Transcriptome Response to Glyphosate in Sensitive and Resistant Soybean

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The majority of soybeans planted in the United States are resistant to glyphosate due to introduction of a gene encoding for a glyphosate-insensitive 5-enolypyruvylshikimate-3-phosphate synthase. Gene expression profiling was conducted using cDNA microarrays to address questions related to potential secondary effects of glyphosate. When glyphosate-sensitive plants were treated with glyphosate, 3, 170, and 311 genes were identified as having different transcript levels at 1, 4, and 24 h post-treatment (hpt), respectively. Differentially expressed genes were classified into functional categories, and their possible roles in response to glyphosate are briefly discussed. Gene expression profiling of glyphosate-resistant plants treated with glyphosate indicated that the plants were marginally affected at 1 hpt and then quickly adjusted to glyphosate treatment. Ten, four, and four genes were identified as differentially expressed at 1, 4, and 24 hpt. When gene expression profiles of cotyledons from developing seed were compared between the near-isogenic resistant and sensitive lines, two genes were identified as significantly differentially expressed out of 27000, which was less than the empirical false-discovery rate determined from a control experiment. Quantitative real-time reverse-transcribed Polymerase Chain Reaction was conducted on selected genes and yielded results consistent with those from the microarrays. Collectively, these data indicate that there are no major transcriptomic changes associated with currently used glyphosate-resistant soybean.

KEYWORDS: Glyphosate; gene expression profiling; herbicide; chloroplast; cytochrome P450

INTRODUCTION

One of the first and most successful contributions of modern agricultural biotechnology was the development of glyphosate-resistant soybeans. These soybeans contain a bacterial gene that encodes a glyphosate-insensitive form of the enzyme 5-enolypyruvylshikimate-3-phosphate synthase (EPSPS) (*1, 2*). EPSPS catalyzes the penultimate step of the shikimate pathway in plants. Inhibition of EPSPS by glyphosate results in the accumulation of shikimate and the shortage of the chorismate-derived essential aromatic amino acids tryptophan, tyrosine, and phenylalanine, leading to eventual death (*3–5*).

Through the use of a glyphosate-resistant EPSPS, modern biotechnology has enabled a nonselective herbicide (i.e., glyphosate is active on nearly all plant species) to be used selectively after soybean emergence. In addition to its broad-spectrum weed control, glyphosate offers several advantages relative to other available herbicide options. In particular, glyphosate exhibits low toxicity to nonplant species and does not accumulate in the environment (*6*). From a grower's perspective, glyphosate-resistant soybean varieties greatly simplify weed management by enabling effective weed control with a single herbicide having a wide postemergence timing window (*7*). Consequently, it is not surprising that glyphosate-resistant soybeans now dominate the market in the United States and elsewhere (*8*).

Because insertion of a foreign gene may have unintended consequences, various techniques have been suggested to evaluate the safety of transgenic crops and their equivalency to nontransgenic counterparts (*9*). Several studies have demonstrated the safety and equivalency of glyphosate-resistant soybean relative to conventional (nontransgenic) soybean (*1, 2, 10, 11*). These studies generally have focused on comparison of seed composition between transgenic and conventional seed.

In the case of a herbicide-resistant transgenic crop, there additionally is the potential that the resistance transgene may not overcome all effects of the herbicide. For example, glyphosate may interact with cellular targets other than EPSPS. Any such "off-target" effects could imply that secondary effects

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Table 1. Summary of Experiments

expt	description	glyphosate	tissue	~ no. of cDNA	no. of sig genes
1	sensitive soybean ± glyphosate	±	leaves and shoots[a]	27000	464
2	empirical fdr[b] (with sensitive soybean)	−	leaves and shoot[a]	9000	1
3	resistant soybean ± glyphosate	±	leaves and shoots[a]	27000	18
4	sensitive vs resistant soybean	−	cotyledons[c]	27000	2

[a] Pool of fully expanded leaf, folded leaf, and shoot apical meristem. [b] fdr, false-discovery rate. [c] Pool of green cotyledons of developing seed in the range of 175−200 mg.

of glyphosate, even if marginal, may be manifested in plants that contain glyphosate-insensitive EPSPS. In the present study, we utilize soybean cDNA microarrays (*12*) to investigate potential secondary targets of glyphosate. First, to demonstrate the sensitivity of the approach, transcriptome changes were determined in glyphosate-sensitive soybean treated with glyphosate. As expected, numerous genes were identified with altered transcript levels, and the roles of these genes in glyphosate response are discussed. Second, to empirically determine the false-discovery rate (fdr), a control experiment was performed in which transcriptomes between identically treated plants of the same cultivar were compared. In the third experiment, gene expression changes were determined in a transgenic, glyphosate-resistant soybean line treated with glyphosate. In the fourth experiment, gene expression in cotyledons of developing seeds, the edible portion of soybeans, was compared between a glyphosate-resistant line and a sensitive near-isogenic line. Collectively, results of these experiments indicated that commercialized glyphosate-resistant soybean, with or without glyphosate treatment, is associated with few, if any, significant changes in gene transcript levels and that there likely are no major secondary targets for glyphosate.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Glyphosate-sensitive and resistant F_8-derived F_{11} isolines (sister events to the transgenic AG3205 soybean cultivar) were obtained from the Monsanto Co. Glyphosate resistance in this transgenic line is due to the addition of a gene encoding a glyphosate-insensitive EPSPS from *Agrobacterium* sp. strain CP4 (*13, 14*).

