynthetic process (6.21E-04)
	BP:GO:0044550	secondary metabolite biosynthetic process (1.20E-03)
	BP:GO:0004007	growth (1.96E-03)

B

Bin	Name	Elements	LogFC	p-value	LogFC
27	RNA	150	3.833	1.26E-04	3
27.3	RNA.regulation of transcription	135	3.785	1.54E-04	2
10	cell wall	86	-3.685	2.28E-04	1
16	secondary metabolism	107	-3.623	2.91E-04	1
5	fermentation	6	3.443	5.76E-04	1
27.3.3	RNA.regulation of transcription.AP2 -EREBP, APETALA2 -Ethylene-responsive element binding protein family	7	3.261	1.11E-03	1
17.5.2	hormone metabolism.ethylene.signal transduction	16	3.058	2.23E-03	1
27.3.32	RNA.regulation of transcription.WRKY domain transcription factor family	9	2.963	3.05E-03	1
28.1	DNA.synthesis -chromatin structure	18	-2.953	3.15E-03	-1
16.1	secondary metabolism.simple phenols	11	-2.947	3.21E-03	-1
5.3	fermentation.ADH	4	2.897	3.77E-03	1
34.2	transport.sugars	9	2.873	4.07E-03	1
17.5	hormone metabolism.ethylene	28	2.835	4.58E-03	1
26.3	misc.gluco-, galacto- and mannosidases	18	-2.741	6.13E-03	-1
16.8.4	secondary metabolism.flavonoids.flavonols	8	-2.697	6.99E-03	-1
16.1.5	secondary metabolism.isoprenoids.terpenoids	12	-2.682	7.32E-03	-1
26.16	misc.myrosinases-lectin-jacalin	8	2.610	9.05E-03	1
30.3	signalling.calcium	31	2.590	9.59E-03	1
20.2.2	stress.abiotic.cold	8	-2.584	9.76E-03	-1
10.5	cell wall.cell wall proteins	16	-2.581	9.86E-03	-1
29.5.5	protein.degradation.serine protease	15	2.570	1.02E-02	1
26	misc	212	-2.502	1.23E-02	-1
20.2.4	stress.abiotic.touch -wounding	5	2.475	1.33E-02	1
16.8.3	secondary metabolism.flavonoids.dihydroflavonols	21	-2.473	1.34E-02	-1
2.2.1.5	major CHO metabolism.degradation.sucrose.Susy	3	2.374	1.76E-02	1
17.2.1	hormone metabolism.auxin.synthesis-degradation	4	-2.310	2.09E-02	-1
16.2.1.10	secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD	3	2.284	2.24E-02	1
16.1	secondary metabolism.isoprenoids	20	-2.219	2.65E-02	-1
35.2	not assigned.unknown	460	2.211	2.70E-02	1
29.5.11.4	protein.degradation.ubiquitin.E3	45	2.175	2.96E-02	1
17.1.1.1.10	hormone metabolism.abscisic acid.synthesis-degradation.synthesis.9-cis-epoxycarotenoid dioxygenase	2	-2.168	3.02E-02	-1
10.5.1	cell wall.cell wall proteins.AGPs	10	-2.148	3.17E-02	-1
10.5.1.1	cell wall.cell wall proteins.AGPs.AGP	10	-2.148	3.17E-02	-1
11	lipid metabolism	34	-2.120	3.40E-02	-1
2.1	major CHO metabolism.synthesis	7	-2.115	3.44E-02	-1
2.1.2	major CHO metabolism.synthesis.starch	7	-2.115	3.44E-02	-1
34.22	transport.cyclic nucleotide or calcium regulated channels	3	-2.077	3.78E-02	-1
30.2.25	signalling.receptor kinases.wall associated kinase	3	-2.064	3.91E-02	-1
18	Co-factor and vitamins metabolism	4	2.035	4.19E-02	1
27.3.27	RNA.regulation of transcription.NAC domain transcription factor family	4	2.028	4.26E-02	1
29.5.11.4.3.2	protein.degradation.ubiquitin.E3.SCF.FBOX	15	2.016	4.38E-02	1
29.5	protein.degradation	102	1.988	4.68E-02	1
2.2.2.9	major CHO metabolism.degradation.starch.limit dextrinase - pullulanase	2	-1.988	4.68E-02	-1
16.8	secondary metabolism.flavonoids	38	-1.964	4.96E-02	-1
29	protein	186	1.963	4.96E-02	1

**Figure 3.** Waterlogging reprogramming transcriptome of *Jatropha* roots.

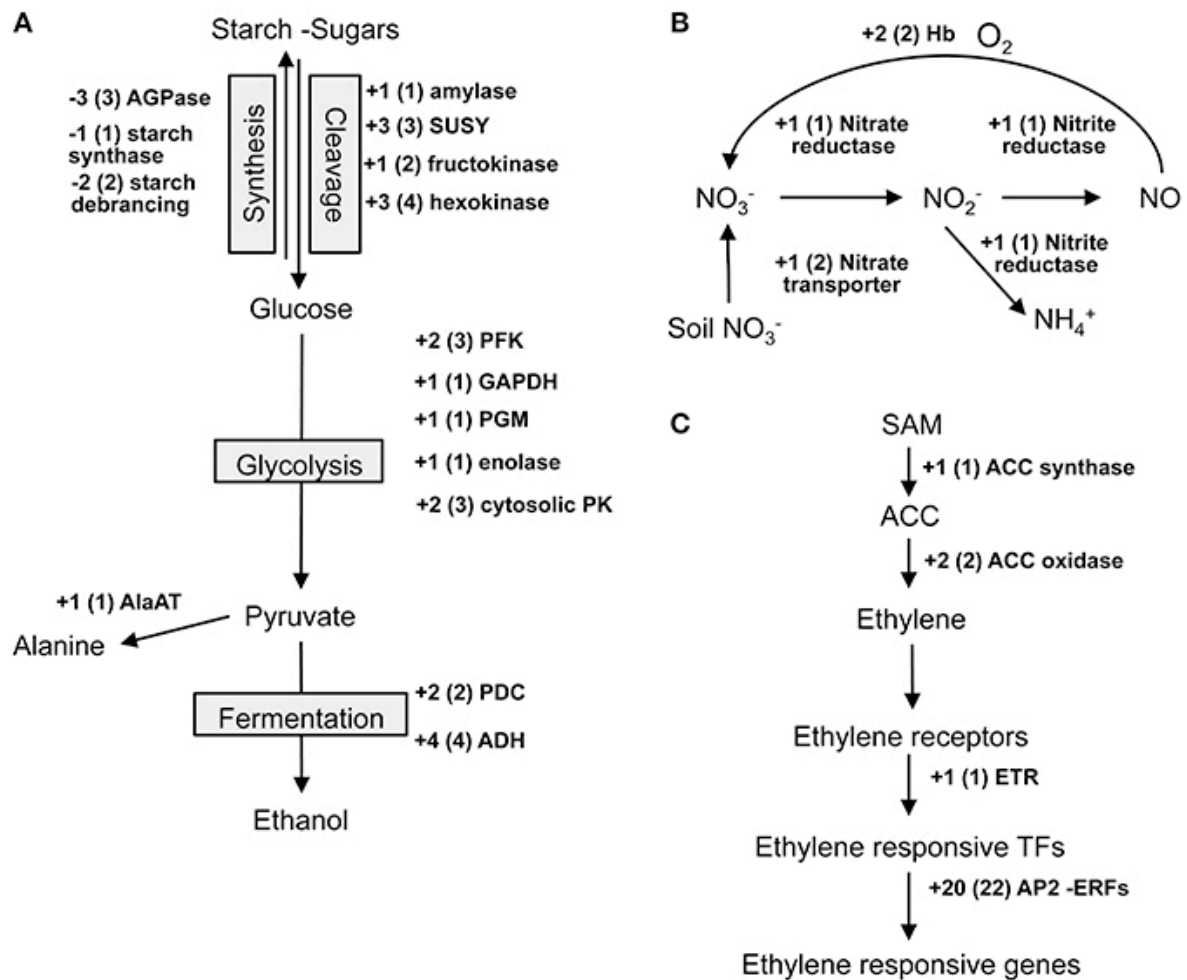
(A) Selected gene ontology (GO) categories (p-values calculated by GOHyperG) from up-regulated (Up) and down-regulated (Down) groups are shown. (B) PAGEMAN enrichment analysis with significant up/down-regulation (Wilcoxon rank sum test; p-value < 0.05).

## 5.2 Differential regulation of genes associated with anaerobic fermentation and glycolysis, nitrate metabolism, nitric oxide production, ethylene synthesis and perception, and genes encoding for transcription factors.

Because the low oxygen conditions could promote glycolysis and fermentation, we further examined regulation of the DEGs encoding central carbon metabolism enzymes (Figure 4A). We found that waterlogging promoted the expression of the genes involved in sugar and starch cleavage [e.g., *alpha-amylase* and *sucrose synthase (SUSY)*]. In contrast, waterlogging inhibited the accumulation of mRNAs encoding for enzymes controlling starch synthesis [e.g., *ADP-glucose pyrophosphorylase (AGPase)* and *starch synthase*]. Moreover, the expression of the genes associated with glycolysis was up-regulated by waterlogging [e.g., *phosphofructokinase (PFK)*, *pyruvate kinase (PK)*, and *glyceraldehyde phosphate dehydrogenase (GAPDH)*]. As expected, an increase in the expression of fermentative genes (*PDC* and *ADH*) was evident. Furthermore, the expression of *alanine transaminase (AlaAT)*, an enzyme responsible for conversion of pyruvate to alanine, was found up-regulated in our analysis. Overall the results revealed that waterlogging promoted catabolism of carbohydrates and a switch from oxidative to anaerobic respiration in *Jatropha* roots.

We evaluated the regulation of the genes involved in nitrate metabolism and found that a set of genes was up-regulated in response to waterlogging in *Jatropha* roots (Figure 4B). These included genes encoding nitrate reductase (NR), a key enzyme responsible for conversion of nitrate ( $\text{NO}_3^-$ ) to nitrite ( $\text{NO}_2^-$ ) and nitric oxide (NO), nitrite reductase (NIR), an enzyme responsible for reduction of  $\text{NO}_2^-$  to ammonium and conversion of  $\text{NO}_2^-$  to NO, and  $\text{NO}_3^-$  transporter. Besides this, waterlogging induced the expression of non-symbiotic hemoglobins, which have been found to be associated with NO removal. These results suggest the regulation of NO<sub>3</sub> metabolism and that the modulation of endogenous NO levels might be important for waterlogging acclimation in *Jatropha*.

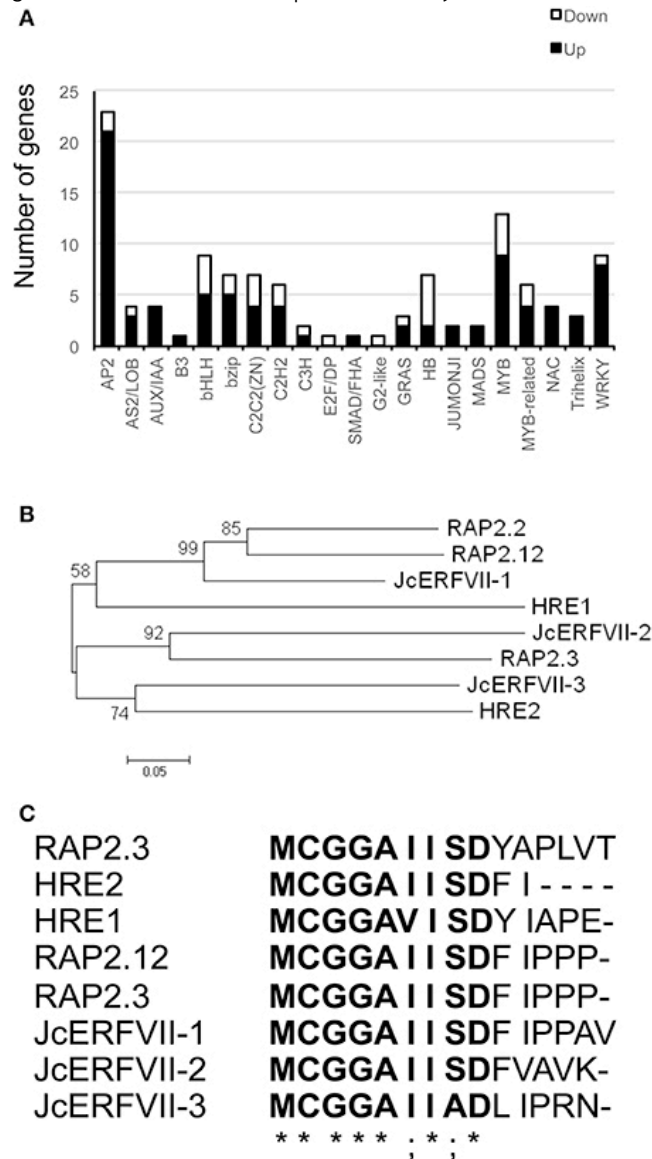
Our analysis demonstrated the differential regulation of DEGs encoding for proteins functioning in ethylene synthesis and response (Figure 4C). The expression of two key-genes controlling ethylene synthesis, *1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS)* and *ACC oxidase (ACO)*, was up-regulated by waterlogging. In addition, we found that waterlogging induced accumulation of transcripts encoding for ethylene receptor (*ETR*). Interestingly, several genes encoding for ERF (ethylene responsive factors) transcription factors (TFs) were up-regulated by waterlogging (Figure 5A). These marks highlighted the roles of ethylene in acclimation to waterlogging in *Jatropha*.



