Original Article

Microarray analysis of differentially expressed gene responses to bisphenol A in *Arabidopsis*

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ABSTRACT — Environmental levels of bisphenol A (BPA) are a global concern because the compound can cause damage to reproductive organs, the thyroid gland, and brain tissues at developmental stages. Plants are important in removing BPA from the atmosphere, soil, and water. However, knowledge on the mechanism by which plants respond to this compound is limited. To determine the response mechanism of plants to BPA, we used a microarray system to analyze the gene expression patterns of *Arabidopsis thaliana* after irrigation with 3.0 mM BPA. We identified 651 genes that were differentially expressed upregulated and 470 genes that were downregulated by BPA. These genes may specifically contribute to BPA uptake, transformation, conjugation, and compartmentation in plants. The potential function of upregulated genes in plant defense against BPA was also determined.

Key words: *Arabidopsis*, Microarray, RT-PCR, BPA, Toxicity

INTRODUCTION

Bisphenol A {BPA; 4-[2-(4-hydroxyphenyl)propan-2-yl]} is an industrially important compound widely used for the production of polycarbonates and other plastics (Ben-Jonathan and Steinmetz, 1998). Over the past few years, many issues have been raised all over the world regarding the use of BPA. BPA possesses estrogenic activities; hence, this compound mimics the function of estrogen upon entering living systems. BPA has been placed in the category of compounds called endocrine disruptors. The compound can cause damage to reproductive organs, the thyroid gland, and brain tissues at developmental stages and has been linked to cancer development in humans (Welshons *et al.*, 2006).

Human exposure to BPA occurs mainly through the diet. BPA molecules are linked through ester bonds to produce polycarbonate plastics, which are used in the manufacture of plastic containers. These ester bonds undergo hydrolysis under high temperature, such as when food stored in plastic containers is microwaved, and pH conditions, such as when acidic or basic food is stored

in plastic containers for long durations, causing leaching out of BPA (Welshons *et al.*, 2006). BPA then mixes with the food or beverage stored in the plastic container, and consumption of the food stored in the container results in BPA exposure (Krishnan *et al.*, 1993).

A significant quantity of waste containing BPA is generated from plastic manufacturing facilities. This waste is discharged into water bodies (Ye *et al.*, 2009) and finally settles in lakes or rivers, causing accumulation of BPA on the surface of the water. The compound can cause severe and adverse effects on human lives (Hu *et al.*, 2013). Several studies have focused on biodegradation of BPA, and recent research indicates that fungi and bacteria are efficient in degrading this compound (Hirano *et al.*, 2000; Tsutsumi *et al.*, 2001; Fukuda *et al.*, 2001). However, the use of bacteria to degrade BPA in polluted sites may cause adverse ecological impacts caused by unlimited propagation.

Phytoremediation is a useful method for removing BPA pollutants (Lindholm *et al.*, 2001); using this system, plants can grow independently using sunlight, water, and inorganic ions and be cultivated through seed germination

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or vegetative propagation, causing minimal disturbance to contaminated sites. However, plant genes responsible for BPA uptake, degradation, and conjugation remain unknown. The mechanisms of BPA toxicity to plants are also poorly understood. As molecular-level research on the plants stress response and defense mechanisms to BPA has rarely been reported, elucidating plant transcriptional responses to BPA is necessary and useful for determining appropriate phytoremediators for the compound. In the present study, we investigated transcriptional changes in *Arabidopsis* in response to BPA using microarray analysis. Several BPA-induced and BPA-repressed genes were identified, and the potential function of upregulated genes in plant defense against BPA was discussed.

MATERIALS AND METHODS

Arabidopsis culture conditions and BPA response

The *Arabidopsis thaliana* cv. Columbia seeds were surface sterilized and spread on solid Murashige and Skoog (MS) medium supplemented with 3% sucrose. To investigate *Arabidopsis* response to BPA, BPA was added into each plate of solid MS medium, with the concentration of 0, 100, 200, 300 and 500 μ M. Acetone was added into the control plate (0 mg/L BPA concentration) for eliminating the inequality caused by solvent. After jarovization at 4°C for 2 d, the plates were placed vertically in a greenhouse kept at 22°C under 16 hr light/8 hr dark. The growth responses to BPA were analyzed by measuring the primary root length after growth for 12 d. The *Arabidopsis* seedlings were also grown in pots containing a mixture of vermiculite, peat moss, and perlite (18:6:1 v/v). The 4-week-old seedlings were exposed to 0, 1, 2 and 3 mM BPA. For avoiding BPA volatilization, we poured the same volume (200 ml) of different concentrations of BPA into the plastic bag, put the pot with seedlings into the liquor, and then used elastic string to bundle the bag and pot together. One month later, the growth status was observed.

