

## Detoxification and Transcriptome Response in *Arabidopsis* Seedlings Exposed to the Allelochemical Benzoxazolin-2(3H)-one (BOA)

Scott R. Baerson<sup>‡</sup>, Adela Sánchez-Moreiras<sup>§\*</sup>, Nuria Pedrol-Bonjoch<sup>¶</sup>, Margot Schulz<sup>||</sup>, Isabelle A. Kagan<sup>‡\*\*</sup>, Ameeta K. Agarwal<sup>‡‡</sup>, Manuel J. Reigosa<sup>§</sup>, and Stephen O. Duke<sup>‡</sup>

From the <sup>‡</sup>United States Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit, P.O. Box 8048, University, MS 38677, USA, <sup>§</sup>Laboratorio de Ecofisiología Vegetal, Facultad de Ciencias, Universidade de Vigo, Vigo, Spain, <sup>¶</sup>Área de Nutrición, Pastos y Forrajes, SERIDA, Estación Experimental "La Mata", Grado, Spain, <sup>||</sup>Institut für Molekulare Physiologie und Biotechnologie der Pflanzen, Universität Bonn, Biozentrum Karlrobert Kreiten Str. 13, 53115 Bonn, Germany, <sup>‡‡</sup>National Center for Natural Products Research, University of Mississippi, University, MS 38677, USA

Running Title: Benzoxazolinone Detoxification Response in *Arabidopsis*

Address correspondence to: Scott R. Baerson, USDA, ARS, NPURU, P. O. Box 8048, University, MS 38677, USA, Tel: 662-915-7965, Fax: 662-915-1035, E-mail: sbaerson@olemiss.edu

\*Current address: Department of Biochemistry, Max-Planck-Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, Germany

\*\*Current address: USDA-ARS-FAPRU, N220 Agricultural Sciences North, Lexington, KY 40546, USA

Benzoxazolin-2(3H)-one (BOA), is an allelochemical most commonly associated with monocot species, formed from the *O*-glucoside of DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one) by a two step degradation process. The capacity of *Arabidopsis* to detoxify exogenously supplied BOA was analyzed by quantification of the major known metabolites BOA-6-OH, BOA-6-*O*-glucoside, and glucoside carbamate, revealing that detoxification occurs predominantly through *O*-glucosylation of the intermediate BOA-6-OH, most likely requiring the sequential action of as-yet-unidentified cytochrome P450 and UDP glucosyltransferase activities. Transcriptional profiling experiments were also performed with *Arabidopsis* seedlings exposed to two different BOA concentrations, representing  $I_{50}$  and  $I_{80}$  levels based on root elongation inhibition assays. One hundred-fifty eight genes were induced, and 30 were repressed in both the  $I_{50}$  and  $I_{80}$  treatments, totaling approximately 0.8% of all genes represented on the ATH1 gene chip. One of the largest functional categories observed for BOA-responsive genes

corresponded to protein families known to participate in cell rescue and defense, with the majority of these genes potentially associated with chemical detoxification pathways. Further experiments using a subset of these genes revealed that many are also transcriptionally induced by a variety of structurally diverse xenobiotic compounds, suggesting they comprise components of a coordinately-regulated, broad-specificity xenobiotic defense response. In addition, several novel transcriptional responses to xenobiotics, including the auxenic herbicide 2,4-D and two different herbicide safeners, were observed. The data significantly expand upon previous studies examining plant transcriptional responses to allelochemicals and other environmental toxins, and provide novel insights into xenobiotic detoxification mechanisms in plants.

Allelopathy, the chemical inhibition of one plant species by another, represents a form of chemical warfare between neighboring plants competing for limited light, water, and nutrient

resources (1-3). Allelopathic interactions have been proposed to have profound effects on the evolution of plant communities through the loss of susceptible species via chemical interference, and by imposing selective pressure favoring individuals resistant to inhibition from a given allelochemical (e.g., 4, 1). In addition, allelochemicals released by grain crop species such as barley, rye, and sorghum are thought to play a significant role in their efficacy as weed suppressants when used as cover crops or within intercropping systems (5, 3).

Despite the ecological and agronomic importance of this class of natural products, relatively little is known concerning the molecular target sites mediating their toxicity or the adaptive strategies mounted by plants in defense against these compounds. Furthermore, in comparison to other areas of chemical ecology, molecular and genomics-based approaches have seldom been employed in the field of allelopathy. One recent exception is the use of DNA microarrays to characterize the transcriptome response of *Arabidopsis* seedlings exposed to (-)-catechin, an allelochemical identified in root exudates of *Centaurea maculosa* (6). Benzoxazinoids and their benzoxazinone derivatives represent one of the more intensively studied classes of allelochemicals (7, 8). For example, the genes encoding all of the enzymes required for the biosynthesis of the benzoxazinoid DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one) have been identified in corn via transposon tagging, and represent the first known example of a plant secondary metabolic pathway organized as a gene cluster (9). The biosynthesis of benzoxazinoids, particularly in young seedlings, is generally associated with cereals such as corn, rye, and wheat, but have also been identified in species of *Acanthaceae*, *Ranunculaceae*, and *Scrophulariaceae* (7). Benzoxazinoids and benzoxazinones act as defense compounds against microbial pathogens as well as insect herbivores, and within the rhizosphere play an important role as allelochemicals (7, 8). Formation of the benzoxazinone BOA (benzoxazin-2(3H)-one) results from a two-step degradation of the glucoside of DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one; Fig. 1). Numerous plant species exhibit tolerance to benzoxazinoids such as BOA, and can rapidly

metabolize them to less phytotoxic glucoside and glucoside carbamate derivatives (Fig. 1), potentially due to having co-evolved in association with allelopathic species within the same communities (4).

A primary response to the presence of xenobiotic compounds in both prokaryotic and eukaryotic organisms involves the induction of detoxifying enzymes and transporters which facilitate the inactivation and elimination of toxins, and the associated metabolic processes can be divided into discrete phases (10, 11, 12). In phase I, compounds are typically modified such that a functional group such as a hydroxyl moiety is added or exposed through the action of hydrolases, cytochrome P450s, or peroxidases. The availability of functional groups then facilitates the formation of glucosyl, glutathione, and malonyl conjugates in phase II, through the action of specific glucosyltransferases, glutathione S-transferases, and less frequently, malonyltransferases. Conjugated forms of xenobiotics can then be recognized by specific membrane-associated transporters such as ABC transporters (13-15) in phase III detoxification, resulting in their vacuolar sequestration or release into the apoplasmic space via exocytosis. In addition, subsequent less well-characterized processes can also occur, resulting in further enzymatic modification in vacuoles or deposition of metabolites as cell wall-associated bound residues (11), sometimes referred to as 'phase IV' detoxification.

The signaling pathways associated with the response to xenobiotic stress in mammals have been extensively studied, and involve both xenobiotic ligand-activated transcription factors and redox sensing proteins (16, 17). In plant systems, it is also well established that the expression of specific genes involved in processes related to the detoxification of allelochemicals and synthetic herbicides can be induced in the presence of these compounds (18), however evidence for the existence of integrated, coordinately regulated chemical detoxification gene networks such as those identified in animal systems is scarce. Furthermore, the signaling components involved in plant responses to xenobiotic stress have not been identified at present. An important class of agrichemicals, the herbicide safeners, may act as potent inducers of

these signaling pathways, thereby rendering crops less susceptible to herbicide-induced injury (19, 20).

*Arabidopsis thaliana* represents an excellent model for the study of plant responses to allelochemicals and other environmental toxins, as its genome has been fully sequenced and well characterized. The commercial availability of nearly full-genome oligonucleotide microarrays (21), opens the possibility for identifying specific enzyme isoforms within complex gene families such as cytochrome P450s, glutathione S-transferases, and UDP-glucosyltransferases (22-24) representing potential components of a coordinated xenobiotic defense response network. In addition to gaining basic insight into plant xenobiotic stress responses, molecular ecotoxicological studies in plants are also of considerable importance to human health and agriculture, given the incidents of soil and groundwater contamination occurring worldwide from industrial pollutants (11, 25).

In the present work we have focused on the analysis of the physiological response, detoxification pathways, and transcriptome responses in *Arabidopsis* seedlings exposed to the model benzoxazolinone allelochemical BOA. Feeding studies using exogenously supplied BOA revealed that detoxification in *Arabidopsis* occurs predominantly through *O*-glucosylation of the intermediate BOA-6-OH, most likely requiring the sequential action of as yet unidentified cytochrome P450 and UDP glucosyltransferase activities. Transcriptional profiling experiments using microarrays representing approximately 24,000 transcripts identified a significant number of genes potentially involved in Phase I, II, and III detoxification processes which are induced following exposure to this allelochemical. Using a subset of these genes, we further demonstrate their induction in response to a variety of structurally diverse xenobiotic compounds, suggesting they comprise components of a general xenobiotic response network. These data significantly expand upon previous studies examining plant transcriptional responses to allelochemicals and other environmental toxins, and provide a foundation for elucidating both the enzymes and regulatory mechanisms involved.

## EXPERIMENTAL PROCEDURES

*BOA Metabolite Studies*--Seeds of *Arabidopsis thaliana* (Col-0) were germinated in a mixture of sand, mould and Perlite (4:4:2), then maintained in a greenhouse at 20°C for a period of three weeks. Under these conditions, plants did not initiate flowering during this time period. Approximately 30 plants per experimental group were carefully removed from the potting media to avoid root damage, then washed extensively to remove soil particles. Incubations with BOA (2-Benzoxazolinone; Sigma-Aldrich, St. Louis, MO) were performed for 24 h as described previously (4) using 10, 100, 250 and 500  $\mu$ M treatment solutions. After incubation, plants were rinsed, dried between paper sheets, then weighed. Plant material was ground in 100% methanol using a mortar and pestle with quartz sand. Homogenates were then centrifuged for 15 min at 4°C, 10,000  $\times$  g, and the volumes of the supernatants determined.

The extracts were analyzed for detoxification products by HPLC using a Beckman Coulter (Beckman Coulter, Inc., Fullerton, CA) model 126 Chromatograph equipped with a diode array detector (model 168), and an Ultrasphere ODS RP 18 column. Compounds were eluted with the following gradient: 1 min 100% eluent A (0.1% TFA in H<sub>2</sub>O), 1-21 min 20% B (methanol) linear, 21-41 min 80% B linear, 41-43 min 100% B linear, using a flow rate of 1 ml/min. The detection wavelengths used were 280 and 405 nm. Major metabolites were identified by co-chromatography with natural BOA-6-O-glucoside and with synthetic BOA-6-OH and glucoside carbamate prepared as described previously (26, 4). These compounds were also used as external standards for quantification.

*BOA Treatments for Growth Inhibition and Microarray Studies*--For all BOA growth inhibition and microarray experiments, aseptically germinated *Arabidopsis thaliana* (Col-0) seedlings were maintained in a growth chamber at 21°C under a 16-h photoperiod and light intensity of 150  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>. Seeds were surface sterilized in 70% ethanol for 5 min, then rinsed 2 times in sterile, distilled water, followed by treatment with 0.5 X bleach (3% sodium hypochlorite) and 0.05% Tween-20 for 10 min, then finally rinsed 4 times in sterile, distilled water. Following surface-

sterilization, seeds were placed on top of an approximately 2.0 cm-high stack of 9.0 cm #4 Whatman filter discs and allowed to air dry in a sterile hood. Liquid and semi-solid germination media used for all experiments contained 0.5 X Murashige and Skoog salts, 1X Gamborg's B5 vitamins, and 1.0% sucrose (w/v), adjusted to pH 5.7 with KOH. Semi-solid media also contained 1.0% (w/v) agar.

For root elongation assays, seeds were placed in a horizontal line approximately 2.0 cm from the edge in 9.0 x 9.0 x 1.5 cm square petridishes containing semi-solid germination media supplemented with different concentrations of BOA. Control plates contained solvent alone (0.1% ethanol). Plates were cold-treated for three days, then transferred to a growth chamber and maintained in a vertical position for 10 days, at which time root lengths were scored. All BOA and control treatments were performed in duplicate; each replicate consisted of 25 seedlings.

For BOA treatments prior to microarray analyses, approximately 200 seeds were scooped into a microspatula, then distributed evenly over the surface of a sterile 0.3  $\mu$ m microporous membrane raft supported by a buoyant float (Osmotek Ltd., Rehovot, Israel). Seeds, rafts, and floats were placed on liquid germination media in Lifeguard tissue culture vessels with 4.0 cm vented lids (Osmotek Ltd., Rehovot, Israel), cold-treated for three days, then transferred to a growth chamber. After ten days, BOA (or 0.1% ethanol for control treatments) was added to the media, then the vessels were returned to the growth chambers until harvest. At the end of the treatment period, seedlings were removed from the vessels, flash-frozen in liquid nitrogen, then stored at  $-80^{\circ}\text{C}$  prior to analysis.

*Chemical Treatments for Real-Time PCR Assays*--Follow-up chemical treatments for quantitative real-time PCR experiments were performed as described above for microarray experiments. Ten-day-old seedlings grown on floating microporous membrane/raft assemblies were exposed two different concentrations of fenclorim (Toronto Research Chemicals, Inc., Ontario, Canada), benoxacor (Sigma-Aldrich, St. Louis, MO), 2,4-dichlorophenoxyacetic acid (Sigma-Aldrich, St. Louis, MO), phenobarbital (Sigma-Aldrich, St. Louis, MO), *p*-hydroxybenzoic acid (sodium salt; Sigma-Aldrich,

St. Louis, MO), and 4-dimethylaminoantipyrine (free base; Sigma-Aldrich, St. Louis, MO). For 2,4,5-trichlorophenol (Sigma-Aldrich, St. Louis, MO), due to extensive injury observed on seedlings exposed to 10 mM treatments, only 100  $\mu$ M treatments were used. Stock solutions were prepared in either DMSO or ethanol, then added to seedlings growing on liquid media as described above in duplicate treatments. Control treatments (0.25% ethanol and 0.5% DMSO) were also performed in duplicate. At 24 h post-treatment, seedlings were flash-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  prior to analysis.

*RNA Isolation*--Total RNAs for use in microarray experiments were isolated from 0.5 g flash-frozen, pulverized 10-day-old seedling tissues using the Trizol reagent (Invitrogen Corp., Carlsbad, CA), with an additional homogenization step of 30 s at 25,000 rpm using a handheld homogenizer. The RNA recovered was then re-purified with a RNeasy Plant Mini-Kit (Qiagen Inc., Valencia, CA) per manufacturer's instructions. RNA recovery and purity were determined spectrophotometrically, and sample integrity was assessed by agarose gel electrophoresis.

Total RNAs for real-time PCR experiments shown in Fig. 5 were isolated from 50 mg of flash-frozen, pulverized 10-day-old seedling tissues using a RNeasy Plant Mini-Kit, with an additional homogenization step of 30 s at 25,000 rpm as described above. The RNA samples were also treated with DNase I "on column" using a RNase-Free DNase kit as per manufacturer's instructions (Qiagen, Inc., Valencia, CA) to remove residual DNA contamination. RNA recovery and purity were also determined spectrophotometrically for these samples, and sample integrity was also assessed by agarose gel electrophoresis.

*Microarray Hybridization and Analysis*--Microarray hybridizations were performed for three independent replicates with Affymetrix *Arabidopsis* ATH1 Genome Arrays, using protocols described by Affymetrix, Inc. (Santa Clara, CA). GeneTraffic software (Iobion Informatics, La Jolla, CA) was used to conduct a two-class comparison analysis on normalized and log-transformed signal values obtained from Affymetrix Microarray Suite software version 5.0. A significance analysis test (unpaired *t*-test with Benjamini-Hochberg false discovery rate

correction) was performed to test the equality of the mean signal values between the two classes. Means for each class were then inverse-transformed to provide a geometrical mean as an overall estimate of expression. In this manner, a more robust estimate of overall expression, less impacted by outliers or skewed expression levels (as compared to a simple arithmetic average of the raw signal values from each array), was obtained. Fold-change was then calculated as the simple ratio of overall signal values from the two classes. Genes with  $p$ -values of  $\leq 0.05$  were considered to be significantly differentially expressed. Genes that were common to both the  $I_{50}$  and  $I_{80}$  data were identified. A floor adjustment of 64 was applied to genes with very low signal values to avoid artifactually large fold-change calculations. Genes that were induced or repressed by at least 2.5-fold in either the  $I_{50}$  or  $I_{80}$  data were identified and retained for further analysis. Differentially expressed genes, thus identified, were then annotated using the NetAffx database provided by Affymetrix ([www.affymetrix.com](http://www.affymetrix.com)). In some cases the annotations of unknown genes were further refined by performing additional BLAST searches, or updated as additional literature reports became available.

