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Transcriptional responses of *Arabidopsis thaliana* to oil contamination

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Highlights:

1) The expression of 340 genes was modulated by water soluble fraction of marine fuel.

2) The stress leads to a fast initial (2h) transcriptome response.

3) *HSP* genes were up-regulated by the stress.

4) Iron deficiency genes were down-regulated by the stress.

5) WSF-MF380 encompasses a general abiotic stress response.

ABSTRACT

The growing demand for fossil-based energy sources has increased oil pollution worldwide. Oil pollution is harmful because 75% of its components are polycyclic aromatic hydrocarbons, which are molecules known to present carcinogenic, mutagenic and teratogenic effects. Mangrove areas are commonly affected by petroleum accidents, making these ecosystems particularly sensitive to oil pollution. In order to elucidate the molecular response of a petroleum mixture on plants, the global gene expression analysis of 10-day-old *Arabidopsis thaliana* exposed to the water soluble fraction of the marine fuel MF380 (WSF-MF380) was evaluated by a 24 h time-course microarray. The microarray results revealed that 340 genes were modulated by WSF-MF380 stress; these genes were distributed in 12 clusters according to their expression profile. Different classes of biological processes were overrepresented, such as the response to heat, hypoxia, oxidative and osmotic stresses. The expression pattern of five transcription factors and 16 possible targets with enriched *cis*-regulatory elements was further investigated by qPCR at three selected time-points, revealing that the expression of selected target genes are in accordance with the expression profile of their possible regulator. We further demonstrate that WSF-MF380 stress gives rise to a high induction of genes at the initial time-point t2h, indicating a rapid molecular response in plants. At t2h, many *HSP* genes were induced, showing a similar expression profile in WSF-MF380 and heat stresses. In contrast to the initial induction, many iron deficiency genes, such as *bHLH38*, *bHLH39*, *bHLH100* and *bHLH101*, were down regulated from t3h to t24h, with no GUS activity observed in the roots of *pbHLH38::GUS* transgenic *A. thaliana* after 16 h of exposure. Taken together, these analyses demonstrate that WSF-MF380 exposure seems to encompass a general response to abiotic stresses in plants, which could be an evidence of the complex chemical composition of the oil.

Keywords: Petroleum; Marine oil pollution; oxidative stress; Abiotic stress; heat stress; hypoxia.

1

[1]Abbreviations:

1- INTRODUCTION

Plants are constantly confronted with unfavorable growth conditions, such as drought, high salinity and extreme temperature. Their defenses to these abiotic stresses hinge on the reprogramming of gene expression through the regulation of transcription, which results in several effects including the inhibition of cell division and expansion, the disruption of ion homeostasis and the production of reactive oxygen species (Kant et al., 2008; Vaahtera and Brosché, 2011).

Although many abiotic stresses have been widely studied, little is known about the molecular responses of plants to oil contamination. The growing demand for energy sources has resulted in several oil spills, which has increased oil pollution worldwide. For instance, 1300 m^3 of marine fuel MF-380 were discharge in Guanabara Bay (Rio de Janeiro, Brazil) owing to the pipeline rupture at Duque de Caxias in 2000 (Reduc-Petrobras). Mangrove areas are commonly affected by these accidents, making this ecosystem one of the most sensitive with both lethal and sublethal effects (Getter, 1981; Noaa, 2010).

Oil is a complex mixture of many chemical elements, but it is estimated that approximately 75% of its components are polycyclic aromatic hydrocarbons (PAHs) (Ralph and Burchett, 1998). PAHs are a family of hydrophobic environmental toxins introduced into aquatic environments mainly by oil spills and discharges from industrial, municipal and urban operations (Cho and Kim, 2006). Processes such as evaporation, photo-oxidation and biodegradation may mask the distribution of hydrocarbons in the sediment, making the identification of spilled material a difficult

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BP: Biological Processes
FC: Fold-change values
GO: Gene Ontology
GSEA: Gene Set Enrichment Analysis
GT: Glycosyl Transferase
GUS: β-glucuronidase
HSFs: Heat Shock Transcriptional Factors
HSPs: Heat Shock Proteins
PAHs: Polycyclic Aromatic Hydrocarbons
ROS: Reactive Oxygen Species
TFs: Transcriptional Factors
TFBS: Transcription Factor Binding Sites
WSF-MF380: Water Soluble Fraction of Marine Fuel MF380

task (Kingston, 2002). PAHs are carcinogenic, mutagenic and teratogenic, and pose a high risk to human health (Habe and Omori, 2003). In plants, they cause injuries in all growth stages, from germination to reproduction (Pasková et al., 2006). Previous studies have shown that PAHs induce oxidative stress, reduce growth, and cause leaf deformation and tissue necrosis in *Arabidopsis thaliana* (Liu et al., 2009; Alkio et al., 2005). Plants subjected to phenanthrene, a PAH composed of three fused benzene rings, exhibited disturbs in signaling and metabolic pathways that regulate reactive oxygen species (ROS) and responses related to pathogen defense (Weisman et al., 2010).

However, it is unclear if petroleum mixtures trigger stress signaling pathways common to other stresses, or if specific oil responses exist. Although plants have evolve different molecular pathways to respond to various forms of abiotic stresses during evolution, oil stress is a relative recent event that probably did not have time to activate specific responses. In order to elucidate the molecular effects of petroleum on plants, the global gene expression analysis of 10-day-old *A. thaliana* exposed to the water soluble fraction of the marine fuel MF380 (WSF-MF380) was evaluated using a time-course whole genome microarray analysis. This analysis revealed 340 genes modulated by WSF-MF380 stress and several overrepresented classes of biological processes that correspond to different types of abiotic stresses. We hypothesized that oil stress encompass a general response to abiotic stresses and that the molecular effects of oil exposure in plants should induce gene expression responses similar to other abiotic stresses such as heat, hypoxia, oxidative and osmotic stresses.

Given that *A. thaliana* is a model species, and that its seedlings can initially develop in aquatic environments, our study could be translate to other plant species, such as mangrove flora, and might contribute to the knowledge on how early petrochemical pollution affects gene expression patterns. Management approaches to damaged areas might also benefit from a better understanding of the consequences of marine oil exposure in plants. In addition this analysis will be also useful to highlight specific marker genes of petrochemical contamination in plants that could be implemented to evaluate the oil disturbance.

2- MATERIALS AND METHODS

2.1- WSF-MF380 preparation

The water soluble fraction of the marine fuel MF-380 (WSF-MF380) was obtained by 24 h of rapid stirring (Fisatom 752, Perdizes, Brazil) of the oil and autoclaved MilliQ® water at a proportion of 20% (v/v) in a beaker protected from light and sealed with parafilm. The mixture was stored for one hour to allow phase separation and the supernatant was removed with a 20 mL disposable syringe. The WSF-MF380 was transferred to another container and stored at room temperature protected from light (adapted from Ralph & Burchett, 1998). All glassware was pre-treated with a mild detergent (Extran®), soaked in a 2 M HNO_3 acid-bath for 24 h, rinsed in distilled water and sterilized in an oven.