**Figure 4.** Waterlogging caused differential expression of transcripts encoding proteins involved in energy, nitrate, and ethylene metabolisms. (A) Starch metabolism (Starch), glycolysis, and fermentation (B) Nitrate metabolism (C) Ethylene production and perception. A plus sign represents induction and a minus sign represents reduction. Numbers in brackets represent numbers of DEGs found in this analysis.

Since low oxygen induced TFs could mediate the expression of anaerobic responsive genes, we analyzed for TFs that were differentially regulated by waterlogging. One-hundred and fifteen waterlogging-regulated TFs were classified based on their assigned protein families (Figure 5A). Of these, 85 TFs were up-regulated and 30 TFs were down-regulated. Remarkably, TFs accounted for nearly six percent of the DEGs analyzed in this study. ERFs (21 genes), MYBs (9 genes), and WRKYs (8 genes) were mostly pronounced from the up-regulated group, whereas Homeobox TFs (HBs; 5 genes) were mostly noticeable from the down-regulated group. Recent studies on *Arabidopsis* demonstrated that group-VII ERFs are modulators of the anaerobic response under low oxygen conditions (Gibbs et al., 2011; Licausi et al., 2011). Therefore, we examined the expression of the group-VII ERFs in *Jatropha*. Firstly, we searched the *Jatropha* genome using the HMM profile of the *Apetalla2* (AP2)/ERF domain in order to identify all AP2/ERF genes. As a result, 133 putative AP2/ERF genes were identified. Secondly, we performed

sequence alignment and phylogenetic analyses to compare Arabidopsis and Jatropha ERFs. Finally, 14 groups of AP2/ERF genes, including 11 ERF subgroups, AP2, RAV, and soloist were successfully identified. This analysis revealed that the Jatropha group-VII ERFs comprised three members, all possessing a conserved N-terminal domain [NH<sub>2</sub>-MCGGAI(A/S)D] (Figures 5B,C). Remarkably, two of them (*Jcr4S00982.160* and *Jcr4S01651.60*; *JcERFVII-2* and *JcERFVII-3*, respectively) were up-regulated in our transcriptome analysis.

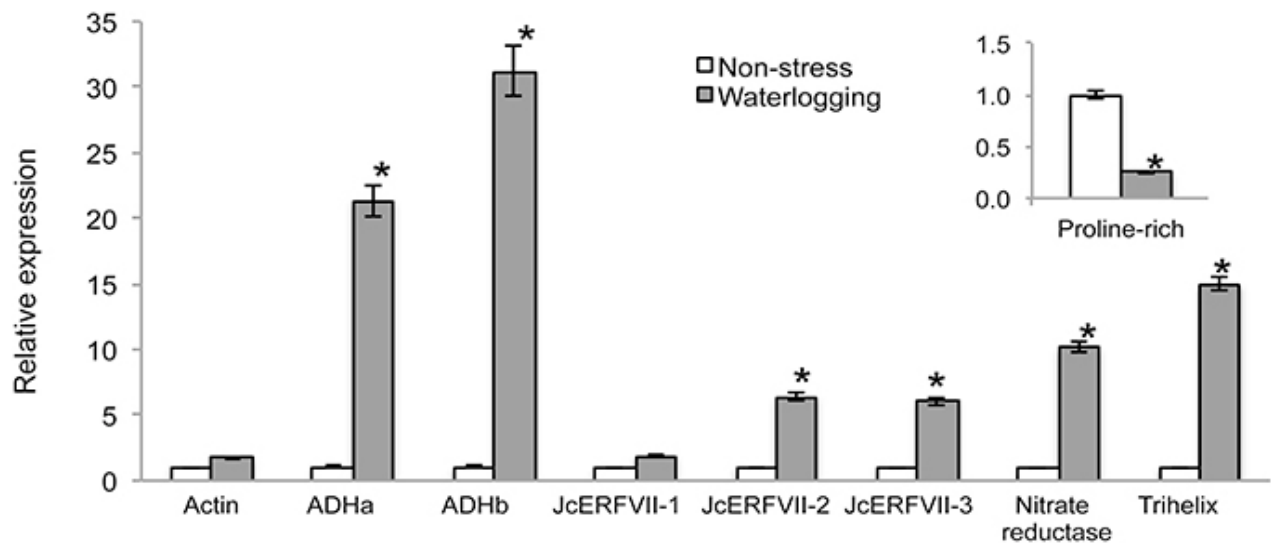


**Figure 5.** The group-VII ERFs of Jatropha. (A) Graphical representation of waterlogging-regulated transcription factors based on their assigned protein families. “Up” and “Down” represent up-regulation and down-regulation in this analysis. (B) Phylogenetic tree of group-VII ERF proteins.

The full-length protein sequences were analyzed with a Neighbor-joining method. Numbers above branches represent the bootstrapped value from 1000 replicates. (C) Multiple sequence analysis of N-terminal sequences of group-VII ERFs. Bold letters indicate a conserved motif at the N-terminus initiated with Met1-Cys2, as identified by multiple sequence alignment. Asterisks and semi-colons indicate identical and conserved substitution, respectively.

### 5.3 Confirmation of differentially regulated genes by qRT-PCR

To verify the transcriptome data, 10 genes were selected for analysis by quantitative real-time PCR to determine their relative expression in response to waterlogging. These selected genes included 6 waterlogging-induced DEGs [*ADHa*, *ADHb*, *JcERFVII-2*, *JcERFVII-3*, *NR*, and *trihelix TF (trihelix)*], two non-DEGs with limited changes in response to waterlogging (*actin* and *JcERFVII-1*), one waterlogging-repressed DEG (*proline-rich*), and one control for fold change analysis (*UBC*). Real-time PCR results of the same samples used for RNA-seq clearly confirmed that waterlogging induced the accumulation of *ADHa*, *ADHb*, *JcERFVII-2*, *JcERFVII-3*, *NR*, and *trihelix* but not *actin* and *JcERFVII-1* (Figure 6). By contrast, mRNA accumulation of *proline-rich*, a gene encoding a cell-wall protein, was clearly reduced upon waterlogging (Figure 6).



**Figure 6.** Quantitative real-time PCR validation of transcriptome data for selected genes. Relative expression was normalized to the abundance of Ubiquitin (UBC; Jcr4S00238.120). Data represent mean  $\pm$  SE (n = 3). \*p-value < 0.01 (t-test).

### 5.4 Comparative analysis of transcriptome response to waterlogging in Arabidopsis, Jatropha, gray poplar and rice

In order to identify common or specific changes in transcriptional regulation between waterlogging intolerant Arabidopsis and Jatropha and tolerant gray poplar and rice, we first analyzed public microarray data to identify DEGs in response to waterlogging or submergence in Arabidopsis, gray poplar and rice. Additionally, we performed a PAGEMAN over-representation analysis (ORA) using Fisher's exact test and a cut-off value of two to obtain an overview of comparative comparison. PAGEMAN ORA analysis allowed comparison of genes with similar functions. We observed an over-represented induction of genes encoding for anaerobic fermentation in Arabidopsis, Jatropha, and rice (Figure 7). In addition, AP2/ERF DNA binding TFs were up-regulated in all species. The down-regulation of genes involved in cell wall processes and secondary metabolism was common in both tolerant and intolerant species (Figure 7).

Over-represent

Z-score	Bin Name	UP										DOWN									
		JCA		ATH				PTR		OSA		JCA		ATH				PTR		OSA	
		Root24h	Root7h -Light	Root24h -Light	Root7h -Dark	Root24h -Dark	Root5h	Root24h	Root96h	Leaf-M202-24h	Leaf-Sub1-24h	Root24h	Root7h -Light	Root24h -Light	Root7h -Dark	Root24h -Dark	Root5h	Root24h	Root96h	Leaf-M202-24h	Leaf-Sub1-24h
3	major CHO metabolism	0	2.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	minor CHO metabolism	0	2.8	0	0	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0	0	0
1	fermentation	2.7	3.8	3.7	0	2.2	0	0	0	3.1	2.8	-2.2	0	0	0	0	0	0	0	0	0
0	gluconeogenesis - glyoxylate cycle	0	3.0	3.0	0	0	0	0	0	3.4	3.6	0	0	0	0	0	0	0	0	0	0
-1	OPP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.9
-2	cell wall	-4.3	0	-2.2	0	0	0	0	0	0	0	-4.7	8.1	9.1	0	0	0	-4.2	0	0	0
-3	lipid metabolism	-2.6	0	0	0	0	0	0	0	-3.2	-2.9	2.2	0	0	0	0	0	0	0	0	0
	N-metabolism.nitrate metabolism	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.3	2.7
	- Nitrate reductase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.1	2.4
	amino acid metabolism	0	5.8	5.2	0	0	0	0	0	0	0	-2.2	-2.6	0	0	0	0	0	0	0	2.0
	S-assimilation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.3	3.1
	metal handling.acquisition	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.3
	secondary metabolism	0	0	0	0	0	0	0	0	0	0	2.8	3.0	4.3	0	0	0	0	0	4.7	4.8
	- isoprenoids.non-mevalonate pathway	2.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	hormone metabolism	0	2.8	0	0	0	0	0	0	0	0	0	2.2	2.0	0	0	0	0	0	0	2.1
	- ethylene	3.8	3.9	0	0	0	0	2.1	0	0	0	-3.3	0	0	0	0	0	0	0	0	0
	Co-factor and vitamin metabolism	2.1	2.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	stress	0	0	0	0	0	0	0	0	4.2	5.8	0	2.0	2.9	0	0	0	0	0	0	0
	- heat	2.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	- cold	-2.5	0	0	0	0	0	2.9	0	0	0	3.0	0	0	0	0	0	0	0	0	0
	- touch -wounding	2.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	redox.dismutases and catalases	0	2.6	2.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	polyamine metabolism	0	0	0	0	0	0	0	0	0	0	0	2.6	2.4	0	0	0	0	0	0	0
	RNA	4.5	0	0	0	0	0	0	0	3.2	-4.7	-2.9	-5.7	0	0	0	-2.0	-2.3	0	-3.9	-4.9
	- AP2 -EREBP	3.0	3.1	2.8	0	0	2.4	0	0	4.7	5.5	-2.4	0	0	0	0	0	-2.1	0	0	-2.1
	- NAC	2.1	0	0	0	0	0	0	0	2.5	2.4	0	0	0	0	0	0	0	0	0	0
	- WRKY	2.7	0	0	0	0	0	0	0	3.6	-2.1	0	0	0	0	0	0	0	0	0	0
	- Pseudo ARR	0	3.3	3.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	DNA.synthesis -chromatin structure	-3.3	0	0	0	0	0	0	0	0	0	2.9	0	0	0	0	0	0	0	0	0
	protein.synthesis.ribosomal protein	2.1	-2.0	-2.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	protein.degradation.ubiquitin.E3	2.0	0	2.2	0	0	0	0	0	0	0	-3.5	-4.8	-6.7	0	0	0	-2.3	0	-4.4	-4.0
	- E3.SCF	2.0	0	0	0	0	0	0	0	0	0	-3.5	-3.1	-4.4	0	0	0	0	0	-2.9	-3.2
	- E3.SCF.FBOX	2.1	0	0	0	0	0	0	0	0	0	-3.3	-3.0	-4.2	0	0	0	0	0	-2.9	-3.2
	- E3.BTB -POZ Cullin3	0	0	3.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	- E3.BTB -POZ Cullin3.BTB -POZ	0	0	3.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	signalling	0	0	-2.1	0	0	-3.0	0	0	6.5	6.5	0	0	0	0	0	4.3	0	0	-3.9	-3.8
	- receptor kinases	0	0	0	0	0	-2.5	0	0	9.2	9.4	2.4	0	0	0	0	4.2	0	0	-4.5	-4.5
	- receptor kinases.leucine rich repeat III	-3.1	0	0	0	0	0	0	0	0	0	3.0	0	0	0	0	0	0	0	0	0
	- receptor kinases.leucine rich repeat XI	0	0	0	0	0	-2.4	0	0	2.8	3.3	0	0	0	0	0	5.2	0	0	-2.6	0
	- receptor kinases.DUF 26	0	0	0	0	0	0	0	0	7.4	8.2	0	0	0	0	0	0	0	0	0	-2.3
	- receptor kinases.wall associated kinase	0	0	0	0	0	0	0	0	3.6	3.5	0	0	0	0	0	0	0	0	-2.2	-2.2
	- receptor kinases.misc	0	0	0	0	0	0	0	0	4.5	3.3	0	0	0	0	0	0	0	0	-2.2	0
	- calcium	2.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	cell	-3.4	-3.0	0	0	0	0	0	0	0	0	3.1	0	0	0	0	0	0	0	-2.3	-3.3
	- organisation	-3.6	0	0	0	0	0	0	0	0	0	3.0	0	0	0	0	0	0	0	-2.0	-2.3
	development	0	0	2.8	0	0	0	0	0	3.3	3.5	0	0	0	0	0	0	0	0	0	0
	transport	0	0	0	0	0	0	0	0	-2.7	-2.0	0	2.6	0	0	2.5	0	0	0	4.5	4.6
	- sugars	2.7	2.4	0	0	0	0	0	0	0	0	-2.1	0	0	0	0	0	0	0	0	0
	- amino acids	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-2.5	0	0	2.4	0
	- sulphate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.3
	- metal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.0	0	0	0	2.1	0
	- unspecified cations	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.3	2.0
	- potassium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.0	0
	- ABC transporters and multidrug resistance systems	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.0	2.4	0	0	2.2	2.6
	- Major Intrinsic Proteins	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.7	2.4
	- Major Intrinsic Proteins.PIP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.1	2.9

**Figure 7.** Overview of transcriptome response for selected functional categories to waterlogging or submergence in Arabidopsis (ATH), Jatropha (JCA), gray poplar (PTR), and rice (OSA). PAGEMAN analysis of the gene expression data ( $|\log_2$  fold change| > 1; FDR < 0.05). Statistical analysis of



over-represented functional bins was carried out using Fisher method. Z-scores indicate over/under representation (Numbers indicate z-scores; Green, over-represented; Red, under-represented).

