Effects of Photosystem-II-Interfering Herbicides Atrazine and Bentazon on the Soybean Transcriptome

Jin Zhu, William L. Patzoldt, Osman Radwan, Patrick J. Tranel, and Steven J. Clough*

Abstract
Atrazine and bentazon are both photosystem-II (PSII)–inhibiting herbicides that interfere with photosynthetic electron transport, provoking oxidative stress. While atrazine is lethal to soybean (Glycine max [L.] Merr.), bentazon does not kill soybean because of the capability of soybeans to metabolize the herbicide. Gene expression profiling was conducted using cDNA microarrays to understand the responses of soybeans to PSII interruption and concomitant stress caused by atrazine and bentazon by monitoring expression at 1, 2, 4, and 8 h after treatment (HAT). The microarray study revealed that 6646 genes were differentially expressed with high statistical significance over the experiment, with 88% of them sharing similar expression pattern between the atrazine and bentazon treatments. Many genes related to xenobiotic detoxification and antioxidation, such as cytochrome P450s, glutathione-S-transferases, superoxide dismutases, catalases, and tocophero cyclases, were induced by the herbicides. The study also discovered plants treated with bentazon started to recover between 4 and 8 HAT as reflected in the decreased amplitude of fold changes of most genes from 4 to 8 HAT. The 12% of the genes that were differentially expressed between atrazine and bentazon were largely related to cell recovery, such as genes related to ribosomal components.

Herbicides Control Weeds by inhibiting a variety of metabolic systems and are categorized based on their mode of action. A significant portion of commercial herbicides target the inhibition of essential plant-specific processes, such as photosynthesis, to minimize possible harmful effects on humans and the environment (Ashton and Crafts, 1973). For example, several herbicides specifically interrupt photosystem II (PSII) by antagonizing plastoquinone function and inhibiting electron transport (Hess, 2000).

One common PSII-inhibiting herbicide is atrazine (2-chloro-4-ethylamino-6-isopropyl amino-1,3,5-triazine). Atrazine is effective for the control of many broadleaf and grass weeds (Gast, 1970). Atrazine inhibits photosynthesis by competitively associating with the plastoquinone B (Q$_b$) binding site of the D1 subunit of PSII, and therefore blocks chloroplast electron flow from plastoquinone A to Q$_b$, greatly reducing the production of ATP, nicotinamide adenine dinucleotide phosphate

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Abbreviations: APX, ascorbate peroxidase; cytochrome P450; cytochrome P450 monoxygenase; EST, expressed sequence tag; GPX, glutathione peroxidase; GST, glutathione-S-transferase; HAT, hours after treatment; NAD(P)H:quinone oxidoreductase; PSI, photosystem I; PSII, photosystem II; Q$_b$, plastoquinone B; qRT-PCR, quantitative real-time reverse-transcribed polymerase chain reaction; ROS, reactive oxygen species; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.
(NADPH), and CO\(_2\) fixation (Mullet and Arntzen, 1981; Steinback et al., 1981; Hess, 2000). However, studies have shown that plant death caused by atrazine is not the direct result of starvation. Instead, it is due to the toxic effect of the rapid accumulation of a number of highly reactive radicals, including triple-state chlorophyll and singlet oxygen, that result from the blockage of photosynthetic electron transfer (Bowyer et al., 1991; Hess, 2000; Rutherford and Krieger-Liszky, 2001). This rapid and prolonged production of reactive oxygen species (ROS) exceeds the quenching capacity of photo-protective components such as carotenoids, xanthophylls, and tocopherol in the vicinity of PSII (Krasnovsky, 1998; Trebst, 2003; Loll et al., 2005), causing severe oxidative damage of proteins, lipids, and pigments, and eventually cell membrane destruction and plant death (Hess, 2000).

Bentazon [3-isopropyl-2,1,3-benzothiadiazin-4-one-2,2-dioxide (3-(methylthio)-(1H)-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide)] is another PSII inhibitor and is primarily a postemergence herbicide used for selective control of broadleaf weeds in crop production, such as for soybean [Glycine max (L.) Merr.], rice [Oryza sativa L.], corn [Zea mays L.], and peanut [Arachis hypogaea L.] (Mine et al., 1975; Han and Wang, 2002). Bentazon is generally believed to interact at the same site of PSII as atrazine; however, bentazon may have a different binding site in the Q\(_b\) niche or binding association than that of atrazine according to competitive binding experiments (Nimbal et al., 1996). Indeed, it was recognized that atrazine inhibits photosynthesis at different PSII sites according to revised classification of herbicides by site of action (Mallory-Smith and Retzinger, 2003).

The tolerance of plants to herbicides depends on a variety of physiological processes, including uptake, translocation, and metabolism. Atrazine is readily absorbed into leaves, but there is essentially no basipetal translocation out of the treated leaves if applied as a foliar application (Vencill, 2002). Metabolism of atrazine in the leaves involves multiple mechanisms, varying with plant species (Vencill, 2002). In tolerant corn, the primary factor providing tolerance is the activity of a primary factor providing tolerance is the activity of a soluble glutathione S-transferase (GST) that detoxifies atrazine by forming an atrazine–glutathione conjugate (Shimabukuro et al., 1971). Glutathione S-transferase enzymes have been shown to detoxify various herbicides in different plant species such as wheat (Triticum aestivum L.), black grass (Alopecurus myosuroides Huds.), and soybean (Romano et al., 1993; Tal et al., 1993; Cummins et al., 1997). Meanwhile, in soybean, which is highly susceptible to atrazine, detoxification has been noted in some resistant plants, involving the dealkylation of atrazine by producing a nonlethal isopropyl or ethyl derivative (Shimabukuro et al., 1970).

