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Mammalian cytochrome CYP2E1 triggered differential gene regulation in response to trichloroethylene (TCE) in a transgenic poplar

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Abstract

Trichloroethylene (TCE) is an important environmental contaminant of soil, groundwater, and air. Studies of the metabolism of TCE by poplar trees suggest that cytochrome P450 enzymes are involved. Using poplar genome microarrays, we report a number of putative genes that are differentially expressed in response to TCE. In a previous study, transgenic hybrid poplar plants expressing mammalian cytochrome P450 2E1 (CYP2E1) had increased metabolism of TCE. In the

vector control plants for this construct, 24 h following TCE exposure, 517 genes were upregulated and 650 genes were downregulated over 2-fold when compared with the non-exposed vector control plants. However, in the transgenic CYP2E1 plant, line 78, 1,601 genes were upregulated and 1,705 genes were downregulated over 2-fold when compared with the non-exposed transgenic CYP2E1 plant. It appeared that the CYP2E1 transgenic hybrid poplar plants overexpressing mammalian CYP2E1 showed a larger number of differentially expressed transcripts, suggesting a metabolic pathway for TCE to metabolites had been initiated by activity of CYP2E1 on TCE. These results suggest that either the over-expression of the CYP2E1 gene or the abundance of TCE metabolites from CYP450 2E1 activity triggered a strong genetic response to TCE. Particularly, cytochrome p450s, glutathione S-transferases, glucosyltransferases, and ABC transporters in the CYP2E1 transgenic hybrid poplar plants were highly expressed compared with in vector controls.

Keywords

Cytochrome P450; Microarray; Phytoremediation; Poplar; TCE (trichloroethylene)

Introduction

Industrialization has led to increased concentrations of synthetic chemicals in the environment, many of which pose a threat to human health. One of the most common environmental pollutants is trichloroethylene (TCE). In the USA, 54% of the US EPA (Environmental Protection Agency) Superfund sites contain TCE (Lee et al. 2006). This chlorinated solvent has been widely used as a cleaning agent and solvent for military, commercial, and industrial applications. Widespread use, along with improper handling, storage, and disposal, has resulted in frequent detection of TCE in groundwater, sometimes at high levels, TCE has the potential to cause liver damage and malfunctions in the central nervous system, and it is considered a likely human carcinogen (Milton et al. 1998). Conventional engineering techniques to remove TCE include excavation and transport, soil washing, extraction, pumping, and treating of contaminated water, addition of oxidants, or incineration (McCutcheon and Schnoor 2003). Anaerobic bioremediation is an effective method for degrading TCE and other chlorinated contaminants in soil and groundwater (Ferguson and Pietari 2000). These methods, however, are often too expensive to establish and manage.

Phytoremediation uses plants to detoxify or degrade pollutants from the environment. There is interest in the use of poplar trees for phytoremediation of soil and groundwater pollutants since they can grow rapidly and have extensive root systems and high water uptake rates. Other advantageous characteristics of *Populus* species include a small genome, ease of vegetative propagation, availability of genetic transformation systems, and genetic resources (Tuskan et al. 2006). *Populus* species are capable of taking up and degrading organic pollutants. Significant uptake and degradation of TCE in hybrid poplar have been reported in several studies (Gordon et al. 1998; Newman et al. 1997; Orchard et al. 2000). Komives et al. (2003) demonstrated the ability of poplar to detoxify chloroacetanilide herbicides. Ma et al. (2004) also reported the uptake of methyl *tert*-butyl ether in hybrid poplar.

Most research has focused on identifying the TCE degradation pathway in plants by chemical observation. Shang et al. (2001; 2002) and Newman et al. (1997) showed TCE oxidative metabolism in plants by chemical identification and the fate of TCE in plants and axenic poplar tissue cultures using radiolabeled TCE. However, the gene expression level of TCE metabolism has not been studied extensively in any other plants. Various plant enzymes are responsible for metabolizing foreign organic compounds. Generally, the

process of metabolism can be divided into three phases. In the initial stage of metabolism, cytochrome P450s typically activate foreign compounds to a more reactive metabolite (Khatishvili et al. 1997). Then the transformed compounds are conjugated with glutathione or glucose by glutathione S-transferases or glycosyltransferases, respectively (Schröder 2002). These conjugated compounds are transferred to the vacuole or cell wall materials by ATP-binding cassette (ABC) transporters (Sandermann 1994; Van Aken 2008). Ekman et al. (2003) found that cytochrome P450 enzymes, glutathione S-transferases, and ABC transporters were induced by 2,4,6-trinitrotoluene (TNT) exposure.