*Quantitative Real-Time RT-PCR Assays*--First strand cDNAs were synthesized from 2  $\mu\text{g}$  of total RNA in a 100  $\mu\text{L}$  reaction volume using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. Quantitative real-time PCR reactions were performed in triplicate using the GenAmp® 5700 Sequence Detection System (Applied Biosystems). Independent PCR reactions were performed using the same cDNA for both the gene of interest and 18S rRNA, using the SYBR® Green PCR Master Mix (Applied Biosystems). Gene-specific primers were designed for the gene of interest and 18S rRNA using Primer Express® software (Applied Biosystems) and the Amplify program (27). Closely related sequences within the *Arabidopsis* (Col-0) genome were identified via BLASTN queries of the AGI Transcripts database using the BLAST server at The *Arabidopsis* Information Resource (<http://www.arabidopsis.org/Blast/>). All sequences thus identified were then aligned using the ClustalW alignment function of MegAlign software (DNASTAR, Inc. Madison, WI). Gene-

specific primer pairs were then manually selected such that at least one primer per pair contained a minimum of two consecutive mismatches at the 3' end when compared against all related *Arabidopsis* transcripts. For almost all genes analyzed, both primers within a pair fulfilled the above criteria, and contained numerous additional mismatches when compared against related sequences. The PCR conditions consisted of denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the GenAmp® 5700 sequence detection system. A negative control reaction in the absence of template (no template control) was also routinely performed in triplicate for each primer pair. The change in fluorescence of SYBR® Green I dye in every cycle was monitored by the GenAmp® 5700 system software, and the threshold cycle ( $C_T$ ) above background for each reaction was calculated. The  $C_T$  value of 18S rRNA was subtracted from that of the gene of interest to obtain a  $\Delta C_T$  value. The  $C_T$  value of an arbitrary calibrator (e.g., untreated sample in the case of upregulated genes) was subtracted from the  $\Delta C_T$  value to obtain a  $\Delta\Delta C_T$  value. The fold-changes in expression level relative to the calibrator were expressed as  $2^{-\Delta\Delta C_T}$ . For determination of statistical significance, pairwise comparisons were performed between treated and control sample  $\Delta\Delta C_T$  values using an independent two-tailed  $t$ -test, assuming common variance. Differences associated with  $p$ -values  $\leq 0.05$  were considered significant.

*Motif Searches*--5' upstream sequences from -1500 to -1 (relative to potential transcription start sites if available) were retrieved using RSA-tools (28, <http://rsat.ulb.ac.be/rsat/>), by selecting the 'mRNA' feature type, and 'prevent overlap with upstream ORFs'. The program Motif Sampler (29, <http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html>) was then used for the identification of over-represented motifs within the retrieved sequences. All searches were performed using a precompiled 3<sup>rd</sup> order Markov background model based on *Arabidopsis* upstream sequences (29), prior probability of finding 1 motif instance = 0.5, maximum number of motif instances per sequence

= 0 (no limit), and maximum allowed overlap between different motifs = 2. Each data set was analyzed 10 times using the same parameters to reduce local optima (30), and only cases where an identical or similar consensus sequence was returned from multiple runs were further considered. In addition, two independent statistical tests were employed for motif validation. For the first, searches were performed on 28,577 *Arabidopsis* -1500 to -1 (see above) 5' upstream sequences to determine the total number of motif instances for all predicted *Arabidopsis* genes using the RSA-tools 'genome-scale DNA-pattern' search function (28). These values were used to estimate the probability (**p**) of occurrence for a given motif within the total number of nucleotides searched on both DNA strands, then *p*-values for each experimental result were determined based on calculated binomial probabilities using the 'PROBBNML' function within the SAS v. 9.1 statistical analysis software package (SAS Institute, Inc., Cary, NC). The second test involved a bootstrap analysis using the POBO program (31). For each motif, 1000 bootstrap pseudo-clusters were generated by random sampling with replacement from the input promoter data set, then compared with 1000 pseudo-clusters generated in a similar manner from a background data set consisting of all predicted *Arabidopsis* 5' upstream regions. The number of sequences in each pseudo-cluster generated was equal to the number in the input data set, and all background data set sequences were 1500 bp in length. An independent *t*-test was then performed using pseudo-cluster values to estimate the probability for the number of motifs observed in the input data set to occur by chance. A *p*-value  $\leq 0.05$  was considered significant for both of these tests.

## RESULTS AND DISCUSSION

*Detoxification of BOA in Arabidopsis*--Two major pathways leading to the formation of BOA metabolites exhibiting reduced phytotoxicity have been identified in plants (Fig. 1). BOA-6-*O*-glucosides are formed via the intermediate BOA-6-OH, which is subsequently *O*-glucosylated. The second pathway involves direct *N*-glucosylation of BOA, which undergoes spontaneous isomerization

to form the glucoside carbamate (1-(2-hydroxyphenylamino)-1-deoxy- $\beta$ -glucoside 1,2-carbamate) shown in Fig. 1 (26). An additional metabolite, gentiobioside carbamate (1-(2-hydroxyphenylamino)-1-deoxy- $\beta$ -gentiobioside 1,2-carbamate) derived from the glucoside carbamate, has also been identified in *Zea mays*, but was undetectable in several other *Poaceae* species analyzed (32, 26).

Sensitivity to BOA is highly species-dependent, although dicot species are in general less tolerant than monocots (33). This difference could be explained, at least in part, by the observation that dicots tend to detoxify BOA via *O*-glucosylation, whereas in several monocot species examined a significant proportion of exogenously applied BOA is metabolized via *N*-glucosylation, and for *Zea mays*, virtually all metabolism occurs via *N*-glucosylation (4). The glucoside carbamate exhibits dramatically reduced phytotoxicity relative to BOA-6-*O*-glucoside in bioassays, whereas the *O*-glucoside precursor, BOA-6-OH, is approximately twice as phytotoxic as the parent compound BOA (4). The pathway leading to the formation of the glucoside carbamate via *N*-glucosylation of BOA therefore represents a more efficient mechanism for detoxification of the allelochemical, and would presumably confer increased tolerance relative to species that favor accumulation of *O*-glucoside.

The capacity of *Arabidopsis* to detoxify exogenously supplied BOA was analyzed by quantification of the known metabolites BOA-6-OH, BOA-6-*O*-glucoside, and glucoside carbamate, and these results are shown in Table I. Significant levels of all three metabolites were observed in plants treated with solutions containing 100, 250, and 500  $\mu$ M concentrations of BOA, however the accumulation of glucoside carbamate was not observed in any of the four trials performed using 10  $\mu$ M treatments (Table I). Significantly, for all of the treatments performed, much higher accumulation levels of BOA-6-OH and BOA-6-*O*-glucoside were observed as compared with levels of glucoside carbamate, and the molar ratios of total *O*-glucosylation pathway metabolites to glucoside carbamate per gram fresh weight were approximately 11:1 for both the 250  $\mu$ M and 500  $\mu$ M treatments. Taken together, these data clearly indicate that BOA detoxification in *Arabidopsis* occurs mainly via the *O*-glucosylation

route, likely requiring the sequential action of as yet unidentified cytochrome P450 and UDP glucosyltransferase activities. The surprisingly high levels of BOA-6-OH which accumulate in *Arabidopsis* as compared with other species examined (4), suggests either an inefficient conversion of BOA-6-OH to BOA-6-*O*-glucoside, or alternatively, competing, higher levels of  $\beta$ -glucosidase which markedly increase steady-state levels of BOA-6-OH. While these data strongly suggest that BOA detoxification in *Arabidopsis* is a relatively inefficient process, vacuolar sequestration and/or apoplastic extrusion mechanisms must also be taken into account when considering whole-plant tolerance levels against allelochemicals and other xenobiotic compounds.

*Transcriptome Response to BOA*--To determine appropriate conditions for conducting microarray experiments, dose-response studies were first performed using seedlings grown on vertical agar plates containing varying concentrations of BOA (Fig. 2A). At inhibitory concentrations, pronounced effects were seen on the development of both root and shoot systems of 10-day-old seedlings grown continuously in the presence of BOA. A reduction in cotyledon expansion and the absence of true leaves were observed at the higher concentrations tested, accompanied by severe chlorosis. Root system development was also significantly impaired, resulting in dramatically reduced root lengths and a complete absence of lateral root formation. Root lengths represented the most reproducible parameter tested (not shown), and were therefore used for the generation of  $I_{50}$  and  $I_{80}$  values. Based on these studies, the BOA concentrations necessary to inhibit root elongation in 10-day-old seedlings by 50% ( $I_{50}$ ) and 80% ( $I_{80}$ ) were estimated at approximately 540  $\mu$ M and 1 mM, respectively (Fig. 2B). Similar dose-dependent responses for root elongation have been reported for other species grown in the presence of BOA (e.g., 33), indicating the suitability of *Arabidopsis thaliana* as a model species for examining plant responses to this allelochemical.

Transcriptome responses were next analyzed using commercially prepared oligonucleotide arrays (Affymetrix, Inc., Santa Clara, CA.) representing approximately 81% of the 29,454 predicted *Arabidopsis* genes (34). Approximately 200 seedlings per treatment were germinated

aseptically on membrane rafts floated on germination media, stratified for 3 days, then maintained for 10 days in a growth chamber under a 16-h photoperiod (see 'Experimental Procedures'). After 10 days, BOA was added to the media at a final concentration of 540  $\mu$ M or 1.0 mM, and seedlings were harvested 24 h later. Mock treatments were also performed which consisted of ethanol alone (final 0.1% v/v), followed by a 24-h incubation. Three experimental replicates were prepared for all treatments and controls, grown in separate culture vessels to account for experimental variation, then total RNAs were isolated from each replicate and used for hybridizations with Affymetrix ATH1 arrays.

Complete results from these transcriptional profiling experiments are shown in Supplemental Table I. A total of 188 genes were differentially expressed in both the  $I_{50}$  and  $I_{80}$  treatments for all replicates performed ( $p \leq 0.05$ ), which includes a 2.5-fold cut-off for change in expression observed in at least one of the two treatments. One hundred-fifty eight genes were induced, and 30 were repressed in both the  $I_{50}$  and  $I_{80}$  treatments, representing in total approximately 0.8% of all *Arabidopsis* genes included on the ATH1 array.

To further confirm the reliability of the observed changes, quantitative real-time PCR assays were developed for 16 differentially expressed genes identified by microarray analysis, and transcript levels were measured using the same RNA samples used for the microarray hybridizations. The gene-specific primer pairs designed for these assays are shown in Table II. As shown in Fig. 3, all of the selected genes indicated as being differentially expressed by transcriptional profiling were confirmed by quantitative real-time PCR. Remarkably, even differences between expression levels in the  $I_{50}$  and  $I_{80}$  treatments relative to controls were confirmed by these analyses. The magnitude of the fold-changes determined for most of the genes tested were similar for the two methods, however for four of the genes (At4g12490, At1g15520, At4g12500, and At2g15490) the increases were substantially larger when analyzed by real-time PCR. Relative fold-changes seen in microarray data are often larger when analyzed by real-time PCR, predominantly due to limitations in the dynamic range and sensitivity of microarray

experiments, and global normalization methods applied to microarray data sets. In addition, cross-hybridization may occur in microarray experiments with probe sets designed for closely-related genes, resulting in complex signals which will not occur in real-time PCR assays using appropriately designed gene-specific primers. In the case of a differentially expressed putative ABC-type transporter At1g15520 (Fig. 3), the discrepancy is likely attributable to the fact that the Affymetrix software assigned an "absent" call to the corresponding probe set in untreated samples, thus a true measure of fold-induction could not be determined.

The 158 genes induced by BOA were distributed into 12 different functional categories (Fig. 4), based on FunCat assignments available through the MIPS *Arabidopsis thaliana* database (MATDB; <http://mips.gsf.de/proj/thal/db/>), BLAST searches, and literature reports. The largest categories identified were 'metabolism' and 'cell rescue, defense, and virulence', representing approximately 27.9% and 26.2% of all functions assigned, respectively (Fig. 4). The 'metabolism' category included genes potentially involved in chemical detoxification processes, thus there is significant overlap with genes assigned to 'cell rescue, defense, and virulence', as each gene could be assigned to multiple categories. Also of significant interest are putative transcription factors (4.1%), and genes associated with cellular communication and signal transduction mechanisms (7.8%), which could potentially mediate responses for other genes identified in the data set.

A striking number of the most highly up-regulated genes represent enzymes potentially involved in the modification, conjugation, or elimination of xenobiotics (Supplemental Table I). Included in this category would be various quinone oxidoreductases, aldo-keto reductases, peroxidases, cytochrome P450-like proteins, glutathione S-transferases, glucosyltransferases, and representatives of several families of multidrug efflux transporters including the ATP binding cassette (ABC), major facilitator superfamily (MFS), and multidrug and toxic compound extrusion (MATE) transporters. A list of selected up-regulated genes potentially associated with detoxification processes, as well as putative signal transduction components which

could potentially be involved in regulating their expression is shown in Table III.

As mentioned, a number of putative signal transduction components including receptor-like protein kinases, protein kinases, and transcription factors were also significantly upregulated following allelochemical exposure (Table III). Thus the microarray data conceivably represent the entire spectrum of signaling components, enzymes and transporters that would be required for the perception, metabolism, and sequestration/disposal of phytotoxic compounds. In the transcription factor category, of particular interest was the observation that mRNA levels for five different NAC domain family members were upregulated, representing the only family of transcription factors from which multiple members were induced (Table III). The *Arabidopsis* genome encodes 90 putative members of this plant-specific family, several of which have been implicated in various developmental processes (35). Recently, a study performed in *Brassica napus* demonstrated that specific NAC domain gene family members also respond via steady-state mRNA increases to mechanical wounding, insect feeding, pathogen infection, cold treatment, and dehydration (36), indicating an important role for these factors in mediating transcriptional responses to diverse biotic and abiotic stresses. Our microarray studies suggest an additional potential role for this family in mediating xenobiotic defense responses following exposure to phytotoxins such as benzoxazinoid allelochemicals.

*Induction of Putative Phase I-Related Proteins--Enzymes associated with phase I-related detoxification processes primarily perform oxidative modifications, frequently resulting in decreased toxicity and increased solubility of parent molecules (11). A significant body of evidence suggests that cytochrome P450s perform the majority of oxidative phase I reactions in plants, and this may in part explain the complexity of P450-related protein families which exist in most plant species. For example, a recent survey of the Arabidopsis genome suggested that as many as 286 genes comprise a superfamily of P450-related sequences (22). Cytochrome P450s are heme-proteins located in the endoplasmic reticulum, and catalyze a wide array of oxidative reactions using diverse substrates. Thus, in*

addition to their general metabolic role, the large families of plant P450 proteins potentially represent an arsenal of degradative activities for the detoxification of foreign and endogenous compounds. Results from the microarray analysis revealed six cytochrome P450 sequences significantly up-regulated in response to I<sub>50</sub> and I<sub>80</sub> BOA treatments (Table III) whose physiological roles and substrate specificities are not known at present.

Less well studied in plant species, but well known to participate in xenobiotic detoxification pathways in animals, are members of the aldo-keto reductase (AKR) superfamily (reviewed in 37, 38). The *Arabidopsis* genome encodes at least 18 genes related to AKRs (<http://www.arabidopsis.org>) whose functions remain unknown. AKRs have been identified in all kingdoms, and metabolize structurally diverse substrates including monosaccharides, steroids, aromatic aldehydes and hydrocarbons, and aliphatic aldehydes, utilizing nicotinamide cofactors. Unlike other detoxifying protein families, relatively little is known in plants or animals regarding the transcriptional responses for AKR genes in tissues exposed to toxins or other inducers. Four *Arabidopsis* AKR-like sequences were significantly induced by I<sub>50</sub> and I<sub>80</sub> BOA treatments (Table III). Interestingly, an AKR-like sequence, *In2-2*, was isolated from corn roots by differential screening of safener-treated tissues (39). Northern analyses revealed that *In2-2* expression increased from undetectable levels within 30 min of exposure to the substituted benzenesulfonamide safener 2-CBSU, and that this induction was not observed following various stress treatments. Subsequent experiments using *In2-2* promoter::reporter gene fusions in transformed tobacco also demonstrated inducibility by the herbicide chlorsulfuron and exogenously supplied branched-chain amino acids, both known to inhibit the enzyme acetolactate synthase (40). Further experiments will be necessary to definitively assign a role for AKR enzymes in plant xenobiotic detoxification, as has been demonstrated for animals (37), however the transcriptional responses observed in *Arabidopsis* (see below) for AKR-like sequences to safeners, herbicides, and other xenobiotics suggests they may in fact play such a role.