Bentazon is readily absorbed by leaves, with minimal basipetal translocation following foliar application (Vencill, 2002). Like atrazine, the absorption and translocation rate also depends on plant species. In tolerant plants, the absorption and translocation of bentazon may be slower than in susceptible plants (Bradshaw et al., 1992). However, tolerance to bentazon seems to be largely due to increased metabolic breakdown (Sterling and Balke, 1989). To date, most of the studies on bentazon metabolism focused on detoxification via cytochrome P450 monooxygenases (cytochrome P450) that hydroxylate bentazon into 6-hydroxyl bentazon or 8-hydroxyl bentazon (or both), which can be further conjugated with glucose to generate glucosyl bentazon (Sterling and Balke, 1989; Gronwald and Connelly, 1991; Burton and Maness, 1992). For soybean, plant tissue is initially damaged by bentazon, but then the plants rapidly adjust to tolerate the chemical because of induction of genes involved in glucoside conjugation of acyl hydroxybentazon, which is not toxic to plants (Sterling and Balke, 1989).

Most of the physical and biochemical studies on herbicide-plant interactions focus on their specific metabolism and detoxification pathways in plants. A limited number of studies have been conducted to monitor molecular changes induced by the stress of an herbicide, especially on a genomic or proteomic scale. Changes at a proteome level in response to the herbicide flumioxazin, which inhibits protoporphyrinogen oxidase, was studied in grapevine (Vitis vinifera L.) plants (Castro et al., 2005). The flumioxazin-induced stress led to an accumulation of a number of pathogenesis-related proteins in both roots and shoots. Additionally, photosynthesis-related proteins and their breakdown products such as RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), the oxygen-evolving enhancer protein, and light harvesting complex of PSII type III chlorophyll \(a/b\) binding protein were identified. Meanwhile, induction of several enzymatic antioxidant systems was observed, indicating an oxidative stress caused by the herbicide (Castro et al., 2005). An investigation of the transcriptomic response to glyphosate on sensitive and resistant soybean was conducted (Zhu et al., 2008). In glyphosate-sensitive plants, the most consistent genes changing during a 24-h time course were those related to protein turnover, gene regulation, cytochrome P450 chemistry, glycolysis, and calcium signaling, whereas glyphosate induced no major transcriptomic change in glyphosate-resistant soybean. Transcriptomic survey studies can also be an effective tool to discover and classify new herbicides (Eckes et al., 2004). Overall, gene expression profiling is a powerful tool to understand plant responses to herbicides at the molecular level.

Soybean microarray technology has been used to monitor gene expression in a variety of studies, such as different tissues (Maguire et al., 2002), tissue development (Thibaud-Nissen et al., 2003), in response to pathogens (Moy et al., 2004; Zou et al., 2005; Alkharouf et al., 2006; van de Mortel et al., 2007), insect feeding (Casteel et al., 2008; Li et al., 2008), and symbiont interaction (Brechemmacher et al., 2008), as well as in response to the abiotic factors carbon dioxide (Ainsworth et al., 2006), ozone (Bilgin et al., 2008), and glyphosate (Zhu et al., 2008). By taking advantage of the soybean microarray...
technology, we conducted a large-scale investigation of transcription levels of ~36,000 genes in soybean leaf tissue treated with atrazine or bentazon, to understand the effect caused by PSII-inhibiting herbicides, including lethal (atrazine) and nonlethal (bentazon) herbicides. We expected that atrazine and bentazon would initially cause nearly identical transcriptome changes because they share the same protein target. Because soybean is tolerant to bentazon, however, we also expected to observe leaf tissue recovery from the effect of bentazon with time. The present microarray study identified 6646 genes significantly increased or decreased due to treatment, with results consistent with the above stated expectations. A majority of the 6646 genes (88%) were modulating in the same or similar manner whether the treatment was atrazine or bentazon, and the experiment captured the period of tissue recovery from bentazon treatment as reflected in decreases in the fold-change amplitude of the majority of modulating genes between four and eight hours after treatment (HAT). In addition, several hundred genes showed distinct differential gene expression patterns between atrazine and bentazon treatment. By dissecting common and distinct gene expression patterns between these two PSII-interfering herbicides, a deeper understanding of the molecular mechanism of soybean tolerance to bentazon and susceptibility to atrazine at the transcriptional level was attained.

Materials and Methods

Plant Materials and Herbicide Treatment

Soybean seeds (cultivar William 82) were germinated in a greenhouse at the University of Illinois Urbana-Champaign campus at ~25°C under a 16-h light regime with three seeds in a pot. Pots were thinned to contain just a single plant before the unifoliate stage. When the first trifoliate leaves were fully expanded, plants were placed in a chemical mist chamber and sprayed with atrazine or bentazon dissolved in commercial adjuvant (Herbimax, Loveland Industries, Greeley, CO) at 1% (v/v), or control (commercial adjuvant at 1% [v/v]), and returned to the greenhouse bench within 5 min. The dosage of herbicide was comparable to that used commercially, which was 1.1 kg ha⁻¹ (0.45 kg a.i. acre⁻¹; equivalent to ~22–25 mM). Treatments were applied using a compressed air, moving-nozzle laboratory sprayer equipped with an 80° flat-fan nozzle (Teetjet, Spray Systems Inc., Wheaton, IL), and the nozzle was maintained approximately 45 cm above the plant canopy. One set of trifoliate leaflets was collected for RNA extraction at time points 1, 2, 4, and 8 HAT. Three biological replicates from individual plants were collected for each treatment.

cDNA Microarray Analysis

The microarray study, including RNA extraction and labeling, was conducted essentially as described previously (Zou et al., 2005). Frozen tissue in liquid nitrogen was ground to a powder and total RNA extracted by treatment with TRIzol reagent (Invitrogen, Carlsbad, CA) in tubes containing Phase Lock Gel-Heavy (Brinkmann Instruments, Inc., Westbury, NY). RNA samples were checked with a BioAnalyzer 2100 (Agilent Technologies, Palo Alto, CA) to verify that RNA had not degraded, and their concentration determined with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Approximately 30 μg of purified (RNasey kit, Qiagen, Valencia, CA) total RNA was reverse transcribed, incorporating amino-allyl-dUTP (Sigma, St. Louis, MO) into cDNA using SuperScript III (Invitrogen). Purified (Qiagen PCR clean-up kit) cDNA was fluorescently labeled with Cy3 or Cy5 monofunctional dyes (Amersham Pharmacia Biotech, Piscataway, NJ), cleaned (Qiagen PCR clean-up kit), and hybridized to a two-set cDNA microarray with 36,760 different cDNA clones (Vodkin et al., 2004). Hybridization, washing, scanning, image analysis, and data acquisition were performed as previously described (Zou et al., 2005).