Soybeans were grown in a greenhouse maintained at 28/22 °C day/ night with supplemental light (minimum of 800 μmol m^{-2} s^{-1} photon flux at the plant canopy) provided by mercury halide and sodium vapor lamps programmed for a 16 h photoperiod. Soybean seeds from each line were sown in 12 cm plastic pots containing 800 mL of a 1:1:1 mixture of soil/peat/sand. Two seeds were sown in each pot and then thinned to one plant per pot following seedling emergence. Expression of the CP4 EPSPS transgene was verified when the plants reached the unifoliate growth stage. A leaf sample was taken from each putative glyphosate-resistant plant using a 6 mm cork borer. Leaf disks were homogenized with pestles in 1.5 mL microcentrifuge tubes in the presence of liquid nitrogen and then suspended in 0.5 mL of H$_2$O. Colorimetric test strips (Trait RUR, Strategic Diagnostics, Inc.) were placed in each tube to assay for the expression of the CP4 EPSPS transgene. All glyphosate-resistant plants utilized in the study tested positive for the transgene using this assay. In addition, random plants from the glyphosate-sensitive line were used as negative controls for the assay (data not shown).

Treatments. Glyphosate-sensitive and -resistant soybeans were utilized in four separate experiments (**Table 1**) as follows (*1*): comparison between glyphosate-treated and nontreated sensitive soybean (*2*), within cultivar comparison between two separate sets of identically grown sensitive soybean (as a control to empirically determine the fdr) (*3*), comparison between glyphosate-treated and nontreated glyphosate-resistant soybean, and (*4*) comparison between cotyledons of developing seeds from sensitive versus resistant soybean lines in the absence of glyphosate. In experiments 1 and 3, sensitive and resistant soybeans were treated with glyphosate (Monsanto Co.,

Roundup Ultra, 356 g of ae L^{-1}) at 0.75 kg of ae ha^{-1} or the formulation blank (solvent and surfactant only, provided by Monsanto Co.) when plants had two fully expanded trifoliates. Treatments were applied using a compressed air, moving-nozzle laboratory sprayer equipped with an 80° flat-fan nozzle (Teetjet, Spray Systems Inc.) delivering 187 L ha^{-1} at 207 kPa. The nozzle was maintained approximately 45 cm above the plant canopy. Soybeans were returned to the greenhouse immediately after treatment. Both glyphosate-sensitive and -resistant soybeans were treated at the same time and with the treatment solutions (Roundup Ultra or the formulation blank). The second and third trifoliate, plus the apical meristem, from each plant were harvested at 1, 4, or 24 h post-treatment (hpt). Tissue was pooled from 10 plants per biological replication for each treatment and time point. Soybean plants from experiments 1 and 3 were grown together in a completely randomized design. For the comparison of cotyledons from developing seed, soybean seedlings from each line were transplanted into 23 cm plastic pots containing 10 L of soil. Plants were allowed to flower, and immature seed cotyledons ranging between 175 and 200 mg/seed were pooled from 10 plants. Experiment 2 was conducted in the same was as experiment 1 except that no glyphosate was applied. For each treatment, tissues were immediately placed in liquid nitrogen after harvest, freeze-dried, and then stored at −80 °C until RNA extractions.

Preparation of Labeled Probes and Microarray Hybridization. RNA was extracted from soybean tissue using a modified version described by McCarty (*15*) and further purified to mRNA according to the manufacturer's instructions (Promega, PolyATtract mRNA Isolation System). For each probe, 2.0 μg of mRNA was reverse transcribed in the presence of Cy3- or Cy5-dUTP, as described in Thibaud-Nissen et al. (*16*). Labeled probes were hybridized to soybean cDNA microarrays representing approximately 27000 unigenes (*12*). Preparation of slides and methods for hydridization were performed as described in Thibaud-Nissen et al. (*16*). Slides were scanned with a ScanArray Express and quantified with associated software (Perkin-Elmer Life Sciences). Separate hybridizations were performed using RNA from each of two biological replications of each treatment, including a dye-swap experiment (technical replication) for each biological replication, for a total of four hybridizations per treatment.

Microarray Data Analysis. Raw data were normalized, flagged, and transformed using in-house software (*16*). Normalized data were analyzed with the maanova package in R (*17*). A mixed effect linear model was used, which included dye and sample as fixed effect terms and array as a random effect term. The F1 test, which is a gene-specific test based on the variance components of a single gene, and the F3 test, which assumes common variance among the genes, were conducted in R (*18*). Genes were considered to be significantly differentially expressed if the *p* values from both of the *F* tests were <0.005, a cutoff value that selected a minimal number of false positives while still allowing selection of several hundred significant genes in glyphosate-treated sensitive plants.

