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

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Benzoxazolin-2(3*H*)-one (BOA) is an allelochemical most commonly associated with monocot species, formed from the *O*-glucoside of 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-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 BOA concentrations, representing I_{50} and I_{80} levels based on root elongation inhibition assays. 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. 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 (1, 4). 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 (3, 5).

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 benzoxazolinone 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 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one have been identified in corn via transposon tagging, and they 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 benzoxazolinones act as defense compounds against microbial pathogens as well as insect herbivores, and within the rhizosphere they play an important role as allelochemicals (7, 8). Formation of the benzoxazolinone, benzoxazolin-2(3*H*)-one (BOA),[1] results from a two-step degradation of the glucoside of DIBOA (2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one; see Fig. 1). Numer-

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[S] The on-line version of this article (available at www.jbc.org) contains Table I.

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[1] The abbreviations used are: BOA, benzoxazolin-2(3*H*)-one; DIBOA, 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one; ABC, ATP binding cassette; MATE, multidrug and toxic compound extrusion; MFS, major facilitator superfamily; AKR, aldo-keto reductase; GSTs, glutathione *S*-transferases; UGTs, UDP-glucosyltransferases; AhR, aryl hydrocarbon receptor; RT, reverse transcription; 2,4-D, 2,4-dichlorophenoxyacetic acid.

ous 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 (10–12). 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 ~24,000 transcripts identified a significant number of genes potentially involved in phase I, II, and III detoxification processes that are induced following exposure to this allelochemical. By 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 *A. thaliana* (Col-0) were germinated in a mixture of sand, mold, and Perlite (4:4:2) and then maintained in a greenhouse at 20 °C for a period of 3 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 and then washed extensively to remove soil particles. Incubations with BOA (Sigma) were performed for 24 h, as described previously (4), using 10, 100, 250, and 500 μM treatment solutions. After incubation, plants were rinsed, dried between paper sheets, and 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 were determined.

The extracts were analyzed for detoxification products by high pressure liquid chromatography using a model 126 chromatograph (Beckman Instruments, Fullerton, CA) equipped with a diode array detector (model 168) and an Ultrasphere ODS RP 18 column. Compounds were eluted with the following gradients: 1 min, 100% eluent A (0.1% trifluoroacetic acid in H_2O); 1–21 min, 20% eluent B (methanol) linear; 21–41 min, 80% eluent B linear; 41–43 min, 100% eluent 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 (4, 26). 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 *A. thaliana* (Col-0) seedlings were maintained in a growth chamber at 21 °C under a 16-h photoperiod and light intensity of 150 μmol m^{-2} s^{-1}. Seeds were surface-sterilized in 70% ethanol for 5 min and then rinsed two times in sterile distilled water, followed by treatment with 0.5× bleach (3% sodium hypochlorite) and 0.05% Tween 20 for 10 min, and then finally rinsed four times in sterile distilled water. Following surface sterilization, seeds were placed on top of an ~2.0-cm-high stack of 9.0-cm Whatman No. 4 filter disks and allowed to air-dry in a sterile hood. Liquid and semi-solid germination media used for all experiments contained 0.5 × Murashige and Skoog salts, 1× 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 ~2.0 cm from the edge in 9.0 × 9.0 × 1.5-cm square Petri dishes containing semi-solid germination media supplemented with different concentrations of BOA. Control plates contained solvent alone (0.1% ethanol). Plates were cold-treated for 3 days, transferred to a growth chamber, and then 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, ~200 seeds were scooped into a microspatula and then distributed evenly over the surface of a sterile 0.3-μm microporous membrane raft supported by a buoyant float (Osmotek Ltd., Rehovat, Israel). Seeds, rafts, and floats were placed on liquid germination media in Lifeguard tissue culture vessels with 4.0-cm vented lids (Osmotek Ltd., Rehovat, Israel), cold-treated for 3 days, and then transferred to a growth chamber. After 10 days, BOA (or 0.1% ethanol for control treatments) was added to the media and 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, and then stored at −80 °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 to two different concentrations of fenclorim (Toronto Research Chemicals, Inc., Ontario, Canada), benoxacor (Sigma), 2,4-dichlorophenoxyacetic acid (Sigma), phenobarbital (Sigma), *p*-hydroxybenzoic acid (sodium salt; Sigma), and 4-dimethylaminoantipyrine (free base; Sigma). For 2,4,5-trichlorophenol (Sigma), due to extensive injury observed on seedlings exposed to 10 mM treatments, only 100 μM treatments were used. Stock solutions were prepared in either Me_2SO or ethanol and then added to seedlings growing on liquid media as described above in duplicate treatments. Control treatments (0.25% ethanol and 0.5% Me_2SO) were also performed in duplicate. At 24 h post-treatment, seedlings were flash-frozen in liquid nitrogen and stored at −80 °C prior to analysis.