A loop design was utilized (Supplementary Fig. 1), involving three treatments (atrazine, bentazon, and control) and four time points (1, 2, 4, and 8 HAT). The 12 samples were replicated (separate plants) three times and hybridized on two slide sets, for a total of 72 hybridizations. Microarray data were collected, formatted, and cleaned using in-house PERL programs that flagged spots where the combined Cy3 and Cy5 values were less than the combined average of the negative control spot, a human myosin cDNA (GeneBank ID X13899), following general analysis procedures outlined in Brechenmacher et al. (2008). Raw median spot intensity values were normalized in the freeware program R running the maanova module (Wu et al., 2003) and the GLOWESS procedure. Normalized intensity values were statistically analyzed using the following model:

\[
\text{log}_{10}\text{intensity} = Dye + \text{Treatment} + \text{Time} + \text{Time} \times \text{Treatment} + E
\]

by running the PROC MIX command in SAS v9.1 (SAS Institute, Cary, NC). The overall treatment effect was used to define transcripts as being significantly changing in response to either atrazine or bentazon using a false discovery rate (fdr) (Benjamini and Hochberg, 1995) corrected P-value cutoff of ≤0.005. Least-squares means were calculated and used to determine expression ratios after log₂ transformation. Significance of direct comparisons between two samples was determined using t test and an fdr-corrected P-value cutoff of ≤0.005.

Quantitative RT-PCR

Quantitative real-time reverse-transcribed polymerase chain reaction (qRT-PCR) was performed essentially as described by Zhu et al. (2008). Briefly, 2.5 μg of purified total RNA was reverse transcribed into cDNA, which was subsequently quantified by real-time RT-PCR using an Mx3005p Thermal Cycler (Stratagene, La Jolla, CA). The primers for the target genes (Supplementary Table S1) were designed based on either The Institute for Genomic
Research (TIGR) tentative consensus sequences (http://compbio.dfci.harvard.edu/tgi/ [verified 6 May 2009]) with emphasis on the unique oligomer region, or the singleton expressed sequence tag (EST) sequence, using Primer 3.0 software (Rozen et al., 2000). 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 the forward primer 5′-TCCAAGGGGACCTAACGGAGA-3′ and reverse primer 5′-TGTTGTCAGAGCTGGATGGT-3′. The specificity of the primers was verified by the presence of a single peak in the dissociation curve analysis after the qRT-PCR run and sequencing the RT-PCR products.

The qRT-PCR data were analyzed using the relative quantification $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Three biological and three technical replicates were conducted on every comparison between treatment and control.

Results and Discussion

General Responses to the Herbicides

Soybean plants treated with atrazine displayed severe chlorosis and necrosis by 24 HAT and plants appeared to be dead by 48 HAT (data not shown). Plants treated with bentazon showed symptoms of chlorosis and necrosis by 24 HAT but rapidly recovered, showing healthy new growth by 48 HAT (Fig. 1).

Our interest was to acquire gene expression changes that occurred well before visible symptoms manifested. Therefore, a microarray analysis was conducted on trifoliolate leaflets collected at 1, 2, 4, and 8 HAT. Statistical analysis of the data identified 6646 gene transcripts as being significantly increased or decreased in abundance due to the application of atrazine or bentazon across all time points (detailed in Supplementary Table S2), some of which are detailed and discussed below. To determine gene annotation calls for a given spotted cDNA, we analyzed annotation details obtained from National Center for Biotechnology Information and TIGR tentative consensus corresponding to both 5′ and 3′ EST sequences, as the cDNA that were used as polymerase chain reaction templates to construct the microarrays have both 5′ and 3′ EST annotations. To simplify discussion, all spotted cDNA are referred to as genes.

The majority of the differentially expressed 6646 genes showed similar expression patterns between atrazine and bentazon treatment during the 8-h time course as depicted by Fuzzy K-means clustering (Fig. 2) (Gasch and Eisen, 2002). Separating the Fuzzy K-means clusters into one for atrazine and one for bentazon (Fig. 3) showed that plant tissue treated with bentazon began to recover by 8 HAT, as fold-change values of many modulating genes decreased to values more similar to control levels (reflected in the lower intensity of the colors in the heat map).

Herbicides imposed a profound overall impact as seen by differential expression of 525 primary metabolism-related genes. The 525 genes were distributed in a variety of subcategories including amino acid, carbohydrate, lipid, nucleic acid, nucleotide-sugar, and nitrogen metabolism. The regulation included decreased transcript level of methionine synthase and S-adenosylmethionine synthetase in amino acid metabolism; increased gene transcript level of aldehyde dehydrogenase, beta-amylase, beta-D-xylosidase, and galactosidase in carbohydrate metabolism; and increased gene expression of acetyl-CoA carboxylase and beta oxidation in lipid metabolism.

Photosynthesis Regulation Induced by Photosynthesis Inhibitors

Both atrazine and bentazon are PSII inhibitors associated with interruption of electron flow from PSII to photosystem I (PSI). Interrupting electron transfer and disturbing photosynthesis would presumably signal a coordinated expression of photosynthetic genes. The present experiment provoked significant modulation of 45 photosynthesis-related genes in common between the two herbicide treatments (Supplementary Table S3).

The majority of the modulating photosynthetic genes were associated with light reaction components, such as PSI and PSII. One gene encoding PSI-associated ferredoxin (Gm-r1088-3390) increased in both atrazine and bentazon treatments. In PSII, gene expression of five type I chlorophyll a/b binding proteins and two type III chlorophyll a/b binding proteins generally decreased in both herbicide treatments. Transcripts encoding PSI subunit proteins, psbR (Gm-r1083-1311) and psbS (Gm-r1088-4787) increased in abundance, and the oxygen evolving complex (OEC23, Gm-r1070-1025) transcript also increased (Table 1).

