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Assays of dioxins and dioxin-like compounds in actually contaminated soils using transgenic tobacco plants carrying a recombinant mouse aryl hydrocarbon receptor-mediated β-glucuronidase reporter gene expression system

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The transgenic tobacco plant XD4V-26 carrying the recombinant mouse aryl hydrocarbon receptor XD4V-mediated β-glucuronidase (GUS) reporter gene expression system was used for assay of dioxins and dioxin-like compounds consisting of polychlorodibenzo-*p*-dioxins, polychlorinated dibenzofurans, and coplanar polychlorinated biphenyls (Co-PCBs) in actually contaminated soils. The transgenic tobacco plant XD4V-26 showed a significant dose-dependent induced GUS activity when cultured on MS medium containing PCB126 [toxic equivalency factor (TEF) = 0.1]. In contrast, PCB169 and PCB180, which have 0.03 of TEF and unassigned TEF values, respectively, did not significantly induce GUS activity under the same conditions as with PCB126. When the tobacco plants were cultivated for up to 5 weeks on actually contaminated soils with dioxins and dioxin-like compounds collected from the periphery of an incinerator used for disposal of life and industrial wastes, GUS activity in the leaves was dose-dependently increased. The plants clearly detected 360 pg-TEQ g^{-1} of dioxins and dioxin-like compounds in this assay. There was a positive correlation between GUS activity and TEQ value of dioxins and dioxin-like compounds in the plants. This assay does not require any extraction and purification processes for the actually contaminated soil samples.

Keywords: Biochemical assay, dioxin, aryl hydrocarbon receptor, transgenic plants, polychlorinated biphenyl.

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (Co-PCBs), the so-called dioxins and dioxin-like compounds, are highly lipophilic and persistent in the environment. These compounds widely contaminated the environment. These were deposited in sediments in aquatic environments, and then highly accumulated at the tops of food chains, including humans. This contamination mainly first occurred at the sites of incinerators of life and industrial wastes. Therefore, it is important to continuously monitor dioxins and dioxin-like compounds in the periphery of incinerators from the standpoint of risk assessment and management. PCDDs, PCDFs, and Co-PCBs each consist of a number of congeners with different numbers and positions of chlorines attached on two benzene rings and are represented by a toxic equivalency factor (TEF), which was updated by the World Health Organization in 2005.[1] The TEF is a relative toxicity value based on the value 1 of the most toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD). A mixture of these congeners is represented by the total toxic equivalency (TEQ), which is the sum of concentration of each of the congeners multiplied by its TEF.

High-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) is used to identify and quantify residues of PCDDs, PCDFs, and Co-PCBs in

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environmental samples. This method is sensitive and accurate for measuring extremely low amounts of the residues in a variety of environmental samples. However, extraction and purification of these compounds from samples are imperative and result in a high cost of analysis. In contrast, biochemical assays based on molecular mechanisms of modes of actions of chemicals are suitable for rapid screening of a number of different kinds of samples and are advantageous for assessing the toxicity of these residues in mammals. Most of the biochemical assays estimate toxicity levels of dioxins and dioxin-like compounds in recombinant mammalian hepatoma cells expressing aryl hydrocarbon receptors (AhRs), since AhRs are primarily responsible for toxicity in mammals towards these compounds.[2,3] However, like instrumental analyses, these methods require several steps for extraction and purification of the samples.

The transgenic tobacco plants carrying a gene encoding the recombinant AhR, XDV, consisting of the ligand-binding domain of mouse AhR, the DNA-binding domain of bacterial repressor protein LexA, and the transactivation domain of the virus VP16 as well as β-glucuronidase (GUS) reporter gene were genetically engineered. These transgenic tobacco plants showed a significantly increased GUS activity when treated with the AhR ligands such as indigo, β-naphthoflavone, and 3-methylcholanthrene (MC).[4] The transgenic tobacco plants seemed to be useful for a biochemical assay of dioxins and dioxin-like compounds toxic to mammals. The assay did not need any extraction and purification of chemicals, since the plants took up these chemicals in medium through their developed roots by passive diffusion.

