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Transcriptome response of the foundation plant *Spartina alterniflora* to the *Deepwater Horizon* oil spill

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Abstract

Despite the severe impacts of the *Deepwater Horizon* oil spill, the foundation plant species *Spartina alterniflora* proved resilient to heavy oiling, providing an opportunity to identify mechanisms of response to the anthropogenic stress of crude oil exposure. We assessed plants from oil affected and unaffected populations using a custom DNA microarray to identify genome-wide transcription patterns and gene expression networks that respond to crude oil exposure. Additionally, we used T-DNA insertion lines of the model grass *Brachypodium distachyon* to assess the contribution of four novel candidate genes to crude oil response. Responses in *S. alterniflora* to hydrocarbon exposure across the transcriptome as well as xenobiotic specific response pathways had little overlap with those previously identified in the model plant *Arabidopsis thaliana.* Among T-DNA insertion lines of *B. distachyon*, we found additional support for two candidate genes, one (ATTPS21) involved in volatile production, and the other (SUVH5) involved in epigenetic regulation of gene expression, that may be important in the response to crude oil. The architecture of crude oil response in *S. alterniflora* is unique from that of the model species *A. thaliana*, suggesting that xenobiotic response may be highly variable across plant species. In addition, further investigations of regulatory networks may benefit from more information about epigenetic response pathways.

Introduction

Human-induced environmental change is now a dominant evolutionary force, altering populations and ecological communities through direct and indirect effects of development and commerce (Palumbi 2001; Halpern 2008; Gedan *et al.* 2009). Coastal ecosystems, which have great commercial value and provide valuable ecosystem services (Pennings & Bertness 2001), are among the most vulnerable environments to human-mediated impacts (Halpern

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2008; Gedan *et al.* 2009). Within coastal systems, salt marshes, in particular, have long been the target of exploitation and development, and pollution arising from economic pressures threatens these productive communities worldwide (Gedan *et al.* 2009). Pollutant releases, like oil spills, pose an immediate threat to organisms through chemical effects, such as polycyclic aromatic hydrocarbon (PAH) toxicity, and physical effects, such as coating (Pezeshki *et al.*, 2000). Over longer periods of time, pollution toxicity degrades habitat and potentially alters evolutionary trajectories in natural populations via selection and genotoxicity (Lin & Mendelssohn 2012; Silliman *et al.* 2012; Reid *et al.* 2016).

In 2010, the *Deepwater Horizon* (*DWH*) oil spill released an estimated 4.9 million barrels of oil into the Gulf of Mexico (National Commission on the BP *Deepwater Horizon* oil spill 2011). This oil eventually made landfall along 1,773 kilometers of the shorelines of Louisiana, Mississippi, and Alabama (Mendelssohn *et al.* 2012), nearly half of which was salt marsh habitat dominated by the grass *Spartina alterniflora* (Michel *et al.* 2012). As a foundation species, *S. alterniflora* provides crucial ecosystem functions by serving as refuge for invertebrates, as nurseries for birds and fish, and as a buffer from storm and wave action (Day *et al.* 2007; Mendelssohn *et al.* 2012). *Spartina alterniflora* also shows remarkable resilience to a variety of stressors (Baisakh *et al.* 2008; Baisakh & Subudhi 2009; Pennings & Bertness 2001; Silliman *et al.* 2012). Oil-affected populations showed up to 100% recovery within seven months of the *DWH* spill, despite the immediate effects of reduced carbon fixation and transpiration (Lin & Mendelssohn 2012; RamanaRao *et al.* 2012; Silliman *et al.* 2012; Lin *et al.* 2016), and evidence for genetic divergence of oil exposed populations from nearby uncontaminated populations (Robertson *et al.* 2017). Prior studies in *S. alterniflora* under controlled conditions have examined expression of candidate genes in response to heat, salt or oil stressors (Baisakh *et al.* 2008; Baisakh & Subudhi 2009; RamanaRao *et al.* 2012), and Bedre *et al.* (2016) recently characterized the full transcriptome response to salinity.

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However, the particular molecular mechanisms that regulate the remarkable resilience of *S. alterniflora* remain understudied. Understanding the molecular underpinnings of oil stress response *in natura* (Shimizu *et al.* 2011; Kudoh 2016) may provide not only valuable information for the conservation and management of these threatened ecosystems, but also a novel understanding of the mechanisms of extreme stress response to an anthropogenic stressor.

Molecular biologists have developed the xenome concept to understand the genetic machinery that underlies the detection, transport and detoxification of toxic compounds, or 'xenobiotics' (Edwards *et al.* 2010). Although genetic resources are scarce in non-model species such as *S. alterniflora*, in the model plant *Arabidopsis thaliana*, accumulating evidence supports the involvement of six multigenic families in the activation, metabolizing, storage or excretion of xenobiotics: cytochrome P450s (CYPs), alpha/beta hydrolases, glycosyltransferases (GTs), glutathione transferases (GSTs), malonyltransferases (MTs) and ATP-binding cassette (ABC) transporters (Edwards *et al.* 2010; Fig. 1a). In the cell, xenobiotics are first activated by cytochrome P450s (CYPs) or alpha/beta hydrolases. Activated xenobiotics may be moved out of the cell, or conjugated with low molecular weight molecules such as glucose (via GTs) or the tripeptide glutathione (via GSTs). Glycosylated xenobiotics are excreted or conjugated with malonate (via MTs). Finally, xenobiotics are stored in the vacuole via ABC-transporters (El Amrani *et al.* 2015). Although studies have investigated the xenome response of *A. thaliana* exposed to one component of crude oil, phenanthrene (Weisman *et al.* 2010; Dumas *et al.* 2016), the xenome response to complex crude oil has not been quantified either in controlled conditions or *in situ*. Further, we know of no investigation of the xenome in non-model plants. However, targeted gene expression assays in *S. alterniflora* have identified several oil responsive candidate genes, including cell wall proteins and regulatory genes, which may participate in the detoxification

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process more broadly (RamanaRao *et al.* 2012). The degree to which detoxification and tolerance pathways are conserved across evolutionary divergence remains understudied. Species-specific xenome features may reflect the differential ability to respond to challenging conditions under the selective pressure of toxic molecules (El Amrani *et al.* 2015). Therefore, measuring xenome and stress-response activity in non-model species under complex, natural conditions is critical to understanding the evolution of the xenome.

To understand the mechanisms of response and resilience to oil stress in *S. alterniflora*, we assessed (1) differential gene expression in individuals from natural *S. alterniflora* populations exposed to the *DWH* oil spill, (2) the xenome and stress response of the oil-resilient *S. alterniflora* compared to that of PAH-sensitive *A. thaliana*, and (3) the phenotypic effects of four candidate oil response genes. Because native *S. alterniflora* populations harbor high levels of genetic diversity (Richards *et al.* 2004; Hughes & Lotterhos 2014; Foust *et al.* 2016; Robertson *et al.* 2017), we expected expression differentiation among populations. However, we also anticipated that large differences in expression due to oil exposure, particularly in the xenome and in genes involved in stress response pathways, would allow us to identify novel candidate genes since complex environments have exposed novel transcript behaviors in other studies (Colbourne *et al.* 2011; Whitehead *et al.* 2012), and *S. alterniflora* has a complex hexaploid genome (Ainouche *et al.* 2012) with potential for diversification of duplicated gene copies (Fortune *et al.* 2007; Roulin *et al.* 2013; Boutte *et al.* 2016).

