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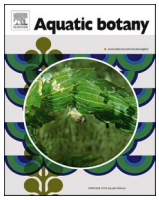

Transcriptional responses to 2,4-D herbicide treatment of a Eurasian (*Myriophyllum spicatum*) and a hybrid (*M. spicatum* × *M. sibiricum*) genotype of watermilfoil that differ in their sensitivity to 2,4-D

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ABSTRACT

Aquatic plant managers frequently treat Eurasian watermilfoil (*Myriophyllum spicatum* L.; EWM) and hybrid watermilfoil (*Myriophyllum spicatum* L. × *Myriophyllum sibiricum* Komarov) with 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide. However, watermilfoil genotypes can differ in their response to 2,4-D. In this study, we compared facultative and constitutive gene expression differences for two watermilfoil genotypes (one Eurasian and one hybrid) that differ in their sensitivity to 2,4-D. To do this, we compared between control and 0.5 mg L^{-1} 2,4-D treated plants at four time points after treatment. We also assembled the first *de novo* watermilfoil transcriptome. We found that while qualitatively similar, the facultative transcriptional response of the EWM genotype to 2,4-D treatment was much stronger than the hybrid genotype, indicated by a greater number and log-fold-change of differentially expressed genes at all time points after treatment. Further, we found that the EWM and hybrid genotype differed in their 9-cis-epoxycarotenoid dioxygenase (NCED) and abscisic acid (ABA) gene response, and that there was a greater amount of photosynthesis gene downregulation (both in number and log-fold-change) in the EWM than the hybrid genotype. At the constitutive level, overall, the hybrid expressed genes at a higher level than the EWM genotype, but not the genes of the 2,4-D response pathway. These differences in gene expression match with the degree of phenotypic difference in growth observed between these genotypes when exposed to 2,4-D. The hybrid genotype used here mitigates the effects of 2,4-D treatment better than the EWM genotype at both the molecular and phenotypic level. More study is needed to understand the mechanism (s) of mitigation and whether this is a cause of hybridity, or the specific genotypic backgrounds used here.

1. Introduction

Worldwide, approximately 366 million hectares of weeds are treated with synthetic auxin herbicides every year (Busi et al., 2018). The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was the first synthetic auxin herbicide developed (in 1945) and is still commonly used to control terrestrial and aquatic weeds (Peterson et al., 2016). Like the rest of the synthetic auxins, 2,4-D mimics the natural plant hormone indole-3-acetic acid (IAA) and causes an over stimulation of this hormone pathway (Peterson et al., 2016; Busi et al., 2018). However, herbicide resistance evolution threatens the effective use of auxinic herbicides to control weeds. There are currently populations of 21 species of terrestrial weeds and populations of four species of aquatic weeds resistant to 2,4-D (Heap, 2021). Given the small number of approved aquatic herbicides, it is important to understand mechanisms of resistance to enact resistance tracking and management to preserve these important management tools.

Understanding the mechanisms of 2,4-D response is complicated because it induces the same signaling pathways as the naturally occurring hormone IAA. In fact, high concentrations of IAA stimulate the same

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herbicide-like effect as 2,4-D (Grossman, 2010). There are several auxin/IAA receptors in plants, and it is possible that mutations in any one of them may confer resistance to 2,4-D (Peterson et al., 2016), although only one instance has been identified to date (LeClere et al., 2018). Studies in *Arabidopsis thaliana* and other species have found several non-target site pathways for 2,4-D resistance, including differences in absorption, translocation, metabolism, and gene expression compared to wildtype plants (Teixeira et al., 2007; Peterson et al., 2016; Figueiredo et al., 2017; Busi et al., 2017).

Ethylene accumulation and subsequent hydrogen cyanide production was thought to be the cause of plant necrosis and death from auxinic herbicides (Grossman, 2010). However, not all synthetic auxin herbicides induce ethylene production, and instead increased levels of abscisic acid (ABA) have been proposed as sufficient for the down-regulation of photosynthesis and growth that ultimately kills the plants (McCauly et al., 2020; Gaines, 2020). While the mechanism of plant death from auxinic herbicides is still unclear, there is consensus regarding the events that take place after a plant is exposed to auxinic herbicides. Once auxins enter the plant cell, they bind to and remove Aux/IAA proteins, the repressors for the 9-cis-epoxycarotenoid dioxygenase (NCED) gene (Grossman, 2010; McCauly et al., 2020). NCED is then upregulated, the rate limiting step in the production of ABA, which leads to an accumulation of ABA in the plant. Finally, photosynthesis is downregulated (Grossman, 2010; McCauly et al., 2020; Gaines, 2020).

In the US, the invasive aquatic macrophyte Eurasian watermilfoil (*Myriophyllum spicatum* L. [EWM]), including interspecific hybrids with native northern watermilfoil (*Myriophyllum sibiricum* Komarov × *M. spicatum*) are heavily managed with 2,4-D (Bartodziej and Ludlow, 1998). However, experimental studies in watermilfoil show that there is variation in growth rates between genotypes of invasive watermilfoil in control and 2,4-D treated environments (Thum et al., 2012; LaRue et al., 2013; Netherland and Willey, 2017; Taylor et al., 2017; Hoff and Thum, 2022). For example, a hybrid genotype isolated from Hayden Lake (Idaho) exhibited relatively fast growth in control and 2,4-D treatments compared to the other genotypes in a study by Taylor et al. (2017). In contrast, a EWM genotype isolated from Coeur D′Alene Lake (Idaho) was a relatively slow grower in control and 2,4-D treatments compared to the other genotypes tested by Hoff and Thum (2022). Therefore, we hypothesized that these two genotypes would respond to 2,4-D differentially at the molecular level, specifically in the genes they express.

Gene expression differences may be constitutive, occurring in the presence and absence of herbicide, or facultatively stimulated by herbicide treatment. Across multiple weed species, gene expression differences between herbicide susceptible and resistant genotypes tend to be constitutive (Giacomini et al., 2018). However, there are well-defined cases of facultative expression conferring resistance to herbicides. For example, expression of transporter genes is increased in glyphosate resistant individuals of horseweed (*Conyza canadensis*) but not in susceptible individuals when treated with glyphosate (Nol et al., 2012). Here, we determine the sensitivity of the two genotypes listed above (hybrid from Hayden Lake, ID and EWM from Coeur D′Alene Lake, ID) to four concentrations of 2,4-D treatment. Next, we assessed these genotypes for constitutive and facultative gene expression over time in response to 2,4-D treatment using RNA-seq.

2. Methods

2.1. Sample collection

A single meristem of each genotype (a hybrid watermilfoil genotype collected from Hayden Lake, Idaho, and a Eurasian genotype collected from Coeur D′Alene Lake, Idaho) was collected from the field and vegetatively propagated in 208 L drums in the Montana State University Plant Growth Center (Bozeman, MT) until there were enough healthy meristems for the experiment. – From here on, we refer to the Hayden Lake genotype as 'hybrid', and the Coeur D′Alene genotype as 'EWM' for simplicity.

2.2. Growth assay

Prior information on 2,4-D response for the two genotypes came from two separate studies. Therefore, we conducted a simple dose-response study to directly compare their 2,4-D response and to select the dose at which the largest difference between genotypes occurs. We followed similar methods as described in Hoff and Thum (2022). Briefly, three 10 cm meristems of each genotype were planted in separate 210 mL Deepot Cell Cone-tainers™ (Stuewe and Sons Inc. Tangent, OR) and then pressed into the soil lined (1:1:1 topsoil:peat:sand) bottom of a 208 L drum filled with 170 L of Smart and Barko (1985) buffered water. We used five drums each with three replicates of each genotype per drum. The herbicide assay took place in a growth room in the Plant Growth Center at Montana State University (Bozeman, MT) equipped with LED lights, a photoperiod of 16 hr light:8 hr dark, a light intensity of 450 μmol m^{-2} s^{-1} at the water surface, and a constant air and water temperature of 21 °C. Plants were allowed to establish for four weeks after which four of the five tanks were randomly chosen to receive treatments – 0.25, 0.50, 1.00, and 2.00 mg L^{-1} 2,4-D and the fifth tank was used as an untreated control. We used a powdered chemical grade 2,4-D (Fisher Scientific Waltham, MA) solubilized in 100% ethanol before adding to water. We chose these concentrations because 2,4-D is commonly used in the field at these applications rates. After 48 h in treatment, tanks were drained and flushed with 94 L and then refilled with 190 L of Smart and Barko (1985) buffered water. Plants were grown for another four weeks after treatment flushing, at which time the remaining above and below ground biomass was harvested and dried to a constant weight at 44 °C in a drying room in the Plant Growth Center at Montana State University (Bozeman, MT).

