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ORIGINAL ARTICLE

Transcriptomic changes in *Echinochloa colona* in response to treatment with the herbicide imazamox

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Abstract

Main conclusion **Presented here is the first *Echinochloa colona* leaf transcriptome. Analysis of gene expression before and after herbicide treatment reveals that *E. colona* mounts a stress response upon exposure to herbicide.**

Herbicides are the most frequently used means of controlling weeds. For many herbicides, the target site is known; however, it is considerably less clear how plant gene expression changes in response to herbicide exposure. In this study, changes in gene expression in response to herbicide exposure in imazamox-sensitive (S) and- resistant (R) junglerice (*Echinochloa colona* L.) biotypes was examined. As no reference genome is available for this weed, a reference leaf transcriptome was generated. Messenger RNA was isolated from imazamox-treated- and untreated R and S plants and the resulting cDNA libraries were sequenced on an Illumina HiSeq2000. The transcriptome was assembled, annotated, and differential gene expression analysis was performed to identify transcripts that were upregulated or downregulated in response to herbicide exposure for both biotypes. Differentially expressed transcripts included transcription factors, protein-modifying enzymes, and enzymes involved in metabolism and signaling. A literature search revealed that members of the families represented in this analysis were known to be involved in abiotic stress response in other plants, suggesting that imazamox exposure induced a stress response. A time course study examining a subset of transcripts showed that expression peaked within 4–12 h and then returned to untreated levels within 48 h of exposure. Testing of plants from two additional biotypes showed a similar change in gene expression 4 h after herbicide exposure compared to the resistant and sensitive biotypes. This study shows that within 48 h junglerice mounts a stress response to imazamox exposure.

Keywords Abiotic stress · Imazamox · RNA-seq · Transcriptomics

Abbreviations

ALS Acetolactate synthase
NIS Non-ionic surfactant

Introduction

Weed control is a critical component of agriculture. If left unchecked, weeds compete with crops for resources and can interfere with harvest, thereby reducing yield (Oerke 2006). Herbicides are the tools most often used by growers

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to control weeds. While the target sites of many herbicides are known, it is not known for some of these chemicals what other enzymes and pathways are affected. Large-scale gene expression studies, such as microarray and RNA-seq analyses, are beginning to explore plant responses to herbicide exposure at the gene expression level.

In *Arabidopsis*, two studies have used microarrays to examine gene expression response to herbicide exposure (Manabe et al. 2007; Das et al. 2010). In the first study, response to treatment with imazapyr, an acetolactate synthase (ALS) inhibitor, was examined in a wild-type biotype and in a biotype with resistance due to an alteration of the target site (Manabe et al. 2007). Only the wild-type plants exhibited changes in gene expression following herbicide treatment. Many of the transcripts that were differentially expressed in the wild-type following herbicide exposure were also known to be elicited by other abiotic stressors (Manabe et al. 2007). This type of response was similar to that found by Das et al. (2010). Microarrays were used to assay changes in transcription following treatment with glyphosate or ALS inhibitors. The response to each herbicide varied, but there were some transcripts that were differentially expressed following all herbicide treatments. As in the first study, many of the transcripts that were differentially expressed encoded enzymes involved in stress responses (Das et al. 2010). Quantitative PCR showed a similar response to herbicide treatment in rapeseed (Das et al. 2010). Microarray analysis of glyphosate-treated and untreated soybean (*Glycine max* L. Merr.) plants also showed a stress response (Zhu et al. 2008).

Gene expression studies can provide insight into how weeds respond to herbicides and, in doing so, may provide information on additional targets for future herbicide development (Duhoux et al. 2015; Gardin et al. 2015). Crops and model species such as *Arabidopsis* have the advantage of sequenced genomes, but most agronomically important weeds currently do not. However, an efficient and cost-effective means of producing a catalog of the expressed sequences within a particular plant/tissue is through sequencing and assembly of mRNAs (in the form of cDNAs) and assembly of resulting sequencing reads into a transcriptome. A transcriptome represents a set of gene coding sequences against which RNA-seq data can be aligned and compared for differential gene expression analysis. This technology has been used in blackgrass (*Alopecurus myosuroides* L.) (Gardin et al. 2015), annual ryegrass (*Lolium rigidum* Gaud.) (Gaines et al. 2014; Duhoux et al. 2015), purple morning glory [*Ipomoea purpurea* (L.) Roth] (Leslie and Baucom 2014), and goosegrass (*Eleusine indica* L. Gaertn.) (An et al. 2014). While these studies have mostly focused on herbicide resistance, the wealth of data they provide can also be used to investigate herbicide responses. Two studies have examined herbicide responses. An annual ryegrass study noted that transcripts involved in transport, gene expression regulation, and metabolism were upregulated following penoxsulam exposure (Duhoux et al. 2015). A study investigating blackgrass (Gardin et al. 2015) found that the plant response to a sulfonylurea mixture was similar to that observed for wild-type *Arabidopsis* in the study by Manabe et al. (2007).