Six class III peroxidase-like sequences were upregulated by BOA (Table III), which could

potentially play a role in oxidative phase I detoxification schemes similar to that established for cytochrome P450s (41-44), although far fewer studies have been performed in plant systems for these enzymes. More effort has been focused on their role in cell wall modification, where they are involved in the biosynthesis of lignins and mediating oxidative cross-linking of cell wall-associated carbohydrates and glycoproteins (reviewed in 45). Anilines, which result from the enzymatic degradation of acyl aniline, phenylcarbamate, and phenylurea herbicides, may be primarily metabolized by soluble peroxidases (42). Additionally, the xenobiotic substrates N-nitrosodimethylamine (NDMA), N-nitrosomethylaniline (NMA), aminopyrine, and 1-phenylazo-2-hydroxynaphthalene (Sudan I) have been shown to be metabolized by peroxidase-like activities in tulip, although cytochrome P450s also present were shown to be more active against NMA and Sudan I (44). Interestingly, DIMBOA, a benzoxazinoid related to BOA, has been shown to induce cell wall peroxidase activity in oat coleoptiles (46), which could account for the growth inhibitory effects of benzoxazinoids due to reduced cell wall plasticity in exposed seedlings. It is therefore possible that the observed upregulation of peroxidase sequences in the present study (Table III) could result specifically from exposure to BOA or other benzoxazinoids, rather than as a general xenobiotic defense response.

*Induction of Putative Phase II and Phase III-Related Proteins*--A significant portion of genes transcriptionally induced by BOA represent protein families such as glutathione S-transferases (GSTs), UDP-glucosyltransferases (UGTs), quinone oxidoreductases, and various multidrug efflux transporters potentially involved in phase II and phase III detoxification processes (Table III). The induction of a glutathione S-transferase (GST) at the level of transcription in response to herbicide safener exposure was first reported by Wiegand and coworkers (47), and numerous reports have subsequently described the regulation of GSTs in response to a variety of agrichemicals and other xenobiotics (reviewed in 48). GSTs play a central role in phase II reactions, catalyzing the transfer of glutathione to a substrate to form S-glutathione conjugates, which are then substrates for ABC transporters for removal (13). Not

unexpectedly, the seven putative GSTs shown to be upregulated in the present study include some of the most highly induced transcripts in BOA-treated *Arabidopsis* seedlings (Table III).

Plant UDP-glucosyltransferases (UGTs) play an equally well-established role in phase II metabolism, and catalyze the transfer of glucose to diverse xenobiotic substrates (18), producing both *N*- and *O*-glucoside derivatives of the parent compounds. A recent survey of the *Arabidopsis* genome revealed the presence of 107 glucosyltransferase genes (24), and significant progress has been made toward elucidating the biochemical activities of this complex family (49, 24). In contrast to glutathionylated compounds, less is known regarding the transport of glucosides, and it is possible that both ABC transporters and tonoplast-associated H<sup>+</sup> antiporters are involved (50).

In BOA-treated *Arabidopsis* seedlings, UGTs were the most highly represented family of upregulated genes, with eleven different members being significantly induced (Table III). Most striking is the almost 70-fold induction observed by real-time PCR in the I<sub>80</sub> treatments for UGT73B4 (At2g15490; Fig. 3), which was below the limit of detection for microarray analysis in untreated samples (i.e., was assigned an 'absent' call; data not shown). The induction of UGT enzymatic activities in plants in response to xenobiotic compounds has been previously documented (e.g., 51), however increases in steady-state levels for the corresponding mRNAs have not to our knowledge been directly demonstrated prior to this study. Given that the glycoside of BOA is the predominant metabolite formed in plants, including *Arabidopsis* (32), the possibility cannot be excluded that plant UGT gene promoter activities are specifically induced following exposure to BOA and related compounds.

Fifteen membrane transporter-like sequences were induced by BOA, including six putative ABC-type transporters, a MFS-type transporter (At5g13750), and a MATE-type transporter (At2g04040; Table III). ABC-, MFS-, and MATE-type transporters represent different multidrug efflux protein super-families associated with resistance to antibiotics and other toxins in microorganisms (52). In contrast to MFS and MATE-type transporters, induction of ABC-type

transporters at the level of steady-state mRNA accumulation in response to xenobiotic compounds has been previously reported for plants (53-56). In studies performed by van den Brùle and Smart (55), the BOA-induced ABC transporter AtPDR12 (At1g15520; Table III) was shown to be highly induced by cycloheximide and the antifungal diterpene sclareol, but not by hormone, cadmium, cold, or salt stress treatments. In addition, a recent study performed by Campbell et al. (15) showed induction of AtPDR12 transcript levels in response to pathogen infection, salicylic acid, ethylene, and methyl jasmonate. Using various signal transduction mutants, it was also demonstrated that induction of AtPDR12 by pathogen infection requires salicylic acid, NPR1 function, and sensitivity to jasmonic acid and ethylene. A T-DNA insertion line for AtPDR12 was more sensitive to sclareol than wild-type seedlings in germination assays, thus collectively these data suggested a potential role for AtPDR12 in transporting antifungal compounds accumulating in response to pathogen infection (15). The data generated in the present study suggests that this transporter may also have a more general role in the elimination of phytotoxins unrelated to pathogen defense (see discussion below).

Also of significance among BOA-upregulated genes include putative quinone oxidoreductases (NADP-dependent oxidoreductases), dehydrogenases, a nitrilase-like sequence (At5g22300), a class III lipase (At5g24210), and a fatty acyl-CoA ligase-like sequence (At5g27600) (Table III; see also Supplemental Table I). As was the case for peroxidases, a role for many of these enzyme classes has yet to be established in plants, yet all are known to participate in detoxification processes in non-plant systems (e.g., 57-60, 37). With the exception of quinone oxidoreductases, which have been shown to be rapidly induced in *Triphysaria* species treated with various allelopathic quinones (61), the induction of mRNAs encoding members of these protein families in response to phytotoxins has not previously been reported.

*Response of Selected Genes to Xenobiotics, and Inducers of Xenobiotic Metabolism*--The genome-wide responses revealed by transcriptional profiling of BOA-treated *Arabidopsis* seedlings suggests that exposure to

this allelochemical induces the expression of a battery of defense genes involved in the detoxification of xenobiotic compounds (Table III). Presumably, the signal transduction pathway(s) involved in this response would not be highly specific, given the structural diversity of environmental and endogenous toxins typically encountered during the life cycle of a plant (62). Alternatively, the expression levels for these genes could be coordinately increased in response to a wide variety of chemical agents through the action of broad-specificity xenobiotic sensing mechanisms, as has been observed in other organisms (63-65). A more detailed examination of the response of select BOA-responsive genes following exposure to a variety of xenobiotics, and proposed inducers of plant chemical detoxification pathways, could therefore provide additional insights concerning the nature of plant xenobiotic defense responses, and potentially identify key target genes and regulatory factors involved as well.

To further explore this, *Arabidopsis* seedlings were exposed to different concentrations of the herbicide 2,4-D, *p*-hydroxybenzoic acid (an allelochemical agent in wheat and potentially *S. bicolor* – 66, 67), the environmental pollutant 2,4,5-trichlorophenol, the herbicide safeners fenclorim and benoxacor, and the drugs aminopyrine and phenobarbital which have been shown to induce putative detoxification pathways involving ABC transporters, glutathione S-transferases, or P450s in both plants and animals (e.g., 68, 69, 56). Chemical treatments were performed in an identical manner to the BOA treatments described above, then plants were harvested at 24 h post-treatment, and RNA levels were monitored for 20 selected BOA-responsive genes by real-time PCR (Table III). The results from these experiments are shown in Fig. 5.

Overall, steady-state mRNA levels were increased in response to multiple treatments for a majority of the genes analyzed, although the fold-changes observed for a given treatment as well as the relative responsiveness to different treatments varied significantly from gene to gene. Importantly, a subset of the genes analyzed were strongly induced by a majority of the compounds tested, suggestive of a physiological role involving defense against toxins. For example, steady-state mRNA levels of ABC transporter AtPDR12

(At1g15520) exhibited greater than 5-fold increases following exposure to all of the xenobiotics tested, with the exception of aminopyrine, where only a modest (3.1-fold) increase was observed at the highest concentration (Fig. 5). Benoxacor and 2,4,5-trichlorophenol elicited surprisingly dramatic increases in AtPDR12 expression: transcript levels were induced more than 5,000-fold by 1mM benoxacor treatments and more than 400-fold following exposure to 100  $\mu$ M 2,4,5-trichlorophenol. Glucosyltransferase UGT73B4 (At2g15490) transcript levels were induced more than 30-fold by 5 out of 7 compounds tested, and increased approximately 17-fold and 14-fold at the highest concentrations tested for phenobarbital and aminopyrine, respectively. Dramatic responses were also observed for UGT73B4 following exposure to safeners and 2,4,5-trichlorophenol: approximately 100-fold increases were observed in response to 100  $\mu$ M fenclorim and 2,4,5-trichlorophenol treatments, and approximately 800-fold for 1mM benoxacor treatments. Interestingly, 100  $\mu$ M *p*-hydroxybenzoate treatments induced UGT73B4 transcript levels approximately 32-fold, and this compound has also been shown to be a substrate for this enzyme in addition to 3,4-dihydroxybenzoate and the hydroxycoumarin allelochemicals esculetin and scopoletin (49, 24). For both of these genes (AtPDR12 and UGT73B4) it is interesting to note that basal expression levels (i.e., expression levels in control-treated seedlings) were below the threshold of detection by microarray analysis (not shown), yet were induced to high levels in response to the treatments performed. These observations would be consistent with physiological roles for AtPDR12 and UGT73B4 principally involving chemical defense, as opposed to more general housekeeping functions. Similarly, 'marginal' or 'absent' calls were assigned by the Affymetrix software for basal expression levels of quinone oxidoreductase At5g16980 which, as discussed below, was also highly induced by a majority of the treatments performed.

In addition to AtPDR12 and UGT73B4, steady-state mRNA levels for nine other genes analyzed were induced approximately five-fold or greater following exposure to at least five out of the seven compounds tested (Fig. 5). These included glutathione S-transferase AtGSTU24

(At1g17170), cytochrome P450 CYP81D11 (At3g28740), an aldo-keto reductase (At1g60730), a short-chain type dehydrogenase/reductase (At3g04000), two different quinone oxidoreductases (At5g16980, At5g16970), glucosyltransferase UGT73B5 (At2g15480), a malonyl transferase-related sequence (At5g39050), and At4g12490, a sequence of undetermined function related to *Arabidopsis* pEARLI 1 (70). As observed for AtPDR12 and UGT73B4, steady-state mRNA levels increased dramatically (more than 100-fold) in response to specific treatments for many of these genes, particularly those involving herbicide safeners (Fig. 5). For the class III peroxidase sequences tested, PER62 (At5g39580) and PER49 (At4g36430), neither exhibited a general pattern of chemical responsiveness, nor were they significantly up-regulated following exposure to either herbicide safener.

All of the compounds tested in the present work caused some level of induction for the majority of the genes analyzed, although striking differences in their overall effectiveness were observed (Fig. 5). The safener benoxacor was clearly the most effective agent, with 1 mM treatments increasing steady-state mRNA levels five-fold or greater for 18 of the 20 selected genes. The median level of induction for the 18 benoxacor-responsive genes was approximately 21-fold, nearly three times that observed for the safener fenclorim (Fig. 5). Previous studies using *Arabidopsis* have demonstrated the ability of benoxacor to induce the expression of specific phi-, theta-, and tau-class GST gene family members (71), and modest increases for several ABC transporter mRNA sequences have also been reported for benoxacor-treated *Arabidopsis* cell suspensions (54). The data shown in Fig. 5 significantly expand upon existing information concerning the molecular action of this important class of agrichemicals. For example, the data strongly suggest that, at least for benoxacor and fenclorim, herbicide safening action involves the induction of a battery of genes potentially involving all aspects of xenobiotic metabolism and elimination, representing a wide range of enzymatic and transport-related functions. In addition, the observation that transcript levels for many of these genes also increase following exposure to numerous, structurally-unrelated

xenobiotic compounds further suggests that these safeners activate broad-specificity xenobiotic defense pathways responsive to a wide array of compounds.

Recent studies in mammalian systems have revealed that transcriptional responses of genes involved in phase I, II, and III detoxification pathways are mediated through receptor-xenobiotic ligand interactions as well as redox-sensitive factors associated with the actin cytoskeleton (16, 17). For example, the aryl hydrocarbon nuclear receptor (AhR) stimulates the expression of a battery of detoxifying enzymes by binding promiscuously to structurally diverse xenobiotic compounds (64). Following binding to ligand, complexed AhR is translocated to the nucleus and subsequently dimerizes with the co-activator Arnt, then the heterodimeric ligand:AhR:Arnt complex stimulates transcription via xenobiotic response elements (XREs) in target gene promoters. A second important mechanism involves members of the steroid family of nuclear receptors, the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), which are activated by binding to xenobiotic ligands, and subsequently activate transcription of target promoters by binding as heterodimers with the *9-cis* retinoic acid receptor (RXR, 63, 72). As is the case observed for the AhR, PXR binds promiscuously to structurally diverse xenobiotic ligands, and thus serves as a broad-specificity xenobiotic sensor which can induce the expression of a battery of detoxification-related genes (63).

While ligand-activated transcription factors such as AhR and PXR have no apparent plant counterparts, a third major mammalian xenobiotic sensing system involving the basic leucine zipper transcription factor Nrf2 and its repressor Keap1 has recently been elucidated (73, 17). In the absence of chemical inducers, Nrf2 is localized cytoplasmically via interaction with the kelch domain-containing protein Keap1, which is itself tethered to the actin cytoskeleton. In the presence of a wide range of structurally diverse sulfhydryl-reactive electrophilic compounds, two critical highly reactive cysteine residues on the Keap1 protein (C273 and C288) form an intermolecular disulfide bridge between two Keap1 monomers, which then releases Nrf2 for transport into the nucleus. In association with other transcription factors, possibly small maf proteins, Nrf2

subsequently activates target promoters containing the antioxidant response element (ARE), which include a battery of phase II detoxifying enzymes such as GSTs, glucosyltransferases, and quinone oxidoreductases (65). Thus the Keap1 sensor-Nrf2 system provides a relatively simple feed-forward mechanism for responding to a large diversity of xenobiotic electrophiles, aspects of which could readily be envisaged to occur in plant cells. For example, binding of the *Arabidopsis* transcription factor TGA1 with its co-activator NPR1 has recently been shown to be redox-regulated through critical cysteine residues within the protein (74). Interestingly, mRNA levels for a kelch repeat-containing F-box protein family member with sequence similarity to Keap1 were increased by exposure to BOA in the present study (At2g44130; see Supplemental Table I). Members of this plant-specific protein family (75) could conceivably fulfill some of the functions attributed to Keap1, including cytoskeletal anchoring via the kelch repeats, and facilitating the ubiquitin-mediated degradation of its interacting partners via the F-box motif (75, 73, 17). Of further interest is the observation that chemical structures for all known herbicide safeners contain at least one 'soft' electrophilic center which could potentially modify protein sulfhydryl groups (76, 77). Given their potency as transcriptional inducers of plant phase I, II, and III detoxifying enzymes as demonstrated in the present study as well as by others, it is conceivable that the effects of these compounds are mediated via as-yet-unidentified protein redox sensors analogous to the mammalian Keap1 sensor-Nrf2 system.

Emerging evidence suggests that broad-specificity chemosensory mechanisms such as those described above are commonplace among both prokaryotes and eukaryotes (64, 17). Their ability to coordinately up-regulate batteries of xenobiotic detoxifying enzymes enables organisms to expeditiously metabolize and eliminate a wide array of xenobiotics and endotoxins, and this capacity is often further enhanced through the induction of enzymes exhibiting broad substrate specificities (63). The identification of specific *Arabidopsis* genes potentially representing components of a xenobiotic detoxifying 'arsenal', as in the present study, provides the tools necessary to elucidate the signal transduction pathway(s) underlying the xenobiotic defense

response in *Arabidopsis* via molecular genetic and bioinformatics-based approaches. Potent elicitors for these pathways, such as the herbicide safener benoxacor, will undoubtedly serve as important pharmacological probes for these studies as well.