We used the dry biomass measurements to generate a dose response curve for the two genotypes using the drc package (Ritz et al., 2015) in R (R Core Team, 2021). We first tested a set of three-parameter models with lower limits fixed at zero – log-logistic (LL.3), Weibull type 1 (W1.3), Weibull type 2 (W2.3) and four-parameter models with lower limits allowed to vary – log-logistic (LL.4), Weibull type 1 (W1.4), Weibull type 2 (W2.4). Because we used the proportion of mean control biomass as the predicted variable, we fixed all model upper limits to one. We then used the 'mselect' function in the drc package to determine the best fitting dose response model for each genotype. All models had very similar fits within a genotype according to their IC values. Biomass was never zero at the upper concentrations of 2,4-D, so we chose to plot both genotype dose response models as a four parameter log-logistic model with the upper limit fixed at one and the lower limit allowed to vary (Fig. 1).

2.3. Differential expression experiment

To determine differential gene expression of the two genotypes, we planted 30 – 8 cm meristems of each genotype into individual 50 mL glass beakers (VWR International Radnor, PA) with sediment described in Organisation for Economic and Co-operation Development TG 239 (2014). Each 50 mL beaker was then placed into a 1.5 L glass beaker (VWR International Radnor, PA) filled with Smart and Barko (1985) buffered water.

To reduce variation among experimental units, we grew the beaker-planted watermilfoil meristems and performed herbicide treatments in a growth chamber in the Plant Growth Center at Montana State University (Bozeman, MT). The incubator was equipped with LED lights and was set to a photoperiod of 16 hr light: 8 hr dark, a light intensity of 120 μmol m^{-2} s^{-1} at the water surface, and a constant water and air temperature of 21 °C. All 30 beakers of each genotype (n = 60 total) were randomly arranged on the same plane in the incubator and were rotated daily to reduce positional effects. After a nine-day establishment period, six of each genotype were harvested to check for roots to ensure

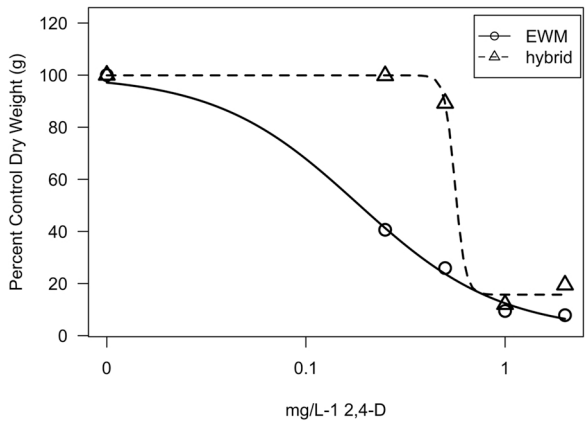

Fig. 1. A plot of the dose response model for each watermilfoil genotype – a four parameter log-logistic model with the upper limit fixed at one. The dashed line represents the fitted model for a hybrid genotype from Hayden Lake, Idaho (hybrid) and the solid line represents the fitted model for a Eurasian genotype collected from Coeur d'Alene Lake, Idaho (EWM). Each model predicted the percent response (each dry weight (g) / the mean control dry weight (g) * 100) for each concentration (0.250, 0.500, 1.000, and 2.000 mg L^{-1}) of 2,4-D treatment. Each point (triangles = hybrid and circles = EWM) represents the mean of three replicates at that treatment level for each genotype.

that the plants had established.

To capture facultative expression response of the two genotypes, half (n = 12) of the beakers of each genotype were treated with 0.5 mg L^{-1} 2,4-D. We chose to examine gene expression at 0.5 mg L^{-1} 2,4-D because that is where we saw a large difference in growth between the two genotypes in the dose response assay but did not want either genotype to die before collecting samples. We used a powdered chemical grade 2,4-D (Fisher Scientific Waltham, MA) solubilized in 100% ethanol before adding to water. The control beakers were also given a mock dose of 100% ethanol. Because we wanted to see how 2,4-D affected facultative expression differences over time, we harvested three beakers of each treatment and control for each genotype at timepoints that successively doubled in duration - 12, 24, 48, and 96 h after treatment (HAT; see Fig. S1). Auxins stimulate growth, and because 2,4-D is a synthetic auxin, we wanted to determine gene expression in the actively growing meristems. We harvested meristem tissue at each of the time points after treatment by dissecting the meristem from the rest of the plant and immediately flash freezing in liquid nitrogen to preserve total RNA.

2.4. Molecular methods

We extracted total RNA from each frozen meristem using a Qiagen RNeasy Plant Mini kit (Valencia, CA). Total RNA of each sample was quantified on a Nanodrop spectrophotometer (ThermoFisher Waltham, MA) and Qubit 4 fluorometer (ThermoFisher Waltham, MA). We sent 2 μg of RNA for each sample to Novogene Corp. (Sacramento, CA) for quantification on an Agilent 2100 Bio Analyzer (Santa Clara, CA), after which they performed the library prep using poly-A selection and cDNA synthesis. The 150 bp size selected fragments were then paired end sequenced using Illumina sequencing technologies (San Diego, CA). Each sample per direction (paired end) was sequenced to a minimum depth of 20 million raw reads, and two control samples from each genotype were also sequenced to a minimum depth of 40 million raw reads per direction for *de novo* transcriptome assembly.

2.5. Transcriptome assembly and annotation

Because there is no sequenced genome for watermilfoil or any closely related species, we assembled a *de novo* watermilfoil transcriptome. First, we removed adaptors and common Illumina sequencing artifacts (e.g., phi X) from the four samples more deeply sequenced with BBDuk using default settings (http://jgi.doe.gov/data-and-tools/bb-tools/). Next, we verified fragment sizing and quality using FastQC v0.11.8 (Andrews, 2010). To ensure that only watermilfoil reads were being assembled, we filtered contaminant reads using BBMap with default settings (http://jgi.doe.gov/data-and-tools/bb-tools/) by removing reads that aligned to non-plant species' (reference files included in BBMap). After filtering, samples ranged from 43,356,672 to 50,529,019 reads of size 150 bp.

We assembled the reference transcriptome using Trinity v2.8.4 with default parameters (Grabherr et al., 2011). To ensure that differential expression between treatments and genotypes was reliable, and not due to reference alignment biases, we constructed transcriptomes from EWM only samples, hybrid only samples, and combined EWM and hybrid samples. We then aligned all experimental samples to all transcriptomes using Hisat2 v2.2.1 (Daehwan et al., 2019) and determined that samples from both taxa had the highest alignment rate to the combined transcriptome (Supp. Table 1). Because of this, we used the combined EWM and hybrid transcriptome for differential expression analyses. Additionally, this ensured that we were looking at the same gene between taxa in differential expression analyses. The final combined transcriptome contained 334,917 assembled genes averaging 923 bases and 535,628 transcripts averaging 595 bases (Supp Table 2). Next, we annotated the combined transcriptome using the full Trinotate pipeline (Bryant et al., 2017). Briefly, the Trinotate pipeline determines homology between the assembled genes and the UniProt database, protein domain identification (Pfam), protein signal peptides, and transmembrane domains. At the end of the pipeline, the *de novo* watermilfoil transcriptome contained predicted Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and eggnog annotations for ~50% of the transcripts. This annotated watermilfoil transcriptome was then used as the reference for the differential gene expression analyses below.

2.6. Differential expression analyses

To determine the differential expression of the 48 experimental samples, we started by removing sequencing adaptors and trimming to an average minimum Phred score of 10 with BBDuk. Quality was then visually verified with FastQC v0.11.8. Next, we aligned sample reads to the transcriptome and quantified them using Salmon v1.6.0 with default parameters (Patro et al., 2017). We then used the tximport v1.14.2 package (Soneson et al., 2015) in R to import and aggregate the quantification files into gene level counts. The differential expression was done by calculating a full model and performing paired contrasts in edgeR v3.28.1 (Robinson et al., 2010). To identify genes demonstrating facultative differential expression, we compared three treated samples (0.5 mg L^{-1} 2,4-D) at all timepoints (12, 24, 48, and 96 HAT) relative to the three control samples at the same time point. To determine constitutive expression differences, we compared between genotypes in only the control environment, so that facultative expression did not obscure these comparisons. We also grouped all control samples from the four time points (12 total/genotype) and made one contrast between the two genotypes to determine constitutive differences in expression (see Supp Fig S1 for experimental design and contrasts). Significant differences in expression were determined as those genes with a p-value less than 0.05 after Benjamini-Hochberg (1995) False Discovery Correction. To summarize overall expression differences, we calculated the total numbers of significantly up- and downregulated genes in response to treatment at all time points after treatment.

To visualize the differential expression of auxin herbicide pathway genes, we started by determining enriched gene ontology (GO) terms using the GOseq v1.38.0 package (Young et al., 2010) in R. GO terms were considered significantly enriched if the p-value after Benjamini--Hochberg (1995) False Discovery Correction was less than 0.05. From

Table 1
Table of the ratio of differentially expressed genes in each Gene Ontology group due to 0.5 mg L^{-1} 2,4-D treatment at 12 h after treatment. Gene ontologies are broken into biological processes (BP), cellular components (CC), and molecular functions (MF). 'EWM' represents a Eurasian watermilfoil genotype collected from Coeur d'Alene Lake, Idaho and 'hybrid' represent a hybrid genotype of watermilfoil collected from Hayden Lake, Idaho.