Echinochloa spp. are problematic weeds in rice, soybean and other crops world-wide (Muenscher 1955). If not controlled, they can cause severe yield reductions in rice yield (Smith 1968; Gibson et al. 2002). Control of this genus has become increasingly difficult as they have evolved resistance to several classes of herbicides. Resistant populations have been reported in six *Echinochloa* spp. for 11 classes of herbicides (Heap 2016). Some of these populations exhibit multiple herbicide resistance (Heap 2016). In Sunflower County, Mississippi, USA, a population of junglerice [*Echinochloa colona* (L.) Link] was found whose members have evolved resistance to imazamox, fenoxaprop-*P*-ethyl, quinclorac, and propanil. These four herbicides represent different classes of herbicides with different mechanisms of action. The objective of this research was to understand how these plants respond to herbicide exposure by treating resistant and sensitive plants with imazamox and comparing the expression profiles of both untreated and treated plants from each biotype.

Materials and methods

Plant growth conditions and RNA-seq

The resistant biotype (R) of junglerice (*Echinochloa colona* L.) originated from a rice field in Sunflower County, Mississippi, USA. The sensitive biotype (S) was propagated from a single plant from Chicot County, Arkansas, USA. The sensitive biotype has no known prior exposure to herbicides. This plant was confirmed to be sensitive to all four herbicides (imazamox, fenoxaprop-*P*-ethyl, quinclorac, and propanil). Plants of the R and S biotypes were grown in Metromix 360® potting soil (Sun Gro Horticulture, Bellvue, WA, USA) and kept in a greenhouse with a 12-h photoperiod and day and night temperatures of 24 and 21 °C, respectively. Once S and R plants reached the two to four leaf stage, they were each divided into two groups of five to six plants each (i.e., two groups of S plants with six plants each and two groups of R plants with five to six plants each). The second leaf of a single plant did not generate enough RNA for RNA-seq, thus for each sample a pool of five to six plants was required. One group for each biotype remained untreated, while the other group was treated with herbicide in a moving nozzle spray chamber (Spraying Systems Co., Wheaton, IL, USA). The spray chamber had an 8002E nozzle set at a pressure of 220 kPa, delivering a spray volume of

187 L ha^{-1}. The treated plants were sprayed with imazamox (Beyond®, BASF, Research Triangle Park, NC, USA) at a rate of 53 g ai ha^{-1}. Even though the plants showed a failure to respond in the field to imazethapyr, imazamox was chosen because it is a more potent imidazolinone ALS inhibitor for junglerice. A non-ionic surfactant (NIS), Induce® (Helena Chemical Company, Collierville, TN, USA), was included at 0.25% v/v. Three trials were performed, creating three biological replicates separated in time. This generated 12 samples (each containing tissue from five to six plants): 3 R treated, 3 R untreated, 3 S treated, and 3 S untreated.

RNA was isolated from both treated and untreated plants 1 h after spraying. The second leaves of the plants in each treatment group were combined and ground in a mortar and pestle in 900 µL of RNeasy Lysis Buffer (RLT buffer, Qiagen). Once homogenized, the tissue was transferred to a QiaShredder column. From this point forward, the Qiagen RNEasy plant mini kit protocol was followed. After extraction, an off-column DNase treatment was performed. Reactions consisted of RNA (60 µL), 1 × RDD buffer (Qiagen), and 2.7 units DNase and H_2O was added to produce a final volume of 70 µL. DNAse digestion proceeded at room temperature for 30 min and then RNA was purified on column again using the same Qiagen kit. The RNA was added to 700-µL RW1 buffer and the Qiagen kit protocol was followed from that point forward. Following extraction, the quantity and quality of RNA was checked by A_{260} and $A_{260/280}$ readings and agarose gel electrophoresis. All samples had an excess of 200 ng μL^{-1} RNA and an $A_{260/280}$ reading of 2.1–2.2. Gel electrophoresis did not indicate degradation of the RNA. The twelve RNA samples for RNA-seq were shipped to the Institute of Genomics, Biocomputing, and Biotechnology (IGBB) at Mississippi State University (Mississippi State, MS, USA) for library preparation and bar-coding.

The samples were shipped to Global Biologics NextGen-Prep Service (Colombia, MO, USA), where HiSeq libraries were prepared for each sample. TruSeq RNA directional libraries were made for each sample and all samples were barcoded. The libraries were returned to IGBB at Mississippi State University for sequencing. Sequencing was performed using a HiSeq2000. Samples were pooled by biotype and treatment and run on four lanes of a PE100 flowcell (i.e., one lane had the three R untreated samples, another the three R treated samples, etc.).

