RESEARCH NOTE

Preliminary investigation of glyphosate resistance mechanism in giant ragweed using transcriptome analysis [version 1; peer review: 1 approved with reservations, 1 not approved]

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Abstract

Giant ragweed (*Ambrosia trifida*) is a highly competitive annual weed prevalent mainly in the United States across the eastern Corn Belt. Glyphosate has been a key herbicide to help tackle the spread of giant ragweed in the past few decades. Recently, there have been reports of widespread resistance to glyphosate in giant ragweed, with the mechanism of resistance yet to be determined. We designed a single-replicate RNA sequencing experiment to study the genes differentially expressed between glyphosate-resistant and glyphosate-sensitive biotypes of giant ragweed. We used a *de novo* assembly of the giant ragweed transcriptome to determine key marker genes that could help explain the mechanism of resistance.

Keywords

Herbicide resistance , giant ragweed , glyphosate , RNA-Seq

Open Peer Review

Approval Status

	1	2
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Any reports and responses or comments on the article can be found at the end of the article.

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Introduction

Giant ragweed (*Ambrosia trifida*) is a problematic annual weed in the United States and Canada, particularly in fields where corn and soybean are grown^{1,2}. Due to the extensive use of weed control measures such as the growth of genetically-modified glyphosate-resistant crops, giant ragweed populations have been kept in check³. But due to the overuse of glyphosate and strong selective pressure, resistant weeds have been reported across the world (<http://www.weedscience.org>)^{4,5}. The mechanism of resistance to glyphosate in other common weeds such as Malaysian goosegrass, Italian ryegrass, and rigid ryegrass have been identified⁶⁻⁸. However, the glyphosate resistance mechanism in giant ragweed is still unknown. In this study, we compare gene expression differences between the glyphosate resistant and sensitive giant ragweed plants using a time course experiment and identify genes that could be involved in glyphosate resistance.

Methods

Glyphosate-resistant and glyphosate-sensitive seeds of giant ragweed were collected from Noble County, Indiana (N41° 28.470 W85° 29.371) and Darke County, Ohio (N40° 15.9 W84° 42.7) respectively. After seed germination, plants at the five-node growth stage were selected for herbicide treatment. mRNA was extracted from leaf disks following a protocol adapted from Eggermont *et al.* 2 cm diameter leaf disks from the first fully developed leaf were punched out, frozen in liquid nitrogen, and total RNA was extracted in a 2 ml test tube. mRNA was sequenced using Illumina TruSeq for four time points – pre-treatment (0 hour), 3 hours, 8 hours and 12 hours after treatment with glyphosate at a rate of 0.7kg ae ha⁻¹ (recommended field rate) sprayed using a compressed-air bench top track sprayer with a nozzle pressure of 249 kPa delivering a volume of 187 L of spray solution ha⁻¹¹⁹. RNA sequences were assembled using the Trinity package (version r2012-10-05) from paired-end reads¹⁰.

RNA-seq reads were mapped to the transcriptome assembly, and the counts per million transcripts (CPM) value was determined using RSEM (version 1.2.8)¹¹. Since we observed clear systemic changes in gene expression even at the first time point, we used a set of genes previously published in rice analyses as controls to normalize the expression values¹². The consistent expression of these genes with respect to each other across the time course was verified (Table 1). Gene level counts that were less than 1 CPM in all time points were excluded from further analysis. Expression ratios were then calculated for each assembly, comparing the expression levels in the glyphosate resistant and sensitive strains at each time point. Numbers larger than 1 therefore reflect genes (transcript assemblies) with higher expression in the resistant variety. Sampling variation was controlled by the addition of a pseudo count of 0.5 CPM before calculating expression ratios. Assemblies with expression

ratios greater than 4, or less than -4 were considered to be differentially expressed and were further examined. Annotations of the transcriptome were done using Trinotate (version r2013-02-25)¹³.

Results

There is a clear difference in gene expression patterns between the resistant and sensitive plants even before plants were treated with herbicide (Table 2). The top differentially expressed transcripts in resistant and sensitive plants before treatment are shown in Table 3 and Table 4. The response to glyphosate is rapid, and a large number of genes are significantly differentially expressed within the first three hours after treatment compared to pre-treatment expression levels (Table 5).