			EWM			hybrid		
Category	Ontology	Term	Over P-value	Under P-value	Ratio DE in Catalog	Over P-value	Under P-value	Ratio DE in Catalog
GO:0031323	BP	regulation of cellular metabolic process	-	-	-	0.00	1.00	0.01
GO:0080090	BP	regulation of primary metabolic process	-	-	-	0.00	1.00	0.01
GO:0065007	BP	biological regulation	0.00	1.00	0.01	0.00	1.00	0.01
GO:0050789	BP	regulation of biological process	0.00	1.00	0.01	0.00	1.00	0.01
GO:0050794	BP	regulation of cellular process	0.00	1.00	0.01	0.00	1.00	0.01
GO:0009889	BP	regulation of biosynthetic process	-	-	-	0.00	1.00	0.01
GO:0051171	BP	regulation of nitrogen compound metabolic process	-	-	-	0.00	1.00	0.01
GO:0031326	BP	regulation of cellular biosynthetic process	-	-	-	0.00	1.00	0.01
GO:0050896	BP	response to stimulus	0.00	1.00	0.01	0.00	1.00	0.01
GO:0010556	BP	regulation of macromolecule biosynthetic process	0.00	1.00	0.01	0.00	1.00	0.01
GO:2000112	BP	regulation of cellular macromolecule biosynthetic process	0.00	1.00	0.01	0.00	1.00	0.01
GO:0019219	BP	regulation of nucleobase-containing compound metabolic process	0.00	1.00	0.01	0.00	1.00	0.01
GO:0051252	BP	regulation of R- metabolic process	0.00	1.00	0.01	0.00	1.00	0.01
GO:2001141	BP	regulation of R- biosynthetic process	0.00	1.00	0.01	0.00	1.00	0.01
GO:1903506	BP	regulation of nucleic acid-templated transcription	0.00	1.00	0.01	0.00	1.00	0.01
GO:0006355	BP	regulation of transcription, D–templated	0.00	1.00	0.01	0.00	1.00	0.01
GO:0042221	BP	response to chemical	0.00	1.00	0.02	0.00	1.00	0.01
GO:0009791	BP	post-embryonic development	-	-	-	0.00	1.00	0.01
GO:0007165	BP	sig-l transduction	0.00	1.00	0.02	0.00	1.00	0.01
GO:0033993	BP	response to lipid	0.00	1.00	0.02	0.00	1.00	0.01
GO:0010033	BP	response to organic substance	0.00	1.00	0.02	0.00	1.00	0.01
GO:0048589	BP	developmental growth	-	-	-	0.00	1.00	0.01
GO:0032989	BP	cellular component morphogenesis	0.00	1.00	0.02	0.00	1.00	0.01
GO:0099402	BP	plant organ development	0.00	1.00	0.02	0.00	1.00	0.02
GO:0016049	BP	cell growth	0.00	1.00	0.02	0.00	1.00	0.02
GO:0000902	BP	cell morphogenesis	0.00	1.00	0.02	0.00	1.00	0.02
GO:0040008	BP	regulation of growth	0.00	1.00	0.02	0.00	1.00	0.02
GO:1901617	BP	organic hydroxy compound biosynthetic process	-	-	-	0.00	1.00	0.02
GO:0060560	BP	developmental growth involved in morphogenesis	0.00	1.00	0.02	0.00	1.00	0.02
GO:0009719	BP	response to endogenous stimulus	0.00	1.00	0.03	0.00	1.00	0.02
GO:0009725	BP	response to hormone	0.00	1.00	0.04	0.00	1.00	0.02
GO:0048364	BP	root development	0.00	1.00	0.03	0.00	1.00	0.02
GO:0009308	BP	amine metabolic process	0.00	1.00	0.03	0.00	1.00	0.02
GO:0010817	BP	regulation of hormone levels	0.00	1.00	0.03	0.00	1.00	0.02
GO:0009826	BP	unidimensio-l cell growth	0.00	1.00	0.02	0.00	1.00	0.03
GO:0006720	BP	isoprenoid metabolic process	0.00	1.00	0.03	0.00	1.00	0.03
GO:0009755	BP	hormone-mediated sig-ling pathway	0.00	1.00	0.05	0.00	1.00	0.03
GO:0008299	BP	isoprenoid biosynthetic process	0.00	1.00	0.04	0.00	1.00	0.03
GO:0006721	BP	terpenoid metabolic process	0.00	1.00	0.04	0.00	1.00	0.03
GO:0044770	BP	cell cycle phase transition	-	-	-	0.00	1.00	0.03
GO:0044772	BP	mitotic cell cycle phase transition	-	-	-	0.00	1.00	0.03
GO:0000079	BP	regulation of cyclin-dependent protein serine/threonine ki-se activity	-	-	-	0.00	1.00	0.04
GO:0034754	BP	cellular hormone metabolic process	0.00	1.00	0.05	0.00	1.00	0.04
GO:0016114	BP	terpenoid biosynthetic process	0.00	1.00	0.05	0.00	1.00	0.04
GO:1905393	BP	plant organ formation	0.00	1.00	0.05	0.00	1.00	0.04
GO:0042445	BP	hormone metabolic process	0.00	1.00	0.04	0.00	1.00	0.04
GO:0009736	BP	cytokinin-activated sig-ling pathway	-	-	-	0.00	1.00	0.04
GO:0009788	BP	negative regulation of abscisic acid-activated sig-ling pathway	0.00	1.00	0.05	0.00	1.00	0.04
GO:1901420	BP	negative regulation of response to alcohol	0.00	1.00	0.05	0.00	1.00	0.04
GO:1905958	BP	negative regulation of cellular response to alcohol	0.00	1.00	0.05	0.00	1.00	0.04
GO:0016101	BP	diterpenoid metabolic process	0.00	1.00	0.06	0.00	1.00	0.05
GO:0010311	BP	lateral root formation	0.00	1.00	0.08	0.00	1.00	0.06
GO:0042446	BP	hormone biosynthetic process	0.00	1.00	0.04	0.00	1.00	0.06
GO:0009733	BP	response to auxin	0.00	1.00	0.10	0.00	1.00	0.06
GO:0009734	BP	auxin-activated sig-ling pathway	0.00	1.00	0.11	0.00	1.00	0.06
GO:0042214	BP	terpene metabolic process	0.00	1.00	0.10	0.00	1.00	0.07
GO:0009741	BP	response to brassinosteroid	0.00	1.00	0.06	0.00	1.00	0.07
GO:0010252	BP	auxin homeostasis	0.00	1.00	0.13	0.00	1.00	0.07
GO:0016102	BP	diterpenoid biosynthetic process	0.00	1.00	0.10	0.00	1.00	0.07
GO:0009685	BP	gibberellin metabolic process	0.00	1.00	0.10	0.00	1.00	0.07
GO:0009690	BP	cytokinin metabolic process	0.00	1.00	0.09	0.00	1.00	0.07
GO:0009686	BP	gibberellin biosynthetic process	0.00	1.00	0.11	0.00	1.00	0.08
GO:0006714	BP	sesquiterpenoid metabolic process	0.00	1.00	0.11	0.00	1.00	0.08
GO:1905421	BP	regulation of plant organ morphogenesis	0.00	1.00	0.07	0.00	1.00	0.08
GO:0009687	BP	abscisic acid metabolic process	0.00	1.00	0.13	0.00	1.00	0.08
GO:0043288	BP	apocarotenoid metabolic process	0.00	1.00	0.13	0.00	1.00	0.08
GO:1902644	BP	tertiary alcohol metabolic process	0.00	1.00	0.13	0.00	1.00	0.08
GO:0016106	BP	sesquiterpenoid biosynthetic process	0.00	1.00	0.14	0.00	1.00	0.10
GO:0009688	BP	abscisic acid biosynthetic process	0.00	1.00	0.15	0.00	1.00	0.10
GO:0043289	BP	apocarotenoid biosynthetic process	0.00	1.00	0.15	0.00	1.00	0.10

(*continued on next page*)

Table 1 (*continued*)

