On the discovery of genes involved in metabolism-based resistance to herbicides using RNA-Seq transcriptome analysis in *Lolium rigidum*

Zur Entdeckung der beteiligten Gene an der metabolischen Herbizidresistenz in Lolium rigidum durch RNA-Seq Transkriptom Analyse.

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Abstract

Weed control failures due to herbicide resistance are an increasing and worldwide problem significantly impacting crop yields. Herbicide resistance due to increased herbicide metabolism in weeds is not well characterized at the genetic level. An RNA-Seq transcriptome analysis was used to identify genes conferring metabolism-based herbicide resistance (MBHR) in a population (R) of a major global weed (*Lolium rigidum*), in which resistance to the herbicide diclofop-methyl was experimentally evolved through recurrent selection from a susceptible (S) progenitor population. A reference transcriptome of 19,623 contigs was assembled using 454 sequencing technology on a cDNA library and annotated using UniProt and Pfam databases. Transcriptomic-level gene expression was measured using Illumina 100 bp reads from untreated control, mock, and diclofop-methyl treatments of R and S. Due to the established importance of cytochrome P450 (CytP450), glutathione-S-transferase (GST), and glucosyltransferase (GT) genes in MBHR, 11 contigs with these annotations and higher constitutive expression in untreated R than in untreated S were selected as candidate genes for hypothesis testing, along with 17 additional differentially expressed contigs with annotations related to metabolism or signal transduction. In a forward genetics validation experiment, higher constitutive expression of nine contigs co-segregated with the resistance phenotype in an F_2 population, including 3 CytP450, 3 GST, and 1 GT. At least nine genes with heritable increased constitutive expression are associated with MBHR trait. In a physiological validation experiment where 2, 4-D pre-treatment induced diclofop-methyl protection in S individuals due to increased metabolism, seven of the nine genetically-validated contigs were significantly induced. These data help explain accumulation of resistance-endowing genes and rapid evolution of MBHR, and provide the opportunity to improve diagnostics of MBHR using molecular tools such as transcriptional markers.

Keywords: 2,4-Dichlorophenoxyacetic acid (2,4-D), diclofop-methyl, evolution, herbicide metabolism, herbicide resistance, next-generation sequencing, transcriptional markers

Zusammenfassung

Herbizidresistenz ist weltweit ein zunehmendes Problem in der Landwirtschaft, vor allem der enzymatische Abbau von Wirkstoffen bzw. die metabolische Herbizidresistenz (MBHR) ist bislang noch weitgehend unbekannt, besonders auf genetischer Ebene. Um die an einer MBHR beteiligten Gene zu identifizieren wurde das Transkriptom von Herbizid-resistenten und sensitiven Weidelgräsern (*Lolium rigidum*) in einem RNA-Seq-Ansatz verglichen. Die verwendete Diclofop-Methyl herbizidresistente Population wurde experimentell aus einer sensitiven Population rekurrent selektiert. Ein 19623 Contig umfassendes Referenztranskriptom wurde aus einer cDNA Bibliothek der resistenten Pflanzen durch 454 Sequenzierung erstellt und mit Hilfe von UniProt und Pfam annotiert. Mit 100 bp Illumina-Reads wurde die Genexpression in unbehandelten, mit Blindformulierung und mit formuliertem Herbizid Diclofop-methyl behandelten resistenten und sensitiven Pflanzen untersucht. Es ist bekannt, dass Cytochrom P450 (CytP450), Glutathion-S-transferasen (GST) oder Glycosyltransferasen (GT) eine wichtige Rolle in der MBHR spielen. Elf Contigs, die als solche annotiert und in unbehandelten resistenten Pflanzen konstitutiv höher exprimiert sind als in Sensitiven, wurden als Kandidatengene ausgewählt und getestet. Zusätzlich wurden 17 weitere Contigs untersucht, die zwischen resistenten und sensitiven Pflanzen unterschiedlich exprimiert und durch ihre Annotation in Metabolismus oder Signaltransduktion eingebunden sind. Eine höhere konstitutive Expression konnte in neun selektierten Contigs (u.a. 3 CytP450, 3 GST, 1 GT) auch in der F2 Generation bestätigt werden, cosegregierend mit den resistenten Phänotypen. Daher ist die Expression von mindestens neun Genen mit metabolischer Diclofop-