Additionally, we attempted to compare changes in expression of orthologs in the genomes of Arabidopsis, Jatropha, gray poplar and rice. OrthoMCL (OMCL) cluster analysis using a Markov Cluster algorithm was applied in order to group orthologs.

To examine whether there is a link between tolerance levels and specific molecular mechanisms, we further inspected the PAGEMAN ORA results. We found that multiple processes were specifically regulated in Jatropha. For example, the genes involved in non-mevalonate pathway biosynthesis of isoprenoids, response to stress including heat and touch/wounding, and calcium signaling were up-regulated, while lipid metabolism, cold stress response, and cell organization were down-regulated (Figure 7). In rice, we found several protein kinases genes were specifically up-regulated (Figure 8). Interestingly, we also found that the down-regulation of genes controlling NO<sub>3</sub> metabolism, including NR, was specific to rice (Figure 7). An OMCL cluster analysis also confirmed that, while NRs were specifically down-regulated in rice, this was not the case in Arabidopsis and Jatropha (Figure 8). Additionally, the analysis also showed that up-regulation of NIR was specific to Jatropha. Evidently, the up-regulation of class I nsHbs (OMCL3239), putative NO scavengers, was found in Arabidopsis, Jatropha, and rice. However, the induction levels were much higher in Jatropha than in Arabidopsis and rice (Figure 8).

OMCL family	Gene ID	Species	mean logFC
OMCL2496 Nitrate reductase	AT1G37130	ATH	1.539
	AT1G77760	ATH	-3.882
	Jcr4S02321.10	JCA	5.811
	LOC_Os02g53130	OSA	-5.739
	LOC_Os08g36480	OSA	-3.730
	LOC_Os08g36500	OSA	NA
	Potri.005G172400	PTR	NA
OMCL7779 Nitrite reductase	Potri.002G088600	PTR	NA
	AT2G15620	ATH	-1.750
	Jcr4S01372.10	JCA	4.292
	LOC_Os01g25484	OSA	-1.778
	LOC_Os02g52730	OSA	NA
OMCL3239 Non-symbiotic hemoglobin (Class I)	Potri.004G140800	PTR	NA
	AT2G16060	ATH	2.983
	Jcr4S02362.60	JCA	7.966
	LOC_Os03g12510	OSA	1.046
	LOC_Os03g13140	OSA	NA
	LOC_Os03g13150	OSA	NA
	LOC_Os03g13160	OSA	NA
OMCL18700 Non-symbiotic hemoglobin (Class II)	Potri.009G110800	PTR	NA
	AT3G10520	ATH	-1.278
	Jcr4S01154.10	JCA	NA
OMCL10097 Non-symbiotic hemoglobin (Class III)	AT4G32690	ATH	1.057
	Jcr4S01353.90	JCA	3.897
	LOC_Os06g39140	OSA	NA
	Potri.006G244300	PTR	NA

**Figure 8.** Response of OMCL clusters with genes involved in nitrogen metabolism and NO production to waterlogging. Yellow, Up-regulated DEGs; Blue, Down-regulated DEGs; White, Orthologs not differentially expressed. Numbers represent mean log<sub>2</sub> fold change values.



## 6. Conclusion and discussion

In this study, high-throughput RNA-sequencing technology was employed to compare differential gene expression profiles of *Jatropha* roots subjected to 24 h waterlogging stress. Our study provides a new insight into understanding the molecular mechanisms underlying the response to waterlogging in *Jatropha*. WS reprogrammed expression of 1968 DEGs; seven of these were further confirmed to be differentially expressed by quantitative real-time PCR. By applying bioinformatic analyses for non-model species, our study demonstrated that a number of cellular processes in *Jatropha* roots were affected by waterlogging. These included anaerobic fermentation, carbohydrate metabolism, glycolysis, ethylene synthesis and perception,  $\text{NO}_3^-$  metabolism, NO production, regulation of transcription, protein degradation, transport, and signaling, suggesting that diverse physiological processes were affected by waterlogging stress.

Low oxygen conditions in plants promote the utilization of  $\text{NO}_3^-$  and the production of NO to facilitate anaerobic survival (Horchani et al., 2010; Oliveira et al., 2013; Oliveira and Sodek, 2013). Alternative to anaerobic fermentation,  $\text{NO}_3^-$  could be used as NADH acceptors. This allows  $\text{NAD}^+$  to be reused in glycolysis. It has been observed that longer periods of low oxygen survival can be achieved when  $\text{NO}_3^-$  is provided (Allegre et al., 2004; Horchani et al., 2010). In waterlogged *Jatropha* roots, the expression of NR, NiR, and nitrate transporter was induced, emphasizing the roles of  $\text{NO}_3^-$  in waterlogging acclimation. Future studies should focus more on investigating the significant of NR, NiR, and  $\text{NO}_3^-$  on tolerance to waterlogging in *Jatropha*.

Accumulation of the gas hormone ethylene is extremely important for the induction of plant responses to low oxygen. Since waterlogging inhibits gas diffusion, ethylene can be trapped in the waterlogged plant organs. Our transcriptome data revealed that root waterlogging promoted mRNA accumulation of two key enzymes in ethylene biosynthesis, ACS and ACO. Concomitantly, the expression of *ETR*, was enhanced in waterlogged *Jatropha* roots. These findings suggested that waterlogging might increase ethylene synthesis and perception in *Jatropha* roots. Our study recognized three group-VII ERFs from the *Jatropha* genome. Two of these, designated *JcERFVII-2* and *JcERFVII-3*, were noticeably induced in response to waterlogging. Previous studies identified the two group-VII ERF genes, *Snorkel* and *Sub1*, as key players in the submergence response in deepwater rice and lowland rice varieties, respectively, (Xu et al., 2006; Hattori et al., 2009). These two rice varieties utilize two contrasting strategies that allow adaptive growth responses to submergence. *Snorkel* promotes the internode elongation in the deepwater rice, whereas, *Sub1* restricts the shoot elongation in the lowland rice. In Arabidopsis, studies have also shown that the group-VII ERFs function in the low-oxygen response (Hinz et al., 2010; Gibbs et al., 2011; Licausi et al., 2011). Taken together, we propose that the group-VII ERFs are promising candidates for engineering of waterlogging tolerant *Jatropha*. In support of this idea, overexpression of the Arabidopsis group-VII ERFs (*HRE1*, *HRE2*, *RAP2.2*, and *RAP2.12*) significantly improved low oxygen survival by promoting expression of the genes involved in low oxygen adaptation (Hinz et al., 2010; Gibbs et al., 2011; Licausi et al., 2011).

Enhanced NO production by low oxygen conditions has been documented in plants (Rockel et al., 2002; Dordas et al., 2003). NO is believed to be a signaling molecule. As previously mentioned, NO can be synthesized from  $\text{NO}_3^-$  via NR and NiR (Stohr et al., 2001). In Arabidopsis, it was reported that accumulation of NO could induce AOX expression via inhibition of aconitase resulting in accumulation of citrate and a shift of metabolism toward nitrogen assimilation under hypoxia (Gupta et al., 2012). A recent study revealed that NO could control the stability of Arabidopsis group-VII ERFs via proteolytic modulation (Gibbs et al., 2014). We speculate that enhanced NO accumulation in *Jatropha* roots under low oxygen conditions could destabilize the group-VII ERF proteins and, therefore, decrease low oxygen tolerance. Modulation of NO accumulation via nsHbs in roots under low oxygen conditions has been reported (Dordas et al., 2003, 2004; Perazzolli et al., 2004; Hebelstrup et al., 2006). Our study demonstrated that waterlogging could induce the expression of nsHbs in *Jatropha* roots. The function of nsHbs possibly involves fine-tuning the accumulation of NO in waterlogged roots of *Jatropha*. Future research to improve waterlogging tolerance should emphasize genetic engineering to modulate NO levels.

Nearly six percent of DEGs encoding for TFs was identified in this study, implying that transcriptional regulation plays an important role in the waterlogging response. In agreement with the finding that ethylene synthesis and perception were activated, this study recognized AP2/ERF TFs as the most pronounced TFs in response to waterlogging. Waterlogging induced AP2/ERFs included two of group-VII ERFs and an additional 19 ERF, whose function might be associated with waterlogging acclimation.

In this study, the expression of *trihelix* TF (*Jcr4S02762.30*: OMCL11085), an ortholog of Arabidopsis *HYPOXIA RESPONSE ATTENUATOR1* (*HRA1*), was found to be strongly up-regulated by waterlogging. Recently, Giuntoli et al. (2014) demonstrated that HRA1 negatively regulates RAP2.12 through protein-protein interaction and proposed that the modulation of the aerobic response by group-VII ERFs and HRA1 is important to control plant responses to low oxygen stress. In addition, our analysis demonstrated that waterlogging enhanced the expression of several members of MYBs and WRKYs. An in-depth functional analysis of waterlogging-induced TFs is required to further identify candidate genes for growth improvement of waterlogged *Jatropha*.

Previous studies reported the use of comparative transcriptome comparison for identification of conserve and species-specific responses to low oxygen stress (Mustroph et al., 2010; Narsai et al., 2011; Narsai and Whelan, 2013). In this study, comparative transcriptome analysis between *Jatropha* and other plant species identified a common response to waterlogging including the up-regulation of carbohydrate cleavage, glycolysis and fermentative genes and the down-regulation of genes involved in cell-wall and secondary metabolite biosynthesis. These findings also confirmed previous reports that the alteration of carbohydrate metabolism appeared to be a common response to low oxygen stress in plants. Additionally, Arabidopsis, gray poplar and *Jatropha* showed the up-regulation of genes involved in ethylene production and

signaling, which were commonly found in response to low oxygen in several plants. The up-regulation of AP2/ERF genes by waterlogging was commonly identified in all species examined. These results strongly emphasized the roles of AP2/ERF genes in low oxygen adaptation. While comparative analysis exposed the common response to waterlogging in plants, the analysis showed that various processes were specifically regulated in each species. Contrary to what has been observed in waterlogged *Jatropha*, the expression of NR was down-regulated in submerged rice suggesting the down-regulation of the NR gene may be a characteristic of waterlogging-tolerant species.

In conclusion, *Jatropha* orchestrates a complex transcriptional adjustment in response to WS that yields changes in its root physiological response to waterlogging stress. This study highlights several possibilities for future investigation, including the roles of specific pathways and genes involved in the waterlogging response. Thus, a detailed characterization of individual genes must be carried out as the first step to understand their specific functions. Due to the high similarity among global *J. curcas* accessions, as reported by Popluechai et al. (2009), the improvement of waterlogging-tolerant *Jatropha* through traditional breeding, can be difficult to achieve. With biotechnology advances, genetic engineering and gene transformation into *Jatropha* are now feasible (Mukherjee et al., 2011). A recently developed protocol by Jaganath et al. (2014) enables *Agrobacterium*-mediated in planta transformation into *Jatropha* that does not require in vitro-based multiplication of transformed plants. Clearly, this method has the potential to facilitate the genetic modification of *Jatropha*. Successfully validated candidate genes could be targeted for the engineering of waterlogging-tolerant *Jatropha*.

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- International Journal Publication

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# Elucidation of the molecular responses to waterlogging in *Jatropha* roots by transcriptome profiling

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*Jatropha* (*Jatropha curcas*) is a promising oil-seed crop for biodiesel production. However, the species is highly sensitive to waterlogging, which can result in stunted growth and yield loss. To date, the molecular mechanisms underlying the responses to waterlogging in *Jatropha* remain elusive. Here, the transcriptome adjustment of *Jatropha* roots to waterlogging was examined by high-throughput RNA-sequencing (RNA-seq). The results indicated that 24 h of waterlogging caused significant changes in mRNA abundance of 1968 genes. Comprehensive gene ontology and functional enrichment analysis of root transcriptome revealed that waterlogging promoted responses to hypoxia and anaerobic respiration. On the other hand, the stress inhibited carbohydrate synthesis, cell wall biogenesis, and growth. The results also highlighted the roles of ethylene, nitrate, and nitric oxide in waterlogging acclimation. In addition, transcriptome profiling identified 85 waterlogging-induced transcription factors including members of AP2/ERF, MYB, and WRKY families implying that reprogramming of gene expression is a vital mechanism for waterlogging acclimation. Comparative analysis of differentially regulated transcripts in response to waterlogging among *Arabidopsis*, gray poplar, *Jatropha*, and rice further revealed not only conserved but species-specific regulation. Our findings unraveled the molecular responses to waterlogging in *Jatropha* and provided new perspectives for developing a waterlogging tolerant cultivar in the future.