The rate-limiting step of TCE metabolism is likely to be the activation step initiated by a cytochrome P450 (Cai and Guengerich 2001). In support of this hypothesis, metabolism and removal of TCE was significantly increased by overexpressing mammalian cytochrome P450 2E1 (CYP2E1) in transgenic hybrid poplar (*Populus tremula* × *Populus alba*) plants (Doty et al. 2007). P450 2E1 is a key enzyme in the metabolism of a variety of halogenated compounds including TCE.

Molecular studies of the genetic responses to TCE are needed to further understand the process of TCE metabolism in plants. Poplar genome arrays, based on the *Populus trichocarpa* genome sequence, can be used to study gene expression in the *Populus* species (Tuskan et al. 2006). The analysis of the *Populus* transcriptome in response to TCE can reveal the genes that play roles in detoxification. Here, we report a detailed whole-genome survey of the detoxification genes transcribed in poplar in response to TCE exposure and demonstrate the key role played by CYP450 2E1 in the degradation of TCE.

Materials and methods

Plant materials and TCE treatments

Plants were grown in Murashige and Skoog (MS) tissue culture medium (Murashige and Skoog 1962) in vitro at 24°C with a photoperiod of 16 h light and 8 h dark cycle. Twelve-week-old plants were used for the experiment. To determine if any genes are differentially regulated in response to TCE, triplicate cuttings of transgenic poplar, either the null vector control plants that were transformed with pKH200 or the rabbit CYP2E1, line 78 (Doty et al. 2007), were exposed to trichloroethylene for 24 h with untreated control plants. All samples were incubated in sterile 40 ml VOA (Volatile Organics Analysis) vials, sealed with Teflon-lined septum valve caps, and then the MS medium was dosed to a level of 50 µg/ml TCE (99.5% purity; Sigma-Aldrich). After each time exposure, plants were frozen with liquid nitrogen.

RNA extraction

Total RNA was extracted from the leaves of TCE-treated and untreated plants of the null vector control plant, and transgenic CYP2E1 plant. Frozen leaves (approximately 0.5 g) were homogenized with a mortar and pestle in liquid N₂. RNA was purified from each sample, using the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) and stored at -70°C. The quality of total RNA was evaluated using a model 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA). RNA yields were quantified by measuring the absorbance of a 1:50 dilution of the samples with an ultraviolet spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) at 260 and 280 nm. Only total RNA and subsequent biotin-labeled cRNA samples with appropriate size distribution and an A260:A280 ratio of 1.8–2.1 were used for microarray analysis.

Microarray analysis

Microarray analysis was carried out four Affymetrix GeneChip® Poplar Genome Arrays. Although the microarray probes were designed for the genome sequence of *P. trichocarpa*, it was necessary to use hybrid poplar INRA 717-1B4 (*P. tremula* × *P. alba*) for this study since the genetic background of the transgenic poplars was this hybrid.

Triplicate RNA samples from the null vector plants and transgenic CYP2E1 plants (TCE-treated, untreated) were pooled with equal amounts of RNA from three individual plants from 24 h TCE treatment. The RNA was investigated by microarray analysis (Peng et al. 2003). Total RNA was processed for use on the Affymetrix GeneChip® Poplar Genome Arrays according to the manufacturer's protocol. In brief, 3 µg of total RNA was used in a reverse transcription reaction to generate first-strand cDNA, using the One-Cycle cDNA synthesis Kit (Affymetrix, Santa Clara, CA) with oligo(dT) 24 primer used to T7 RNA polymerase promoter. After second-strand synthesis, biotin-labeled target complementary RNA (cRNA) was prepared using the IVT Labeling Kit (Affymetrix, Santa Clara, CA) in the presence of biotinylated UTP and CTP. After purification and fragmentation, 15 µg of cRNA was used in a 300 µl hybridization mixture containing added hybridization controls. A total of 200 µl of the mixture was hybridized on arrays for 16 h at 45 C. Standard posthybridization wash and double-stain protocols were used on an Affymetrix Gene Chip fluidics station 450. Arrays were scanned on an Affymetrix GeneChip® Scanner 3000 (Affymetrix, Santa Clara, CA).