RNA Isolation—Total RNAs for use in microarray experiments were isolated from 0.5 g of flash-frozen, pulverized 10-day-old seedling tissues using the Trizol reagent (Invitrogen), with an additional homogenization step of 30 s at 25,000 rpm using a hand held homogenizer. The RNA recovered was then re-purified with an RNeasy plant mini-kit (Qiagen, Valencia, CA) per the 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 an 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 an RNase-free DNase kit as per the 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 with 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 ≤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 data base provided by Affymetrix (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 μg of total RNA in a 100-μl reaction volume using the TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. Quantitative real time PCRs were performed in triplicate using the GenAmp® 5700 sequence detection system (Applied Biosystems). Independent PCRs were performed using the same cDNA for both the gene of interest and 18 S rRNA, using the SYBR® Green PCR Master Mix (Applied Biosystems). Gene-specific primers were designed for the gene of interest and 18 S 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 data base using the BLAST server at The *Arabidopsis* Information Resource (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 GeneAmp® 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 18 S rRNA was subtracted from that of the gene of interest to obtain a ΔC_T value. The C_T value of an arbitrary calibrator (*e.g.* untreated sample in the case of up-regulated genes) was subtracted from the ΔC_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 ≤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 (see Ref. 28; rsat.ulb.ac.be/rsat/), by selecting the "mRNA" feature type, and preventing overlap with upstream open reading frames. The program Motif Sampler (see Ref. 29; 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 3rd 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, and then *p* values for each experimental result were determined based on calculated binomial probabilities using the "PROBBNML" function within the SAS version 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 and 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 ≤ 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-β-glucoside 1,2-carbamate) shown in Fig. 1 (26). An additional metabolite, gentiobioside carbamate (1-(2-hydroxyphenylamino)-1-deoxy-β-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 (26, 32).

Sensitivity to BOA is highly species-dependent, although dicot species are in general less tolerant than monocots (33).

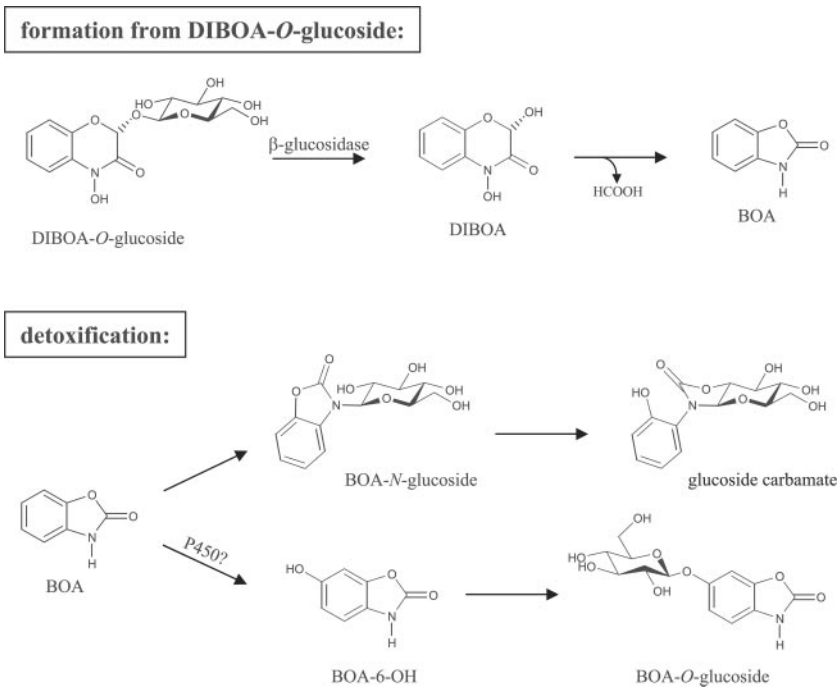

FIG. 1. **Generation of BOA from DIBOA-*O*-glucoside and common BOA detoxification mechanisms in plants.** Enzymatic and chemical degradation of DIBOA-*O*-glucoside resulting in the formation of BOA is shown, along with the two prevalent detoxification schemes (*O*- and *N*-glucosylation) known to occur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