**Keywords:** *Jatropha curcas*, genomics, low oxygen, ERFs, transcriptional control, RNA-seq

## INTRODUCTION

Waterlogging is an adverse abiotic stress that can heavily damage crop production worldwide. The condition can be defined as the saturation of soils with water. Waterlogging occurs during the heavy rainy season in lowland areas, including Thailand. Due to the limited diffusion of gas under water, waterlogging creates low oxygen (hypoxia) environments in the root areas, causing a shortage of ATP from the inhibition of oxidative phosphorylation. Upon experiencing long-term waterlogging stress (WS), stomatal closure can lead to impaired root hydraulic conductivity, thereby reducing the photosynthetic rate and the nutrient and water uptake of the plant.

*Jatropha curcas* (common names: *Jatropha*, physic nut, and purging nut) is a promising crop for the generation of biodiesel. *Jatropha* seeds contain between 40 and 50% of high quality oil (Basha and Sujatha, 2009). Based on dominant marker analysis among worldwide populations, *Jatropha* possesses a narrow genetic base (Popluechai et al., 2009). Indigenous to South America, *Jatropha* is drought tolerant, however, the species is highly sensitive to waterlogging. Gimeno et al. (2012) reported that 10 days of waterlogging caused an approximately 30% reduction in *Jatropha* biomass. Moreover, a decrease in leaf

photosynthetic rate and a decline in carbohydrate concentration in both leaves and roots were observed in waterlogged *Jatropha* (Gimeno et al., 2012). Despite these lines of evidence, the molecular mechanism underlying the waterlogging response in *Jatropha* remains unknown.

In recent years, studies in *Arabidopsis* (*Arabidopsis thaliana*), gray poplar (*Populus × canescens*), cotton (*Gossypium hirsutum*), and rice (*Oryza sativa*) have revealed that low oxygen can cause drastic changes in transcription, translation, and metabolite levels (Branco-Price et al., 2008; Kreuzwieser et al., 2009; Mustroph et al., 2009; Narsai et al., 2009; Christianson et al., 2010; Lee et al., 2011; Juntawong et al., 2014). The low oxygen condition results in limited ATP production and negatively affects cellular energy status. Therefore, a key feature for the acclimation to low oxygen environment is to activate genes encoding proteins and enzymes for anaerobic fermentation, glycolysis, transcription factors, and signaling pathways in order to allow biological and physiological adjustments to the low oxygen conditions (Bailey-Serres et al., 2012). While several published studies of model plants have provided some fundamental clues, quite a few studies were performed in hypoxic chambers, in which the entire plants were subjected to low oxygen conditions, grown on artificial media, or

subjected to complete darkness. These conditions would not imitate the impact observed in plants exposed to soil waterlogging. Recently, high-throughput sequencing technology has been used as a powerful tool for genomic analysis in both model and non-model species. This method can provide quantitative descriptions of gene expression at the genome-scale level, accompanying high accuracy, low background noise, and large dynamic ranges. High-throughput transcriptome analysis could help provide a basic understanding of the molecular responses to waterlogging in *Jatropha*.

The goal of this study is to provide new insights into the molecular responses of *Jatropha* to waterlogging. Here, we profiled transcriptome changes in *Jatropha* roots subjected to 24 h of waterlogging, using high-throughput sequencing by the Ion Proton platform. Bioinformatic analysis of transcriptome data was performed to allow identification and functional annotation of differentially expressed genes (DEGs). Additionally, we comparatively analyzed waterlogging transcriptomes from *Arabidopsis*, *Jatropha*, gray poplar and rice to identify conserved and species-specific responses. Lastly, we discussed the use of genetic engineering to target some candidate genes for generation of waterlogging-tolerant *Jatropha*.

## MATERIALS AND METHODS

### PLANT MATERIALS AND STRESS CONDITIONS

*Jatropha curcas* seeds (cv. “Chai Nat”—a Thai local variety) were germinated in soil containing 50% (v/v) peat moss, 25% (v/v) perlite, and 25% (v/v) coconut fiber with a daily water supply. Plants were grown outdoors between July and August of 2013 and 2014 at Kasetsart University, Bang Khen campus. Thirty day-old, six-leaf-stage plants were used in the waterlogging treatment. In brief, plant pots were placed in plastic containers filled with tap water. The level of water was set at 3 cm above the soil. WS began at mid-day and continued for 24 h. For the control, non-treated plants were placed in a container with no water. Root tissue was harvested at the end of the treatment, immediately placed in liquid nitrogen, ground into a fine powder, and kept at  $-80^{\circ}\text{C}$ .

### RNA EXTRACTION, LIBRARY PREPARATION, AND SEQUENCING

Total RNA was extracted with TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Total RNA samples were subjected to DNase treatment and RNA cleanup by a GF-1 RNA extraction kit (Vivantis). Poly(A)<sup>+</sup> mRNAs were isolated from the total RNAs by using an Absolutely mRNA purification kit (Agilent). *Jatropha* mRNA (200 ng) was used to construct a sequencing library, by using a Ion Total RNA seq kit (Life Technologies). For each treatment, two independent biological replicates were sequenced on a Ion Proton sequencer (Life Technologies). FASTQ files were obtained with the base caller provided by the instrument and subjected to quality control filtering and 3' end trimming using the FASTX-toolkit ([http://hannonlab.cshl.edu/fastx\\_toolkit/index.html](http://hannonlab.cshl.edu/fastx_toolkit/index.html)) included in the Torrent Suite™ Software (Life Technologies). The preprocessed reads with a minimum of 35 nt and a quality value of 17, were deposited in the NCBI GEO database under the accession number GSE57428.

### TRANSCRIPTOME ANALYSIS

FASTQ reads were mapped to the *J. curcas* genome release 4.5, downloaded from <http://www.kazusa.or.jp/jatropha/> (Sato et al., 2011; Hirakawa et al., 2012) with TMAP mapping program (<https://github.com/iontorrent/TMAP>), using the default settings and “mapall” command. A binary format of sequence alignment files (BAM) was generated and subsequently used to create read count tables using HTseq library (Anders et al., 2014) for differential gene expression analysis. Statistical analysis of DEGs was calculated in the R environment (R Development Core Team, 2005), by using a generalized linear model (GLM) approach from the edgeR package (Robinson et al., 2010). Genes with the count per million (CPM) values  $\geq 1$  in at least two library samples were included in this analysis (Supplementary Table S1). In brief, the calcNormFactors function was applied for data normalization using the trimmed mean of M-values (TMM) method. Subsequently, the GLMTagwiseDisp function was used to generate the estimated tagwise dispersion. Next, the glmFit function was applied to fit the negative binomial GLM for each tag. Finally, the glmLRT function was applied to carry out the likelihood ratio test. Significant DEGs were filtered, based on false discovery values (FDR < 0.05).

Assignment of the DEGs into functional bins and the creation of a mapping file from the *Jatropha* genome were performed using the Mercator pipeline for automated sequence annotation (Lohse et al., 2013). The inputs for the Mercator were *Jatropha* protein sequences supplied by <http://www.kazusa.or.jp/jatropha/>. This analysis was performed with a blast score cutoff > 80. For visualization, the MapMan (Thimm et al., 2004) and the PageMan (Usadel et al., 2006) programs were used.

Gene ontology (GO) enrichment analysis was performed in the R environment. Firstly, the GO terms for each gene were derived from homology searching of plant protein databases using GOANNA with an *e*-value cutoff <  $E^{-10}$  (McCarthy et al., 2006). Subsequently, a gene annotation file was generated. GO term enrichment analysis in up or down regulated DEGs was performed by the GOHyperGALL function (Horan et al., 2008). Significant GO terms were filtered based on the adjusted *p*-value < 0.05.

### IDENTIFICATION OF JATROPHA AP2/ERF GENES

For mining of AP2/ERF genes from the *Jatropha* genome, the hidden Markov model (HMM) profile of the AP2/ERF superfamily was extracted from the Pfam and used to search *Jatropha* protein sequences.

To perform phylogenetic analysis comparing *Jatropha* and *Arabidopsis* AP2/ERF genes, the amino acid sequences of the *Arabidopsis* AP2/ERF genes were downloaded from the TAIR database (<http://www.arabidopsis.org/>). A multiple alignment was performed using the MUSCLE alignment in the MEGA5 software (Tamura et al., 2011). A phylogenetic tree of AP2/ERF genes was constructed by the Neighbor Joining method, with a bootstrap number set to 1000 replicates. A list of predicted *Jatropha* AP2/ERF genes can be found in Supplementary Table S2.



## QUANTITATIVE REAL-TIME PCR

cDNA was synthesized from 1.2  $\mu$ g of total RNAs using oligo(dT)20 and superscript III reverse transcriptase (Invitrogen), according to the manufacturer's protocol. Real-time PCR was assayed in a 20  $\mu$ L reaction, containing KAPA SYBR FAST qPCR master mix (KAPABIOSYSTEMS) using the Stratagene Mx3000P real-time PCR system (Agilent Technologies). Amplification specificity was validated by melt-curve analysis at the end of each PCR experiment. Relative gene expression was calculated using the comparative  $\Delta\Delta C_T$  method (Livak and Schmittgen, 2001). *Ubiquitin protein (UBC; Jcr4S00238.120)* was used as a normalization control. Primer sequences and annealing temperatures were listed in **Supplementary Table S3**.

## ANALYSIS OF PUBLICLY AVAILABLE MICROARRAY DATA

Publicly available microarrays were downloaded from the Gene Expression Omnibus (GEO) database (details listed in **Supplementary Table S4**). Microarray data were normalized using the Robust Multi-chip Average (RMA) method. Differential expression analysis was performed on RMA-normalized data. False discovery rates (FDR) for significant differences between genes in each comparison were generated using *p*-value distributions (Smyth, 2004). For each comparison, DEGs were selected based on criteria of  $FDR < 0.05$  and the absolute values of  $\log_2$  fold change ( $\log FC$ )  $\geq 1$ .

## ORTHOLOG IDENTIFICATION

Orthologs among *Jatropha*, *Arabidopsis*, rice, and poplar were identified based on protein sequence homology using ORTHOMCL (OMCL) version 1.4 (Li et al., 2003). The inflate number used to perform this analysis was set to 1.2. Protein sequences used in this step were downloaded from *Jatropha*, *Arabidopsis*, rice, and poplar genome databases ([ftp://ftp.kazusa.or.jp/pub/jatropha/JAT\\_r4.5.aa.gz](ftp://ftp.kazusa.or.jp/pub/jatropha/JAT_r4.5.aa.gz), [ftp://ftp.arabidopsis.org/home/tair/Sequences/blast\\_datasets/TAIR10\\_blastsets/TAIR10\\_pep\\_20110103\\_representative\\_gene\\_model\\_updated](ftp://ftp.arabidopsis.org/home/tair/Sequences/blast_datasets/TAIR10_blastsets/TAIR10_pep_20110103_representative_gene_model_updated), [ftp://ftp.jgi-psf.org/pub/compngen/phytozome/v9.0/Osativa/annotation/Osativa\\_204\\_protein\\_primaryTranscriptOnly.fa.gz](ftp://ftp.jgi-psf.org/pub/compngen/phytozome/v9.0/Osativa/annotation/Osativa_204_protein_primaryTranscriptOnly.fa.gz), and [ftp://ftp.jgi-psf.org/pub/compngen/phytozome/v9.0/Ptrichocarpa/annotation/Ptrichocarpa\\_210\\_protein\\_primaryTranscriptOnly.fa.gz](ftp://ftp.jgi-psf.org/pub/compngen/phytozome/v9.0/Ptrichocarpa/annotation/Ptrichocarpa_210_protein_primaryTranscriptOnly.fa.gz), respectively). A list of OMCL clusters can be found in **Supplementary Table S5**.

## ANALYSIS OF LEAF CHLOROPHYLL CONTENT

Chlorophyll content was measured using the atLEAF+ chlorophyll meter (FT Green LLC, Wilmington, DE). The youngest fully expanded leaves of approximately 30 day-old plants were measured three times and the averages were used in subsequent analysis. Six plants were analyzed for each time point. The total chlorophyll content of the leaves was obtained by converting the atLEAF+ values in SPAD using an online webtool: <http://www.atleaf.com/SPAD.aspx>.

## CARBOHYDRATE ASSAY

One hundred mg of frozen root tissue was used to quantify the total carbohydrate content using a method described by Sadasivam and Manickam (2007). Non-structural carbohydrates

were extracted and hydrolyzed by adding 5 mL of 2.5 N HCl and incubated in a boiling water bath for 3 h. The extract was neutralized by adding 0.75 g of  $\text{Na}_2\text{CO}_3$ . The anthrone method was used to determine total carbohydrate content relative to a standard series of glucose. In brief, the extract (300  $\mu$ L) and distilled water (700  $\mu$ L) were mixed with 4 mL of 0.14% (w/v) anthrone solution in 95%  $\text{H}_2\text{SO}_4$ , incubated in a boiling water bath for 8 min, and rapidly cooled on ice. The absorbance was quantified at 630 nm.