In this study, the transgenic tobacco plant XD4V-26 was examined for practical assays of PCDDs, PCDFs, and Co-PCBs in actually contaminated soils collected from the periphery of an incinerator used for disposal of life and industrial wastes, since the transgenic plants showed low background activity and dose- and time-dependent GUS activity induced in response to MC.

Materials and Methods

Chemicals

The compounds3,3′,4,4′,5-Pentachlorobiphenyl (PCB126; TEF = 0.1), 3,3′,4,4′,5,5′-hexachlorobiphenyl (PCB169; TEF = 0.03), and 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (PCB180; TEF value was not assigned) were purchased from AccuStandard Inc. (New Heaven, CT, USA). They were each dissolved in dimethyl sulfoxide (DMSO) for application to Murashige and Skoog (MS) medium. The final concentration of DMSO was 0.1 % in MS medium. The compounds 4-Methyl-umbelliferyl-β-D-glucuronide (4MUG) for a substrate of GUS and 4-methyl-umbelliferone (4MU) as a product of GUS reaction were purchased from Nacalai Tesque (Kyoto, Japan).

Plants

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were transformed by the use of *Agrobacterium tumefaciens* carrying the plasmid pGPΩXD4VGUS as previously described.[4] The resultant transgenic tobacco strain XD4V-26 carries the gene encoding recombinant AhR, which consists of the DNA-binding domain of bacterial LexA [amino acids (AA) 1 to 202], the ligand-binding domain of mouse AhR (a.a. 83 to 494), and the transactivation domain of virus VP16 (a.a. 413 to 490), as well as the gene encoding the reporter GUS. The transgenic tobacco plant XD4V-26 was aseptically and separately incubated in MS medium containing each of the PCB congeners in a growth chamber at 25 °C under 16-h light/8-h dark cycle conditions or grown on the soils contaminated with PCDDs, PCDFs, and Co-PCBs in a closed greenhouse under natural day-length light conditions.

Soils

Weathered contaminated soils with PCDDs, PCDFs, and Co-PCBs (5100 pg-TEQ g^{-1}) were collected from the periphery of an incinerator used for disposal of life and industrial wastes in Japan. Table 1 shows concentrations of PCDD, PCDF, and Co-PCB congeners in the collected soils. The soils were diluted with uncontaminated soils (1.1 pg-TEQ g^{-1}) purchased from Suntory Flowers Ltd. (Tokyo, Japan) prior to assays. The uncontaminated soils were also used as the control soils.

Fluorometric GUS assay of transgenic tobacco plants

Axillary buds of the transgenic tobacco plants were cultured on MS medium containing 3.0 % (w/v) sucrose, 0.7 % (w/v) agar, and 0.1 to 1000 ng mL^{-1} PCB126, PCB169, or PCB180. After two weeks, either the second or third leaf from the top of the plant, or both, were used for fluorometric GUS assay as previously described.[5] Soluble fractions were prepared from leaves and incubated with 4MUG. After stopping GUS reaction, fluorescence at 360 nM excitation and 450 nM emission was measured with a microplate reader (MTP-100F; CORONA, Katsuta, Japan). GUS activity was determined from a standard curve of the fluorescence of 4MU, and data were presented as means ± standard deviation (SD).

Axillary buds were also aseptically cultured on MS medium for about a month. Approximately 10 cm high plants were individually transferred into 1/5000-acre pots filled with the soils contaminated with dioxins and dioxin-like compounds or the uncontaminated soils. The surface of the soils was covered with the uncontaminated soils or aluminum foil. The tobacco plants were grown in a closed greenhouse for several weeks, and then either the second or third leaf from the top of the plants, or both, were assayed for GUS activity as described above.

Table 1. Concentrations of PCDD, PCDF, and Co-PCB congeners in the contaminated soils containing 5100 pg-TEQ g^{-1}.

