Weed Science 2010 58:109–117

WSSA
WEED SCIENCE SOCIETY OF AMERICA

Functional Genomics Analysis of Horseweed (*Conyza canadensis*) with Special Reference to the Evolution of Non–Target-Site Glyphosate Resistance

Joshua S. Yuan, Laura L. G. Abercrombie, Yongwei Cao, Matthew D. Halfhill, Xin Zhou, Yanhui Peng, Jun Hu, Murali R. Rao, Gregory R. Heck, Thomas J. Larosa, R. Douglas Sammons, Xinwang Wang, Priya Ranjan, Denita H. Johnson, Phillip A. Wadl, Brian E. Scheffler, Timothy A. Rinehart, Robert N. Trigiano, and C. Neal Stewart, Jr.*

The evolution of glyphosate resistance in weedy species places an environmentally benign herbicide in peril. The first report of a dicot plant with evolved glyphosate resistance was horseweed, which occurred in 2001. Since then, several species have evolved glyphosate resistance and genomic information about nontarget resistance mechanisms in any of them ranges from none to little. Here, we report a study combining iGentifier transcriptome analysis, cDNA sequencing, and a heterologous microarray analysis to explore potential molecular and transcriptomic mechanisms of nontarget glyphosate resistance of horseweed. The results indicate that similar molecular mechanisms might exist for nontarget herbicide resistance across multiple resistant plants from different locations, even though resistance among these resistant plants likely evolved independently and available evidence suggests resistance has evolved at least four separate times. In addition, both the microarray and sequence analyses identified non–target-site resistance candidate genes for follow-on functional genomics analysis.

Nomenclature: Glyphosate; horseweed, *Conyza canadensis* (L.) Cronq. ERICA.
Key words: Bioinformatics, herbicide resistance, phylogeography, systems biology, transcriptomics.

The development of herbicide-resistant crops has revolutionized weed control for modern agriculture because they enable the application of effective concentrations of broad-spectrum herbicides, such as glyphosate, for weed control (Duke and Powles 2008b). The adoption of glyphosate-resistant crops increased dramatically from 1996, especially in the United States, which resulted in significant increases of glyphosate application in the field (Dill 2005; Duke and Powles 2008a). However, increased herbicide applications have presumably also led to a dramatic increase of herbicide-resistant weed biotypes. More than 323 resistant biotypes have been reported in 183 weedy species (Heap 2010). Among the different herbicide-resistant cases, glyphosate resistance is a particularly important problem (Duke and Powles 2008b). Glyphosate is considered to be an environmentally benign herbicide with broad activity (Williams et al. 2000). It was originally argued that the evolution of glyphosate resistance via the requisite target-site 5-enolpyruvylshikimate-3-phosphate synthase mutation would be improbable (Bradshaw et al. 1997). Indeed, although target-site resistance is rare for glyphosate, non–target-site resistance has evolved rapidly, which was an unexpected development (Yuan et al. 2007). The debate about whether glyphosate resistance can quickly evolve ended in 2001, less than 6 yr after wide-scale deployment of transgenic glyphosate-resistant soybean, when the first case of glyphosate-resistant horseweed, the first evolved glyphosate-resistant dicot species, was reported in glyphosate-resistant soybean in Delaware (VanGessel 2001).

Various aspects of glyphosate resistance in weed biology has been a topic of increasing interest in recent years (Baucom and Mauricio 2008; Dill et al. 2008; Duke and Powles 2008a,b; Funke et al. 2009; Gressel 2009; Gressel and Valverde, 2009; Mallory-Smith and Zapiola 2008; Powles 2008; Preston and Wakelin 2008; Sammons et al. 2007; Vila-Aiub et al. 2008; and references found therein). Resistance mechanisms can be generally classified as target-site and non–target-site resistance based on the mechanisms (reviewed in Hu et al. 2009; Sammons et al. 2007; Yuan et al. 2007). Target-site resistance is endowed by a mutation in the herbicide target gene and changing the protein's proper binding of the herbicide (reviewed in Preston et al. 2009), and is therefore known to be monogenic. Non–target-site herbicide resistance is more complex and could involve several metabolic, conversion, and sequestration processes, including oxidation, conjugation, or compartmentation of the herbicide molecules (or all of these) (Yuan et al. 2007). Hypothetically, because the evolution of non–target-site resistance could be polygenic, it might be more advantageous to the weed to evolve non–target-site vs. single-gene target-site resistance. It is feasible that hybridization among non–target-site resistant plants could lead to increasingly resistant hybrids (Wakelin and Preston 2006). Because the non–target-site resistance is likely more complicated with regards to mechanism, and perhaps evolution, genomic characterization is needed to elucidate the mechanism (or mechanisms) of non–target-site resistance for different herbicides. For glyphosate resistance, non–target-site mechanisms might be the major cause for most resistant biotypes (Dill 2005; Koger and Reddy 2005; Shaner 2009; Yuan et al. 2007).

Several biotypes of glyphosate-resistant horseweed have non–target-site resistance mechanisms, and, in these biotypes, the target-site resistance mechanism is not present (Feng et al. 2004; Koger and Reddy 2005; Main et al. 2004; Mueller et al. 2003; Owen and Zelaya 2005; Zelaya et al. 2004). It is unclear if only one or multiple molecular mechanisms are responsible for resistance among biotypes. A non–target-site mechanism

DOI: 10.1614/WS-D-09-00037.1

* 1st, 5th, and 7th authors: Department of Plant Pathology and Microbiology, Institute of Plant Genomics and Microbiology, Texas A&M University, College Station, TX 77843; 2nd, 4th, 6th, 7th, 8th, 13th, and 19th authors: Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996; 4th author: Department of Biology, St. Ambrose University, Davenport, IA 52803; 3rd, 9th, 10th, and 11th authors: Monsanto Company, Saint Louis, MO 63167; 12th, 14th, 15th, and 18th authors: Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN 37996; 13th author: Environmental Science Division, Oak Ridge National Laboratory, Oak Ridge, TN; 16th author: U.S. Department of Agriculture–Agricultural Research Service–Gemonics and Bioinformatics Research Unit; Mid-South Area, Genomics Laboratory, 141 Experiment Station Rd., Stoneville, MS 38776; 17th author: U.S. Department of Agriculture–Agricultural Research Service Southern Horticultural Laboratory, 810 Highway 26 West, Poplarville, MS 39470. Corresponding author's E-mail: nealstewart@utk.edu

that is based on altered translocation has been proposed from data derived from the analysis of glyphosate transport (Feng et al. 2004; Mueller et al. 2003). Furthermore, resistance seems to be dominant or semi-dominant and of single-locus origin (Owen and Zelaya 2005), but the gene (or genes) involved in resistance are unknown. In addition, it is unknown whether all resistant horseweed biotypes from different locations have evolved independently or even share the same molecular mechanisms for the resistance. Without genomic information for weedy species, forward genetics to elucidate the evolution of weediness approaches is challenging (Basu et al. 2004). By starting to develop genomics tools in horseweed, we should begin to obtain genetic information useful for new weed management strategies (Stewart et al. 2009).