Data Verification by qRT-PCR. Quantitative real-time reverse-transcribed PCR (qRT-PCR) was performed essentially as described by Zou et al. (*19*). In brief, 2.5 μg of purified total RNA was treated with DNase I, amplification grade (Invitrogen) and reverse transcribed using Super III Reverse Transcriptase (Invitrogen) in a 20 μL volume reaction. The resulting cDNA was adjusted to final a concentration of 30 ng/μL and quantified by PCR using an Mx3005p Thermal Cycler (Strategene). The primers for the target genes (**Table 2**) were designed on the basis of either TIGR tentative consensus sequences (http:// compbio.dfci.harvard.edu/tgi/) with emphasis on the unique oligomer

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Table 2. Primers Used in qRT-PCR[a]

microarray clone ID	forward primer	reverse primer
Gm-r1083-1418	CAAACTCTTCTCCCGCTTCCA	CCAGCCCCTTTTTCATCACC
Gm-r1070-5838	CGGAGAGAAACCCATTCCAC	AATAGCACCTCCAACGGACA
Gm-r1021-880	CATCGCCAAAGCTGGTTACA	CTCCCAGTCATCCTGGTCAA
Gm-r1088-3841	TCTGCCATCTCCATCTCATTC	CAGGGCTAGTGTGCATTGTG
Gm-r1088-8829	TCTGTGTTGTGTTTGTTAGGGTTAGTC	CAGTCACCTTTGCGTTGGTT
Gm-r1070-8746	GCCCTCACACACACTGATCTT	TTCCAGCCGCTATTACTTCAC

[a] Sequences are written in the direction from 5′ to 3′.
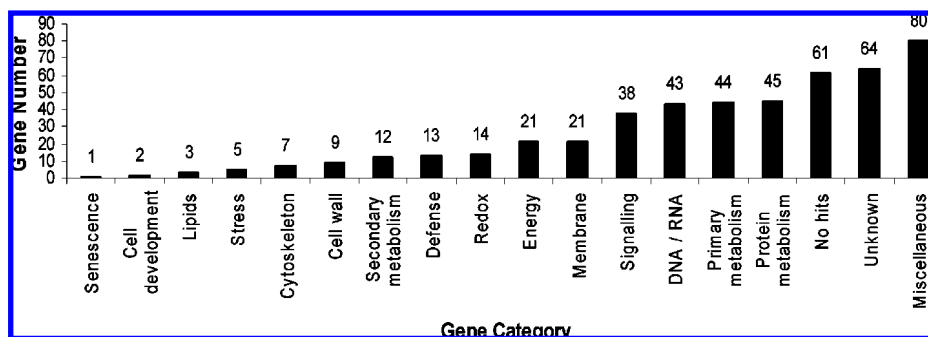
Figure 1. Bar chart of categories of genes found to be differentially expressed from microarray experiments.

region or singleton EST sequence using Primer 3.0 software (Applied Biosystems, Foster City, CA). The expression of a soybean β-actin gene (Gm-r1021-3839) was used as the internal standard to normalize the small difference in template amounts, with forward primer 5′-TCCAAGGGGACCTAACGGAGA-3′ and reverse primer 5′-TGGGTCAAGAGCTGGATGGTG-3′ (*19*). QRT-PCR reactions were carried out in a total volume of 20 μL, containing 2 μL of cDNA template, 0.8 μL (4 μM) of each primer (forward and reverse), 8.9 μL of Brilliant SYBR Green PCR master mix (Stratagene), and 7.5 μL of water, using the following thermocycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s, 72 °C for 30 s. The specificity of the primers was validated by the presence of a single peak in the dissociation curve analyses after the qRT-PCR and by sequencing the PCR products. The qRT-PCR data were analyzed using the relative quantification $2^{-\Delta\Delta CT}$ method (*20*). The difference in the cycle numbers at which the amplified gene amount reaches the threshold, $\Delta\Delta CT$, was used to determine the differential gene expression. First, the ΔCT was calculated as the difference between the tested genes and the reference actin gene to normalize the template quantities across the treatments. Then the $\Delta\Delta CT$ was obtained by comparing the difference in the ΔCTs of two treatments. The arithmetic fold change was calculated as $2^{-\Delta\Delta CT}$ and reported as log2 fold change ($-\Delta\Delta CT$). Two biological and three technical replicates were conducted on every comparison between treatment and control.

RESULTS AND DISCUSSION

Four experiments (**Table 1**) were conducted that were designed to address (1) the effect of glyphosate on glyphosate-sensitive plants, (2) within-cultivar variation to determine baseline of variation independent of treatment (i.e., an empirical fdr), (3) the effect of glyphosate on glyphosate-resistant transgenic plants, and (4) variation in transgenic plants versus nontransgenic in the edible part of the soybean—the seed cotyledons.

In total, 483 genes were determined to have a significant change in relative expression during these experiments. Translations of the 5′ and 3′ ESTs of the cDNAs were compared to protein databases from NCBI and *Arabidopsis* MIPS using BLASTx to obtain annotations. Additional annotation was also obtained from TIGR TCs containing these 5′ and/or 3′ ESTs. Annotations from all three sources were compared for each gene to determine final annotation calls and to assign functional
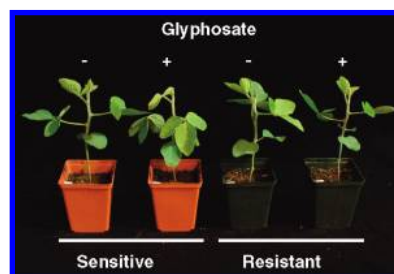
Figure 2. Whole-plant responses to glyphosate (0.75 kg of ae ha^{-1}) at 24 h post-treatment (hpt). The first apparent symptom of glyphosate was a reduction in diurnal leaf movement: leaves of glyphosate-sensitive soybeans treated with glyphosate failed to return to a horizontal orientation at the onset of the light period. No injury symptoms were observed on glyphosate-resistant soybean.

categories (Table 1, Supporting Information). Distribution of functional categories revealed that the most abundant categories (ignoring "miscellaneous", "unknown", and "no hit") were in the order protein metabolism > primary metabolism > DNA/RNA > signaling (**Figure 1**).