Assembly, annotation, expression estimation, and differential gene expression analysis

For assembly, the sequencing data from the untreated and treated samples for both sensitive and resistant plants were pooled. The bar codes were removed and the transcriptome was assembled using the open-source method Trinity (Haas et al. 2013). The quality of the assembly was assessed using contig N50 values, within-sample correlation, and calculation of total transcripts above the 1 transcripts per kilobase million (TPM) expression threshold. The assembled junglerice transcriptome was then annotated using the following resources and methods: InterPro, BLAST homologs to Swiss-Prot database, SignalP, TargetP, and TMHMM (Emanuelsson et al. 2000; Krogh et al. 2001; Petersen et al. 2011; Mitchell et al. 2015). The Swiss-Prot annotations are available in Table S1. The remaining annotations are available upon request. The transcriptome has been submitted to NCBI (Accession number GFJI00000000).

Expression estimation was performed using the RSEM method (Li and Dewey 2011). Differential gene expression analysis to compare untreated and treated samples within each biotype (treated S compared to untreated S and treated R compared to untreated R) to the junglerice transcriptome was performed using Genedata Analyst v9.1 (Genedata, Lexington, MA, USA). Using the count algorithm in Genedata Analyst, the relative abundance of each transcript was determined for imazamox-treated samples and- untreated samples for each biotype allowing for identification of differentially expressed transcripts. Specifically, the TPM values were analyzed using a negative binomial method with the two group exact test algorithm. Only transcripts that showed a fold ratio of 3 or greater and had a P value less than 0.05 were included in subsequent analysis.

Time-course and biotype comparison

Plants were grown as described above. R and S plants were divided into groups of five to six plants and were either not treated, treated with NIS (Induce®) only (0.25% v/v), or treated with herbicide (53 g ai ha^{-1} imazamox with 0.25% NIS). Treatments were performed in the spray chamber as described above. RNA was isolated from different sets of treated plants at 1, 4, 12, 24, and 48 h after treatment. RNA was isolated from the untreated samples at the 1 h time point. Isolations were performed as described above. For a comparison of different biotypes, plants from the R and S biotypes, and plants from two additional biotypes, 1998 and 2002 (from Azlin Seed Company, Leland, MS, USA), were not treated, treated with NIS only, or treated with the herbicide and NIS as described above. RNA was isolated from the untreated, NIS-treated-, and herbicide-treated plants at 4 h after treatment. Treatments for the time course and population comparison experiments were performed in duplicate.

RNA was converted to cDNA for qPCR using the High Capacity cDNA Reverse Transcription kit (Fisher Scientific). Reactions consisted of 2-µg RNA, 4-mM dNTPs, 1 × random primers, and 1 × buffer and H_2O was added to reach a final volume of 20 µL. Cycle conditions were 25 °C 10 min, 37 °C 2 h, and 85 °C 5 s.

The sequences of primers used in qPCR are shown in Table 1. Primers were designed using the sequences of the upregulated transcripts. For genes with multiple isozymes, all sequences were aligned and primers were designed within conserved regions of the coding sequence. For all primers, optimization was performed to determine efficiency and melt curve analyses were performed to confirm production of a single PCR product. Reactions consisted of 25 ng cDNA, 15 µM primers, 2 × Power Sybr Green Master Mix (Fisher Scientific), and H_2O added to a final volume of 50 µL. Cycle conditions were 50 °C 2 min, 95 °C 10 min, and 40 cycles of 95 °C 15 s, 55 °C 30 s, and 72 °C 30 s. All reactions were performed on an ABI7500 real-time PCR instrument (Thermo Fisher Scientific). Malate dehydrogenase was used as a reference gene for all samples. This gene has been used as a reference in *Vitis vinifera* L. (Reid et al. 2006). Data were analyzed using the standard curve method and standard error was calculated according to the methods described in the ABI User Bulletin #2 (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf).

Results

The junglerice leaf transcriptome

HiSeq data from twelve samples, three replicates each of R and S untreated and imazamox-treated, were pooled and assembled using Trinity to generate the junglerice leaf transcriptome. The assembly consists of 435,239 contigs with an average contig length of 1178 bp and a contig N50 value of 1701 (Table 2). These contigs represent 196,496 transcripts. Of the 196,496 transcripts, 113,282 were present in at least 2 or more of the 12 samples, while 45,108 transcripts had an expression level of greater than 1 TPM in at least one sample. The transcriptome was annotated as described above.

Table 2 Statistics for junglerice transcriptome

Contigs	435,239
Average	1178 bp
N50	1701 bp
Longest	17,287 bp
Greater than 500 bp	72.17%
Greater than 1 kb	40.35%

RNA-seq analysis

Expression estimation was performed using the RSEM method (Li and Dewey 2011). Differential gene expression analysis was performed to compare untreated samples to imazamox-treated samples for each biotype. The analysis was performed using Genedata Analyst software and only transcripts with a P value < 0.05 and a fold change greater than 3 were selected for further analysis. For the R plants, 133 transcripts exhibited differential expression: 115 were upregulated and 18 were downregulated following herbicide exposure. In the S plants, 136 were differentially expressed: 82 upregulated and 54 downregulated. Some transcripts were differentially expressed following herbicide treatment in both R and S plants: 12 were downregulated and 62 were upregulated. Overall, the change in gene expression ranged from 3- to 34-fold.