The objective of our research was to compare transcriptomic responses among biotypes and glyphosate treatments to mine genomic data for the purpose of identifying candidate nontarget resistance genes as well as to examine the phylogeographic patterns of resistance. Transcriptome analysis was performed using two methods to assess gene expression differences among glyphosate-sensitive and -resistant biotypes as well as to assess the transcriptomic effect of glyphosate treatment on a resistant biotype. Our end goal is to identify, clone, and characterize gene (or genes) responsible for non–target-site glyphosate resistance.

Material and Methods

Horseweed Accessions, Breeding, Glyphosate Treatment, and Sequence Analysis of Transcriptome. The study involved both F_2 progeny from crosses of two different biotypes and various resistant and sensitive biotypes from multiple locations across the United States and Canada. The F_2 progeny from the crosses of accessions from Delaware (DE) and California (CA)—$DE1_S \times CA1_R$ and $DE1_S \times DE3_R$—were sampled and assayed for the level of glyphosate resistance. Resistant and sensitive biotypes were crossed and progenies were scored for resistance. Plants that were at the rosette stage, approximately 3 mo old and 6 to 8 cm in diam, were treated with glyphosate (field rate: 0.84 kg ha^{-1} ae[1]) using a backpack sprayer and were subjected to transcriptome profiling with iGentifier technology originally developed by Axaron (Fischer et al. 2007). In addition, a partial horseweed expressed sequence tag (EST) library was also sequenced to serve as the reference for the iGentifier analysis. The two types of sequence data allowed both the quantification of gene expression upon the glyphosate treatment and the accumulation of transcript information for horseweed. The glyphosate-treated plants were considered to be resistant if they were living at the end of 3 wk.

Annotation of the cDNA Library. An automatic sequence annotation tool as described by Zhou et al. (2009) was used for the annotation of the cDNA library. Basically, the cDNA sequence was first assembled into unigenes. The unigene sequence space was searched against National Center for Biotechnology Information (NCBI) *Arabidopsis* REF sequences using BLASTn. The REF library is conservative for gene annotation relative to function annotation. A 10^{-6} cut off was set for the annotation and the annotation of the most similar sequence was parsed for the annotation of the cDNA sequences.

Cluster Analysis of iGentifier Sequence Tags. Cluster analysis using the counts of the sequence tags were analyzed using TIGR MEV 4.0 software.[2] Hierarchy cluster analysis using methods described above were used to deconvolute patterns and the number of the counts was represented using a heat map and color scheme ranging from blue (low) to red (high).

Cross-Platform Analysis. The cross-platform analysis tool developed by Zhou et al. (2009) was employed for the cross-platform analysis of the different methods used to collect transcriptome data. Basically, the sequence tags were searched against the unigene sequence and the exact match pattern allowed the annotation of the iGentifier sequence tag with the unigene function.

Phylogeographic Analysis Using Simple Sequence Repeat (SSR) Markers. Data from eight horseweed SSR loci described in Abercrombie et al. (2009) were compiled for 22 horseweed accessions from across the United States and Canada that represented distinct spatially isolated populations and analyzed for shared allelic frequencies. SSR–polymerase chain reaction (PCR) reactions were performed using published methods (Abercrombie et al. 2009). Gene diversity estimates were produced using Nei's 1987 estimator for heterozygosity and unbiased gene diversity per population was determined using FSTATS and CERVUS software (Goudet 1995; Saitou and Nei 1987). A dendrogram and principle components analysis (PCoA) display were produced based on Nei's minimum-genetic-distance matrix (Nei 1972). Populations version 1.2.28 was used to create dendrograms (Langella 2002). Neighbor-joining with 100 bootstrap replicates for statistical support was used to show clustering of genetically similar samples (Saitou and Nei 1987). Dendrograms were visualized with TreeView (Page 1996). PCoA analysis was performed using NTSYS software.[3]

Assay for Genes Up-Regulated by Glyphosate via Heterologous Microarray Analysis. Whole-genome mouse-ear cress [*Arabidopsis thaliana* (L.) Heynh.] oligonucleotide arrays[4] were probed with *A. thaliana* ecotype Columbia leaf-derived cDNAs (with one fluorescent dye) and horseweed leaf-derived cDNAs (with a second fluorescence dye), in a dye-swap protocol to compare cross-species hybridization (see Rao et al. 2009) for detailed microarray protocols. A second experiment was conducted to probe the response to glyphosate treatment of a single Tennessee accession of glyphosate-resistant horseweed. Growth-chamber-grown plants at the 6-cm rosette stage (three pooled samples of eight individual plants) were treated with glyphosate (field rate: 0.84 kg ha ae^{-1}) or water (control) using a backpack sprayer. Meristematic tissue and young leaves were harvested 24 h posttreatment and subjected to RNA isolation and subsequent mRNA purification using TRIZOL reagent. Pools of mRNA were directly labeled with Cy3 or Cy5 dyes using the Superscript III direct labeling kit.[5] Glyphosate-treated vs. control horseweed cDNA was hybridized to *Arabidopsis* oligonucleotide arrays as above, with three biological replicates and two technical replicates (six arrays). After hybridization and washing, slides were scanned utilizing a GenePix 4000B microarray scanner[6] and images were analyzed utilizing GenePix Pro 4.1 software.[7] Microarray data were analyzed using rank products in the Bioconductor RankProd package (Hong et al. 2006). Increased gene expression from glyphosate treatment of glyphosate-resistant horseweed is reported on a linear scale (fold-change) in Table 1.

Table 1. Microarray analysis of differentially expressed and up-regulated genes after glyphosate treatment of resistant horseweed. The most significantly ($P < 0.0001$) differently up-regulated genes with *Arabidopsis thaliana* gene ID are listed. The analysis occurred at 24 hr post–glyphosate treatment. The false discovery rate (FDR) of all these genes was less than 0.0001. FDR was calculated using a permutational analysis.

ID	Gene name	Fold change
At3g13080	ABC transporter protein family	29.64
At4g08920	Cryptochrome 1 apoprotein (CRY1)/flavin-type blue-light photoreceptor (HY4)	13.59
At1g05060	Expressed protein	7.34
At3g16340	ABC transporter protein family similar to PDR5-like ABC transporter GI:1514643 from [*Spirodela polyrhiza*]	6.66
At5g06640	Proline-rich extensin-like protein family	6.24
At3g48850	Mitochondrial phosphate transporter	5.80
At3g54580	Proline-rich extensin-like protein family	5.70
At4g15233	ABC transporter protein family	5.55
At3g47340	Glutamine-dependent asparagine synthetase 1	5.24
At4g20200	Terpene synthase/cyclase protein family 5-epi-aristolochene synthase	5.10
At5g60390	Elongation factor 1-alpha/EF-1-alpha	5.03
At3g62150	Multidrug-resistant ABC transporter	4.67
At5g40010	AAA-type ATPase protein family contains Pfam profile: ATPase family PF00004	4.66
At3g54590	Proline-rich extensin-like protein family	4.57
At5g10880	tRNA synthetase-related/tRNA ligase-related	4.53
At3g11710	Lysyl-tRNA synthetase, putative/lysine–tRNA ligase	4.23
At4g34050	Caffeoyl-CoA 3-O-methyltransferase	4.22
At5g49080	Proline-rich extensin-like family protein	4.16
At4g15160	Protease inhibitor/seed storage/lipid transfer protein family	4.12
At3g62120	tRNA synthetase class II (G, H, P, and S) protein family	4.07