FtsH proteins in plants are a family of ATP-dependent metalloproteinase homologs to bacterial FtsH and are a family of proteases involved in clean-up proteolysis of unassembled, misassembled, or unfolded proteins (Ostertzter and Adam, 1997; Suno et al., 2006). In chloroplasts, FtsH proteases have been associated with removal of unassembled Rieske Fe-S protein, as well as in the secondary cleavage of D1, a key step required for D1 turnover and PSI maintenance (Spetea et al., 1999; Silva et al., 2003; Suno et al., 2006). In response to the D1-interfering herbicides, we observed six cDNAs encoding FtsH or FtsH-like proteases with altered expression levels, with four increased and two decreased in response to treatment. The transcript reduction of FtsH was observed in plants experiencing hypersensitive responses in tobacco (Nicotiana tabacum L.) (Seo et al., 2000), Nicotiana benthamiana Domin (Matsumura et al., 2003), and soybean (Zou et al., 2005). The six FtsH homologs showing differential expression in the present study have yet to be functionally characterized. Of these six FtsH homologs, only one (Gm-r1070-8221) is considered as a putative homolog to the chloroplastic FtsH protease, Variegated 2, which is involved in repair of PSII components (Takechi et al., 2000), and this FtsH homolog decreased in response to the herbicides (Table 1). It is assumed that a decreased transcript abundance of the chloroplastic...
FtsH protease would reflect a reduced rate of D1 protein turnover when D1 is bound with atrazine or bentazon.

In addition to genes associated with the light reactions, 12 genes involved in the Calvin cycle significantly modulated in response to both herbicides. While three genes encoding RuBisCO activase (Gm-r1089-3620, Gm-r1089-2123, and Gm-r1089-2196) and two encoding RuBisCO-associated proteins (Gm-r1089-3694, Gm-r1021-1539) displayed mixed expression patterns, two encoding RuBisCO methylase (Gm-r1070-1002, Gm-r1089-6111) increased across all time points. Meanwhile, one gene encoding carbonic anhydrase 2 (Gm-r1070-4267), an enzyme enriching CO2 concentration to promote carbon fixation (Fett and Coleman, 1994), significantly increased as well as one gene encoding NADP+ glyceraldehyde-3-phosphate dehydrogenase, the enzyme catalyzing the production of NADPH (Table 1).

Herbicide Detoxification-Related Gene Modulations

The tolerance of plants to herbicides depends mainly on the ability to metabolize and detoxify the toxic chemicals. Metabolism of herbicides may involve attempts to chemically modify the toxins into less toxic substances through conversion and conjugation reactions (Shimabukuro, 1985; Hatzios, 1991, 1997). Many significantly modulated genes may have been related to possible attempts at herbicide conversion (e.g., cytochrome P450s) and conjugation (e.g., GSTs).

Herbicide metabolism includes chemical oxidation, reduction, or hydrolysis, resulting in a molecule that becomes more water soluble and, most likely, less toxic than the parent herbicide itself (Hatzios, 1997). The primary class of enzymes functioning in herbicide metabolism is cytochrome P450–dependent monooxygenase, which is involved in substrate oxidation (Hatzios, 1997; Barrett, 2000). Cytochrome P450s are a large group of heme-containing proteins playing an important role in the oxidative metabolism of various substances (Schuler, 1996; Chapple, 1998). The first report regarding cytochrome P450 metabolism of an herbicide was on the metabolism of monuron by a microsomal fraction in cotton (Gossypium hirsutum L.) seedlings (Frear et al., 1969). In the subsequent decades, the role of cytochrome P450 in herbicide detoxification has been well established. The examples include their involvement in detoxification of chlortoluuron in wheat, corn, and cotton (Cole and Owen, 1987; Mougin et al., 1990); pyrazosulfuron ethyl in rice (Yun et al., 2001); and 2,4-dichlorophenoxyacetic acid in wheat cells (Mougin et al., 1991). In soybean, an in vivo study suggests that cytochrome P450s are involved in the metabolism of bentazon (Sterling and Balke, 1990) and the expression of a soybean cytochrome P450 (CYP7A10) in yeast (Saccharomyces cerevisiae) and tobacco enhanced the metabolism of phenylurea herbicides including fluometuron, linuron, chlortoluuron, and diuron by converting them into more polar compounds (Siminszky et al., 1999). Microarray studies on Arabidopsis have shown that 29 out of 49 cytochrome P450 genes increased in abundance at the transcript level due to biotic and abiotic stresses, including the stress induced by the herbicide paraquat (Narusaka et al., 2004). In the present study, 54 cDNAs annotated as cytochrome P450s showed differential expression in response to both atrazine and bentazon treatment and half of them were increased in abundance (Supplementary Table S2). Due to the enormous diversity of cytochrome P450 isofoms and unknown substrates or specific functions for a large number of these enzymes, it is not clear from transcriptomic data alone which, if any, cytochrome P450s might be utilizing atrazine or bentazon as a substrate in our study.

After the conversion phase of detoxification efforts, plants have a battery of enzymes that could make additional detoxification steps through chemical conjugation with a sugar, amino acid, or glutathione. The conjugated form of the herbicide may result in increased water solubility that might enhance subsequent compartmentalization. Glutathione (GSH) conjugation via GSTs is one of the most studied mechanisms of herbicide conjugation. Glutathione S-transferases are multifunctional enzymes
that are capable of catalyzing the conjugation of the tripeptide GSH to a variety of hydrophobic, electrophilic, and usually cytotoxic substrates (Pickett and Lu, 1989). It was first shown nearly 40 years ago that GSH conjugation is responsible for atrazine detoxification in corn and sorghum [Sorghum bicolor (L.) Moench] (Frear et al., 1969; Lamoureux et al., 1970; Shimabukuro et al., 1971) and, since then, plant GSTs have been intensively studied because of their ability to detoxify various herbicides and confer herbicide tolerance in many crop plants (Shimabukuro et al., 1971; Marrs 1996; Cummins et al., 1997; Dixon et al., 1998; McGonigle et al., 2000). For atrazine-tolerant plants such as sorghum and corn, GSTs catalyze atrazine–GSH conjugation to convert atrazine into a nontoxic and water-soluble form, contributing substantially to detoxification of atrazine (Shimabukuro et al., 1971). Our study identified 26 GSTs differentially expressed due to herbicide treatment with 24 GSTs strongly increased in bentazon, and 20 GSTs in atrazine treatment (Supplementary Table S4). It has been reasoned that atrazine-sensitive plants such as pea (Pisum sativum L.), oat (Avena sativa L.), and many broadleaf weeds do not have similar GST activity (Marrs, 1996). Our study indicates that atrazine-sensitive soybean increase expression of many GSTs, but none capable of adequately nullifying the toxic effect of atrazine.