The genes with at least a four-fold change in expression level were identified in resistant and sensitive plants, and pathways associated with significantly over-represented genes identified using agriGO (cutoff $P < 1e-7$)^{14,15}. Pathways with terms such as “response to other organisms” and “lipid biosynthetic process”, both of which are known to be related to pathogen response, were the most significantly over-represented¹⁶. Contrastingly, pathways that are typically over-represented in the sensitive biotype are annotated with terms like “response to stress”, “response to oxidative stimulus” and “lignin biosynthesis”, which are known stress response indicators¹⁷. This leads us to speculate that, not only do resistant giant ragweed plants react to glyphosate treatment in a manner resembling pathogen defense reactions, but they are already primed by alterations in stress response processes to hyper-react. This is consistent with the rapid necrosis reaction observed in resistant giant ragweed biotypes used in this study.

Conclusion

The complete transcriptome assembly of giant ragweed has been deposited in the NCBI BioProject database (<http://www.ncbi.nlm.nih.gov/bioproject/>) and is publicly available under accession PRJNA267208. The preliminary time-course experiment presented here identified groups of genes that may explain glyphosate resistance in giant ragweed. A more extensive transcriptome analysis study, with multiple replicates of sensitive and resistant giant ragweed biotypes, from a broader range of geographic sources, and with shorter time intervals will be useful to overcome the limitations of this preliminary study.

Dataset 1. Raw data of glyphosate resistance mechanism in giant ragweed using transcriptome analysis

<http://dx.doi.org/10.5256/f1000research.8932.d125530>

This dataset contains two data files (csv) and a text file providing description of the data.

Table 1. Normalization of expression values using control genes from rice. 12 genes from the list of 25 genes identified by Jain (2009) showed relatively stable expression across all time points, and thus were used for determining the scaling factor for the normalization¹².

	Initial average expression (CPM)								Scaled Expression values							
NCBI Gene ID	R0	R3	R8	R12	S0	S3	S8	S12	R0	R3	R8	R12	S0	S3	S8	S12
LOC_Os07g34589	92.97	130.37	153.91	138.51	90.76	78.69	121.71	142.69	1.000	1.402	1.655	1.490	1.000	0.867	1.341	1.572
NM_001065286	182.57	244.85	103.47	136.94	175.15	133.20	202.35	240.41	1.000	1.341	0.567	0.750	1.000	0.760	1.155	1.373
LOC_Os04g35910	10.90	13.04	19.94	20.00	9.49	14.38	19.88	21.08	1.000	1.196	1.829	1.835	1.000	1.515	2.095	2.221
LOC_Os01g05490	87.30	100.14	33.86	25.81	98.41	68.50	73.38	62.60	1.000	1.147	0.388	0.296	1.000	0.696	0.746	0.636
LOC_Os08g03290	519.16	487.42	235.41	209.67	749.70	664.05	768.51	622.39	1.000	0.939	0.453	0.404	1.000	0.886	1.025	0.830
LOC_Os01g70780	33.59	32.51	41.98	31.06	51.52	38.63	52.90	64.43	1.000	0.968	1.250	0.925	1.000	0.750	1.027	1.251
LOC_Os07g11290	5.84	7.65	10.93	9.83	4.00	5.96	15.40	11.38	1.000	1.310	1.872	1.683	1.000	1.490	3.850	2.845
LOC_Os04g53620	1147.96	1395.11	1766.81	1772.33	1549.44	1577.01	1345.80	929.58	1.000	1.215	1.539	1.544	1.000	1.018	0.869	0.600
LOC_Os08g03390	53.36	57.83	58.61	56.47	50.29	56.69	95.99	86.41	1.000	1.084	1.098	1.058	1.000	1.127	1.909	1.718
NM_001057599	9.88	8.04	2.23	2.70	6.07	5.46	6.15	6.71	1.000	0.814	0.226	0.273	1.000	0.900	1.013	1.105
LOC_Os08g12750	7.29	7.00	5.08	7.51	9.30	10.57	14.39	15.06	1.000	0.960	0.697	1.030	1.000	1.137	1.547	1.619
LOC_Os04g51370	13.88	17.12	20.60	21.42	11.35	15.18	10.89	16.06	1.000	1.233	1.484	1.543	1.000	1.337	0.959	1.415
Final Scale Factor									1.000	1.134	1.088	1.069	1.000	1.040	1.461	1.432