Results

Progeny Analysis from Resistant and Sensitive Biotype Hybrids. The general research schema is as shown in Figure 1. $DE1_S \times CA1_R$ and $DE1_S \times DE3_R$ F_2 progenies had variable phenotypes with regard to glyphosate sensitivity, but each plant could be scored as either resistant or susceptible in all cases of glyphosate treatment. For the downstream molecular and genomic analysis, both resistant and sensitive progeny were randomly selected for the iGentifier analyses along with several other representative resistant and sensitive biotypes. Plants selected allowed for the association among molecular responses across a broad spectrum of genotypes using iGentifier analysis. Single plants representing segregating crosses or ecotypes were used as representatives in transcriptome experiments. In the instance of hybrids, several F_2 plants (selfed) that segregated for resistance were produced from F_1 hybrids

Annotation of EST Data and iGentifier Data Analysis. iGentifier is a novel sequencing technique combining the transcript fragment display (such as in differential-display PCR) with tag sequencing (e.g., massively parallel signature sequencing) to enable the high-throughput parallel sequencing of multiple samples using Sanger sequencing techniques (Fischer et al. 2007). It is a novel method using a traditional sequencing platform, which involves a short primer-based sequencing of 17-base sequence tags from cDNA pools. The technique allows a semiquantitative analysis of transcriptomes across different samples (Fischer et al. 2007). However, the interpretation of the data largely depends on genome information available for the target species. Therefore, in parallel to the iGentifier analysis, EST libraries were sequenced as the reference for annotating iGentifier data.

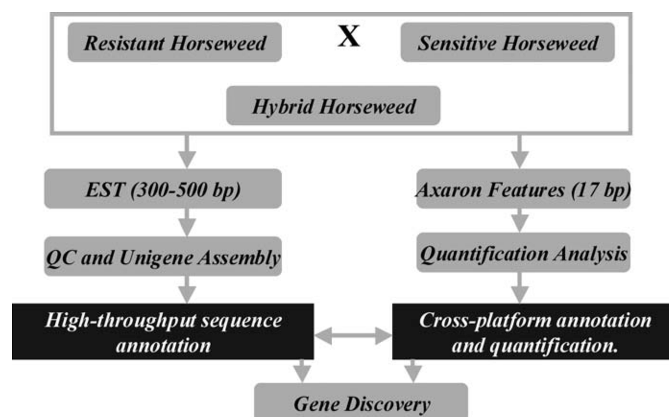

Figure 1. The schema of experimental design for sequence-based transcriptome analysis. The experiment includes two different types of sequence analysis for the transcriptome profiling, iGentifier and cDNA. The data from the two sources were analyzed with cross-platform analysis tools. For all hybrid accessions, F_2 plants segregating for resistance were analyzed.

The automatic sequence analysis and the cross-platform analysis tools were developed to analyze these sequence data as described elsewhere (Zhou et al. 2009). Preliminary analysis indicated that the direct annotation of the 17-base iGentifier sequence tag with public databases is not possible because of the short sequence length. We therefore first annotated the sequences from the EST library. A total of 4,024 EST sequences were available and were assembled into 2,786 unigenes including 2,254 singletons and 532 contigs (see online supplemental material). The unigene sequences were annotated based on sequence similarity with the REF sequence library from NCBI. We employed stringent criteria for the analysis, in which the well-annotated and characterized dicot model species *Arabidopsis* REF sequences were used for the annotation. The overview of the gene ontology annotation profile of the ESTs is displayed (Figure 2). The 17-base sequence tag can, in many cases, represent a unique sequence (gene) in the data (Figure 3). The cluster analysis can be interpreted from both sequence-tag clusters and the clustering of the samples themselves. The cluster of sequence tags shows that most sequence tags share a similar pattern among different samples, which is indicative of similar gene expression patterns among horseweed accessions under glyphosate treatment. However, we were most interested in the sequence tags that show differential expression pattern across different samples, and in particular, between resistant and sensitive biotypes. Some sequence tags had differential patterns between sensitive and resistant biotypes. It is therefore important to annotate the function of the genes represented by these sequence tags for downstream gene discovery. Generally speaking, replicates of the same sample clustered together, which indicated data robustness and reproducibility among similar samples and treatments.

Comparison of Phylogeographic Analysis and Transcriptome Analysis. The clustering of the iGentifier data by sample (Figures 3 and 4B) shows a moderate pattern of separation of resistant and sensitive biotypes, although these data are inconclusive. In particular, for the biotypes from different locations, the location effect is apparently less important than resistance as demonstrated by clustering together of some sensitive biotypes. The clustering pattern

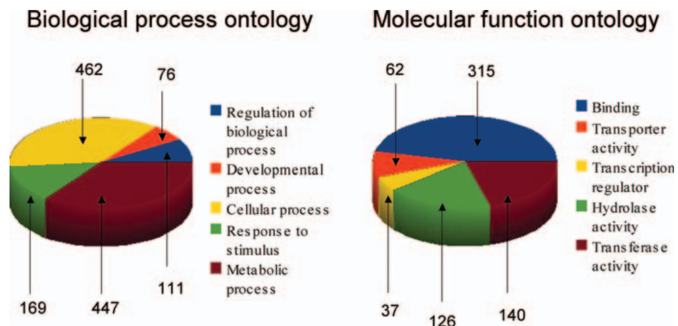

Figure 2. The distribution of gene ontology annotation of unigene sequences.

indicates that there could be similar molecular mechanisms for glyphosate resistance among accessions from different locations, although the results are not definitive. On the other hand, cluster and PCoA analyses of 22 different accessions using SSR markers (Abercrombie et al. 2009) were useful to build phylogeographic hypotheses addressing the spread of resistance and its evolution (Figures 4A and 5). Biotypes from proximate locations generally share the same clades. In addition, the PCoA analysis (Figure 5) corroborates the cluster analysis (Figure 4A) in showing that glyphosate resistance has evolved at least four different times: in Delaware, Tennessee, Ohio/Indiana, and California (Figure 5); e.g., locally adapted populations have evolved resistance and spread locally. If a single evolution event had spread via seeds, then resistant accessions would be expected to cluster together. However, DE-R clusters with the DE susceptible accessions, TN-R clusters with TN susceptible, and the nearest neighbor for CA-R is the CA sensitive accession.

Cross-Platform Annotation of iGentifier Data and Potential Genes Involved in Glyphosate Resistance. The iGentifier sequence tag cluster analysis allowed an overview of the glyphosate-induced responses in resistant and sensitive biotypes. However, the detailed understanding of molecular mechanisms of horseweed resistance to glyphosate first

Figure 3. The cluster analysis of the iGentifier data. The color in the figure represents counts of each sequence tag. Red indicates a larger number of sequence tags, hence higher transcript count (blue, fewer) in the analysis.