2.2- Growth conditions, WSF-MF380 treatment of *A. thaliana* seedlings and plants biomass measurement

Seeds of *A. thaliana* ecotype Columbia (Col-0) were sterilized for ten minutes with 70% ethanol plus 0.05% TWEEN®20 detergent, washed with absolute ethanol and air dried to ensure that no ethanol remained on the seeds. Fifty seeds were transferred to 250 mL Erlenmeyer flasks containing 50 mL of half-strength Murashige and Skoog (MS) liquid medium (Murashige and Skoog, 1962) with 45 mM sucrose and vitamins (pH 5.8) (adapted from Beynon et al., 2009). The flasks were kept at 4°C for three days, and they were then transferred to a growth room at 22 ±1°C with a 16 h/8 h photoperiod (100 µmol m^{-2} s^{-1}). Flasks were maintained on a rotary shaker at 125 rpm throughout the experiment (Certomat MOII, B. Braun Biotech International, Germany) (Beynon et al., 2009).

Ten-day-old seedlings had their medium replaced with a new MS½ medium with 45 mM sucrose and vitamins (the control treatment) or with 25 mL of MS medium with 45 mM sucrose and vitamins plus 25 mL of WSF-MF380 (the WSF-MF380 treatment). The plants were maintained under identical growth conditions and were collected at a start time control (t0h) and ten points after WSF-MF380 treatment: t1h, t2h, t3h, t5:15h, t7:30h, t9:45h, t12h, t16h, t20h and t24h. At each sampling point, two flasks (one control and one WSF-MF380 treatment) were removed from the growth chamber; the samples were immediately soaked in liquid

nitrogen and stored at -80°C. The assay was performed again in order to obtain biological replicates.

Total dry weight of control and WSF-MF380 treated plants at t12h and t24h was determined by oven dry plants at 60°C until constant weight for measurement of plants biomass.

2.3- Histochemical analysis

Seeds with the promoter of the *bHLH38* gene fused to *β-glucuronidase* (GUS) reporter gene were kindly provided by Dr. Petra Bauer's group. Control and WSF-MF380 treated seedlings of the *pbHLH38::GUS A. thaliana* lineages were collected after 2 h and 16 h of oil exposure in 1.5 mL tubes for GUS histochemical analysis. Later, 300 µL of 90% acetone was added, and the tubes were maintained in -20°C for 30 min. The whole seedlings were washed twice in sodium phosphate 0.1 M (pH 7.0) and incubated overnight at 37°C in GUS solution [400 µL of sodium phosphate 0.1 M (pH 7.0), 4 µL of $K_3Fe(CN)_6$ 0.5 mM, 1 µL of $K_4Fe(CN)_6$ 0.5 mM and 4 µL of X-Gluc 200 mM]. Then, GUS solution was replaced by 70% ethanol for de-staining. The experiment was performed twice to obtain biological replicates, and the material was stored in 50% glycerol at 4°C.

2.4- RNA extraction and DNA microarray hybridization

RNA was extracted using the "Plant RNeasy" kit (QIAGEN) according to the manufacturer's protocols with on-column DNAse digestion to remove residual genomic DNA. The quality of the total RNA was determined using a Nanodrop™ ND-1000 (Thermo Scientific, Inc., Wilmington, DE, USA) and a Bioanalyzer using the Agilent Nano kit (Agilent Technologies, Inc., Santa Clara, USA). The amplification process was carried out with the Aminoalil MessageAmp aRNA II™ kit (Ambion) from 1 µg of total RNA following the manufacturer's protocol. Overall, 10 µg of each amplified aRNA was labeled with cyanine-3 (Cy3) or cyanine-5 (Cy5) (GE) in a coupling reaction of fluorophores for two hours at 16°C and purified. The marked aRNA was again measured to calculate of normalized amounts of RNA from the samples to be hybridized in the same slide, following the manufacturer's protocol (Ambion). Cross markings were made on 4X44K Arabidopsis microarray glass slides,

containing all genes of *A. thaliana* genome, customized by Dr. Riechmann's group in the Center for Research in Agricultural Genomics (Barcelona, Spain) on the Agilent Technologies platform. The experimental set-up used three slides, each with four blocks, thereby allowing 12 hybridizations. Two of these blocks were hybridized with t0h control samples (Cy3) x t0h (Cy5), and the remaining ten blocks were hybridized with t0h samples (Cy5) x WSF-MF380 (Cy3) samples following the extended reference design (or augmented reference design) (Kerr and Churchill, 2001). From this point, the Two-Color Microarray-Based Gene Expression Analysis protocol (Agilent) was followed. The slides were scanned in an Agilent Microarray Scanner (G2565BA) (Agilent Technologies, Inc., Santa Clara, USA), and the intensity data were extracted with Agilent Feature Extraction program (version 10.7.3.1), using the standard options of the protocol.

2.5- Bioinformatics microarray analysis

The pre-processing and differential expression analysis of the gene expression data was performed in R software using BioConductor's libraries (Gentleman et al., 2004). The data were preprocessed using the BioConductor library Agi4x44PreProcess (López-Romero et al., 2010). We selected MeanSignal to signal the foreground, among the collection of signals provided by the image analysis program Agilent Feature Extraction (AFE). This signal was used for data normalization and scaling. Before that, hybridization control probes and those that did not contain TAIR identification (The Arabidopsis Information Resource; available at http://www.arabidopsis.org/) were removed. Internal replication probes within the slide blocks were summarized by the foreground signal arithmetic mean. The data were then normalized by the global loess method, implemented in the LIMMA BioConductor library (Smyth, 2004) without prior background subtraction according to Zahurak et al. (2007). Then, the normalized data were scaled by the quantile method (Bolstad et al., 2003), also implemented in the LIMMA library (Smyth, 2004).

The arithmetic means of the probes of control blocks (t0h x t0h) were calculated. The functional annotation of the probes was constructed from TAIR identifiers corresponding to each probe, a customized library, that we called "Arabidopsis.db", for the Agilent array developed by Dr. Riechmann's group. For each gene expression profile relative to the t0h stage (Fold-change values; FC), the

coefficient of variation was calculated. Those with a coefficient of variation of FC-values ≥ 0.7 or ≤ -0.7 were characterized as "modulated genes" used in a Bayesian hierarchical clustering analysis (Savage et al., 2009). Beforehand, FC-values were categorized in five levels of modulation relative to the t0h stage: (1) highly repressed, (2) suppressed, (3) normal-expressed, (4) induced, and (5) very induced, and these were used as a criterion for categorizing the FC-value percentiles 0-20, 20-40, 40-60, 60-80 and 80-100, respectively. With the categorized data, clusters were selected after the Bayesian uncertainty given by the Dirichilet process (infinite mixtures), in which clusters are interactively joined (bottom-up clustering) based on a posterior log-odds of union between adjacent clusters.