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Methods

Study species and population

In August 2010, four months after the *DWH* oil spill, we collected plant material from four intertidal locations near Grand Isle, Louisiana and two locations in Bay St. Louis, Mississippi. These sites were naturally highly variable in conditions, but all sites supported monocultures of *S. alterniflora* and were moderately protected, as *S. alterniflora* grows predominantly in areas that are not exposed to full energy coastal conditions. In each of three contaminated and three uncontaminated populations of *S. alterniflora,* we collected leaf tissue from nine individuals spaced 10 meters apart (Fig. 2a). Contamination was confirmed by the visual presence of oil on the sediment in populations with substantial above ground dieback of *S. alterniflora* on the leading edge of the marsh. Nearby uncontaminated populations did not have any visual signs of the presence of oil or noticeable dieback of the above ground portions of *S. alterniflora*. From each plant, we collected the 3rd fully expanded leaf to standardize age and minimize developmental bias in sampling. Leaf samples were immediately frozen in liquid nitrogen to prevent RNA degradation, and kept frozen during transport to the University of South Florida for processing and analysis.

RNA extraction and microarray hybridization

We extracted total RNA from each of nine plants separately per population from homogenized leaf tissue using RNeasy Plant Mini Kits (QIAGEN). The Interdisciplinary Center for Biotechnology Research at the University of Florida standardized RNA concentrations and created three pools of three randomly chosen individuals from within each population: a total of 54 samples were combined into 18 population-specific RNA pools. Pooling is a common strategy in ecological research that sacrifices measures of individual-

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level variation to increase sample size and capture population-level response (Alvarez *et al.* 2015). We reverse transcribed twenty RNA pools (18 sample pools and 2 technical replicates) into cDNA and hybridized them to custom 4x44k Agilent DNA microarrays containing 17,049 unique 60-mer probes corresponding to 16,608 unique annotations and 441 unannotated contigs. Of these, 9,356 probes were designed from the *S. alterniflora* Roche-454 reference transcriptome (Ferreira de Carvalho *et al.* 2013), 7170 probes from the co-assembly of 5 *Spartina* species transcriptomes (A. Salmon, J. Ferreira, H. Chelaifa & M. Ainouche, unpublished), and 523 from the *Spartina maritima* Roche-454 reference transcriptome (Ferreira de Carvalho *et al.* 2013). The reference transcriptomes used in this study were assembled with a minimum of 90% identity to account for the redundancy due to recent genome duplication events in these species, and were functionally annotated with sequence and protein homology searches using complementary datasets from Poaceae sequenced species, and *A. thaliana* (Ferreira de Carvalho *et al.* 2013). The samples used to create the transcriptome did not include plants exposed to oil, and therefore genes that are specifically up- or down-regulated during this stress response may be under-represented on the array. Probes were designed using the e-array web-portal (https://earray.chem.agilent.com/earray/) following the manufacturer requirements and printed on 4*44k chips with 2 sets of probes per 44k array. The microarray was designed to maximize the capture of overall expression of a transcript regardless of how many functional copies there are in the genome, which may have resulted in the collapse of homeologs into single probes.

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Microarray data analysis

We imported expression data into the statistical program JMP/Genomics (Version 6 for Windows; SAS Institute, Cary, NC, USA) for analysis after initial image processing by Agilent. We log-transformed the data and filtered out intensity values less than two and subsequent probes that appeared in less than half of the pools (<10). Of the total 17,049 probes, 15,950 passed our filtering protocol. Despite the potential for environmental variation in field studies, as well as possible variation introduced during the pooling process, our technical replicate pools were highly correlated with each other (r=0.995). We median normalized the raw data prior to downstream analysis, and visualized differences between individuals and populations using multidimensional scaling, implemented in the metaMDS function of the Vegan package (Oksanen *et al.* 2017).

We visualized the relative contribution of oil exposure, state (Louisiana or Mississippi), population, and slide using a principal variance components analysis (PVCA) on the probe-level data. This strategy uses a principal components analysis (PCA) to reduce the dimensionality of the data before calculating variance components via a linear model for each principal component (Richards *et al.* 2012). To understand the effects of oil exposure, population and state on gene expression, we fit a mixed-model analysis of variance (ANOVA) to these data using a model that incorporated oil exposure, state, population, and microarray slide, with population nested within oil exposure and designated as a random effect (expression = oil + state + population-within-oil + slide). Populations were nested within oil because, due to the nature of the oiling event, we were not able to collect individuals affected by oil stress and individuals unaffected by oil stress from the same populations. State was included to model the effects of the comparatively large geographic distance between the four Louisiana populations and the two Mississippi populations, which also separated out on a visualization of the PCA of the expression data (Fig. S1). We

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corrected for false discovery using q-values (Storey *et al.* 2015), and used values of less than or equal to 0.05 to identify significantly differentially expressed transcripts.

Technical confirmation of microarray

Using the same RNA as we used in the microarray hybridization, we reverse transcribed total RNA using RetroScript kits (Ambion) to create cDNA for qPCR confirmation of the microarray. We generated primers with the primer3 program (Rozen & Skaletsky 2000) for three target genes as well as for *a-tubulin*, which has been validated as an endogenous control (Baisakh *et al.* 2008, RamanaRao *et al.* 2012). We ran the qPCR reactions in duplicate using template from three individuals from each population (for a total subsample of 18). We quantified differential expression using the delta-delta Ct method with corrections for primer efficiency (Schmittgen & Livak 2008), and confirmed the expression results of our DNA microarray for all three genes, which were up- or down-regulated in the same direction as they were in our microarray (Fig. S2).

Enrichment, stress annotation, and xenome comparison

To explore the functionality of genes differentially expressed in response to oil ($Q<0.05$), we used five different techniques based on both previous annotations and previously identified hydrocarbon-responsive genes. First, we performed a Gene Ontology (GO) enrichment test in JMP/G based on annotation data from the model species *A. thaliana* (TAIR 10), as measured by the Fisher exact test, to identify overrepresented GO terms within the oil-responsive genes ($Q<0.05$). Second, we searched for enriched Pfam annotations (Finn *et al.* 2016) via a Fisher's exact test in R ($Q<0.05$) using HMMER (Finn *et al.* 2011) and custom R scripts (github.com/AlvarezMF/DWHoilspill_transcriptome).

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Third, we compared transcriptome-wide response to crude oil in *S. alterniflora* to transcriptome-wide response to the PAH phenanthrene in two previous studies of *A. thaliana.* Weisman *et al.* (2010) germinated seeds in phenanthrene and harvested the plants at 21 days, which identified 1074 genes that responded to phenanthrene. Dumas *et al*. (2016) ran a time series experiment exposing 15 day old seedlings to phenanthrene for 30 minutes, 2, 4, 8 and 24 hours identifying 467 genes that responded to phenanthrene in at least one of these time points. Combined, these studies captured gene expression response to phenanthrene that was affected in either the short term or the long-term in 1426 genes. While these two studies differed somewhat in their approach, plants were grown under similar abiotic conditions, and both assessed the response to phenanthrene of the same genotype of *A. thaliana* (Col-0). We looked for enrichment of gene categories with GO analysis on the 1426 differentially expressed genes in these two *A. thaliana* studies using the BioMaps function in Virtual Plant with a Fisher's exact test (FDR<0.05; Katari *et al.* 2010; File S1). We also looked for overlap between genes that were differentially expressed in *S. alterniflora* and homologs that were differentially expressed in either the Weisman *et al.* (2010) study or the Dumas *et al.* (2016) study. To facilitate the comparison with *S. alterniflora*, we only used genes that appeared on the custom *S. alterniflora* microarray and on either the ATH1 or CATMA chips. In total, we used 7,566 out of the 15,950 *Spartina* probes, all of which were homologous to *A. thaliana* loci and were represented on either the ATH1 or CATMA chips (Fig. S3).