Category	Ontology	Term	EWM			hybrid		
			Over P-value	Under P-value	Ratio DE in Catalog	Over P-value	Under P-value	Ratio DE in Catalog
GO:1902645	BP	tertiary alcohol biosynthetic process	0.00	1.00	0.15	0.00	1.00	0.10
GO:0008300	BP	isoprenoid catabolic process	0.00	1.00	0.18	0.00	1.00	0.11
GO:0016128	BP	phytosteroid metabolic process	-	-	-	0.00	1.00	0.11
GO:0016131	BP	brassinosteroid metabolic process	-	-	-	0.00	1.00	0.12
GO:1901371	BP	regulation of leaf morphogenesis	-	-	-	0.00	1.00	0.13
GO:0048830	BP	adventitious root development	0.00	1.00	0.14	0.00	1.00	0.14
GO:0010050	BP	vegetative phase change	-	-	-	0.00	1.00	0.14
GO:0009691	BP	cytokinin biosynthetic process	-	-	-	0.00	1.00	0.14
GO:0010268	BP	brassinosteroid homeostasis	-	-	-	0.00	1.00	0.15
GO:0016119	BP	carotene metabolic process	0.00	1.00	0.24	0.00	1.00	0.16
GO:0016121	BP	carotene catabolic process	0.00	1.00	0.40	0.00	1.00	0.27
GO:0046247	BP	terpene catabolic process	0.00	1.00	0.40	0.00	1.00	0.27
GO:0080191	BP	secondary thickening	-	-	-	0.00	1.00	0.33
GO:0009852	BP	auxin catabolic process	0.00	1.00	0.67	0.00	1.00	0.67
GO:0048756	BP	sieve cell differentiation	0.00	1.00	1.00	0.00	1.00	1.00
GO:0009914	BP	hormone transport	0.00	1.00	0.05	-	-	-
GO:0060918	BP	auxin transport	0.00	1.00	0.06	-	-	-
GO:0010315	BP	auxin efflux	0.00	1.00	0.13	-	-	-
GO:0009415	BP	response to water	0.00	1.00	0.03	-	-	-
GO:0010928	BP	regulation of auxin mediated sig-ling pathway	0.00	1.00	0.07	-	-	-
GO:0009414	BP	response to water deprivation	0.00	1.00	0.02	-	-	-
GO:0009628	BP	response to abiotic stimulus	0.00	1.00	0.01	-	-	-
GO:0009606	BP	tropism	0.00	1.00	0.04	-	-	-
GO:0042447	BP	hormone catabolic process	0.00	1.00	0.22	-	-	-
GO:2000280	BP	regulation of root development	0.00	1.00	0.04	-	-	-
GO:0010411	BP	xyloglucan metabolic process	0.00	1.00	0.07	-	-	-
GO:0048766	BP	root hair initiation	0.00	1.00	0.17	-	-	-
GO:0009630	BP	gravitropism	0.00	1.00	0.04	-	-	-
GO:0009958	BP	positive gravitropism	0.00	1.00	0.08	-	-	-
GO:0010082	BP	regulation of root meristem growth	0.00	1.00	0.09	-	-	-
GO:0009629	BP	response to gravity	0.00	1.00	0.04	-	-	-
GO:0016053	BP	organic acid biosynthetic process	0.00	1.00	0.01	-	-	-
GO:0046394	BP	carboxylic acid biosynthetic process	0.00	1.00	0.01	-	-	-
GO:0065008	BP	regulation of biological quality	0.00	1.00	0.01	-	-	-
GO:0048528	BP	post-embryonic root development	0.00	1.00	0.05	-	-	-
GO:1901700	BP	response to oxygen-containing compound	0.00	1.00	0.01	-	-	-
GO:0010035	BP	response to inorganic substance	0.00	1.00	0.01	-	-	-
GO:0009787	BP	regulation of abscisic acid-activated sig-ling pathway	0.00	1.00	0.03	-	-	-
GO:1901419	BP	regulation of response to alcohol	0.00	1.00	0.03	-	-	-
GO:1905957	BP	regulation of cellular response to alcohol	0.00	1.00	0.03	-	-	-
GO:0090696	BP	post-embryonic plant organ development	0.00	1.00	0.04	-	-	-
GO:0048878	BP	chemical homeostasis	0.00	1.00	0.01	-	-	-
GO:0042546	BP	cell wall biogenesis	0.00	1.00	0.03	-	-	-
GO:0045162	BP	clustering of voltage-gated sodium channels	0.00	1.00	0.50	-	-	-
GO:0019228	BP	neuro-l action potential	0.00	1.00	0.50	-	-	-
GO:0045760	BP	positive regulation of action potential	0.00	1.00	0.50	-	-	-
GO:0098902	BP	regulation of membrane depolarization during action potential	0.00	1.00	0.50	-	-	-
GO:1900825	BP	regulation of membrane depolarization during cardiac muscle cell action potential	0.00	1.00	0.50	-	-	-
GO:1900827	BP	positive regulation of membrane depolarization during cardiac muscle cell action potential	0.00	1.00	0.50	-	-	-
GO:0009738	BP	abscisic acid-activated sig-ling pathway	0.00	1.00	0.02	-	-	-
GO:1902259	BP	regulation of delayed rectifier potassium channel activity	0.00	1.00	0.40	-	-	-
GO:1902260	BP	negative regulation of delayed rectifier potassium channel activity	0.00	1.00	0.40	-	-	-
GO:0005634	CC	nucleus	0.00	1.00	0.01	0.00	1.00	0.01
GO:1902554	CC	serine/threonine protein ki-se complex	-	-	-	0.00	1.00	0.03
GO:0000307	CC	cyclin-dependent protein ki-se holoenzyme complex	-	-	-	0.00	1.00	0.04
GO:0016328	CC	lateral plasma membrane	0.00	1.00	0.08	-	-	-
GO:0003677	MF	D- binding	-	-	-	0.00	1.00	0.01
GO:0003690	MF	double-stranded D- binding	0.00	1.00	0.02	0.00	1.00	0.01
GO:0043565	MF	sequence-specific D- binding	0.00	1.00	0.02	0.00	1.00	0.01
GO:1990837	MF	sequence-specific double-stranded D- binding	0.00	1.00	0.02	0.00	1.00	0.01
GO:0001067	MF	regulatory region nucleic acid binding	0.00	1.00	0.02	0.00	1.00	0.01
GO:0044212	MF	transcription regulatory region D- binding	0.00	1.00	0.02	0.00	1.00	0.01
GO:0000976	MF	transcription regulatory region sequence-specific D- binding	0.00	1.00	0.02	0.00	1.00	0.01
GO:0003700	MF	D–binding transcription factor activity	0.00	1.00	0.02	0.00	1.00	0.02
GO:0016705	MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.00	1.00	0.02	0.00	1.00	0.02
GO:0051213	MF	dioxyge-se activity	0.00	1.00	0.04	0.00	1.00	0.02
GO:0016879	MF	ligase activity, forming carbon-nitrogen bonds	0.00	1.00	0.05	0.00	1.00	0.04
GO:0016538	MF	cyclin-dependent protein serine/threonine ki-se regulator activity	-	-	-	0.00	1.00	0.04
GO:0016881	MF	acid-amino acid ligase activity	0.00	1.00	0.13	0.00	1.00	0.11
GO:0102682	MF		-	-	-	0.00	1.00	0.15

(*continued on next page*)

Table 1 (*continued*)

Category	Ontology	Term	EWM			hybrid		
			Over P-value	Under P-value	Ratio DE in Catalog	Over P-value	Under P-value	Ratio DE in Catalog
		N6-(Delta2-isopentenyl)-adenosine 5′-monophosphate phosphoribohydrolase activity						
GO:0008395	MF	steroid hydroxylase activity	-	-	-	0.00	1.00	0.20
GO:0010436	MF	carotenoid dioxyge-se activity	0.00	1.00	0.38	0.00	1.00	0.25
GO:0045549	MF	9-cis-epoxycarotenoid dioxyge-se activity	0.00	1.00	0.43	0.00	1.00	0.29
GO:0010279	MF	indole-3-acetic acid amido synthetase activity	0.00	1.00	1.00	0.00	1.00	0.67
GO:0051777	MF	ent-kaurenoate oxidase activity	-	-	-	0.00	1.00	1.00
GO:0045543	MF	gibberellin 2-beta-dioxyge-se activity	0.00	1.00	0.26	-	-	-
GO:0052635	MF	C-20 gibberellin 2-beta-dioxyge-se activity	0.00	1.00	0.44	-	-	-
GO:0016762	MF	xyloglucan:xyloglucosyl transferase activity	0.00	1.00	0.12	-	-	-
GO:0052634	MF	C-19 gibberellin 2-beta-dioxyge-se activity	0.00	1.00	0.22	-	-	-
GO:0015295	MF	solute:proton symporter activity	0.00	1.00	0.10	-	-	-
GO:0016702	MF	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	0.00	1.00	0.06	-	-	-
GO:0016706	MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	0.00	1.00	0.04	-	-	-
GO:0010329	MF	auxin efflux transmembrane transporter activity	0.00	1.00	0.09	-	-	-
GO:0005351	MF	carbohydrate:proton symporter activity	0.00	1.00	0.11	-	-	-
GO:0005402	MF	carbohydrate:cation symporter activity	0.00	1.00	0.11	-	-	-
GO:0080161	MF	auxin transmembrane transporter activity	0.00	1.00	0.09	-	-	-
GO:0016701	MF	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	0.00	1.00	0.05	-	-	-
GO:0005365	MF	myo-inositol transmembrane transporter activity	0.00	1.00	0.21	-	-	-
GO:0019139	MF	cytokinin dehydroge-se activity	0.00	1.00	0.20	-	-	-
GO:0015562	MF	efflux transmembrane transporter activity	0.00	1.00	0.05	-	-	-
GO:0140031	MF	phosphorylation-dependent protein binding	0.00	1.00	0.67	-	-	-
GO:0015166	MF	polyol transmembrane transporter activity	0.00	1.00	0.18	-	-	-
GO:0015294	MF	solute:cation symporter activity	0.00	1.00	0.05	-	-	-
GO:0015665	MF	alcohol transmembrane transporter activity	0.00	1.00	0.16	-	-	-

the list of significantly enriched GO terms we chose terms from the 2,4-D response pathway to examine log-fold-change between treatment and control at all time points, and constitutively between genotypes. GO terms '9-cis-epoxycarotenoid dioxygenase (NCED) activity' (GO:0045549), 'abscisic acid signaling pathway' (GO:0009738), 'photosynthesis' (GO:0015979), and 'ethylene activated signaling' (GO:0009873) were plotted using pheatmaps v1.0.12 in R.