PCDD, PCDF, and Co-PCB	*TEF*	*Concentration (pg g^{-1})*	*TEQ (pg-TEQ g^{-1})*
PCDDs			
1,3,6,8-TetraCDD	0	1005.06	0
1,3,7,9-TetraCDD	0	346.84	0
2,3,7,8-TetraCDD	1	22.71	22.71
1,2,3,7,8-PentaCDD	1	476.47	476.47
1,2,3,4,7,8-HexaCDD	0.1	983.23	98.32
1,2,3,6,7,8-HexaCDD	0.1	1235.29	123.53
1,2,3,7,8,9-HexaCDD	0.1	1155.27	115.53
1,2,3,4,6,7,8-HeptaCDD	0.01	17355.54	173.56
1,2,3,4,5,6,7,8-OctaCDD	0.0003	23222.10	6.97
Total PCDDs		79735.53	–
PCDFs			
1,2,7,8-TetraCDF	0	186.37	0
2,3,7,8-TetraCDF	0.1	111.29	11.13
1,2,3,7,8-PentaCDF	0.03	1193.18	35.80
2,3,4,7,8-PentaCDF	0.3	2565.58	769.67
1,2,3,4,7,8-HexaCDF	0.1	4962.89	496.29
1,2,3,6,7,8-HexaCDF	0.1	6015.95	601.59
1,2,3,7,8,9-HexaCDF	0.1	2237.86	223.79
2,3,4,6,7,8-HexaCDF	0.1	12378.06	1237.81
1,2,3,4,6,7,8-HeptaCDF	0.01	48617.88	486.18
1,2,3,4,7,8,9-HeptaCDF	0.01	9901.31	99.01
1,2,3,4,5,6,7,8-OctaCDF	0.0003	83064.51	24.92
Total PCDFs		326048.35	–
Co-PCBs			
3,3′,4,4′-TetraCB(#77)	0.0001	110.46	0.01
3,4,4′,5-TetraCB(#81)	0.0003	27.45	0.01
3,3′,4,4′,5-PentaCB(#126)	0.1	919.70	91.97
3,3′,4,4′,5,5′-HexaCB(#169)	0.03	936.83	28.11
2,3,3′,4,4′-PentaCB(#105)	0.00003	480.92	0.01
2,3,4,4′,5-PentaCB(#114)	0.00003	126.31	0.00
2,3′,4,4′,5-PentaCB(#118)	0.00003	941.48	0.03
2′,3,4,4′,5-PentaCB(#123)	0.00003	129.42	0.00
2,3,3′,4,4′,5-HexaCB(#156)	0.00003	938.53	0.03
2,3,3′,4,4′,5′-HexaCB(#157)	0.00003	557.97	0.02
2,3′,4,4′,5,5′-HexaCB(#167)	0.00003	1436.44	0.04
2,3,3′,4,4′,5,5′-HeptaCB(#189)	0.00003	1340.37	0.04
2,2′,3,3′,4,4′,5-HeptaCB(#170)	0	5238.69	0
2,2′,3,4,4′,5,5′-HeptaCB(#180)	0	940.58	0
Total Co-PCBs		7945.89	–
Total TEQ			5123.54

HRGC/HRMS analysis of PCDDs, PCDFs, and Co-PCBs in tobacco plants

As previously described, PCDDs, PCDFs, and Co-PCBs were extracted from the aerial parts of the tobacco plants.[6] Congeners of PCDDs, PCDFs, and Co-PCBs were each quantified by HRGC/HRMS (HP6890/Micromass Autospec-Ultima, Micromass Ltd., Manchester, UK) under the same conditions as previously reported.[7]

Statistical analysis

Statistical analysis used Student's *t*-test for GUS activity in the transgenic tobacco plants in the response to PCB congeners in the transgenic tobacco plants as well as differences between uncontaminated and contaminated soils. Pearson's correlation coefficient was used for analysis of correlation.