*Analysis of 5' Upstream Sequences*--It is possible that many or a subset of the genes responsive to BOA share common *cis*-regulatory elements in their promoter sequences, which could mediate either general transcriptional responses to stresses associated with xenobiotic exposure, or alternatively, responses specific to benzoxazinoids such as BOA. With the exception of the involvement of TGA transcription factors, basic/leucine zipper (bZIP) protein family members which mediate responses to the auxenic herbicide 2,4-D via interaction with *as-1*-like elements present in some GST promoter sequences (78, 79), little is known regarding other transcription factors and *cis*-regulatory elements involved in responses to xenobiotic stress in plants.

To examine this question, the 5' upstream regions from genes identified by our microarray studies were analyzed for 8-, 10-, and 12-mer statistically over-represented motifs using the program Motif Sampler (29), then identified motifs were compared with known *cis*-regulatory elements in the PlantCARE database (80). The 5' upstream sequences analyzed were grouped by those most highly induced in the microarray results, as well as by enzyme family (Table IV). As only the 8-mer searches yielded promising motifs observed in multiple runs of Motif Sampler (29), the 10- and 12-mer results were not considered further. Importantly, all of the motifs listed in Table IV were significantly over-represented in the data sets analyzed ( $p \leq 0.0043$ ), as compared with the number of motif occurrences observed in searches performed with 28,577 *Arabidopsis* predicted upstream regions.

As shown in Table IV, W-box-like motifs containing the sequence 'TTGACTT' were significantly over-represented in upstream regions of the most highly induced genes, as well as in GST gene upstream sequences. WRKY proteins, a family of plant zinc-finger transcription factors containing the conserved sequence 'WRKYGQK', bind to W-box motifs which contain an invariant TGAC core within the hexamer (T)TGAC(C/T) (81). W-boxes frequently occur in clusters in the

promoters of pathogen-responsive genes and can act synergistically (82-84). Interestingly, one of the genes containing closely-linked W-box-like motifs corresponds to the receptor-like protein kinase SIRK (At2g19190 - Table III), which has been shown to be a regulatory target of the WRKY transcription factor WRKY6 (At1g62300) listed in Table III (85). Inspection of the distribution of 'TTGACTT'-containing motifs, as well as the W-box 'TTGAC(C/T)' consensus motif in these promoter sets revealed that a subset of the upstream regions did contain closely-linked W-box-like elements, however for the majority of the sequences analyzed these motifs were not tightly clustered (not shown). Within the six P450 gene upstream sequences analyzed (Table III), CYP81D8 (At4g37370) also contained three clustered W-Boxes, although the motif was not statistically over-represented in this group ( $p>0.05$ ). It is possible that adjacent 'TGAC'-containing motifs serve as additional WRKY factor binding sites, or alternatively, non-clustered elements could retain functionality and mediate more modest changes in promoter activity (see for example 86, 82). Information currently available suggests that WRKY factors play diverse roles in mediating transcriptional responses to both abiotic and biotic stresses, as well as physiological processes such as leaf senescence (81). Whether they also play a significant role in gene expression changes associated with stresses imposed by exposure to xenobiotic compounds merits further investigation.

As mentioned, TGA transcription factors have been implicated in the activation of some xenobiotic-responsive promoters, therefore we were also interested in examining upstream regions in selected groups of promoters for the presence of *as-1-like* elements. Characteristic of these elements are two imperfect TGACGTC A palindrome motifs whose centers are separated by 12 bp (87). Upstream regions for all genes analyzed by Motif Sampler were also scanned for the presence of *as-1-like* elements using the pattern finding algorithm within RSA-Tools (28). Among the GST genes found to be up-regulated (Table III), two of the gene upstream sequences (AtGSTU7; At2g29420 and AtGSTU1; At2g29490), contain TGAC half sites exhibiting the 12 bp spacing consistent with an *as-1-like* element (not shown). One out of the six P450

gene upstream regions (CYP81D11; At3g28740 - Table III), contained an *as-1-like* element, and corresponding mRNA levels increased following 2,4-D exposure (Fig. 5), suggesting this motif may be active. In addition, the glucosyltransferase UGT73B1 (At4g34138) identified by our microarray studies contains an *as-1-like* element in its upstream region, and was also shown recently to be induced by 2,4-D and salicylic acid (88), suggesting a potential role for these elements in the activation of some *Arabidopsis* glucosyltransferase promoters during xenobiotic stress. Inspection of the upstream regions for the remaining 10 UGTs shown in Table III revealed only one additional UGT gene (UGT75B1; At1g05560) containing an *as-1-like* element on the '-' strand (not shown), however steady-state mRNA levels for this UGT were unresponsive to 10  $\mu$ M 2,4-D treatments, and exhibited only modest increases (approximately 3-fold) in response to 100  $\mu$ M treatments (Fig. 5), suggesting that this motif may possess only minimal activity. *As-1-like* elements were identified in the upstream regions for two additional genes selected for the experiments shown in Fig. 5: ABC transporter AtPDR12 and a putative FAD-linked oxidoreductase (At4g20860). AtPDR12 transcript levels increased approximately 7-fold and 5-fold following exposure to 10  $\mu$ M and 100  $\mu$ M 2,4-D, respectively (Fig. 5), potentially due to the presence of the identified *as-1-like* element. Only modest increases (approximately 2.5-fold) were observed for transcript levels for the putative oxidoreductase following exposure to 10  $\mu$ M 2,4-D, suggesting minimal or no functionality for the *as-1* element identified in its upstream region. The absence of obvious *as-1-like* elements in the majority of the upstream sequences identified in our results, coupled with their responsiveness to herbicide safeners (Fig. 5), indicates that other mechanisms play a more central role in their activation following exposure to xenobiotics.

An important caveat concerning data generated by promoter motif discovery algorithms is that the functionality of an over-represented motif must be determined experimentally, and that motif detection *in silico* can only provide likely candidates for such follow-up studies (30). In addition, it is possible that motifs identified in the present study are functional but not associated

with physiological processes related to phytotoxin exposure. Nevertheless, the list of statistically over-represented motifs shown in Table IV generated from these analyses provides a potentially useful framework for investigating transcriptional control mechanisms influencing a critical biological process.

In summary, in the present study we have identified *O*-glucosylation as the predominant detoxification mechanism in *Arabidopsis* seedlings exposed to the allelochemical BOA. Using nearly full-genome transcriptional profiling studies and quantitative real-time RT-PCR, we have further identified specific genes which may represent integrated components of a diverse broad-specificity chemical defense network. These data significantly expand upon previous studies examining plant transcriptional responses to

environmental toxins, and provide a foundation for further biochemical and genetic experiments to more fully elucidate plant xenobiotic detoxification pathways, and the chemo-sensory mechanisms critical to plant survival in the presence of allelochemicals and other environmental toxins.

*Acknowledgments*--We thank Dr. Divyen Patel of Genome Explorations, Inc. (Memphis, TN) for conducting the Affymetrix gene chip hybridizations, Ms. Melanie Mask for excellent technical assistance, and Drs. Zhiqiang Pan and Melissa Jacob for critical reviews of this manuscript. The work was in part supported via a fellowship under the OECD Cooperative Research Programme: Biological Resource Management for Sustainable Agriculture Systems (to N.P.-B.).

#### REFERENCES

1. Bais, H. P., Park, S. W., Weir, T. L., Callaway, R. M., and Vivanco, J. M. (2004) *Trends Plant Sci.* **9**,26-32
2. Inderjit, and Duke, S. O. (2003) *Planta* **217**, 529-539
3. Weston, L. A., and Duke, S. O. (2003) *Crit. Rev. Plant Sci.* **22**, 367-389
4. Schulz, M., and Wieland, I. (1999) *Chemoecology* **9**, 133-141
5. Duke, S. O., Rimando, A. M., Baerson, S. R., Scheffler, B. E., Ota, E., and Belz, R. G. (2002) *J. Pestic. Sci.* **27**, 298-306
6. Bais, H. P., Vepachedu, R., Gilroy, S., Callaway, R. M., and Vivanco, J. M. (2003) *Science* **301**, 1377-1380
7. Sicker, D., Frey, M., Schulz, M., and Gierl, A. (2000) *Internat. Rev. Cytol.* **198**, 319-346
8. Friebe, A. (2001) *J. Crop Prod.* **4**, 379-400
9. Frey, M., Chomet, P., Glawischnig, E., Stettner, C., Grun, S., Winklmaier, A., Eisenreich, W., Bacher, A., Meeley, R. B., Briggs, S. P., Simcox, K., and Gierl, A. (1997) *Science* **277**, 696-699
10. Parkinson, A. (1996) in *Toxicology: The Basic Science of Poisons* (Klaassen, C. D., ed) pp. 113-186, McGraw-Hill, New York, NY
11. Coleman, J. O. D., Blake-Kalff, M. M. A., and Emyr Davies, T. G. (1997) *Trends Plant Sci.* **2**, 144-151
12. Sandermann, H. Jr. (1999) in *Plant Biotechnology and In Vitro Biology in the 21<sup>st</sup> Century* (Altman, A., ed) pp. 321-328, Kluwer Academic Publishers, Netherlands
13. Martinoia, E., Klein, M., Geisler, M., Bovet, L., Forestier, C., Kolukisaoglu, U., Muller-Rober, B., and Schulz, B. (2002) *Planta* **214**, 345-355
14. Windsor, B., Roux, S.J., and Lloyd, A. (2003) *Nat. Biotechnol.* **21**, 428-433
15. Campbell, E. J., Schenk, P. M., Kazan, K., Penninckx, I. A., Anderson, J. P., Maclean, D. J., Cammue, B. P., Ebert, P. R., and Manners, J. M. (2003) *Plant Physiol.* **133**, 1272-1284
16. Rushmore, T. H., and Kong, A. N. (2002) *Curr. Drug Metab.* **3**, 481-490
17. Wakabayashi, N., Dinkova-Kostova, A. T., Holtzclaw, W. D., Kang, M. I., Kobayashi, A., Yamamoto, M., Kensler, T. W., and Talalay, P. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 2040-2045

18. Cole, D. and J., Edwards, R. (2000) in *Metabolism of Agrochemicals in Plants* (Roberts, T., ed) pp. 108-154, John Wiley and Sons Ltd, Hoboken, NJ
19. Davies, J., and Caseley, J. C. (1999) *Pestic. Sci.* **55**, 1043-1058
20. Ramsey, R. J. L., Mena, F. L., and Stephenson, G. R. (2001) in *Pesticide Biotransformation in Plants and Microorganisms* (Hall, J. C., Hoagland, R. E., and Zablutowicz, R. M., eds) pp. 310-332, ACS Symposium Series No. 777, Oxford University Press, New York, NY
21. Town, C., Haas, B., Redman, J., and Tanimoto, G. (2002) in *Proceedings, 13<sup>th</sup> International Conference on Arabidopsis Research* pp. 1-21, Seville, Spain
22. Xu, W., Bak, S., Decker, A., Paquette, S. M., Feyereisen, R., and Galbraith, D. W. (2001) *Gene* **272**, 61-74
23. Wagner, U., Edwards, R., Dixon, D. P., and Mauch, F. (2002) *Plant Mol. Biol.* **49**, 515-532
24. Lim, E. K., Baldauf, S., Li, Y., Elias, L., Worrall, D., Spencer, S. P., Jackson, R. G., Taguchi, G., Ross, J., and Bowles, D. J. (2003) *Glycobiology* **13**, 139-145
25. Cobbett, C. S., and Meagher, R. B. (2002) in *The Arabidopsis Book* (Somerville, C. R., and Meyerowitz, E. M., eds), pp. 1-22, American Society of Plant Biologists, Rockville, MD, doi/10.1199/tab.0032, <http://www.aspb.org/publications/arabidopsis/>
26. Sicker, D., Schneider, B., Hennig, L., Knop, M., and Schulz, M. (2001) *Phytochemistry* **58**, 819-825
27. Engels, W. R. (1993) *Trends Biochem. Sci.* **18**, 448-450
28. van Helden, J. (2003) *Nucleic Acids Res.* **31**, 3593-3596
29. Thijs, G., Marchal, K., Lescot, M., Rombauts, S., De Moor, B., Rouze, P., and Moreau, Y. (2002) *J. Comput. Biol.* **9**, 447-464
30. Rombauts, S., Florquin, K., Lescot, M., Marchal, K., Rouze, P., and van de Peer, Y. (2003) *Plant Physiol.* **132**, 1162-1176
31. Kankainen, M., and Holm, L. (2004) *Nucleic Acids Res.* **32** (web server issue), W222-9
32. Sicker, D., Hao, H., and Schulz, M. (2004) in *Allelopathy: Chemistry and Mode of Action of Allelochemicals* (Macias, F. A., Galindo, J. C. G., Molinillo, J. M. G., and Cutler, H. G., eds) pp. 77-102, CRC Press, New York, NY
33. Barnes, J. P., Putnam, A. R., and Burke, B. A. (1986) in *The Science of Allelopathy* (Putnam, A. R., and Tang, C. S., eds) pp. 271-286, Wiley-Interscience, New York, NY
34. Alonso, J. M., Stepanova, A. N., Lisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C., and Ecker, J. R. (2003) *Science* **301**, 653-657
35. Duval, M., Hsieh, T. F., Kim, S. Y., and Thomas, T. L. (2002) *Plant Mol. Biol.* **50**, 237-248
36. Hegedus, D., Yu, M., Baldwin, D., Gruber, M., Sharpe, A., Parkin, I., Whitwill, S., and Lydiate, D. (2003) *Plant Mol. Biol.* **53**, 383-397
37. Oppermann, U. C., and Maser, E. (2000) *Toxicology* **144**, 71-81
38. Jez, J. M., Bennett, M. J., Schlegel, B. P., Lewis, M., and Penning, T. M. (1997) *Biochem. J.* **326**, 625-636
39. Hershey, H. P., and Stoner, T. D. (1991) *Plant Mol. Biol.* **17**, 679-690
40. De Veylder, L., Van Montagu, M., and Inze, D. (1997) *Plant Cell Physiol.* **38**, 568-577
41. Sandermann, H. Jr. (1992) *Trends Biochem. Sci.* **17**, 82-84
42. Cole, D. J. (1994) *Pestic. Sci.* **42**, 209-222
43. Pflugmacher, S., and Sandermann, H. Jr. (1998) *Plant Physiol.* **117**, 123-128
44. Stiborová, M., Schmeiser, H. H., and Frei, E. (2000) *Phytochemistry* **54**, 353-362
45. Darley, C. P., Forrester, A. M., and McQueen-Mason, S. J. (2001) *Plant Mol. Biol.* **47**, 179-195
46. Gonzalez, L. F., and Rojas, M. C. (1999) *Phytochemistry* **50**, 931-937
47. Wiegand, R. C., Shah, D.M., Mozer, T. J., Harding, E. I., Diaz-Collier, J., Saunders, C., Jaworski, E. G., and Tiemeier, D. C. (1986) *Plant Mol. Biol.* **7**, 235-243