3. Results

3.1. Dose-response assay

As hypothesized, treatments with 2,4-D were not as effective at reducing biomass of the hybrid genotype compared to the EWM genotype. Plants of the hybrid genotype remained approximately the same biomass as untreated controls out to ~0.45 mg L^{-1} 2,4-D, whereas the EWM genotype was reduced by more than 50% at only 0.25 mg L^{-1} 2,4-D (Fig. 1). The modeled effective dose confirms this as the two genotypes had different estimates to reduce biomass by 50% (ec50; hybrid = 0.561 mg L^{-1}, EWM = 0.182 mg L^{-1}; see Supp Table 3). However, the ec50 of the two genotypes were not significantly different from one another when using models where the lower limit was not fixed at zero. Our findings comport with what others have already documented in these two genotypes (Taylor et al., 2017; Hoff and Thum, 2022). Taylor et al. (2017) showed that the hybrid genotype used in this study had a higher growth rate relative to the other genotypes in their study and Hoff and Thum (2022) showed that the EWM genotype used in this study had a low growth rate relative to the other genotypes in their study.

3.2. Overall differential expression

The 2,4-D treatment stimulated the expression of many more genes in the EWM genotype relative to the hybrid genotype at all time points. However, the facultative response to 2,4-D treatment over time was qualitatively similar between the two genotypes (Fig. 2). Differential expression of both up- and downregulated genes increased in both genotypes between 12 and 48 h after treatment (HAT; Fig. 2). Differential expression peaked at 48 HAT, followed by a reduction in differential expression between the treated and control groups by 96 HAT (Fig. 2).

The two genotypes also differentially express many of the same genes in response to 2,4-D treatment, but many of differentially expressed genes in the EWM were specific to this genotype (Fig. 2 B). At all timepoints after treatment, the majority of genes differentially expressed in response to 2,4-D treatment in the hybrid genotype were also shared by the EWM genotype (Fig. 2 B). Conversely, while the EWM shares many of the differentially expressed genes with the hybrid, the majority of differentially expressed genes in the EWM genotype at all timepoints after treatment are specific to the EWM genotype (Fig. 2 B).

Although both genotypes showed similar dynamics of differential gene expression over time, the 2,4-D treatment did not affect the hybrid genotype's gene expression as much as the EWM genotype (Fig. 2). The EWM genotype differentially expressed a greater number of genes relative to the hybrid genotype at all time points after treatment with ~5.4 times more genes differentially expressed in EWM than the hybrid at 48 HAT (Fig. 2). Because of this reduced facultative response by the hybrid, we hypothesized that genes may be constitutively up regulated in the hybrid genotype relative to the EWM.

Constitutive gene expression did tend to be higher in the hybrid genotype. In the control environment, 28,549 genes were more highly expressed in the hybrid genotype compared to 10,419 that were more highly expressed in the EWM genotype. After we removed the 5814 genes that were present in the hybrid but not in EWM genotype and the 1180 genes that were present in the EWM but not in the hybrid, the hybrid still more highly expressed the large majority of the genes between the two genotypes (hybrid = 22,735 and EWM = 9239). However, this difference seems to be driven mostly by lowly expressed genes in both genotypes (Supp. Fig. S3).

Finally, treatment caused almost all the same GO terms to be enriched in both genotypes at 12 HAT (Table 1). However, there were several GO terms related to auxin/hormone transport and efflux that

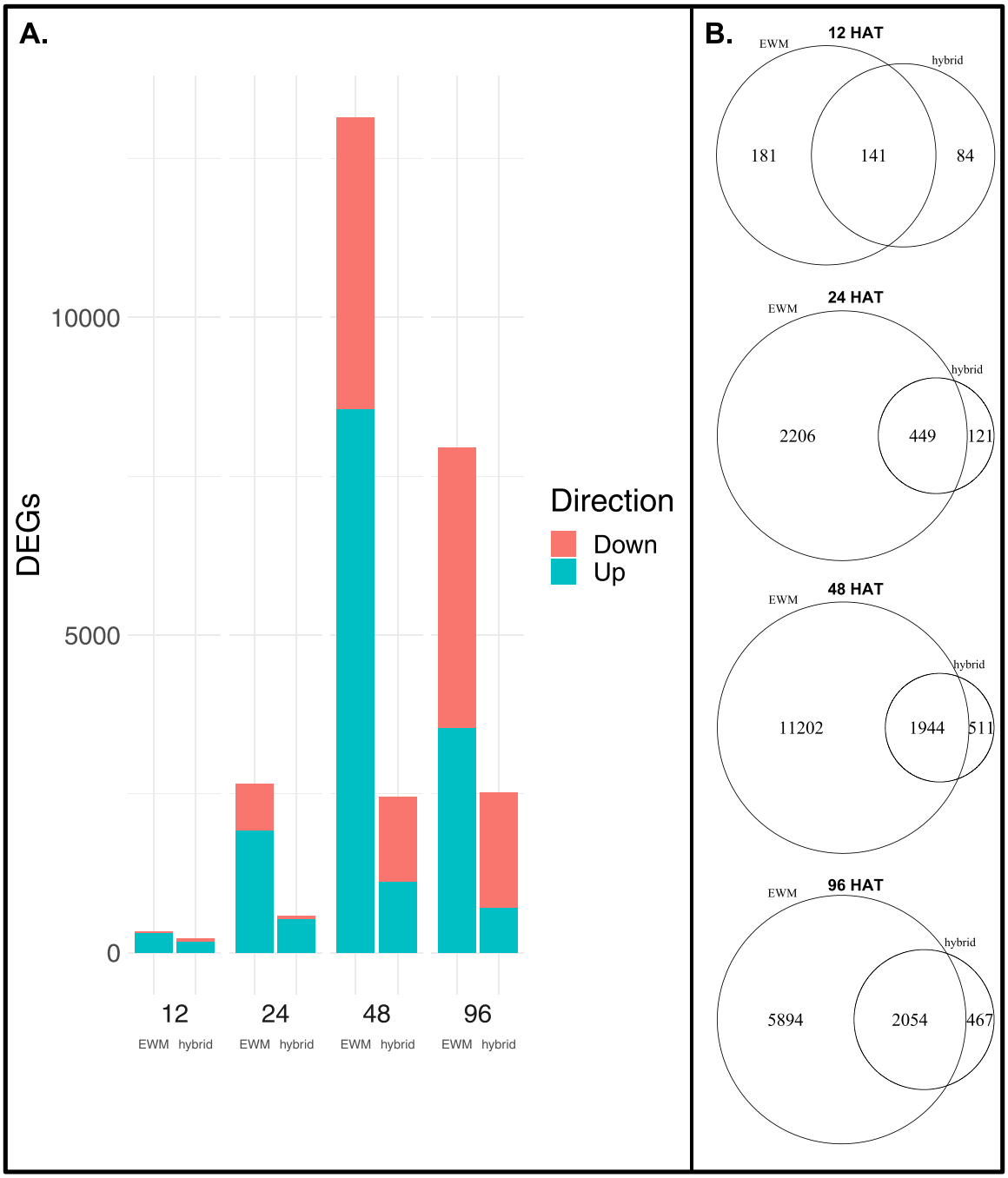

Fig. 2. Plot of the number of genes that are significantly (Benjamini-Hochberg FDR < 0.05) differentially expressed in the control vs treatment group at all time points (hours after treatment) in response to treatment with 0.5 mg L^{-1} 2,4-D. Panel A.) shows the total number of genes differentially expressed either up- (green bars) or downregulated (red bars) for each genotype at each time point. Panel B.) shows the overlap between the genotypes in number of differentially expressed genes between the two genotypes at each timepoint in hours after treatment (HAT). The EWM genotype is a Eurasian watermilfoil collected from Coeur d'Alene Lake, Idaho and the hybrid genotype of watermilfoil was collected from Hayden Lake, Idaho.

were enriched in the EWM genotype but not in the hybrid (Table 1). Overall, the EWM genotype also tended to differentially express a higher proportion of genes from each GO term relative to the hybrid genotype. There were relatively few cellular component genes enriched by treatment when compared to the molecular function and biological processes ontologies (Table 1). These patterns held throughout the time course (see Supp. Tables 4–6).