Gene Set Enrichment Analysis (GSEA) was performed for the overall “modulated genes” and those belonging to each significant cluster for the identification of overrepresented biological process ontologies (GO - gene ontology, available at http://www.geneontology.org/) when compared to the biological processes represented in all genes of the array by hypergeometric tests implemented in the BioConductor libraries GOstats and Category (Alexa et al., 2006).

2.6- *Cis*-regulatory elements analysis

In order to better understand the regulatory mechanisms involved in WSF-MF380 stress in plants, analysis of Transcription Factor Binding Sites (TFBS) in the promoter region of each modulated gene was performed with PlantPAN software (Chang et al., 2008), which identified the local occurrence of each TFBS. Then the number of occurrences of each TFBS was provided by PLANTCIS software (http://microsatellite.org/). Transcription factor genes with significant *cis*-regulatory elements in their promoter region were grouped into families together with their probable targets. These genes were clustered by expression using the Cluster 3.0v (Hoon et al., 2004) and later visualized in a TreeView image (Eisen et al., 1998). Possible WSF-MF380 responses were highlighted by EGAN (The Exploratory Gene Association Networks) (Paquette and Tokuyasu, 2010), and 4 families of transcription factors were selected for expression pattern analysis by qPCR (Supplemental figure 1).

2.7- Microarray validation and *cis*-regulatory element analysis confirmation by qPCR

Single-stranded cDNA was synthesized from 1 µg of total RNA, 2.5 µM poly-T primer Oligo (DT20), 0.5mM of each dNTP, 200 units of reverse transcriptase SuperScript III™ (Invitrogen), 5 mM dithiothreitol (DTT) and synthesis buffer (1x) (Invitrogen) in a final volume of 20 µL and incubated for 60 minutes at 50°C. Primers were designed using the Web Tool Primer3 (Rozen and Skaletsky, 2000) to specifically amplify a fragment of 70-220 nucleotides and to have a melting temperature of 60 ± 1 °C (Supplemental table 1). The primers were designed in different exons, and the specificity of the primers was evaluated by BLASTN © (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nr/nt database. Reaction mixtures contained 10 µL of diluted cDNA (1:50), 0.2 µM of each primer, 25 µM of each dNTP, 1X PCR Buffer (Invitrogen), 3 mM $MgCl_2$, 2 µL of SYBR®GreenI diluted in water (1:10000) and 0.25 units of Platinum Taq DNA polymerase (Invitrogen) at a total volume of 20 µL. qPCR technical triplicates reactions were carried out in optical 96-well plates with a 7500 Fast Real-Time PCR system (Applied Biosystems). Reactions without cDNA were also performed for each gene as a negative control. Reaction mixtures were incubated for five minutes at 94°C, followed by 40 amplification cycles of 15 s at 94°C, 10 s at 60°C and 15 s at 72°C, and then 35 s at 60°C (step plate reading data for the measurement of the emitted fluorescence). The melting curves were estimated using the standard program of the machine to verify primers specificity and gDNA contamination. Furthermore, before the differential expression analysis, the average efficiency of each primer pair were estimated by the Miner software (v2.2) (Zhao and Fernald, 2005) (Supplemental table 1).

The fluorescence accumulation data from triplicate qPCR reactions for each sample were used to fit four-parameter sigmoid curves to represent each amplification curve using the qPCR library (Ritz and Spiess, 2008) and the R statistical package version 3.1.2 (R Development Core Team, 2014). The quantification cycle, identified by a characteristic point or crossing point, Cp, was determined for each amplification by the maximum of the second derivative of the fitted sigmoid curve. The efficiency of each amplification reaction was calculated as the ratio between the fluorescence of the quantification cycle and the fluorescence of the cycle immediately preceding that. The estimated efficiency of each gene was obtained by averaging all efficiencies calculated for that gene. Normalized expression

values were then calculated according to geNorm normalization flow chart (Vandesompele et al., 2002). We compared the means of normalized gene expression values among groups with nonparametric one-way ANOVA synchronized permutation tests (B = 1,000 permutations) followed by pair-wise comparisons with Bonferroni adjustment (Basso et al., 2009).

The reference genes REF1 and REF2 were used for normalization between the different amplified samples and were previously defined by our group as the best for oil stress studies in *A. thaliana* (Nardeli et al. submitted). After a thorough selection, using NormFinder (Andersen et al., 2004) and geNorm (Vandesompele et al., 2002) algorithms and validation of indicated reference genes, we found that the pair of genes used here was stably expressed in the control and WSF-MF380-treated samples. For better visualization, the data were plotted in heatmaps using the BAR Heatmapper Tool (http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper.cgi).