Results

GUS activity in the transgenic tobacco plant XD4V-26 cultured on MS medium containing PCB congeners

The transgenic tobacco plant XD4V-26 was cultured on MS medium containing each of PCB126 (TEF = 0.1), PCB169 (TEF = 0.03), and PCB180 (not assigned TEF

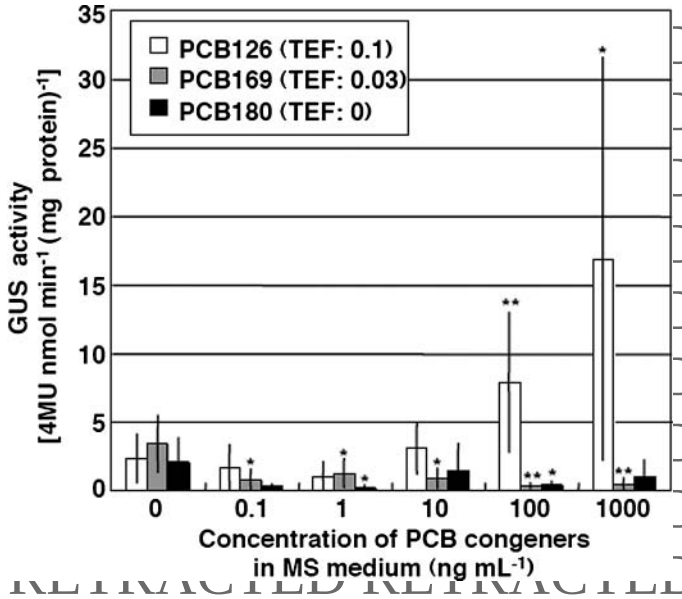

Fig. 1. GUS activity in leaves of the transgenic tobacco plant XD4V-26 cultured for 2 weeks on MS medium containing PCB126, PCB169, or PCB180. Values are means ± SDs (PCB126, $n = 9–12$; PCB169, $n = 6–8$; PCB180, $n = 3–9$). Significant difference (Student's *t*-test): **, $p < 0.01$; *, $p < 0.05$.

value) for 2 weeks, and then leaves were subjected to GUS assay. As shown in Figure 1, a dose-dependent increased GUS activity was clearly observed when the tobacco plants were treated with PCB126, with significant differences at 100 and 1000 ng mL^{-1} ($P < 0.01$ and $P < 0.05$, respectively, relative to no treatment with PCB126, Student's *t*-test). The GUS activity at 1000 ng mL^{-1} was 7.4 times higher than that in the plants treated with DMSO alone. In contrast, no dose-dependent increased GUS activity was observed when treated with PCB169 and PCB180. Background GUS activity was detected in tobacco plants treated with DMSO and was significantly decreased by the treatment with increasing doses of PCB169 and PCB180 ($P < 0.05$). Thus, the present study indicated that the transgenic tobacco plant XD4V-26 showed induced GUS activity in response to the agonist PCB126 and reduced GUS activity in response to the antagonists PCB169 and PCB180.

GUS activity and uptake of PCDDs, PCDFs, and Co-PCBs in the transgenic tobacco plant XD4V-26 cultured on the actually contaminated soils

The transgenic tobacco plant XD4V-26 was cultured in pots containing the soils actually contaminated with PCDDs, PCDFs, and Co-PCBs. The GUS activity in their leaves was significantly induced after 5 weeks of culture on the soils containing 360 pg-TEQ g^{-1} of these congeners (Fig. 2a). On the other hand, the GUS activity in the tobacco plants cultured on the uncontaminated soils containing 1.1 pg-TEQ g^{-1} was not induced so high. The transgenic tobacco plant XD4V-26 was also cultured in the soils containing 510 and 5100 pg-TEQ g^{-1} for 33 days. The leaves were subjected to assay of GUS activity, and the aerial parts were analyzed in HRGC/HRMS for PCDD, PCDF, and Co-PCB congeners. The GUS activity in the tobacco plants cultured on the soils containing 5100 pg-TEQ g^{-1} was twice higher than that in the plants cultured on the soils of 510 pg-TEQ g^{-1} (Fig. 2b). The concentrations of PCDDs, PCDFs, and Co-PCBs in the plants cultured on the soils containing 5100 pg-TEQ g^{-1} were higher than those of the plants cultured on the soils of 510 pg-TEQ g^{-1} (Fig. 2c). Particularly, the concentrations of 1,2,3,4,5,6,7,8-octaCDD and 2,3′,4,4′,5-pentaCB (PCB118) in the plants were the highest, followed by 2,3,3′,4,4′-pentaCB (PCB105) and others (Fig. 3).