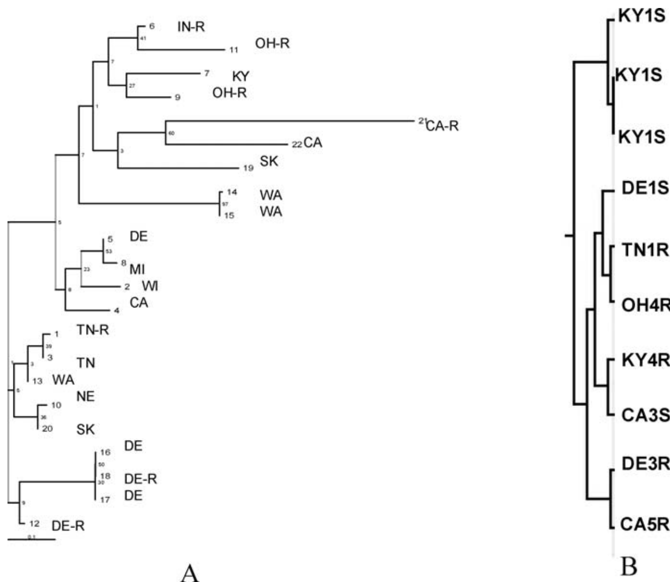

Figure 4. The comparison of phylogeography and transcriptomic cluster analyses. (A) Phylogenetic tree using Nei's minimum distance (Nei 1972) was constructed from simple-sequence-repeat horseweed data. Origins of accessions are represented by standard abbreviations of U.S. states or Canadian province followed by either R (glyphosate-resistant) or nothing, the latter indicating glyphosate susceptibility. (B) Cluster analysis of different horseweed biotypes based on the iGentifier data. R, resistant biotypes; S, sensitive biotypes.

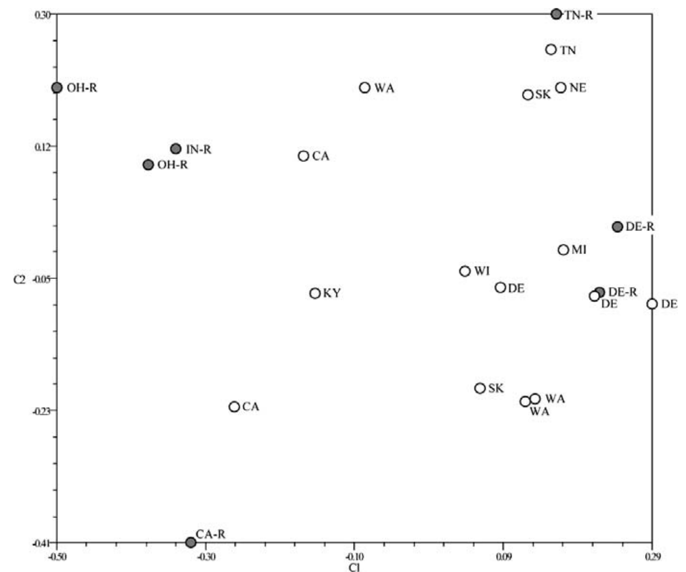

Figure 5. Phylogeographic principle components analysis using Nei's minimum distance (Nei 1972) was constructed from simple-sequence-repeat horseweed data. Origins of accessions are represented by standard abbreviations of U.S. states or Canadian province followed by either R and filled circles (glyphosate-resistant) or nothing and open circles, the latter indicating glyphosate susceptibility. The first two components, C1 and C2, represent 33.5 and 20.9%, respectively, of the observed genetic variation.

requires annotating sequence tags. The EST and unigene sequences can be used for cross-platform analysis to annotate the iGentifier sequence tags. Sequence tag searches were performed against the unigene library. A perfect sequence match allowed the annotation of sequence tag with the unigene sequence. Once a unigene was annotated, we then identified the potential gene function for the iGentifier sequence tag. Because iGentifier sequence tags consist of 17 bases of known sequence, the length should be sufficient to enable the identification of a unique transcript. Based on the cross-platform analysis, only 72 iGentifier sequence tags were matched to the unigene sequence, among which 54 tags are annotated with a function. The small number of the annotated sequence tags was the result of low coverage of transcriptome by both unigene and iGentifier sequence tag.

Even though the number of annotated iGentifier tags was limited, we still obtained much information from the analysis of the 72 sequence tags of interest. As in the cluster analysis of all iGentifier sequence tags, most of the iGentifier tags showed a similar pattern among different biotypes under the glyphosate treatment. However, four sequence tags as shown in Figure 6 were induced in certain glyphosate-resistant biotypes. Among these, the tonoplast intrinsic protein (TIP) homolog was most relevant to the hypothesized function of glyphosate transport to vacuoles (Shaner 2009). TIPs are submembers of part of the major-intrinsic-protein gene family, which is localized in the cell to the tonoplast membrane and is expected to be involved in water transport (Maurel et al. 2008), and possibly glyphosate transport. A WD40-like protein and unknown proteins also had interesting expression patterns among accessions. WD proteins are ubiquitous among organisms and have varied functions (Smith et al. 1999) and therefore the role it and the unknown protein might have with regards to glyphosate resistance would be speculative. Data are equivocal in providing strong and universal gene leads because the same genes are not differentially regulated in all resistant accessions.

Heterologous Microarray Analysis. Approximately 15,000 to 18,000 horseweed probe spots yielded positive hybridization on the *Arabidopsis* microarrays at over 50% background ($\pm$ 2 SD), not statistically different from Arabdiopsis–*Arabidopsis* chip hybridization for the four replicates ($P < 0.05$), a result not unlike those from similar studies (e.g., Lee et al. 2004). As compared to the sequence analysis with low coverage of the transcriptome, the microarray analysis allowed for a more comprehensive understanding of the transcriptomic level responses of glyphosate-resistant horseweed. The microarray analysis revealed a general up-regulation of detoxification genes as shown in Table 1. In particular, ABC transporters were the largest group of genes from a single superfamily shown to be up-regulated. The up-regulation of the ABC transporter genes is congruent with the model of glyphosate being sequestered in vacuoles (Shaner 2009). An ABC transporter could feasibly be involved in glyphosate transport across the tonoplast. In addition to ABC transporters, several cytochrome P450 genes were also found to be up-regulated, although no P450s are known to metabolize glyphosate.

Discussion

Evolution of Glyphosate Resistance in Horseweed. The patterns and mechanisms of the evolution of the glyphosate resistance in horseweed are important for understanding resistant weed spread and management. If the resistant biotypes were all from the same ancestry, then the molecular mechanisms would be expected to be similar because of identical descent. In that case, weed management could consist of an effective strategy of controlling sources of seed or

