RNA-Seq transcriptome analysis to identify genes involved in metabolism-based

diclofop resistance in *Lolium rigidum*

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S.B.P., and R.B. analyzed data; and T.A.G., L.L., R.B., Q.Y., S.B.P, and R.B. wrote the paper.

Abbreviations: cytochrome P450 monooxygenase (CytP450); glutathione transferase (GST); glucosyltransferase (GT); nitronate monooxygenase (NMO); resistant (R); susceptible (S); acetyl-CoA carboxylase (ACCase).

Accession Numbers for Sequence Data:

This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GAYU00000000. The version described in this paper is the first version, GAYU01000000.

BioProject: PRJNA239942

BioSample: SAMN02671742

Running Title: RNA-Seq transcriptome analysis in *Lolium rigidum*

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SUMMARY

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Weed control failures due to herbicide resistance are an increasing and worldwide problem significantly impacting crop yields. Metabolism-based herbicide resistance (referred to as metabolic resistance) in weeds is not well characterized at the genetic level. An RNA-Seq transcriptome analysis was used to find candidate genes conferring metabolic resistance to the herbicide diclofop in a diclofop-resistant population (R) of the major global weed *Lolium rigidum*. A reference cDNA transcriptome (19,623 contigs) was assembled and assigned

putative annotations. Global gene expression was measured using Illumina reads from

untreated control, adjuvant-only control, and diclofop treatment of R and susceptible (S).

Contigs showing constitutive expression differences between untreated R and untreated S

were selected for further validation analysis, including 11 contigs putatively annotated as

cytochrome P450 (CytP450), glutathione transferase (GST), or glucosyltransferase (GT), and

17 additional contigs with annotations related to metabolism or signal transduction. In a

forward genetics validation experiment, nine contigs had constitutive up-regulation in R

individuals from a segregating F_2 population, including 3 CytP450, one nitronate

monooxygenase (NMO), 3 GST, and 1 GT. Principal component analysis using these nine

contigs differentiated F_2-R from F_2-S individuals. In a physiological validation experiment

where 2,4-D pre-treatment induced diclofop protection in S individuals due to increased

metabolism, seven of the nine genetically-validated contigs were significantly induced. Four

contigs (2 CytP450, NMO, and GT) were consistently highly expressed in nine field-evolved

metabolic resistant *L. rigidum* populations. These four contigs were strongly associated with

the resistance phenotype and are major candidates for contributing to metabolic diclofop

resistance.

INTRODUCTION

Weed control in modern cropping systems is vital to protect crop yields, maintain

profitable farming, and meet global food demands. Herbicides are major tools to control

weeds and weed control failure caused by herbicide resistance is an increasing and significant

problem worldwide (Heap, 2013). The evolution of herbicide resistance has rapidly occurred

when large and genetically variable weed populations have been subjected to intensive

herbicide selection (reviewed in Powles & Yu, 2010). While in many cases target-site based

herbicide resistance mechanisms endow resistance only to a selecting herbicide

chemistry/mode-of-action, the greatest threat is posed by metabolic resistance mechanisms, as there can be resistance across diverse herbicide classes. Metabolic resistance, while evident in several species, has been repeatedly documented in the important grass weed *Lolium rigidum*. Since first apparent (Heap & Knight, 1986) subsequent studies have established that herbicide cross-resistance in *L. rigidum* involves enhanced rates of herbicide metabolism that can be reversed in vivo by known cytochrome P450 monooxygenase (CytP450) inhibitors (Holtum et al., 1991; Christopher et al., 1994; Preston et al., 1996; Preston & Powles, 1998; Preston, 2004). Collectively CytP450 are the largest family of plant enzymes and plant genomes contain a large number of CytP450 genes, in most cases from 300 to 400 (Werck-Reichhart et al., 2000; Omura, 2013). The diverse functions of CytP450 include detoxification of xenobiotic compounds, and this function enables some CytP450 enzymes to also serendipitously metabolize herbicides (Werck-Reichhart et al., 2000). Metabolic resistance in *L. rigidum* has been characterized as multi-genic and conferred by at least three loci (Preston, 2003; Busi et al., 2011). However, the high number of CytP450 genes in plant genomes makes characterization of individual CytP450s difficult, and the specific genes conferring metabolic resistance and their roles remain unknown.

The acetyl-coA carboxylase (ACCase)-inhibiting herbicide diclofop controls susceptible *L. rigidum* and other grass weeds. Wheat is tolerant to diclofop due to metabolic mechanisms, involving CytP450-catalyzed rapid aryl hydroxylation and subsequent glucosyl transferase (GT)-catalyzed glucose conjugation (Figure 1), that render the herbicide molecule non-phytotoxic (Shimabukuro et al., 1979; Zimmerlin & Durst, 1992; Helvig et al., 1996). *L. rigidum* is a cross-pollinated, genetically diverse species and individuals vary in their capacity to metabolize certain herbicides (such as diclofop). Recurrent low rate diclofop selection using an initially herbicide susceptible (S) progenitor population and cross-pollination among survivors rapidly (after 3 generations) resulted in a diclofop-resistant

population (R), involving 2-3 gene loci (Neve & Powles, 2005; Busi et al., 2013). The resistance is due to enhanced diclofop metabolism rates, likely involving CytP450 (Yu et al., 2013), and there is cross-resistance to the dissimilar herbicide chlorsulfuron which can be reversed by the CytP450 inhibitor malathion (Busi et al., 2013). Therefore, we used this R population to test the hypothesis (Gressel, 1987; Gressel, 1988; Kemp et al., 1990; Powles et al., 1990) that metabolic resistance is endowed by heritable, increased expression of several CytP450 and other relevant enzymes.

Next-generation sequencing technologies provide many advantages for characterizing transcriptome-wide gene expression, allowing digital quantification of known reference sequences (Morozova & Marra, 2008). Such technology can now be applied to non-model species such as weeds, as reference transcriptomes necessary for expression quantification can be obtained even when no previous transcriptome data are available. The 454 pyrosequencing technology is useful for generating *de novo* reference transcriptomes (Peng et al., 2010; Riggins et al., 2010) due to its longer reads (400-500 bp). Illumina HiSeq technology provides shorter reads and higher coverage, and is useful for an approach known as RNA-Seq that enables transcript quantification and detection of lower abundance transcripts (Lister et al., 2009). Using RNA-Seq transcriptome analysis of *L. rigidum* and validation experiments, our objective was to identify and validate differential expression of specific genes between R and S individuals, with the hypothesis that expression changes contribute to metabolic resistance.