## RESULTS

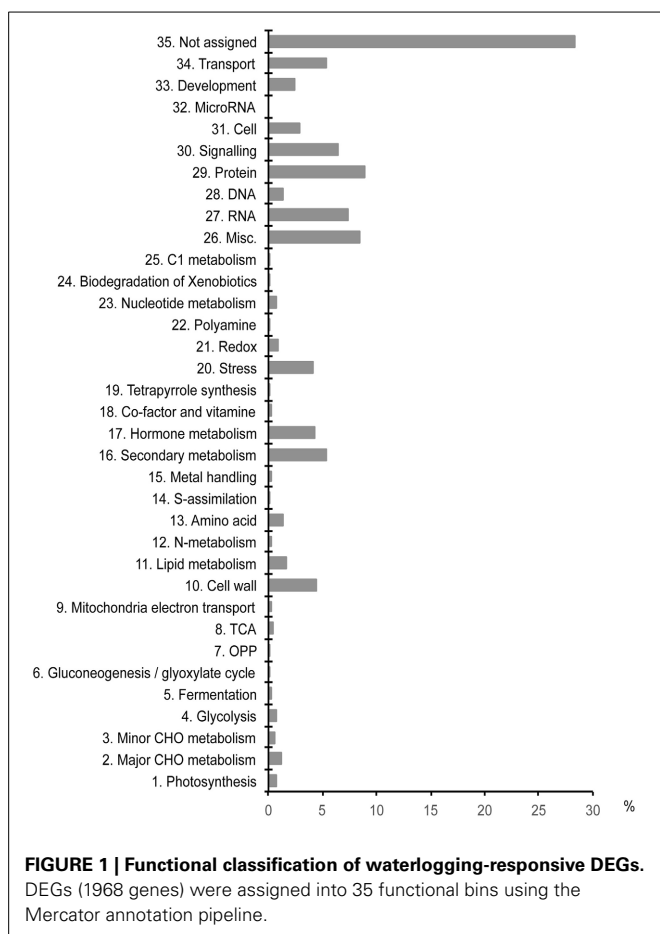
### WATERLOGGING STRESS TRIGGERED TRANSCRIPTOME READJUSTMENT IN JATROPHA ROOTS

In our observation, we found that long-term waterlogging in young *Jatropha* seedlings resulted in leaf chlorosis (**Supplementary Figure S1A**) and reduction of total root carbohydrate content (**Supplementary Figure S1B**). To determine the molecular responses of *Jatropha* to waterlogging, six-leaf-stage plants (30-days old) were tested by applying 24 h of waterlogging. First, we examined the response of *Jatropha* leaves and roots to waterlogging by using the semi-quantitative reverse-transcription PCR. We found waterlogging induced the expression of low oxygen responsive marker genes, *alcohol dehydrogenases (ADHs)* and *pyruvate decarboxylase (PDC)* in roots, but not in leaves (**Supplementary Figure S2**). Therefore, our transcriptome analysis was focused only on the response in root organs.

To compare the effects of waterlogging on the *Jatropha* roots, we quantitatively profiled transcriptome from WS and non-stress (NS) samples. Over 4 million reads were obtained for each of the two independent biological replicates. The majority of reads (93–96%) mapped to the *Jatropha* genome (**Supplementary Figure S3A**). Biological replicate transcriptome data were highly correlated as shown by Pearson's correlation coefficient of CPM values ( $r = 0.93$  and  $0.85$  from NS and WS libraries, respectively; **Supplementary Figure S3B**). A multi-dimension scaling plot of the gene expression data demonstrated that samples were clearly separated by treatments (**Supplementary Figure S3C**).

Transcriptome analysis identified 1968 DEGs with significant changes in expression evaluated by the false discovery rate ( $FDR < 0.05$ ) (**Supplementary Table S1**). Of these, 931 genes (47%) were up-regulated and 1037 genes (53%) were down-regulated. Assignment of the DEGs to functional bins, using a well-established Mercator pipeline (Lohse et al., 2013), categorized the DEGs into 35 functional bins (**Figure 1; Supplementary Table S1**). Approximately 28% of the DEGs fell into a "not-assigned" bin. Most of the genes in this bin were annotated as "short," "partial," and "transposable element (TE)" by Sato et al. (2011) and Hirakawa et al. (2012). Nevertheless, the genes that related to cell wall, secondary metabolism, hormone metabolism, stress, RNA, protein, signaling, and transport were abundant in the DEG dataset.

To further evaluate the response to waterlogging, we considered the biological function of the DEGs that were co-regulated in response to waterlogging. GO enrichment analysis was performed with the up-regulated or down-regulated DEGs. Co-regulation of DEGs with similar functions was observed



(Figure 2A; Supplementary Table S1). The up-regulated DEGs were involved in the response to stress (adjusted  $p$ -value:  $1.45\text{E-}08$ ), response to hypoxia (adjusted  $p$ -value:  $2.37\text{E-}03$ ), and response to ethylene (adjusted  $p$ -value:  $7.34\text{E-}03$ ). In addition, we also observed enrichment of genes encoding transcription factors (adjusted  $p$ -value:  $4.08\text{E-}02$ ) following WS. The down-regulated DEGs were enriched for cell wall organization or biogenesis (adjusted  $p$ -value:  $9.03\text{E-}10$ ), cellular carbohydrate biosynthetic process (adjusted  $p$ -value:  $6.21\text{E-}04$ ), secondary metabolite biosynthetic process (adjusted  $p$ -value:  $1.20\text{E-}03$ ), and growth (adjusted  $p$ -value:  $1.96\text{E-}03$ ).

To confirm the results from the GO enrichment analysis, we performed functional enrichment analysis using PAGEMAN tools (Usadel et al., 2006) (Figure 2B). The PAGEMAN results were highly correlated with those derived from the GO enrichment analysis. In fact, results from PAGEMAN further demonstrated that DNA synthesis/chromatin structure related genes were down-regulated ( $p$ -value:  $3.15\text{E-}03$ ). On the other hand, the expression of the genes involved in protein degradation, calcium signaling, and sugar transport were enhanced ( $p$ -value:  $4.68\text{E-}02$ ,  $9.59\text{E-}03$ , and  $4.07\text{E-}03$ , respectively). Altogether, the GO enrichment and PAGEMAN results confirmed that waterlogging caused global gene expression reprogramming in *Jatropha* roots.

## DIFFERENTIAL REGULATION OF GENES ASSOCIATED WITH ANAEROBIC FERMENTATION AND GLYCOLYSIS

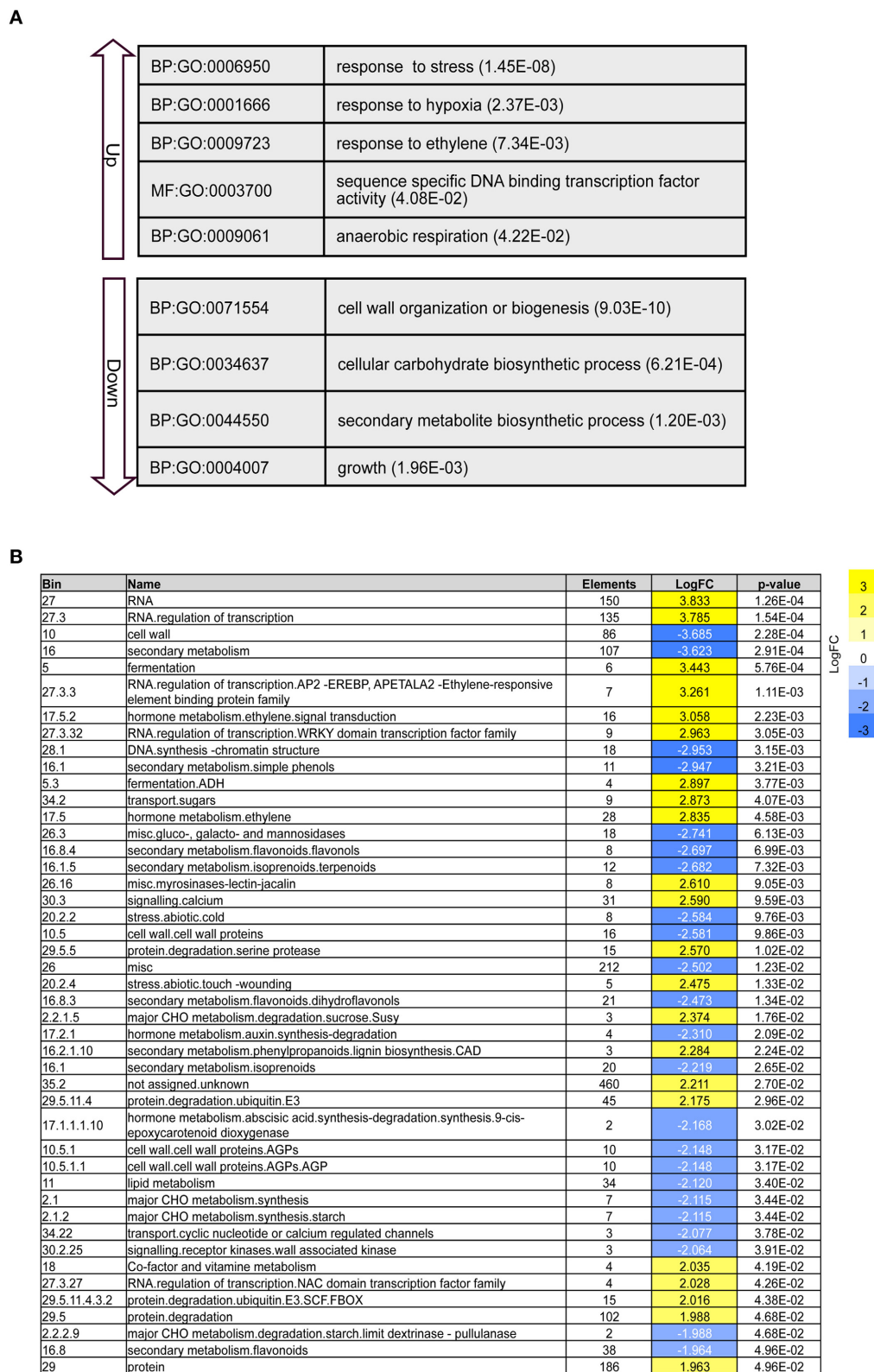
Because the low oxygen conditions could promote glycolysis and fermentation, we further examined regulation of the DEGs encoding central carbon metabolism enzymes (Figure 3A; Supplementary Table S1). We found that waterlogging promoted the expression of the genes involved in sugar and starch cleavage [e.g., *alpha*-amylase and *sucrose synthase (SUSY)*]. In contrast, waterlogging inhibited the accumulation of mRNAs encoding for enzymes controlling starch synthesis [e.g., *ADP-glucose pyrophosphorylase (AGPase)* and *starch synthase*]. Moreover, the expression of the genes associated with glycolysis was up-regulated by waterlogging [e.g., *phosphofructokinase (PFK)*, *pyruvate kinase (PK)*, and *glyceraldehyde phosphate dehydrogenase (GAPDH)*]. As expected, an increase in the expression of fermentative genes (*PDC* and *ADH*) was evident. Furthermore, the expression of *alanine transaminase (AlaAT)*, an enzyme responsible for conversion of pyruvate to alanine, was found up-regulated in our analysis. Overall the results revealed that waterlogging promoted catabolism of carbohydrates and a switch from oxidative to anaerobic respiration in *Jatropha* roots.

## DIFFERENTIAL REGULATION OF GENES INVOLVED IN NITRATE METABOLISM AND NITRIC OXIDE PRODUCTION

Changes in nitrate metabolism were observed upon waterlogging in several plant species (Horchani et al., 2010; Oliveira et al., 2013; Oliveira and Sodek, 2013). Therefore, we evaluated the regulation of the genes involved in nitrate metabolism and found that a set of genes was up-regulated in response to waterlogging in *Jatropha* roots (Figure 3B; Supplementary Table S1). These included genes encoding nitrate reductase (NR), a key enzyme responsible for conversion of nitrate ( $\text{NO}_3^-$ ) to nitrite ( $\text{NO}_2^-$ ) and nitric oxide (NO), nitrite reductase (NIR), an enzyme responsible for reduction of  $\text{NO}_2^-$  to ammonium and conversion of  $\text{NO}_2^-$  to NO, and  $\text{NO}_3^-$  transporter. Besides this, waterlogging induced the expression of *non-symbiotic hemoglobins (nsHbs)* (Supplementary Table S1), which have been found to be associated with NO removal (Heckmann et al., 2006). These results suggest the regulation of  $\text{NO}_3^-$  metabolism and that the modulation of endogenous NO levels might be important for waterlogging acclimation in *Jatropha*.