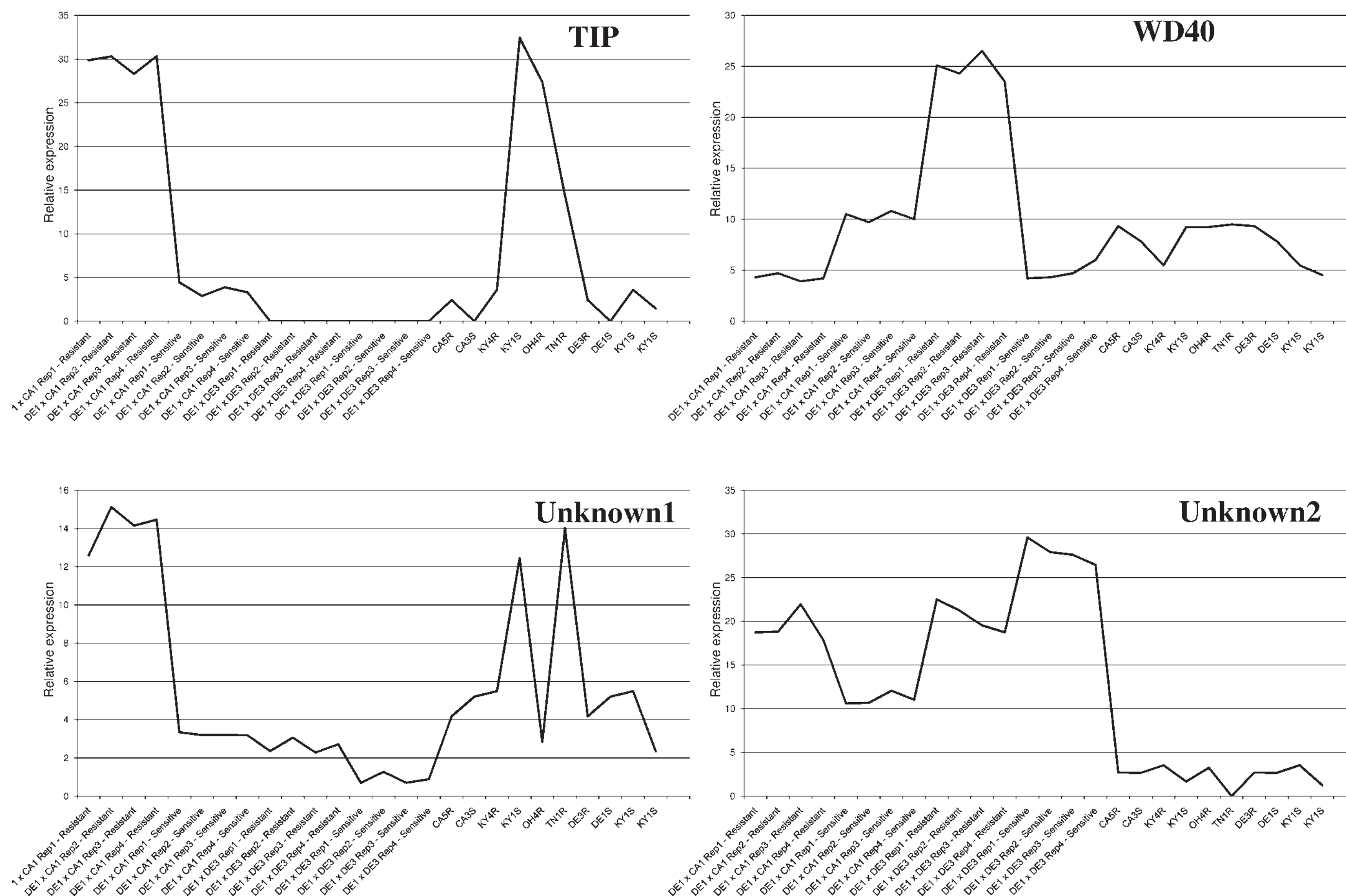

Figure 6. Detailed expression analysis of three genes showing differential expression profiles in resistant and sensitive phenotypes: a tonoplast intrinsic protein, a WD40 transcription factor and a gene coding for a protein of unknown function. The Y axis shows the relative gene expression level as quantified by the iGentifier technology. The X axis shows the different biological samples. The samples ending with R indicate the resistant biotypes, and the samples ending with S indicate the sensitive biotypes.

pollen. A study of 22 independent accessions of horseweed, a mixture of resistant and sensitive biotypes, showed that independent evolution is likely and that it has happened at least four separate times (Figures 4A and 5). If there is significant propensity or ability of weeds to evolve resistance independently of gene flow, which is apparently the case, then focusing on the mechanism and not pollen or seed spread is more crucial for management of resistance.

The transcriptome data (Figure 4B) showed a different clustering pattern compared to the SSR data. One interpretation is that there might be similar mechanisms among resistant biotypes, indicating that although resistance could have evolved independently several times, feasibly there are only a few mechanisms to endow nontarget resistance. This was a surprising result. Horseweed predominantly self-pollinates, but has plumuled seeds that can potentially travel long distances on air currents. The longest seed movement that has been determined experimentally is more than 500 m; however, 99% of horseweed seeds settled less than 100 m from the source (Dauer et al. 2007). Shields et al. (2006) collected horseweed seeds in the planetary boundary layer in the atmosphere, enabling the authors to speculate that long-range seed dispersal (say, up to 500 km) is possible. Our data do not support the hypothesis of long-range rapid spread of resistance from a single source. If a single or few evolution events occurred, followed by long-distance seed dispersal, we would expect to see proximate samples with different SSR profiles, but this was not observed. The nearest neighbor, analytically, of each resistant accession was a susceptible accession from each geographic locale (Figures 4A and 5).

The evolution of glyphosate resistance was first expected to be slow, if not improbable (Bradshaw et al. 1997). However, taking the non–target-site mechanisms into consideration, it appears that glyphosate resistance has evolved multiple times and quite rapidly in horseweed. Increased application of glyphosate in agriculture could lead to the evolution of more resistant biotypes (Duke and Powles 2008a,b). Therefore, a strategy of herbicide rotation should be implemented (Dill 2005); chemical enhancement to glyphosate herbicide and other management strategies should also be considered. The results also pose important questions about genomic and molecular mechanisms of glyphosate resistance. Is there a shared mechanism of glyphosate resistance for biotypes that evolved independently? If not, how many mechanisms exist for glyphosate resistance, at least for the existing resistant biotypes? What genes and pathways are involved in the glyphosate resistance? How fast can we expect evolution to occur in other weedy species? Can we predict resistance evolution based on genome and transcriptome information? These questions require additional research and genomics resources to be effectively addressed (Stewart 2009; Stewart et al. 2009).

Genomic Mechanisms of Glyphosate Resistance in Horseweed. Our study represents the first genome-level study of herbicide resistance in any weed. The study, one of an exploratory nature, is a first step towards a transcriptome- and genome-level understanding of a weed's response to glyphosate treatment that could be useful to identify candidate resistance genes that will be used for downstream functional genomics analysis. Transcriptomic analysis has been performed once before to assess glyphosate response, but for a crop: transgenic glyphosate-resistant soybean (Zhu et al. 2008). Because these soybeans contain an engineered target-site resistance, little to no differential gene regulation was observed in glyphosate-treated plants. This is to be expected, because the target-site pathway remains unperturbed. The same paper describes 170 genes that were differentially expressed in a sensitive soybean (Zhu et al. 2008). Resistant horseweed reacts more comparably to the sensitive rather than the glyphosate-resistant soybean.

The transcriptome analyses we performed were not definitive in converging on a single resistance mechanism, but rather indicated that although there might be a few mechanisms for glyphosate resistance in different resistant biotypes, that an in-depth follow-up analysis of a few resistant biotypes might be the best use of scarce research resources (Stewart et al. 2009). For example, up-regulation of a TIP gene in multiple resistant biotypes along with the cluster analysis of iGentifier data by horseweed biotypes indicated there were similar molecular responses among resistant biotypes treated with glyphosate. However, there were exceptions to this pattern among resistant biotypes, both in single-gene expression patterns and transcriptome clustering. For example, the California resistant horseweed biotype clustered with the sensitive biotypes in the iGentifier analysis. These results also supported certain level of diversity in the mechanisms of glyphosate resistance in horseweed, which would not be surprising given that resistance has likely evolved many times and independently from one another.