3.3. *Differential expression of the auxin pathway*

Because the hybrid received half of its genome from northern watermilfoil, many genes may have homologous functions, and each gene variant may not be present in each genotype. This confounds gene comparisons across the two genotypes. Therefore, we elected to examine all genes associated with a GO term and describe the overall patterns of them. The proposed progression of an auxin herbicide response should begin with NCED upregulation, followed by an ABA signaling response as ABA accumulates in the plant, and finally, downregulation of the photosystem (Grossman, 2010; McCauly et al., 2020; Gaines, 2020). Indeed, we saw this expected response for genes within these GO terms (Figs. 3–5). Both the hybrid and EWM responded to auxin with NCED upregulation (Fig. 3), ABA signaling upregulation (Fig. 4), and

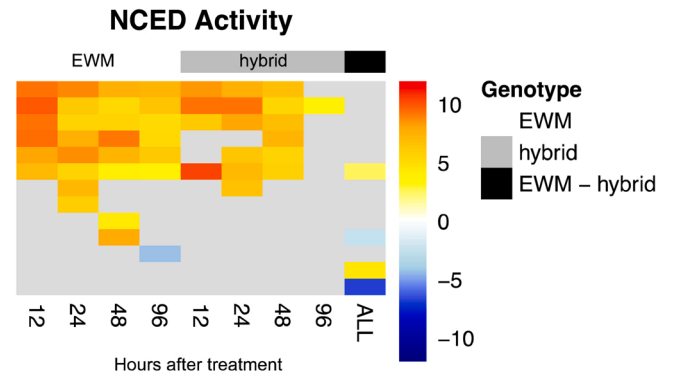

Fig. 3. Heatmap of log-fold-change in expression for genes in the '9-cis-epoxycarotenoid dioxygenase activity' Gene Ontology group (GO:0045549). Each row represents a gene, and each column represents a control versus treatment (0.5 mg L^{-1} 2,4-D) comparison for a genotype at a timepoint in hours after treatment. The last column represents the log-fold-change in expression of the same genes between the two genotypes in the untreated control (constitutive expression). Genotypes are one hybrid genotype from Hayden Lake, Idaho (hybrid) and one Eurasian genotype collected from Coeur d'Alene Lake, Idaho (EWM). Positive values ('red' colors) indicate that a gene was upregulated in response to 2,4-D treatment or higher expressed in the EWM genotype in the untreated control. Negative numbers ('blue' colors) indicate that gene was downregulated in response to 2,4-D treatment or higher in the hybrid genotype in the untreated controls. The more saturated the color the greater the log-fold-change between the control and treatment or EWM and hybrid genotype. Grey boxes indicate that gene was not significantly ($P <= 0.05$ after Benjamini-Hochberg correction) differentially expressed in that contrast.

photosynthesis downregulation (Fig. 5). Further, both ABA signaling differences and photosynthesis downregulation increased in log-fold-change and associated gene number over time (Figs. 4–5). However, the hybrid genotype differentially expressed fewer genes and at lower log-fold-changes in all of these gene sets relative to the EWM genotype (Figs. 3–5). It is possible the hybrid did not facultatively respond as much as the EWM genotype because it was already constitutively expressing these genes at the same level as the EWM was facultatively stimulated to. However, while we found overall higher constitutive expression in the hybrid, we did not find any obvious constitutive differences between the two genotypes in the auxinic herbicide pathway GO sets (last column Figs. 3–6). This suggests that the difference in facultative response between the two genotypes is not a function of the higher constitutive expression in the hybrid genotype. Although, there may be constitutive expression of other genes that we are not aware of, besides the auxin pathway, that allow this hybrid to mitigate 2,4-D's affects.

While the recently proposed pathway of generalized auxin-mediated plant death does not include ethylene accumulation (McCauly et al., 2020; Gaines, 2020), previous models did (Grossman, 2010). Further, although not all synthetic auxin herbicides stimulate ethylene production, 2,4-D does (McCauly et al., 2020). Therefore, we examined the 'ethylene activated signaling' GO term. Indeed, we found several genes were upregulated in response to 2,4-D treatment (Fig. 6). However, we observed differences between the hybrid and EWM genotypes in the log-fold-change and number of genes upregulated at all time points. In the EWM genotype, the number of genes upregulated increased over time, whereas the hybrid had a lower number of upregulated genes at each time point and did not change much over time (Fig. 6).

4. Discussion

In this study, we compared constitutive and facultative gene expression of two watermilfoil genotypes in response to 0.5 mg L^{-1} 2,4-D treatment over time. The two genotypes differed in their phenotypic (growth) response to 2,4-D treatment, with the hybrid less sensitive

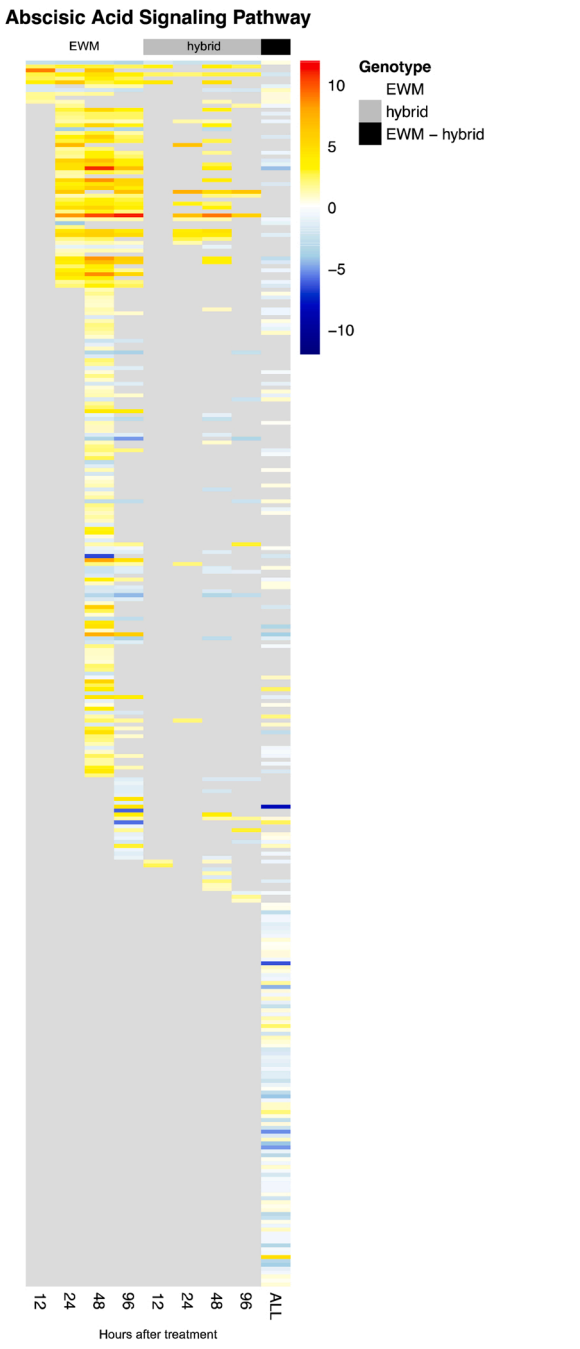

Fig. 4. Heatmap of log-fold-change in expression for genes in the 'abscisic acid signaling pathway' Gene Ontology group (GO:0009738). Each row represents a gene, and each column represents a control versus treatment (0.5 mg L^{-1} 2,4-D) comparison for a genotype at a timepoint in hours after treatment. The last column represents the log-fold-change in expression of the same genes between the two genotypes in the untreated control (constitutive expression). Genotypes are one hybrid genotype from Hayden Lake, Idaho (hybrid) and one Eurasian genotype collected from Coeur d'Alene Lake, Idaho (EWM). Positive values ('red' colors) indicate that a gene was upregulated in response to 2,4-D treatment or higher expressed in the EWM genotype in the untreated control. Negative numbers ('blue' colors) indicate that gene was downregulated in response to 2,4-D treatment or higher in the hybrid genotype in the untreated controls. The more saturated the color the greater the log-fold-change between the control and treatment or EWM and hybrid genotype. Grey boxes indicate that gene was not significantly ($P <= 0.05$ after Benjamini-Hochberg correction) differentially expressed in that contrast.