Experiment 1: Effects of Glyphosate on Sensitive Plants. The goal for this experiment was to identify glyphosate-responsive genes prior to severe symptom development. **Figure 2** illustrates that a response to glyphosate was beginning to be visible in the sensitive line at the whole-plant level by 24 hpt. However, injury symptoms were minor at this point and were not present at 1 or 4 hpt. Therefore, we chose 1, 4, and 24 h sampling times to capture initial responses to the effect of glyphosate application on gene expression.

Microarray analysis of glyphosate-sensitive plant responses to glyphosate identified 464 genes differently expressed during the time course with 3, 170, and 311 genes changing at 1, 4, and 24 hpt, respectively (Table 1, Supporting Information). Of these 464 genes, 20 were changing at both 4 and 24 hpt (**Table 3**). These 20 genes can be summed up as being involved in protein turnover, gene regulation, cytochrome P450 chemistry, glycolysis, and calcium signaling. No gene was significantly affected across each of the three time points.

At 1 hpt, only three genes were identified as differentially expressed in response to glyphosate application in sensitive

Table 3. Genes Differentially Expressed in Glyphosate-Sensitive Soybean at both 4 and 24 h Post-treatment (hpt) with Glyphosate

| clone ID | fold change | | category | subcategory A | subcategory B |
	4 hpt	24 hpt			
Gm-r1088-8667	0.54	0.55	DNA/RNA	binding-DNA	DNA binding
Gm-r1070-3892	1.30	1.57	DNA/RNA	binding-DNA	DNA-binding protein RAV1
Gm-r1070-5134	0.82	0.58	DNA/RNA	gene regulation	homeodomain transcription factor
Gm-r1088-3478	0.29	0.35	DNA/RNA	helicase	ATP-dependent DNA-binding helicase
Gm-r1083-1418	2.98	2.00	miscellaneous	cytochrome P450	similar to CYP82A3
Gm-r1088-2632[a]	15.15	13.10	miscellaneous	cytochrome P450	similar to CYP82A3
Gm-r1088-6079	0.66	0.51	miscellaneous	possible chimeric	
Gm-r1088-1539	0.39	0.33	miscellaneous	possible chimeric	
Gm-r1088-8177	0.17	0.21	miscellaneous	possible chimeric	
Gm-r1088-3798	0.28	0.36	miscellaneous	possible chimeric	
Gm-r1070-5900	0.81	0.49	no hits		
Gm-r1021-880	3.85	5.99	primary metabolism	carbohydrate	2-phosphoglycerate dehydratase
Gm-r1021-981	2.74	3.23	primary metabolism	carbohydrate	2-phosphoglycerate dehydratase
Gm-r1070-5903	0.66	0.57	protein	degradation	subtilisin-like serine protease AIR3
Gm-r1088-1520	0.38	0.46	protein	degradation	matrix metalloproteinase MMP2
Gm-r1088-3525	0.22	0.25	protein	degradation	matrix metalloproteinase MMP2
Gm-r1088-4079	0.27	0.39	protein	degradation	matrix metalloproteinase MMP2
Gm-r1070-8746	2.44	3.07	protein	synthesis	translation initiation factor 6 (EIF-6)
Gm-r1070-5838	2.72	2.69	signaling	calcium	Ca^{2+}-binding EF-hand
Gm-r1088-3688	0.48	0.45	unknown	expressed	

[a] This P450 gene was also significantly increased in abundance in glyphosate-resistant soybean at 1 h post treatment (hpt).

plants, consistent with the delay required for glyphosate to reach its target site. Of these three early affected genes, two increased in transcript levels (a WRKY transcription factor homologue, Gm-r1070-1657, and a gene of unknown function, Gm-r1088-8166), whereas the third gene decreased in transcript abundance. The gene with decreased expression in response to glyphosate was homologous to an early light-induced gene (Gm-r1070-5167) that has been associated with light signaling. In contrast to the weak response at 1 hpt, by 4 hpt the number of significant differentially expressed genes dramatically increased to 170, indicating that the glyphosate-sensitive plants were responding to glyphosate.

Glyphosate inhibits the shikimate biosynthetic pathway that leads to the production of chorismate, the precursor of the aromatic amino acids. Therefore, inhibition of the shikimate pathway by glyphosate treatment may lead to differential gene expression for enzymes related to chorismate and aromatic amino acid biosynthesis. In a study involving potato suspension cells, glyphosate-induced starvation of aromatic amino acids led to an increase of transcript levels corresponding to 3-deoxy-7-phosphoheptulonate synthase [EC 2.5.1.54; also commonly referred to as 2-keto-3-deoxy-D-arabino-heptonic acid 7-phosphate synthetase (DHS) or 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase], the enzyme that catalyzes the first and committed step in the shikimate pathway (*21*). In addition to DHS/DAHP synthase, studies in *Arabidopsis* reported other transcripts to accumulate in glyphosate-treated plants including anthranilate synthase (AS), the committed step in tryptophan biosynthesis; phosphoribosylanthranilate transferase (PAT), the second step in tryptophan biosynthesis; and tryptophan synthase (TS), the last step of tryptophan biosynthesis (*22, 23*). In our study, evidence to support inhibition of the shikimate pathway could be observed by a significant 1.3-fold increase in transcript level of DHS/DAHP synthase gene (Gm-r1070-6471) at 4 hpt. Several genes encoding AS, PAT, and TS were all slightly induced in at least one time point; however, their degree of change was not strong enough to meet our cutoff criteria and therefore did not show up in our differentially expressed gene list (Table 1, Supporting Information).