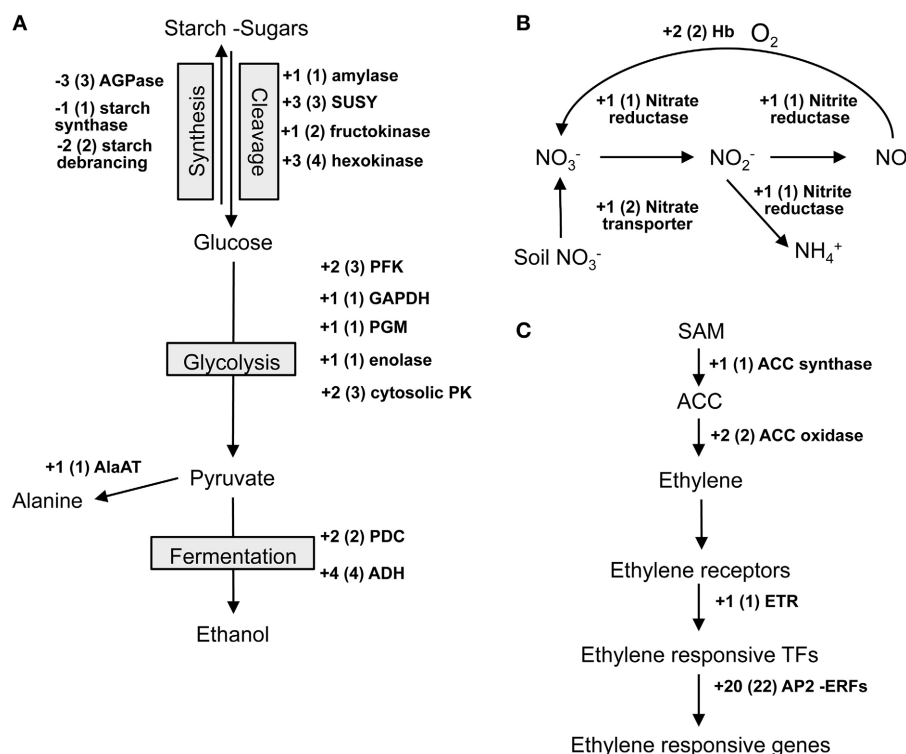
## DIFFERENTIAL REGULATION OF GENES RELATED TO ETHYLENE SYNTHESIS AND PERCEPTION

Accumulation of a plant stress hormone, ethylene, in response to low oxygen has been reported (Voesenek et al., 1993; Vriezen et al., 1999; Rzewuski et al., 2007; Hinz et al., 2010). Our analysis demonstrated the differential regulation of DEGs encoding for proteins functioning in ethylene synthesis and response (Figure 3C; Supplementary Table S1). The expression of two key-genes controlling ethylene synthesis, *1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS)* and *ACC oxidase (ACO)*, was up-regulated by waterlogging. In addition, we found that waterlogging induced accumulation of transcripts encoding for ethylene receptor (ETR). Interestingly, several genes encoding for



**FIGURE 2 | Waterlogging reprogramming transcriptome of *Jatropha* roots. (A)** Selected gene ontology (GO) categories (*p*-values calculated by GOHyperG) from up-regulated (Up) and down-regulated

(Down) groups are shown. **(B)** PAGEMAN enrichment analysis with significant up/down-regulation (Wilcoxon rank sum test; *p*-value < 0.05).



**FIGURE 3 | Waterlogging caused differential expression of transcripts encoding proteins involved in energy, nitrate, and ethylene metabolisms. (A)** Starch metabolism (Starch), glycolysis, and fermentation **(B)** Nitrate metabolism **(C)** Ethylene production and

perception. A plus sign represents induction and a minus sign represents reduction. Numbers in brackets represent numbers of DEGs found in this analysis. Data can be found in **Supplementary Table S1**.

ERF (ethylene responsive factors) transcription factors (TFs) were up-regulated by waterlogging (**Figure 4A**). These marks highlighted the roles of ethylene in acclimation to waterlogging in Jatropha.

#### DIFFERENTIAL REGULATION OF GENES ENCODING TFs

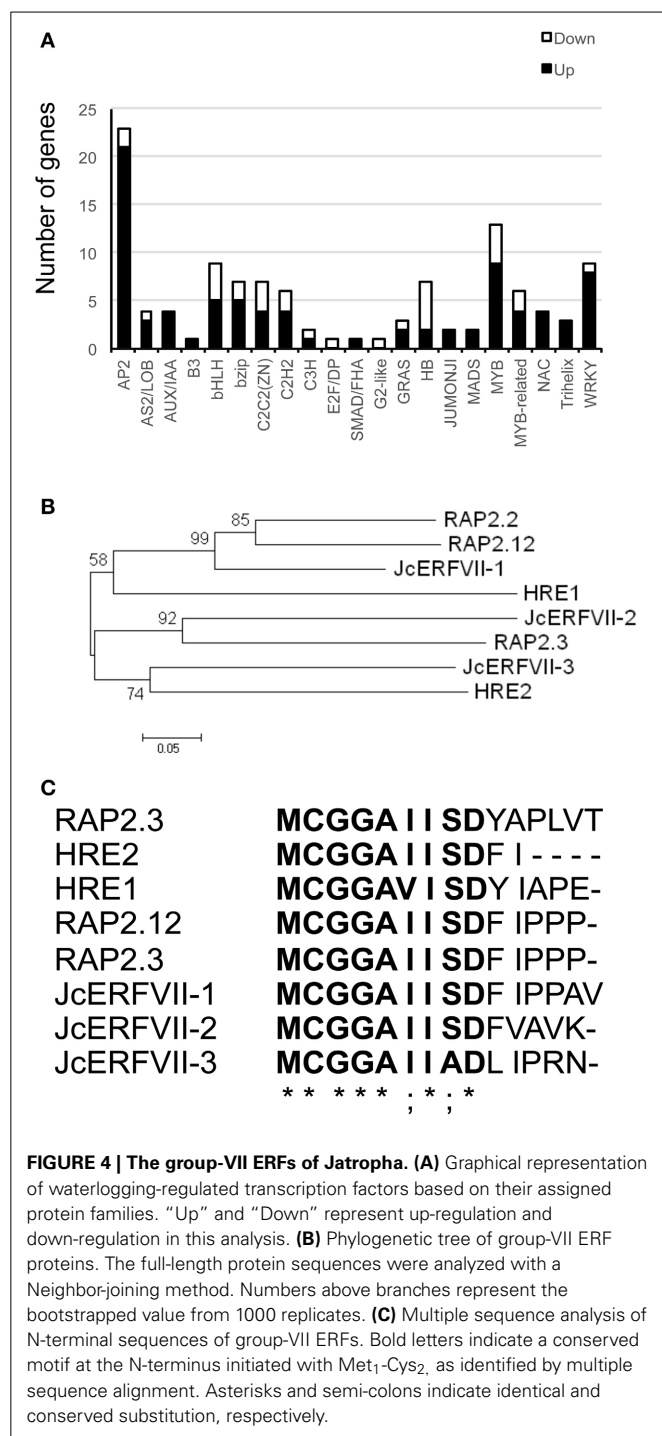
Since low oxygen induced TFs could mediate the expression of anaerobic responsive genes, we analyzed for TFs that were differentially regulated by waterlogging. One-hundred and fifteen waterlogging-regulated TFs were classified based on their assigned protein families (**Figure 4A**; **Supplementary Table S1**). Of these, 85 TFs were up-regulated and 30 TFs were down-regulated. Remarkably, TFs accounted for nearly six percent of the DEGs analyzed in this study. ERFs (21 genes), MYBs (9 genes), and WRKYs (8 genes) were mostly pronounced from the up-regulated group, whereas Homeobox TFs (HBs; 5 genes) were mostly noticeable from the down-regulated group. Recent studies on Arabidopsis demonstrated that group-VII ERFs are modulators of the anaerobic response under low oxygen conditions (Gibbs et al., 2011; Licausi et al., 2011). Therefore, we examined the expression of the group-VII ERFs in Jatropha. Firstly, we searched the Jatropha genome using the HMM profile of the Apetalla2 (AP2)/ERF domain in order to identify all AP2/ERF genes. As a result, 133 putative AP2/ERF genes were identified. Secondly, we performed sequence alignment and phylogenetic analyses to compare Arabidopsis and Jatropha ERFs. Finally, 14 groups of

AP2/ERF genes, including 11 ERF subgroups, AP2, RAV, and soloist were successfully identified (**Supplementary Table S2**). This analysis revealed that the Jatropha group-VII ERFs comprised three members, all possessing a conserved N-terminal domain [ $\text{NH}_2$ -MCGGAI(A/S)D] (**Figures 4B,C**). Remarkably, two of them (*Jcr4S00982.160* and *Jcr4S01651.60*; *JcERFVII-2* and *JcERFVII-3*, respectively) were up-regulated in our transcriptome analysis (**Supplementary Table S1**). Together, these data implicated the essence of transcriptional regulation in response to waterlogging.

#### VALIDATION OF DIFFERENTIALLY REGULATED GENES BY QUANTITATIVE REAL-TIME PCR

To verify the transcriptome data, 10 genes were selected for analysis by quantitative real-time PCR to determine their relative expression in response to WS. These selected genes included 6 waterlogging-induced DEGs [*ADHa*, *ADHb*, *JcERFVII-2*, *JcERFVII-3*, *NR*, and *trihelix TF (trihelix)*], two non-DEGs with limited changes in response to waterlogging (*actin* and *JcERFVII-1*), one waterlogging-repressed DEG (*proline-rich*), and one control for fold change analysis (*UBC*). Real-time PCR results of the same samples used for RNA-seq clearly confirmed that waterlogging induced the accumulation of *ADHa*, *ADHb*, *JcERFVII-2*, *JcERFVII-3*, *NR*, and *trihelix* but not *actin* and *JcERFVII-1* (**Figure 5**). By contrast, mRNA accumulation of *proline-rich*, a gene encoding a cell-wall protein, was clearly reduced upon waterlogging (**Figure 5**). These data provided additional support





**FIGURE 4 | The group-VII ERFs of Jatropha. (A)** Graphical representation of waterlogging-regulated transcription factors based on their assigned protein families. “Up” and “Down” represent up-regulation and down-regulation in this analysis. **(B)** Phylogenetic tree of group-VII ERF proteins. The full-length protein sequences were analyzed with a Neighbor-joining method. Numbers above branches represent the bootstrapped value from 1000 replicates. **(C)** Multiple sequence alignment of N-terminal sequences of group-VII ERFs. Bold letters indicate a conserved motif at the N-terminus initiated with Met<sub>1</sub>-Cys<sub>2</sub>, as identified by multiple sequence alignment. Asterisks and semi-colons indicate identical and conserved substitution, respectively.

that transcriptome analysis by RNA-seq could be used to quantify genome-scale gene expression in the roots of waterlogged Jatropha.

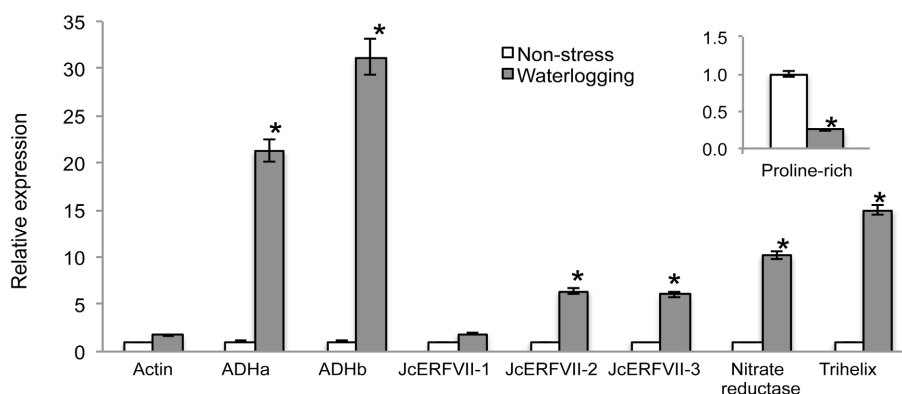
#### COMPARATIVE ANALYSIS OF TRANSCRIPTOME RESPONSE TO WATERLOGGING IN ARABIDOPSIS, JATROPHA, GRAY POPLAR AND RICE

The variation in tolerance to the low oxygen conditions among plant species has been reported (Bailey-Serres and Voesenek,

2008). In order to identify common or specific changes in transcriptional regulation between waterlogging intolerant Arabidopsis and Jatropha and tolerant gray poplar and rice, we first analyzed public microarray data to identify DEGs in response to waterlogging or submergence in Arabidopsis, gray poplar and rice (**Supplementary Table S4**). Additionally, we performed a PAGEMAN over-representation analysis (ORA) using Fisher's exact test and a cut-off value of two to obtain an overview of comparative comparison. PAGEMAN ORA analysis allowed comparison of genes with similar functions. We observed an over-represented induction of genes encoding for anaerobic fermentation in Arabidopsis, Jatropha, and rice (**Figure 6; Supplementary Table S5**). In addition, AP2/ERF DNA binding TFs were up-regulated in all species. The down-regulation of genes involved in cell wall processes and secondary metabolism was common in both tolerant and intolerant species (**Figure 6; Supplementary Table S5**).