Fourth, we compared the *A. thaliana* xenome with the putative *S. alterniflora* xenome by examining loci found in the six xenome gene families in *Spartina* (our study) and *A. thaliana* (Weisman *et al.* 2010; Dumas *et al.* 2016; i.e. represented on either ATH1 or CATMA chips). We defined a list of putative *A. thaliana* xenome genes, which was composed of 945 *A. thaliana* candidate loci (191 α/β hydrolases, 53 GST, 319 GT, 130 ABC transporters, 245 CYP450 and 7 related-malonyltransferases), based on published studies

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(Edwards *et al.* 2010; Weisman *et al.* 2010; Skipsey *et al.* 2011; Dumas *et al.* 2016). Large families (GST, GT, ABC transporters and CYP450) were downloaded from the TAIR10 database (http://www.arabidopsis.org/browse/genefamily), whereas loci coding for α/β hydrolases protein superfamily were directly retrieved from the TAIR10 *A. thaliana* genome annotation file, and related-malonyltransferases from Taguchi *et al.* (2010). The *in silico* survey of the whole putative genes set of the xenome revealed that 239 candidate loci were represented in *A. thaliana* arrays (including 61 α/β hydrolases, 13 GST, 87 GT, 49 ABC transporters, 29 CYP450 and 0 related-malonyltransferases) related to 437 candidate loci in our *S. alterniflora* array (99 α/β hydrolases, 18 GST, 193 GT, 92 ABC transporters, 35 CYP450 and 0 related-malonyltransferases). We identified the phenanthrene responsive *A. thaliana* xenome by combining differentially expressed xenome genes in the Weisman *et al.* (2010) and Dumas *et al.* (2016) studies. We then compared only the loci whose homologs were present on the *Spartina* array and were also represented on either the ATH1 or CATMA. Hence, 22 loci met these criteria in *A. thaliana* (3 α/β hydrolases, 4 GSTs, 7 GTs, 4 ABC transporters, 4 CYP450 and 0 related-malonyltransferases) and 99 in *S. alterniflora* (26 α/β hydrolases, 6 GST, 49 GT, 14 ABC transporters, 3 CYP450 and 0 related-malonyltransferases, Table S1). We used χ^2 and Fisher exact tests (R Core Team 2015) to compare differentially expressed genes in *A. thaliana* and *S. alterniflora* by gene families.

Finally, to understand the relationship between oil-responsive genes in *S. alterniflora* and stress response genes in general, we compared oil-responsive genes to a previously generated list of environmental stress annotations for *A. thaliana* (Richards *et al.* 2012). The stress annotation identified whether genes were previously shown to respond to abiotic or biotic stresses, including high light (Rossel *et al.* 2002), cold, drought, heat, osmotic stress, oxidative stress, salt, genotoxins, UV-B exposure, wounding (Kilian *et al.* 2007), infection by RNA virus (Whitham *et al.* 2003; Babu *et al.* 2008), bacterial pathogens, fungi and

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herbivores (De Vos *et al.* 2005). Across these data sets, more than half of the genes on the ATH1 array had been associated with at least one stress (13,153 of 22,800 genes). For this comparison, we only used genes that appeared on both the custom *S. alterniflora* microarray and the ATH1 array on which the stress annotation was based (6770 genes).

Gene interaction networks

We used two forms of gene networks to identify specific genes that may be involved in crude oil response in *S. alterniflora.* First, we used a weighted gene co-expression network analysis (WGCNA; Langfelder & Horvath 2008) to assess relationships between genes and identify co-expressed clusters of genes that respond to crude oil exposure. To do this, we used the same filtered and normalized expression values that we used for our gene-by-gene linear mixed modeling (n = 15,950) and further filtered the gene set using the function goodSamplesGenes to remove 83 additional genes missing in exactly 50% of samples, which ensured that an adjacency matrix could be calculated for subsequent coexpression analysis (n = 15,867). We used the function pickSoftThreshold to quantify and plot values of scale independence and mean connectivity across a vector of possible soft thresholds in order to identify the optimal value of the soft threshold that minimized spurious noise within our network (Fig. S4a). Our scale-free topology index remained below 0.8 for all assessed values and we therefore used a soft threshold of 6 as recommended by the WGCNA creators. Next, we created a weighted adjacency matrix with the blockwiseModules function using biweight midcorrelation to represent the relationships between genes (Fig. S4a). The blockwiseModule function clustered the adjacency values into a dendrogram (Fig. S4b), which was separated using a dynamic cut algorithm to yield co-expressed clusters (File S2). For each cluster, the blockwiseModules function performed a PCA on the gene expression values for each cluster

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and defined the first principal component of each cluster as the cluster eigengene, which represented the average expression of that cluster. The relationship of clusters to crude oil response was assessed using the generalized linear mixed model (eigengene = oil + state + population nested within oil) and corrected for multiple testing using a Holm correction. For this model, we removed the term for slide because it explained very little of the variance. We assessed Gene Ontology enrichment for each cluster with TopGO (Alexa & Rahnenfuhrer 2010), using genes with *A. thaliana* homologs as a background for Kolmogorov-Smirnov (KS) tests. We then summarized the most highly enriched GO term for each module, along with its corresponding q-value for a KS test (Table S2), and examined the protein sequences of genes within any oil-responsive clusters using HMMER (Finn *et al.* 2011) and custom R scripts (github.com/AlvarezMF/DWHoilspill_transcriptome). Finally, we used binomial tests to assess for enrichment of differentially expressed genes in each cluster.

We created a second gene interaction network using the software Virtual Plant (Katari *et al.* 2010). We used the 1410 differentially expressed genes (Q<0.05) with homologs in *A. thaliana,* after removing duplicates and genes with no known connection (File S3). Interactions, or edges, were parameterized with data on microRNA binding populations, protein-to-protein interactions, transcriptional regulation (which includes transcription factors, enhancers, and repressors), and transport interactions. The Virtual Plant software created additional edges using data from the metabolic interaction databases Aracyc and KEGG, and using published literature interactions (Katari *et al.* 2010). Although these edges were parameterized with data from *A. thaliana,* we hypothesized that interactions between highly connected genes may be conserved, allowing us to identify important genes and pathways among the oil responsive genes in *S. alterniflora*. We visualized the resulting network using Cytoscape (Shannon *et al.* 2003) and counted the number of connections using Virtual Plant without additional ranking. We used information from the WGCNA and Virtual

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Plant networks to identify four genes that might be hubs, important regulators, or otherwise key genes in orchestrating the response to crude oil.

Functional confirmation in Brachypodium distachyon

We attempted to confirm the function of four genes in controlled experiments using the model grass *Brachypodium distachyon.* We chose four lines that were altered in target genes whose expression was correlated to hydrocarbon exposure in *S. alterniflora* with the understanding that these genes may represent the function of one or more potential homeologs within the *S. alterniflora* genome. We selected three genes (homologs of KCS11, ATMCB1, and ATTPS21) that were highly connected within our Virtual Plant interaction network, as measured by number of connections. Two of these genes, KCS11 and ATMCB1, were also part of the co-expression cluster that was correlated with oil exposure in our co-expression analysis (File S2). For the fourth gene, we identified an epigenetic regulator (SUVH5) that was correlated with oil exposure in the gene-by-gene analysis. SUVH5 was not part of the Virtual Plant interaction network or our oil-responsive co-expression cluster, but may participate in oil response by regulating gene expression.

We obtained seeds for wild-type (B21-3) and four previously generated and sequence-confirmed T-DNA insertion lines (Fig. 3a) from the Western Regional Resource Center (Bragg *et al.* 2012), and stored seeds in ambient conditions before stratifying them for two weeks. We then sowed one replicate each of wild-type and T-DNA insertion lines in each of five oil treated trays, and five untreated trays, which were all grown in a single growth chamber. After all seeds had bolted, each treatment tray received 500ul of 2.5% crude oil in tap water every other day, which is a sub-lethal concentration that we found induced phenotypic response in *S. alterniflora* and in *B. distachyon*. Untreated trays received 500ul of

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only tap water every other day. We grew seedlings for 27 days until a majority of plants had flowered and senesced. On the 27th day, we measured the height and number of leaves and stems of each plant as proxies for above-ground biomass. We also measured the number of inflorescences as a component of fitness. Finally, we used a LI-6400 portable photosynthesis system (LI-COR) to measure photosynthetic rate and stomatal conductance, and to derive instantaneous water use efficiency (WUE).