Correlation between GUS activity and TEQ values of PCDDs, PCDFs, and Co-PCBs taken up into the transgenic tobacco plant XD4V-26

There was a significant positive correlation [0.681 (Person's correlation coefficient), $P < 0.01$, Fig. 4] between the GUS activity and TEQ values of PCDDs, PCDFs, and Co-PCBs in the tobacco plant. In contrast, the transgenic tobacco plants grown on the uncontaminated soils that contained low level of PCDDs, PCDFs, and Co-PCBs including toxic and non-toxic congeners, accumulated as indicated by 0.5 to 1.0 pg-TEQ g^{-1}, although the GUS activity was very low. These results suggested that the induced GUS activity may be due to TEF values of the congeners, although the uptake of congeners of PCDDs, PCDFs, and Co-PCBs in the plants seemed to be under passive diffusion mechanism. The effects of certain congeners, in which a TEF value was not assigned, were examined on GUS activity in the transgenic plants. The relative amounts are defined as the amounts of PCDD and PCDF congeners without TEF value in the plants divided by the amounts of PCDD and PCDF congeners with TEF values of 0. These relative amounts were negatively correlated with GUS activity [−0.486 (Person's correlation coefficient), $P < 0.05$, data not shown]. These results suggested that relatively low GUS activity in the transgenic plants grown in the uncontaminated soils seemed to be due to antagonistic effects of certain congeners in which TEF values were not assigned.

Discussion

The transgenic tobacco plant XD4V-26 carrying the recombinant mouse AhR-mediated GUS reporter gene expression system exhibited a dose-dependent GUS activity towards PCB126 (TEF = 0.1), but not towards PCB169 (TEF = 0.03) and PCB180 (TEF was not assigned.). It suggested that the plant took up PCB126, which activated the recombinant mouse AhR XD4V, and then induced GUS activity. Uptake of PCB169 and PCB180 also occurred,