The transcripts that were upregulated following herbicide exposure were examined more closely to determine what groups of transcripts might have been activated in response to herbicide exposure. These transcripts are diverse (Table S2), representing enzymes involved in metabolism, transcription, protein modification, signaling, cell wall

Table 1 Primers used in qPCR for time course and biotype comparison experiments

Primer name	Sequence	Target transcript
AAW42	GCTCAACAAGCTCAAGCTC	CYP734A6
AAW43	GATTGGGATCGTCAAGATCG	CYP734A6
AAW66	CAGATGGAGGAGGCATC	Malate dehydrogenase
AAW67	GAGGGTTCATCTTCATCAGTAG	Malate dehydrogenase
AAW90	GAGTACCAGTTGAGCGTG	3-Ketoacyl
AAW91	CAGCTACAACCTCGGC	3-Ketoacyl
AAW94	GAGGACTGACTGTTGCTTC	Cobra
AAW95	CAATGCCACCGCTGTTG	Cobra
AAW100	GTACCAAGTACGCCAGC	Ankyrin
AAW101	GTGGATAGCATCGGTTTG	Ankyrin
AAW120	CGACAGCGAGAAGTGCT	Oxysterol-binding protein
AAW121	GCTGCTGAAGAAGGACTG	Oxysterol-binding protein
AAW126	CAGAGATGTCCAATGAGACGA	Kinase
AAW127	CACACTGAAGCTCATACCAATAC	Kinase

modification, and transport. Many of the transcripts could not be assigned a function because there were no hits in the InterPro database (Mitchell et al. 2015) or when a BLASTx search was performed against rice. These represent nearly a third of the upregulated genes (Fig. 1). Almost a third of the upregulated genes could not be classified in the groups listed above (Fig. 1). Of the transcripts involved in metabolism, five cytochrome P450 transcripts were upregulated. Among the cytochrome P450s, TR73895 was highly upregulated in both biotypes following herbicide treatment: 21.33-fold in S and 16.21-fold in R. This transcript was studied in further detail in the time-course and biotype comparison studies described below. Three UDP-glycosyltransferases were upregulated; two were upregulated in both biotypes. Regulatory enzymes that were upregulated included protein-modifying enzymes and transcription factors. Protein-modifying enzymes included six protein kinases, two MAP kinases, and four E3 ubiquitin ligases. Interestingly, the MAP kinases were only upregulated in R biotype. One E3 ubiquin ligase, TR25174, was upregulated just over tenfold in both the R and S biotypes. Transcription factors that were upregulated included members of the WRKY, GATA, and bHLH families. These varied among biotypes and only the WRKY transcription factor was upregulated in both biotypes: 4.49 in S and 5.37 in R. In addition to the kinases, other enzymes involved in signaling were upregulated. These included six transcripts with homologs to calcium-binding

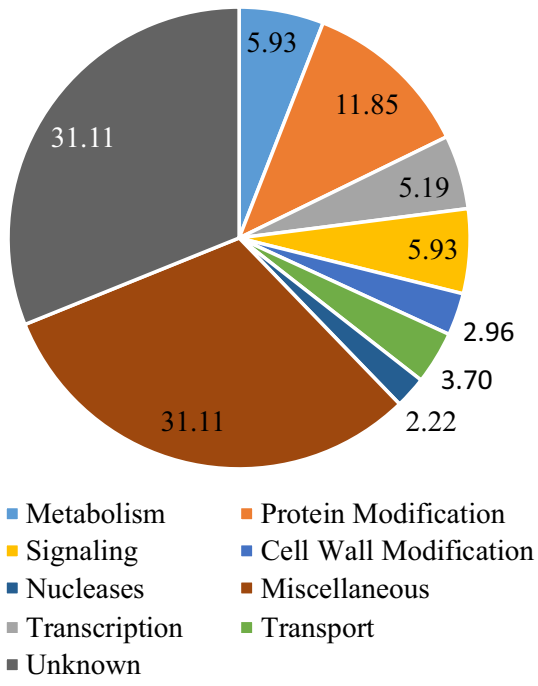

Fig. 1 Distribution across functional categories of transcripts that were upregulated following imazamox treatment of junglerice biotypes

proteins. Five of these were upregulated in both biotypes with fold increases ranging from 3.34- to 9.5-fold for S and 3.85- to 15.67-fold for R. Two transcripts associated with plant hormones were upregulated; specifically exordium and 1-aminocyclopropane-1carboxylate (ACC) oxidase (Hyodo et al. 1993; Coll-Garcia et al. 2004). Additional transcripts of interest included nucleases, cell wall-modifying enzymes, and transporters. The two most highly upregulated transcripts in S were not differentially expressed in R. These encoded a zinc finger CCCH domain and a dnaJ homolog and were upregulated 34.54- and 29.81-fold, respectively. The dnaJ homolog transcript was very short, only 295 bp in length. How these transcripts fit in with herbicide response and/or herbicide resistance is unknown.