methyl Herbizidresistenz gekoppelt und vererbbar. Eine Vorbehandlung sensitiver Weidelgras-Pflanzen mit 2,4-D induziert metabolische Resistenz gegenüber dem ACCase Inhibitor Diclofop-methyl. Dies wurde verwendet, um die Expression der 28 selektierten Contigs zusätzlich physiologisch zu validieren. Sieben der neun zuvor beschriebenen Contigs sind in der physiologischen Validierung auch in sensitiven Pflanzen durch 2, 4-D induziert. Diese Ergebnisse bieten neue Diagnosemöglichkeiten und verdeutlichen den Zusammenhang zwischen einer vererbbaren Expressionsregulierung von Resistenz vermittelnden Genen und der schnellen Entwicklung von MBHR.

Stichwörter: 2,4-Dichlorophenoxyessig Säure (2, 4-D), Diclofop-Methyl, Evolution, Herbizidmetabolisierung, Herbizidresistenz, Next-Generation Sequenzierung, transkriptionelle Marker, Transkriptomic

Introduction

Herbicides are major tools to control weeds and weed control failure caused by herbicide resistance is an increasing and worldwide problem (HEAP, 2013). The evolution of herbicide resistance involved several mechanisms and has rapidly occurred when large and genetically variable weed populations have been subjected to intensive herbicide selection (POWLES AND YU, 2010). Target-site based herbicide resistance mechanisms confer resistance only to a selecting herbicide chemistry/mode-of-action. In contrast metabolism-based herbicide resistance (MBHR) mechanisms can induce resistance across diverse herbicide chemical classes having different modes-of-action. Several studies in *L. rigidum* have shown that 1) herbicide cross-resistance involves enhanced herbicide metabolism (PRESTON, 2004), 2) MBHR can be selected through several generations by the use of recurrent low rate of herbicides, in particular the acetyl-coA carboxylase (ACCase) inhibitor diclofop-methyl (DFM; NEVE AND POWLES, 2005), 3) cytochrome P450 monooxygenases (CytP450) might be involved since inhibitors like malathion can reverse MBHR (BUSI *et al.*, 2013), 4) several loci are involved in MBHR (BUSI *et al.*, 2013). Therefore MBHR is a heritable trait under the control of several genes involving probably several *CytP450* genes (largest gene family in plants) and other unknown relevant genes.

Next-generation sequencing technologies provide many advantages for quantitative transcriptome-wide gene expression analyses (MOROZOVA and MARRA, 2008) and has the great advantage to 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. Long length reads (400-500 bp) obtained using the 454 pyrosequencing technology are useful for generating *de novo* reference transcriptomes (PENG *et al.*, 2010; RIGGINS *et al.*, 2010). Illumina HiSeq technology provides shorter reads and higher coverage, and is useful for the so-called RNA-Seq that enables transcript quantification even for genes expressed at a low rate (LISTER *et al.*, 2009). Using RNA-Seq transcriptome analysis of *L. rigidum* and genetic and physiological validation experiments, our objective was to identify and validate specific genes expressed in plants showing MBHR.

Material and Methods

The R population used for the reference transcriptome and for the RNA-Seq expression quantification experiment was reported by NEVE and POWLES (2005) and produced by recurrent selection at initially low doses of DFM. The Australian S population used in all studies was VLR1, also described for DFM response by NEVE and POWLES (2005). The R population was selected from its progenitor S population over three generations of recurrent selection (NEVE and POWLES, 2005). This provided a much higher level of similarity in genetic background 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 MBHR traits of interest (VILA-AIUB *et al.*, 2011). Individuals were vegetatively cloned by separating tillers and transplanting into separate pots. All plants were grown and treatments performed as described in GAINES *et al.* (submitted, Plant J.).

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}) DFM dose, lacking any ACCase

or ALS target site resistance mutations (measured using a pyrosequencing assay), and having increased DFM metabolism relative to S (measured through an in-vivo ^{14}C-DFM assay) (BEFFA *et al.*, 2012; YU *et al.*, 2013). Plant preparation, RNA extraction and cDNA library was performed as described in GAINES *et al.* (submitted). 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 ACCase and ALS genotyping and DFM metabolism. S individuals were selected on the basis of not surviving a 1/2× (188 g ha^{-1}) DFM dose and having low DFM 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, surfactant (mock) only, and 1× (375 g ha^{-1}) DFM treatments. Leaf samples for the untreated control were collected at time point 0, and leaf samples from the adjuvant only control and DFM 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, followed by read processing and analyses as described in detail (GAINES *et al.*, submitted).