3- RESULTS

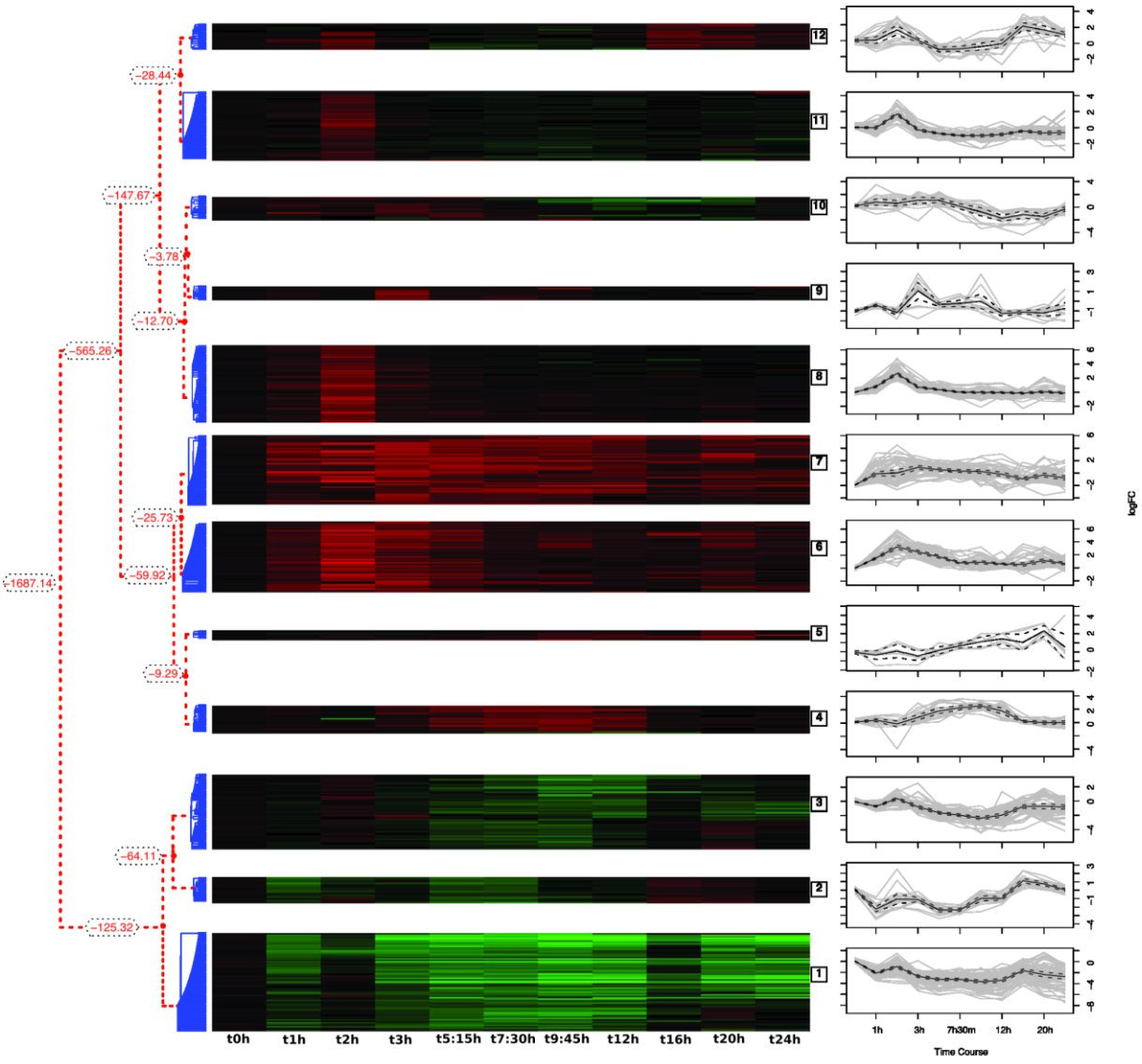
3.1- Transcriptional response to WSF-MF380 exposure

The molecular responses of *A. thaliana* subjected to the water soluble fraction of marine fuel MF380 (WSF-MF380) was evaluated during a 24 h time-course assay. Seedlings after 1 h, 2 h, 3 h, 5:15 h, 7:30 h, 9:45 h, 12 h, 16 h, 20 h and 24 h of WSF-MF380 exposure were compared to the t0h control time-point in a microarray experiment. After Bayesian hierarchical clustering analysis, a coefficient of variation for FC-values ≥ 0.7 or ≤ -0.7 was used to characterize modulated genes (Savage et al., 2009). In total, 340 genes were modulated by WSF-MF380 stress (Supplemental table 2). From these, 46 are transcription factors belong to 12 different families, of which bHLH, AP2/EREBP, MYB-related, and C2C2-Co-like are the most represented (Supplemental table 3).

The WSF-MF380 responsive genes were grouped in 12 clusters according to their expression pattern (Figure 1). GO and GSEA were performed for all 340 modulated genes as well as for each cluster gene set. The top five GO categories for all modulated genes were i) response to heat, ii) abiotic stimulus, iii) high light intensity, vi) response to hydrogen peroxide and v) oxidative stress (Figure 2). Similar GO categories are present in clusters 6, 8 and 11, which had a peak of induction at

t2h, indicating an initial response to oil exposure. Cluster 12 also showed induction of transcripts at t2h and had a GO enriched class related to the hypoxia response (Figure 1 and Supplemental table 4).

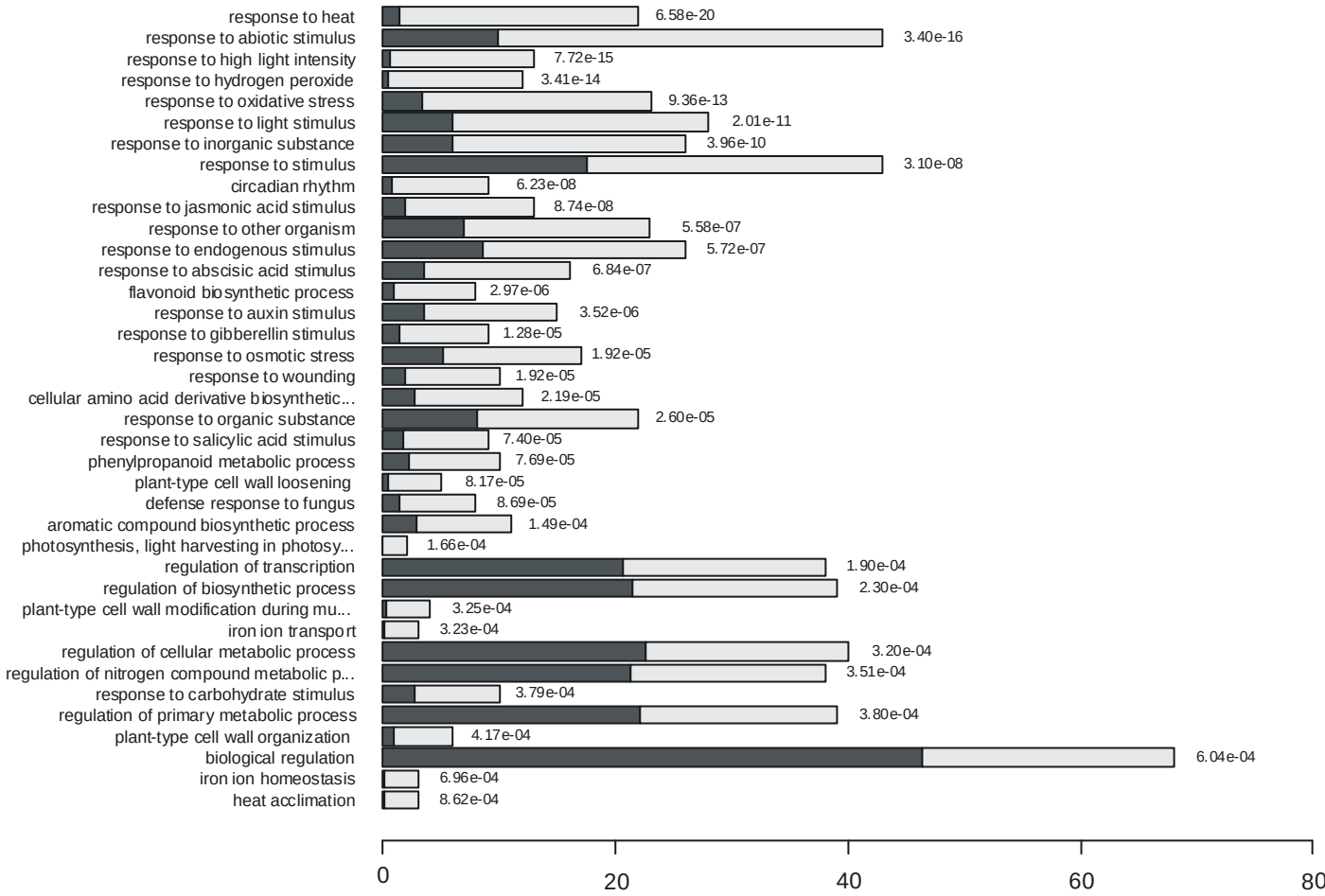
Among the enriched biological processes (BP) in WSF-MF380 stress, responses to oxidative stress and to other organisms were significantly overrepresented (Figure 2). Genes such as *APX2 (ASCORBATE PEROXIDASE 2),* which was induced in all time-points but most strongly at t2h (log2FC= 4.27), and *PR1 (PATHOGENESIS RELATED PROTEIN 1)*, which showed a 4.06-fold increase at t3h, were modulated by WSF-MF380 exposition.