Previous studies also have shown glyphosate-induced changes in expression of non-shikimate pathway genes. For example, a

key enzyme in ammonia assimilation, glutamine synthetase, was reduced in expression in *Arabidopsis* (*22*). It has also been reported that glyphosate-induced blockage of the shikimate pathway in carrot suspension cells resulted in the accumulation of ammonia and that this ammonia increase may be an important factor in the toxicity of glyphosate (*24*). To minimize the toxic effects of ammonia, plants assimilate ammonia utilizing both glutamine synthetase and glutamate synthase (*25*). Our observed increased abundance of glutamate synthase (Gm-r1083-629) and a tRNA glutamine synthetase (Gm-r1088-2428) at 4 hpt suggests a possible detoxification response to increasing ammonia concentrations.

In addition to amino acid metabolism, eight genes encoding various ribosomal components were also significantly changing at 4 hpt. Seven of the eight had increased transcript abundance, suggesting a general effort to initiate repair and/or defend against possible damage caused by glyphosate-induced stress. Interestingly, all eight of these genes were for ribosomes of the cytosol, whereas at 24 hpt, only four ribosomal genes were changing in abundance and three of the four were for plastid ribosomes. Of the ribosomal genes changing at 24 hpt, the three annotated as plastid ribosomes were reduced and the one cytosolic was increased in abundance.

The impact of glyphosate on the shikimate pathway also extends to other related metabolic pathways as hundreds of primary and secondary aromatic compounds are derived from the shikimate pathway (*26*). The present study identified glycolysis as an additional pathway affected by glyphosate treatment. Five cDNAs annotated as enolases were all increased at 4 hpt, and three were increased at 24 hpt. Enolase is the ninth enzyme in the glycolysis pathway and catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, the penultimate step in the conversion of glucose to pyruvate. The accumulation of phosphoenolpyruvate will result in increased abundance of pyruvate, the starting molecule of the TCA cycle. In our data, a 3.9-fold increase in a dihydrolipoamide *S*-acetyltransferase (Gm-r1088-2346) was observed. Dihydrolipoamide *S*-acetyltransferase is the E2 component of pyruvate dehydrogenase, which connects glycolysis to the TCA cycle. Its increased abundance reflects a more active conversion of pyruvate in the TCA cycle.

In addition to alterations of glycolysis and the TCA cycle, photosynthesis was a major process affected by glyphosate, based on gene expression data. Although photosynthesis is not considered to be the primary site of action for glyphosate, its alteration could be the consequence of affecting chloroplast function as suggested by rapid, reduced abundance of plastid-associated ribosome transcripts. At 4 hpt, three genes (Gm-r1088-2682, Gm-r1088-8318, and Gm-r1088-692) encoding photosystem components were reduced. The number of differentially expressed photosynthesis genes increased to 16 by 24 hpt and, similarly, all were reduced in transcript abundance. The majority of these repressed photosynthesis-related genes encode components of the photosystems, such as the photosystem II subunits, light-harvesting antennae, chlorophyll-associated proteins, oxygen-evolving enhancer proteins, and the electron transfer component, plastocyanin. Besides genes involved in the light reactions, one gene (Gm-r1088-3266) encoding phosphoribulokinase, which phosphorylates ribulose 5-phosphate into ribulose 1,5-bisphosphate in the Calvin cycle, was also reduced in transcript abundance. The overall decrease of photosynthesis transcripts from both the light and dark reactions suggests a severe physical impact on soybean chloroplasts by 24 hpt. Similar to photosynthesis, genes involved in carbohydrate metabolism were differentially reduced in expression at 24 hpt. Glyphosate also influenced genes related to carbohydrate metabolism, such as ADP-glucose pyrophosphorylase (Gm-r1088-3118 and Gm-r1088-3368) and amylase (Gm-b10BB-53, Gm-r1088-3368, and Gm-r1070-5613).

Changes in secondary metabolites are a common response in plants to biotic and abiotic stresses. Therefore, it would be expected to find genes associated with secondary metabolism to be affected by glyphosate, especially because aromatic amino acids from the shikimate pathway are essential components of many secondary metabolites, such as flavones, isoflavones, and anthocyanins in soybean. Interestingly, in our study, the gene for the key enzyme chalcone synthase (CHS) of the phenylpropanoid pathway (which stems from the shikimate pathway derived product phenylalanine) was induced at both 4 and 24 hpt. These findings suggest this gene may be regulated independent of the shikimate pathway products. In *Arabidopsis*, glyphosate was also reported to induce CHS expression, showing a 20-fold increase at 4 hpt (23). It would be advantageous to plants not to link regulation of key stress-responsive genes like those of the phenylpropanoid pathway in soybean (19, 27) to functional pathways of primary metabolism. Additionally, it is often observed that some branches of the phenylpropanoid pathway will be inhibited while others are active, presumably to conserve metabolically costly resources (27). In this study, two genes encoding anthocyanin rhamnosyl transferase (AMT) were reduced at 4 hpt. AMT catalyzes the glycosylation of anthocyanin precursor, which allows the accumulation of the anthocyanin pigment. Other examples of changes in secondary metabolism include transcript reductions of chalcone reductase (CHR), which functions in the biosynthesis of isoflavonones, and cinnamoyl-CoA reductase (CCR), a key enzyme converting hydroxycinnamoyl CoA esters into hydroxycinnamaldehydes in the lignin biosynthesis pathway.