In addition to detoxifying foreign chemicals, GSTs also function on endogenous substrates such as phenylpropanoids and other secondary metabolite products in plants. Marrs (1996) suggested that some naturally synthesized plant metabolites can be recognized, transported, and metabolized in similar ways as herbicides and other xenobiotics. It was observed in maize that GSTs catalyze the GSH conjugation to anthocyanin, cinnamic acid, and other phenylpropanoids that may be cytotoxic (Dean et al., 1995; Marrs et al., 1995). In our study, the application of herbicides significantly affected 170 genes involved in secondary metabolism, with most of them decreasing in transcript levels, such as those playing a role in the anthocyanin, flavanone, lignin, and terpene synthesis pathways. Strikingly, seven genes encoding phenylalanine ammonia-lyase, a rate-limiting enzyme in the entrance of phenylpropanoid pathway, were all reduced in abundance in both herbicide treatments. Similar decreases occurred for chalcone synthase, chalcone reductase, and chalcone isomerase (Supplementary Table S2). Induction of phenylpropanoid products has been found in plants under a variety of environmental stresses, such as cold, ultraviolet light, and pathogen attack (Christie et al., 1994; Solec, 1997; Zabala et al., 2006). It is interesting to see an extensive reduction of phenylpropanoid biosynthesis–related genes in this study. The possible explanation is that it may be beneficial for plants to reduce GST alternative substrate pool and, hence, increase the availability of GSTs for the detoxification of the toxic herbicides. In addition, the reduced production of these energy-costly secondary metabolites would make more energy available for xenobiotic detoxification if needed. The reduction of this universal biotic stress-responsive pathway suggests plants may distinguish biotic stress from xenobiotic stresses.

### Activated Antioxidant Systems on Herbicides Treatment

Oxidative stress, not nutrient starvation, is commonly recognized as the most damaging stress induced by photosynthesis-inhibiting herbicides and the cause of cell death (Hess, 2000; Rutherford and Krieger-Liszkay, 2001). A primary ROS produced in plants treated with atrazine and bentazon is triplet chlorophyll, which is relatively long lived and has the potential to react with O2 to form toxic singlet oxygen (Rutherford and Krieger-Liszkay, 2001; Krieger-Liszkay, 2005). In PSII, the quenching of singlet oxygen is mainly performed by tocopherols existing in the neighborhood of PSII (Hess, 2000; Telfer, 2002; Krieger-Liszkay, 2005). Our study identified one gene encoding tocopherol cyclase (Gm-r1070-3553), an enzyme catalyzing the biosynthesis of tocopherol (Porfiriova et al., 2002; Kanwischer et al., 2005), strongly induced through all time points in both atrazine and bentazon treatments. As tocopherol is the major singlet oxygen scavenger in PSII of the thylakoid membranes (Porfiriova et al., 2002; Kruk et al., 2005), the increased transcription of tocopherol further supports a positive response of plants to severe oxidative stress in PSII induced by the herbicides.

The derivative radical products of triplet chlorophyll and singlet oxygen are the lipid radicals or lipid...
Table 1. Significant genes specifically mentioned in the text.

<table>
<thead>
<tr>
<th>CloneID</th>
<th>Annotation†</th>
<th>Log₂-fold change in gene expression vs. control</th>
<th>Atrazine</th>
<th>Bentazon</th>
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<tr>
<td></td>
<td></td>
<td>1 HAT</td>
<td>2 HAT</td>
<td>4 HAT</td>
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<td>−1.19</td>
<td>−1.26</td>
<td>−1.73</td>
</tr>
<tr>
<td>Gm-r1070-3553</td>
<td>Tocopherol cyclase</td>
<td>2.16</td>
<td>2.29</td>
<td>3.18</td>
</tr>
<tr>
<td>Gm-r1070-4267</td>
<td>Carbonic anhydrase 2</td>
<td>0.59</td>
<td>0.57</td>
<td>1.20</td>
</tr>
<tr>
<td>Gm-r1070-6041</td>
<td>dirg1</td>
<td>−0.08</td>
<td>0.22</td>
<td>1.72</td>
</tr>
<tr>
<td>Gm-r1083-3000</td>
<td>Phytochelatin synthetase</td>
<td>−0.38</td>
<td>−0.67</td>
<td>−1.17</td>
</tr>
<tr>
<td>Gm-r1083-3439</td>
<td>Phytochelatin synthetase</td>
<td>0.57</td>
<td>0.39</td>
<td>0.30</td>
</tr>
<tr>
<td>Gm-r1083-3508</td>
<td>Phytochelatin synthetase</td>
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<td>0.56</td>
<td>0.22</td>
</tr>
<tr>
<td>Gm-r1083-4126</td>
<td>Phytochelatin synthetase</td>
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<td>−1.93</td>
</tr>
<tr>
<td>Gm-r1083-6089</td>
<td>Superoxide dismutase</td>
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<td>0.23</td>
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<tr>
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<td>CYP89A2</td>
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<td>0.56</td>
<td>0.22</td>
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<tr>
<td>Gm-r1083-6502</td>
<td>GST 22</td>
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<td>0.22</td>
<td>0.60</td>
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<tr>
<td>Gm-r1083-7470</td>
<td>Glutathione peroxidase</td>
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<tr>
<td>Gm-r1083-7497</td>
<td>CYP94A1</td>
<td>0.25</td>
<td>1.06</td>
<td>1.71</td>
</tr>
</tbody>
</table>