RNA preparation

Using microarray to study the genes related to BPA, 4-week-old seedlings were irrigated with 3 mM BPA for 24 hr and harvested. Acetone-treated-seedlings serve as control for eliminating the inequality caused by solvent. Plants were treated during the light period, and three replications with 12 plants in each were included. After treatments, all 12 plants in a pot were harvested and mixed, immediately frozen in liquid nitrogen, and stored at -70°C for RNA isolation. Total RNA from pooled plants

was isolated and purified using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed by agarose gel electrophoresis for two-color microarrays and by Agilent Bioanalyzer for gene expression microarrays.

Microarray experiment

Microarray analyses were carried out using a competitive hybridization method (i.e. dye-flip method) using the Agilent microarray system (Agilent Technologies, Palo Alto, CA, USA). All procedures were carried out according to the manufacturer's protocols. Briefly, 2 μ g of total RNA from each sample was used to synthesize cRNA and was labeled with cyanine-3 (Cy3)-labeled CTP (Perkin Elmer/NEN Life Sciences, Tokyo, Japan). The labeled cRNAs (including a treatment and a control sample) were competitively hybridized to the Agilent Arabidopsis (V4) gene expression microarray (4x44K), and then washed. The hybridized slides were scanned using Agilent DNA Microarray Scanner and data points were extracted using Agilent Feature Extraction software. Expression profile trends for the selected genes were examined by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers.

Data analysis

The hybridization signals were quantified and analysed using Agilent Feature Extraction software (Agilent Technologies, Inc.). Statistical analysis was performed using Cluster 3.0 software (University of Tokyo). In brief, those transcripts that had detection call 'P', signal value ≥ 25 and change P value < 0.05 in all the three replicates of each treatment were selected in the identification of BPA relative genes. Only the transcripts with a minimum twofold increase or decrease in signal over the control in at least two of the three biological replicates and a coefficient of variation (CV) $< 10\%$ were identified as BPA relative genes. The signal log ratio is the change in the expression level of a transcript between the control and experimental. samples, expressed as the log₂ ratio. The fold change was calculated as $2^{(\text{signal log ratio})}$ when the signal log ratio was ≥ 0 .

RT-PCR for validity of the microarray data

Gene-specific primers were synthesized for selected probe sets, and RT-PCR was carried out to verify the microarray results (Primers are listed in Supplementary Table S1). 3 mM BPA solution was used to treat the one-month-old seedlings for 12, 24 and 48 hr. Total RNA from *Arabidopsis* treated with different time was extracted by using the Trizol Kit (Watson biotechnology Inc., New Taipei, Taiwan). DNase I (Promega, Madison,

WI, USA) was used to remove the genomic DNA contamination. The first strand of cDNA was synthesized by using 2 μ g total RNA as template with Reverse Transcription System (TOYOBO, Osaka, Japan) in a 20 μ l reaction volume. The *Arabidopsis actin* gene *ACT2* (GenBank No. U41998) was used as the internal standard. PCR conditions were as follows: 94°C for 5 min, different cycles of 94°C for 20 sec, 54°C for 20 sec, 72°C for 30 sec, and with a final extension at 72°C for 10 min. DNA intensity ratio of the target genes to *ACT2* was analyzed with Shine Tech Gel Analyze (Shanghai Shine Science of Technology Co. Ltd., Shanghai, China). The same results were obtained for three independent experiments, only the result from one experiment is presented. Real-time PCR was performed in a Mini Option Real-time PCR System (Bio-Rad, Hercules, CA, USA). The reaction mix (10 μ l) contained cDNA from 20 ng total RNA (3 mM BPA-treated for 24 hr), 0.2 μ M of each primer, 0.2 μ M SYBR, 3 mM MgCl₂, 200 μ M dATP, dCTP and dGTP, 400 μ M dUTP, 1 unit Taq DNA polymerase. Fold change in the expression of RNA was estimated using

threshold cycles. All analyses were analyzed in triplicate. Mean values were calculated for relative expression ratios.