Molecular Mechanisms of Resistance: TIPs and ABC Transporters as Nontarget Candidate Genes. Previous studies have indicated that glyphosate transport and relocation might be the physiologic mechanisms for non–target-site resistance in weedy species (Feng and Chiu 2005; Feng et al. 1999, 2000, 2003a,b; Koger and Reddy 2005; Mueller et al. 2003; Ryerse et al. 2004). In addition, the target-site mechanism can be excluded for glyphosate-resistant horseweed (Feng et al. 2004; Yuan et al. 2007). In this study, we have shown the up-regulation of several transporter genes in the glyphosate-treated horseweed. As shown by the data, glyphosate resistance could involve the same general nontarget resistance pathways such as cytochrome P450 and ABC transporters (Yuan et al. 2007) or TIPs. However, the in-depth understanding of molecular mechanisms requires more genome information, which will allow a better annotation of the genes and a deeper coverage of the transcriptome. Also needed is a functional analysis of candidate genes. Cross-platform analysis was not useful in microarray analysis because the latter consisted of heterologous (*Arabidopsis*) sequence and not horseweed sequence.

The up-regulation of TIP could be relevant to the horseweed resistance from two perspectives. First, water transport is an important response for abiotic stress, and the overexpression of TIP protein could help to relieve the stress in horseweed under the glyphosate treatment (Maurel et al. 2008). Second, a TIP, or TIP-like protein, is expected to consist of 12 transmembrane domains, which could be involved in the direct transport of glyphosate or glyphosate breakdown products (Ma and Yamaji 2008; Maurel et al. 2008). Further studies will need to be performed to determine the molecular mechanism for TIP involvement in glyphosate resistance. Overall, our study indicated that the cross-platform analysis of sequence tags coupled with the EST–unigene library might be helpful to narrow the field of candidate genes involved in glyphosate resistance in a weedy species with limited genome information.

Among the top 20 up-regulated genes found in the microarray analysis, there were four ABC transporter genes that were up-regulated by glyphosate; the most highly up-regulated gene was an ABC transporter (Table 1). ABC transporter genes code for membrane-associated active transport proteins that utilize adenosine triphosphate and serve to move a variety of plant metabolites and xenobiotics across membranes. Subdivided amongst nine subfamilies, there are 129 members in *Arabidopsis* (Sanchez-Fernandez et al. 2001). Plants seem to have greater ABC transporter diversity than any other type of organism, which might aid in their wide abiotic adaptation. ABC transporters are of interest with regard to glyphosate resistance because of their diverse substrates (Linton and Higgins 2007) and the potential to sequester substrates into the vacuole. ABC transporters are responsible for a wide range of functions in plants including export of toxins, sequestration of plant secondary metabolites, translocation of fatty acids and phospholipids, and cell homeostasis (Sanchez-Fernandez et al. 2001; Schulz and Kolukisaglu 2006). Also of interest is transport of xenobiotics and variability amongst substrates (see, for example, Mentewab and Stewart 2005). ABC transporters can be targeted to any component of the endomembrane system, but of particular interest here would be tonoplast targeting; glyphosate pumped into vacuoles would be rendered harmless and such a mechanism corresponds with the described physiologic effects of glyphosate transport (e.g., Feng et al. 2004; Shaner 2009). Even though little glyphosate-resistance work has been elucidated in this gene family, there exist physiological studies showing affinity of ABC transporters to some other herbicides. For example, multiple plant ABC transporters have been shown to transport herbicides and herbicide metabolites (Klein et al. 2006; Liu et al. 2001; Schulz and Kolukisaglu 2006). Although we examined gene regulation when assessing potential gene targets, we cannot exclude point mutations in coding sequence, which could cause substrate changes (e.g., Ito et al. 2001; Özvegy et al. 2002). Indeed, for ABC transporters, substrate specificity could be variable and adaptive depending on peptide sequence in key areas of transporters (Sanchez-Fernandez et al. 2001; Schulz and Kolukisaglu 2006; and references found therein). On a related note, there might be taxonomic differences in substrate specificity in identical ABC transporters. For example, Kang et al. (2010) found that when they over-expressed *Atwbc19* in poplar (*Populus* spp.) it conferred antibiotic resistance to a broader suite of chemicals than when the same gene was overexpressed in tobacco (*Nicotiana tabacum* L.) (Mentewab and Stewart 2005).

Conclusions

Our cross-platform analysis included a limited number of annotated sequence tags for functional genomics, mainly from

the limited coverage of the transcriptome by both the EST–unigene library and the iGentifier sequence tags. Next-generation sequencing techniques represent the best option for future genomic data collection and analysis (Ahmadian et al. 2006; Margulies et al. 2005; Shendure et al. 2005). We expect to employ both GS-FLX and Illumina technologies to perform transcriptome analysis for the same types of the samples in this study. These two types of next-generation sequence analyses produce very similar type of the data as ESTs and iGentifier, but much more of it. Our cross-platform sequence analysis strategies can also be used for the analysis of the GS-FLX and Illumina data to discover the important genes involved in the horseweed glyphosate resistance. In addition to the sequence analysis, comprehensive metabolite analysis will also be valuable. The combined sequence and metabolite profiling will enable a systems biology approach to elucidate glyphosate resistance utilizing a gene-to-metabolite network (Yuan et al. 2008). In particular, four ABC transporters were found to be up-regulated by the microarray data, which suggests that vacuole metabolomics will give in-depth understanding of the compartmentation of glyphosate.

Also needed is functional analysis of existing candidate genes. We are cloning the TIP gene and other genes discovered in this study, especially ABC transporters for overexpression in an otherwise susceptible biotype of horseweed. Horseweed transformation is facile (Halfhill et al. 2007; Scheiber et al. 2006) and is an appropriate approach to study functional genomics in horseweed. Overall, the combination of the "–omics-level" study and the gene function study will help to eventually dissect the genes and pathways involved in the glyphosate resistance in horseweed, which will help the development of new weed management strategies in the post-genome era.

Sources of Materials

[1] Roundup Weathermax, Monsanto, St. Louis, MO.

[2] TIGR MEV 4.0 software.

[3] NTSYS - PC Numerical Taxonomy and Multivariate Analysis System, version 1.70. Exeter Software, Setauket, NY.

[4] 26K spotted Qiagen-Operon *A. thaliana* Genome Oligo Set, Version 1.0, University of Arizona, Tucson, AZ.

[5] Superscript III direct labeling kit, Invitrogen, Carlsbad, CA.

[6] GenePix 4000B microarray scanner, Molecular Devices, Sunnyvale, CA.

[7] GenePix Pro 4.1 software, Molecular Devices, Sunnyvale, CA.

Acknowledgments

This work was supported by U.S. Department of Agriculture agreement 58-6404-2-0057, a grant from Monsanto Company, and funds from the Tennessee Agricultural Experiment Station and Hatch Grants. Thanks to many cooperating laboratories across the United States and Canada for collecting and donating horseweed seeds and to people in the laboratories of Tom Mueller, John McElroy, and Greg Armel for help with glyphosate treatments and useful conversations about weed biology.