†NADPH, nicotinamide adenine dinucleotide phosphate; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.
‡HAT, hours after treatment.
peroxides, which is the origin of membrane destruction as seen in plants as necrosis caused by herbicides (Hess, 2000). Lipid peroxides are highly cytotoxic and they can be eliminated by tocopherols (Munne-Bosch and Alegre, 2002) or by GSTs via GSH conjugation (Bartling et al., 1993). In addition to the above-mentioned ability of GSTs to use xenobiots as substrates and directly detoxify them, it has been shown that GSTs can protect plants against general oxidative stresses generated from herbicides, ozone, and pathogen attack (Mauch and Dudler, 1993; Sharma and Davis, 1994). It has been hypothesized that plant GST gene promoters have multiple regulatory elements that respond differently to specific or more general stress-related signals (Droog, 1997). Moreover, GSTs probably have common mechanisms of signal transduction to activate gene expression (Tenhaken et al., 1995). Another antioxidant gene, glutathione peroxidase (GPX; Gm-r1083-1294), also responded to the herbicide treatment. Glutathione peroxidases are involved in reducing hydrogen peroxide and inducing GSH synthesis by feedback control (Ursini et al., 1995; Arthur, 2000). It was discovered that transgenic bacteria with a citrus [Citrus sinensis (L.) Osbeck] GPX-like gene displayed increased survival rates in the presence of paraquat (Holland et al., 1994). Glutathione peroxidase also showed increased expression when plants were under various abiotic and biotic stresses (Criqui et al., 1992; Sugimoto and Sakamoto, 1997; Depege et al., 1998; Milla et al., 2003). Additionally, GPXs are considered to be up-regulated by singlet oxygen but only weakly expressed by exposure to superoxide or peroxide (Leisinger et al., 2001).

Our study identified several antioxidant enzymes directly involved in the scavenging of ROS (Table 1): superoxide dimutase (SOD; Gm-r1070-9021 and Gm-r1088-6809), an important enzyme detoxifying ROS in almost all cellular compartments by converting superoxide to H$_2$O$_2$ (Mittler et al., 1995); thioredoxin peroxidase (Gm-r1021-1467); catalase (e.g., Gm-r1088-8229), which reduces H$_2$O$_2$ (Baier and Dietz, 1996); and alternative oxidase (e.g., Gm-r1088-6638), an oxidase diverting electron flow through electron transport chains and reducing O$_2$ to water in mitochondria and chloroplasts (Vanlerberghene and McIntosh, 1997; Rizhsky et al., 2002). We also noted an increased abundance of gene transcripts associated with protection from oxidative stress through disulfide oxidoreductase, glutaredoxin, and thioredoxin (Moller, 2001; Buchanan and Balmer, 2005; Cheng et al., 2006).

Interestingly, seven ascorbate peroxidases (APXs) were decreased in both atrazine and bentazon treatment. Ascorbate peroxidases play key roles to convert H$_2$O$_2$ into water in various subcellular locations. Reduced abundance of APX in herbicide treatment was also found when soybean was treated with glyphosate (Zhu et al., 2008). The reason behind the reduction of APX transcript is not well understood; one possibility is that catalases are located in peroxisomes and these seven APXs are cytosolic; therefore, the subcellular location of APXs might be reflected in the expression patterns. Consistent with this hypothesis, it was found in the present study that one peroxisomal APX displayed increased abundance in both atrazine and bentazon treatment (Supplementary Table S5).

**Herbicide-Induced Defense, Stress, and Senescence Responses**

This study identified 145 defense-related genes differentially expressed due to the herbicide treatments, and 90% of them shared a similar trend between atrazine and bentazon treatment with both increases and decreases in gene expression. The transcripts that increased across most of the time points included five genes encoding snakin, a cysteine-rich antimicrobial molecule. Snakin-2 has been found to be induced in potato (Solanum tuberosum L.) due to wounding and infection with a compatible fungus and other pathogens (Segura et al., 1999; Berrocal-Lobo et al., 2002). Additional defense-related genes that were induced included hypersensitive-response-associated genes, syringolide, Avr9/Cf-9-induced, pathogen resistance (PR) protein homologs, In2-1 and PR4, some R genes, nonexpresser of PR, defense-related transcription factor LOL1, and disease resistance regulator SGT1.

The most striking decreases in expression level for defense-related genes were the eight genes encoding dirigent proteins. Dirigent proteins are reported to be involved in the synthesis of lignans and an important defense layer in the plant cell wall (Davin and Lewis, 2000). A well-characterized dirigent-like protein gene, DRR206 was originally cloned from pea (Fristensky et al., 1988; Culley et al., 1995), and it was strongly induced in response to both fungal and bacterial pathogens and elicitors (Fristensky et al., 1988; Hadwiger et al., 1992). The decreased expression of dirigent genes suggests a reduced production of lignin, which would be consistent with the reduced lignin biosynthesis–related genes in our study (Supplementary Table S2).

One of the prominent features was the modulation of apoptosis-related genes in response to herbicides. Four genes encoding adenosine nucleotide translocator (ANT) decreased in abundance due to herbicide treatment. Adenine nucleotide translocators are mitochondrial proteins cooperating with Bax, an apoptosis molecule to increase mitochondrial membrane permeability and to trigger cell death (Marzo et al., 1998). Meanwhile, genes related to apoptosis, Bax inhibitor (Gm-r1088-5052 and Gm-r1088-390), and ACCELERATED CELL DEATH (ACD; Gm-r1021-4094) were increased (Table 1). Together with the decreased expression of ANT, it seems that plants were trying to prevent apoptosis induced by herbicides. Another possible cell death–related gene, metacaspase (Gm-r1070-4634), a cysteine endopeptidase involved in the induction of programmed cell death (Hoeberichts et al., 2003), increased at 4 and 8 HAT in atrazine treatment, which may contribute to cell death induced by atrazine (Table 1).

Herbicide treatment also provoked significant modulation to stress response pathways, as 66 abiotic-
stress-related genes were significantly perturbed in our study. Nine universal stress-related genes increased significantly, as did four wound-induced genes. It was also noticed that cold-stress-related genes, such as an antifreeze gene (Gm-r1021-1974) and a cold-acclimation-related gene W COR413 (Gm-r1070-6041), increased in abundance (Table 1). However, drought-stress-related genes displayed mixed patterns in both atrazine and bentazon treatment. Another prominent feature was that all nine of the differentially expressed genes related to senescence increased in response to herbicide treatment. The induction of these leaf-senescence and ripening-related proteins may indicate that herbicides advanced cell death by promoting senescence.