Time course

The RNA-seq study examined only one time point following imazamox exposure. To expand on this, a time course was performed in duplicate and RNA was isolated from untreated plants and plants treated with either Induce®, a non-ionic surfactant (NIS), alone or with imazamox at 1, 4, 12, 24, and 48 h after treatment. A subset of the upregulated genes identified by the RNA-seq analysis were selected for qPCR. These included a cytochrome P450, 3-ketoacyl-CoA synthase 11, COBRA 7, an ankyrin repeat-containing protein, an oxysterol-binding protein, and a kinase (Fig. 2). These transcripts were selected for the time course study because they represent enzymes that are diverse in function. The cytochrome P450, TR73895, exhibited the greatest change in expression of the five cytochrome P450 s that were upregulated following herbicide exposure. The 3-ketoacyl-CoA synthase was also highly upregulated with 8.63- and 15.07-fold increases for the S and R biotypes. The COBRA protein, kinase, anykyrin repeat protein transcripts were upregulated in both biotypes. The oxysterol-binding protein was only upregulated in the R biotype.

The two trials are presented separately and expression was measured as a percent of the untreated control. Although the transcripts differ in the numerical change in gene expression, they do show the same trend. In most instances, expression induced by the NIS peaked early and then dropped to the level of the untreated. The cytochrome P450 and the oxysterol-binding protein peaked in expression at 4 h, the 3-ketoacyl-CoA synthase and kinase peaked between 4 and 12 h, and the COBRA and ankyrin repeat-containing protein peaked at 12 h after treatment. Although the oxysterol-binding protein was flagged as being differentially expressed only for the R biotype at 1 h, in the qPCR analysis it was upregulated for both biotypes by 4 h. With the exception of the second trial for the oxysterol-binding protein, all seem to have returned to the expression level of the control by