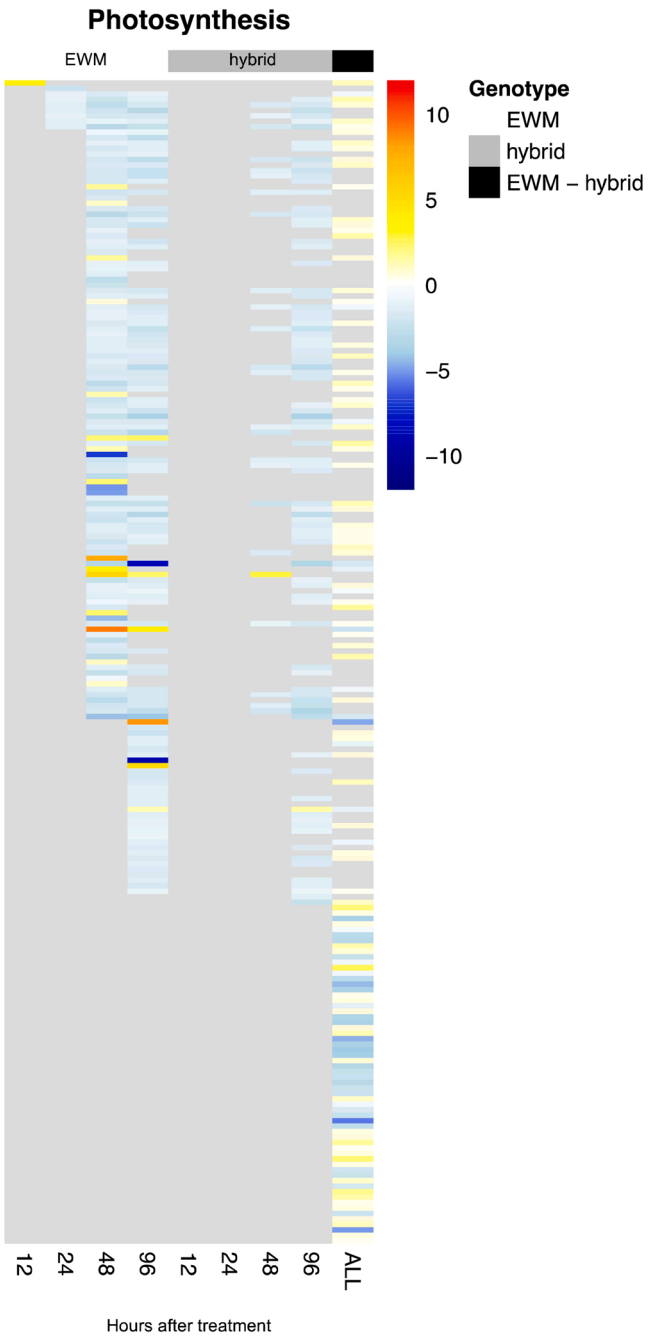

Fig. 5. Heatmap of log-fold-change in expression for genes in the 'photosynthesis' Gene Ontology group (GO:0015979). Each row represents a gene, and each column represents a control versus treatment (0.5 mg L^{-1} 2,4-D) comparison for a genotype at a timepoint in hours after treatment. The last column represents the log-fold-change in expression of the same genes between the two genotypes in the untreated control (constitutive expression). Genotypes are one hybrid genotype from Hayden Lake, Idaho (hybrid) and one Eurasian genotype collected from Coeur d'Alene Lake, Idaho (EWM). Positive values ('red' colors) indicate that a gene was upregulated in response to 2,4-D treatment or higher expressed in the EWM genotype in the untreated control. Negative numbers ('blue' colors) indicate that gene was downregulated in response to 2,4-D treatment or higher in the hybrid genotype in the untreated controls. The more saturated the color the greater the log-fold-change between the control and treatment or EWM and hybrid genotype. Grey boxes indicate that gene was not significantly ($P <= 0.05$ after Benjamini-Hochberg correction) differentially expressed in that contrast.

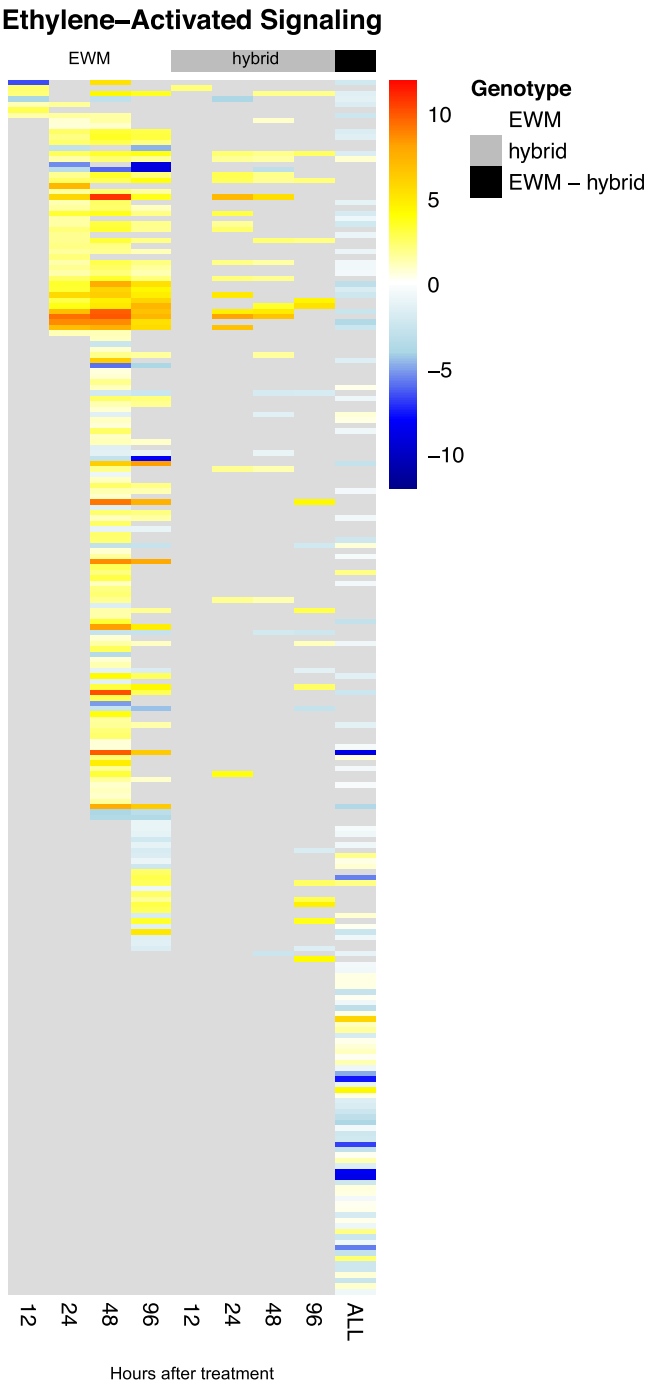

Fig. 6. Heatmap of log-fold-change in expression for genes in the 'ethylene activated signaling' Gene Ontology group (GO:0009873). Each row represents a gene, and each column represents a control versus treatment (0.5 mg L^{-1} 2,4-D) comparison for a genotype at a timepoint in hours after treatment. The last column represents the log-fold-change in expression of the same genes between the two genotypes in the untreated control (constitutive expression). Genotypes are one hybrid genotype from Hayden Lake, Idaho (hybrid) and one Eurasian genotype collected from Coeur d'Alene Lake, Idaho (EWM). Positive values ('red' colors) indicate that a gene was upregulated in response to 2,4-D treatment or higher expressed in the EWM genotype in the untreated control. Negative numbers ('blue' colors) indicate that gene was downregulated in response to 2,4-D treatment or higher in the hybrid genotype in the untreated controls. The more saturated the color the greater the log-fold-change between the control and treatment or EWM and hybrid genotype. Grey boxes indicate that gene was not significantly ($P <= 0.05$ after Benjamini-Hochberg correction) differentially expressed in that contrast.

relative to the EWM genotype (Fig. 1). We also found that the phenotypically less sensitive hybrid (Fig. 1) differentially expressed fewer genes and at lower log-fold change than the EWM genotype at all time points after treatment and this pattern was true for both overall gene expression (Fig. 2) and the auxinic herbicide stimulated pathway (Figs. 3–6). Through this process, we also assembled the first *de novo* transcriptome of Eurasian watermilfoil (*sensu lato*), a potentially important resource for understanding this invasive species (transcriptome available at: doi:10.5061/dryad.547d7wmcx).

4.1. Overall differential expression

We found that the overall facultative response in gene expression was much higher at all time points after treatment in the EWM genotype relative to the hybrid genotype (Fig. 2). The lower facultative gene expression response in the hybrid genotype relative to the EWM genotype suggests that the hybrid is less affected by the 2,4-D treatment than the EWM genotype. Because the hybrid genotype still reacts in a similar manor (increasing DE in response to treatment over time) as the more sensitive EWM genotype, this suggests that it is still perceiving and responding to the herbicide. However, it could have a blunted perception of the herbicide due to reduced binding at the target site of the herbicide - Aux/IAA receptors. Some potential non-target site explanations for this difference may be that the hybrid absorbs or translocates less of the 2,4-D throughout the plant; additionally, it could have a higher desorption or metabolism rate of 2,4-D than the EWM genotype. Any one or combination of these mechanisms could be the mechanism of reduced sensitivity to 2,4-D seen in this hybrid genotype. While we did not measure any of these processes in the two genotypes here, Ortiz et al. (2021) studied the Hayden Lake hybrid genotype used in our study and found that absorption was higher and metabolism was the same between this hybrid genotype and a 2,4-D sensitive EWM genotype. This study also found that desorption was higher, and translocation was lower in the hybrid genotype compared to the sensitive EWM genotype. Ortiz et al. (2021) concluded the reduced sensitivity of the Hayden Lake hybrid genotype was likely related to the target site and pathway of 2, 4-D, but it is possible that the reduced translocation (less 2,4-D transported throughout the plant) and increased desorption (shunting 2,4-D out faster) are what leads to the reduced sensitivity and therefore, the lower facultative gene expression response seen in the hybrid genotype relative to the more sensitive EWM genotype.