RESULTS AND DISCUSSION

Growth of *Arabidopsis* under BPA treatment

To investigate the toxicity of BPA to *Arabidopsis*, we sterilized seeds with chlorinated lime and then sowed them in MS solid medium containing 0, 100, 200, 300, and 500 μ M BPA. Seed growth after 10 d is shown in Fig. 1A. All BPA concentrations affected the growth of treated seeds compared with that of the control (0 μ M BPA). At 100 and 200 μ M BPA, seedling cotyledons turned yellow. At 300 μ M BPA, seed germination was gradually restricted. At 500 μ M BPA, seed germination was seriously restricted. To determine root growth in BPA-containing plates, we added different concentrations of BPA to the MS solid medium. The plates with *Arabidopsis* seeds were then positioned vertically. After 10 d of growth, the root length of seedlings at each concentration was recorded; relevant results are illustrated in Figs.

Fig. 1. Effect of BPA treatment on *Arabidopsis* seedling growth. A) Seedling growth. Seeds were sowed on 0, 100, 200, 300 or 500 μ M BPA plates. The pictures were captured after 10 d growth. B) Root growth on BPA containing plates. The pictures were captured after 10 d growth. C) Root length was measured after 10 d growth. The bars represent the standard deviation of one experiment, $n \geq 35$. D) One-month-old plants grew in soil were irrigated with 0, 1, 2, 3 mM BPA solution. The picture was captured after 7 d treatment.

1B and 1C. The average root length of seedlings grown on the control plate was 3.4 cm; by contrast, root lengths of 2.5, 1.6, and 0.7 cm were observed in seedlings grown on plates with 50, 100, and 200 μ M BPA, respectively. The corresponding phenomenon was observed in seedlings grown in soil (Fig. 1D). To determine the relative biomass of pot-grown seedlings, we selected 1, 2, and 3 mM BPA solutions for treatment. After 7 d, the leaves of seedlings treated with BPA became yellow. More evident toxicosis was observed with increasing BPA concentration. On the basis of the growth of 4-week-old seedlings (Fig. 1D), we considered 3.0 mM BPA as the sub-lethal concentration and used this concentration for subsequent microarray experiments.

RT-PCR to validate the microarray data

Eighteen genes were selected to validate the microarray data using RT-PCR. The expression patterns obtained through RT-PCR were consistent with those obtained

from microarray analysis (Fig. 2). RT-PCR indicated that the CTF2B in the “upregulated gene” group (Supplementary Table S2) evidently increased after BPA treatment; by contrast, PIP1A in the “downregulated gene” group (Supplementary Table S3) was extremely inhibited after BPA treatment.

Eight (fold-change > 5.0 in expression) of the eighteen genes were further verified using real-time PCR. The average fold-change of all transcripts analyzed through real-time PCR was higher than that obtained from the microarray data (Fig. 3).

***Arabidopsis* gene response to BPA**

An *Arabidopsis* (V4) gene expression microarray (4X44K) containing probes for the *Arabidopsis* genome was used to identify transcriptome differences in response to BPA. “P” detection and a minimum fold-change of 3 were applied. A total of 651 and 470 genes were considered up- and downregulated, respectively (Supplementary

Fig. 2. Reverse transcriptase polymerase chain reaction (RT-PCR) analyses of changes in gene expression in response to BPA. *Arabidopsis* plants were treated with 3 mM BPA for 12, 24 and 48 hr. A, up-regulated gene; B, down-regulated gene; 1, WT; 2, 12 hr; 3, 24 hr; 4, 48 hr; 5, negative control (H_2O).

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Fig. 3. Comparison of eight genes (fold change > 5.0 in expression) expression data from microarray hybridization and real-time PCR. Changes in gene expression were estimated as the fold-change over the control. The genes tested are indicated below each group of bars. The bars represent the average fold-change and standard error in transcript changes estimated from three biological replications for both real-time PCR and microarray hybridization.

Tables S2 and S3).

BPA-up- or downregulated genes were categorized according to their biological functions (Tables 1 and 2, Fig. 4). Results strikingly featured downregulation of photosynthesis-related mRNA levels (Supplementary Table S3). Moreover, photosynthesis, chlorophyll, and proteins targeting chloroplasts decreased (Supplementary Table S3). BPA-induced downregulation of other metabolic processes stimulated senescence and programmed cell death. Peroxidase increased by 5.3-fold; this enzyme can activate cell death in response to pathogen invasion and xenobiotic uptake (Blokhina and Fagerstedt, 2010).