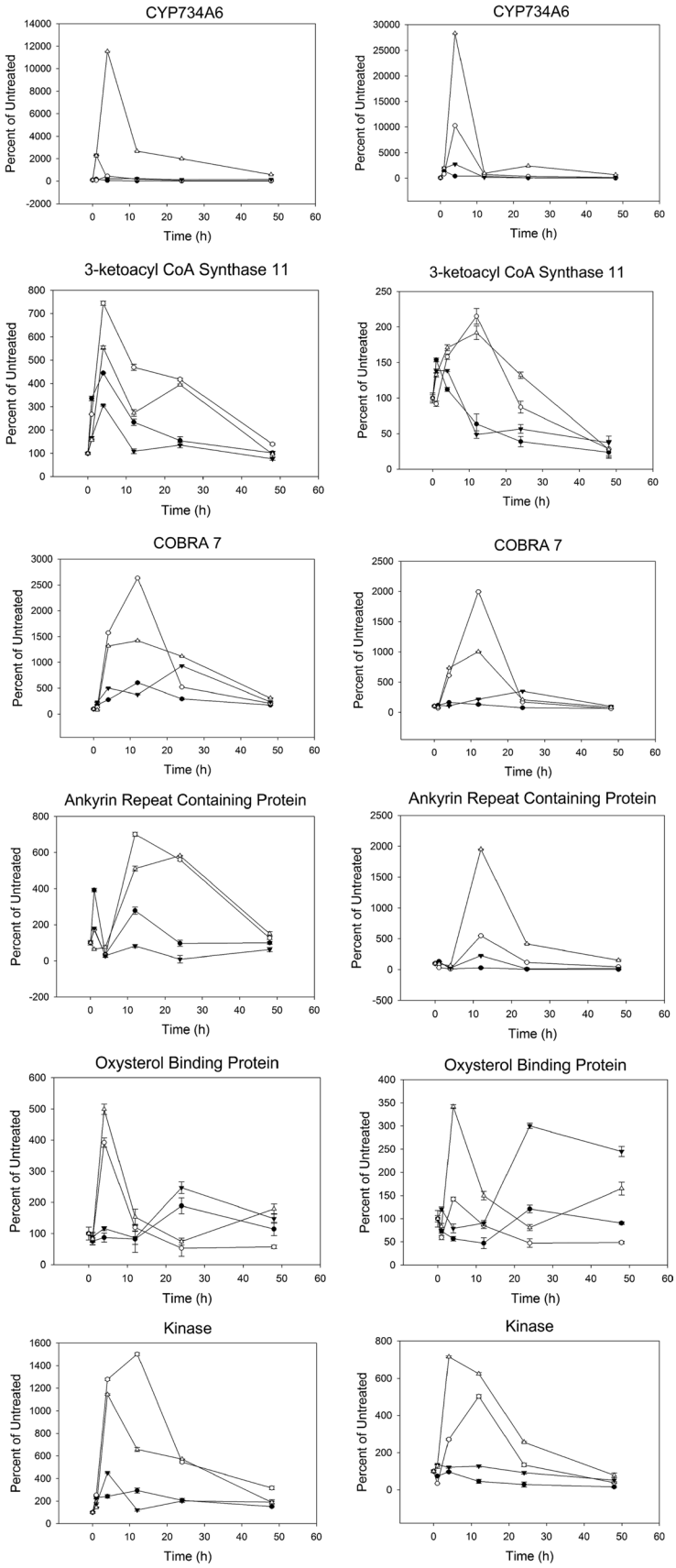

Fig. 2 qPCR analysis of gene expression during an herbicide treatment time course. Changes in gene expression are measured as a percent of the untreated control. The two trials are presented in separate graphs, the first trial on the left and the second on the right. The triangles and circles represent R and S junglerice biotypes, respectively, and closed and open are NIS treatment only or herbicide treatment, respectively

48 h indicating that much of the response for these genes at least, is contained with the first 48 h of herbicide exposure.

Biotype comparison

In the RNA-seq experiment, only the R and S plants were compared. To determine if plants from other biotypes exhibited a similar response to herbicide exposure, the R and the S plants and plants from two additional biotypes, 1998 and 2002, were treated with either NIS alone or imazamox. At 4 h after treatment, RNA was isolated from the untreated, NIS-treated-, and imazamox-treated plants for each biotype. This was done in duplicate. qPCR was performed for each of the six transcripts included in the time-course study. The results of the two trials are presented separately (Fig. 3). The change in expression was measured as a percent of the untreated control for each biotype. For the cytochrome P450, the ankyrin repeat domain-containing protein, and the kinase, all plants in both trials showed an increase in response to imazamox treatment. There was also a response to the NIS, as was observed in the time course (Fig. 3). The oxysterol-binding protein and the kinase mostly showed an increase in expression in response to herbicide exposure. COBRA 7 was the most variable; in the time-course expression of the gene peaked at 12 h.

Discussion

The junglerice leaf transcriptome

This study presents the first leaf transcriptome of *Echinochloa colona*. A total of 196,497 transcripts were assembled. This large number of transcripts (and isozymes) is likely due to the hexaploid nature of this weed species. As for many other weed species, there are limited genomic and transcriptomic resources available for this weed. The transcriptome has been made available through NCBI (Accession number GFJI00000000) and the searchable annotation table (Table S1) will allow for further examination of transcripts of interest to other researchers.

RNA-seq analysis

Differential gene expression analysis was performed to examine changes in gene expression following imazamox exposure in both biotypes. Many different types of transcripts were upregulated in both biotypes (Fig. 1, Table S2). These included metabolic enzymes such as cytochrome P450s, which comprise a large family whose members are involved in metabolic processes (Mizutani 2012). Three UDP-glycosyltransferases were upregulated—these enzymes modify molecules by the addition of an UDP-glucose to the recipient molecule (Ross et al. 2001). Regulatory enzymes were upregulated such as protein kinases, E3 ubiquitin ligases, and transcription factors. Protein kinases constitute a large and diverse family—942 have been identified in *Arabidopsis* (Zulawski et al. 2014). A subset of these, the MAP kinases, have been known to exhibit increased gene expression in response to various abiotic stresses, including salt stress, temperature stress, and drought (Agrawal et al. 2002; Wen et al. 2002; Sun et al. 2013). E3 ubiquitin ligases are also a large family of enzymes that can target proteins for degradation and are also activated in response to stress (Yee and Goring 2009). WRKY transcription factors are often involved in response to both abiotic and biotic stresses (Banerjee and Raychoudhuri 2015). Signaling enzymes included five transcripts with homologs to calcium-binding proteins, which are also induced by stress (Zeng et al. 2015). 1-Aminocyclopropane-1carboxylate (ACC) oxidase, which is involved in ethylene synthesis, is stress responsive as it can be induced by wounding (Hyodo et al. 1993). CAF1 deadenylases, one of the three nucleases that were upregulated, belong to the CCR-CAF1 complex and have roles in plant growth and pathogen resistance (Walley et al. 2010). Cobra proteins are GPI-anchored proteins that are important in cell expansion (Schindelman et al. 2001). MATE transporters have been involved in detoxification (Omote et al. 2006). Trehalose phosphatase, HSP70, chitinase, a PLAC8 family member, an ankyrin repeat protein, and an oxysterol-binding protein were also upregulated and can be induced by stress (Avrova et al. 2004; Wang et al. 2004; Ge et al. 2008; Li et al. 2008; Song et al. 2011; Grover 2012; Yuan et al. 2013).