Interestingly, three genes encoding phytochelatin synthetases (Gm-r1083-4126, Gm-r1083-3439, and Gm-r1083-300), which function to detoxify heavy metals in plants (Howden et al., 1995), decreased significantly in transcript levels due to herbicide treatments (Table 1). The reduction of phytochelatin biosynthesis would be beneficial for plants because it reduces the consumption of GSHs, as GSH is the precursor of phytochelatin (Meister and Anderson, 1983; De Vos et al., 1992). Decreased consumption of GSH would save more GSHs for xenobiotic detoxification and oxidative stress relief. Therefore, the reduction of phytochelatin synthesis is consistent with the reduction of phenylpropanoid biosynthesis as discussed above regarding enhancing GSH availability to address the xenobiotic attack.

Miscellaneous Differential Gene Expression in Response to Both Herbicides

Other noteworthy patterns that were also observed in response to the herbicides included differential expression of 80 protein chaperones, 70 of which increased in abundance in both treatments. Dramatic effects on phytohormones were reflected in differential expression of 49 auxin-, 24 ethylene-, and 13 gibberellic acid–related genes that either decreased or increased due to treatment. Additionally, eight genes encoding aquaporins increased in transcript abundance in both atrazine and bentazon treatments at almost all the time points, with another six aquaporins being more abundant in response to atrazine treatment.

Identification of Genes Whose Expression Differed in Response to Atrazine and Bentazon

Soybeans in our study developed severe chlorosis and necrosis 24 h after atrazine treatment, and appeared to be dead by 48 HAT. However, plants treated with bentazon recovered, showing new and healthy trifoliate leaves by 48 HAT (Fig. 1). The different symptoms caused by these two different herbicides were reflected in gene expression modulation (Supplementary Table S6). A major difference in expression patterns was observed for ribosome-related genes in response to atrazine and bentazon treatments. A direct comparison of 46 significant differentially expressed cytosolic ribosomal proteins or ribosomal-RNA-related genes between the two treatments showed that expression levels increased in greater amplitude in the bentazon treatment (Supplementary Table S7). Interestingly, although the plastid ribosomal components increased both in atrazine and bentazon, direct comparison of the data from two treatments revealed that they were generally less abundant in bentazon-treated plants than in atrazine-treated ones. This differential expression between cytosolic and chloroplastic ribosome-related proteins was also observed in soybean tissue treated with the herbicide glyphosate (Zhu et al., 2008).

Although most cytochrome P450 genes shared the same or similar expression patterns between the two herbicide treatments, a few cytochrome P450 homologs, such as CYP82C1P (Gm-r1083-2569), CYP89A2 (Gm-r1083-3508), and CYP89A9 (Gm-r1088-5191), tended to be more abundant in bentazon-treated plants (Table 1). The function that these enzymes might play in their response to bentazon treatment is uncertain due to unknown specific substrates of these cytochrome P450s, but their differential expression patterns make them candidates as possible gene products contributing to the overall detoxification mechanism in bentazon-treated soybean.

As discussed above, most of the significantly differentially induced genes annotated as being involved in detoxification and antioxidation were induced by both herbicides. Hence, most differences that were noted between atrazine and bentazon were quantitative instead of qualitative. The comparison of gene expression data between bentazon and atrazine directly showed that most GST genes were significantly more abundant in bentazon-treated plants. Furthermore, three GSTs (Gm-r1089-6502, Gm-r1088-5106, Gm-r1021-12) were reduced in atrazine-treated tissue but increased in bentazon treatment (Table 1). As in the case for the differential expression of select cytochrome P450 genes, the differential expression of these GSTs indicates that soybean is differentiating these two herbicides and that detoxification and antioxidation conferred by GSTs could be a mechanism contributing to the tolerance to bentazon. Other genes related to glutathione metabolism that showed significantly higher levels in bentazon-treated leaves were a GPX (Gm-r1088-5727) that was induced at later time points, and a gene encoding glutathione reductase (Gm-r1089-7470) that was strongly induced by bentazon but not atrazine (Table 1). Glutathione reductases catalyze the reduction of oxidized GSH to form reduced GSH, which is critical for repletion of GSH for ascorbate–GSH cycle (Noctor et al., 1998).

Another antioxidation-related gene encoding NAD(P)H quinone oxidoreductase (NQO; Gm-r1021-299), a flavoprotein that utilizes NAD(P)H as an electron donor, was strongly increased in bentazon-treated plants at 4 HAT (log R = 1.91) and 8 HAT (log R = 0.59) but only slightly in atrazine-treated plants. NAD(P)H NQOs catalyze two-electron reduction and detoxification of quinones and their derivatives (Gaikwad et al., 2001). In Arabidopsis, NQO is induced by oxidative stress and is...
Figure 4. Quantitative real-time reverse-transcribed polymerase chain reaction (qRT-PCR) verification of microarray results. (A) Genes induced in both atrazine and bentazon treatments. (B) Genes reduced in both atrazine and bentazon treatments. (C) Genes differentially expressed between atrazine and bentazon treatments. Solid bars: microarray data. Open bars: qRT-PCR data. Error bars represent standard error from three biological replicates.
believed to scavenge highly toxic, lipid peroxide- derived α, β unsaturated aldehydes (Mano et al., 2002). The significant increase of NQO at the later time points in bentazon treatment suggests its possible contribution to the overall detoxification of bentazon in soybeans.

Although many of the genes that were differentially expressed between atrazine and bentazon differed in only a quantitative manner, 55 genes were identified that showed completely opposite expression patterns between the two treatments across all time points (Supplementary Table S6). Of these oppositely expressed genes, 41 increased in bentazon- but decreased in atrazine-treated plants, and the other 14 were the reverse. That a high number of the induced genes were in response to the bentazon treatment reflects a more robust overall expression and is believed to be related to soybean tolerance to bentazon.