Table 2. Gene expression differences between resistant and sensitive biotypes of giant ragweed before treatment with glyphosate. The number of genes that are expressed > 4-fold higher in glyphosate-resistant giant ragweed (Resistant +) or > 4-fold higher in glyphosate-sensitive giant ragweed (Sensitive +) are shown.

Pre-treatment	
Resistant +	318
=	35079
Sensitive +	70

Table 3. Genes with greater than four-fold higher expression in resistant plants compared to sensitive plants. Genes expressed higher in resistant plants tend to play important roles in pathogen response regulation.

Trinity transcript identifier	Gene Annotation	GO Annotation	Expression ratio R/S
comp148939_c0	Glycosyl hydrolase superfamily protein	GO:0009725 response to hormone stimulus	26.846
comp166081_c1	Alpha/beta-hydrolases superfamily protein	GO:0005515 protein binding	26.344
comp149865_c0	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	GO:0012505 endomembrane system	7.665
comp142951_c0	Lipid transfer protein 12	GO:0008289 lipid binding	6.532
comp159731_c0	Glutathione S-transferase family protein	GO:0006457 protein folding	4.906
comp167561_c0	Protein kinase superfamily protein	GO:0006468 protein amino acid phosphorylation	4.777
comp158185_c0	Ethylene-forming enzyme	GO:0009815 1-aminocyclopropane-1-carboxylate oxidase activity	4.581
comp171245_c0	Pleiotropic drug resistance 7	GO:0005886 plasma membrane	4.076

Table 4. Genes with greater than four-fold higher expression levels in sensitive plants compared to resistant plants. Genes expressed at a higher level in sensitive plants seem to impact control of stress response.

Trinity transcript identifier	Annotation	GO annotation	Expression ratio S/R
comp161591_c0	Metallathionein 2B	GO:0006508 proteolysis	12.926
comp165624_c0	Thioredoxin superfamily protein	GO:0009535 chloroplast thylakoid membrane	9.706
comp144176_c0	NADH-ubiquinone oxidoreductase (complex I), chain 5 protein	GO:0009507 chloroplast	7.694
comp150391_c0	Cysteine-rich domain superfamily protein	GO:0009507 chloroplast	4.972
comp165059_c0	Fe superoxide dismutase 2	GO:0019430 removal of superoxide radicals	4.702
comp163658_c0	Unknown protein involved in response to salt stress	GO:0003677 DNA binding	4.258

Table 5. Gene expression differences between resistant and sensitive biotypes of giant ragweed after treatment with glyphosate. After treatment with glyphosate, the number of differentially expressed genes increases rapidly within the first three hours, and continues to increase at later time points.

		3 hours PT			8 hours PT			12 hours PT		
		Sensitive			Sensitive			Sensitive		
		+	=	-	+	=	-	+	=	-
Resistant	+	62	550	31	101	3342	323	412	5471	329
Resistant	=	552	33020	1014	2643	26654	597	1273	25339	696
Resistant	-	18	181	39	58	1632	117	22	1710	215

+ at least four-fold higher expression level; = similar expression level; - at least four-fold lower expression level; PT post-treatment

Data availability

The transcriptome assembly is available in the NCBI BioProject database under accession PRJNA267208.

F1000Research: Dataset 1. Raw data of glyphosate resistance mechanism in giant ragweed using transcriptome analysis, [10.5256/f1000research.8932.d125530](https://doi.org/10.5256/f1000research.8932.d125530)¹⁸

Author contributions

SCW, BS and KS grew the giant ragweed plants in the greenhouse and extracted samples for RNA-seq. KRP and MG did the transcriptome assembly, annotation and expression analysis. KRP wrote the

draft of the manuscript. All authors contributed to revision and discussion of the manuscript.