48. Dixon, D. P., Laphorn, A., and Edwards, R. (2002) *Genome Biol.* 3:Reviews3004
49. Lim, E. K., Doucet, C. J., Li, Y., Elias, L., Worrall, D., Spencer, S. P., Ross, J., and Bowles, D. J. (2002) *J. Biol. Chem.* **277**, 586-592
50. Klein, M., Weissenbock, G., Dufaud, A., Gaillard, C., Kreuz, K., and Martinoia, E. (1996) *J. Biol. Chem.* **271**, 29666-29671
51. Loutre, C., Dixon, D.P., Brazier, M., Slater, M., Cole, D.J., and Edwards, R. (2003) *Plant J.* **34**, 485-493
52. Putman, M., vanVeen, H. W., and Konings, W. N. (2000) *Microbiol. Mol. Biol. Rev.* **64**, 672-693
53. Tommasini, R., Vogt, E., Schmid, J., Fromentau, M., Amrhein, N., and Martinoia, E. (1997) *FEBS Lett.* **411**, 206-210
54. Sánchez-Fernández, R., Ardiles-Díaz, W., Van Montagu, M., Inze, D., and May, M. J. (1998) *Mol. Gen. Genet.* **258**, 655-662
55. van den Brûle, S., and Smart, C. C. (2002) *Planta* **216**, 95-106
56. Theodoulou, F. L., Clark, I. M., He, X. L., Pallett, K. E., Cole, D. J., and Hallahan, D. L. (2003) *Pestic. Manag. Sci.* **59**, 202-214
57. Zhang, L., and Birch, R.G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 9984-9989
58. Haselden, J. N., Hutson, D. H., and Dodds, P. F. (1998) *Biochem. Pharmacol.* **56**, 1591-1598
59. Vessey, D. A., Kelley, M., and Warren, R. S. (1999) *Biochim Biophys. Acta* **1428**, 455-462
60. Nebert, D. W., Roe, A. L., Vandale, S. E., Bingham, E., and Oakley, G. G. (2002) *Genet. Med.* **4**, 62-70
61. Matvienko, M., Wojtowicz, A., Wrobel, R., Jamison, D., Goldwasser, Y., and Yoder, J. I. (2001) *Plant J.* **25**, 375-387
62. Sandermann, H. Jr. (2004) *Trends Plant Sci.* **9**, 406-413
63. Kliewer, S. A., Goodwin, B., and Willson, T. M. (2002) *Endocr. Rev.* **23**, 687-702
64. Denison, M. S., and Nagy, S. R. (2003) *Annu. Rev. Pharmacol. Toxicol.* **43**, 309-334
65. Nguyen, T., Sherratt, P. J., and Pickett, C. B. (2003) *Annu. Rev. Pharmacol. Toxicol.* **43**, 233-260
66. Roth, C. M., Shroyer, J. P., and Paulsen, G. M. (2000) *Agron. J.* **92**, 855-860
67. Wu, H., Haig, T., Pratley, J., Lemerle, D., and An, M. (2000) *J. Agric. Food. Chem.* **48**, 5321-5325
68. Batard, Y., LeRet, M., Schalk, M., Robineau, T., Durst, F., and Werck-Reichhart, D. (1998) *Plant J.* **14**, 111-120
69. Sueyoshi, T., and Negishi, M. (2001) *Annu. Rev. Pharmacol. Toxicol.* **41**, 123-143
70. Richards, K. D., and Gardner, R. C. (1995) *Plant Physiol.* **109**, 1497
71. DeRidder, B. P., Dixon, D. P., Beussman, D. J., Edwards, R., and Goldsbrough, P. B. (2002) *Plant Physiol.* **130**, 1497-1505
72. Rosenfeld, J. M., Vargas, R. Jr., Xie, W., and Evans, R. M. (2003) *Mol. Endocrinol.* **17**, 1268-1282
73. Nguyen, T., Yang, C. S., and Pickett, C. B. (2004) *Free Radic. Biol. Med.* **37**, 433-441
74. Després, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., and Fobert, P. R. (2003) *Plant Cell* **15**, 2181-2191
75. Andrade, M. A., Gonzalez-Guzman, M., Serrano, R., and Rodriguez, P. L. (2001) *Plant Mol. Biol.* **46**, 603-614
76. Komives, T., and Hatzios, K. K. (1991) *Z. Naturforsch.* **46c**, 798-804
77. Coles, B. (1985) *Drug Metab. Rev.* **15**, 1307-1334
78. Singh, K., Foley, R.C., and Onate-Sanchez, L. (2002) *Curr. Opin. Plant Biol.* **5**, 430-436
79. Johnson, C., Glover, G., and Arias, J. (2001) *J. Biol. Chem.* **276**, 172-178
80. Lescot, M., Dehais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouze, P., and Rombauts, S. (2002) *Nucleic Acids Res.* **30**, 325-327
81. Eulgem, T., Rushton, P. J., Robatzek, S., and Somssich, I. E. (2000) *Trends Plant Sci.* **5**, 199-206
82. Eulgem, T., Rushton, P. J., Schmelzer, E., Hahlbrock, K., and Somssich, I. E. (1999) *EMBO J.* **18**, 4689-4699
83. Fukuda, Y. (1997) *Plant Mol. Biol.* **34**, 81-87

84. Rushton, P. J., Torres, J. T., Parniske, M., Wernert, P., Hahlbrock, K., and Somssich, I. E. (1996) *EMBO J.* **15**, 5690-5700
85. Robatzek, S., and Somssich, I. E. (2002) *Genes Dev.* **16**, 1139-1149
86. Rushton, P. J., Reinstadler, A., Lipka, V., Lippok, B., and Somssich, I. E. (2002) *Plant Cell* **14**, 749-762
87. Krawczyk, S., Thurow, C., Niggeweg, R., and Gatz, C. (2002) *Nucleic Acids Res.* **30**, 775-781
88. Uquillas, C., Letelier, I., Blanco, F., Jordana, X., and Holuigue, L. (2004) *Mol. Plant Microbe Interact.* **17**, 34-42

## FIGURE LEGENDS

**FIG. 1. Generation of BOA from DIBOA-*O*-glucoside, and common BOA detoxification mechanisms in plants.** Enzymatic and chemical degradation of DIBOA-*O*-glucoside resulting in the formation of BOA is shown, along with the two prevalent detoxification schemes (*O*- and *N*-glucosylation) known to occur in plants.

**FIG. 2. Dose-response of *Arabidopsis* seedlings grown in the presence of BOA.** *A*, *Root elongation assays*. Representative results are shown for 10-day-old seedlings germinated on vertical MS-agar plates in the presence of 0, 240, 360, 540, 810, or 1215  $\mu$ M BOA. Control treatments consisted of solvent only (0.1% ethanol). *B*, *Determination of  $I_{50}$  and  $I_{80}$  values*. Pooled results from two independent root elongation assay experiments, performed as shown above in *A*, are indicated. Each treatment consisted of approximately 25 seedlings per MS-agar plate, and primary root lengths were measured after ten days of growth at 21°C under a 16-h photoperiod. Each data point represents mean root length from two independent replicates  $\pm$  S.D. The results were used to determine  $I_{50, BOA}$  (average root lengths reduced 50% relative to control treatments), and  $I_{80, BOA}$  (average root lengths reduced 80% relative to control treatments) concentrations for transcriptional profiling experiments.

**FIG. 3. Verification of microarray-based gene expression changes by quantitative real-time RT-PCR analysis.** Sixteen representative genes identified as differentially expressed by microarray analysis were also analyzed by quantitative real-time RT-PCR using the gene-specific primer pairs shown in Table III. *A*, *Transcriptional profiling results*. Shown are relative gene expression values obtained from microarray experiments with BOA-treated *Arabidopsis* seedlings. The data represent selected genes up-regulated in both  $I_{50}$  (open bars) and  $I_{80}$  (closed bars) BOA treatments, and had a *p*-value  $\leq 0.05$  in a two-class comparison performed to identify differentially expressed genes (see 'Experimental Procedures'). Values for control samples, treated with solvent-only (0.1% ethanol), were arbitrarily set to 1.0. *B*, *Quantitative real-time RT-PCR results*. The RNA samples used were identical to those used for transcriptional profiling results shown in panel *A*. Assays were performed in triplicate with SYBR® Green I dye as described in 'Experimental Procedures'. Data were normalized to an internal 18S ribosomal RNA control, and the  $\Delta\Delta C_T$  method was used to obtain the relative expression levels for each gene. Data are shown as mean  $\pm$  S.D., and control samples are as described above in panel *A*. Error bars were generated by transformation of mean  $\Delta\Delta C_T$  values  $\pm$  one standard deviation unit, taken to 2<sup>-1</sup>. The TAIR locus IDs (<http://www.arabidopsis.org>) for genes included in this comparison are indicated on the x-axis.

**FIG. 4. Distribution of BOA-induced genes into functional categories.** Genes were assigned to various functional categories based on the FunCat scheme devised by the Munich Information Center for Protein Sequences (MIPS).

**FIG. 5. Effect of various chemical treatments on selected BOA-responsive genes.** Twenty genes identified as differentially induced by microarray analysis (see ‘Experimental Procedures’), were examined by quantitative real-time RT-PCR for responsiveness to various chemical treatments with xenobiotics, and proposed inducers of xenobiotic metabolism (see text). Assays were performed in triplicate with SYBR® Green I dye, using RNAs pooled from two independent experiments. RNAs were isolated from flash-frozen tissues, harvested at  $t = 24$  h. The  $\Delta\Delta C_T$  method was used to obtain expression levels for each gene relative to solvent-only (0.2 % ethanol or 0.5% DMSO) control treatments, which were arbitrarily set to 1.0. Data are shown as mean  $\pm$  S.D., and error bars were generated by transformation of mean  $\Delta\Delta C_T$  values,  $\pm$  one standard deviation unit, taken to  $2^{-n}$ . The y-axis represents relative fold-increase in gene expression, and the chemical treatments performed are indicated along the x-axis for each panel. Control treatments are indicated by open bars; statistically significant differences in gene expression levels relative to control treatments are indicated by black bars ( $p \leq 0.05$ ); non-significant differences are indicated by gray bars.

TABLE I

*BOA detoxification products in Arabidopsis*

Metabolites of BOA were quantified by HPLC for three-week-old plants exposed to 10, 100, 250, and 500  $\mu\text{M}$  concentrations of BOA for a period of 24 h. A minimum of 30 plants were used per treatment. Each data point represents the mean from three replicates  $\pm$  S.D.

[BOA]	Metabolite (nmol/g fresh weight)			
	BOA-6-OH	BOA-6-O-glucoside	Glucoside carbamate	O:N
10 $\mu\text{M}$	20.3 $\pm$ 4.0	23.8 $\pm$ 5.3	n.d.	-
100 $\mu\text{M}$	66.0 $\pm$ 10.0	121.5 $\pm$ 41.8	33.5 $\pm$ 32.8	5.6 : 1
250 $\mu\text{M}$	174.8 $\pm$ 58.1	249.3 $\pm$ 90.0	38.7 $\pm$ 20.0	11.0 : 1
500 $\mu\text{M}$	212.3 $\pm$ 35.6	381.3 $\pm$ 147.7	54.8 $\pm$ 10.0	10.8 : 1

TABLE II  
Primers used for real-time RT-PCR assays

Locus ID	Gene	Primer Pairs
At1g15520	AtPDR12	F: 5'-CTTTCGCTCAGGTTTTCATCG-3' R: 5'-CTATCGCTTGACGAGAACG-3'
At1g60730	Aldo-keto reductase	F: 5'-CGTCTGAGTTTCTCTGATTGTTGTG-3' R: 5'-TTAGACCACTTAAATGACATGAACCC-3'
At3g28740	CYP81D11	F: 5'-TGCTTCTGTCCATAACTTTTCATCA-3' R: 5'-CTATCGAAATTGTTATACAAGAGGTTCTG-3'
At3g04000	Dehydrogenase/reductase	F: 5'-TGAAAAGAAACATCAATAATCCATGTATC-3' R: 5'-GAGCTTGAGGTTGTATTTGACTGG-3'
At1g17170	AtGSTU24	F: 5'-CTACTTTGTTATGTTGATCTGTTGTTGC-3' R: 5'-CATAGACCTCAAAGAAAATAGAACAAAGC-3'
At5g13750	MFS antiporter	F: 5'-ACAGTCCGAGGAAATATGGAGG-3' R: 5'-AACCAACATCTGCAGTGGAGTATC-3'
At5g16980	Quinone oxidoreductase	F: 5'-CCGGCCAATGTTATCACTTG-3' R: 5'-CAGTATGTTGGATCTTGAATGCTC-3'
At4g20860	FAD-linked oxidoreductase	F: 5'-TTGTAAAAGTGAGATGTACCCAATACG-3' R: 5'-TGACTATTCTCGGTCGGCTTAAAC-3'
At5g16970	Quinone oxidoreductase	F: 5'-TGAAACTATGTTTGATACTTGTACTTTATCCA-3' R: 5'-CCACCCACACAACTCTAATTGG-3'
At5g39580	PER62	F: 5'-CAATAGAATAGAAAGGATATGAGAGAAACC-3' R: 5'-TTTATCAGCAACCCACTTTTGG-3'
At4g36430	PER49	F: 5'-TCGGACCAAGTTCTGTTCAGC-3' R: 5'-TCACAAGCTCTCTCGATTCTCG-3'
At2g19190	Protein kinase	F: 5'-CGCTCTTGCTGTACCGAGC-3' R: 5'-TGACTCATCGTTGGCCTCTG-3'
At5g27420	Transcription factor	F: 5'-TTAGAGGAAATTCAGTAACAAGTCCG-3' R: 5'-GGTCTGCTCGTACCGAGTCAC-3'
At4g34135	UGT73B2	F: 5'-AGCAACCTCTACCATAGACATCACTC-3' R: 5'-AGAGGATTATGCTGAAGATAATAGAGACTTAG-3'
At1g05560	UGT75B1	F: 5'-GGCGTTCCGATGTGGTC-3' R: 5'-CCAGTAGCTTCGCGTTCGTC-3'
At2g15480	UGT73B5	F: 5'-TCTGAGATAGATTATTGACTTTGTGTTC-3' R: 5'-TGACATAAAGAACAACCTCAAAGAGG-3'
At2g15490	UGT73B4	F: 5'-GATGGATTGTTAACTTTCTTAATCTTTGA-3' R: 5'-TTAATTATATAAGAACACACCACGAACATC-3'
At5g39050	Malonyl transferase	F: 5'-TTAGCTGCTGCGAGAATGGTTAG-3' R: 5'-TTCTCATCCAATGCCTCAACAG-3'
At4g12490	pEARLI 1-like	F: 5'-TGTTTCAATCTTTGTTTAAAGTGTGAGG-3' R: 5'-TATATGCGCGAAAAAGATAACG-3'
At4g12500	pEARLI 1-like	F: 5'-ACTGTTTGAATAAATGCATGAATGG-3' R: 5'-ACAAATTCAACTGAAACATTTAAACCTAC-3'

TABLE III  
Selected genes induced  $\geq 2.5$ -fold in response to  $I_{50}$  or  $I_{80}$  BOA treatments

Locus ID	Name	Gene Description	Fold Change ( $I_{50}$ )	Fold Change ( $I_{80}$ )
<b>ABC transporters</b>				
At1g15520	AtPDR12	ABC transporter family protein	2.1	10.8
At3g59140	AtMRP14	ABC transporter family protein	3.6	6.3
At3g53480	AtPDR9	ABC transporter family protein	2.9	3.4
At2g34660	AtMRP2	glutathione S-conjugate ABC transporter (AtMRP2)	2.5	3.2
At3g21250	AtMRP6	ABC transporter family protein	2.4	2.9
At1g30410	AtMRP13	ABC transporter-related	2.5	2.9
<b>Aldo-keto reductases</b>				
At1g60730		aldo-keto reductase family	3.9	8.9
At2g37770		aldo-keto reductase family	4.3	8.9
At2g37760		aldo-keto reductase family	2.8	4.9
At1g60750		aldo-keto reductase family	1.3	4.4
<b>Cytochrome P450s</b>				
At3g28740	CYP81D11	cytochrome P450 family	8.9	16.9
At4g37370	CYP81D8	cytochrome P450, putative	2.5	5.6
At3g14620	CYP72A8	cytochrome P450, putative	2.9	5.1
At5g58860	CYP86A1	cytochrome P450 86A1	2.4	4.4
At3g26210	CYP71B23	cytochrome P450 71B23	4.1	4.2
At5g57220	CYP81F2	cytochrome P450, putative	3.4	4.0
<b>Dehydrogenases/Reductases</b>				
At3g04000		short-chain type dehydrogenase/reductase-related	5.1	15.0
At1g72680	AtCAD1	cinnamyl-alcohol dehydrogenase (CAD), putative	2.7	5.6
At4g13180		short-chain dehydrogenase/reductase family protein	3.4	5.0
At1g54100		aldehyde dehydrogenase, putative (ALDH)	2.6	3.0
At1g21400		branched-chain alpha keto-acid dehydrogenase-related	2.3	3.0
<b>Glutathione transferases</b>				
At1g17170	AtGSTU24	glutathione transferase, putative	6.9	18.1
At1g17180	AtGSTU25	glutathione transferase, putative	3.8	16.2
At2g29490	AtGSTU1	glutathione transferase, putative	5.9	12.8
At2g29420	AtGSTU7	glutathione transferase, putative	5.5	9.2
At1g78340	AtGSTU22	glutathione transferase, putative	3.3	5.2
At3g09270	AtGSTU8	glutathione transferase, putative	3.1	5.0
At1g02930	AtGSTF6	glutathione transferase, putative	3.8	4.8
<b>Other transporters</b>				
At4g23700	CHX17	cation/hydrogen exchanger, putative (CHX17)	4.0	4.3
At1g76520		auxin efflux carrier protein family	2.6	4.3
At2g04040		MATE efflux protein family	2.0	4.0
At5g13750		MFS antiporter	2.5	3.9
At1g79410		transporter-related	2.7	3.7
At5g26340	MSS1	hexose transporter, putative	3.2	3.4
At5g45380		urea active transporter-related protein	2.9	3.2
At1g68570		peptide transporter-related	1.9	3.2
At5g17860	CAX7	cation exchanger, putative (CAX7)	2.2	2.8
<b>Oxidoreductases</b>				
At1g30700		FAD-linked oxidoreductase family	10.4	10.9
At5g16980		quinone oxidoreductase, putative	3.2	9.4
At4g20860		FAD-linked oxidoreductase family	8.4	9.3
At1g76690	OPR2	12-oxophytodienoate reductase (OPR2)	5.8	8.0
At5g16970		quinone oxidoreductase, putative	3.1	4.9
At4g20830		FAD-linked oxidoreductase family	2.9	4.4
At5g24530		oxidoreductase, 2OG-Fe(II) oxygenase family	2.2	3.2
At5g54500		quinone reductase, putative	1.9	3.0
At1g30720		FAD-linked oxidoreductase family	5.5	2.8
At5g38900		fmE protein-like	2.1	2.7