Cysteine protease (AT3G49340) was upregulated by BPA by about fourfold relative to the control. Cysteine proteases perform several functions in plant physiology and development, including mediation of senescence and programmed cell death (Coupe *et al.*, 2003). Ubiquitin-protein ligase (AT2G35000), which also performs a prominent function in the control of apoptosis by conjugating and committing proteins for breakdown (Brancolini, 2008), was upregulated by 3.9-fold in the present study. During phosphate transport in the phloem between the shoots and roots, ubiquitin-protein ligase induces cell death by destroying the phosphate transporter and preventing phosphate accumulation in the cell (Lin *et al.*, 2008).

Strong over-representation of genes involved in detoxification systems was observed among the differentially expressed transcripts (Table 1). Glutathione transferases (GSTs) and UDP-glucuronosyl/UDP-glucosyl transferases (UGTs) are important enzymes responsible for the degradation of toxic pollutants, such as polycyclic aromatic hydrocarbons and BPA (Nakajima *et al.*, 2002; Dixit *et al.*, 2011). GSTs tagging xenobiotics with glutathione for transport into vacuoles (Marrs, 1996; Wagner *et al.*, 2002) were upregulated in the present study. For instance, GST-AtGSTF6 was strongly upregulated by BPA. Microarray probes recognizing AtGSTF7 and AtGSTU4 also indicated 2.5-fold increases in these transcripts induced by BPA. Similar to GSTs, UGTs perform important functions in the detoxification of endogenous and xenobiotic compounds. UGTs transfer carbohydrate residues into hydrophilic compounds containing an available nucleophilic center, such as hydroxyl, carboxyl, amino, or thiol groups, to regulate their activity, toxicity, or amenability for transport (Owens *et al.*, 2005). In the present study, genes of UGT1 and UGT73d1 were strongly upregulated by BPA.

A previous study showed that BPA induces production of various reactive oxygen species (ROS), including highly reactive hydroxy radicals (Ooe *et al.*, 2005). ROS function as bridges for triggering defense responses in plants (Vranova *et al.*, 2002; Mittler *et al.*, 2004; Laloi *et al.*, 2001). The defense system in various subcellular compartments forms an integrated network with extensive crosstalk that can be triggered by ROS. Several genetic studies have recently revealed that plant NADPH oxidases perform important functions in ROS formation (Kwak *et al.*, 2003; Li and Shah, 2003; Boldogh *et al.*, 2005). The NADPH oxidase AT4G11230, a systemic signal, was upregulated by BPA in the present study. Positive feedback amplification of the signal may occur through calcium binding and promote ROS production after BPA treatment for long durations. Phosphorylation of residues located at the N-terminal region of NADPH oxidases by calcium-dependent protein kinases (CDPKs) contributes to signal amplification. Intracellular free calcium is decoded by different types of CDPKs, CDPK-related kinases, and CBL-interacting protein kinases (CIPKs, also called snf1-related kinase) (Kobayashi *et al.*, 2007). In the present study, three CDPKs (CPK34, CPK18, and 29) and two CIPKs (CIPK1 and CIPK22) were transcriptionally activated by BPA to amplify signals for generating ROS.

BPA induced the expression of a large number of genes encoding transcription factors. Some of these genes are members of the WRKY family. For example, WRKY50,

Table 1. List of biological function of up-regulated genes in response to BPA

Name	Hits	Total	Percent
molecular_function	609	29448	2.07%
catalytic activity	231	8137	2.84%
cyclase activity	1	15	6.67%
oxidoreductase activity	38	1420	2.68%
transferase activity	104	2722	3.82%
hydrolase activity	64	2933	2.18%
lyase activity	3	333	0.90%
isomerase activity	9	220	4.09%
ligase activity	15	453	3.31%
structural molecule activity	1	501	0.20%
structural constituent of eye lens	1	1	100.00%
transporter activity	32	1222	2.62%
drug transporter activity	3	67	4.48%
transmembrane transporter activity	27	930	2.90%
substrate-specific transporter activity	25	865	2.89%
binding	278	10009	2.78%
nucleotide binding	66	2177	3.03%
pattern binding	2	17	11.76%
nucleic acid binding	51	3939	1.29%
protein binding	100	2640	3.79%
drug binding	1	24	4.17%
lipid binding	10	267	3.75%
oxygen binding	5	231	2.16%
vitamin binding	1	63	1.59%
carbohydrate binding	11	153	7.19%
peptide binding	1	28	3.57%
ion binding	78	2255	3.46%
tetrapyrrole binding	6	320	1.88%
cofactor binding	13	264	4.92%
electron carrier activity	17	495	3.43%
antioxidant activity	5	126	3.97%
peroxidase activity	5	109	4.59%
enzyme regulator activity	6	342	1.75%
enzyme inhibitor activity	5	174	2.87%
enzyme activator activity	1	74	1.35%
transcription regulator activity	48	1885	2.55%
transcription factor activity	48	1677	2.86%
transcription cofactor activity	1	28	3.57%
transcription activator activity	3	134	2.24%
transcription repressor activity	1	48	2.08%
protein tag	1	4	25.00%
nutrient reservoir activity	2	58	3.45%
molecular transducer activity	24	375	6.40%
signal transducer activity	24	375	6.40%