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 *Z. 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 μM concentrations of BOA; however, the accumulation of glucoside carbamate was not observed in any of the four trials performed using 10 μM treatments (Table I). Most 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 g of fresh weight were ~11:1 for both the 250 and 500 μM 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), suggest either an inefficient conversion of BOA-6-OH to BOA-6-*O*-glucoside or alternatively, competing, higher levels of β-glucosidase, which markedly increase steady-state levels of BOA-6-OH. Although 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. 2*A*). 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 ~540 μM and 1 mM, respectively (Fig. 2*B*). Similar dose-dependent responses for root elongation have been reported for other species grown in the presence of BOA (33), indicating the suitability of *A. 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 ~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, and 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 μM 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 and grown in separate culture vessels to account for experimental variation, and then total RNAs were isolated from each replicate and

TABLE I
BOA detoxification products in Arabidopsis

Metabolites of BOA were quantified by high pressure liquid chromatography for 3-week-old plants exposed to 10, 100, 250, and 500 μM 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 ± S.D. ND, not detected.

[BOA]	Metabolite (nmol/g fresh weight)			*O*-glucosylation:*N*-glucosylation
	BOA-6-OH	BOA-6-*O*-glucoside	Glucoside carbamate	
10 μM	20.3 ± 4.0	23.8 ± 5.3	ND	
100 μM	66.0 ± 10.0	121.5 ± 41.8	33.5 ± 32.8	5.6:1
250 μM	174.8 ± 58.1	249.3 ± 90.0	38.7 ± 20.0	11.0:1
500 μM	212.3 ± 35.6	381.3 ± 147.7	54.8 ± 10.0	10.8:1

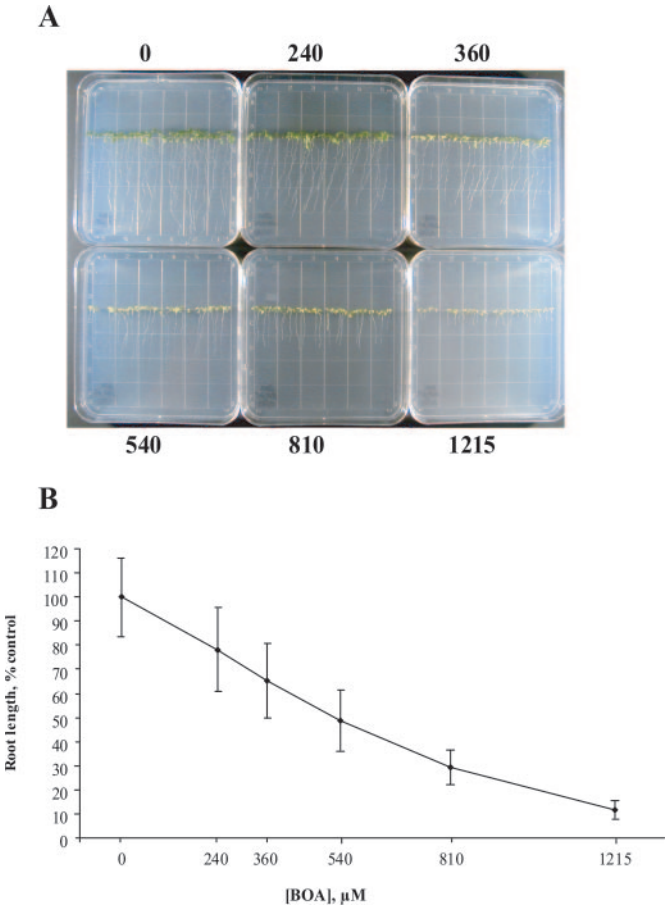

FIG. 2. **Dose-response of *Arabidopsis* seedlings grown in the presence of BOA.** *A,* root elongation assays. Representative results are shown for 10-day-old seedlings germinated on vertical MS-agar plates in the presence of 0, 240, 360, 540, 810, or 1215 μM BOA. Control treatments consisted of solvent 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 ~25 seedlings per MS-agar plate, and primary root lengths were measured after 10 days of growth at 21 °C under a 16-h photoperiod. Each data point represents mean root length from two independent replicates ± 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.