Changes in transcript abundance were observed in genes related to oxidative stress responses following glyphosate treatment. Increased transcript levels of an alternative oxidase (Gm-r1088-6638) and decreased abundance of transcripts corresponding to two ascorbate peroxidases (APs) were identified at 4 hpt. Alternative oxidase induction is an indication of mitochondrial oxidative stress (28). AP functions to eliminate

hydrogen peroxide (H_2O_2) and its reduction upon glyphosate treatment are not understood. One possible explanation is that APs have much lower affinity for H_2O_2 (micromolar range) than catalases (CATs) (millimolar range). Therefore, it has been hypothesized that APs might be more involved in fine modulation of H_2O_2 as a signaling molecule, whereas CATs might be essentially responsible for the removal of excess H_2O_2 during stress (29). Additionally, CATs mainly exist in peroxisomes, whereas APs are located in the cytosol. The subcellular location difference may be another factor leading to differential regulation.

Another effective strategy for a plant to defend against a toxin such as glyphosate would be to activate genes encoding metabolic enzymes related to its detoxification. As evidence that glyphosate-sensitive soybeans were attempting to chemically modify and/or nullify its toxic effects, several genes related to herbicide metabolism were increased in abundance. At 4 hpt, three cytochrome P450 genes (Gm-r1083-1418, Gm-r1088-2632, and Gm-r1070-2675) were substantially induced with fold changes of 2.9, 15, and 2.4, respectively. At 24 hpt, the number of induced cytochrome P450s increased to six, including allene oxide synthase, CYP81E8, CYP82A3, and CYP93A1. Cytochrome P450s are a large gene family of monooxygenases playing an important role in the metabolism of a variety of substances (30–32). One of their functions is to oxidize xenobiotics and promote the detoxification of herbicides (33). However, despite the fact that some cytochrome P450s have been proven to metabolize several herbicides, such as diclofop-methyl (34), chlorotoluron (35), and 2,4-dichlorophenoxyactic acid (36), the specific substrates and functions of the vast majority of cytochrome P450s are still unknown (32). Data have been presented in support of a glyphosate oxidoreductase-like enzyme in soybean that may degrade glyphosate to aminomethylphosphonic acid (37). The three cytochrome P450s identified at 4 hpt all belong to the CYP82A subfamily. The CYP82 family of cytochrome P450 is involved in defense mechanisms in plants following wounding and pathogen infections (38, 39). The induction of these genes by glyphosate suggests that they may function to metabolize xenobiotics or they may be more generally responding to common stress signals, such as reactive oxygen species. It has also been postulated that glyphosate may inhibit the enzyme activity of cytochrome P450s, thereby increasing its toxicity (40, 41). Therefore, it is possible that transcriptional induction of these cytochrome P450s is part of the feedback control provoked by the glyphosate-induced inhibition of some cytochrome P450 activity.

Glutathione *S*-transferases (GSTs) may be involved in either detoxification of xenobiotics or the protection of cells from lipid oxidation resulting from oxidative stresses. GSTs conjugate glutathione (GSH) to various molecules including xenobiotics, which is often an effective step for metabolic detoxification and elimination of xenobiotics from the cytoplasm (42–46). In response to glyphosate, we observed an increase in GST transcript levels at 24 hpt. The induction of the expression of GSTs indicates an active detoxification effort from soybean plants. In *Arabidopsis*, it was found that glyphosate induced expression of GSTs by 4 hpt (23). It was argued that the induction was due to the aromatic amino acid starvation caused by the inhibition of glyphosate on EPSPS and, possibly, the amino acid starvation is similar to other biotic and abiotic stresses (23).

As time progressed over the study to 24 hpt, the effect of glyphosate increased as reflected by the significant increase in the number of differentially expressed genes to 311. At 24 hpt transcripts from the signal transduction category largely in-

Table 4. Genes Differentially Expressed in Glyphosate-Resistant Soybean 1, 4, or 24 h Post Glyphosate Treatment (hpt)

clone ID	fold change[a]			category	subcategory A	subcategory B
	1 hpt	4 hpt	24 hpt			
Gm-r1070-4692	1.95*	0.84	1.09	membrane	transport	heavy metal
Gm-r1088-2632[b]	1.80*	1.21	0.97	miscellaneous	cytochrome P450	similar to CYP82A3
Gm-r1083-2476	1.70*	1.04	1.14	miscellaneous	prenylated protein	FP6
Gm-r1083-734	1.64*	0.78	0.93	defense	candidate resistance protein KR1	
Gm-r1088-5884	1.50*	1.14	1.00	defense	disease resistance protein-like MsR1	
Gm-r1088-8690	1.49*	0.98	0.96	no hits		
Gm-r1088-7400	1.45*	1.08	0.95	miscellaneous	phloem protein	AtPP2-B10
Gm-r1088-8903	1.35*	1.00	0.96	signaling	kinase	receptor kinase
Gm-r1021-2781	1.32*	1.03	1.09	unknown	hypothetical protein	
Gm-r1088-2476	1.32*	0.89	1.04	senescence	SAG101	
Gm-r1070-8914	1.07	1.24*	0.90	unknown	hypothetical	AT4g3698/C7A1_38
Gm-r1070-5643	1.22	0.83*	1.06	no hits		
Gm-r1070-3141	1.01	0.82*	1.08	membrane	transport	coatomer protein-like
Gm-r1070-3757	1.01	0.81*	0.97	DNA/RNA	RNA helicase	DEAD BOX RNA helicase
Gm-r1088-4938	0.95	1.08	0.84*	no hits		
Gm-r1088-3999	1.16	0.97	0.84*	primary metabolism	carbohydrate	carbohydrate kinase
Gm-r1088-8726	1.02	1.06	0.82*	no hits		
Gm-r1083-3711	1.12	1.03	0.75*	unknown	expressed	