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Table 2. List of biological function of down-regulated genes in response to BPA

Name	Hits	Total	Percent
molecular_function	433	29448	1.47%
catalytic activity	216	8137	2.65%
cyclase activity	2	15	13.33%
oxidoreductase activity	67	1420	4.72%
transferase activity	48	2722	1.76%
hydrolase activity	77	2933	2.63%
lyase activity	12	333	3.60%
isomerase activity	7	220	3.18%
ligase activity	6	453	1.32%
structural molecule activity	1	501	0.20%
structural constituent of cell wall	1	39	2.56%
transporter activity	19	1222	1.55%
drug transporter activity	3	67	4.48%
transmembrane transporter activity	19	930	2.04%
substrate-specific transporter activity	10	865	1.16%
binding	156	10009	1.56%
nucleotide binding	25	2177	1.15%
nucleic acid binding	41	3939	1.04%
protein binding	31	2640	1.17%
lipid binding	7	267	2.62%
oxygen binding	12	231	5.19%
carbohydrate binding	3	153	1.96%
ion binding	46	2255	2.04%
amine binding	1	23	4.35%
tetrapyrrole binding	17	320	5.31%
cofactor binding	10	264	3.79%
metal cluster binding	3	40	7.50%
electron carrier activity	23	495	4.65%
antioxidant activity	4	126	3.17%
peroxidase activity	4	109	3.67%
enzyme regulator activity	9	342	2.63%
enzyme inhibitor activity	7	174	4.02%
kinase regulator activity	2	42	4.76%
transcription regulator activity	35	1885	1.86%
two-component response regulator activity	1	35	2.86%
transcription factor activity	29	1677	1.73%
RNA polymerase II transcription factor activity	1	34	2.94%
transcription activator activity	2	134	1.49%
transcription repressor activity	1	48	2.08%
transcription initiation factor activity	1	20	5.00%
nutrient reservoir activity	2	58	3.45%
molecular transducer activity	3	375	0.80%
signal transducer activity	3	375	0.80%

Fig. 4. Functional physiological categorisation of differentially expressed genes respond to 3 mM BPA. A, up-regulated gene; B, down-regulated gene.

WRKY63, and WRKY75 were upregulated by 6.4-fold, 5.0-fold, and 4.0-fold, respectively, compared with corresponding controls. Transcription factors induced by BPA and their downstream target genes form a network to increase plant resistance to environmental stresses. Among BPA upregulated genes, 9 cytochrome P450 monooxygenase genes, including CYP714A1, CYP81F2, CYP710A1, CYP704A1, CYP71A15, CYP710A3, and CYP89A6, were upregulated by over 2.5-fold (Supplementary Table S2). The biological functions of cytochrome P450 monooxygenases are based on their ability to catalyze oxygen insertion into various compounds. Mammalian P450 proteins are critical for detoxification of PAHs and BPA (Raucy and Allen, 2001; Sasaki *et al.*, 2005; Stiborova *et al.*, 2003), and their catalytic functions are extremely diverse (Durst and Nelson, 1995). Thus, we speculate that upregulated WRKY genes induce the expression of cytochrome P450 genes under BPA stress by recognizing and binding special *cis*-elements in abiotic and biotic stress conditions.

In conclusion, we identified several genes encoding known or putative proteins that are induced under BPA stress conditions. Considering the continuous development of *Arabidopsis* functional genomics, the function of a protein identified through expression profiling analysis can be determined rapidly. These genes were classified according to their functions in response to BPA treatments. The potential involvement of several genes in BPA metabolism and plant defense was demonstrated by

the induced expression of genes during stress treatments. The characterization of differential gene regulation in this study provides relevant data for selecting phytoremediation and phytosensing components. Further studies using other approaches, such as reverse genetics, are necessary to verify the functions of these genes in the metabolism of BPA and in plant stress responses.

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