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 ~0.8% of all *Arabidopsis* genes included on the ATH1 array.

To confirm further 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 that 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 *A. thaliana* data base (mips.gsf.de/proj/thal/db/), BLAST searches, and literature reports. The largest categories identified were "metabolism" and "cell rescue, defense, and virulence," representing ~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-ketoreductases, peroxidases, cytochrome P450-like proteins, glutathione *S*-transferases, glucosyltransferases, and representatives of several families of multidrug efflux transporters, including the ABC, major facilitator superfamily (MFS), and multidrug and toxic compound extrusion (MATE) transporters. A list of selected up-regulated genes potentially associated with detoxification

TABLE II
Primers used for real time RT-PCR assays

Locus identification	Gene	Primer pairs[a]
At1g15520	*AtPDR12*	F: 5′-CTTTCGCTCAGGTTTTCATCG-3′ R: 5′-CTATCGCTTGCACGAGAACG-3′
At1g60730	Aldo-ketoreductase	F: 5′-CGTCTGAGTTTCTCTGATTGTTGTG-3′ R: 5′-TTAGACCACTTAAATGACATGAACCC-3′
At3g28740	*CYP81D11*	F: 5′-TGCTTCTTGTCCATAACTTTCATCA-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′-CAGTATGTTGGATCTTGAAATGCTC-3′
At4g20860	FAD-linked oxidoreductase	F: 5′-TTGTAAAAGTGAGATGTACCCAATACG-3′ R: 5′-TGACTATTCTCGGTCCGCTTAAAC-3′
At5g16970	Quinone oxidoreductase	F: 5′-TGAAACTATGTTTGATACTTGTACTTTATCCA-3′ R: 5′-CCACCCACACAAACTCTAATTGG-3′
At5g39580	*PER62*	F: 5′-CAATAGAATAGAAAGGATATGAGAGAAACC-3′ R: 5′-TTTATCAGCAACCCACTTTTGG-3′
At4g36430	*PER49*	F: 5′-TCGGACCAAGTTCTGTTCAGC-3′ R: 5′-TCACAAGCTCTCTCGATTTCTCG-3′
At2g19190	Protein kinase	F: 5′-CGCTCTTGCTTGTACCGAGC-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′-GGCGTTTCCGATGTGGTC-3′ R: 5′-CCAGTAGCTTCGCGTTCGTC-3′
At2g15480	*UGT73B5*	F: 5′-TCTGAGATAGATTATTGACTTTGTGTTCC-3′ R: 5′-TGACATAAAGAACAACTCCAAAGAGG-3′
At2g15490	*UGT73B4*	F: 5′-GATGGATTGTTAACTTTTCTTAATCTTTGA-3′ R: 5′-TTAATTATATAAGAACACACCACGAACATC-3′
At5g39050	Malonyltransferase	F: 5′-TTAGCTGCTGCGAGAATGGTTAG-3′ R: 5′-TTCTCATCCAATGCCTCAACAG-3′
At4g12490	*pEARLI 1*-like	F: 5′-TGTTTCAATCTTTTGTTTAAGTGTGAGG-3′ R: 5′-TATATGCGCGGAAAAAGATAACG-3′
At4g12500	*pEARLI 1*-like	F: 5′-ACTGTTTGAATAAATGCATGAATGG-3′ R: 5′-ACAAATTCAAACTGAAACATTAAAACCTAC-3′

[a] F indicates forward and R indicates reverse.

processes, as well as putative signal transduction components that 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 up-regulated 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 up-regulated, 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 that 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 hemeproteins 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_{50} and I_{80} 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-ketoreductase (AKR) superfamily (reviewed in Refs. 37 and 38). The *Arabidopsis* genome encodes at least 18 genes related to AKRs (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