A common theme among this diverse group of transcripts is that many have been reported to be upregulated in response to different biotic and abiotic stresses. This would suggest that the observed change in gene expression following imazamox treatment is a stress response. It is not surprising that an herbicide would elicit this type of response as its purpose is to kill the plant. This is also in line with other studies that have examined herbicide response, which observed many of the same types of genes to be upregulated following herbicide exposure (Manabe et al. 2007; Zhu et al. 2008; Das et al. 2010; Duhoux et al. 2015; Gardin et al. 2015). Manabe et al. (2007), in examining the response of wild-type and ALS inhibitor-resistant *Arabidopsis* to imazpyr treatment found that in the sensitive plants, several transcripts were upregulated, including transcription factors, cytochrome P450s, MATE efflux proteins, kinases, and trehalose-6-phosphate phosphatase. This is similar to what is observed in both the R and S junglerice biotypes presented here. Manabe et al. (2007) did not observe changes in gene expression in the resistant biotype following exposure, however, the resistant biotype contained a point mutation in the gene-encoding ALS, rendering the enzyme insensitive to

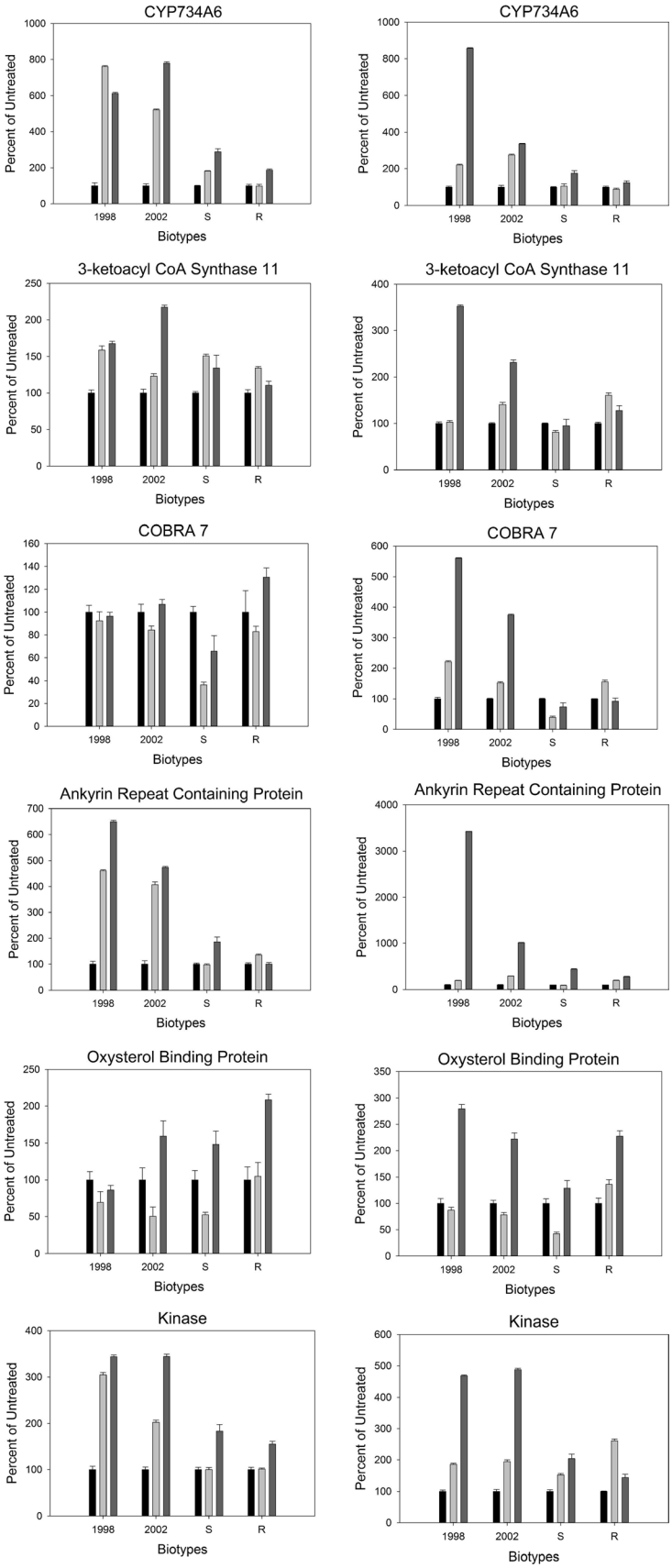

Fig. 3 Comparison of R, S and two additional junglerice biotypes (1998 and 2002) at 4 h after herbicide treatment. Black bars are untreated, light grey bars are NIS alone, and dark grey bars are imzamox treated. Data are presented as a percent of untreated within each biotype. The two trials are presented separately with the first on the left and the second on the right