Additionally, we attempted to compare changes in expression of orthologs in the genomes of Arabidopsis, Jatropha, gray poplar and rice. OrthoMCL (OMCL) cluster analysis using a Markov Cluster algorithm was applied in order to group orthologs (Li et al., 2003). This analysis identified 19,103 OMCL clusters (**Supplementary Table S5**). Over 7000 OMCL clusters were differentially regulated upon waterlogging (**Supplementary Figure S4**). We found that using a list of 49 core hypoxia genes reported to be up-regulated in response to low oxygen stress in shoots and roots of Arabidopsis seedlings (Mustroph et al., 2009), we were able to identify 37 OMCL clusters among Arabidopsis, poplar, Jatropha, and rice (**Supplementary Figure S5**). Of these, 30 OMCL clusters were induced in at least two species. OMCL236 (wound-responsive proteins), OMCL442 (pyruvate decarboxylase), OMCL450 (alcohol dehydrogenase), and OMCL1101 (ethylene receptor) were induced in all species. We further compared the expression of OMCL clusters involved in carbohydrate cleavage, glycolysis, and fermentation and, in general, found up-regulation of OMCL clusters in these categories (**Supplementary Figure S6**). All together, the results suggested that, regardless of the variation in waterlogging tolerance among plant species, common responses could still be identified.

To examine whether there is a link between tolerance levels and specific molecular mechanisms, we further inspected the PAGEMAN ORA results. We found that multiple processes were specifically regulated in Jatropha. For example, the genes involved in non-mevalonate pathway biosynthesis of isoprenoids, response to stress including heat and touch/wounding, and calcium signaling were up-regulated, while lipid metabolism, cold stress response, and cell organization were down-regulated (**Figure 6**). In rice, we found several protein kinases genes were specifically up-regulated (**Figure 6**). Interestingly, we also found that the down-regulation of genes controlling NO<sub>3</sub><sup>-</sup> metabolism, including NR, was specific to rice (**Figure 6**). An OMCL cluster analysis also confirmed that, while NRs were specifically down-regulated in rice, this was not the case in Arabidopsis and Jatropha (**Figure 7**). Additionally, the analysis also showed that up-regulation of NIR was specific to Jatropha. Evidently, the up-regulation of class I *nsHbs* (OMCL3239), putative NO scavengers,



**FIGURE 5 | Quantitative real-time PCR validation of transcriptome data for selected genes.** Relative expression was normalized to the abundance of Ubiquitin (UBC; *Jcr4S00238.120*). Data represent mean  $\pm$  SE ( $n = 3$ ). \* $p$ -value  $< 0.01$  ( $t$ -test).

was found in *Arabidopsis*, *Jatropha*, and rice. However, the induction levels were much higher in *Jatropha* than in *Arabidopsis* and rice (Figure 7).

Comparative analysis of TFs in response to waterlogging revealed that the expression pattern of TFs was generally conserved in all plant species examined (Supplementary Table S5). OMCL clusters of ERFs, bZIPs, NACs and WRKYs were induced by waterlogging. Interestingly, OMCL clusters of trihelix TFs were found to be up-regulated in *Arabidopsis*, *Jatropha*, and gray poplar but not in rice (Supplementary Table S5). For protein kinases, OMCL clusters of leucine rich repeat kinases XI and XII and wall-associated kinases were induced in much greater quantities in rice (Supplementary Table S5). Examination of OMCL clusters of heat stress and redox regulation revealed that the induction of alternative oxidase was found only in *Jatropha*. Up-regulation of heat-shock proteins (HSPs), were commonly found in both intolerant and tolerant species (Supplementary Table S5). Interestingly, OMCL clusters of peroxidases were greatly induced in rice (Supplementary Table S5).

## DISCUSSION

In this study, high-throughput RNA-sequencing technology was employed to compare differential gene expression profiles of *Jatropha* roots subjected to 24 h WS. Our study provides a new insight into understanding the molecular mechanisms underlying the response to waterlogging in *Jatropha*. WS reprogrammed expression of 1968 DEGs; seven of these were further confirmed to be differentially expressed by quantitative real-time PCR (Figure 5). By applying bioinformatic analyses for non-model species, our study demonstrated that a number of cellular processes in *Jatropha* roots were affected by WS. These included anaerobic fermentation, carbohydrate metabolism, glycolysis, ethylene synthesis and perception,  $\text{NO}_3^-$  metabolism, NO production, regulation of transcription, protein degradation, transport, and signaling, suggesting that diverse physiological processes were affected by WS.

Plants subjected to low oxygen conditions shift their metabolism from oxidative phosphorylation to anaerobic

fermentation to maintain ATP production (Bailey-Serres et al., 2012). Our results supported a longstanding notion that waterlogging promotes anaerobic respiration as observed by the up-regulation of DEGs encoding enzymes in glycolysis and fermentation (Figure 3; Supplementary Table S1). Remarkably, the expression of *Alternative oxidase* (AOX) was specifically enhanced in roots of waterlogged *Jatropha* (*Jcr4S02312.80*, Supplementary Table S1) implying that AOX may function as an alternative to cytochrome oxidase under low oxygen conditions. The increase in AOX gene expression could prevent reactive oxygen species (ROS) formation from the over-reduction of the ubiquinone pool. Plants can initiate additional responses to low oxygen conditions, including down-regulation of energy consuming processes such as storage metabolism (Geigenberger et al., 2000) and switching from invertase to sucrose synthase for hydrolysis of sucrose (e.g., Guglielminetti et al., 1995, 1997; Biemelt et al., 1999; Zeng et al., 1999; Albrecht and Mustroph, 2003; Bologna et al., 2003; Bieniawska et al., 2007). Here, we observed in the roots of waterlogged *Jatropha*, the down-regulation of genes involved in the synthesis of the cell wall, DNA, secondary metabolites, and starch (Figure 2) and the up-regulation of sucrose synthase (Figure 3A). These results, together with the finding that waterlogging resulted in the decrease in total root carbohydrate (Supplementary Figure 1B) provided a strong conclusion that *Jatropha* roots responded to waterlogging via the regulation of energy consumption and production.

Low oxygen conditions in plants promote the utilization of  $\text{NO}_3^-$  and the production of NO to facilitate anaerobic survival (Horchani et al., 2010; Oliveira et al., 2013; Oliveira and Sodek, 2013). Alternative to anaerobic fermentation,  $\text{NO}_3^-$  could be used as NADH acceptors. This allows  $\text{NAD}^+$  to be reused in glycolysis. It has been observed that longer periods of low oxygen survival can be achieved when  $\text{NO}_3^-$  is provided (Allegre et al., 2004; Horchani et al., 2010). In waterlogged *Jatropha* roots, the expression of *NR*, *NiR*, and *nitrate transporter* was induced (Figure 4B; Supplementary Table S1), emphasizing the roles of  $\text{NO}_3^-$  in waterlogging acclimation. Future studies should focus more on investigating the significant

Over-represent		UP												DOWN											
Z-score	3	JCA		ATH				PTR			OSA			JCA		ATH				PTR			OSA		
	2																								
	1																								
	0																								
-1																									
-2																									
-3																									
	Bin Name	Root24h	Root7h -Light	Root24h -Light	Root7h -Dark	Root24h -Dark	Root5h	Root24h	Root96h	Leaf-M202-24h	Leaf-Sub1-24h	Root24h	Root7h -Light	Root24h -Light	Root7h -Dark	Root24h -Dark	Root5h	Root24h	Root96h	Leaf-M202-24h	Leaf-Sub1-24h				
	major CHO metabolism	0	2.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	minor CHO metabolism	0	2.8	0	0	0	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0	0				
	fermentation	2.7	3.6	3.7	0	2.2	0	0	0	3.1	2.8	-2.2	0	0	0	0	0	0	0	0	0				
	gluconeogenesis - glyoxylate cycle	0	3.0	3.0	0	0	0	0	0	3.4	3.6	0	0	0	0	0	0	0	0	0	0				
	OPP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.9				
	cell wall	-4.3	0	-2.2	0	0	0	0	0	0	0	4.7	8.1	9.1	0	0	0	4.2	0	0	0				
	lipid metabolism	-2.6	0	0	0	0	0	0	0	-3.2	-2.9	2.2	0	0	0	0	0	0	0	0	0				
	N-metabolism.nitrate metabolism	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.3	2.7				
	- Nitrate reductase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.1	2.4				
	amino acid metabolism	0	5.6	5.2	0	0	0	0	0	0	0	-2.2	-2.6	0	0	0	0	0	0	0	2.0				
	S-assimilation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.3	3.1				
	metal handling.acquisition	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.3				
	secondary metabolism	0	0	0	0	0	0	0	0	0	0	2.8	3.0	4.3	0	0	0	0	0	4.7	4.8				
	- isoprenoids.non-mevalonate pathway	2.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	hormone metabolism	0	2.8	0	0	0	0	0	0	0	0	2.2	2.0	0	0	0	0	0	0	0	2.1				
	- ethylene	3.8	3.9	0	0	0	0	2.1	0	0	0	-3.3	0	0	0	0	0	0	0	0	0				
	Co-factor and vitamin metabolism	2.1	2.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	stress	0	0	0	0	0	0	0	0	4.2	5.8	0	2.0	2.9	0	0	0	0	0	0	0				
	- heat	2.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	- cold	-2.5	0	0	0	0	0	2.9	0	0	0	3.0	0	0	0	0	0	0	0	0	0				
	- touch -wounding	2.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	redox.dismutases and catalases	0	2.6	2.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	polyamine metabolism	0	0	0	0	0	0	0	0	0	0	0	2.6	2.4	0	0	0	0	0	0	0				
	RNA	4.5	0	0	0	0	0	0	0	3.2	-4.7	-2.9	-5.7	0	0	0	-2.0	-2.3	0	-3.9	-4.9				
	- AP2-EREBP	3.0	3.1	2.8	0	0	2.4	0	0	4.7	5.5	-2.4	0	0	0	0	0	-2.1	0	0	-2.1				
	- NAC	2.1	0	0	0	0	0	0	0	2.5	2.4	0	0	0	0	0	0	0	0	0	0				
	- WRKY	2.7	0	0	0	0	0	0	0	3.6	-2.1	0	0	0	0	0	0	0	0	0	0				
	- Pseudo ARR	0	3.3	3.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	DNA.synthesis -chromatin structure	-3.3	0	0	0	0	0	0	0	0	0	2.9	0	0	0	0	0	0	0	0	0				
	protein.synthesis.ribosomal protein	2.1	-2.0	-2.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	protein.degradation.ubiquitin.E3	2.0	0	2.2	0	0	0	0	0	0	0	-3.5	-4.8	-6.7	0	0	0	-2.3	0	-4.4	-4.0				
	- E3.SCF	2.0	0	0	0	0	0	0	0	0	0	-3.5	-3.1	-4.4	0	0	0	0	0	-2.9	-3.2				
	- E3.SCF.FBOX	2.1	0	0	0	0	0	0	0	0	0	-3.3	-3.0	-4.2	0	0	0	0	0	-2.9	-3.2				
	- E3.BTB -POZ Cullin3	0	0	3.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	- E3.BTB -POZ Cullin3.BTB -POZ	0	0	3.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	signalling	0	0	-2.1	0	0	-3.0	0	0	6.5	6.5	0	0	0	0	0	4.3	0	0	-3.9	-3.8				
	- receptor kinases	0	0	0	0	0	-2.5	0	0	9.2	9.4	2.4	0	0	0	0	4.2	0	0	-4.5	-4.5				
	- receptor kinases.leucine rich repeat III	-3.1	0	0	0	0	0	0	0	0	0	3.0	0	0	0	0	0	0	0	0	0				
	- receptor kinases.leucine rich repeat XI	0	0	0	0	0	-2.4	0	0	2.8	3.3	0	0	0	0	0	5.2	0	0	-2.6	0				
	- receptor kinases.DUF 26	0	0	0	0	0	0	0	0	7.4	8.2	0	0	0	0	0	0	0	0	0	-2.3				
	- receptor kinases.wall associated kinase	0	0	0	0	0	0	0	0	3.6	3.5	0	0	0	0	0	0	0	0	-2.2	-2.2				
	- receptor kinases.misc	0	0	0	0	0	0	0	0	4.5	3.3	0	0	0	0	0	0	0	0	-2.2	0				
	- calcium	2.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
	cell	-3.4	-3.0	0	0	0	0	0	0	0	0	3.1	0	0	0	0	0	0	0	-2.3	-3.3				
	- organisation	-3.6	0	0	0	0	0	0	0	0	0	3.0	0	0	0	0	0	0	0	-2.0	-2.3				
	development	0	0	2.8	0	0	0	0	0	3.3	3.5	0	0	0	0	0	0	0	0	0	0				
	transport	0	0	0	0	0	0	0	0	-2.7	-2.0	0	2.6	0	0	2.5	0	0	0	4.5	4.6				
	- sugars	2.7	2.4	0	0	0	0	0	0	0	0	-2.1	0	0	0	0	0	0	0	0	0				
	- amino acids	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-2.5	0	0	2.4				
	- sulphate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.3				
	- metal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.0	0	0	0	2.1	0				
	- unspecified cations	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.3	2.0				
	- potassium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.0	0				
	- ABC transporters and multidrug resistance systems	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.0	2.4	0	0	2.2	2.6				
	- Major Intrinsic Proteins	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.7	2.4				
	- Major Intrinsic Proteins.PIP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.1	2.9				

**FIGURE 6 | Overview of transcriptome response for selected functional categories to waterlogging or submergence in *Arabidopsis* (ATH), *Jatropha* (JCA), gray poplar (PTR), and rice (OSA).** PAGEMAN analysis of the gene expression data ( $|\log_2$  fold change| > 1; FDR < 0.05). Statistical

analysis of over-represented functional bins was carried out using Fisher method. Z-scores indicate over/under representation (Numbers indicate z-scores; Green, over-represented; Red, under-represented). Data used to generate this figure can be found in **Supplementary Table S5**.