RESULTS

Reference Transcriptome

In order to conduct RNA-Seq experiments in *L. rigidum*, a reference transcriptome sequence was obtained using 454 pyrosequencing of a cDNA library developed from RNA of young seedling and vegetatively-cloned tissues from a single R individual in both untreated and diclofop-treated conditions. Sequencing of one pico-titer plate produced 1,069,238 reads of average length 448 bp, with 405,875,904 bases sequenced in total (Table 1). Assembly with Newbler (454 Life Sciences) resulted in 883,595 full assembled reads. In total 19,623 contigs greater than 100 bp in length were obtained and 12,450 contigs greater than 500 bp long, with an average contig size of 1,049 bp and an N50 contig size of 1,150. The total number of bases contained in the reference was 15,040,525, and 97.43% had a quality score higher than Q40 (0.01% chance of error). These contigs were assigned putative annotations using both UniProtKB (Uniprot Consortium, 2012) and Pfam (Punta et al., 2012) databases with a cutoff value of $E \leq 1 \times 10^{-4}$, and the top 3 hits from each database were returned. Using these criteria, 56.7% of the 19,623 contigs had a hit in the Pfam database of protein families, and 74.5% had a hit in the UniProt database. Among gene families with particular relevance for metabolic resistance, 57 CytP450, 56 glutathione transferase (GST), and 7 GT contigs were assigned putative annotations using Pfam. Species with the highest sequence similarity to *L. rigidum* were identified, with *Brachypodium distachyon* (34.4% of total contigs), *Hordeum vulgare* (25.7%), *Oryza stativa* (4.3%), and *Sorghum bicolor* (1.6%) gene sequences having the highest probability BLAST results to the *L. rigidum* reference transcriptome contigs.

RNA-Seq Expression Quantification

An RNA-Seq experiment was then performed using the R and S populations. Treatments included an untreated control, adjuvant only control, and diclofop plus adjuvant

(1× labeled field rate), applied to vegetative clones of each individual, with 4 biological

replicates for each treatment. The 24 RNA libraries were sequenced using Illumina HiSeq

and 100 bp paired-end reads, producing 1.4 billion reads ranging from 33.1 to 102.6 million

reads per sample. Reads were aligned to the reference transcriptome using the Bowtie short

read aligner (Langmead et al., 2009), FPKM (fragments aligned per thousand bases per

million reads) values for each contig were calculated, and differential expression statistical

analysis was conducted using the DESeq package in the statistical software 'R' (Anders &

Huber, 2010).

Constitutive differential expression (FPKM > 3 in at least one treatment group, fold

change ≥2, P≤0.05) was apparent between untreated R and S samples at time point 0, with

458 contigs constitutively up-regulated in R, and 318 contigs up-regulated in S. Differential

expression between R and S in all three treatments was found for 278 contigs (Figure S1).

More contigs with differential expression due to the adjuvant only control were identified in

both R and S than contigs with differential expression due to diclofop treatment relative to

the adjuvant only control (Table S1). When comparing the diclofop treatment to the adjuvant

only control (measuring the effects on transcription due specifically to diclofop and

controlling for transcriptional effects due to spraying with water and adjuvant), 11 contigs

were down-regulated in R, and 47 contigs were up-regulated in S. Comparison of contigs up-

regulated in S specifically by the diclofop treatment to contigs constitutively up-regulated in

untreated R relative to S (458) revealed an overlap of 13 contigs, including contigs putatively

annotated as CytP450s, GSTs, and a GT. Other contigs up-regulated in S specifically by

diclofop treatment were annotated with Gene Ontology terms in the Biological Process

category including defense response, toxin catabolic process, response to stress, and response

to abiotic stimulus (Table S1). This suggests a transient stress response occurs in S due to the

phytotoxic effects of diclofop at 24 h after treatment, and the stress response does not occur in R due to rapid diclofop metabolism and ensuing prevention of phytotoxic effects.

Candidate Metabolic Resistance Contig Selection

Based on our knowledge that this R population more rapidly metabolizes diclofop in comparison to S (Yu et al., 2013), we tested the hypothesis that contigs differentially expressed between R and S with predicted annotations related to metabolism and signaling pathways would be prognostic transcriptional markers for metabolic diclofop resistance. Differentially expressed contigs were selected for further evaluation based on UniProt and/or Pfam putative assignment to one of the major gene families with known roles in metabolic resistance (CytP450, GST, and GT), or having an assigned Gene Ontology molecular function that could be involved in any one of the three described phases of pesticide metabolism (Van Eerd et al., 2003). Contigs selected included those putatively annotated as CytP450s (which have oxidoreductase molecular function) and all differentially expressed contigs having oxidoreductase molecular function. In addition, all differentially expressed contigs annotated as GSTs, GTs, and ABC transporters were selected (Table 2). All CytP450 annotated contigs with >2-fold up-regulation in R, but a non-significant *P*-value in the DESeq analysis were also selected (contigs 07659 and 12788). In addition, contigs were selected with predicted annotations related to signaling pathways (e.g., protein kinase, phosphatase) and transcription factors, along with contigs having the largest fold-change expression differences but unknown function (no assigned annotation). In total, 28 contigs were selected based on these criteria (Figure 2, Table 2) for confirmation of RNA-Seq results using quantitative real-time Reverse Transcription Polymerase Chain Reaction (qRT-PCR). All selected contigs showed significant differential expression in RNA-Seq between R and S in the untreated condition, and 7 contigs were up-regulated by diclofop treatment in S (Figure 2; Table 2). qRT-PCR results were normalized as $2^{-\Delta Ct}$ using the method of Schmittgen &

Livak (2008) and two internal control genes determined to be stably expressed in all conditions using BestKeeper (r^2=0.886, P=0.001) (Pfaffl et al., 2004). Observed differential expression from the RNA-Seq experiment was validated by qRT-PCR for 24 of the 28 selected contigs (Table 2).