Gene expression patterns from plants treated with bentazon seemed to reflect active recovery from herbicide damage from 4 HAT. The gene expression profile showed that the effect of bentazon clearly decreased by 8 HAT as the fold-change intensities of gene expression decreased from 4 to 8 HAT as depicted by the lower color intensities in the clustering heat map (Fig. 3). Looking at the bentazon data more closely, it was observed that ~80% of the genes that had increased at 4 HAT were less induced at 8 HAT and that ~70% of genes reduced at 4 HAT were less reduced at 8 HAT. However, in atrazine-treated tissue, the corresponding number of genes was approximately 45 and 46%, respectively. This suggests that the effect of bentazon on soybean faded between time point 4 HAT and time point 8 HAT and transcriptome changes caused by the effect were transient. Likewise, it also indicated that the effect of atrazine was persistent and prompted plants to continue the gene expression pattern started at early hours, presumably until death.

The transient gene expression modulation in bentazon-treated soybean led us to suspect that active metabolism of bentazon began near the 4-HAT time point in this study. Induction of cytochrome P450s have been associated with resistance to chemical assault. For example, CYP94A1, a plant cytochrome P450–catalyzing fatty acid omega-hydroxylase, was induced by chemical stress in Vicia sativa L. seedlings, and was associated with protection of the plants from chemical injury (Benveniste et al., 2005). In our study with bentazon treatment, a homolog of CYP94A1 (Gm-r1089-7497) was induced at a peak at T4 (log2 R = 5.8) and then the induction decreased at T8 nearly 16-fold (log2 R = 5.8 down to log2 R = 2.1). A few other cytochrome P450 homologs, such as CYP82A2 (Gm-r1021-41), CYP71D8 (Gm-b1088-19), and CYP81E8 (Gm-r1088-1287), shared a similar expression pattern (Table 1). Furthermore, the fold change of all induced GST homologs reached their highest levels at 4 HAT and declined substantially at 8 HAT (Supplementary Table S4). These results are consistent with the gradual reduction of the xenobiotic stress and the accompanying oxidative stress caused by bentazon near the 4-HAT time point, with the stress under control by 8 HAT.

Verification of Microarray Data with qRT-PCR

To verify the microarray results, 16 genes showing differential expression were further studied by qRT-PCR using the same biological samples used in the microarray study. Three biological replicates with three technical replicates of each sample were performed and the data was presented as the average of the three biological replicates (Fig. 4). The qRT-PCR results displayed similar trends of increased or decreased abundance in response to herbicide treatment, to that of microarray results, supporting the validity of microarray data (Table 1). The genes studied included those increased in both atrazine and bentazon treatment, such as GST (Gm-r1038-4181), cytochrome P450 (Gm-r1070-3553), and catalase (Gm-r1021-445). Genes with decreased transcript levels in both treatments, such as methionine synthase (Gm-r1021-1184), dirigent gene (Gm-r1070-1177), and phytochelatin synthetase (Gm-r1083-4126), were also tested. To verify differential expression of some genes in two treatments, two genes, cinnamyl alcohol dehydrogenase (Gm-r1088-7671) and one cytochrome CYP9A2 (Gm-r1083-3508), were also examined.

Conclusions

When plants are inflicted with an abiotic stress that interferes with a vital physiological system, such as man-made photosynthesis-disrupting chemical herbicides like atrazine and bentazon, what the plants most likely sense is not the exact chemical identity of the assaulting chemical, but that photosynthesis has been disrupted, leading to a serious redox imbalance. Therefore, plants appear to respond with a wide battery of defense strategies, including the gene expression activation of numerous enzymes capable of chemically modifying a wide range of chemical substrates.

It has been argued that bentazon may have a different binding amino residue of Qβ pocket of D1 protein.
from atrazine (Nimbal et al., 1996). Our gene expression data suggest that, if atrazine and bentazon utilize different binding niches, this difference does not lead to a significant difference on global gene expression. Of the 6646 genes differentially expressed in our study, the number of significantly expressed genes shared between atrazine and bentazon was 5230 at 1 HAT, 5231 at 2 HAT, 5692 at 4 HAT, and 5695 at 8 HAT. This result supports that a similar stress is caused by both atrazine and bentazon in soybean plants.

Two classes of enzymes have often been associated with herbicide tolerance: cytochrome P450s and GSTs. We identified candidates of both that may play a role in bentazon tolerance. As the major stress caused by the two photosynthesis inhibitors is oxidative stress, it was not surprising that we identified a wide range of antioxidant enzymes such as GSTs, catalases, and SODs in response to both herbicides as well as the increase in tocopherol cyclase expression levels, as tocopherol is the major pigment scavenger of singlet oxygen in PSII.

Although most of the gene expression patterns were similar in response to atrazine and bentazon, some differences were observed. The main response differences seemed to be indicative of bentazon detoxification near 4 HAT and the lack of this detoxification for atrazine. In this type of global transcriptomic study, it is not always clear which of the many differentially expressed genes were truly key for determining a given response. For example, it is impossible to pinpoint which gene might be responsible for bentazon tolerance in soybean in our study. However, in spite of this limitation, several genes have been identified and are considered to be prime candidates for governing bentazon tolerance.

The present study revealed that only a small number of genes (12%) were differentially expressed between atrazine- and bentazon-treated soybean plants. These genes, however, convey important information about how plants respond to different chemicals. Ribosomal components in bentazon-treated plants were more abundant than in atrazine-treated ones. Similarly, a few GST and cytochrome P450s increased in transcript levels due to bentazon treatment but decreased due to atrazine treatment. Meanwhile, we noticed that among the genes that were expressed in opposite direction between two treatments, most of these genes increased in response to bentazon. It is reasonable to speculate that these genes are directly or indirectly involved in metabolism of bentazon or secondary product of bentazon.

Active metabolism of bentazon was reflected by the amplitude of fold changes that decreased from 4 to 8 HAT. In light of this, one can reason that the gene(s) responsible for benzton metabolism was adequately expressed and functioning during the first 4 HAT and that many of the differently expressed genes after 4 HAT would be involved in recovery. In atrazine treatment, the expression was still related to continual futile efforts to survive.

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References