Candidate MBHR Contig Selection and Relative Gene Expression Quantification

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 PCR quantification of gene expression (GAINES *et al.*, submitted).

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 DFM 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× (188 g ha^{-1}) and 1× (375 g ha^{-1}) DFM. Individuals were classified as susceptible (F_2-S) if they did not survive the 1/2× treatment, and individuals were classified as resistant (F_2-R) if they survived the 1× 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 (GAINES *et al.*, submitted). Hierarchical clustering was conducted using Genedata Analyst (Genedata AG, Basel, Switzerland) with normalized qRT-PCR data (subtract contig $2^{-\Delta Ct}$ mean and divide by contig standard error), Euclidean distance, and complete linkage.

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 DFM-treatment was used to induce protection against DFM 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 from the control and from the

2,4-D treatment (24 HAT). RNA extraction and relative expression quantification were performed as previously described using qRT-PCR. The experiment included seven biological replications.

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 cloned tissues from a single R individual in both untreated and DFM-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. 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 (less than 0.01% chance of error). These contigs were annotated using both UniProtKB (Uniprot Consortium, 2012) and Pfam (PUNTA *et al.*, 2012) databases with a cutoff value of $E \leq 1\times10^{-4}$, 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.

Quantitative Rt-PCR and Forward Genetic Validation

RNA-Seq analyses showed differential expression (DE) between R and S in all three treatments (untreated, mock, and DFM) for 278 contigs (GAINES *et al.*, submitted). Selected contigs included CytP450s and other contigs with oxidoreductase Gene Ontology molecular function, GSTs, GTs, and ABC transporters (Tab. 1). Any CytP450 contigs with >2-fold higher expression 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 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 annotations. DE was confirmed for 24 of them using quantitative real-time Reverse Transcription Polymerase Chain Reaction (qRT-PCR). All selected contigs showed significant DE in RNA-Seq between R and S in the untreated condition, and 7 contigs were induced by DFM treatment in S (Tab. 2). qRT-PCR results were normalized as $2^{-\Delta Ct}$ using the method of SCHMITTGEN and 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 DE from the RNA-Seq experiment was validated by qRT-PCR for 24 of the 28 selected contigs (Tab. 1).

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 DFM resistance with multi-genic inheritance Expression of the 24 contigs was evaluated in untreated clones of the seven F_2-S and nine F_2-R individuals. Within the 24 contigs with RNA-Seq results validated by qRT-PCR, 9 contigs had significant differences between F_2-R and F_2-S (Tab. 1). None of the 9 contigs were highly expressed in any F_2-S individuals. These contigs included three CytP450s, a GT (UDP GT 73C6), three GSTs, a nitronate monooxygenase (NMO), and a contig with unknown function.

Tab. 1 Identification of differentially expressed contigs between diclofop-methyl 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-ΔCt method of Schmittgen and Livak (2008). For validation experiments, fold change in 2-ΔCt between F2-R and F2-S untreated samples, and fold-change in 2-ΔCt between 24 h after 2,4-D treatment and untreated. Control untreated (n), and diclofop-methyl 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 (F2 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.

Tab.1 *Unterschiedlich exprimierte Contigs zwischen Diclofop-methyl resistenten (R) and susceptiblen (S) L. rigidum in der RNA-Seq Transkriptom Analyse, in der Validierung durch „Forward Genetic" sowie in der physiologischen Validierung. Unterschied (x-facher Unterschied) zwischen den FPKM-Werten (Fragmente pro Tausend-Basen pro Millionen Reads) und Unterschied in der Validierung der RNA-Seq Daten durch die relative Gen Expression überprüft durch qRT-PCR, berechnet durch die $2^{-\Delta Ct}$ Methode von Schmittgen und Livak (2008). In den Validierungsexperimenten: Unterschied in $2^{-\Delta Ct}$ zwischen unbehandelten F2-R und F2-S Proben, sowie Unterschied in $2^{-\Delta Ct}$ 24 h nach 2, 4-D Behandlung zwischen unbehandelten und mit 2, 4-D behandelten Proben. Kontrolle unbehandelt (n), und Diclofop-Methyl behandelten (t). Nitronat Monooxygenase (NMO), Glutathion-S-Transferase (GST), Transkriptions Faktor (TF). P-Wert < 0.05 (*), 0.01 (**), 0.001 (***) und 0.0001 (****) durch DESeq Analyse (FPKM Daten) oder GenePattern Analyse (F_2 und 2,4-D Daten). Ein 1-facher Unterschied zeigt gleiche Expression, negative Werte zeigen eine niedrigere Expression an, qRT-PCR Werte die die RNA-Seq Daten nicht bestätigen sind in kursiver Schrift dargestellt.*