Forward Genetics Validation

The 24 validated contigs were next evaluated using a forward genetics approach with an F_2 population derived from a cross of the R by S individuals. This F_2 population segregates for diclofop resistance with multi-genic inheritance (Busi et al., 2013). Vegetative clones of individuals were evaluated for diclofop resistance, and the most susceptible F_2-S individuals (n=7) were identified using the criteria that they did not survive a 1/2× diclofop application. Resistant F_2-R individuals (n=9) were identified on the basis that they survived a 1× diclofop application and exhibited robust growth following the treatment. Expression of the 24 contigs was evaluated in untreated clones of the seven F_2-S and nine F_2-R individuals. Differences in gene expression between the two groups were evaluated using the GenePattern Comparative Marker Selection suite (Gould et al., 2006; Reich et al., 2006) to test for significant differences in the mean $2^{-\Delta Ct}$ between F_2-S and F_2-R. Within the 24 contigs with RNA-Seq results validated by qRT-PCR, nine contigs had significant differences between F_2-R and F_2-S and appeared to co-segregate with resistance (Table 2; Figures S2, S3). None of the nine contigs were highly expressed in any untreated F_2-S individuals (Table 2, Figure S3). These contigs had predicted annotations including CytP450 (3), GT, GST (3), nitronate monooxygenase (NMO), and a contig with unknown function. The predicted GSTs are members of the Tau and Phi classes, but do not share high sequence similarity (from 33 to 42%). Two validated CytP450s were both classified as CYP72A based on BLAST similarity to known CytP450 genes (contigs 01604 and 02218), and the two contigs had high (85.7%) sequence similarity with each other. The third validated CytP450 was classified as CYP716A

(contig 06783). Two CytP450s (classified as CYP71B and 89A) were constitutively up-regulated in R in the RNA-Seq experiment, but were not associated with resistance in the F_2 (Table 2). The CYP89A was up-regulated in F_2-S relative to F_2-R, highlighting the importance of hypothesis testing using forward genetics for candidate genes identified by RNA-Seq.

Gene expression data for the nine contigs with up-regulation in F_2-R relative to F_2-S (Table 2, Figure S3) were evaluated as potential metabolic diclofop resistance transcriptional markers to visually separate R and S samples using principal component analysis (Riedmaier & Pfaffl, 2013). Gene expression FPKM data from the RNA-Seq experiment (Figure 3A, 3B) and qRT-PCR data from the F_2-R and F_2-S (Figure 3C, 3D) provided clear separation of R and S in the principal component analysis. Sample F_2-S2 was least similar to all other F_2-S samples (Figure 3C). This sample did not have high (above average) expression for any of the contigs annotated as CytP450 (3) or NMO, but rather mid-level (average) expression of 2 CytP450-annotated contigs, providing an explanation for its distance from the other F_2-S samples. Sample F_2-S2 also had low expression of the GT-annotated contig, potentially an important factor for diclofop resistance. The principal component analysis results from the F_2 population also suggest that segregation for several genes is occurring, and the genes may contribute to metabolic diclofop resistance in various combinations.

Physiological Validation

We have established that protection against diclofop phytotoxicity due to increased diclofop metabolism is inducible by pre-treatment with 2,4-dichlorophenoxyacetic acid (2,4-D) in the S *L. rigidum* population used in our studies (Han et al., 2013). Most grasses, including *L. rigidum*, are tolerant to 2,4-D due to CytP450-mediated aryl hydroxylation (Coupland, 1994). The phenotype following induction of diclofop protection with 2,4-D is similar to that observed in the R population, and due to the inducible nature, must be

conferred by induced gene expression and not by any altered enzymatic activity due to mutations in genes. Therefore, susceptible individuals were treated with 2,4-D and expression of the 24 contigs validated from the RNA-Seq experiment was evaluated 24 h after treatment in comparison to an untreated control sampled at the same time point. Eight contigs were highly and significantly (P<0.05) induced by 2,4-D treatment (Table 2), including contigs annotated as CytP450 (2), NMO, GT, and GST (4). Two contigs associated with diclofop resistance in the F_2 were not induced by 2,4-D, including the contig annotated as CYP716A (Table 2).

Population Validation

Expression of the genetically validated contigs was measured in eight French *L. rigidum* populations with metabolic resistance to diclofop (confirmed diclofop resistance and lacking any ACCase target-site resistance mutations, Table S2) and compared to the S population VLR1, using qRT-PCR as previously described. Four contigs (annotated as CYP72A-01604, CYP72A-02218, NMO, and GT) were each significantly up-regulated (fold-change $\geq$ 2, P < 0.05) in seven of eight metabolic diclofop resistant populations (Table 3). Five of the eight French metabolic diclofop resistant populations had up-regulation of all four contigs. The three GST-annotated contigs were up-regulated in between one and six populations. Expression was also measured in the Australian SLR31-P450 metabolic diclofop resistant population (Preston & Powles, 1998; Vila-Aiub et al., 2005), and contigs annotated as CYP72A-01604, NMO, GT, and two GST were up-regulated (Table 3). The contig annotated as CYP716A-06783 was not up-regulated in any field population, and the unknown function contig 00035 was down-regulated in most populations, with up-regulation only in two. Additional susceptible populations were measured, and no field S populations had significant up-regulation of CytP450s or NMO annotated contigs (Table 3). The contigs annotated as GT and one GST were up-regulated in a French S population. In a susceptible

sub-population selected out of SLR31 (S-SLR31) that is less susceptible to diclofop than

VLR1 (Table S2), contigs annotated as CYP72A-01604 and GT were 2- and 3-fold up-

regulated, respectively (Table 3).

DISCUSSION

Twenty-eight candidate contigs, selected based on a global RNA-Seq transcriptome

analysis of a *L. rigidum* population with metabolic resistance to the herbicide diclofop, were

subjected to three validation experiments. Four contigs (with putative annotations of two

CYP72A, one NMO, and one GT) were linked to the metabolic diclofop resistance phenotype

in all three experiments, using forward genetics, 2,4-D induced protection against diclofop,

and population validation. These four contigs were constitutively and highly expressed in the

Australian R population used for RNA-Seq, and in genetically unrelated French metabolic

diclofop resistant *L. rigidum* populations, suggesting that increased expression of these four

genes contributes to metabolic diclofop resistance. Consistent with inheritance studies (Busi

et al., 2011; Busi et al., 2013), metabolic diclofop resistance is under quantitative genetic

control with high expression of multiple genes required to confer diclofop resistance. The

high expression of GT alone or a small increase in GT and CytP450 observed in two S

populations were insufficient to confer resistance. As genetic diversity in metabolic diclofop

resistance is expected, other *L. rigidum* populations are expected to have additional and/or

different sets of genes contributing to metabolic diclofop resistance. Several contigs were

identified with large constitutive expression difference between R and S, but were not

validated by subsequent experiments, including contigs annotated as transcription factor,

protein kinase, cytochrome b5 (an effector of CytP450), oxygenase, and phosphatase. The

possibility remains that genes such as these may have complex interactions with other genes

in contributing to metabolic diclofop resistance, despite the lack of clear evidence for their role in validation experiments. In addition, three GST-annotated contigs were validated using forward genetics and 2,4-D induced protection, and highly expressed in some unrelated populations, suggesting that GST may also play a role in metabolic diclofop resistance in *L. rigidum*.