Literature Cited

Abercrombie, L. G., C. M. Anderson, B. G. Baldwin, et al. 2009. Permanent genetic resources added to Molecular Ecology Resources database 1 January 2009–30 April 2009. Mol. Ecol. Resourc. 9:1375–1379.

Ahmadian, A., M. Ehn, and S. Hober. 2006. Pyrosequencing: history, biochemistry and future. Clin. Chim. Acta 363:83–94.

Basu, C., M. D. Halfhill, T. C. Mueller, and C. N. Stewart, Jr. 2004. Weed genomics: new tools to understand weed biology. Trends Plant Sci. 9:391–398.

Baucom, R. S. and R. Mauricio. 2008. Constraints on the evolution of tolerance to herbicide in the common morning glory: resistance and tolerance are mutually exclusive. Evolution 62:2842–2854.

Bradshaw, L. D., S. R. Padgette, S. L. Kimball, and B. H. Wells. 1997. Perspectives on glyphosate resistance. Weed Technol. 11:189–198.

Dauer, J. T., D. A. Mortensen, and M. J. VanGessel. 2007. Temporal and spatial dynamics of long-distance *Conyza canadensis* seed dispersal. J. Appl. Ecol. 44:105–114.

Dill, G. M. 2005. Glyphosate-resistant crops: history, status and future. Pest Manag. Sci. 61:219–224.

Dill, G. M., C. A. Cajacob, and S. R. Padgette. 2008. Glyphosate-resistant crops: adoption, use and future considerations. Pest Manag. Sci. 64:326–331.

Duke, S. O. and S. B. Powles. 2008a. Glyphosate-resistant weeds and crops. Pest Manag. Sci. 64:317–318.

Duke, S. O. and S. B. Powles. 2008b. Glyphosate: a once-in-a-century herbicide. Pest Manag. Sci. 64:319–325.

Feng, P.C.C. and T. Chiu. 2005. Distribution of C^{14} glyphosate in mature glyphosate-resistant cotton from application to a single leaf or over-the-top spray. Pestic. Biochem. Physiol. 82:36–45.

Feng, P.C.C., T. Chiu, and R. D. Sammons. 2003a. Glyphosate efficacy is contributed by its tissue concentration and sensitivity in velvetleaf (*Abutilon theophrasti*). Pestic. Biochem. Physiol. 77:83–91.

Feng, P.C.C., T. Chiu, R. D. Sammons, and J. S. Ryerse. 2003b. Droplet size affects glyphosate retention, absorption, and translocation in corn. Weed Sci. 51:443–448.

Feng, P.C.C., J. E. Pratley, and J. A. Bohn. 1999. Resistance to glyphosate in *Lolium rigidum*. II. Uptake, translocation, and metabolism. Weed Sci. 47:412–415.

Feng, P.C.C., J. J. Sandbrink, and R. D. Sammons. 2000. Retention, uptake, and translocation of C^{14}-glyphosate from track-spray applications and correlation to rainfastness in velvetleaf (*Abutilon theophrasti*). Weed Technol. 14:127–132.

Feng, P.C.C., M. Tran, T. Chiu, R. D. Sammons, G. R. Heck, and C. A. Cajacob. 2004. Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): retention, uptake, translocation, and metabolism. Weed Sci. 52:498–505.

Fischer, A., A. Lenhard, and H. Tronecker, et al. 2007. iGentifier: indexing and large-scale profiling of unknown transcriptomes. Nucleic Acids Res. 35:4640–4648.

Funke, T., Y. Yang, H. Han, M. Healy-Fried, S. Olesen, A. Becker, and E. Schonbrunn. 2009. Structural basis of glyphosate resistance resulting from the double mutation Thr97 → Ile and Pro101 → Ser in 5-enolpyruvylshikimate-3-phosphate synthase from *Escherichia coli*. J. Biol. Chem. 284:9854–9860.

Goudet, J. 1995. FSTAT (Version 1.2): a computer program to calculate F-statistics. J. Heredity 86:485–486.

Gressel, J. 2009. Crops with target-site herbicide resistance for *Orobanche* and *Striga* control. Pest Manag. Sci. 65:560–565.

Gressel, J. and B. E. Valverde. 2009. A strategy to provide long-term control of weedy rice while mitigating herbicide resistance transgene flow, and its potential use for other crops with related weeds. Pest Manag. Sci. 65:723–731.

Halfhill, M. D., L. L. Good, C. Basu, J. Burris, C. L. Main, T. C. Mueller, and C. N. Stewart, Jr. 2007. Transformation and segregation of GFP fluorescence and glyphosate resistance in horseweed (*Conyza canadensis*) hybrids. Plant Cell Reports 26:303–311.

Heap, I. 2010. International Survery of Herbicide Resistant Weeds. www.weedscience.org. Accessed: February 15, 2010.

Hong, F. X., R. Breitling, C. W. McEntee, B. S. Wittner, J. L. Nemhauser, and J. Chory. 2006. RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. Bioinformatics 22:2825–2827.

Hu, J., P. J. Tranel, C. N. Stewart, Jr., and J. S. Yuan. 2009. Molecular and genomic mechanisms of non-target site herbicide resistance. Pages 149–161 *in* C. N. Stewart, Jr., ed. Genomics of Weedy and Invasive Plants. Ames, IA: Blackwell Scientific.

Ito, K., C. J. Oleschuk, C. Westlake, M. Z. Vasa, R. G. Deeley, and S.P.C. Cole. 2001. Mutation of Trp^{1254} in the multispecific organic anion transporter, multidrug resistance protein 2 (MRP2) (ABCC2), alters substrate specificity and results in loss of methotrexate transport activity. J. Biol. Chem. 276:38,101–38,114.

Kang, B.-G., X. Ye, L. D. Osburn, C. N. Stewart, Jr., and Z.-M. Cheng. 2010. Transgenic hybrid aspen overexpressing the *Atwbc19* gene encoding an ATP binding cassette transporter confers resistance to four aminoglycoside antibiotics. Plant Cell Rep. In press.

Klein, M., B. Burla, and E. Martinoia. 2006. The multidrug resistance-associated protein (MRP/ABCC) subfamily of ATP-binding cassette transporters in plants. FEBS Lett. 580:1112–1122.

Koger, C. H. and K. N. Reddy. 2005. Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). Weed Sci. 53:84–89.

Langella, O. 2002. Populations, a Free Population Genetics Software. https://launchpad.net/~olivier-langella/+archive/ppa. Accessed: September 14, 2009.

Lee, H. S., J. Wang, and L. Tian, et al. 2004. Sensitivity of 70-Mer oligonucleotides and cDNAs for microarray analysis of gene expression in *Arabidopsis* and its related species. Plant Biotechnol. J. 2:45–57.

Linton, K. J. and C. F. Higgins. 2007. Structure and function of ABC transporters: the ATP switch provides flexible control. Pflügers Arch. Eur. J. Physiol. 453:555–567.

Liu, G., R. Sanchez-Fernandez, Z. S. Li, and P. A. Rea. 2001. Enhanced multispecificity of *Arabidopsis* vacuolar multidrug resistance-associated protein-type ATP-binding cassette transporter, AtMRP2. J. Biol. Chem. 276:8648–8656.