(color) (2-column) Figure 1: Bayesian hierarchical clustering analysis of the 340 WSF-MF380 modulated genes. The axis represents log_2FC (ordinate) and the time

of exposition in hours (abscissae). Red and green colors indicate up-regulated and down-regulated genes, respectively. Red dotted lines in the dendrogram (left) demonstrate weak unions discouraged by the Bayesian clustering analysis. Values appointed in the dendrogram branches correspond to the log-odds of the union of corresponding branches. The expression profiles of different clusters are shown in graphics at the right side. Gray lines indicate the log fold-change expression of each gene relative to t0h, while black solid and dotted lines represent the mean and 95% confidence interval CI95% of the mean for all genes belonging to the cluster.

Genes from the bHLH family of transcription factors had their expression regulated by WSF-MF380 treatment, such as *bHLH38*, *bHLH39, bHLH100* and *bHLH101,* which were down regulated from t2h to t24h. These above mentioned *bHLH* genes are related to iron deficiency responses, and in accordance with iron ion transport and iron ion homeostasis biological processes, were overrepresented in the experiment (Figure 2).



(black-and-white) (2-column) Figure 2: Gene ontology (GO) enrichment analysis of WSF-MF380 modulated genes in 24 h time-course microarray assay. Graphical representation of enriched biological processes (BP) (described in y axis) during WSF-MF380 stress and number of modulated genes (described in x axis). Dark bars: expected count of modulated genes in each biological process (BP); light bars: observed BP enrichment; values alongside the bars: hypergeometric test p-values. Overrepresentation was assessed with a statistical score based on hypergeometric tests with p-values ≤ 0.001.

Another interesting feature is the overrepresentation of GO classes related to cell wall modification as shown in Figure 2. Expansins have an important role during this process because they participate in cell wall loosening and enlargement (Perrot-rechenmann et al., 2010). Six expansin genes modulated by WSF-MF380 displayed a general repression pattern from the t5.15h to t12h time-points (Supplemental table 2). We also observed, measuring the plants biomass, that the seedlings treated with

WSF-MF380 grew significantly less than control seedlings (p<0.001) after 24 h of contamination (Supplemental table 5).

The interpretation of other pathways related to oil contamination was complemented with software tools that allow the visualization of high-throughput experiments. EGAN revealed a network containing 64 genes, well over the 340 identified modulated genes, which respond to inorganic substances. This analysis includes the already mentioned *bHLH* genes related to iron uptake and also other main regulators in this process, such as *FIT*, *IRT1* and *FRO2* (Supplemental figure 2).

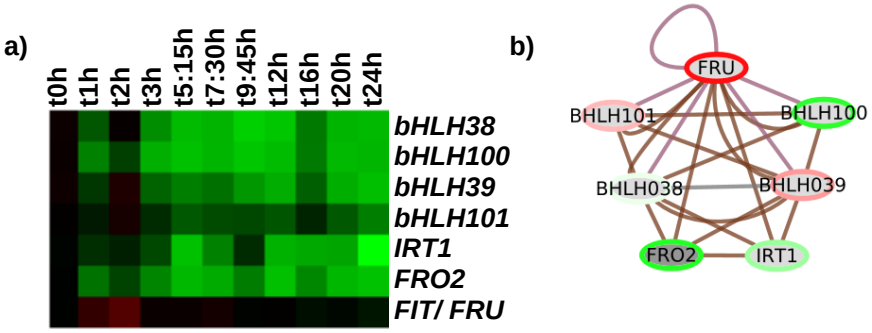
Furthermore, MapMan figures highlighted the biotic and abiotic responses in WSF-MF380 treated plants. Abiotic stress included hormone signaling, indicating that the expression levels of many hormone-responsive genes were changed as shown in Figure 2. In general, auxin, abscisic acid and ethylene responsive genes were up-regulated at t2h. Increased expression of *HSP* genes were also observed at this time-point. In total, 21 genes encoding HSPs were up-regulated at t2h, with *HSP22* showing greater changes with a 6.50-fold increase at this time-point (Supplemental table 2). An overview of these regulatory processes is provided in Supplemental figure 3.

3.2-Validation of the microarray experiment

The microarray experiment was validated by qPCR using 19 genes and seven selected time-points (t1h, t2h, t3h, t9:45h, t16h, t20h and t24h). The qPCR expression of the 19 selected genes was compared with our microarray expression data and a correct correlation between both expressions was observed, validating the results presented herein (Supplemental figure 4).

3.3- WSF-MF380 interference on iron uptake

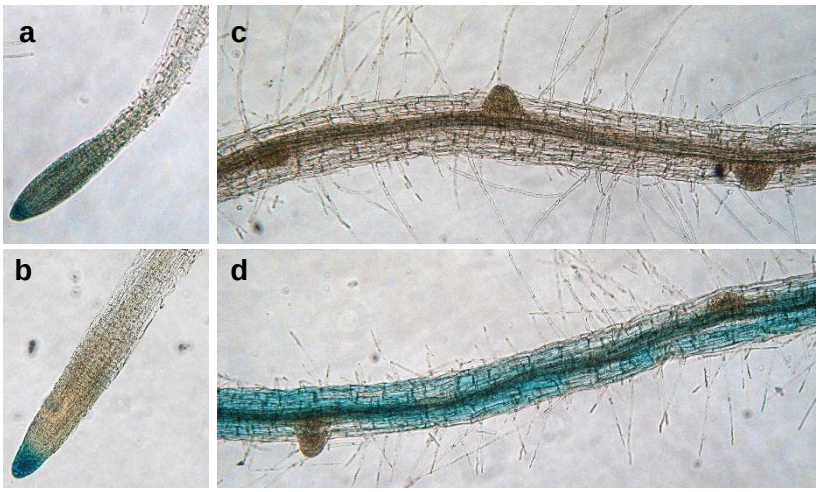
Our microarray analysis indicated that WSF-MF380 exposure triggers the repression of iron deficiency genes from time t2h to t24h, with the exception to *FIT*, which had less variable expression and showed an induction at t2h (log2FC= 2.17) (Figure 3a and 3b).