We observed that six validated contigs were also induced in S 24 h after diclofop treatment. The induction in S by diclofop treatment was transient and did not provide diclofop protection, while pre-treatment with a high dose of 2,4-D induced up-regulation of contigs prior to diclofop treatment and thus provided diclofop protection. Similar to the experiment where plants were pre-treated with 2,4-D, the constitutive up-regulation of the same contigs was present in untreated R compared to untreated S at the time of diclofop treatment. Presumably functional proteins, such as CytP450, must be translated and biochemically competent to metabolize diclofop starting from the beginning of diclofop treatment, especially as diclofop is expected to rapidly penetrate the cuticle and reach the ACCase target site; therefore, transient gene expression induced by diclofop in S does not provide timely and sufficient protection against diclofop. Collectively, multiple genes related to diclofop metabolism are inducible in response to 2,4-D treatment and have been selected for heritable constitutive up-regulation in the R population. Constitutive up-regulation of these genes appears critical to confer metabolic diclofop resistance.

The biochemical role of CytP450-mediated herbicide metabolism has been well established in field-evolved resistant *L. rigidum* populations using CytP450 inhibitors, showing strong evidence that several distinct CytP450 isoforms are involved in metabolic resistance to ACCase and acetolactate synthase (ALS)-inhibiting herbicides (Christopher et al., 1994; Preston et al., 1996; Preston & Powles, 1998; Busi et al., 2011). Several CytP450 genes, including two CYP72A, were also up-regulated in a multiple herbicide resistant

Echinochloa phyllopogon population (Iwakami et al., 2013). In addition, a handful of CytP450s have been identified in crop species conferring herbicide resistance. In rice, the role of a CYP81A6 gene in conferring resistance to photosystem II and ALS-inhibiting herbicides has been established (Pan et al., 2006). The CytP450s we have identified by RNA-Seq could contribute to ALS metabolic resistance, as the R population in this study is also resistant to the ALS herbicide chlorsulfuron and the resistance is reversed by application of the CytP450 inhibitor malathion (Busi et al., 2013). Wheat CYP71C6v1 has been cloned, expressed in yeast, and purified as a 52.5 kDa recombinant protein which specifically metabolizes chlorsulfuron, triasulfuron and metsulfuron-methyl via phenyl ring hydroxylase (Xiang et al., 2006a; Xiang et al., 2006b).

The biochemical role of GSTs in metabolic herbicide resistance is less clear. Both Tau and Phi class GSTs have demonstrated roles in herbicide detoxification for several compounds (Cummins et al., 2011), although this has not been previously shown for diclofop. The GSTs could function to protect against general side-effects of diclofop phytotoxicity, such as anti-oxidant stress and/or lipid peroxidation (Cummins et al., 2011). The GST-annotated contig 16302 is highly similar (94.5% similarity in 145 bp) to the *Lr*GSTF1 homologue of *Am*GSTF1, reported to play a role for fenoxaprop-ethyl resistance in *Alopecurus myosuroides* (Cummins et al., 2013). The GST-annotated contig 04546 (up-regulated in Rn relative to Sn, induced by 2,4-D, but no difference in expression between F_2-R and F_2-S) is highly similar (BLAST score 205, E-value 1.6×10^{-53}) to wheat *Ta*GSTU4, a GST that is induced by the safener fenchlorazole-ethyl in wheat and that can conjugate fenoxaprop-ethyl and dimethenamid with glutathione (Thom et al., 2002). Contig 05390 (up-regulated in Rn relative to Sn, induced by 2,4-D, and up-regulated in F_2-R relative to F_2-S) is similar (BLAST score 78, E-value 9.3×10^{-15}) to an *Arabidopsis* GST (At1g10870) reported

to have a role in light signaling, glutathione levels, and as a negative regulator of salt and drought stress signal transduction (Jiang et al., 2010; Chen et al., 2012).

Further evaluation using heterologous expression in yeast (for CytP450s) and bacteria, or ideally transgenic expression in *Lolium*, is necessary to define the biochemical roles in diclofop (and chlorsulfuron) metabolism of the two CYP72A, NMO, three GST (two Tau class and one Phi class), and GT-annotated contigs found in this study. Both NMO and GT genes have been associated with metabolic detoxification of xenobiotics in *Arabidopsis*. The GT-annotated contig identified here in *L. rigidum* has some similarity (BLAST score 40, E-value $4.4{\times}10^{-2}$) to an *Arabidopsis* UDP-GT locus (At2g15490), one of multiple GT associated with metabolic detoxification of the explosive 2,4,6-trinitrotoluene (Gandia-Herrero et al., 2008). The NMO-annotated contig identified in *L. rigidum* is similar (BLAST score 50, E-value $3.8{\times}10^{-5}$) to an *Arabidopsis* NMO gene (At5g64250) associated with metabolic detoxification of the allelochemical benoxazolin (Baerson et al., 2005). NMO, formerly referred to as 2-nitropropane dioxygenase (NPD) (EC 1.13.12.16), contains FMN as a non-covalently bound cofactor, is a flavin-depedent monooxygenase with nitroalkanes and alkyl nitronates as substrates in yeast and bacteria, and catalyzes an oxidative denitrification reaction (Gadda & Francis, 2010). NMO was selected for additional expression validation because the gene shares a Gene Ontology molecular function term with cytochrome P450, oxidoreductase activity. While no previous reports have implicated NMO as having a role in metabolic herbicide resistance, NMO was found to detoxify propionate-3-nitronate in *E. coli* and yeast, providing protection against nitronate poisoning in vivo and suggesting a role for NMO in resistance to allelopathy (Francis et al., 2012). Oxidative transformations involving nitroaromatic xenobiotic compounds have been described in bacteria (Van Eerd et al., 2003), but as diclofop does not contain nitrogen, it is intriguing and remains to be experimentally

determined whether the *L. rigidum* NMO-annotated contig has diclofop metabolic activity or other roles in metabolic diclofop resistance.