TABLE III - *continued*

Locus ID	Name	Gene Description	Fold Change (I <sub>50</sub> )	Fold Change (I <sub>80</sub> )
<b>Peroxidases</b>				
At5g39580	PER62	peroxidase, putative	9.3	6.3
At4g36430	PER49	peroxidase, putative	2.5	5.0
At5g64120	PER71	peroxidase, putative	7.4	5.0
At2g35380	PER20	peroxidase family	2.7	3.5
At3g49120	PER34	peroxidase, putative	2.4	2.9
At4g37530	PER51	peroxidase, putative	3.6	2.9
<b>Protein kinases</b>				
At2g19190		light repressible receptor protein kinase, putative	8.4	7.6
At5g25930		receptor-related protein kinase-like	3.1	4.2
At2g37710		receptor lectin kinase, putative	2.6	4.2
At3g22060		receptor protein kinase-related	3.1	3.8
At1g70530		protein kinase-related	2.8	3.7
At1g51800		leucine rich repeat protein kinase, putative	5.4	3.7
At2g05940		protein kinase, putative	3.1	3.5
At1g51890		leucine rich repeat protein kinase, putative	4.0	3.4
At5g47070		protein kinase, putative	2.3	3.0
At1g51850		leucine rich repeat protein kinase, putative	4.4	2.8
<b>Transcription factors</b>				
At5g27420		RING-H2 zinc finger protein-related	4.6	6.6
At3g10500		NAC domain protein family	3.0	5.2
At2g17740		CHP-rich zinc finger protein, putative	7.0	4.5
At1g01720	AtAF1	NAC domain protein family	2.6	4.2
At5g63790		NAC domain protein family	3.4	4.1
At4g38620	AtMYB4	transcription factor (MYB4)-related	2.4	3.8
At1g62300	WRKY6	WRKY family transcription factor	3.9	3.7
At3g04070		NAC domain protein family	3.2	3.4
At5g05410	DREB2A	DRE binding protein (DREB2A)	2.1	3.3
At5g39610		NAC domain protein family	2.4	3.1
<b>UDP Glucosyltransferases</b>				
At4g34135	UGT73B2	glucosyltransferase-related protein	9.4	18.3
At1g05560	UGT75B1	UDP-glucose transferase(UGT1)	6.4	14.6
At2g15480	UGT73B5	glucosyltransferase-related	5.2	12.0
At2g15490	UGT73B4	glucosyltransferase-related	3.3	10.4
At1g05680	UGT74E2	glucosyltransferase-related	3.1	9.0
At4g34138	UGT73B1	glucosyltransferase-related	3.4	6.4
At4g01070	UGT72B1	glucosyltransferase-related	3.6	5.2
At2g36790	UGT73C6	glucosyl transferase-related	2.2	5.0
At2g30140	UGT87A2	glucosyltransferase-related	2.8	4.6
At2g43820	UGT74F2	glucosyltransferase-related	2.4	4.4
At4g15490	UGT73B4	UDP-glucosyltransferase, putative	2.5	4.1

TABLE IV  
*Motifs identified in upstream regions of selected BOA-responsive genes*

Data Set (No. Promoters)	Consensus <sup>a</sup>	Log-likelihood <sup>b</sup>	No. with Motif	Average Motif Frequency / Promoter			PlantCARE	Function
				Observed	Genome <sup>c</sup>	<i>p</i> -value <sup>d</sup>		
Highest induced (50)	<b>TTAyTAkT</b>	268.1	38	1.3	0.8	0.0037	E4-ERE	Ethylene-responsive element
	n <b>TTGACTT</b>	239.3	33	0.86	0.38	<0.001	Box-W3	Fungal elicitor-responsive element
UDP-glucosyl transferase (10)	<b>TAGATAwG</b>	44.6	4	0.5	0.12	0.0030	GATA-motif	Part of a light-responsive element
	GCAATGAC	58.5	3	0.4	0.02	<0.001	-	-
Glutathione transferase (7)	k <b>TTGACTT</b>	50.9	3	0.71	0.23	0.0043	Box-W3	Fungal elicitor-responsive element
Cytochrome P450 (6)	<b>TwGACTwT</b>	31.1	4	1.67	0.37	<0.001	Box-W1 JERE	Fungal elicitor-responsive element Jasmonate and elicitor-responsive element
	n <b>CCACGTG</b>	27.9	3	0.5	0.12	0.0031	G-box ABRE	Element involved in light-responsiveness Element involved in abscisic acid-responsiveness

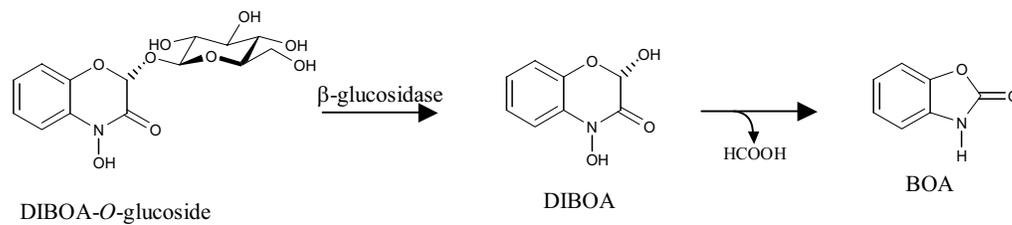
<sup>a</sup> sequence shown in bold used to identify PlantCARE database match

<sup>b</sup> log-likelihood scores were generated by the Motif Sampler software as previously described (26)

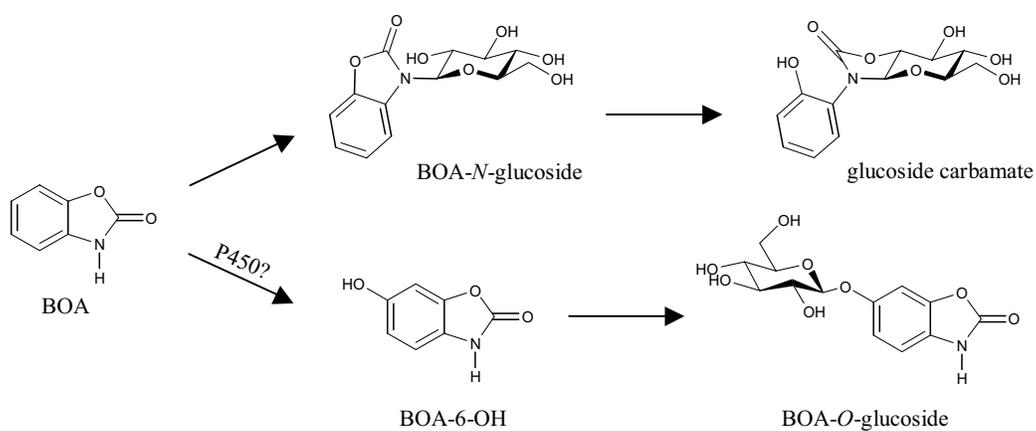
<sup>c</sup> searches were performed with upstream sequences from 28,577 predicted *Arabidopsis* open reading frames using the 'genome-scale dna-pattern' matching function of RSA-tools (<http://rsat.ulb.ac.be/rsat/>); 1,500 bp sequences were retrieved unless an upstream open reading frame was first encountered

<sup>d</sup> *p*-values based on calculated binomial distribution, where **p** (the probability that a motif will occur in a given nucleotide position) is established by the number of occurrences for a given motif within 28,577 upstream sequences retrieved using RSA-tools (see footnote c)

**formation from DIBOA-*O*-glucoside:**

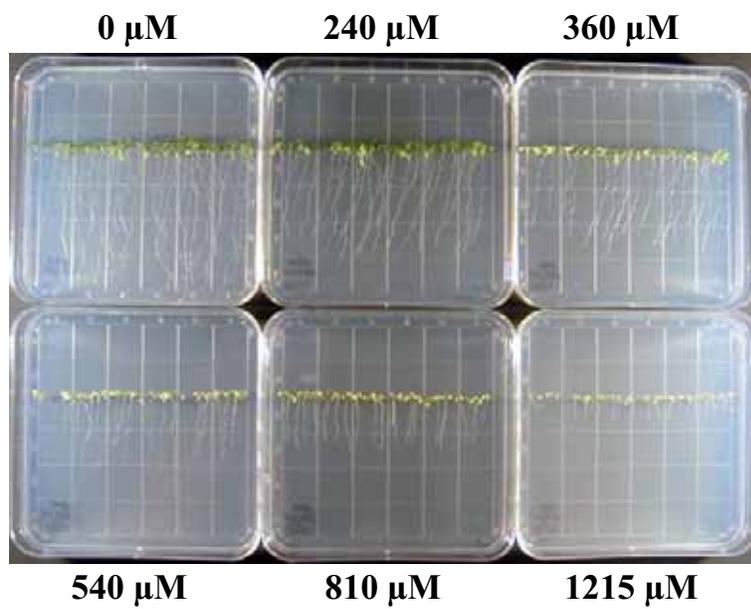


**detoxification:**

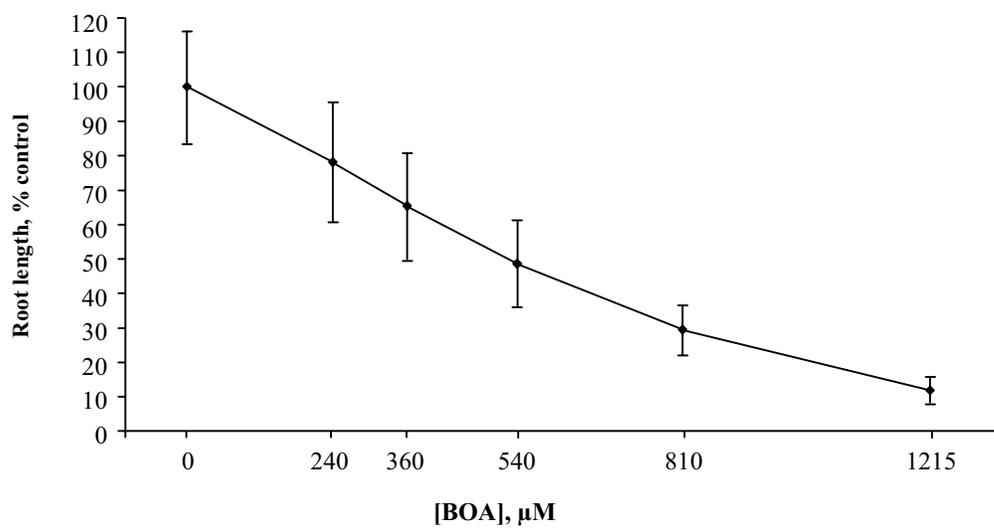


**Figure 1**

**A**



**B**



**Figure 2**

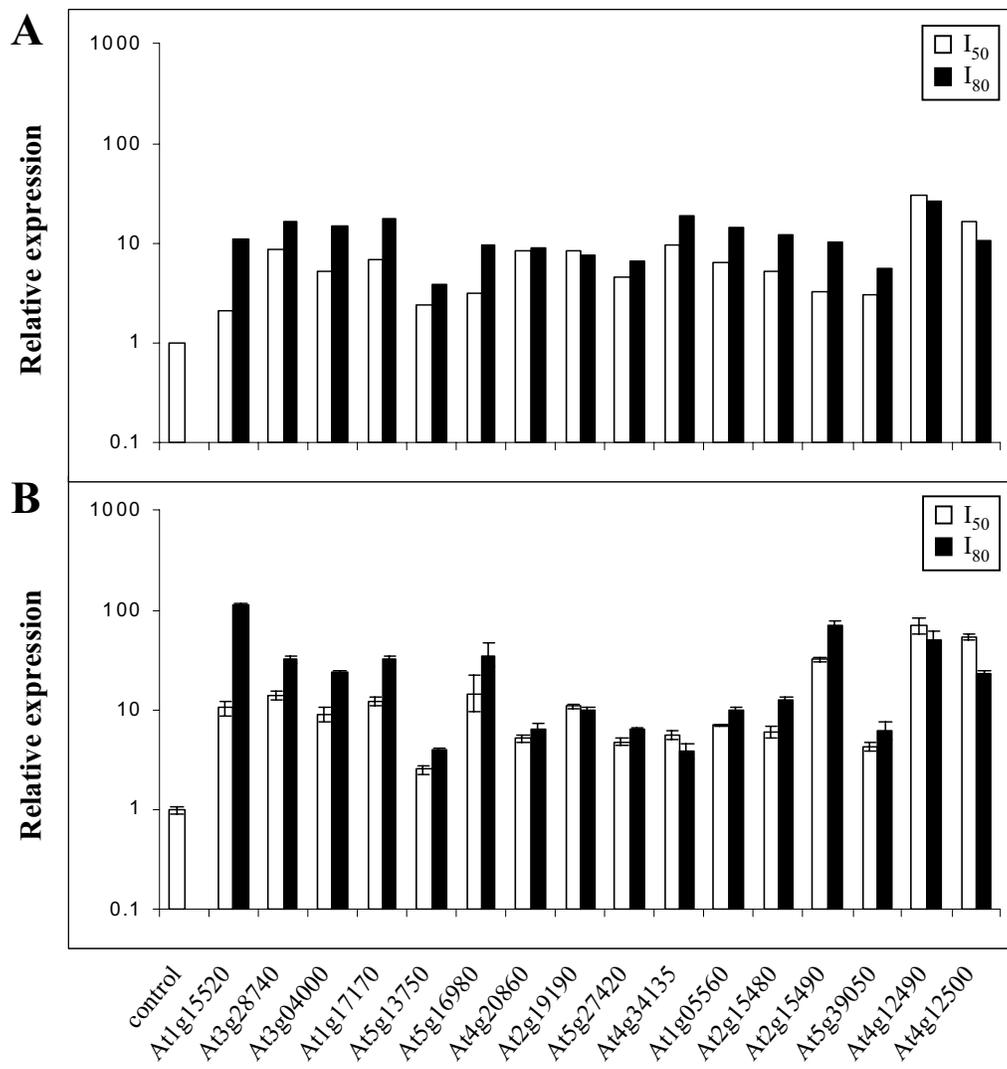
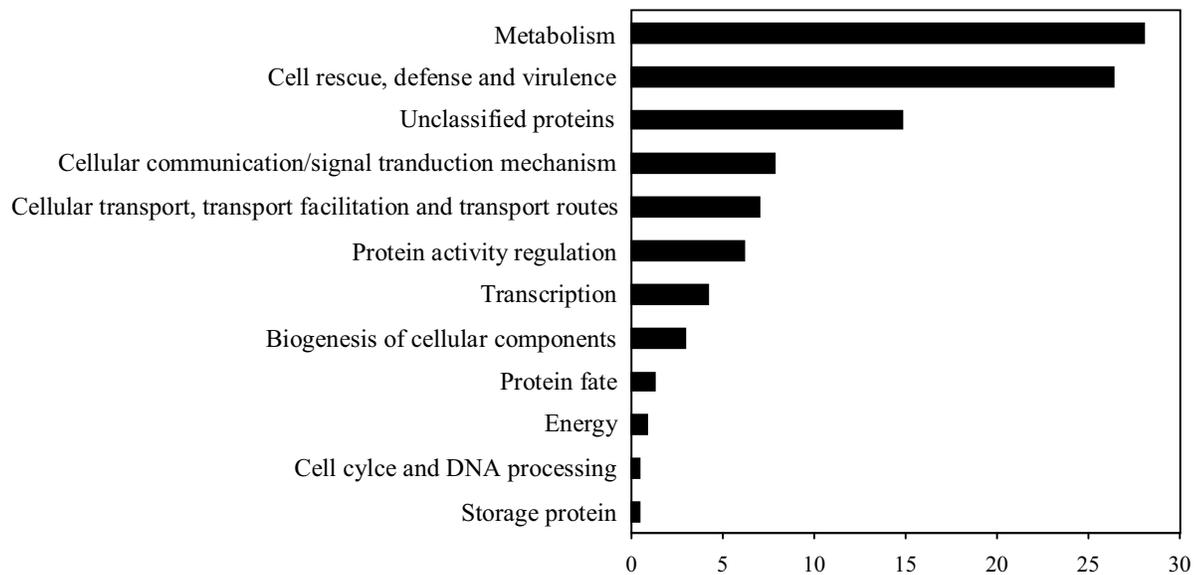


