SEARCH FOR GENE(S) RESPONDING TO THE STRESS OF COPLANAR PCB IN *ARABIDOPSIS THALIANA* USING RT-PCR DIFFERENTIAL DISPLAY AND DNA CHIP

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Introduction

Among the polychlorinated biphenyls, the environmental toxicity of coplanar PCB (Co-PCB) is becoming more serious. In this study, the gene(s) responding to the stress of Co-PCB in the genome of plant, *Arabidopsis thaliana*, was(were) sought to investigate the efficiency of monitoring gene expression as a biomarker in the environmental risk assessment of Co-PCB contamination. Two-week-old seedlings of *Arabidopsis thaliana* were exposed to 0.5 ppb 3,3',4,4',5-pentachlorobiphenyl (PeCB), which has the highest TEF value among the Co-PCBs, for two days. Then, mRNAs of specific genes responding to PeCB were sought by two different methods of RT-PCR differential display[1] and Southern hybridization using the Arabidopsis DNA chip. Four of 46 arbitrary primers analyzed caught the up-regulated gene expression in the mRNA of the PeCB exposed plant by RT-PCR differential display. Southern hybridization using the DNA chip, which has about 2200 cDNA fragments, showed at least 30 genes were up regulated in the expression, to the contrary, about 100 genes were down regulated.

Materials and Methods

Chemicals

3,3',4,4',5-Pentachlorobiphenyl (PeCB) was purchased from Wellington Labs., and the standard solution was prepared in toluene to a concentration of 5 mg/L. The standard solution was diluted to 0.5 ng/ml in distilled water for the exposure experiment to plants. All solvents were pesticide free reagent grade. All other chemicals used were of analytical grade.

Plant material and PeCB exposure

Arabidopsis thaliana ecotype Columbia (The Sendai *Arabidopsis* Seed Stock Center, Japan) were grown on 20 ml of MS solid medium in culture tubes at 26°C under a 16hr light / 8hr dark cycle. Two-week-old seedlings were exposed to 0.5 ml of PeCB (0.5 ng/ml) for two days.

Preparation of mRNA

The seedlings were carefully pulled out of the solid medium, then 80-100 mg of seedlings were ground immediately with a liquid nitrogen in 0.6 ml of 4.2 mol/L guanidine thiocyanate. Total RNA was isolated by phenol/chloroform extraction followed by precipitation using high concentration of lithium chloride as described by Lagrimini *et al.*[2]. Messenger RNA was prepared from total RNA using mRNA purification kit (TaKaRa, Kyoto, Japan)

RT-PCR differential display

cDNA was first synthesized from 0.1 μg of mRNA using Avian Myeloblastosis Virus reverse transcriptase (TaKaRa, Kyoto, Japan) and oligo dT primer, and was used as a template for PCR with several arbitrary primers (NIPPON GENE, Toyama, Japan; BEX, Tokyo, Japan). The PCR was performed in 100 μl of a reaction mixture consisting of 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 50 mM KCl, 200 μM dNTP, 0.4 μM arbitrary primers, 1 μM oligo dT primer and template cDNA. The reaction mixture was heated for 4 min at 94°C before addition of 2.5 units of Ex Taq DNA polymerase (TaKaRa, Kyoto, Japan). After heating at 94°C, 30 cycles of amplification were carried out as follows: 0.5 min denaturation at 94°C, 0.5 min annealing at 35°C, 1.5 min extension at 72°C in one cycle. The reaction was completed by 5-min extension at 72°C. PCR products were separated by 4% (w/v) polyacrylamide gel electrophoresis for 110 min at 100 V or 1.4% (w/v) agarose gel electrophoresis for 60 min at 100 V.

Sequence analysis

PCR products eluted from polyacrylamide gel were cloned into a pGEM 4Z vector (Promega, Madison, USA) according to the manufacturer's instruction, and were partially sequenced using the dye deoxy termination method on the DNA sequencer DSQ-1000L (SHIMADZU, Kyoto, Japan).

DNA chip analysis

One μg of mRNA from the control plant and PeCB exposed plant was fluorescently labeled by AMV reverse transcriptase with the Cy3-UTP and Cy5-UTP, respectively. Oligo DNA fragments on the Arabidopsis DNA CHIP I (TaKaRa, Kyoto, Japan) were hybridized using Cy3 and Cy5 labeled cDNA as a mixed probe in the solution containing 6 x SSC, 5 x Denhardt's solution and 0.2% SDS at 65°C for 14 hours. The fluorescent intensity of hybridized signals in

each spot on the DNA chip was measured by the DNA array scanner at 532 nm and 635 nm for Cy3 and Cy5, respectively.