Our *de novo* reference transcriptome for *L. rigidum* is a critical bioinformatics resource necessary for conducting RNA-Seq. Previous *de novo* transcriptome sequencing projects have been conducted in other weed species including *Amaranthus tuberculatus* (Riggins et al., 2010), *A. hypochondriacus* (Délano-Frier et al., 2011), and *Conyza canadensis* (Peng et al., 2010), resulting in 22,035, 25,998, and 16,102 assembled contigs of average size 434, 971, and 438 bp , respectively, from 1.5, 6, and 1.5 pico-titer plates of 454 sequencing. In comparison, our reference *L. rigidum* transcriptome had a similar number of assembled contigs and longer average size. The 19,623 contigs in our reference transcriptome do not necessarily represent unigenes, as they have been computationally assembled and we do not have genomic sequence for comparison. Multiple contigs may represent different segments of one unigene. Despite these challenges, the *L. rigidum* reference transcriptome enabled an RNA-Seq approach to successfully characterize transcriptomic level gene expression in a non-model species with no previously available sequence information. Other RNA-Seq experiments have also identified differentially expressed genes involved in complex plant phenotypes including phosphorous acclimation, seed germination, and endosperm development (Gao et al., 2013; Liu et al., 2013; O'Rourke et al., 2013).

Changes in plant gene expression have been reported to occur through several mechanisms, including specific promoter motifs and/or transcription factors (e.g., Jepson et al., 1994; Baerson et al., 2005), gene duplication (Gaines et al., 2010), and heritable epigenetic changes in methylation of genes and promoters (Schmitz et al., 2011). Protein activity of the identified genes may be subject to additional controls, such as regulation of protein translation by long non-coding antisense RNA (Jabnoune et al., 2013), or post-

translational modifications. Future research will consider all these possibilities to determine the specific mechanism(s) that alter the heritable gene expression patterns in this metabolic diclofop resistant *L. rigidum* population. The biochemical activity of the putatively-annotated genes will also be experimentally determined using diclofop and herbicides from additional chemical classes. The heritable, constitutively increased expression of multiple genes was selected during recurrent selection at initially low herbicide doses in the R population used for RNA-Seq, supporting the hypothesis that any and all mechanisms that may confer survival are selected in a low herbicide dose environment (Neve & Powles, 2005; Busi et al., 2012). Selection for altered gene regulation in the R *L. rigidum* population occurred in a rapid temporal scale, requiring only three generations to shift the population mean to a high resistance level (Neve & Powles, 2005). Clearly, genetic diversity for gene expression regulation is present in small populations and this diversity is important for evolutionary processes leading to herbicide resistance (Neve et al., 2009). Furthermore, four genes (annotated as 2 CytP450, NMO, and GT) had constitutively increased expression in field-evolved metabolic diclofop resistant populations from France and Australia and will be valuable as transcriptional markers for metabolic diclofop resistance diagnostics. This study represents a milestone towards a greater understanding of evolutionary and ecological functions of genetic traits that have major impacts on plant fitness and life history in the presence of herbicide selection.

EXPERIMENTAL PROCEDURES

Plant Material

The R population used for the reference transcriptome and for the RNA-Seq expression quantification experiment was reported by Neve & Powles (2005) and produced

by recurrent selection at initially low doses of diclofop. The Australian S population used in all studies was VLR1, also described for diclofop response by Neve & Powles (2005). The R population was selected from its progenitor S population over three generations of recurrent selection (Neve & Powles, 2005). This provided a much higher level of similarity in genetic background between R and S populations than would be obtained by comparing a field R population with an unrelated S population, and enabled us to minimize genetic differences that were unrelated to the metabolic resistance traits of interest (Vila-Aiub et al., 2011). Individuals were vegetatively cloned by separating tillers and transplanting into separate pots. All plants were grown in the greenhouse in 4.5 cm Fertilpots containing peat/loam 1:1 soil mixture, and a photoperiod of 16 h light, 22 °C / 8 h dark, 14 °C with a light intensity of at least 220 µE m^{-2} s^{-1} (Phillips Son-T AGRO). Watering and fertilization with 0.4 % Wuxal Super solution were performed as necessary to maintain vigorous plant growth. All herbicides were applied using a laboratory track sprayer calibrated to apply 300 L ha^{-1} spray volume with an 8003EVS nozzle (TeeJet), and diclofop was applied using the commercial formulation (Iloxan, Bayer CropScience) with adjuvant at 0.1% v/v (Biopower, Bayer CropScience).

Reference Transcriptome

Twelve R individuals were vegetatively cloned and a single R individual was chosen for the reference transcriptome on the basis of surviving a 4× (1500 g ha^{-1}) diclofop dose, lacking any ACCase or ALS target site resistance mutations (measured using a pyrosequencing assay described in Collavo et al., 2013), and having increased diclofop metabolism relative to S (measured through an in-vivo ^{14}C-DFM assay) (Collavo et al., 2013; Yu et al., 2013). Vegetative clones were used to generate leaf samples from control untreated clones and from clones 24 HAT that had received a 1× (375 g ha^{-1}) diclofop treatment. Leaf samples were immediately frozen in liquid nitrogen, and total RNA was extracted using the

RNEasy kit and protocols (Qiagen). A reference cDNA library was generated from 2 µg mRNA and cDNA library inserts were prepared for sequencing using one pico-titer plate on the Roche/454 GS FLX+ Titanium platform (LGC Genomics, Berlin, Germany). Contig assembly was performed using Newbler with the following parameters: seed step 12, seed length 16, minimum overlap length 40, minimum overlap identity 90, alignment identity score 2, and alignment difference score -3. Gene Ontology assignment used the best BLAST hit with a cutoff value of $E{\leq}1{\times}10^{-4}$.