OMCL family	Gene ID	Species	mean logFC
OMCL2496 Nitrate reductase	AT1G37130	ATH	1.539
	AT1G77760	ATH	-3.882
	Jcr4S02321.10	JCA	5.811
	LOC_Os02g53130	OSA	-5.739
	LOC_Os08g36480	OSA	-3.730
	LOC_Os08g36500	OSA	NA
	Potri.005G172400	PTR	NA
OMCL7779 Nitrite reductase	Potri.002G088600	PTR	NA
	AT2G15620	ATH	-1.750
	Jcr4S01372.10	JCA	4.292
	LOC_Os01g25484	OSA	-1.778
	LOC_Os02g52730	OSA	NA
	Potri.004G140800	PTR	NA
OMCL3239 Non-symbiotic hemoglobin (Class I)	AT2G16060	ATH	2.983
	Jcr4S02362.60	JCA	7.966
	LOC_Os03g12510	OSA	1.046
	LOC_Os03g13140	OSA	NA
	LOC_Os03g13150	OSA	NA
	LOC_Os03g13160	OSA	NA
	Potri.009G110800	PTR	NA
OMCL18700 Non-symbiotic hemoglobin (Class II)	AT3G10520	ATH	-1.278
	Jcr4S01154.10	JCA	NA
	AT4G32690	ATH	1.057
OMCL10097 Non-symbiotic hemoglobin (Class III)	Jcr4S01353.90	JCA	3.897
	LOC_Os06g39140	OSA	NA
	Potri.006G244300	PTR	NA

**FIGURE 7 | Response of OMCL clusters with genes involved in nitrogen metabolism and NO production to waterlogging.** Yellow, Up-regulated DEGs; Blue, Down-regulated DEGs; White, Orthologs not differentially expressed. Numbers represent mean log<sub>2</sub> fold change values.

of NR, NiR, and NO<sub>3</sub><sup>-</sup> on tolerance to waterlogging in *Jatropha*.

Accumulation of the gas hormone ethylene is extremely important for the induction of plant responses to low oxygen. Since waterlogging inhibits gas diffusion, ethylene can be trapped in the waterlogged plant organs. Our transcriptome data revealed that root waterlogging promoted mRNA accumulation of two key enzymes in ethylene biosynthesis, ACS and ACO (Figure 3C; Supplementary Table S1). Concomitantly, the expression of *ETR*, was enhanced in waterlogged *Jatropha* roots. These findings suggested that waterlogging might increase ethylene synthesis and perception in *Jatropha* roots. Our study recognized three group-VII ERFs from the *Jatropha* genome. Two of these, designated JcERFVII-2 and JcERFVII-3, were noticeably induced in response to waterlogging (Figures 4, 5; Supplementary Table S1). Previous studies identified the two group-VII ERF genes, *Snorkel* and *Sub1*, as key players in the submergence response in deepwater rice and lowland rice varieties, respectively, (Xu et al., 2006; Hattori et al., 2009). These two rice varieties utilize two contrasting strategies that allow adaptive growth responses to submergence. *Snorkel* promotes the internode elongation in the deepwater rice, whereas, *Sub1* restricts the shoot elongation in the lowland rice. In Arabidopsis, studies have also shown that the group-VII ERFs function in the low-oxygen response (Hinz et al., 2010; Gibbs et al., 2011; Licausi et al., 2011). Taken together, we propose that the group-VII ERFs are promising candidates for engineering of waterlogging tolerant *Jatropha*. In support of this idea, overexpression of the Arabidopsis group-VII ERFs (HRE1, HRE2, RAP2.2, and

RAP2.12) significantly improved low oxygen survival by promoting expression of the genes involved in low oxygen adaptation (Hinz et al., 2010; Gibbs et al., 2011; Licausi et al., 2011).

Enhanced NO production by low oxygen conditions has been documented in plants (Rockel et al., 2002; Dordas et al., 2003). NO is believed to be a signaling molecule. As previously mentioned, NO can be synthesized from NO<sub>3</sub><sup>-</sup> via NR and NiR (Stohr et al., 2001). In Arabidopsis, it was reported that accumulation of NO could induce AOX expression via inhibition of aconitase resulting in accumulation of citrate and a shift of metabolism toward nitrogen assimilation under hypoxia (Gupta et al., 2012). A recent study revealed that NO could control the stability of Arabidopsis group-VII ERFs via proteolytic modulation (Gibbs et al., 2014). We speculate that enhanced NO accumulation in *Jatropha* roots under low oxygen conditions could destabilize the group-VII ERF proteins and, therefore, decrease low oxygen tolerance. Modulation of NO accumulation via nsHbs in roots under low oxygen conditions has been reported (Dordas et al., 2003, 2004; Perazzolli et al., 2004; Hebelstrup et al., 2006). Our study demonstrated that waterlogging could induce the expression of nsHbs in *Jatropha* roots (Figure 7; Supplementary Table S1). The function of nsHbs possibly involves fine-tuning the accumulation of NO in waterlogged roots of *Jatropha*. Future research to improve waterlogging tolerance should emphasize genetic engineering to modulate NO levels.

Nearly six percent of DEGs encoding for TFs was identified in this study, implying that transcriptional regulation plays an important role in the waterlogging response. In agreement with the finding that ethylene synthesis and perception were activated, this study recognized AP2/ERF TFs as the most pronounced TFs in response to waterlogging (Figure 4A). Waterlogging induced AP2/ERFs included two of group-VII ERFs and an additional 19 ERF, whose function might be associated with waterlogging acclimation (Supplementary Table S1). The ERF IX group was the most represented waterlogging-induced ERFs in this study (Supplementary Table S1). The function of the group-IX ERFs was associated with hormonal response, such as the responses to ethylene (Wang et al., 2007) or ethylene in combination with jasmonate (Champion et al., 2009). The induction of *JcERFX-6* (*Jcr4S03895.40*; OMCL 2117), an ortholog of Arabidopsis *RAP2.6L*, was evident in this analysis (Supplementary Table S1). Overexpression of *RAP2.6L* in Arabidopsis improved the waterlogging response by delaying waterlogging-induced premature senescence (Liu et al., 2012). In this study, the expression of *trihelix TF* (*Jcr4S02762.30*; OMCL11085), an ortholog of Arabidopsis *HYPOXIA RESPONSE ATTENUATOR1* (*HRA1*), was found to be strongly up-regulated by waterlogging (Supplementary Table S1). Recently, Giuntoli et al. (2014) demonstrated that *HRA1* negatively regulates *RAP2.12* through protein–protein interaction and proposed that the modulation of the aerobic response by group-VII ERFs and *HRA1* is important to control plant responses to low oxygen stress. In addition, our analysis demonstrated that waterlogging enhanced the expression of several members of MYBs and WRKYs. An in-depth functional analysis of waterlogging-induced TFs is required to further identify candidate genes for growth improvement of waterlogged *Jatropha*.



Previous studies reported the use of comparative transcriptome comparison for identification of conserve and species-specific responses to low oxygen stress (Mustroph et al., 2010; Narsai et al., 2011; Narsai and Whelan, 2013). In this study, comparative transcriptome analysis between *Jatropha* and other plant species identified a common response to waterlogging including the up-regulation of carbohydrate cleavage, glycolysis and fermentative genes and the down-regulation of genes involved in cell-wall and secondary metabolite biosynthesis (**Figure 6; Supplementary Table S5**). These findings also confirmed previous reports that the alteration of carbohydrate metabolism appeared to be a common response to low oxygen stress in plants. Additionally, Arabidopsis, gray poplar and *Jatropha* showed the up-regulation of genes involved in ethylene production and signaling (**Figure 6; Supplementary Table S5**), which were commonly found in response to low oxygen in several plants. The up-regulation of AP2/ERF genes by waterlogging was commonly identified in all species examined (**Figure 6; Supplementary Table S5**). These results strongly emphasized the roles of AP2/ERF genes in low oxygen adaptation. While comparative analysis exposed the common response to waterlogging in plants, the analysis showed that various processes were specifically regulated in each species. Contrary to what has been observed in waterlogged *Jatropha*, the expression of *NR* was down-regulated in submerged rice (**Figure 7**) suggesting the down-regulation of the *NR* gene may be a characteristic of waterlogging-tolerant species.

Global warming results in extreme climates, such as drought, flooding and heat stress. Improvement of combined stress tolerance could benefit crop production. It is widely known that stresses including drought, flooding, and high temperatures, could trigger accumulation of ROS, which causes oxidative damage of cellular components. On the other hand, ROS serves as a critical signaling molecule in the oxidative stress response. Pucciariello et al. (2012) demonstrated a connection in Arabidopsis between redox sensing and low oxygen response of seedlings. Evidence from the study of Fukao et al. (2011) showed that submergence-tolerant rice overexpressing *Sub1A* gene exhibited drought-tolerance phenotypes through oxidative stress regulation. Additionally, Loreti et al. (2005) found that several HSPs were induced by low oxygen stress and heat pretreatment enhanced anoxia tolerance of seedlings. Later on, Banti et al. (2008) demonstrated a correlation between the up-regulation of HSP mRNAs and enhanced low oxygen tolerance in Arabidopsis. These findings highlighted the roles of HSPs and ROSs in plant low oxygen acclimation. In this study, we found that waterlogging induced several OMCL clusters of HSPs and ROS enzymes in the roots of *Jatropha* (**Supplementary Table S5**). Taken together, it is possible that genetically engineered, waterlogging-tolerant crops might confer multiple stress tolerance phenotypes.

In summary, *Jatropha* orchestrates a complex transcriptional adjustment in response to WS that yields changes in its root physiological response to WS. This study highlights several possibilities for future investigation, including the roles of specific pathways and genes involved in the waterlogging response. Thus, a detailed characterization of individual genes must be carried out as the first step to understand their specific functions. Due to

the high similarity among global *J. curcas* accessions, as reported by Popluechai et al. (2009), the improvement of waterlogging-tolerant *Jatropha* through traditional breeding, can be difficult to achieve. With biotechnology advances, genetic engineering and gene transformation into *Jatropha* are now feasible (Mukherjee et al., 2011). A recently developed protocol by Jaganath et al. (2014) enables Agrobacterium-mediated in planta transformation into *Jatropha* that does not require *in vitro*-based multiplication of transformed plants. Clearly, this method has the potential to facilitate the genetic modification of *Jatropha*. Successfully validated candidate genes could be targeted for the engineering of waterlogging-tolerant *Jatropha*.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00658/abstract>

**Supplementary Figure S1 | Physiological responses of *Jatropha* seedlings to waterlogging.** (A) Total leaf chlorophyll content. Data represent mean  $\pm$  SE ( $n = 6$ ). (B) Total root carbohydrate content. Data represent mean  $\pm$  SE ( $n = 3$ ). Letters represent significant differences calculated by One-Way ANOVA ( $p$ -value  $< 0.05$ ).

**Supplementary Figure S2 | Change in the expression of selected fermentative genes in response to waterlogging in leaves and roots of *Jatropha*.** Semi-quantitative PCR analysis of fermentative genes (*ADHs* and *PDC*) from 24-h waterlogged (WS) and non-stressed (NS) *Jatropha* roots and leaves.

**Supplementary Figure S3 | Statistic and quality control of RNA-seq data.** (A) Mapping statistics (B) Pearson's correlation derived from CPM values. (C) A multi-dimension scaling plot of gene expression data. NSR, non-stressed root sample; WSR, waterlogged root sample. Numbers represent numbers of replicates.

**Supplementary Figure S4 | Differentially regulated OMCL clusters grouped by the number of organisms per cluster.**

**Supplementary Figure S5 | Waterlogging responsive OMCL clusters containing "49 core hypoxia" genes (Mustroph et al., 2009, 2010).** Arabidopsis (ATH), *Jatropha* (JCA), gray poplar (PTR), and rice (OSA). Numbers indicated numbers of induced genes found in clusters. Yellow, induced orthologs; White, no induced orthologs; Gray (NA), no orthologs identified.

**Supplementary Figure S6 | OMCL clusters with genes involved in glycolysis and fermentation.** OMCL clusters with change in at least one species were included. Up, Up-regulated DEGs; Down, Down-regulated

DEGs. Bright yellow, Cluster has >1 induced DEG; Light yellow, Cluster has 1 induced DEG; Bright blue, Cluster includes >1 reduced DEG in; Light blue, Cluster includes 1 reduced DEG; White, No DEG ortholog identified; Gray, ortholog not identified.

#### Supplementary Table S1 | Analysis of differentially regulated genes.

#### Supplementary Table S2 | A list of *Jatropha* AP2/ERF transcription factors.

#### Supplementary Table S3 | Real-time PCR primer information.

#### Supplementary Table S4 | Analysis of waterlogging transcriptome from *Jatropha*, *Arabidopsis*, gray poplar, and rice.

#### Supplementary Table S5 | Comparative analysis of waterlogging transcriptome.

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