Raw microarray data were processed and analyzed with Bioconductor (Gentleman et al. 2004) and normalized with the Bioconductor GC-RMA package (Wu et al. 2004). From the normalized data genes with significant evidence for differential expression were identified using the Limma Package (Smyth 2004) in Bioconductor.

GO annotation of expressed genes

The Gene Ontology functional classification was obtained from the *Arabidopsis* GO database of The Arabidopsis Information Resource (TAIR, <http://arabidopsis.org>). The proportion (%) of putative genes in each category was calculated (Number of genes annotated to terms in GOslim category × 100/total number of genes from input list annotated to any term in GO category). *Arabidopsis* annotations were taken from BLASTX- hits within the NCBI protein database with an E-value of 1.0E-20 or score >80.

Quantitative real-time PCR analysis

Total RNA was isolated as described above. Triplicate RNA samples used for the quantitative real-time PCR (qRT-PCR) verification of the microarray results were obtained from the leaves of the null vector control plant and the CYP2E1 transgenic plant. The mRNA was converted to cDNA using the SuperScript® II First-Strand Synthesis System (Invitrogen, Carlsbad, CA.). Primer pairs and fluorescently labeled probes to particular upregulated genes from the microarray results were designed as shown in electronic supplementary material Table 1. The expression levels of these selected genes were compared with an internal reference gene (18S rRNA) that served to normalize gene expression levels and to give unit-less values of candidate gene expression. Probes with a fluorescent tag (TaqMan-based) specifically designed to complement an internal region of the 18S rRNA and the genes of interest were used to quantify gene expression. Data were log transformed because it was necessary to achieve equal variance among means. Statistical analysis of data was performed by one-way ANOVA followed by the Least Significant difference (LSD) post-hoc test with $P < 0.05$

Results

Microarray analysis

The microarray experiment was performed to compare the TCE response in null vector control poplar KH200 and CYP2E1 line 78 transgenic poplar after 24 h TCE exposure. Differentially expressed genes in the TCE-dosed transgenic plants (the vector control plants and the CYP2E1 plants) were selected based on greater than 2-fold change (FC) in gene expression. The results revealed that a total of 4,095 genes were differentially regulated in response to TCE. As displayed in the Venn diagram, 790 genes were categorized in the vector control plant; 2,928 genes were categorized in the CYP2E1 transgenic plant; 377 genes were in the intersection (Fig. 1a). In the vector control KH200, 517 genes were upregulated and 650 genes were downregulated. Interestingly, in the transgenic line, 1,601 genes were upregulated and 1,705 genes were downregulated, nearly triple the number in the vector control (Fig. 1b).

Upregulated poplar genes were assigned to the corresponding *Arabidopsis* annotation with BLASTX- hits against the NCBI protein database with an E-value of 1.0E-20 or score >80. From the BLASTX results, 276 genes out of 517 in KH200 plant and 674 genes out of 1,601 genes in CYP2E1 transgenic plant were similar to *Arabidopsis* genes and the rest of genes were unknown or had low similarities with *Arabidopsis*.

To access biological process and molecular function in gene expression patterns of TCE-exposed plants, we classified the upregulated genes in KH200 plants and CYP2E1 plants into 14 functional categories. Electronic supplementary material Fig. 1 shows the categorization of the major biological functions of these genes (Huala et al. 2001). For the null vector control plant KH200, the proportion of metabolic processes (89 of 194 genes, 46%) and protein metabolism (27 of 194 genes, 14%) differentially regulated in response to TCE was similar to that of the transgenic CYP2E1 plant, metabolic processes (216 of 468 genes, 46%) and protein metabolism (59 of 468 genes, 14%). However, the proportion of cellular processes, response to stress, and abiotic or biotic stimulus was lower than the CYP2E1 plant. Although the proportion of each process for the vector control KH200 response was similar to that of the CYP2E1 plant, a larger number of CYP2E1 plant genes were expressed in each category. Also, the number of unknown biological process genes of KH200 plants and CYP2E1 plants was 51 out of 194 (26%) and 78 out of 468 (17%), respectively (Electronic supplementary material, Fig. 1).

qRT-PCR analysis of targeted genes of TCE degradation

To further support the microarray results, and to confirm the expression of TCE metabolism related genes, qRT-PCR was carried out on four selected probes from microarray results that were putatively involved in the xenobiotic detoxification metabolic pathway. The results of the qRT-PCR analysis are presented in Fig. 2. The relative amounts of individual mRNAs were normalized to the expression of 18S rRNA. The levels of expression of targeted genes in the CYP2E1 line 78 were significantly different from other treatments.