RNA-Seq Expression Quantification

Four individuals each of R and S were vegetatively cloned. R individuals were selected for RNA-Seq as described for the reference transcriptome, including verification that no ACCase or ALS target-site resistance mutations were present and verification of enhanced diclofop metabolism (methods described in Collavo et al., 2013). S individuals were selected on the basis of not surviving a $1/2{\times}$ (188 g ha^{-1}) diclofop dose and having low diclofop metabolism. Vegetative clones were used to produce three identically sized clones of each individual. The experimental design included four biological replications of R and S for control untreated, adjuvant only control, and $1{\times}$ (375 g ha^{-1}) diclofop treatments. Leaf samples for the untreated control were collected at time point 0, and leaf samples from the adjuvant only control and diclofop treatments were collected 24 h after treatment application. Each sample consisted of a mixture of 4 cm of the newest emerging leaf, 2 cm of the stem below the whorl, and 2 cm of the first fully expanded leaf. Samples were frozen immediately in liquid nitrogen and pulverized for total RNA extraction. Libraries for Illumina HiSeq 2000 sequencing were prepared with 2 µg RNA following the Illumina TruSeq RNA prep protocols, including selection for main library size of 270-320 bp and an average RIN value of 6 (LGC Genomics). Illumina sequencing of 100 bp paired-end reads was conducted for the 24 samples using bar-coded adapters in 8 channels of the Illumina flow-cell.

Read preprocessing criteria included trimming library adapters and performing quality control checks using FastQC (Version 0.10.1). Bowtie was used to align reads to the reference transcriptome with the following parameters: a 30 bp seed with two allowed mismatches, a maximum mismatch quality sum of 120, only one best alignment reported, and the best alignment guaranteed. Transcript quantification was performed in Genedata Expressionist Refiner Genome (Version 7.6.4) with the Read Condensing activity and the RNA-Seq Feature Quantification activity to calculate FPKM. Gene expression differences were analyzed using the DESeq package (Version 1.12.0) in the statistical software 'R' (Version 2.15.0) using a negative binomial distribution (Anders & Huber, 2010). The criteria for differential expression were defined according to previously established standards, considering only transcriptionally active contigs with FPKM > 3 in at least one treatment group, a fold change ≥2 between compared groups, and statistical significance at $P \leq 0.05$ (O'Rourke et al., 2013). Expression differences were compared between untreated R and untreated S at time point 0; between R and S 24 h after adjuvant only control; and between R and S 24 h after diclofop treatment. Expression differences were also compared within R and S between untreated, adjuvant only control, and diclofop treatments. Genedata Expressionist Analyst (Version 7.6.4) was used to generate heat maps of expression data.

Candidate Metabolic Resistance Contig Selection

Contigs were selected on the basis of statistical significance, magnitude of expression differences, and annotations related to known herbicide metabolism genes and signaling functions. Contig sequences were used to design primers for qRT-PCR quantification of gene expression (Table S3).

Relative Gene Expression Quantification

Total RNA was extracted from leaf samples using Trizol reagent and protocols (Invitrogen). DNA was removed using DNase I (Invitrogen). Following phenol extraction

and precipitation, 2 µg of RNA was used for first-strand cDNA synthesis with SuperScript III

Reverse Transcriptase and oligo(dT)$_{20}$ primer (Invitrogen), followed by digestion with RNase

H. RNA-Seq results were assessed using qRT-PCR on duplicate samples of the templates

used for the RNA-Seq experiment for 28 top contigs (Table 2) from the DESeq analysis. Six

candidate internal control genes were identified in the reference transcriptome on the basis of

predicted annotation (e.g., ubiquitin, tubulin, actin genes) or by using the baySeq package in

"R" to identify contigs with the least variation in expression among all RNA-Seq samples and

treatment conditions (Hardcastle & Kelly, 2010). All primers were assessed for expected

single-product amplification using template from the reference transcriptome RNA

containing all treatment conditions, and to confirm no PCR amplification in (-) template

reaction conditions. All PCR products were sequenced to verify amplification of the

expected contig. Six candidate internal control genes were assessed for stable expression in

cDNA samples of all types (R and S, untreated control and DFM treated). Stability analysis

was conducted using BestKeeper (Pfaffl et al., 2004) and the two best internal control genes

were chosen (contigs 05586 and 06303, annotated as isocitrate dehydrogenase and Ras family

GTPase, respectively; both were identified in baySeq analysis as top candidates for stable

expression in all sample types).

qRT-PCR was conducted using 96-well plates and the LightCycler 480 (Roche).

Reactions were conducted in duplicate, and a negative control consisting of template with no

primers was included for each template. Reactions were conducted in 20 µL volume and

each reaction included 10 µL of SyberGreen Master Mix, 2 µL of 1:10 diluted cDNA, 5 µL

of 0.5 pmol µL^{-1} primers (1:1 mix of forward and reverse primers), and 3 µL of nuclease-free

distilled water. Reaction conditions included 15 min incubation at 95 C, then 45 cycles of 95

C for 30 sec and 60 C for 1 min, followed by a melt-curve analysis to confirm single PCR

product amplification. Threshold-cycle (C_T) values were calculated for each reaction using

the Second Derivative Maximum method in the LightCycler 480 software (Roche). All cDNA templates were first assessed for complete removal of DNA using a control containing only DNase I-processed RNA template and all cDNA synthesis components except for the RT; this minus-RT reaction was used as template for qRT-PCR with internal control gene primers. No amplification was observed in any minus-RT controls. Equivalent slopes for target and internal control genes were observed in amplification plots, so the comparative C_T method was used to calculate relative expression levels as $2^{-\Delta Ct}$ where $\Delta C_T = [C_T$ target gene – geometric mean (C_T internal control gene 1 and 2)], assuming similar PCR efficiencies of target and internal control genes (Schmittgen & Livak, 2008). To validate RNA-Seq results, relative gene expression was measured using qRT-PCR on cDNA from RNA samples extracted from a different tiller that was collected at the same time as the RNA-Seq samples.

Forward Genetics Validation

A forward genetics approach was used to assess the linkage between candidate contig expression and resistance phenotype in a previously described F_2 population segregating for diclofop resistance (Busi et al., 2013). Individuals from the F_2 were vegetatively cloned and assessed for resistance phenotype by treating individual clones with $1/2\times$ (188 g ha^{-1}) and $1\times$ (375 g ha^{-1}) diclofop. Individuals were classified as susceptible (F_2-S) if they did not survive the $1/2\times$ treatment, and individuals were classified as resistant (F_2-R) if they survived the $1\times$ treatment and exhibited robust growth. Leaf samples for RNA extraction were collected from untreated clones of seven F_2-S and nine F_2-R, and qRT-PCR was conducted as previously described. Mean $2^{-\Delta Ct}$ was compared between F_2-S and F_2-R. A principal components analysis on relative expression qRT-PCR data normalized as $2^{-\Delta Ct}$ was conducted using the prcomp package in R (R, 2012) to explore the relationships among expression of all contigs in F_2-R and F_2-S with and without diclofop treatment, along with FPKM data from the RNA-Seq experiment.