Because treatment is applied at the level of trays, this design was a split plot and we modeled the effects of genotype and treatment on gene expression with a linear mixed model implemented in R (R Core Team 2015): (Response ~ Treatment + Genotype + (Treatment * Genotype) + Error (Block*Treatment)), with block as a random effect (Richards *et al.* 2008). We assessed effects using a Type II Wald Chi-squared test implemented in the Car package (Fox & Weisberg *et al.* 2011) in R. We interpreted significant genotype by treatment interactions to indicate that manipulation of the gene of interest affected the response to crude oil.

Results

Oil-contaminated populations were differentiated from uncontaminated populations

Despite substantial differences among populations, oil exposure explained 25% of the variance in overall gene expression (Fig. 2b). In our principal components analysis, PC1 separated samples from the two uncontaminated sites in Louisiana from the samples collected in the uncontaminated Mississippi site and all samples exposed to oil (Fig. S1). The association of the uncontaminated Mississippi site with the contaminated sites in PC1 may be due to unobserved contamination, historical exposure, or underlying population structure. In fact, our principal variance components analysis indicated that differences among populations

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explained the greatest proportion of variation in gene expression (32%, Fig. 2b). Using a gene-by-gene linear mixed model, we found that the expression of 3334 genes significantly differed by oil exposure, and 2287 genes significantly differed by state (869 genes overlapped the two main effects; Q< 0.05, Fig. 2c). We did not assess the significance of the random effect (population nested within oil).

Enrichment and comparison to PAH response in A. thaliana

Through gene set enrichment, we found three overrepresented categories among oil-responsive genes: "plant-type primary cell wall biogenesis", "sucrose biosynthetic process", and "coumarin biosynthetic process" (Q<0.05). Our Pfam enrichment test found three significantly overrepresented families among the oil responsive genes: "leucine-rich repeat", "Spc7 kinetochore protein", and "Cellulose synthase" (Q<0.05).

Of the common *A. thaliana* loci that were represented on our *Spartina* chip and either the ATH1 or CATMA chips (Fig. S3), 3063 (2417 unique *A. thaliana* genes) were responsive to the *DWH* oil spill in *S. alterniflora* and 526 were responsive to phenanthrene in *A. thaliana* (Fig. 4a). We found only 187 genes (224 homology-based *S. alterniflora* loci) that overlapped between these two species, including 12 genes annotated as involved in photosynthesis and 46 genes generally annotated as involved in biosynthetic processes (File S4). The discrepancy of the common number is due to the fact that multiple probes in *S. alterniflora* may be annotated with the same *A. thaliana* gene. Among these 187 genes, only 42 (22%) showed similar patterns of expression in *S. alterniflora* and *A. thaliana*, while most (78%) showed divergent expression patterns in *S. alterniflora* and *A. thaliana* (Fig. 4b), indicating little overlap between the two species' response. We found no significant overrepresentation of GO categories among genes expressed in the same direction, although we note that several

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hydrolases and genes involved in oxidative stress appear among them. We also found that 2839 of the 3063 (93%) oil responsive transcripts in *S. alterniflora* (corresponding to 2230 of 2417 or 92% of the unique *A. thaliana* transcripts) were not responsive in either of the two previous studies of PAH response in *A. thaliana.*

When we assessed the overlap between the *A. thaliana* xenome and oil-responsive genes in *S. alterniflora,* we found six *A. thaliana* loci (seven homology-based *S. alterniflora* loci) that were differentially expressed in both *A. thaliana* and *S. alterniflora* (Fig. 1b,c), indicating that *A. thaliana* shares only 7.6% of the differentially expressed transcripts of the *S. alterniflora* xenome following PAH exposure. Again, the discrepancy of the common number is due to the fact that multiple probes in *S. alterniflora* may be annotated with the same *A. thaliana* gene. χ^2 comparisons by gene families revealed significant enrichment in α/β hydrolases and GT genes in the *S. alterniflora* xenome ($P < 0.001$, Table S1).

In a comparison of oil-responsive *S. alterniflora* genes to a previously generated "stress annotation" in *A. thaliana*, we found that our custom microarray had homologs for 3907 genes that were previously annotated for response to stress in *A. thaliana,* including 356 "super responsive" genes that responded to more than four stressors in the *A. thaliana* studies (File S5). Of these "super responsive" *A. thaliana* genes, 85 gene homologs (including six from the xenome) in *S. alterniflora* were also responsive to the *DWH* crude oil, highlighting the potential importance of this small subset of genes in a diverse array of responses that may be functionally conserved across taxa. Additionally, 149 "stress annotation" genes responded in at least one of the previous *A. thaliana* phenanthrene studies, including 18 "super responders", all of which also responded in *S. alterniflora* (File S5, Weisman *et al.* 2010, Dumas *et al.* 2016).

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Gene interaction networks identify targets for confirmation

Using weighted gene co-expression network analysis (WGCNA; Langfelder & Horvath 2008) with the expression of 15,867 genes across the 18 replicate pools, we identified 39 co-expressed clusters of genes, ranging in size from 36 to 3974 genes (Table S2; File S2). Of the 39 co-expressed clusters, the value of the eigengene from one cluster (#3 in File S2) was significantly predicted by oil exposure after a Holm multiple testing correction. This cluster contains 2054 genes, which were significantly enriched for differentially expressed genes (665/2054, $P<0.001$). The only enriched GO category in this gene cluster after Q-value correction is "biological process". However, the cluster also contains genes with GO annotations that include RNA splicing, response to osmotic stress, response to salt stress, and plant-type cell wall organization (Fig. S5). This gene cluster is also enriched for a single protein family: RNA polymerase Rpb1 C-terminal repeat (PF05001, $Q<0.05$).

In Virtual Plant (Katari *et al.* 2010), 6 of the top 20 most highly connected genes were part of the co-expressed cluster (#3) that was significantly associated with response to oil in our WGCNA analysis. However, many highly connected genes from our Virtual Plant interaction network did not fall within this cluster. For example, a gene homologous to ATMCB1 had 116 connections in our interaction network, but was part of a WGCNA module that was not significantly correlated with oil exposure.

T-DNA insertion genotypes differ in phenotypes from wild-type plants, and in response to oil

After exposing replicates of each of the T-DNA insertion lines to crude oil, we found overall treatment effects for height, number of leaves, number of stems, number of inflorescences, photosynthetic rate and water use efficiency (WUE), but not stomatal

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conductance. We also found a significant effect of T-DNA insertion line for number of leaves, number of inflorescences, photosynthetic rate and WUE (Table 1). Using post-hoc assessments of significance for individual lines, we found a significant effect of a SUVH5 homolog (Bradi3g35330) overexpression on photosynthetic rate, and a KCS11 homolog (Bradi3g07730) overexpression on stomatal conductance and WUE (Table 2).

Finally, we compared response to oil in each line to the wild-type line and found significant T-DNA insertion line-by-treatment effects for two genes. We found that Bradi3g35330, which overexpresses a homolog of SUVH5, resulted in increased number of inflorescences in response to oil compared to the wild type (Fig. 3b; Table 2). We also found that Bradi1g62540, which overexpresses a homolog of ATTPS21, increased the number of leaves produced under crude oil exposure compared to wild-type lines in *B. distachyon* (Fig. 3c, Table 2).