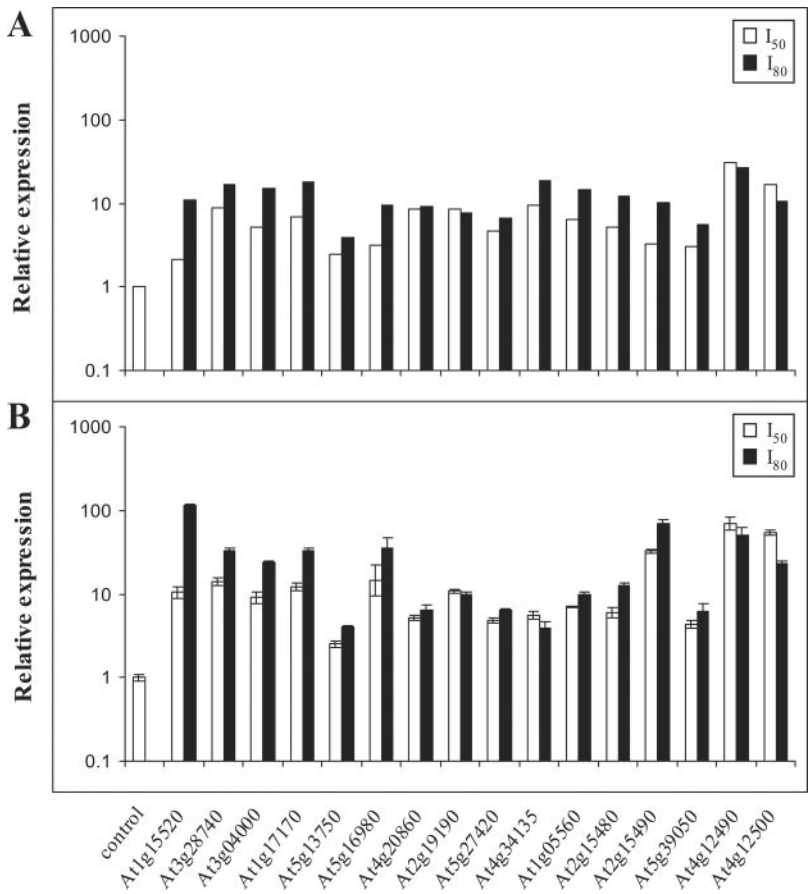

FIG. 3. **Verification of microarray-based gene expression changes by quantitative real time RT-PCR analysis.** Sixteen representative genes identified as differentially expressed by microarray analysis were also analyzed by quantitative real time RT-PCR using the gene-specific primer pairs shown in 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 ≤ 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 *A*. Assays were performed in triplicate with SYBR® Green I dye as described under "Experimental Procedures." Data were normalized to an internal 18 S 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 ± S.D., and control samples are as described above in *A*. *Error bars* were generated by transformation of mean $\Delta\Delta C_T$ values ± 1 S.D. unit, taken to 2^{-n}. The TAIR locus identifications (www.arabidopsis.org) for genes included in this comparison are indicated on the x axis.

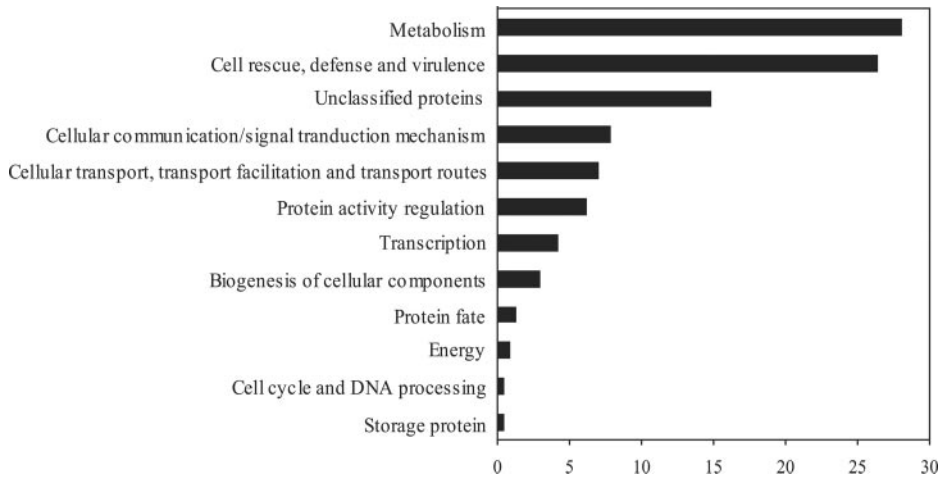

FIG. 4. **Distribution of BOA-induced genes into functional categories.** Genes were assigned to various functional categories based on the FunCat scheme devised by the Munich Information Center for Protein Sequences.