Ma, J. F. and N. Yamaji. 2008. Functions and transport of silicon in plants. Cell. Mol. Life Sci. 65:3049–3057.

Main, C. L., T. C. Mueller, R. M. Hayes, and J. B. Wilkerson. 2004. Response of selected horseweed (*Conyza canadensis* (L.) cronq.) populations to glyphosate. J. Agric. Food Chem. 52:879–883.

Mallory-Smith, C. and M. Zapiola. 2008. Gene flow from glyphosate resistant crops. Pest Manag. Sci. 64:428–440.

Margulies, M., M. Egholm, and W. E. Altman, et al. 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376–380.

Maurel, C., L. Verdoucq, D. T. Luu, and V. Santoni. 2008. Plant aquaporins: membrane channels with multiple integrated functions. Ann. Rev. Plant Biol. 59:595–624.

Mentewab, A. and C. N. Stewart, Jr. 2005. Overexpression of an *Arabidopsis thaliana* ABC transporter confers kanamycin resistance to transgenic plants. Nat. Biotechnol. 23:1177–1180.

Mueller, T. C., J. H. Massey, R. M. Hayes, C. L. Main, and C. N. Stewart, Jr. 2003. Shikimate accumulates in both glyphosate sensitive and glyphosate resistant horseweed (*Conyza canadensis* L. Cronq.). J. Agric. Food Chem. 51:680–684.

Nei, M. 1972. Genetic distance between populations. Amer. Nat. 106:283.

Owen, M. D. and I. A. Zelaya. 2005. Herbicide resistant crops and weed resistance to herbicides. Pest Manag. Sci. 61:301–311.

Özvegy, C., A. Váradi, and B. Sarkadi. 2002. Characterization of drug transport, ATP hydrolysis, and nucleotide trapping by the human ABCG2 multidrug transporter modulation of subtrate specifically by a point of mutation. J. Biol. Chem. 277:47,980–47,990.

Page, R. D. 1996. TreeView: an application to display phylogenetic trees on personal computers. Comp. Appl. Biosci. 12:357–358.

Powles, S. B. 2008. Evolved glyphosate resistant weeds around the world: lessons to be learnt. Pest Manag. Sci. 64:360–365.

Preston, C. 2009. Herbicide resistance: target site mutations. Pages 127–148 *in* C. N. Stewart, Jr., ed. Genomics of Weedy and Invasive Plants. Ames, IA: Blackwell Scientific.

Preston, C. and A. M. Wakelin. 2008. Resistance to glyphosate from altered herbicide translocation patterns. Pest Manag. Sci. 64:372–376.

Rao, M. R., M. D. Halfhill, L. G. Abercrombie, P. Ranjan, J. M. Abercrombie, J. S. Gouffon, A. M. Saxton, and C. N. Stewart, Jr. 2009. Phytoremediation and phytosensing of chemical contaminants, RDX and TNT: identification of the required target genes. Funct. Integr. Genom. 9:537–547.

Ryerse, J. S., R. A. Downer, R. D. Sammons, and P.C.C. Feng. 2004. Effect of glyphosate spray droplets on leaf cytology in velvetleaf (*Abutilon theophrasti*). Weed Sci. 52:302–309.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.

Sammons, R. D., D. C. Heering, N. Dinicola, H. Glick, and G. A. Elmore. 2007. Sustainability and stewardship of glyphosate and glyphosate-resistant crops. Weed Technol. 21:347–354.

Sanchez-Fernandez, R., T.G.E. Davies, J.O.D. Coleman, and P. A. Rea. 2001. The *Arabidopsis thaliana* ABC protein superfamily, a complete inventory. J. Biol. Chem. 276:30,231–30,244.

Scheiber, P., M. Tran, and D. Duncan. 2006. Tissue culture and transient transformation of marestail (*Conyza canadensis* (L.) Cronquist). Plant Cell Rep. 25:507–512.

Schulz, B. and H. U. Kolukisaglu. 2006. Genomics of plant ABC transporters: the alphabet of photosynthetic life forms or just holes in membranes? FEBS Lett. 580:1010–1016.

Shaner, D. 2009. Role of translocation as a mechanism of resistance to glyphosate. Weed Sci. 57:118–123.

Shendure, J., G. J. Porreca, and N. B. Reppas, et al. 2005. Accurate multiplex polony sequencing of an evolved bacterial genome. Science 309:1728–1732.

Shields, E. J., J. T. Dauer, M. J. VanGessel, and G. Neuman. 2006. Horseweed (*Conyza canadensis*) seed collected in the planetary boundary layer. Weed Sci. 54:1063–1067.

Smith, T. F., C. Gaitatzes, K. Saxena, and E. J. Neer. 1999. The WD repeat: a common architecture for diverse functions. Trends Biochem. Sci. 24:181–185.

Stewart, C. N., Jr., ed. 2009. Weedy and Invasive Plant Genomics. Ames, IA: Wiley-Blackwell. 253 p.

Stewart, C. N., Jr., P. J. Tranel, D. P. Horvath, J. V. Anderson, L. H. Rieseberg, J. H. Westwood, C. A. Mallory-Smith, M. L. Zapiola, and K. M. Dlugosch. 2009. Evolution of weediness and invasiveness: charting the course for weed genomics. Weed Sci. 57:451–462.

VanGessel, M. J. 2001. Glyphosate-resistant horseweed from Delaware. Weed Sci. 49:703–705.

Vila-Aiub, M. M., R. A. Vidal, M. C. Balbi, P. E. Gundel, F. Trucco, and C. M. Ghersa. 2008. Glyphosate-resistant weeds of South American cropping systems: an overview. Pest Manag. Sci. 64:366–371.

Wakelin, A. M. and C. Preston. 2006. A target-site mutation is present in a glyphosate-resistant *Lolium rigidum* population. Weed Res. 46:432–440.

Williams, G. M., R. Kroes, and I. C. Munro. 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul. Toxicol. Pharmacol. 31:117–165.

Yuan, J. S., D. W. Galbraith, S. Y. Dai, P. Griffin, and C. N. Stewart, Jr. 2008. Plant systems biology comes of age. Trends Plant Sci. 13:165–171.

Yuan, J. S., P. J. Tranel, and C. N. Stewart, Jr. 2007. Non-target site herbicide resistance: a family business. Trends Plant Sci. 12:6–13.

Zelaya, I. A., M.D.K. Owen, and M. J. VanGessel. 2004. Inheritance of evolved glyphosate resistance in *Conyza canadensis* (L.) Cronq. Theor. Appl. Genet. 110(1):58–70.

Zhou, X., Z. Su, R. D. Sammons, Y. Peng, P. J. Tranel, C. N. Stewart, Jr., and J. S. Yuan. 2009. Novel software package for cross-platform transcriptome analysis (CPTRA). BMC Bioinformatics 10 (Suppl. 11):S16.

Zhu, J., W. L. Patzoldt, R. T. Shealy, L. O. Vodkin, S. J. Clough, and P. J. Tranel. 2008. Transcriptome response to glyphosate in sensitive and resistant soybean. J. Agric. Food Chem. 56:6355–6363.

Received September 16, 2009, and approved December 22, 2009.