Discussion

Large-scale anthropogenic impacts, such as crude oil spills, can be leveraged as natural "treatment and control" designs to understand molecular function in ecologically relevant settings (Whitehead *et al.* 2012; Reid *et al.* 2016). We found 3334 genes that responded to crude oil exposure in *S. alterniflora*, including 2230 genes that were not previously responsive in the model plant *A. thaliana* exposed to the PAH phenanthrene. We found that most (78%) of the shared, differentially expressed transcripts showed divergent expression patterns between *S. alterniflora* and *A. thaliana*. While the two species may share a broadly defined set of stress-responsive genes (including but not limited to the more narrowly-defined xenome), the specific oil-responsive transcriptional pathways in *S. alterniflora*, and the xenome in particular, diverge from those in *A. thaliana*.

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Transcriptional hydrocarbon response in S. alterniflora *may be species specific*

Plants may adapt to cope with toxic molecules (Antonovics & Bradshaw 1970), which may be reflected in the fine-tuning of metabolic detoxification pathways that are transcriptionally activated under challenging toxic conditions (El Amrani *et al.* 2015; Dumas *et al.* 2016). In the Gulf of Mexico, natural oil seeps create the possibility of repeated hydrocarbon exposure in coastal species like *S. alterniflora* over evolutionary time (MacDonald *et al.* 1996). We used the framework of the xenome to compare the regulation of metabolic pathways involved in organic xenobiotic detoxification in *S. alterniflora* and *A. thaliana*. We hypothesized that comparison of the xenome of *S. alterniflora*, which shows hyper tolerance in the presence of phenanthrene (Cavé-Radet & El Amrani, unpublished) and crude oil exposure in laboratory conditions and in the field (Alvarez 2016), to that of the PAH-sensitive model plant species *A. thaliana* exposed to hydrocarbons, would provide insight into the conserved mechanisms that underlie tolerance in plants, as well as non-overlapping divergent components of tolerance. We found remarkably little overlap in genome-wide as well as xenome response between *S. alterniflora* and *A. thaliana*. Only 42 (22%) of the shared, differentially expressed transcripts showed similar direction of expression in *S. alterniflora* and *A. thaliana*, while most (78%) showed divergent expression patterns between *S. alterniflora* and *A. thaliana.* Less than 100 of the 3334 differentially expressed genes in *S. alterniflora* were among the six multigenic families that make up the *A. thaliana* xenome as strictly defined. Gene set enrichment on oil-responsive genes in *S. alterniflora* identified functional categories involved in metabolism (plant-type primary cell wall biogenesis, sucrose biosynthetic process) and defense (coumarin biosynthetic process) that were not enriched in previous *A. thaliana* studies, but may participate in the detoxification process more broadly (Edwards *et al.* 2010, El Amrani *et al.* 2015).

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Highly connected genes are expected to evolve more slowly when they exhibit pleiotropic effects on phenotype (Cork and Purugganan 2004). We expected that highly connected stress-responsive genes would be functionally conserved and potentially play important roles in the response to crude oil. Therefore, we hypothesized that genes that are highly connected in *A. thaliana*, would appear within gene clusters that are most responsive to crude oil exposure, allowing us to identify genes that are more likely to modulate phenotype during crude oil response. However, we found little concordance between highly connected genes in our Virtual Plant network and oil-responsive genes identified in our coexpression analysis. In combination with the lack of overlap in our xenome survey, these results suggest that *S. alterniflora* has a substantially divergent genomic architecture underlying hydrocarbon response relative to *A. thaliana*.

The differences between the two species could be partly because of differences between the response to complex crude oil and response to phenanthrene. In the *DWH* oil spill, Napthalene was the dominant PAH (64% of the total PAHs) while phenanthrene made up only 17% (Liu *et al.* 2012). Additionally, the *DWH* contamination included other toxic compounds, such as methylnaphthalene and dispersants (Liu *et al.* 2012), which may induce different transcriptional responses. However, although there were differences between the field and laboratory conditions, including xenobiotic composition, the hyper-tolerant phenotype of *S. alterniflora* may also be in part due to the fact that *S. alterniflora* detoxification involved more genes for detoxification than were identified in *A. thaliana*. The enrichment of *S. alterniflora* xenome response with more alpha/beta hydrolases and GTs may be related to the fact that, as a hexaploid, *S. alterniflora* has potentially many more functional or subfunctionalized copies of xenome genes (Fortune *et al.* 2007; Boutte *et al.* 2016). The increased copy number of functional genes may also partly explain the overrepresentation of genes annotated as being responsive to cell wall biogenesis among oil-responsive genes in *S.*

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alterniflora. However, enrichment of functional categories in *S. alterniflora*, particularly those involved in cell wall biogenesis in the GO analysis and cellulose synthase in the Pfam analysis, may be also related to a separate, species-specific detoxification process, which includes incorporation of the xenobiotic into cell wall polymers such as lignin (El Amrani *et al.* 2015) as well as the involvement of regulatory elements such as the Rpb1 protein family and CHH methylation, which may modulate gene expression (as discussed below). This behavior, to our knowledge, has not been documented outside of this study, and highlights both limitations in previously published annotations like Gene Ontology as well as the potential for novel behavior in non-model species or in natural settings (Colbourne *et al.* 2011; Whitehead *et al.* 2012). We emphasize, however, that our study compares the response of only two species, one of which is quite tolerant and the other sensitive to hydrocarbons, and which also differ in both physiology and evolutionary history. For example, monocots like *S. alterniflora* differ substantially from dicots, such as *A. thaliana*, in the composition of both primary and secondary cell walls (Vogel 2008), which may contribute to the observed enrichment of these genes in response to crude oil exposure. Ideally, experiments that include a gradient of hydrocarbon-tolerant and hydrocarbon-sensitive species across a broad phylogeny would assess the conservation of hydrocarbon response mechanisms across plant species.

Two candidate genes modulate conserved response to hydrocarbon stress

In our candidate gene screen using knockout lines of *B. distachyon*, we found that overexpression of a homolog of SUVH5 (Bradi3g35330), which was down-regulated in oil exposed *S. alterniflora*, resulted in increased number of inflorescences in response to oil compared to the wild type. SUVH5 is a methyltransferase that broadly contributes to non-CG methylation patterning in *A. thaliana* (At2G35160, Stroud *et al.* 2014). Non-CG methylation, in particular CHH methylation, may be dynamically regulated in response to specific

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stressors and may contribute to the regulation of transcription under stress or novel environments (Dowen *et al.* 2012; Dubin *et al.* 2015). CHH methylation is enriched near transposable elements (TE), and the maintenance of TE silencing may prevent deleterious insertions in protein-coding regions of the genome (Matzke and Mosher 2014). Alternatively, CHH methylation within TEs can also regulate nearby genic regions (Matzke and Mosher 2014), which would allow SUVH5 to function as a global regulator of stress response. Although the putative *S. alterniflora* SUVH5 was downregulated in response to crude oil compared to uncontaminated conditions in the field, our study suggests that individuals that can maintain *relatively higher* SUVH5 expression may display increased reproduction, and perhaps fitness, under oil stress compared to lines that do not. Alternatively, overexpression of SUVH5 may increase number of inflorescences, but reduce a more downstream component of fitness, such as germination success. Further tests with additional mutants will be useful in resolving this ambiguity, particularly with concurrent assessment of TE activity, and increased understanding of the function of DNA methylation variation. For example, manipulation of methylation variation, either through methylation inhibitors or the selective silencing of other methyltransferases, may help resolve the relationship between DNA methylation and fitness during crude oil stress (Richards *et al*. 2017).

We also found that overexpression of a homolog of ATTPS21 (Bradi1g62540), which was up-regulated in oil exposed *S. alterniflora,* increased the number of leaves produced under crude oil exposure compared to wild-type lines in *B. distachyon.* ATTPS21 is a terpene synthase gene involved in the production of volatiles in flower petals in *A. thaliana* (Liu *et al.* 2015). Plant volatiles are often activated during stress, and may play a role in relieving oxidative stress (Holopainen & Gershenzon 2010). Although the mechanism for this relationship remains unclear, the overrepresentation of the coumarin synthesis pathway among differentially expressed genes provides additional evidence for an interaction between

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the maintenance of the production of volatiles and fitness during crude oil exposure in both *B. distachyon* and *S. alterniflora*.