Physiological Validation

Individuals from the S population were vegetatively cloned to produce four identical clones. A treatment with 2,4-D 24 h prior to diclofop treatment was used to induce protection against diclofop as previously described (Han et al., 2013). The experimental design included an untreated control and 2,4-D (3 kg ha^{-1}) treatment. Vegetative clones of each individual were randomly assigned to treatment groups. Leaf samples were collected at the same time, 24 hr after 2,4-D treatment, from both the untreated control and from the 2,4-D treatment. RNA extraction and relative expression quantification were performed as previously described using qRT-PCR. The experiment included seven biological replications.

Population Validation

French *L. rigidum* populations were chosen from Bayer CropScience field surveys based on having high level diclofop resistance (>90% survival at 1× DFM) and wild-type, susceptible genotypes at ACCase target-site positions 1781, 2027, 2041, 2078, 2088, and 2096 (Table S2) (pyrosequencing methods described in Collavo et al., 2013), indicating the populations expressed metabolism-based diclofop resistance. These populations were measured for gene expression using qRT-PCR and compared to gene expression in the S (VLR1) population and a susceptible French population chosen from field survey data. Individuals were cloned as previously described and selected for gene expression measurement based on surviving both 4× (1.5 kg diclofop-methyl ha^{-1}) and 8× (3 kg diclofop-methyl ha^{-1}). Individuals from susceptible populations were selected based on mortality at 2× (0.75 kg diclofop-methyl ha^{-1}). Leaf samples for RNA extraction were collected and gene expression measurements were conducted as described, prior to cloning. The Australian metabolic resistant population SLR31-P450 (Christopher et al., 1994; Preston & Powles, 1998; Vila-Aiub et al., 2005; Busi et al., 2011), a super-susceptible sub-population (SVLR1)

selected from VLR1 (Manalil et al., 2012), a susceptible population WALR1 (Manalil et al., 2011), and a susceptible sub-population (S-SLR31) isolated from SLR31 (Vila-Aiub et al., 2005) were evaluated in a separate experiment using the same methods.

SHORT LEGENDS FOR SUPPORTING INFORMATION

Figure S1. Venn diagram of RNA-Seq results comparing diclofop-resistant (R) and – susceptible (S) *L. rigidum*.

Figure S2. FPKM (fragments per thousand bases per million reads) expression data from the RNA-Seq experiment (excerpted from Figure 2) for nine contigs with significant ($P<0.05$) expression differences between R and S in an F_2 *L. rigidum* population segregating for metabolic diclofop resistance.

Figure S3. Relative expression data for nine contigs with significant ($P<0.05$) expression differences between R and S in an F_2 *L. rigidum* population segregating for diclofop resistance.

Table S1. Contigs with Biological Process Gene Ontology keywords related to stress responses and their transcriptional regulation due to adjuvant only control (a), and due to diclofop treatment (t) in resistant (R) and susceptible (S) *L. rigidum*.

Table S2. French and Australian *L. rigidum* field populations selected for gene expression measurements.

Table S3. Primer sequences used for qRT-PCR relative quantification of gene expression in *L. rigidum*.

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TABLES

Table 1. Results from de novo assembly of *Lolium rigidum* cDNA reference transcriptome
using 454 pyrosequencing.

	Lolium rigidum cDNA Reference Transcriptome
Total reads	1,069,238
Average read length	448
Total bp sequenced	405,875,904
No. contigs >100 bp	19,623
No. contigs >500 bp	12,450
Average contig size	1,049
N50 contig size	1,150
No. bp in reference	15,040,525
bp >Q40	97.43%
No. contigs with Pfam annotation	11,126
No. contigs with UniProtKB annotation	14,619

Table 2. Identification of differentially expressed contigs between diclofop resistant (R) and susceptible (S) *L. rigidum* using RNA-Seq, followed by forward genetics and physiological validation of transcriptional markers. Fold change in FPKM (fragments per thousand bases per million reads) and fold change in qRT-PCR relative gene expression validation of RNA-Seq data, calculated using the $2^{-\Delta Ct}$ method of Schmittgen & Livak (2008). For validation experiments, fold change in $2^{-\Delta Ct}$ between F_2-R and F_2-S untreated samples, and fold-change in $2^{-\Delta Ct}$ between 24 h after 2,4-D treatment and untreated. Control untreated (n), and diclofop treated (t). Nitronate monooxygenase (NMO), Glutathione transferase (GST), transcription factor (TF). *P*-value of < 0.05, 0.01, 0.001, and 0.0001 indicated by *, **, ***, and ****, respectively, from DESeq analysis (FPKM data) or GenePattern analysis (F_2 and 2,4-D data). Fold change of 1 indicates no change, negative indicates down-regulation; italic font indicates qRT-PCR did not confirm RNA-Seq data.