Correlation of gene expression data between microarray and qRT-PCR

The mRNA levels of four probes were quantified in leaves by qRT-PCR. The quantitative data were normalized as a ratio to 18S rRNA expression and then calculated as a ratio of expression from the TCE-dosed plants to the control plants. Microarray data were used as fold change. As shown in electronic supplementary material Fig. 2, there was strong correlation between the qRT-PCR and microarray data with a R^2 of 0.98.

Discussion

Microarray analysis was carried out to identify TCE detoxification genes from poplar. In earlier studies, we compared the responses of TCE in wild-type hybrid poplar at two time points. However, there were neither highly up- or downregulated genes, nor were differentially expressed genes significantly related to detoxification genes (data now shown). To gain a better understanding of how TCE is further metabolized by plants, in this work, we compared normal with transgenic plants with an increased metabolism of TCE.

Van Aken (2008) suggested a hypothetical pathway for metabolism of TCE in plant tissues: (1) oxidative reactions from TCE to trichloroethanol, which are presumed to be catalyzed by cytochrome P450 monooxygenases; (2) conjugation with a plant molecule catalyzed by glutathione S-transferases (GST) or glucosyltransferases; (3) sequestration of the conjugate into the cell wall or within the vacuole by transporters. We focused on these enzymes to look for putative TCE detoxification genes.

Cytochrome P450 proteins are one of the largest superfamily of enzymes and play a significant role in the genomes of all organisms (McLean et al. 2005). In plants, cytochrome P450 enzymes are involved in vital processes such as carbon source assimilation, biosynthesis of hormones and structural components such as lignin synthesis (Ehlting et al. 2006), and chemical defense (Werck-Reichhart and Feyereisen 2000).

There are reports of cloned plant P450s related to phytoremediation. CYP71A10 was the first plant enzyme from soybean shown to metabolize an herbicide (Siminszky et al. 1999). In another study, Didierjean et al. (2002) reported on a successful transgenic approach with a gene for CYP76B1 from *Jerusalem artichoke*. The transgenic plants with CYP71A10 and CYP76B1 both significantly enhanced tolerance to several herbicides.

In our microarray results, six cytochrome P450s were differentially expressed at 24 h TCE exposure in the vector control plants, and 23 P450 genes were differentially expressed at 24 h TCE exposure in the CYP2E1 line 78 (Electronic supplementary material Table 2a). Cytochrome P450s defined as belonging to the CYP 450 family of genes based on >80% amino acid sequence similarity. At 24 h TCE exposure in the vector control plants, Ptp.6116.1.S1_at probe had the highest fold changes (5-fold changes). However in the transgenic line 78, Ptp.6116.1.S1_at probe had the highest differential expression up to 20-fold change. Ptp.6116.1.S1_at is homologous with cytochrome P450 of *P. trichocarpa* (E-value 1e-85, score 318). Three genes out of seven cytochrome P450s are involved in cellular processes, four genes out of seven metabolic processes, and one gene is related to stress responses in the GO biological processes. However, the remaining 17 genes have not been annotated in *Arabidopsis* GO functional categorization.

There are many reports that carcinogens including polycyclic aromatic hydrocarbons are detoxified and conjugated by Phase II enzymes such as GSTs and glucosyltransferases (GTs) in plants and animals (Ada et al. 2007; Dixon et al. 1998; Edwards et al. 2000; Xiang et al. 2001). Rawls (1996) showed that CYP 450 s are involved in epoxide formation in the dechlorination pathway, whereas glutathione S-transferases catalyze conjugation reactions with glutathione. The glutathione S-transferase enzymes have critical roles in the degradation of xenobiotics. They can conjugate various organic compounds such as pesticides and herbicides (Rennenberg 2005). In *Arabidopsis*, a total of 47 GST members have been identified, with 14 belonging to the phi class, 3 to the theta class, 2 to the zeta class, and 28 in the tau class. DeRidder et al. (2002) showed that GSTs of *Arabidopsis* were induced by herbicide safeners. High levels of glutathione and GSH S-transferase activity were detected in poplar leaves exposed to herbicides (Komives et al. 2003).