Conclusions

While these findings show novel patterns of response to hydrocarbon stress, and our experimental results support a role for SUVH5 and ATTPS21 in regulating the response to crude oil, the divergence between *S. alterniflora, B. distachyon,* and *A. thaliana* limits the conclusions that we can draw. The *Spartina* genus (Chloridoideae subfamily) diverged from *Brachypodium* (*Pooideae* subfamily) more than 40 million years ago (Rousseau-Gueutin *et al.* 2015), and from *Arabidopsis* approximately 150 million years ago (Kumar *et al.* 2017). During that time, the *Spartina* genus has undergone several hybridization and polyploidization events (Ainouche *et al.* 2004) that have increased chromosome number and ploidy level from 2n=4x=40 (tetraploid) to 2n=12x=120-124 (dodecaploid). Genome duplication and copy number variation can complicate our understanding of the role of specific genes, and increase the proportion of false positive matches (Fortune *et al.* 2007; Ferreira de Carvalho 2013; Primmer *et al.* 2013; Boutte *et al.* 2016; Ferreira de Carvalho *et al.* 2017). Although we characterized gene function using *B. distachyon* homologs, confirmation of gene function in *S. alterniflora* would require detailed forward and reverse genetic screens, using both transcriptome-wide and single-mutant assays in both field and controlled conditions, to fully characterize gene function in response to crude oil exposure. However, leveraging the genetic resources of model organisms provides a useful baseline and comparison to describe gene function during oil exposure in *S. alterniflora*. Additionally, genomic assays in non-model species that inhabit diverse ecologies will be insightful in the effort to characterize the behavior of genes in natural settings, providing a more complete

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"ecological transcriptome" or "ecological annotation" (Landry & Aubin-Horth 2007; Richards *et al.* 2009; Alvarez *et al.* 2015; Kudoh 2016).

Although we characterize gene function in this manuscript, further dissection of the genomic mechanisms of oil resilience within *S. alterniflora* will be required for understanding the evolutionary fate of oil-exposed populations of *S. alterniflora*. Our field sites possess an unknown ecological history, and latent, unaccounted-for effects may confound and bias our gene expression assays, making controlled follow-up studies important to accurately characterize the functional genomic response to crude oil. We found genetic differentiation among these same populations (Robertson *et al.* 2017), which may explain some of the observed response to oil in gene expression patterns observed in our study. Additionally, the observed variation in gene expression between oil-exposed and unexposed populations may be modulated by epigenetic regulators like SUVH5, which can exert effects in concert with or in addition to the effects of genetic variation (Robertson & Richards 2015; Richards *et al*. 2017). Finally, we note that our sample pooling strategy may miss a number of subtle or cell-specific transcriptional behaviors involved in hydrocarbon response. Future efforts to catalog gene expression variation using RNAseq and additional cell types (including rhizomes and roots) will enhance our capacity to understand the functional response to an increasingly common anthropogenic stressor in this foundation marsh species. Considering that the resilience of *S. alterniflora* may attenuate many types of anthropogenic damage in salt marshes, understanding the molecular underpinnings of oil stress response may provide valuable information for the conservation and management of these ecosystems, as well as a novel understanding of the mechanisms of phenotypic plasticity.

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Tables

Table 1: Results of linear mixed models and generalized linear mixed models for phenotypes assessed. Height, photosynthetic rate, stomatal conductance, and water use efficiency were modeled separately with a normal distribution, while number of leaves, number of stems, and number of inflorescences were modeled with a Poisson distribution. Effects were assessed through Wald type II chi squared tests. Marginal and conditional R^2 values were assessed through the piecewiseSEM R package. df indicates degrees of freedom.

			Oil treatment		**Line**		**Line x Oil treatment**	
			df = 1		df = 4		df = 4	
	Marg. R^2	Cond. R^2	Chi Sq		Chi Sq		Chi Sq	
Final height	0.27	0.42	6.55	*	3.45		4.32	NS
Leaf number	0.43	0.56	9.15	**	11.14	*	7.08	NS
Stem number	0.33	0.42	8.26	**	5.51		3.59	NS
Infl number	0.35	0.54	8.27	**	1.46		5.41	NS
Photosynthesis	0.38	0.65	4.87	*	21.65	***	7.13	NS
Stom conduct	0.23	0.48	0.14		18.29	**	2.43	NS
WUE	0.25	0.34	4.80	*	6.54		3.87	NS

*** P < 0.05; ** P < 0.01; *** P < 0.001; NS P ≥ 0.05.**

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Table 2: Significant terms in pairwise comparisons of T-DNA insertion lines to wild type, and interaction of line x treatment with T values (for linear mixed effects models) and Z values (for generalized linear mixed effects models) using Satterthwaite approximations to estimate degrees of freedom.

Effect	Phenotype	Estimate	t/z
Line (Bradi3g07730)	Stomatal conductance	-0.24	-2.15*
	WUE	15.10	0.013*
Line (Bradi3g35330)	Photosynthetic rate	5.74	2.54*
Interaction of Line x Treatment (Bradi1g62540)	Leaf number	0.45	2.10*
Interaction of Line x Treatment (Bradi3g35330)	Inflorescence number	0.53	1.97*