the herbicide. This is in contrast to the resistant junglerice biotype here which possesses a sensitive ALS and instead appears to have a metabolic mechanism of resistance (Riar et al. 2013). This is similar to what Gardin et al. (2015) observed in blackgrass, where an RNA-seq analysis was performed on ALS inhibitor-resistant and- sensitive blackgrass. The R and S biotypes had overlapping responses to herbicide exposure, including upregulation of cytochrome P450s, UDP-glycosyltransferases, and MATE efflux proteins (Gardin et al. 2015). Das et al. (2010) and Duhoux et al. (2015) also observed in *Arabidopsis* and ryegrass, respectively, that treatment with an ALS inhibitor was followed by increases in expression of cytochrome P450s, UDP-glycosyltransferases, and transcription factors. Working with glyphosate, an herbicide with a different mechanism of action, in soybean, Zhu et al. (2008) observed upregulation of cytochrome P450s, calcium-binding proteins, transcription factors, and transcripts involved in protein turnover. The overlap in classes of transcripts in different plant species exposed to different herbicides, including the junglerice biotypes presented in this study, suggest a general stress response following herbicide treatment.

There were some differences between the responses in the R and S biotypes, however, it is not known what differences can be attributed to resistance and what differences are present due to natural variation among junglerice biotypes (these biotypes are not isogenic). For some transcripts, the differences may be resolved at later time points (i.e., a transcript is slower to be upregulated in one biotype compared to the other). Additional RNA-seq work may resolve this by sampling at time points such as 4, 24, 48 h or even later time points after herbicide treatment. Interestingly, none of the transcripts for branched chain amino acid biosynthesis were flagged in the differential gene expression analysis. Imazamox, as a member of the imidazolinone family, inhibits ALS, which results in a cessation of branched chain amino acid biosynthesis and ultimately kills the plant (Shaner et al. 1984). It may be that these transcripts are differentially expressed at a later time after imazamox treatment.

Time course

The RNA-seq analysis only examined two time points: before imazamox treatment and 1 h after treatment. However, it is possible that changes in gene expression response to herbicide exposure could change with time. In addition, a NIS-only treatment was not included in the RNA-seq analysis. It was important to include this in the time course study as a study that examined surfactant exposure alone also observed upregulation of the same classes of genes 1 h after surfactant exposure (Madhou et al. 2006). Six transcripts representative of different families were chosen for the time course study. Inclusion of the NIS controls showed that most of the change in gene expression was due to herbicide treatment (Fig. 2). Interestingly, expression of these transcripts peaked between 4 and 12 h and generally returned to pretreatment levels by 48 h after exposure (Fig. 2). These transcripts appear to represent an early response to herbicide exposure that occurs within the first 24–48 h of treatment. However, it is still unknown at this time if these transcripts produce a functional enzyme within the cell and how that product affects herbicide response. Even though expression of the transcript generally drops to the untreated levels in 48 h, there may be a prolonged effect due to activity/persistence of the transcript's product. This could be explored further by pairing proteomic and metabolomic experiments with transcriptomic experiments. In addition, the time at which expression of these genes peaked, between 4 and 12 h, is later than the time at which RNA was harvested for RNA-seq (1 h). A second RNA-seq experiment that includes one or more later time points would likely capture additional herbicide-responsive transcripts. Combining additional RNA-seq experiments with proteomic and metabolomic experiments will likely provide a fuller picture of how the plant responds in the hours following herbicide exposure.

Biotype comparison

For the RNA-seq study, only two biotypes were included. To determine if the changes in gene expression observed in R and S following imazamox exposure were representative of junglerice response in general to treatment with this herbicide, qPCR analysis at the 4-h time point using additional junglerice biotypes, 1998 and 2002, was performed. The six transcripts analyzed in the time course were used here and the 4-h time point was chosen as the increase in gene expression was greater at that time point than at the 1-h time point. Although the amount of increase in expression following herbicide treatment varied between trials and plants, the trends were similar, indicating that for these genes, upregulation in response to imazamox exposure is probably part of a normal response to imazamox in junglerice.

Conclusions

This study not only provides the first leaf transcriptome for junglerice but also presents the changes in gene expression that occur soon after imazamox exposure. Differential expression analysis comparing treated and untreated plants revealed the upregulation of several transcripts belonging to families associated with abiotic stress responses. A time course study using qPCR demonstrated that for a subset of transcript expression peaked with 4–24 h and usually returned to untreated levels within 48 h. The biotype

comparison demonstrated that junglerice plants of different genetic backgrounds had similar responses to imazamox treatement. Overall, this work supports recent findings in other plant species that herbicide treatment induces a stress response. Future studies, including additional RNA-seq work with later time points and additional biotypes, may provide more information on how junglerice responds to herbicide treatment, particularly with respect to herbicide resistance.

Author contribution statement AW, DP, VN, JB, JR, and DS designed the research. AW, RS, LK, and MN conducted the research and analyzed the data. AW wrote the manuscript with suggestions and editing from all coauthors. All authors have read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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