	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		$\frac{F_2\text{-}R_n}{F_2\text{-}S_n}$	2,4-D $\frac{}{n}$
Pfam Protein Family	FPKM	$2^{-\Delta Ct}$	FPKM	$2^{-\Delta Ct}$	FPKM	$2^{-\Delta Ct}$		
CytP450, CYP72A	4*	4	2	2	10****	12	2*	32***
CytP450, CYP72A	9***	3	1	2	12****	6	2**	9***
NMO	13**	9	1	1	5***	5	5**	34****
Glucosyltransferase	7***	6	1	1	7***	3	8**	11***
GST, Tau class	7****	5	1	1	4**	2	5**	3**
GST, Phi class	6*	2	1	4	2	11	3**	12****
GST, Tau class	7**	>50	1	2	4*	35	8**	14*
Unknown	24****	>50	1	1	2	2	6**	1
CytP450, CYP716A	4*	5	2	1	1	1	2*	1
GST, Tau class	9*	>50	1	1	2	1	1	7*
GST, Phi class	3**	*1*	1	1	2	1		
Cellulose synthase	3**	*1*	1	1	3**	2		
Protein kinase	13**	>50	1	1	1	1	-2	1
Protein kinase	-13****	>-50	1	1	1	1	-2	-2
Protein kinase	-9***	-17	1	1	1	1	1	1
Protein kinase	7****	2	1	1	1	1	2	1
Protein kinase	-10****	-11	1	1	1	1	1	1
Phosphatase	22****	>50	1	1	1	2	1	1
CytP450, CYP89A	2	12	1	-4	1	1	-5	1
CytP450, CYP71B	5	>50	1	5	1	1	2	2
Unknown	40***	>50	1	1	2	3	1	1
Zn finger TF	85*	>50	1	1	1	1	1	1
Heat shock protein	2*	2	1	2	2	3	1	1
2OG-Fe(II) oxygenase	43*	>50	4	1	1	2	-2	-5
O-methyltransferase	23*	>50	1	1	1	1	-2	-6
Cytochrome b5	8***	*1*	1	1	2	1		
ABC transporter	-20***	-6	1	1	1	1	-2	1
Cold acclimation protein	4***	*1*	1	1	1	1		

Physiological validation

We have established that protection against DFM phytotoxicity due to increased DFM metabolism is inducible by pre-treatment with the auxin mimic 2,4-dichlorophenoxyacetic acid (2,4-D) in the S *L. rigidum* population used in our studies (HAN *et al.*, 2013). The 2,4-D induced DFM protected phenotype is similar to that observed in the R population, and due to the inducible nature, is 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 (Tab. 1), including two CytP450s, NMO, GT, and four GSTs. Two contigs associated with resistance in the F_2 were not induced by 2,4-D, including the CYP716A (Tab. 1).

Discussion

Twenty-eight candidate contigs, selected based on a global RNA-Seq transcriptome analysis of MBHR *L. rigidum*, were subjected to two validation experiments. Seven contigs (two CYP72A, one NMO, one GT, 3 GST) were linked to the MBHR response in both experiments, using forward genetics, 2,4-D induced protection against DFM

Further evaluation using heterologous expression in yeast (CytP450s) and bacteria (others), or ideally transgenic expression in *Lolium*, is necessary to define the biochemical roles in DFM metabolism of the two CYP72A, NMO, three GST (two Tau class and one Phi class), and GT (UDP-GT 73C6) genes found in this study.

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 layers of regulation, such as protein translation regulation by long non-coding antisense RNA (CARRIERI *et al.*, 2012), 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 MBHR *L. rigidum* population. To our knowledge, systematic study of endogenous genes involved in herbicide metabolic resistance was so far not been reported. 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.

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. Our results also highlight the importance of hypothesis testing using forward genetics and physiological experiments for candidate genes identified by RNA-Seq.

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