(color) (single column) Figure 3: Iron deficiency genes modulated by WSF-MF380. a) Heatmap of iron deficiency genes displaying their microarray expression in each time-point **b)** EGAN image showing the network of *bHLH* genes at t2h. Red represents induction and green represents repression. Brown edges indicate co-occurrence in the literature, purple edges indicate protein-protein interactions and grey edges represent chromosomal adjacency.

To better understand the roles of the iron response in WSF-MF380 stress, transgenic plants expressing the β-glucuronidase (GUS) reporter gene fused to the *bHLH38* promoter were analyzed for GUS staining. Tissue differences for *bHLH38* expression in WSF-MF380 treated and non-treated plants were verified at two time-points, t2h and t16h. The *bHLH38* expression was observed in the upper zone of the main root, near the hypocotyl area, and in the lateral roots of control and treated plants at t2h and t16h (data not shown). Treated plants at t2h showed weaker GUS staining at the tip of the root and a diffused signal in the elongation zone compared to non-treated plants, where expression was stronger and concentrated in the tip (Figure 4a and 4b). At t16h, we noticed a repression in *bHLH38* expression with no GUS staining observed in the roots of the hair zone of oil exposed plants in contrast with the control plants (Figure 4c and 4d).

t2h **t16h**



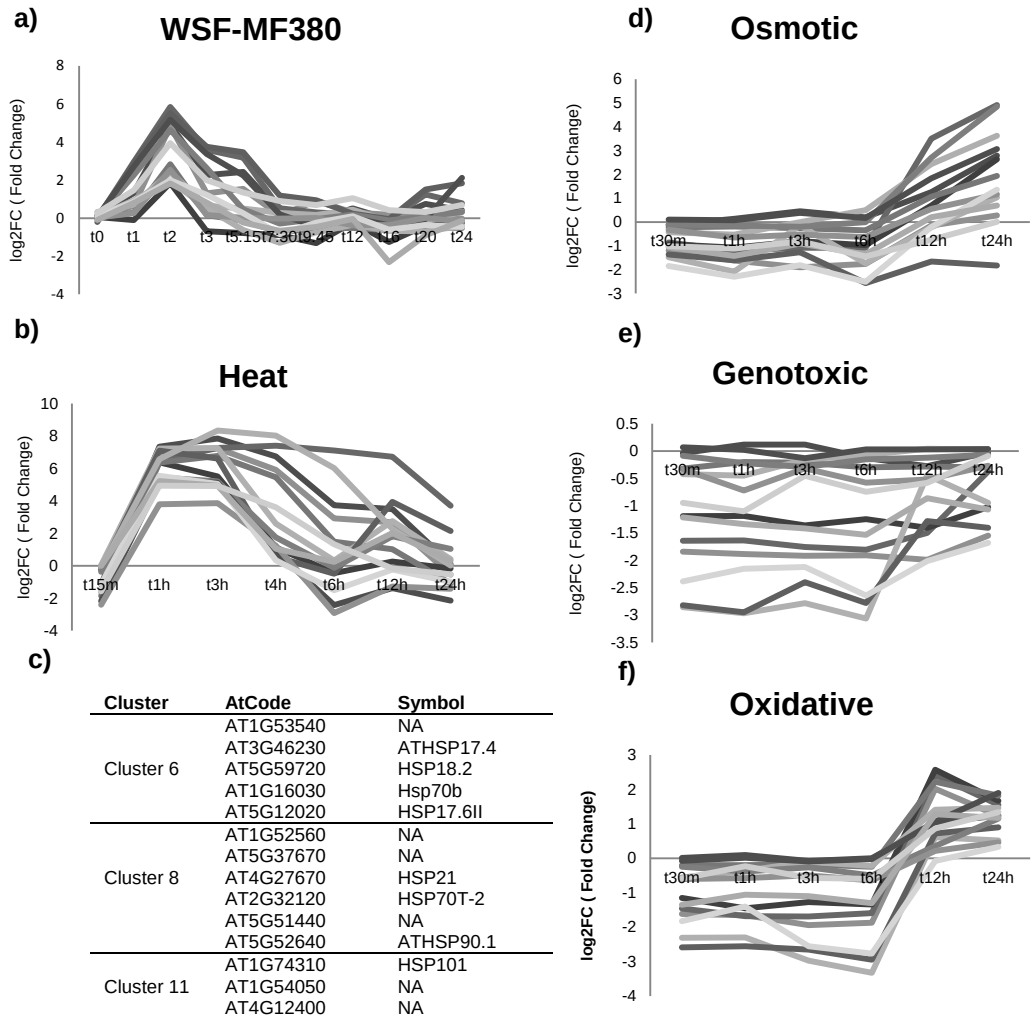
(color) (single column) Figure 4: Histochemical staining of GUS activity in *pbHLH38::GUS* transgenic *A. thaliana* seedlings. Treated plants showed weaker GUS staining at the tip of the root and a diffused signal in the elongation zone (a) compared to the control (b) at t2h. No GUS staining was observed in the root hair zone of oil exposed plants (c) in contrast to the controls (d) at t16h.

3.4- Initial response to oil exposure

The clusters expression profile showed a stronger induction at t2h, indicating a rapid transcriptional response to oil contamination in plants. The four already mentioned clusters with an increase of transcripts at t2h (clusters 6, 8, 11 and 12) had similar enriched GOs related to the response to heat, abiotic stimulus and oxidative stress. Only cluster 12 had a differentially enriched GO: the response to hypoxia and lactate transport. To identify the most abundantly expressed genes associated with the initial response, we ranked them according to their $\log_2$FC at t2h and focused on the top 5% most highly expressed (Supplemental table 6). This list includes 17 genes; among them, ten *HSPs*, *MAPKKK18* and *APX2,* and *ERF71*.

In our analysis, *HSP22* had the highest expression level in the "top list", but another eight HSPs were also remarkably induced, such as *HSP70b* and *HSP21,* which showed 4.91- and 4.64-fold increases, respectively (Supplemental table 6). Currently, several genome-wide microarray datasets have been performed and made publicly available by the AtGenExpress consortium (Kilian et al., 2007), providing an opportunity to profile HSP expression over a wide range of stress conditions.