In our microarray results, glutathione S-transferases were not differentially expressed in the vector control plants after 24 h exposure to TCE, however, 18 glutathione transferases were differentially expressed in the CYP2E1 transgenic line 78 after 24 h TCE exposure (Electronic supplementary material Table 2b). Glutathione S-transferases of the same family were defined as having >80% amino acid sequence identity. Interestingly, most glutathione S-transferases were highly upregulated compared with other genes following TCE treatment. For example, PtpAffx.43231.1.A1_a_at probe had 168-fold upregulation. PtpAffx.43231.1.A1_a_at is a predicted protein of *P. trichocarpa* (E-value 1e-123, score 446), and, it has homology with glutathione S-transferase (class tau) of *Arabidopsis thaliana* (E-value 1e-69, score 266). Most glutathione S-transferases are involved in cellular processes (6/6, 100%), metabolic processes (6/6, 100%), stress responses (2/6, 33.3%), and other biological processes (4/6, 66.7%) in GO biological processes. The remaining 12 genes were not annotated in *Arabidopsis* GO functional categorization.

Plant GTs have a vital role in natural product biosynthesis and the metabolization of xenobiotics (Hefner et al. 2002). The glucosylation of chlorinated phenols and the associated GT activities have been studied in soybean and wheat (Brazier et al. 2002). Gandia-Herrero et al. (2008) showed that GTs play an integral role in the biochemical mechanism of TNT detoxification by plants. The glycosylation of trichloroethanol is responsible for the removal of approximately 90% of the free trichloroethanol in TCE-exposed plants (Shang et al. 2001). Therefore, glycosylation likely constitutes an important survival mechanism for plant cells. Plant glucosyltransferases are part of a multi-gene superfamily with 47 distinct families (Ross et al. 2001). Our microarray results showed that five glucosyltransferases were differentially regulated at 24 h TCE exposure in the vector control plants. Glucosyltransferases defined as belonging to the same *family* based on having >68% amino acid sequence identity. However, 21 glucosyltransferases were differentially regulated in the CYP2E1 transgenic line 78 after 24 h TCE exposure (Electronic supplementary material Table 2c). All five genes were upregulated approximately 2-fold in the vector control plants. But in the CYP2E1 line 78, probe Ptp.6958.1.S1_s_at and PtpAffx.31211.1.A1_at were upregulated 98- and 85-fold, respectively. In the BLASTX result, most probes were predicted proteins in *P. trichocarpa*; also, they had high similarity with other plants glucosyltransferases. Most glucosyltransferases are involved in metabolic processes (15/16, 93.8%) in the GO biological processes. The remaining ten genes have not been annotated in *Arabidopsis* GO functional categorization. These results are consistent with the previously observed removal rate of trichloroethanol in poplar (Shang et al. 2001).

The ABC transporters are one of the largest protein families in plants. They transport substrates such as conjugated organic compounds and xenobiotics out of cells and into vacuoles (Meagher 2000). Insoluble compounds are generally assumed to be stored in the cell wall by ABC transporters. According to our microarray results, ABC transporters were not differentially expressed in the vector control plants after exposure to TCE for 24 h. However, 15 ABC transporters were upregulated in the CYP2E1 line 78 after 24 h TCE exposure (Electronic supplementary material, Table 2d). ABC transporters of the same family were defined as having >80% amino acid sequence identity. PtpAffx.141628.1.S1_at probe showed 17-fold upregulation in CYP2E1 plants exposed to TCE for 24 h. In the BLASTX results, PtpAffx.141628.1.S1_at was identified as a multidrug/pheromone exporter, MDR family, ABC transporter family of *P. trichocarpa* (E-value: 1e-98, score 362). It was also a putative ABC transporter in *A. thaliana* (E-value: 6e-85, score 316). All ABC transporters are involved in transport (8/11, 73%) in the GO biological processes. The remaining six genes were not annotated in *Arabidopsis* GO functional categorization.