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_{50} and I_{80} BOA treatments (Table III). Most 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-*N*-(aminocarbonyl)-2-chlorobenzenesulfonamide 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-

TABLE III
Selected genes induced ≥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})
ABC transporters				
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
Aldo-ketoreductases				
At1g60730		Aldo-ketoreductase family	3.9	8.9
At2g37770		Aldo-ketoreductase family	4.3	8.9
At2g37760		Aldo-ketoreductase family	2.8	4.9
At1g60750		Aldo-ketoreductase family	1.3	4.4
Cytochrome P450s				
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
Dehydrogenases/reductases				
At3g04000		Short chain type dehydrogenase/reductase-related	5.1	15.0
At1g72680	*AtCAD1*	Cinnamyl-alcohol dehydrogenase, putative	2.7	5.6
At4g13180		Short chain dehydrogenase/reductase family protein	3.4	5.0
At1g54100		Aldehyde dehydrogenase, putative	2.6	3.0
At1g21400		Branched-chain α-keto-acid dehydrogenase-related	2.3	3.0
Glutathione transferases				
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
Other transporters				
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
Oxidoreductases				
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		FrnE protein-like	2.1	2.7
Peroxidases				
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
Protein kinases				
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
Transcription factors				
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

TABLE III—*continued*

Locus ID	Name	Gene description	Fold change (I_{50})	Fold change (I_{80})
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
UDP glucosyltransferases				
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*	glucosyltransferase-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

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 suggest they may in fact play such a role.

Six class III peroxidase-like sequences were up-regulated 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 Ref. 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, *N*-nitrosomethylaniline, 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 *N*-nitrosomethylaniline and Sudan I (44). Most interestingly, benzoxazinoid 2,4-dihydroxy-7-methoxy-*2H*-1,4-benzoxazin-3(4*H*)-one, 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 up-regulation 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 GST at the level of transcription in response to herbicide safener exposure was first reported by Wiegand *et al.* (47), and numerous reports have subsequently described the regulation of GSTs in response to a variety of agrichemicals and other xenobiotics (reviewed in Ref. 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 up-regulated in the present study include some of the most highly induced transcripts in BOA-treated *Arabidopsis* seedlings (Table III).

Plant 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 (24, 49). 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^+ antiporters are involved (50).

In BOA-treated *Arabidopsis* seedlings, UGTs were the most highly represented family of up-regulated genes, with 11 different members being significantly induced (Table III). Most striking is the almost 70-fold induction observed by real time PCR in the I_{80} 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 documented previously (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, an MFS-type transporter (At5g13750), and a MATE-type transporter (At2g04040; Table III). ABC-, MFS-, and MATE-type transporters represent different multidrug efflux protein superfamilies 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 reported previously for plants (53–56). In studies performed by van den Brûle 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, and 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,