Figure 3



**Figure 4**

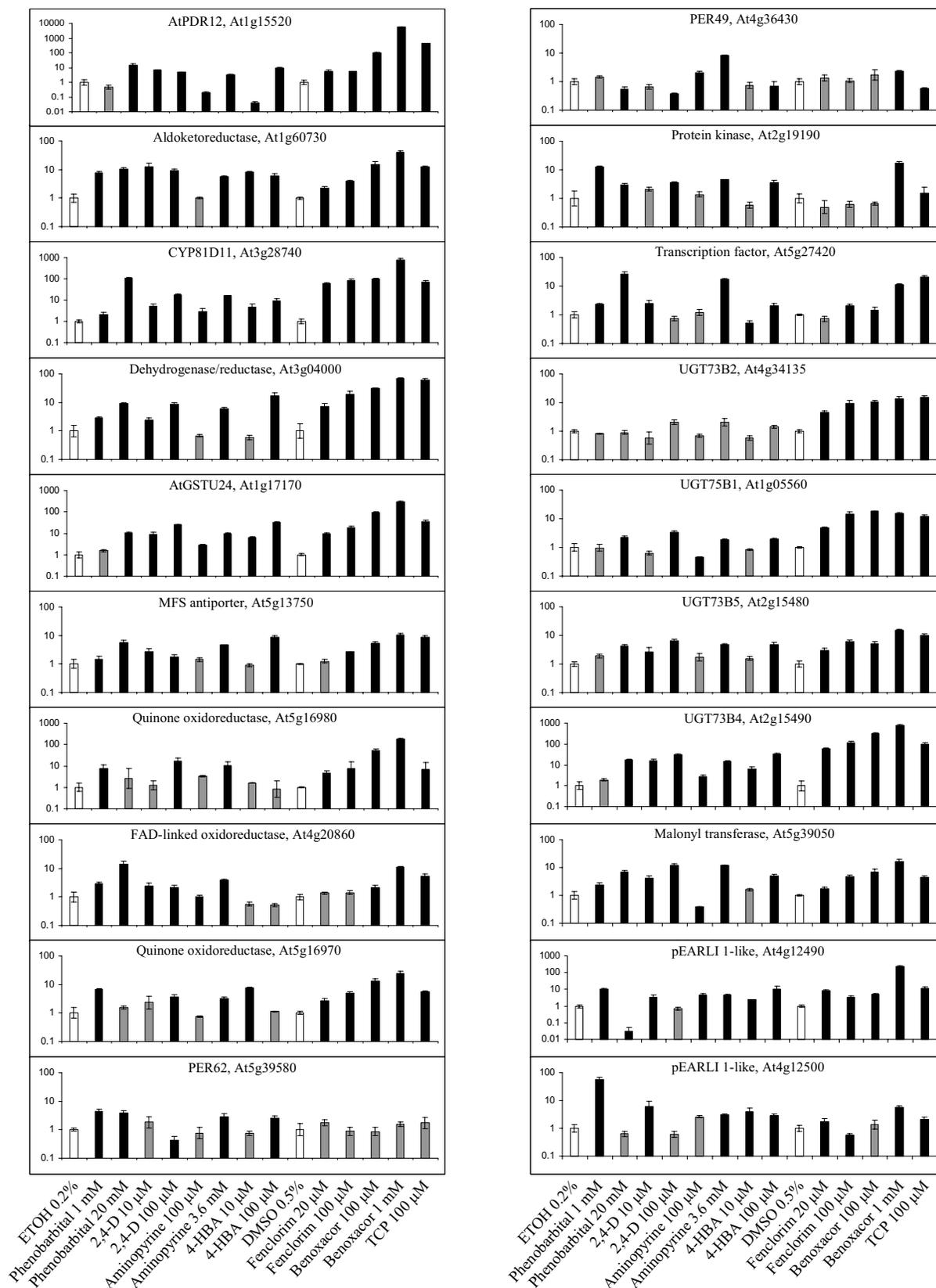


Figure 5

## Supplemental Table I

### A. *thaliana* genes responding to BOA by $\geq 2.5$ -fold in either I50 or I80 treatment

Locus ID	Gene Description	Ratio	p-value
<b><i>I50 treatment upregulated</i></b>			
At4g12490	protease inhibitor/seed storage/lipid transfer protein (LTP) family	30.1	3.14E-14
At4g12500	protease inhibitor/seed storage/lipid transfer protein (LTP) family	16.5	3.14E-14
At1g30700	FAD-linked oxidoreductase family	10.4	3.14E-14
At4g34135	glucosyltransferase -related protein	9.4	8.81E-14
At5g39580	peroxidase, putative	9.3	5.38E-14
At3g28740	cytochrome P450 family	8.9	3.14E-14
At4g20860	FAD-linked oxidoreductase family	8.4	3.14E-14
At2g19190	light repressible receptor protein kinase, putative	8.4	3.14E-14
At2g43620	glycosyl hydrolase family 19 (chitinase)	7.9	9.58E-05
At4g01870	expressed protein	7.8	1.01E-11
At5g64120	peroxidase, putative	7.4	3.96E-09
At2g17740	CHP-rich zinc finger protein, putative	7.0	4.36E-10
At3g46280	expressed protein	7.0	4.28E-14
At3g18250	hypothetical protein	7.0	3.65E-06
At1g17170	glutathione transferase, putative	6.9	3.14E-14
At1g05560	UDP-glucose transferase(UGT1)	6.4	3.14E-14
At2g29490	glutathione transferase, putative	5.9	1.09E-12
At2g44130	Kelch repeat containing F-box protein family	5.9	3.14E-14
At1g76690	12-oxophytodienoate reductase (OPR2)	5.8	4.03E-08
At1g30720	FAD-linked oxidoreductase family	5.5	1.25E-05
At2g29420	glutathione transferase, putative	5.5	3.38E-03
At1g51800	leucine rich repeat protein kinase, putative	5.4	2.33E-07
At2g15480	glucosyltransferase -related	5.2	9.96E-08
At2g39210	nodulin-related protein	5.2	3.14E-14
At3g04000	short-chain type dehydrogenase/reductase -related	5.1	6.15E-07
At3g50480	expressed protein	5.0	1.15E-06
At3g22231	expressed protein	5.0	2.45E-03
At1g02850	glycosyl hydrolase family 1	4.9	3.20E-14
At3g01420	feebly-related protein	4.8	3.14E-14
At5g48540	33 kDa secretory protein-related	4.7	5.03E-04
At4g22470	protease inhibitor/seed storage/lipid transfer protein (LTP) family	4.7	6.64E-06
At5g27420	RING-H2 zinc finger protein-related	4.6	3.68E-08
At5g24210	lipase (class 3) family	4.6	1.54E-12
At5g61820	expressed protein	4.6	7.03E-05
At2g39200	seven transmembrane MLO protein family (MLO12)	4.4	3.14E-14
At1g51850	leucine rich repeat protein kinase, putative	4.4	7.26E-11
At4g11650	osmotin-like protein (OSM34)	4.3	7.18E-03
At2g37770	aldo/keto reductase family	4.3	3.30E-14
At1g65690	expressed protein	4.3	1.35E-05
At1g67810	F12A21.6	4.2	1.23E-10
At3g26210	cytochrome P450 71B23	4.1	3.14E-14
At4g23700	cation/hydrogen exchanger, putative (CHX17)	4.0	2.41E-05
At1g51890	leucine rich repeat protein kinase, putative	4.0	9.36E-13

At1g60730	aldo/keto reductase family	3.9	1.11E-05
At1g62300	WRKY family transcription factor	3.9	3.14E-14
At1g02930	glutathione transferase, putative	3.8	1.61E-02
At5g06860	polygalacturonase inhibiting protein (PGIP1)	3.8	1.08E-03
At5g62630	expressed protein	3.8	3.14E-14
At1g17180	glutathione transferase, putative	3.8	4.58E-04
At2g43510	trypsin inhibitor -related	3.6	1.41E-02
At2g41380	embryo-abundant protein -related	3.6	6.10E-04
At3g59140	ABC transporter family protein	3.6	8.28E-10
At4g01070	glycosyltransferase family	3.6	3.14E-14
At4g37530	peroxidase, putative	3.6	9.08E-12
At1g18970	germin-like protein (GLP1) (GLP4)	3.6	2.56E-13
At2g36220	expressed protein	3.6	3.80E-03
At3g13310	DnaJ protein family	3.4	3.14E-14
At4g21990	5'-adenylylsulfate reductase (APR3)	3.4	3.14E-14
At5g63790	No apical meristem (NAM) protein family	3.4	4.85E-06
At5g57220	cytochrome P450, putative	3.4	2.75E-04
At4g13180	short-chain dehydrogenase/reductase family protein	3.4	5.20E-03
At4g34138	glycosyltransferase family	3.4	3.14E-14
At2g15490	glucosyltransferase -related	3.3	3.57E-14
At1g78340	glutathione transferase, putative	3.3	9.65E-04
At2g14560	expressed protein	3.3	3.14E-14
At5g26340	hexose transporter, putative	3.2	3.14E-14
At1g21250	wall-associated kinase 1	3.2	5.79E-03
At3g04070	No apical meristem (NAM) protein family	3.2	3.14E-14
At5g16980	NADP-dependent oxidoreductase, putative	3.2	3.14E-14
At4g01700	glycosyl hydrolase family 19 (chitinase)	3.2	2.02E-06
At5g25930	receptor-related protein kinase - like	3.1	5.18E-12
At1g05680	glycosyltransferase family	3.1	1.68E-07
At3g14990	putative 4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthes	3.1	4.13E-06
At2g05940	protein kinase, putative	3.1	1.80E-05
At3g22060	receptor protein kinase-related	3.1	6.64E-06
At4g14365	expressed protein	3.1	3.14E-14
At3g09270	glutathione transferase, putative	3.1	1.65E-02
At5g16970	NADP-dependent oxidoreductase (P1), putative	3.1	4.21E-11
At3g10500	expressed protein	3.0	3.14E-14
At2g26560	patatin, putative	3.0	3.14E-14
At5g39050	transferase family	3.0	3.14E-14
At5g09530	proline-rich protein family	3.0	1.64E-02
At1g32940	subtilisin-like serine protease	3.0	3.14E-14
At3g53480	ABC transporter family protein	2.9	1.56E-02
At5g45380	urea active transporter-related protein	2.9	1.81E-07
At4g20830	FAD-linked oxidoreductase family	2.9	2.03E-04
At2g18690	expressed protein	2.9	2.06E-03
At4g12480	protease inhibitor/seed storage/lipid transfer protein (LTP) family	2.9	7.28E-04
At5g03350	expressed protein	2.9	8.45E-06
At3g14620	cytochrome P450, putative	2.9	2.71E-05
At1g68620	expressed protein	2.8	4.98E-07
At1g55850	cellulose synthase family	2.8	1.05E-05
At2g37760	aldo/keto reductase family	2.8	3.26E-04
At1g70530	protein kinase -related	2.8	5.15E-09

At1g14130	2-oxoglutarate-dependent dioxygenase, putative	2.8	1.05E-02
At1g50590	pirin-like protein	2.8	3.14E-14
At2g30140	glycosyltransferase family	2.8	4.47E-14
At4g24160	hydrolase, alpha/beta fold family	2.8	2.77E-02
At4g15530	pyruvate,orthophosphate dikinase	2.7	8.29E-06
At1g79410	transporter - related	2.7	3.14E-14
At5g12420	expressed protein	2.7	3.14E-14
At5g14730	expressed protein	2.7	5.79E-03
At1g72680	cinnamyl-alcohol dehydrogenase (CAD), putative	2.7	1.44E-04
At2g35380	peroxidase family	2.7	1.50E-04
At2g37710	receptor lectin kinase, putative	2.6	2.36E-06
At1g21670	expressed protein	2.6	5.71E-04
At1g51820	leucine rich repeat protein kinase, putative	2.6	3.14E-14
At4g23260	expressed protein	2.6	1.00E-04
At1g76520	auxin efflux carrier protein family	2.6	4.08E-04
At3g56710	SigA binding protein	2.6	2.78E-03
At1g01720	No apical meristem (NAM) protein family	2.6	3.51E-04
At2g35980	harpin-induced protein 1 family (HIN1)	2.6	6.15E-07
At1g54100	aldehyde dehydrogenase, putative (ALDH)	2.6	8.90E-10
At2g37870	protease inhibitor/seed storage/lipid transfer protein (LTP) family	2.5	2.10E-02
At4g36430	peroxidase, putative	2.5	3.14E-14
At2g34660	glutathione S-conjugate ABC transporter (AtMRP2)	2.5	7.12E-03
At4g37370	cytochrome P450, putative	2.5	1.03E-06
At4g27830	glycosyl hydrolase family 1	2.5	1.27E-07
At5g64250	2-nitropropane dioxygenase-related protein	2.5	9.80E-03
At1g30410	ABC transporter -related	2.5	2.45E-04
At5g13750	transporter-related protein	2.5	3.82E-11
At4g15490	UDP-glycosyltransferase, putative	2.5	1.16E-03
At1g55920	serine acetyltransferase	2.5	5.79E-03
At3g49120	peroxidase, putative	2.4	6.12E-04
At2g43820	glycosyltransferase family	2.4	3.53E-02
At5g42050	expressed protein	2.4	1.90E-02
At5g58860	cytochrome P450 86A1	2.4	1.42E-06
At3g21250	ABC transporter family protein	2.4	4.79E-05
At5g39610	NAM / CUC2 - like protein	2.4	2.23E-11
At4g38620	transcription factor (MYB4) -related	2.4	3.40E-05
At4g19880	expressed protein	2.4	8.60E-06
At4g22530	expressed protein	2.3	1.45E-13
At1g51420	hypothetical protein	2.3	4.83E-04
At2g23540	GDSL-motif lipase/hydrolase protein	2.3	3.38E-03
At5g22300	Nitrilase 4 (sp P46011)	2.3	1.96E-12
At5g27600	AMP-binding protein, putative	2.3	2.89E-03
At5g47070	protein kinase, putative	2.3	5.81E-03
At1g21400	branched-chain alpha keto-acid dehydrogenase -related	2.3	1.72E-07
At5g24530	oxidoreductase, 2OG-Fe(II) oxygenase family	2.2	2.89E-03
At2g36790	glucosyl transferase -related	2.2	1.41E-03
At5g17860	cation exchanger, putative (CAX7)	2.2	2.67E-07
At2g43590	glycosyl hydrolase family 19 (chitinase)	2.2	1.14E-02
At1g21680	expressed protein	2.1	8.27E-07
At1g15520	ABC transporter family protein	2.1	9.68E-05
At5g38900	frnE protein - like	2.1	3.20E-14