The KH200-vector control plants dosed with TCE for 24 h displayed 517 genes as upregulated and 650 genes as downregulated. In contrast, in the CYP2E1 transgenic plants

dosed with TCE for 24 h, 1,601 genes were upregulated and 1,705 genes were downregulated. The induction of genes is most likely important for transformation, conjugation, and compartmentation of toxic metabolites in response to the increased metabolism of TCE in the CYP2E1 transgenic line 78. The level change was also higher than that of the control plants. In addition, there are several other genes that may be involved in detoxification, such as laccases (Bollag et al. 1988), peroxidases (Kawano 2003), and dehalogenases (Wang and Chen 2007) in the CYP2E1 transgenic line 78 (Electronic supplementary material, Table 2e).

Four selected probes from microarray results corresponding to genes involved in phase I, II and III drug metabolism, which were chosen for RT-PCR analysis to verify and quantify the expression of detoxification genes, were highly expressed. The Ptp.6116.1.S1_at probe corresponds to a CYP450 gene (95% similarity) that is upregulated 87-fold in the CYP2E1 line. Also, PtpAffx.25444.1.S1_x_at probe, which corresponds to a glutathione S-transferase (98% similarity), had a 59 times greater change in the CYP2E1 line 78 than control poplar. Particularly, PtpAffx.31211.1.A1_at probe which was glycosyltransferase (69% similarity) showed a 681-fold change in the CYP2E1 line 78. PtpAffx.141628.1.S1_at probe, an ABC transporter (95% similarity), showed a 139-fold change in the CYP2E1 line 78.

The expression levels of each treatment showed a large genetic response in the transgenic CYP2E1 line 78, based on the qRT-PCR results. Glutathione S-transferase, glycosyltransferase, and ABC transporter showed significantly increased expression. The microarray and qRT-PCR strongly correlated ($r^2=0.98$). Detoxification genes in CYP 2E1 line 78 appeared to be expressed in both microarray and qRT-PCR data.

One explanation of the lesser response in the vector control plants is that trichloroethylene is not a natural substrate for these endogenous poplar genes and, therefore, might not induce the expression of the relevant detoxification genes. Mammalian CYP450s can degrade diverse xenobiotics (Williams et al. 2003) and specially mammalian CYP2E1 is known to take part in the biotransformation not only of TCE but also of ethanol and acetone and of many small molecule substrates such as halogenated hydrocarbons (Anzenbacher and Anzenbacherová 2001). However, since 312 CYP450s genes are estimated to exist in poplar, it is difficult to assign a catalytic function to the cloned genes, and only a limited number of P450s have been identified and characterized so far. In the case of *Arabidopsis*, the functions of more than 200 out of the 272 P450 genes have yet to be identified (Nelson 2006). The transgenic poplar plants over expressing mammalian cytochrome P450 2E1 showed a stronger genetic response than wild-type hybrid poplar INRA 717-1B4, most likely because the metabolic pathway of TCE had already been initiated by CYP450 2E1 enzyme.

In conclusion, we compared the response of gene expression of hybrid poplar and transgenic poplar exposed to TCE. We identified many detoxification genes including cytochrome P450s, transferases, and transporters that appear to be involved in TCE metabolism. The presence and expression of the mammalian CYP2E1 gene initiated the metabolic pathway for TCE and the abundance of TCE metabolites triggered a strong expression of glutathione-S-transferases, glucosyltransferases, and ABC transporters. In addition, their mRNA expression levels were remarkably higher than those of wild-type hybrid poplar plants. Currently, we are identifying the function of some of these upregulated genes. These genes may have biotechnological potential for degradation of organic pollutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

a Venn diagram representing 4,095 differentially expressed probe sets in transgenic poplar after TCE exposure for 24 h. The *circles* represent vector control KH200 (*left*) and CYP2E1 line 78 (*right*) with the numbers representing the probe sets (fold change ≥ 2). **b** Histograms of the fold change (log2) distribution for all genes in transgenic hybrid poplar after TCE treatment at 24 h. Vector control-KH200 (*left*) and CYP2E1 line 78 (*right*). The histograms show the distribution of fold changes for upregulated genes (*grey bars*), down-regulated genes (*open bars*) and the numbers representing total genes

Fig. 2. Relative mRNA gene expression levels in the transgenic poplar. The qRT-PCR analysis of putative CYP450 (a), glutathione S-transferase (b), UDP glucosyltransferase (c), and ABC transporter (d) is presented. Values represent the mean $\pm$ SE for $n = 3$ plants for each treatment. The *asterisk* indicates significant difference from other treatments ($P < 0.05$)