We compared the expression profile of *HSPs* presented in clusters 6, 8 and 11, which showed overrepresented GOs related to heat and a peak of induction at t2h, in WSF-MF380 stress and in other available abiotic stresses. Heat, oxidative, osmotic and genotoxic time course microarrays from the AtGenExpress microarray data were analyzed considering the same experimental procedure used for WSF-MF380, meaning that all time-points were contraposed to t0h. The expression of the selected 14 *HSPs* in WSF-MF380 were similar to heat stress, showing an induction at the initial time-point and a decrease at t3h. The HSP expression profile was not similar to the other stress conditions analyzed herein (Figure 5).



c)

Cluster	AtCode	Symbol
Cluster 6	AT1G53540	NA
	AT3G46230	ATHSP17.4
	AT5G59720	HSP18.2
	AT1G16030	Hsp70b
	AT5G12020	HSP17.6II
Cluster 8	AT1G52560	NA
	AT5G37670	NA
	AT4G27670	HSP21
	AT2G32120	HSP70T-2
	AT5G51440	NA
	AT5G52640	ATHSP90.1
Cluster 11	AT1G74310	HSP101
	AT1G54050	NA
	AT4G12400	NA

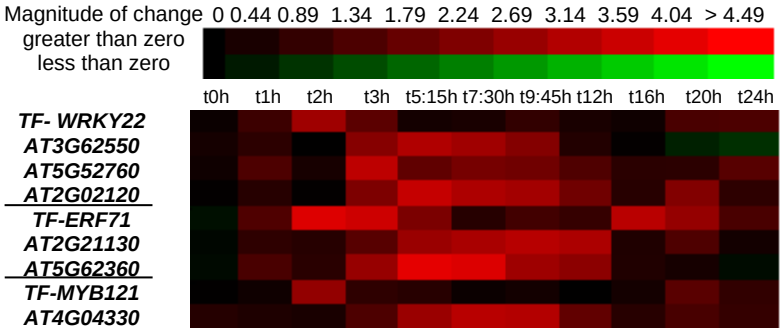
(black-and-white) (2-column) Figure 5: *HSP* genes expression profile in different abiotic stress. *HSPs* from three clusters with overrepresented GO

response to heat were analyzed according to their expression behavior during 24h. Their expression profile in WSF-MF380 stress (a) are similar to the observed in heat stress (b), but not with osmotic (d), genotoxic (e) and oxidative (f) stresses. The list of 14 *HSPs* present in the three clusters which displayed an induction at t2h and their respective Atcode and symbols (c).

3.5- The regulatory network involved in oil response

Transcription factors (TFs) are proteins that recognize DNA in a sequence-specific manner and regulate the frequency of transcriptional initiation. They can act as activators, repressors or even both. Our analysis revealed 46 TFs modulated by WSF-MF380 treatment, and we noticed that some families encompassed a general expression pattern. The AP2/EREBP, MYB, WRKY and AUX/IAA families are induced at t2h, whereas genes from the bHLH family are repressed in the subsequent time-points (Supplemental figure 5).

In order to elucidate the regulatory network involved in WSF-MF380 stress, we scanned the promoter region of all modulated genes for overrepresented DNA elements using PlantPAN software. Genes with the same enriched *cis*-regulatory elements were grouped to form a set with common regulatory mechanisms. The expression patterns of these groups were investigated considering the expression of their regulators. For example, WRKY22 could regulate *At5g52760*, *At3g62550* and *At2g02120* by binding to the “W- box” motif in their promoter region. *WRKY22* was shown to be highly induced by submergence (Hsu et al., 2013) and had a 2.81-fold increase at t2h when treated with WSF-MF380. *WRKY22* induction at t2h can result in the activation of *At5g52760* (a copper transport protein), *At3g62550* (a stress response protein) and *At2g02120* (a predicted pathogen related protein*)* from t3h to t9:45h (Figure 6).



(color) (single column) Figure 6: Transcription factors (TFs) induced at t2h and possible targets genes. Heatmap image of the time-course microarray experiment shows the expression (log_2FC) of *WRKY22*, *ERF71* and *MYB121* transcription factors and their possible targets containing the enriched *cis*-regulatory elements to which they bind.

Taking into consideration enriched *cis*-regulatory elements and gene expression, we also demonstrated that *ERF71* (*At2g47520*) up-regulation at t2h and t3h could lead to *At2g21130* (involved in protein folding) and *At5g62360* (plant invertase/pectin methylesterase inhibitor superfamily protein) activation from t3h to t12h. Furthermore, *MYB121* (*At3g30210*) induction at t2h could regulate the expression of *At4g04330*, which encodes a chloroplast thylakoid localized RbcX protein that acts as a chaperone in the folding of rubisco (Kolesiński et al., 2011) (Figure 6). The expression pattern of five TFs modulated by WSF-MF380 and 16 possible targets genes was further investigated by qPCR in three selected time-points: t2h, t5:15h and t7:30h (Supplemental figure 1). The qPCR expression of the selected possible target genes is in accordance with the expression profile of their regulators.

4- DISCUSSION

The fast rate of industrialization and consumption of energy has led to many oil spills worldwide. In 2000, approximately 1300 m^3 of the marine fuel MF380 was released into the Guanabara Bay, Rio de Janeiro, Brazil, after the pipeline ruptured at

the Duque de Caxias Refinery. The oil was transported by tidal current and wind, was spread over the water, and reached islands, beaches and mangroves (Meniconi et al., 2002). Mangrove plants are highly vulnerable to oil spills, suffering both lethal and sublethal effects from oil exposure (Noaa, 2010). Their damaging effects include inhibition of seed germination, reduction of photosynthetic pigments, decreased nutrient assimilation and shortening of roots and aerial organs (Bona et al. 2011; Zarinkamar et al., 2013).

Oil is a complex mixture of different chemicals that consists of a variety of hydrocarbon-based substances such as alkanes, cyclohexanes and PAHs. Although studies with oil components have been performed, little is known about the molecular effects of oil mixtures in plants. In this regard, we evaluated the transcriptional response of the model plant *A. thaliana* exposed to the WSF-MF380 after different times of treatment. We observed a stronger induction of transcription at the initial time-point t2h, indicating a rapid molecular response to oil contamination in plants (Figure 1). Clusters that displayed the induction profile at t2h showed enriched GO processes related to the response to heat, abiotic stimulus, oxidative stress, the response to hypoxia and lactate transport (Supplemental table 4).