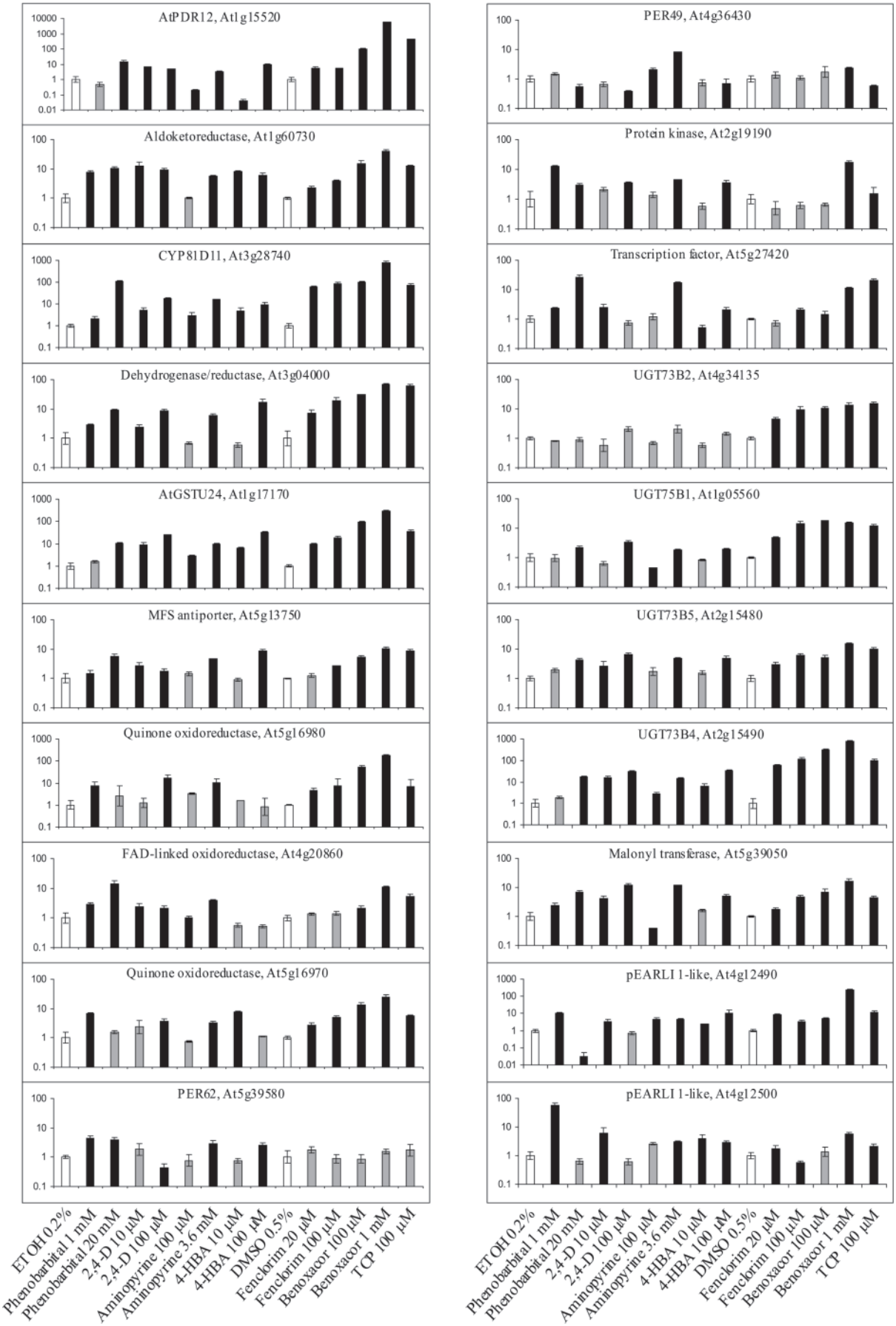

FIG. 5. **Effect of various chemical treatments on selected BOA-responsive genes.** Twenty genes identified as differentially induced by microarray analysis (see "Experimental Procedures") were examined by quantitative real time RT-PCR for responsiveness to various chemical treatments with xenobiotics and proposed inducers of xenobiotic metabolism (see text). Assays were performed in triplicate with SYBR® Green I

salicylic acid, ethylene, and methyl jasmonate. By 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 suggest that this transporter may also have a more general role in the elimination of phytotoxins unrelated to pathogen defense (see below).

Also of significance among BOA up-regulated 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 (37, 57–60). 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 been reported previously.