At5g05410	DRE binding protein (DREB2A)	2.1	9.93E-04
At2g04040	MATE efflux protein family	2.0	4.56E-02
At5g56630	pyrophosphate-dependent phosphofructo-1-kinase-related protein	2.0	6.42E-05
At4g30490	expressed protein	2.0	9.25E-03
At5g54500	quinone reductase, putative	1.9	3.85E-02
At4g18360	glycolate oxidase - like protein	1.9	4.80E-02
At1g68570	peptide transporter -related	1.9	3.47E-03
At3g26470	expressed protein	1.9	1.57E-02
At4g35180	amino acid transporter family	1.3	2.88E-05
At1g60750	aldo/keto reductase family	1.3	1.64E-03
At2g29350	short-chain dehydrogenase/reductase family protein (tropinone reductas	1.2	2.75E-13
At1g10585	expressed protein	1.0	4.90E-03
At1g32960	subtilisin-like serine protease	1.0	2.26E-04

### ***150 treatment downregulated***

At4g15390	acyltransferase family	-8.9	6.77E-07
At4g22212	expressed protein	-7.5	1.03E-10
At4g12550	protease inhibitor/seed storage/lipid transfer protein (LTP) family	-6.1	8.68E-05
At4g33720	pathogenesis-related protein, putative	-4.7	5.84E-03
At1g49860	glutathione transferase, putative	-4.3	2.06E-02
At3g62680	proline-rich protein family	-4.3	1.41E-02
At1g12010	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase), putative	-3.6	3.14E-14
At3g25930	expressed protein	-3.2	5.11E-04
At1g26250	proline-rich extensin, putative	-3.2	4.26E-02
At1g13420	sulfotransferase family	-3.1	3.66E-02
At5g10580	expressed protein	-3.0	2.13E-08
At4g40090	arabinogalactan-protein (AGP3)	-2.9	2.74E-03
At4g26010	peroxidase, putative	-2.8	4.60E-10
At5g25890	auxin-responsive protein IAA28 (Indoleacetic acid-induced protein 28)	-2.7	1.25E-05
At4g19690	iron-regulated transporter (IRT1)	-2.7	8.87E-04
At3g54590	proline-rich protein family	-2.7	2.05E-06
At1g05250	peroxidase, putative	-2.6	3.63E-09
At1g30870	cationic peroxidase, putative	-2.6	2.05E-03
At4g30170	peroxidase, putative	-2.6	1.63E-03
At1g72200	RING-H2 zinc finger protein ATL3 -related	-2.5	7.76E-08
At5g05500	expressed protein	-2.5	2.12E-03
At5g10430	arabinogalactan-protein (AGP4)	-2.4	9.20E-03
At2g24980	proline-rich protein family	-2.3	8.97E-06
At1g23720	proline-rich protein family	-2.3	4.42E-03
At5g03120	expressed protein	-2.2	4.32E-04
At5g59090	subtilisin-like serine protease	-2.2	1.96E-05
At1g52060	jacalin lectin family	-2.1	3.23E-03
At5g35190	proline-rich protein family	-2.1	4.96E-02
Unknown	unknown	-2.1	3.19E-04
At1g76240	expressed protein	-2.0	4.07E-02

### ***180 treatment upregulated***

At4g12490	protease inhibitor/seed storage/lipid transfer protein (LTP) family	26.5	1.71E-14
At4g34135	glucosyltransferase -related protein	18.3	2.94E-14
At1g17170	glutathione transferase, putative	18.1	1.91E-14
At3g28740	cytochrome P450 family	16.9	1.81E-14

At1g17180	glutathione transferase, putative	16.2	4.95E-11
At3g04000	short-chain type dehydrogenase/reductase -related	14.9	1.71E-14
At1g05560	UDP-glucose transferase(UGT1)	14.6	1.81E-14
At2g29490	glutathione transferase, putative	12.8	1.81E-14
At2g15480	glucosyltransferase -related	12.0	1.71E-14
At5g24210	lipase (class 3) family	11.2	1.71E-14
At4g01870	expressed protein	11.0	4.11E-12
At1g67810	F12A21.6	10.9	1.71E-14
At1g30700	FAD-linked oxidoreductase family	10.9	5.04E-11
At1g15520	ABC transporter family protein	10.8	1.89E-14
At4g12500	protease inhibitor/seed storage/lipid transfer protein (LTP) family	10.5	1.71E-14
At2g15490	glucosyltransferase -related	10.4	2.44E-14
At3g50480	expressed protein	9.6	1.18E-13
At1g02850	glycosyl hydrolase family 1	9.6	1.81E-14
At5g16980	NADP-dependent oxidoreductase, putative	9.4	2.01E-14
At4g20860	FAD-linked oxidoreductase family	9.3	1.71E-14
At2g29420	glutathione transferase, putative	9.2	4.55E-05
At1g05680	glycosyltransferase family	9.0	1.71E-14
At1g60730	aldo/keto reductase family	8.9	1.90E-13
At5g61820	expressed protein	8.9	8.35E-11
At2g37770	aldo/keto reductase family	8.9	1.81E-14
At1g76690	12-oxophytodienoate reductase (OPR2)	8.0	4.07E-11
At2g44130	Kelch repeat containing F-box protein family	7.8	1.71E-14
At2g19190	light repressible receptor protein kinase, putative	7.6	1.71E-14
At2g36220	expressed protein	7.1	2.00E-07
At5g48540	33 kDa secretory protein-related	6.8	2.27E-07
At5g27420	RING-H2 zinc finger protein-related	6.6	3.13E-14
At1g14130	2-oxoglutarate-dependent dioxygenase, putative	6.6	5.66E-10
At4g34138	glycosyltransferase family	6.4	1.79E-14
At5g39580	peroxidase, putative	6.3	1.71E-14
At3g59140	ABC transporter family protein	6.3	1.81E-14
At1g65690	expressed protein	6.2	1.14E-09
At3g13310	DnaJ protein family	6.2	1.71E-14
At4g37370	cytochrome P450, putative	5.6	1.71E-14
At1g72680	cinnamyl-alcohol dehydrogenase (CAD), putative	5.6	1.71E-14
At5g39050	transferase family	5.5	1.71E-14
At1g32940	subtilisin-like serine protease	5.5	2.37E-14
At1g68620	expressed protein	5.4	3.81E-10
At4g24160	hydrolase, alpha/beta fold family	5.4	5.83E-06
At3g56710	SigA binding protein	5.4	8.08E-08
At2g37870	protease inhibitor/seed storage/lipid transfer protein (LTP) family	5.3	1.31E-04
At1g78340	glutathione transferase, putative	5.2	3.95E-08
At3g10500	expressed protein	5.2	1.81E-14
At4g01070	glycosyltransferase family	5.2	1.71E-14
At3g14620	cytochrome P450, putative	5.1	1.71E-14
At2g43620	glycosyl hydrolase family 19 (chitinase)	5.0	4.58E-03
At1g21250	wall-associated kinase 1	5.0	8.12E-08
At4g36430	peroxidase, putative	5.0	1.71E-14
At2g36790	glucosyl transferase -related	5.0	1.59E-04
At4g13180	short-chain dehydrogenase/reductase family protein	5.0	5.26E-06
At5g64120	peroxidase, putative	5.0	2.84E-06

At3g09270	glutathione transferase, putative	5.0	5.82E-06
At2g37760	aldo/keto reductase family	4.9	5.97E-10
At3g18250	hypothetical protein	4.9	5.82E-04
At5g22300	Nitrilase 4 (sp P46011)	4.9	1.81E-14
At4g11650	osmotin-like protein (OSM34)	4.9	3.85E-04
At5g16970	NADP-dependent oxidoreductase (P1), putative	4.9	2.29E-10
At1g02930	glutathione transferase, putative	4.8	7.94E-04
At5g64250	2-nitropropane dioxygenase-related protein	4.8	7.78E-08
At5g06860	polygalacturonase inhibiting protein (PGIP1)	4.7	1.63E-06
At4g14365	expressed protein	4.7	6.47E-14
At4g21990	5'-adenylylsulfate reductase (APR3)	4.7	1.71E-14
At2g30140	glycosyltransferase family	4.6	2.87E-14
At2g26560	patatin, putative	4.6	1.81E-14
At2g17740	CHP-rich zinc finger protein, putative	4.5	4.40E-06
At1g32960	subtilisin-like serine protease	4.5	5.21E-11
At2g18690	expressed protein	4.5	2.92E-07
At4g20830	FAD-linked oxidoreductase family	4.4	6.40E-08
At1g60750	aldo/keto reductase family	4.4	5.58E-12
At2g43820	glycosyltransferase family	4.4	4.51E-05
At3g14990	putative 4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthes	4.4	1.59E-13
At5g58860	cytochrome P450 86A1	4.4	1.71E-14
At2g41380	embryo-abundant protein -related	4.3	1.25E-05
At4g23700	cation/hydrogen exchanger, putative (CHX17)	4.3	1.14E-05
At1g76520	auxin efflux carrier protein family	4.3	9.89E-10
At2g39210	nodulin-related protein	4.2	1.71E-14
At5g25930	receptor-related protein kinase - like	4.2	1.71E-14
At2g37710	receptor lectin kinase, putative	4.2	1.71E-14
At3g26210	cytochrome P450 71B23	4.2	1.71E-14
At1g01720	No apical meristem (NAM) protein family	4.2	9.15E-07
At5g14730	expressed protein	4.1	3.78E-05
At2g35980	harpin-induced protein 1 family (HIN1)	4.1	1.72E-12
At4g15490	UDP-glycosyltransferase, putative	4.1	2.04E-11
At5g63790	No apical meristem (NAM) protein family	4.1	9.05E-06
At2g43590	glycosyl hydrolase family 19 (chitinase)	4.0	3.34E-11
At2g04040	MATE efflux protein family	4.0	8.84E-07
At3g46280	expressed protein	4.0	1.81E-14
At5g57220	cytochrome P450, putative	4.0	1.68E-05
At5g13750	transporter-related protein	3.9	1.10E-10
At4g19880	expressed protein	3.8	6.10E-14
At3g22231	expressed protein	3.8	1.98E-02
At4g38620	transcription factor (MYB4) -related	3.8	5.16E-14
At3g22060	receptor protein kinase-related	3.8	1.71E-14
At3g01420	feebly-related protein	3.8	1.81E-14
At1g70530	protein kinase -related	3.7	1.71E-14
At2g23540	GDSL-motif lipase/hydrolase protein	3.7	4.13E-08
At1g62300	WRKY family transcription factor	3.7	1.71E-14
At1g79410	transporter - related	3.7	1.71E-14
At2g14560	expressed protein	3.7	9.31E-09
At2g43510	trypsin inhibitor -related	3.7	1.71E-02
At1g51800	leucine rich repeat protein kinase, putative	3.7	2.15E-12
At5g09530	proline-rich protein family	3.6	1.47E-07

At5g56630	pyrophosphate-dependent phosphofructo-1-kinase-related protein	3.6	1.71E-14
At1g10585	expressed protein	3.6	2.48E-13
At2g05940	protein kinase, putative	3.5	6.65E-08
At4g27830	glycosyl hydrolase family 1	3.5	1.71E-14
At1g50590	pirin-like protein	3.5	1.71E-14
At2g35380	peroxidase family	3.5	1.23E-06
At3g04070	No apical meristem (NAM) protein family	3.4	1.81E-14
At3g53480	ABC transporter family protein	3.4	3.91E-05
At5g12420	expressed protein	3.4	1.98E-14
At5g62630	expressed protein	3.4	1.71E-14
At5g26340	hexose transporter, putative	3.4	1.71E-14
At3g26470	expressed protein	3.4	3.68E-06
At4g22530	expressed protein	3.4	1.71E-14
At1g55850	cellulose synthase family	3.4	6.63E-05
At1g51890	leucine rich repeat protein kinase, putative	3.4	1.18E-13
At5g05410	DRE binding protein (DREB2A)	3.3	1.71E-14
At4g35180	amino acid transporter family	3.2	1.71E-14
At5g45380	urea active transporter-related protein	3.2	8.21E-06
At5g24530	oxidoreductase, 2OG-Fe(II) oxygenase family	3.2	1.76E-06
At1g68570	peptide transporter -related	3.2	5.57E-11
At2g34660	glutathione S-conjugate ABC transporter (AtMRP2)	3.2	3.28E-05
At1g21670	expressed protein	3.2	3.45E-08
At4g22470	protease inhibitor/seed storage/lipid transfer protein (LTP) family	3.2	3.78E-03
At2g29350	short-chain dehydrogenase/reductase family protein (tropinone reductas	3.1	1.81E-14
At5g39610	NAM / CUC2 - like protein	3.1	3.96E-08
At4g15530	pyruvate,orthophosphate dikinase	3.1	2.39E-06
At1g54100	aldehyde dehydrogenase, putative (ALDH)	3.0	7.93E-13
At5g47070	protein kinase, putative	3.0	1.40E-05
At5g54500	quinone reductase, putative	3.0	9.42E-07
At1g21400	branched-chain alpha keto-acid dehydrogenase -related	3.0	1.71E-14
At1g55920	serine acetyltransferase	3.0	3.29E-05
At5g42050	expressed protein	3.0	2.50E-03
At1g51420	hypothetical protein	3.0	4.69E-06
At3g49120	peroxidase, putative	2.9	3.81E-06
At4g37530	peroxidase, putative	2.9	6.28E-09
At3g21250	ABC transporter family protein	2.9	7.31E-10
At1g30410	ABC transporter -related	2.9	1.27E-06
At5g27600	AMP-binding protein, putative	2.9	5.11E-09
At1g51850	leucine rich repeat protein kinase, putative	2.8	1.88E-05
At5g17860	cation exchanger, putative (CAX7)	2.8	3.50E-09
At1g30720	FAD-linked oxidoreductase family	2.8	2.54E-02
At4g18360	glycolate oxidase - like protein	2.7	1.02E-04
At5g38900	frnE protein - like	2.7	1.81E-14
At4g12480	protease inhibitor/seed storage/lipid transfer protein (LTP) family	2.6	1.24E-03
At1g21680	expressed protein	2.6	3.87E-06
At4g01700	glycosyl hydrolase family 19 (chitinase)	2.6	2.47E-04
At4g30490	expressed protein	2.5	5.51E-08
At4g23260	expressed protein	2.5	5.48E-05
At5g03350	expressed protein	2.2	1.51E-04
At1g51820	leucine rich repeat protein kinase, putative	2.1	1.71E-14
At2g39200	seven transmembrane MLO protein family (MLO12)	2.1	2.27E-06

At1g18970	germin-like protein (GLP1) (GLP4)	2.0	8.82E-03
-----------	-----------------------------------	-----	----------

***180 treatment downregulated***

At4g22212	expressed protein	-13.0	5.30E-05
At4g15390	acyltransferase family	-11.6	1.71E-14
At3g62680	proline-rich protein family	-5.8	7.51E-11
At4g12550	protease inhibitor/seed storage/lipid transfer protein (LTP) family	-5.2	5.75E-04
At4g19690	iron-regulated transporter (IRT1)	-5.1	1.68E-10
At1g49860	glutathione transferase, putative	-4.3	9.03E-04
At4g33720	pathogenesis-related protein, putative	-4.2	2.18E-03
At4g26010	peroxidase, putative	-4.2	1.71E-14
At3g25930	expressed protein	-4.1	4.33E-04
At5g05500	expressed protein	-4.0	1.71E-14
At4g40090	arabinogalactan-protein (AGP3)	-3.9	2.50E-10
At1g30870	cationic peroxidase, putative	-3.9	2.17E-06
At3g54590	proline-rich protein family	-3.9	1.71E-14
At4g30170	peroxidase, putative	-3.9	6.19E-07
At1g12010	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase), putative	-3.7	3.32E-14
At1g26250	proline-rich extensin, putative	-3.6	6.26E-05
At5g03120	expressed protein	-3.6	3.69E-07
At2g24980	proline-rich protein family	-3.5	3.40E-06
At1g05250	peroxidase, putative	-3.3	1.71E-14
At1g76240	expressed protein	-3.3	7.02E-06
At5g10430	arabinogalactan-protein (AGP4)	-3.1	1.71E-14
At1g13420	sulfotransferase family	-3.1	6.19E-11
At5g59090	subtilisin-like serine protease	-3.1	1.74E-14
Unknown	unknown	-3.0	1.28E-05
At5g35190	proline-rich protein family	-2.9	1.75E-05
At1g23720	proline-rich protein family	-2.8	1.27E-11
At1g52060	jacalin lectin family	-2.6	6.99E-04
At5g25890	auxin-responsive protein IAA28 (Indoleacetic acid-induced protein 28)	-2.5	1.71E-14
At1g72200	RING-H2 zinc finger protein ATL3 -related	-2.5	4.16E-10
At5g10580	expressed protein	-2.3	2.68E-06