[a] An asterisk (*) following fold changes indicates a significant difference at $P < 0.005$. [b] This P450 gene was also significantly increased in abundance in glyphosate-sensitive soybean at 4 and 24 hpt.

creased. In this category, nine cDNA clones representing auxin down-regulated genes (ADR) were universally reduced in transcript abundance. Auxins are naturally occurring plant hormones regulating the growth and development of plants and are derived from tryptophan. As an important signal transduction pathway, auxins differentially induce and repress various genes. In soybean, three families of ADR genes have been characterized, and they show differential expression in a tissue/organ-specific manner regulated by the auxin level as well as light (*47*). In cotton, it was shown that glyphosate treatment led to auxin accumulation in anthers (*48*). In this study, a reduction of ADR transcripts would presumably be caused by the accumulation of auxin in leaf tissue. However, there are conflicting reports as to whether auxin levels increase or decrease in response to glyphosate (*49, 50*).

There have been few studies investigating the effect of glyphosate on plant transcription changes. Yu et al. identified 24 soybean cDNAs that changed abundance after glyphosate treatment in either sensitive or resistant soybean genotypes (*51*). To compare our data to theirs, the EST sequences of their differential cDNAs were retrieved from the National Center for Biotechnology Information (NCBI) and compared against our soybean EST sequence database of cDNAs printed on the soybean microarrays using BLASTn. The corresponding ESTs represented in our soybean cDNA microarray were obtained, but in our study, none of the highly similar genes identified were significant. It is unclear why inconsistent results between the two studies were obtained, but they may be due to differences in glyphosate application procedures.

Experiment 2: Within-Cultivar Variation. To verify the technical quality of our microarray hybridization and data analysis and to empirically determine an fdr for the study, a dye-swap experiment was performed using cDNA probes derived from different plants of the glyphosate-sensitive soybean line. Using microarray slide set Gm-1021/83 consisting of 9216 gene representatives, only 1 gene (a clone homologous to a 60S ribosomal gene) was identified as being differentially expressed. This very low within-cultivar variation (0.01% of genes screened) indicates the microarray hybridizations and our data analysis procedures were robust and that little variation in gene expression is occurring

independent of treatment effects. Thus, most genes identified as differentially expressed in experiment 1 very likely are the result of glyphosate response and not statistical artifacts.

Experiment 3: Effect of Glyphosate on Glyphosate-Resistant Soybean. This study identified 18 differentially expressed genes in glyphosate-resistant transgenic plants when treated with glyphosate (**Table 4**). Differential regulation of 18 genes of approximately 27000 genes in total across all three time points is considered to be a minor change based on the empirical fdr of 1 of 9216 determined from the within-cultivar experiment involving one time point. Extrapolating from this experimental fdr, one would expect about 3 false positives in 27000 genes for each time point, for a total of 9 across the experiment. Additionally, the fold change of most of these 18 genes was below 1.4. Taken together, this information indicates that glyphosate-resistant soybeans responded minimally to the glyphosate treatment at the transcriptome level. Furthermore, the number of significantly regulated genes decreased with time. Specifically, 10 of these 18 genes were significant at 1 hpt, whereas only 4 of these 18 genes were significant at 4 hpt and another 4 genes at 24 hpt. These data suggest that the glyphosate-resistant plants quickly adjusted to the treatment of glyphosate. In accordance with gene expression data, no injury symptoms were observed following glyphosate treatment of resistant soybeans under the experimental conditions (**Figure 2**). The lack of glyphosate symptoms contrasts to those reported by Reddy et al. (*52*); however, this may be explained by the 9-fold increased application rate of glyphosate when compared to this experiment.

Genes differentially expressed in glyphosate-resistant soybean included a cytochrome P450 homologue similar to CYP82A3 at 1 hpt. This differentially expressed P450 was also increased 10-fold in abundance when glyphosate was applied to the glyphosate-sensitive soybeans at 4 and 24 hpt (**Table 3**). As mentioned above, the up-regulation of cytochrome P450s is considered to be a probable detoxification attempt in response to the glyphosate treatment. It appears that a possible cause of the minor changes in gene expression induced by glyphosate in glyphosate-resistant soybeans would be a nonspecific response to xenobiotics.

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Experiment 4: Differences between Cotyledons of Developing Seeds from Glyphosate-Resistant and -Sensitive Soybean. To determine expression differences between the transgenic glyphosate-resistant and -susceptible isogenic lines of soybean, the transcriptomes of cotyledons from developing seeds were examined. Developing seed tissue in the range of 175−200 mg was selected because the seeds at this stage are in the midmaturation period of rapid seed fill and are highly active both biosynthetically and transcriptionally (*53*).