We also found that overall gene expression was constitutively higher in the hybrid genotype compared to the EWM genotype. It is possible that this constitutively higher gene expression in the hybrid genotype is a feature of hybridity due to the increased heterozygosity and mixing of separately evolved regulatory networks when two divergent genomes come together in a hybrid individual (see Hegarty et al., 2008; Landry et al., 2007). Preliminary data suggests many hybrid genotypes of watermilfoil are likely first-generation hybrids that are likely maintained through clonal reproduction (Thum Lab unpublished). We would expect this pattern to be most pronounced in a first-generation hybrid (LaRue et al., 2013); however, we do not know what generation this hybrid genotype is. Conversely, the higher constitutive expression in the hybrid may just be a feature of this specific genotype. Future studies should examine gene expression levels of a collection of hybrid and parental genotypes of watermilfoil to determine whether hybrid watermilfoil generally exhibits heterosis in their gene expression.

4.2. Differential expression of the auxin pathway

While we could not directly compare across individual genes between the hybrid and pure EWM, the overall differences in expression response between the two genotypes in the auxin pathway gene sets is apparent (Figs. 3–5). Ultimately, downregulation of photosynthesis is the step that causes plant death from auxinic herbicides, as suggested by Gaines (2020), and the overall downregulation of photosynthesis is quantitatively less in the hybrid genotype (Fig. 5). This also explains why the hybrid's growth is less affected by 2,4-D treatments relative to the EWM genotype (Fig. 1). Although the exact mechanism of resistance to 2,4-D in this hybrid genotype is unclear, the resistant hybrid genotype did not facultatively respond to 2,4-D treatment in the auxinic herbicide pathway genes as much as the sensitive EWM genotype (Figs. 3–5). These findings suggest that the mechanism of 2,4-D resistance in this hybrid genotype either lies in the beginning of the auxin pathway and its perception of the herbicide, or that there is some non-target site mitigation of the herbicide, such as the reduced translocation or increased desorption documented by Ortiz et al. (2021). Further, we found that GO terms from the biological process ontology (auxin transport (GO:0060918), hormone transport (GO:0009914), auxin efflux (GO:0010315), and regulation of auxin mediated signaling (GO:0010928)) as well as in the molecular function ontology (auxin efflux transmembrane transport (GO:0010329) and auxin transmembrane transport (GO:0080161)) were all enriched in the EWM but not in the hybrid genotype at 12 HAT (Table 1). When comparing heat maps of these GO terms across all timepoints between the two genotypes, we found that the hybrid does not differentially express these genes as much as the EWM (see Supp. Figs. S3-8). All of these taken together suggest that this hybrid genotype of watermilfoil is less affected by 2,4-D treatment by reducing translocation throughout the plant and desorbing 2,4-D more quickly. These mechanisms may be reducing the time and amount of 2,4-D the plant is experiencing, which may be responsible for the reduced effect of 2,4-D treatment on this genotype. Future studies should investigate translocation and desorption of 2,4-D in a set of watermilfoil genotypes that vary in sensitivity to 2,4-D.

We found that the hybrid genotype did not down regulate photosynthesis as much as the EWM genotype (Fig. 5). The pattern of reduced photosynthesis gene downregulation (Fig. 5) and continued growth in treatment (Fig. 1) seen in this genotype of hybrid watermilfoil also fits with what Riar et al. (2011) found when investigating a resistant biotype of prickly lettuce (*Lactuca serriola* L.). This study found that the susceptible prickly lettuce biotype stopped growing in 2,4-D treatment while the resistant biotype continued to grow throughout the exposure period. They termed the physiological mechanism of resistance, reduced growth deregulation (Riar et al., 2011). While we did not measure growth rate periodically in our study, we did find that the hybrid genotype grew more in the 2,4-D assay than the EWM genotype overall (Fig. 1). Future studies may be able to compare gene expression differences between prickly lettuce and watermilfoil or other species with similar physiological patterns to identify the molecular mechanism(s) of their resistance.

The link between ABA accumulation in the plant and the whole-scale downregulation of photosynthesis due to auxinic herbicide treatment is unclear (Gaines, 2020; McCauly et al., 2020). ABA signaling is used by terrestrial plants to close stomata during drought stress which in turn will slow down photosynthesis (Cai et al., 2017; MacRobbie, 1992). However, it is believed that submersed aquatic macrophytes, like watermilfoil, have lost the ability to open and close stomata (Keeley, 1990). Because we saw a response of increased ABA (Fig. 4) and downregulation of photosynthesis (Fig. 5) in our data, it suggests that ABA signaling stomatal closure is likely not the mechanism that causes photosynthetic down regulation. Further studies are needed to determine the causes of photosynthesis downregulation induced by auxinic herbicides and submersed aquatic macrophytes may be an important system, with their unique stomatal physiology, to help parse out any links between ABA signaling and photosynthesis downregulation.

We also found reduced signaling due to ethylene in the hybrid genotype relative to the EWM genotype (Fig. 6). While we did not measure ethylene directly, this reduced ethylene activated signaling in the hybrid likely means it was accumulating less ethylene than the EWM genotype. This pattern fits with the results from previous studies on Dicamba resistant *Kochia scoparia* that showed ethylene did not accumulate in the resistant biotype like in the susceptible biotype (Howatt et al., 2006).

Our data suggest that there are likely differences in ethylene production between the hybrid and EWM genotypes tested based on gene expression patterns related to ethylene signaling activity (Fig. 6). Future studies should examine the correlation as it relates to 2,4-D sensitivity in watermilfoil.

Finally, our study compared one genotype each of hybrid and pure Eurasian watermilfoil, and it is therefore unclear whether the differences found here represent a general difference between hybrid and Eurasian watermilfoil genotypes (i.e., is a function of hybridity), or if these are genotype-specific differences. Previous studies show that hybrids generally grow better in control and 2,4-D environments compared to pure Eurasian watermilfoil (LaRue et al., 2013; Netherland and Willey, 2017; Taylor et al., 2017; Hoff and Thum, 2022). However, Hoff and Thum (2022) showed that there can be overlap in growth rates between the two taxa in both control and 2,4-D treated environments (see also Poovey et al., 2007). As such, future studies should compare multiple hybrid and Eurasian genotypes to determine if hybrid gene expression is always constitutively higher and less affected by 2,4-D treatment than their parents (i.e., heterosis), or if gene expression distributions also overlap between taxa.

In conclusion, the hybrid differentially expressed fewer genes relative to the EWM genotype at all time points (12, 24, 48, and 96 HAT; Fig. 2). The hybrid also constitutively expressed more genes in the control environment than the EWM genotype (Supp. Fig. S2). Our data suggest that both hybrid and EWM responded qualitatively similar to the 2,4-D treatment overall (Fig. 2) and by up-regulating genes in the auxin response pathway (NCED and ABA signaling; Figs. 3–4), and by down-regulating photosynthesis genes (Fig. 5). Further, both genotypes upregulated signaling in response to ethylene accumulation (Fig. 6). However, facultative expression overall and in the auxin pathway genes was much lower in the hybrid genotype relative to the EWM genotype both in log-fold-change and associated gene number (Figs. 3–6). Molecular response differences among these genotypes of invasive watermilfoil (Figs. 2–6) correlate with the differences in phenotypic response (growth) to 2,4-D treatments (Fig. 1) with the less sensitive hybrid responding less molecularly to treatment. Future studies should determine the extent to which gene expression varies among genotypes within and among taxa of invasive watermilfoil to better understand best management practices of this costly invader.

CRediT authorship contribution statement

Gregory M. Chorak: Conceptualization, Methodology, Data analyses, Writing – original draft, Writing – review & editing, Software, Funding acquisition. **Fernando H. Correr**: Data analyses, Writing – review & editing, Software. **Jennifer Lachowiec**: Conceptualization, Data analyses, Writing – review & editing, Funding acquisition, Supervision. **Gillian Reynolds**: Software, Validation, Writing – review & editing. **Ryan A. Thum:** Conceptualization, Writing – review & editing, Software, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Coding scripts used to generate the data in this manuscript can be found at https://github.com/DrDoom-EvoGen/WMF_DeNovoTranscriptome for the de novo transcriptome assembly and annotation, and https://github.com/DrDoom-EvoGen/WMF_RNAseq_1 for the differential expression analyses. The assembled transcriptome is available at: doi:10.5061/-dryad.547d7wmcx and the raw data is available upon request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2023.103631.

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