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Figures

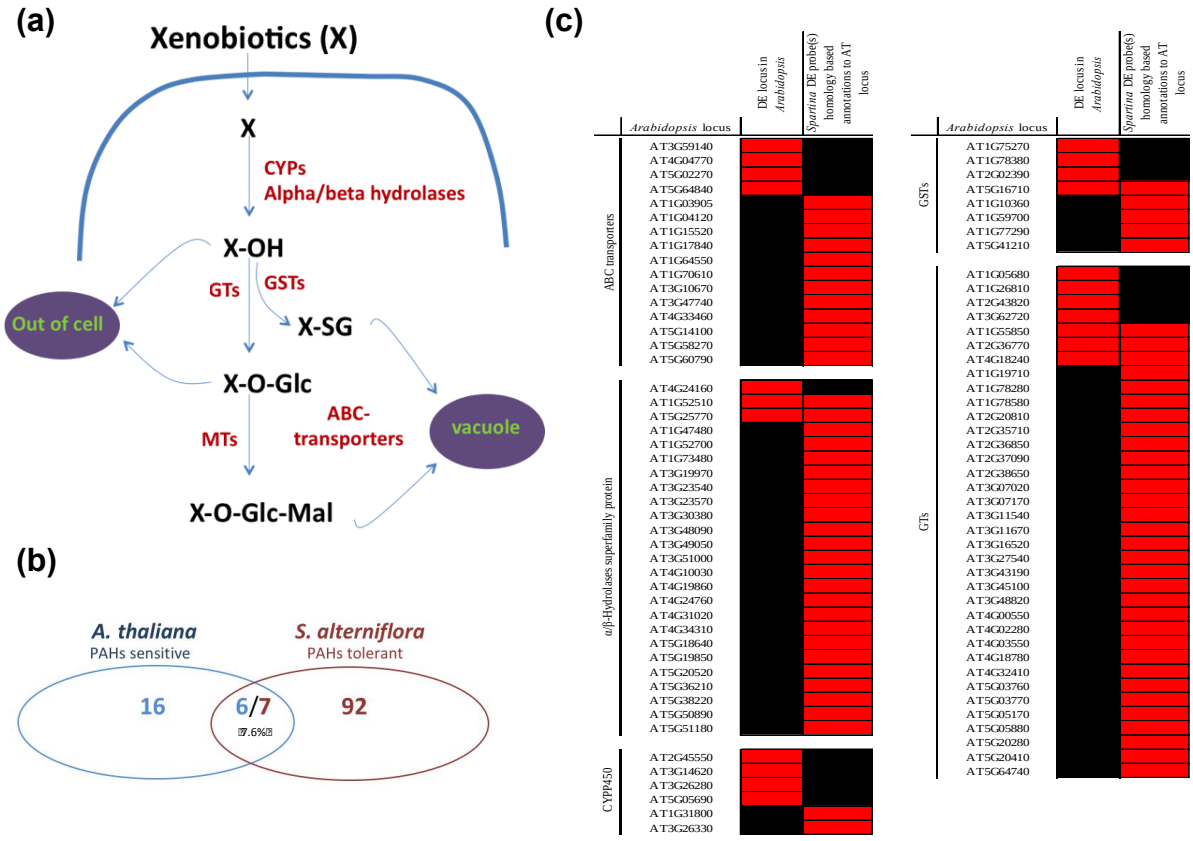


Fig. 1: Xenome comparison between *Spartina alterniflora* and *Arabidopsis thaliana*. (a) Simplified scheme of metabolic pathways of xenobiotic (X) detoxification in plant cells: the thick line indicates the cell membrane, xenome components are indicated in red. (b) The total number of xenome genes expressed in *S. alterniflora* in response to oil contamination in the field, compared to those observed for Col-0 shoot tissue in response to the PAH phenanthrene (Weisman *et al.* 2010; Dumas *et al.* 2016). (c) Graphical matrix depicting differentially expressed xenome loci in response to phenanthrene in *A. thaliana* (Weisman *et al.* 2010; Dumas *et al.* 2016), and in response to the DWH oil spill in *S. alterniflora*. *Arabidopsis thaliana* based gene IDs are categorized by the five xenome gene families on the left. Changes in gene expression were scored as a binary trait: no significant response to PAH are indicated in black, differentially expressed genes are indicated in red.

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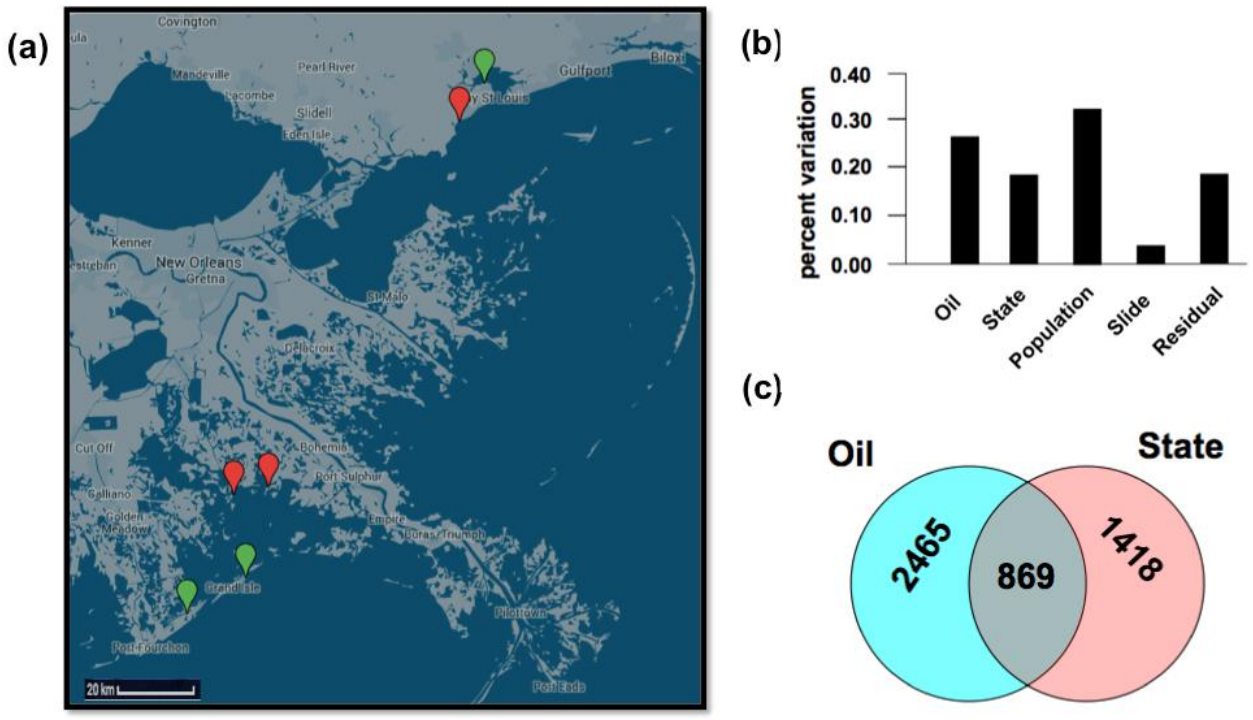


Fig. 2: Response to the *DWH* oil spill in *S. alterniflora* across Gulf of Mexico study sites. (a) Green markers represent sites with no visible oil and red markers represent sites with visible oil in the sediment and substantial dieback of above ground *Spartina alterniflora* stems. (b) The percent of the transcriptional variance explained by oil, state, population, slide and the residual. (c) The number of genes that significantly differ between populations exposed to oil or not, and location (State). Population was modeled as a random effect and not estimable.

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(a)

Spartina alterniflora transcript	*Bd* annotation	*A. thaliana* homolog	*S. alterniflora* response to oil	Modification type
S_alt_contig08149	Bradi1g68290	ATMCB1, MBP1	Decreased	Knockout
S_alt_contig04438_RC	Bradi1g62540	ATTPS21	Increased	Overexpression
S_5sp_contig45211	Bradi3g07730	KCS11	Increased	Overexpression
S_mar_contig02490_RC	Bradi3g35330	SUVH5	Decreased	Overexpression

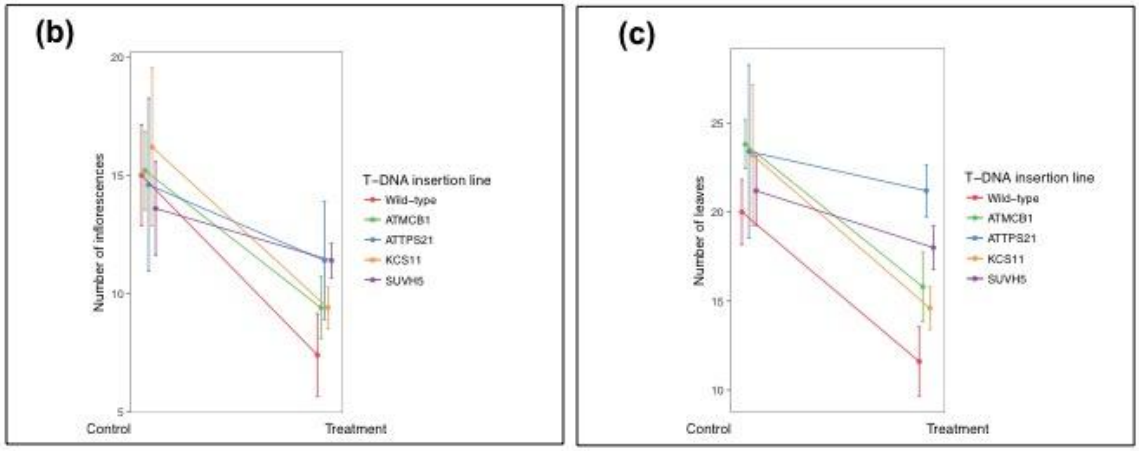


Fig. 3: *Brachypodium distachyon* T-DNA insertion lines used for confirmation of candidate genes. (a) T-DNA lines are listed as the catalog numbers from the WRRC with corresponding *S. alterniflora* contigs from our custom microarray and the closest *A. thaliana* homolog. Modification type represents the effect of the T-DNA insertion, either knocking out the function of the particular gene or tagging the promoter region to induce overexpression. Expression response in *S. alterniflora* is listed along with the justification for choosing each line. Reaction norms for (b) number of inflorescences, and (c) number of leaves for each T-DNA knockout line, referenced by its homolog in *A. thaliana*, in oil treatment and control.