| Contig | Pfam Annotation | Fold Change: RNA-Seq and qRT-PCR Validation | | | | | | Validation Experiments $2^{-\Delta Ct}$ Fold Change | |
| | | R_n/S_n | | R_t/R_n | | S_t/S_n | | | |
		FPKM	$2^{-\Delta Ct}$	FPKM	$2^{-\Delta Ct}$	FPKM	$2^{-\Delta Ct}$	$\dfrac{F_2\text{-}R_n}{F_2\text{-}S_n}$	$\dfrac{2,4\text{-}D}{n}$
01604	CytP450, CYP72A	4^{*}	4	2	2	10^{****}	12	2^{*}	32^{***}
02218	CytP450, CYP72A	9^{***}	3	1	2	12^{****}	6	2^{**}	9^{***}
05345	NMO	13^{**}	9	1	1	5^{***}	5	5^{**}	34^{****}
03513	Glucosyltransferase	7^{***}	6	1	1	7^{***}	3	8^{**}	11^{***}
05390	GST, Tau class	7^{****}	5	1	1	4^{**}	2	5^{**}	3^{**}
16302	GST, Phi class	6^{*}	2	1	4	2	11	3^{**}	12^{****}
13326	GST, Tau class	7^{**}	>50	1	2	4^{*}	35	8^{**}	14^{*}
00035	Unknown	24^{****}	>50	1	1	2	2	6^{**}	1
06783	CytP450, CYP716A	4^{*}	5	2	1	1	1	2^{*}	1
04546	GST, Tau class	9^{*}	>50	1	1	2	1	1	7^{*}
08676	GST, Phi class	3^{**}	*1*	1	1	2	1		
07629	Cellulose synthase	3^{**}	*1*	1	1	3^{**}	2		
00952	Protein kinase	13^{**}	>50	1	1	1	1	-2	1
00769	Protein kinase	-13^{****}	>-50	1	1	1	1	-2	-2
00870	Protein kinase	-9^{***}	-17	1	1	1	1	1	1
02923	Protein kinase	7^{****}	2	1	1	1	1	2	1
05711	Protein kinase	-10^{****}	-11	1	1	1	1	1	1
01201	Phosphatase	22^{****}	>50	1	1	1	2	1	1
07659	CytP450, CYP89A	2	12	1	-4	1	1	-5	1
12788	CytP450, CYP71B	5	>50	1	5	1	1	2	2
00585	Unknown	40^{***}	>50	1	1	2	3	1	1
10549	Zn finger TF	85^{*}	>50	1	1	1	1	1	1
01549	Heat shock protein	2^{*}	2	1	2	2	3	1	1
06084	2OG-Fe(II) oxygenase	43^{*}	>50	4	1	1	2	-2	-5
03180	*O*-methyltransferase	23^{*}	>50	1	1	1	1	-2	-6
12184	Cytochrome b5	8^{***}	*1*	1	1	2	1		
06148	ABC transporter	-20^{***}	-6	1	1	1	1	-2	1
05157	Cold acclimation protein	4^{***}	*1*	1	1	1	1		

Table 3. French and Australian *L. rigidum* field populations with metabolic resistance (R) to diclofop have up-regulation of multiple genes identified through RNA-Seq transcriptome analysis. Fold change in qRT-PCR relative gene expression, calculated using the $2^{-\Delta Ct}$ method of Schmittgen & Livak (2008), relative to herbicide susceptible (S) reference populations (Australian VLR1 for French, SVLR1 for Australian). Contigs CYP1 (01604, CytP450, CYP72A), CYP2 (02218, CytP450, CYP72A), NMO (05345, nitronate monooxygenase), GT (03513, Glucosyl transferase), GST1 (05390, GST, Tau class), GST2 (16302, GST, Phi class), and GST3 (13326, GST, Tau class). Fold change of 1 indicates no change, negative indicates down regulation. *P*-value of < 0.05, 0.01, 0.001, and 0.0001 indicated by [*], [**], [***], and [****], respectively, from GenePattern analysis.

Population	Type	n	Fold Change Relative to Susceptible						
			CYP1	CYP2	NMO	GT	GST1	GST2	GST3
VLR1	S	5	--	--	--	--	--	--	--
French									
FRA10136	S	6	2	1	2	14[*]	3	9[*]	-20
FRA05094	R	4	4[*]	6[*]	2[*]	1	-4	1	-180
FRA06065	R	6	5[*]	2	2[*]	60[***]	2	7	-20
FRA07061	R	6	7[*]	6[***]	4[**]	174[**]	9[***]	47	1
FRA07075	R	6	5[*]	7[*]	5[*]	60[**]	3[*]	14[*]	2
FRA07150	R	5	5	3[*]	3	16[**]	7[*]	18[*]	-16
FRA08169	R	6	23[**]	14[***]	30[*]	292[**]	14[*]	64[*]	20[*]
FRA09066	R	6	9[***]	4[***]	6[*]	107[**]	18[**]	26[*]	1
FRA10036	R	6	3[*]	2[*]	6[**]	25[**]	6[**]	17[*]	-3
Australian									
SVLR1	S	5	--	--	--	--	--	--	--
WALR1	S	5	2	1	1	1	-5[**]	-3[*]	3
S-SLR31	S	5	2[*]	1	1	3[**]	1	-3	-4
SLR31-P450	R	6	8[**]	2	5[****]	11[**]	2[*]	1	26[**]

FIGURE LEGENDS

Figure 1. Diclofop metabolism in wheat. The pro-herbicide diclofop-methyl is hydrolyzed by esterases to the phytotoxic diclofop acid. Cytochrome P450 monooxygenase catalyzes aryl hydroxylation, producing a non-phytotoxic metabolite; this reaction is not reversible and occurs rapidly in wheat. Rapid glucose conjugation by glucosyl transferase produces the aryl-*O*-glucoside conjugate of aryl-hydroxylated diclofop. (Adapted from Shimabukuro et al., 1979; Shimabukuro et al., 1987; Zimmerlin & Durst, 1992; Helvig et al., 1996; Werck-Reichhart et al., 2000)

Figure 2. Heat map of average FPKM (fragments per thousand bases per million reads) values from RNA-Seq experiment for 28 contigs selected for qRT-PCR validation. Resistant (R), Susceptible (S), control untreated (n), adjuvant only control (a), and diclofop treatment (t). FPKM scaled from 0 (black) to ≥85 (red). Photos of R (top) and S (bottom) 40 d after treatment, showing untreated, adjuvant only control, and diclofop treatment effects.

Figure 3. Expression levels of nine contigs differentiate *L. rigidum* individuals with metabolic resistance (R) to diclofop from diclofop-susceptible (S) individuals. Principal component analysis using R and S FPKM data from the RNASeq experiment (untreated, A; diclofop-treated, B) and $2^{-\Delta Ct}$ measured from nine F2-R (r), seven F2-S (s) (untreated, C; diclofop-treated, D). Control untreated (n) and diclofop treated (t). Dashed line circles enclose all S samples. Contigs 1 (01604, CytP450, CYP72A), 2 (02218, CytP450, CYP72A), 3 (05345, nitronate monooxygenase), 4 (03513, glucosyl transferase), 5 (05390, GST, Tau class), 6 (16302, GST, Phi class), 7 (13326, GST, Tau class), 8 (00035, unknown function), and 9 (06783, CytP450, CYP716A).