Competing interests

No competing interests were disclosed.

Grant information

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Glyphosate is a widespread herbicide since 1994, when transgenic crops were commercially released into the environment. Several cases of glyphosate resistance have been reported, after repeated use of this herbicide, since then. Previous cases have included species of Poaceae or Amaranthaceae.

The manuscript by Padmanabhan and colleagues presents an interesting comparison between the transcriptomic profiles of giant ragweed plants collected from populations that are either resistant or sensitive to the use of glyphosate. This paper is relevant, because the authors investigate an agricultural weed that is not a Poaceae or Amaranthaceae. This contribution is useful to addressing if glyphosate resistance follows common metabolic pathways in distantly related plants. Moreover, they do so following an experimental set up, that can potentially be replicated and expanded; this is an additional contribution.

We summarize our main concerns on the present status of the work under review, and provide comments or suggestions that may help improve this work.

A more comprehensive transcriptomic comparison would greatly contribute to the objectives of the work under review.

1. We recommend: Transcriptomic analyses made between resistant and sensitive ragweed individuals sampled at different time points during the experiment –which the authors summarize in Tables 3 and 4 and compare on table 5- with an explicit discussion of their results in light of the transcriptomic profiles (and highly expressed genes) described for other glyphosate-resistant weeds, in order to discuss if common metabolic pathways that are not lineage specific, are being selected upon through pervasive use of glyphosate in the

corn belt in the USA, or not.

2. The paper would also benefit from a more thorough discussion concerning the type and function of genes that were found to be highly expressed in resistant and/or sensitive giant ragweed biotypes. We recommend using other databases, such as KEGG. It would be particularly important to focus on genes involved in secondary growth, lateral meristem activity, or any aspect related to perennial habit, in contrast to non-perennial habit of close relatives, if there are any. Also, comparison to functional information available in other perennial and non perennial herbs that have evolved resistance, would be very useful.
3. The fact that the genes that are highly expressed in sensitive giant ragweed are those involved with chloroplast function, which is the function that is primarily and directly affected by glyphosate, through the inhibition of aromatic aminoacid synthesis. This is not addressed by the authors aside from Table 4. Such discussion would also improve the significance of the paper.
4. In the last paragraph of the discussion, the authors propose a hypothesis concerning the highly expressed genes in resistant ragweed. The following statement made by the authors is confusing and requires clarification: "This is consistent with the rapid necrosis reaction observed in resistant giant ragweed biotypes used in this study". Necrosis is a common response to biotic and abiotic stress but this phrase could be further clarified if the authors maybe by explaining to the reader what is the main physiological response to glyphosate observed in resistant biotypes: necrosis of all leaves? Of some? Necrosis and then new leaf generation? Are any of these observed in non resistant biotypes, ever?
5. In the conclusions the authors suggest how a more thorough analysis could be carried on in the future for giant ragweed resistance. This made it clear that some details were missing in the methods section of the present paper: how many plants did they sample (two for each biotype for biological replicates; same individual plant, but two samplings for technical replicates?). These details should be clarified, please.

A useful review article that may be cited and help tackle questions pertaining the possible metabolic pathways affected by glyphosate:

The Shikimate Pathway and Aromatic Amino Acid Biosynthesis in Plants

Annual Review of Plant Biology

Vol. 63: 73-105 (Volume publication date June 2012)

DOI: 10.1146/annurev-arplant-042811-105439

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Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Reviewer Report 01 August 2016

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Roland Beffa

Weed Resistance Research Bayer AG, Frankfurt, Germany

Preliminary investigation of glyphosate resistance in giant ragweed using transcriptome analysis from Padmanabhan is a very preliminary work, and the differentially expressed genes found have to be further validated, genetically or/and functionally. No isogenic populations of sensitive and resistant glyphosate plants were used. Whereas the paper is well written and the data clearly presented, this work is a good start, but it is in a too early phase to be indexed. The article might be, from my point of view, re-focused, on the transcriptome assembly and annotation. This would be acceptable.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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