Experiment 4 identified only 2 genes of 27000 that were differentially expressed between cotyledons of developing seeds of glyphosate-resistant and glyphosate-sensitive soybeans in the absence of treatment. One of these differentially expressed genes (Gm-r1070-3524) had no homologues in NCBI and was slightly increased in abundance (1.4-fold). The second gene, an Xa21 receptor kinase-like homologue, was also slightly affected, showing a 0.7-fold change in expression level. On the basis of the within-cultivar test of variation that experimentally determined the fdr to be 1/9216 or 0.011%, identifying 2 genes of 27000 (0.007%) falls within the frequency of our empirical fdr and casts uncertainty on the differential expression of these 2 genes. Therefore, the differences between glyphosate-resistant and -sensitive seeds at the level of gene expression were negligible under the experimental conditions. These results are consistent with previous reports describing the composition of glyphosate-resistant soybean seeds as being equivalent to that of conventional seeds (*10, 11*). Our results also are supported by an Affymetrix-based analysis of the differential expression of 37000 genes between leaves of glyphosate-resistant transgenic and conventional soybean cultivars (*54*). Principal component analysis of the Affymetrix expression data revealed that the glyphosate-resistance transgene had no significant effect on global gene expression beyond the natural variation observed in nontransgenic, glyphosate-sensitive cultivars. Further evidence that the gene expression effects were random and nonspecific is provided in that none of the few genes identified as being significantly differentially expressed in each study were significant across both studies.

Verification of Microarray Data with QRT-PCR. To verify the microarray results, six genes showing differential regulation in the glyphosate-sensitive soybean were furthered studied with qRT-PCR. The abundance of gene transcripts was determined using the same biological samples used in the microarray studies. Two biological replicates with three technical replicates of each treatment were conducted, and the data were presented as the average of the two biological replicates (**Figure 3**). The qRT-PCR results displayed similar trends of up- or down-regulation to that of microarray results, supporting the validity of the microarray data. The selected genes with increased transcript levels included a cytochrome P450 that was induced at both 4 and 24 hpt in glyphosate-sensitive soybeans treated with glyphosate. The other genes with increased abundance at 4 and 24 hpt chosen for verification by qRT-PCR included those encoding enolases (Gm-r1021-880), Ca^{2+}-binding EF-hand (Gm-r1070-5838), and the eukaryotic translation initiation factor 6 (EIF-6)-like (Gm-r1070-8746).

Four genes with a decreased transcript level in response to glyphosate in glyphosate-sensitive lines were also selected for qRT-PCR experiment; however, due to the inability to design specific primers, only two yielded successful amplification with qRT-PCR. One of them was a defense-related R gene, Xa21 binding protein (Gm-r1088-3841), and the other a pathogenesis resistance gene (Gm-r1088-8829). Both of the genes were
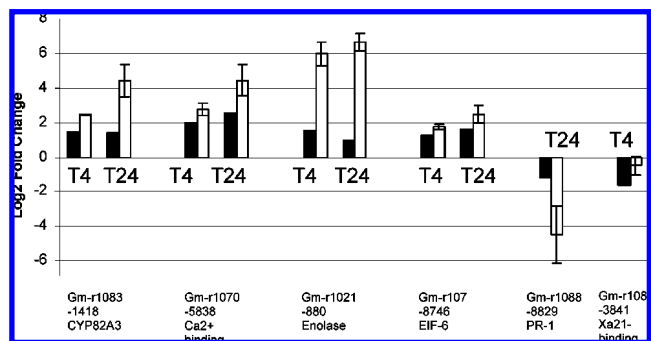

Figure 3. Verification of microarray results by qRT-PCR. Six genes with differentially regulated transcript levels in the glyphosate-sensitive soybean from microarray experiments were further analyzed using qRT-PCR. Black bars represent microarray results, and white bars represent qRT-PCR results. Expression levels are given as log2-transformed fold change of glyphosate-treated soybeans when compared with the formulation blank. QRT-PCR data represent the average values obtained from two independent biological replicates. Error bars represent the standard errors of the two replicates.

confirmed to be reduced at the corresponding time point as in the microarray study.

In summary, cDNA microarrays provided insights into the responses of soybean to glyphosate. As expected, glyphosate-sensitive soybean responded rapidly to glyphosate, with 170 genes having altered transcript abundance by 4 hpt. In contrast, transcript changes were minor and at or near the empirical fdr either when glyphosate-resistant soybeans were treated with glyphosate or when transcriptomes of cotyledons of developing seeds were compared between glyphosate-resistant and -sensitive soybean. The results of this study indicate that there are few, if any, unexpected transcriptome consequences associated with the use of transgenic, glyphosate-resistant soybean in production agriculture.

ABBREVIATIONS USED

AMT, anthocyanin rhamnosyl transferase; ADR, auxin down-regulated genes; APs, ascorbate peroxidases; AS, anthranilate synthase; CCR, cinnamoyl-CoA reductase; cDNA, copy DNA; CHS, chalcone synthase; CHR, chalcone reductase; CATs, catalases; DAHP, 3-desoxyarabinoheptulosenate-7-phosphate; DHS, 2-keto-3-deoxy-D-arabino-heptonic acid 7-phosphate synthetase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; EST, expressed sequence tag; fdr, false-discovery rate; GSSH, glutathione; GST, glutathione-*S*-transferase; hpt, hours post-treatment; PAT, phosphoribosylanthranilate transferase; qRT-PCR, quantitative real-time reverse-transcribed Polymerase Chain Reaction; TS, tryptophan synthase.

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Supporting Information Available: Supplemental Table 1: all cDNA clones that showed differential abundance during the experiment, including significance and fold-change level; included are annotations from 5′ and 3′ ESTs across multiple databases and assignment to functional categories. This material is available free of charge via the Internet at http://pubs.acs.org.

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