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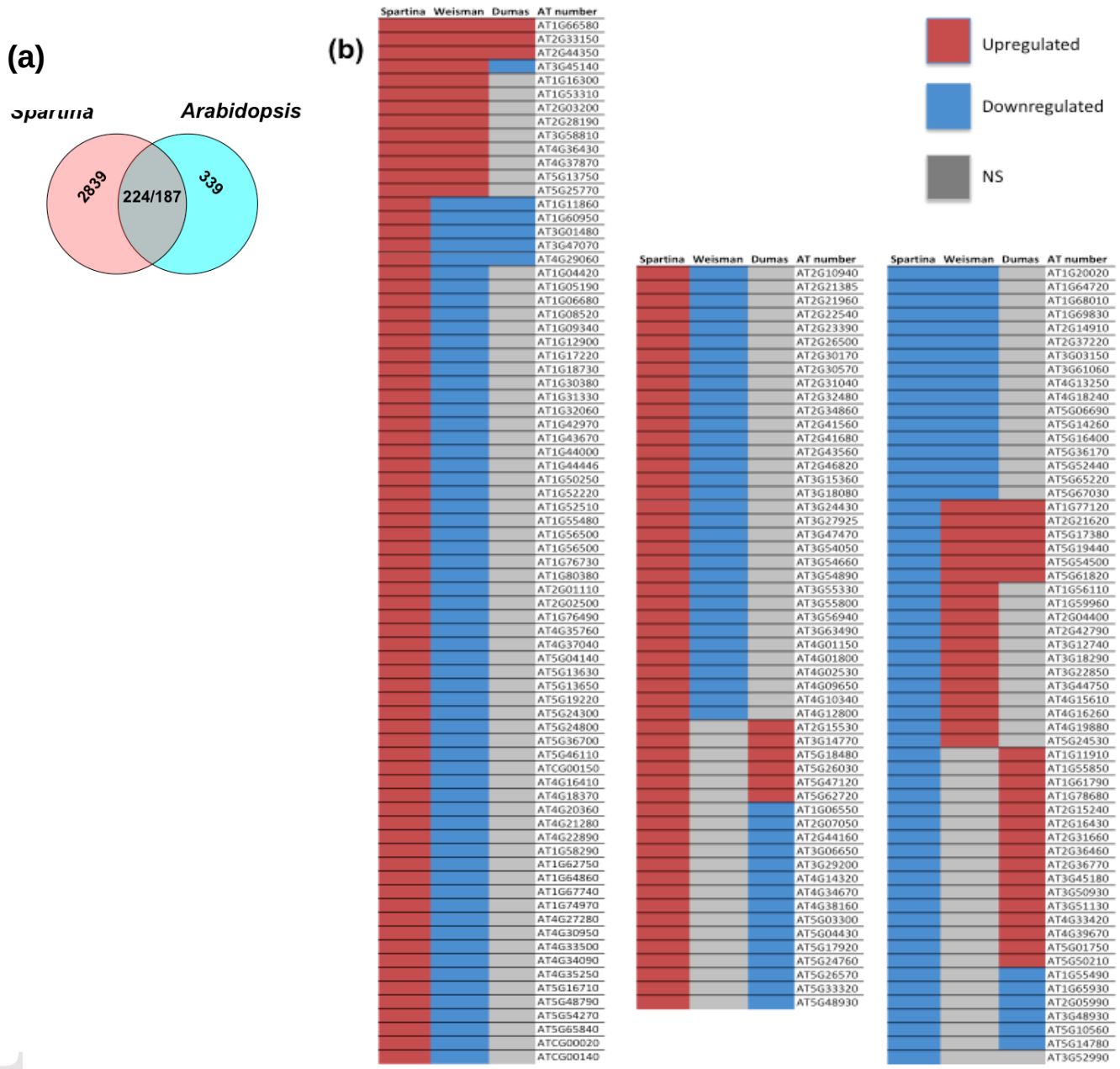


Fig. 4: Comparison of oil responsive genes across studies in *S. alterniflora* and *A. thaliana.* (a) From a consistent set of genes surveyed (represented either on the *Spartina* array and either the ATH1 or CATMA arrays), the total number of genes expressed in *S. alterniflora* in response to oil contamination in the field, compared to those observed for Col-0 shoot tissue in response to the PAH phenanthrene (Weisman *et al.* 2010; Dumas *et al.* 2016). (b) Direction of difference in expression in 187 genes that respond to hydrocarbon stress in both *S. alterniflora* and *A. thaliana*.

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Author Contribution

CLR, AS & MLA designed the research; CLR, MA & SM collected plants, performed greenhouse studies and data collection; AS, JFC, MLA designed the *Spartina* microarray; CLR, MA, AC-R, AEA & TEF performed data analysis. All authors contributed to data interpretation, and writing of the manuscript.

Data accessibility statement

Unprocessed and processed microarray data have been deposited at the Gene Expression Omnibus (GEO) and are available at accession number GSE114635. Other processed data files, R scripts, and site coordinates are available on Dryad at doi:10.5061/dryad.kp0rc5s and on GitHub at https://github.com/AlvarezMF/DWHoilspill_transcriptome/.

Supporting Information

Table S1: Comparison of differentially expressed xenome genes in *A. thaliana* and the custom *S. alternflora*.

Table S2: List of 39 co-expressed gene clusters identified by WGCNA, with the number of genes in each cluster and the most significant enrichment category from Gene Ontology analysis (For clusters with equally significant GO categories, all most significant categories are shown).

Fig. S1: Multidimensional scaling (MDS), showing MDS axes 1 and 2. MSN and MSO represent Mississippi uncontaminated and contaminated sites, respectively. LN1 and LN2 represent Louisiana uncontaminated sites, while LO1 and LO2 represent Louisiana contaminated sites.

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Fig. S2: Average change in expression in both qPCR and microarray for target confirmation genes.

Fig. S3: Comparison of transcripts represented on two *A. thaliana* arrays (ATH1 and CATMA) and the custom *Spartina* array.

Fig. S4: Unique gene expression patterns identified through WGCNA. (A) Scale independence and mean connectivity, as a function of the soft thresholding power β. (B) Clustering dendrogram of dissimilarity showing thirty nine unique clusters represented by different colors.

Fig. S5: A directed acyclic graph of the high-association GO terms to show the relationships between 26 Gene Ontology (GO) categories of genes with A. thaliana homologs in cluster #3. This cluster contains 2,054 genes identified through WGCNA, and is correlated to oil exposure. Higher associations (uncorrected P-values) are colored red.

File S1: Results of Gene Ontology analysis of all differentially expressed genes (n=1426) in response to phenanthrene in *A. thaliana* in either Weisman *et al.* (2010) or Dumas *et al.* (2016).

File S2: Normalized gene expression values for 15,867 genes in WGCNA and corresponding module assignment.

File S3: Number of connections for each of the 1410 highly differentially expressed genes with homologs in *A. thaliana* in the Virtual Plant network.

File S4: Identity and description of 187 oil responsive genes (224 homology-based *S. alterniflora* loci) that overlapped between *A. thaliana* (Weisman *et al.* 2010 and Dumas *et al.* 2016 combined), and *S. alterniflora* (present study).

File S5: Stress-responsive genes (n = 3907) across stresses, the *A. thaliana* xenome and *S. alterniflora* oil responsive genes.

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