Results and Discussion

Analysis of up-regulated genes by RT-PCR

Of 46 arbitrary primers analyzed, 4 primers made new PCR fragments from cDNA of mRNA from the PeCB exposed plant. As a example revealed in Figure 1, a new fragment with the size of about 900 bp appeared in RT-PCR of mRNA from the PeCB exposed plant using the arbitrary sequence 5'-GGAGGATGGCCC-3' and oligo dT as primers. After cloning and sequence analysis of this fragment, each sense and antisense primer was exactly prepared according to the sequence (Figure 2), then the general RT-PCR was performed to confirm the up-regulated gene expression by the exposure of PeCB. As expected from the nucleotide sequence, 270bp fragment was confirmed as shown in Figure 3.

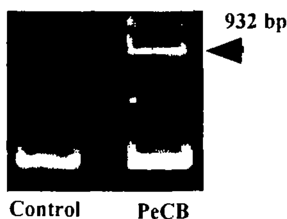

Figure 1. Agarose gel electrophoresis of the up-regulated cDNA expressed by PeCB exposure

Primer: 5' GGAGGATGGCCC 3'
Oligo dT

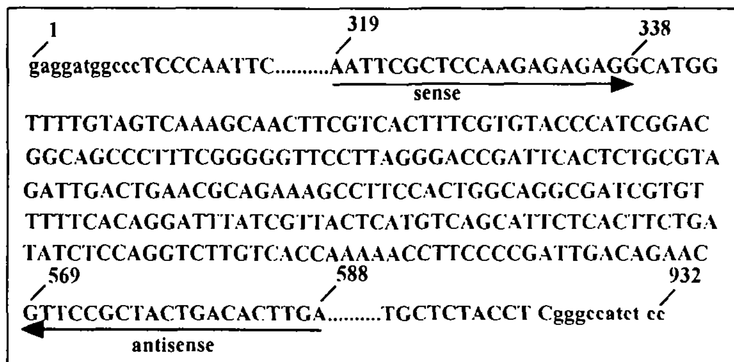

Figure 2. Nucleotide sequence of 932 bp cDNA that appears by PeCB exposure

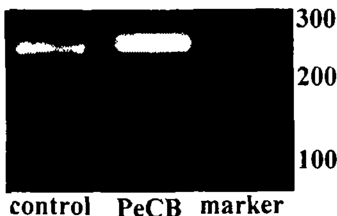

Figure 3. Confirmation of up-regulated gene expression by RT-PCR using a precise primer set on agarose gel

Primer set: sense / 5'AATTCGCTCCAAGAGAGAGG3'
antisense / 5'TCAAGTGTCAGTAGCGGAAC3'

Analysis of the change of gene expression by PeCB exposure using the DNA chip

About 2200 cDNA genes from *Arabidopsis thaliana* fixed on the DNA CHIP I^3 were hybridized with Cy3-cDNA for the control plant and Cy5-cDNA for the PeCB exposed plant as a mixed probe. As a result, the fluorescent intensity ratio of Cy3/Cy5 on many cDNA gene spots became over 3 or below 1/3.

The intensity ratio of Cy3/Cy5 over 3, which were counted more than 100, indicated the expression of these genes was down regulated by the addition of PeCB. On the other hand, the ratio of Cy3/Cy5 below 1/3, which were counted at least 30, revealed the gene expression was up regulated. Many genes in the genome of *Arabidopsis thaliana* seem to be modulated by the exposure of PeCB. Table 1 gave examples of up-regulated or down-regulated genes.

Table 1. Examples of up-regulated and down-regulated genes by PeCB exposure found in DNA chip analysis

Up-regulated genes		Down-regulated genes	
Cy3/ Cy5	protein	Cy3/ Cy5	protein
0.08	alpha1 tubulin.	14.71	unknown protein
0.12	mucin-like protein	12.33	calcium-dependent protein kinase
0.12	polyubiquitin 5	11.87	prolylcarboxypeptidase
0.15	Anthocyanin 5-aromatic acyltransferase	11.45	unknown protein
0.21	low temperature and salt responsive protein	10.44	maize gl1 homolog
0.21	reticuline oxidase homolog	7.44	unknown protein
0.23	ribonuclease	7.02	cytokinin binding protein
0.32	unknown protein	6.48	unknown protein
0.32	MAP kinase	5.29	cell division protein
0.33	peroxidase	4.73	protein phosphatase

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