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 explore this further, *Arabidopsis* seedlings were exposed to different concentrations of the herbicide 2,4-D, *p*-hydroxybenzoic acid (an allelochemical agent in wheat and potentially *Sorghum bicolor*; see Refs. 66 and 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 (56, 68, 69). Chemical treatments were performed in an identical manner to the BOA treatments described above, and 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. More 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 1 mM benoxacor treatments and more than 400-fold following exposure to 100 μM 2,4,5-trichlorophenol. Glucosyltransferase *UGT73B4* (At2g15490) transcript levels were induced more than 30-fold by five of seven compounds tested and increased ~17- 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; ~100-fold increases were observed in response to 100 μM fenclorim and 2,4,5-trichlorophenol treatments and ~800-fold for 1 mM benoxacor treatments. Most interestingly, 100 μM *p*-hydroxybenzoate treatments induced *UGT73B4* transcript levels ~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 (24, 49). 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) and 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 oxidoredutase 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 ~5-fold or greater following exposure to at least five of the seven compounds tested (Fig. 5). These included glutathione *S*-transferase *AtGSTU24* (At1g17170), cytochrome P450 *CYP81D11* (At3g28740), an aldo-ketoreductase (At1g60730), a short chain type dehydrogenase/reductase (At3g04000), two different quinone oxidoreductases (At5g16980 and At5g16970), glucosyltransferase *UGT73B5* (At2g15480), a malonyltransferase-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

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% Me_2SO) control treatments, which were arbitrarily set to 1.0. Data are shown as mean ± S.D., and *error bars* were generated by transformation of mean $\Delta\Delta C_T$ values, ± 1 S.D. 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*.

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 5-fold or greater for 18 of the 20 selected genes. The median level of induction for the 18 benoxacor-responsive genes was ~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, and then the heterodimeric ligand-Ahr-Arnt complex stimulates transcription via xenobiotic-response elements in target gene promoters. A second important mechanism involves members of the steroid family of nuclear receptors, the pregnane X receptor, and the constitutive androstane receptor, 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 (63, 72). As is the case observed for the AhR, pregnane X receptor binds promiscuously to structurally diverse xenobiotic ligands, and thus serves as a broad specificity xenobiotic sensor that can induce the expression of a battery of detoxification-related genes (63).

Although ligand-activated transcription factors, such as AhR and pregnane X receptor, 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 been elucidated recently (17, 73). 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 (Cys-273 and Cys-288) 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, 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 been shown recently to be redox-regulated through critical cysteine residues within the protein (74). Most 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 (17, 73, 75). Of further interest is the observation that chemical structures for all known herbicide safeners contain at least one "soft" electrophilic center that 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 (17, 64). 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 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), and then identified motifs were compared with known *cis*-regulatory elements in the PlantCARE data base (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

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

Data set (no. promoters)	Consensus[a]	Average motif frequency/promoter						
		Log likelihood[b]	No. with motif	Observed	Genome[c]	*p* value[d]	PlantCARE	Function
Highest induced (50)	**TTAyTA**kT	268.1	38	1.3	0.8	0.0037	E4-ERE	Ethylene-responsive element
	n**TTGACT**T	239.3	33	0.86	0.38	<0.001	Box W3	Fungal elicitor-responsive element
UDP-glucosyltransferase (10)	T**AGATAwG**	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)	kT**TGACT**T	50.9	3	0.71	0.23	0.0043	Box W3	Fungal elicitor-responsive element
Cytochrome P450 (6)	**TwGAC**TwT	31.1	4	1.67	0.37	<0.001	Box W1	Fungal elicitor-responsive element
							JERE	Jasmonate and elicitor-responsive
	n**CCACGTG**	27.9	3	0.5	0.12	0.0031	G-box	Element involved in light responsiveness
							ABRE	Element involved in abscisic acid-responsiveness

[a] The sequence shown in boldface was used to identify PlantCARE data base match.
[b] The log likelihood scores were generated by the Motif Sampler software as described previously (26).
[c] The 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 (rsat.ulb.ac.be/rsat/); 1500 bp sequences were retrieved unless an upstream open reading frame was first encountered.
[d] *p* values were 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).

multiple runs of Motif Sampler (29), the 10- and 12-mer results were not considered further. More 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 that 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). Most 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 Refs. 82 and 86). 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 TGACGTCA 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 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 μM 2,4-D treatments and exhibited only modest increases (~3-fold) in response to 100 μM 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 ~7- and 5-fold following exposure to 10 and 100 μM 2,4-D, respectively (Fig. 5), potentially due to the presence of the identified *as-1*-like element. Only modest increases (~2.5-fold) were observed for transcript levels for the putative oxidoreductase following exposure to 10 μM 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 that 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 chemosensory mechanisms critical to plant survival in the presence of allelochemicals and other environmental toxins.

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