Anoxic stress was reported to be an important component of petroleum-induced stress in plants (Peña-Castro et al., 2006), and our analysis of enriched GOs related to hypoxia and lactate transport in WSF-MF380 stress, emphasizing this conclusion (Figure 2). Even if the oil is relatively non-toxic, it can stress or kill plants by forming a thick hydrophobic layer that reduces water availability and prevents gas-exchange (Pezeshki et al., 2000). Anaerobic genes such as *ADH1 (Alcohol Dehydrogenase)* and *AHB1 (Nonsymbiotic Hemaglobin1)* were induced at t2h with 2.68- and 2.85-fold increases, respectively. Members of the AP2/ERF-type family are the most represented up-regulated TFs in hypoxic stress (Geigenberger et al., 2011), and seven members were modulated by WSF-MF380; among them, *ERF71* showed a strong induction at t2h (log_2FC= 4.28) (Supplemental table 6).

If oil coats the leaves of plants, temperature stress may also occur as a consequence of the inhibition of transpiration. Transpiration and photosynthesis are reduced by the physical blockage of the stomata, which will vary according to the amount of the plant that is covered by the oil (Pezeshki et al., 2000). In total, 21 heat shock genes were regulated by WSF-MF380 stress and 10 belonged to the “top list” of genes most strongly expressed at t2h (Supplemental table 6). Among them,

HSP22 showed the highest induction with a 6.50-fold increase. It is known that HSPs are involved in cellular responses to various conditions besides heat, suggesting a considerable cross-talk between heat and non-heat stress (Swindell et al., 2007). Although *HSPs* are also strongly induced by cold, salt and osmotic stresses, we believe that the induction of *HSPs* present in clusters 6, 8 and 11 (14 members) during WSF-MF380 stress may be caused by heat since they showed the same profile of increased expression at the initial time and decreased expression at t3h as those observed in the AtGenExpress heat stress time course (Figure 5).

Phenanthrene, a PAH present in oil, induces oxidative stress and reduces plant growth (Alkio et al., 2005). We found antioxidant genes such as *ASCORBATE PEROXIDASE 2 (APX2),* which was highly induced at t2h (log_2FC= 4.27) (Supplemental table 6), as well as *THIOREDOXIN-DEPENDENT PEROXIDASE 2 (TPX2)* and *GRX480* to be modulated by WSF-MF380 stress (Supplemental table 2). Glycosyltransferases (GT) are enzymes that raise their transcript level in response to oxidative damage-inducing agents. They operate in the face of a xenobiotic stress and act on several chemical structure characteristics of oxidized pollutants, such as phenols, anilines and polycyclic aromatics, which are all present in the complex mixture of petroleum. GTs conjugate sugars to the donor, leading to a less toxic metabolite (Harvey et al., 2002; Peña-Castro et al., 2006; Schaffner et al., 2002). Five GTs genes were modulated by WSF-MF380 treatment; for instance, *AtGOL1* and *AtGOL2* were induced at t2h, showing 2.17- and 3.43-fold increases, respectively (Supplemental table 2). Plants treated with WSF-MF380 showed similarities in their response to pathogen stress, but as they were grown in sterile conditions, it was more likely that the production of ROS from the oil exposure influenced the seedlings. After the initial oxidative burst, WSF-MF380 induced mechanisms common to pathogen defense including the up-regulation of many defense genes, such as *PR1, PR2, WIN3* and *At4g33720* (Supplemental table 2; Supplemental figure 3).

With regards to the morphological symptoms of PAHs exposure, it was previously shown that *A. thaliana* plants treated with phenanthrene exhibited retarded root and seedling growth and showed a decrease in *EXP8* (At2g40610) expression (Alkio et al., 2005) In our time-course microarray analysis, six expansin genes were repressed from t5.15h to t12h; for instance, *EXP8, EXP10* and *EXP17* were the most down-regulated (Supplemental table 2). Indeed, by measuring the seedling dry

weight of controls and WSF-MF380 treated plants after 24 h of exposure, we could observe that the total biomass was severely reduced (Supplemental table 5). The growth reduction may be attributed to a decrease in cell division or cell expansion. The downregulation of expansin genes suggests that this observation is due to the inhibition of cell enlargement.

In contrast to the initial response at t2h, a group of genes from the bHLH family of TFs, namely *bHLH38*, *bHLH39, bHLH100* and *bHLH101,* were down regulated at later time-points. It was demonstrated that high iron, low cooper and low zinc concentrations lead to repression of those genes and that iron is usually one of the major metal contaminants in oil, together with aluminum, sodium, nickel, and vanadium (Wrightson, 1949). Therefore, the iron contamination present in MF-380 may regulate the expression of this group of iron responsive genes. *FIT, FRO2* and *IRT* are key regulators of iron uptake and were also modulated by WSF-MF380 treatment. *FIT* was induced at t2h, but its expression was unchanged in the subsequent time-point. However, *FRO2* and *IRT* were down-regulated from time-points t2h to t24h (Figure 3).

It was demonstrated that bHLH100 and bHLH101 act independently of FIT and do not regulate its target genes, suggesting that they play a non-redundant role with bHLH038 and bHLH039*,* their close homologues, which have been shown to act in concert with *FIT* (Sivitz et al., 2012; Yuan et al., 2008). FIT interacts with bHLH38 and bHLH39 and directly regulates the expression of iron uptake genes, such as *FRO2* and *IRT1* in *A. thaliana* (Yuan et al., 2008). Therefore, the down-regulation of the three transcription factors after t2h in WSF-MF380 exposed plants leads to the repression of *FRO2* and *IRT1*. We also demonstrated that *A. thaliana* seedlings transformed with *pbHLH38::GUS* showed a weaker GUS staining in the roots of WSF-MF380 treated plants at t2h and t16h when compared to the controls (Figure 4).

We also showed that the analysis of overrepresented DNA elements together with gene expression data can constitute a useful tool for understand the gene regulatory network. We revealed 16 possible target genes regulated by five different TFs (Supplemental figure 1) taking into consideration the enriched *cis*-regulatory elements present in their promoter region as well as their microarray expression data. It would be interesting to perform further experiments to confirm their regulatory mechanisms.

5- CONCLUSION

WSF-MF380 stress seems to encompass a general response to abiotic stresses in plants. Heat, hypoxia and oxidative stresses compose the main pathways triggered by water soluble fraction of marine fuel exposure. These results indicate that the major effect of oil exposition to *A. thaliana* at earlier stages might be the formation of an obstructive film covering the plant surface. This coat should interfere to the gas and water vapor exchange to the atmosphere activating gene expression responses to heat, hypoxia and oxidative stresses. The microarray analysis provided a time-course transcriptional profile information in plants treated with oil; these findings are advantageous for the restoration of environmental contaminated areas. Additional molecular studies using different plant species, such as mangrove flora, and the comparison with our results are important to better understand the global response of plants to WSF-MF380. Our results will be of great value for gene biomarker annotation and further comparison of different species stressed by petroleum mixtures.

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