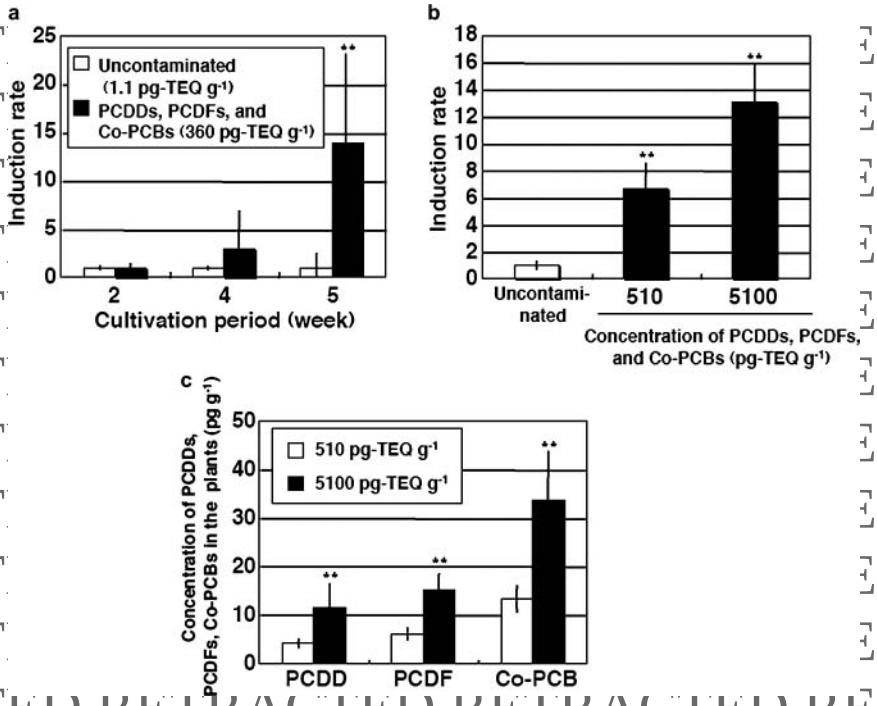

Fig. 2. Time-dependent (a) and dose-dependent (b) GUS activity, and concentrations of PCDD, PCDF, and Co-PCB congeners (c) in the transgenic tobacco plant XD4V-26 cultured on the actually contaminated soils. The transgenic plant XD4V-26 was cultured on the soils containing 510 or 5100 pg-TEQ g^{-1} for 33 days. The induction rate is expressed as the GUS activity in the transgenic plants cultured on the contaminated soils divided by the activity in the plants cultured on the uncontaminated soils. Values are means ± SDs (a: uncontaminated, $n = 11$; dioxins 360 pg-TEQ g^{-1}, $n = 16$–17; b: uncontaminated, $n = 4$; 510 pg-TEQ g^{-1}, $n = 8$; 5100 pg-TEQ g^{-1}, $n = 8$; c: 510 pg-TEQ g^{-1}, $n = 8$; 5100 pg-TEQ g^{-1}, $n = 7$). Significant difference (Student's *t*-test): **, $P < 0.01$ versus uncontaminated soils (a, b) and 510 pg-TEQ g^{-1} (c).

but those compounds did not contribute to induce GUS activity because of very low TEF value and almost no toxicity towards mammals, respectively. Instead, background GUS activity, probably due to endogenous AhR ligands such as indoleacetic acid,[8] was suppressed by PCB169 and PCB180. Concurrent treatment with PCB126 and PCB180 tended to suppress GUS activity induced by PCB126 (data not shown). It was reported that certain congeners with low or not assigned TEF values have shown antagonistic activity toward AhR in rat primary hepatocytes: PCB153 (TEF was not assigned), decreased the induction of CYP1A1 by the treatment with 2,3,7,8-TCDD, but PCB77 (TEF = 0.0001) and PCB156 (TEF = 0.00003) did not.[9] It was also reported that di-, tri-, and tetra-*ortho*–substituted PCBs suppressed the activation of AhR by PCBs with no or one *ortho* chlorine substitution.[10] It was reported that certain persistent organic pollutants (POPs), including *p,p′*-dichloro-diphenyl-trichloroethane (DDT) and dieldrin, were antagonistic toward AhRs.[11,12] In transgenic *Arabidopsis* plants carrying genes encoding a recombinant guinea pig AhR and the GUS reporter, *p,p′*-DDT, *p,p′*-dichloro-diphenyl-dichloroethane (*p,p′*-DDE), and *p,p′*-dichloro-diphenyl-dichloroethylene (*p,p′*-DDD) decreased the GUS activity induced by MC.[13] Antagonism between the agonist MC and dieldrin or *p,p′*-DDT was also observed in an *in vitro* assay using an Ah immunoassay.[13] The present assay responded to both agonists and antagonists among PCB congeners. Therefore, the GUS activity in the assay may reflect the relative toxicity level of a mixture of PCB congeners. It was suggested that the relative toxicity level of a mixture of congeners of PCDD, PCDF, and Co-PCB should not be simply determined by TEQ as the sum of concentrations of congeners multiplied by their TEF values, since TEQ does not count antagonistic effects of certain congeners.

When the transgenic tobacco plant XD4V-26 was cultured on the soils containing dioxins and dioxin-like compounds for 5 weeks, the GUS activities in the leaves were time- and dose-dependently increased. The transgenic plant was thus able to monitor the levels of both 360 pg-TEQ g^{-1} and 5100 pg-TEQ g^{-1}. The environmental standard (less than 1000 pg-TEQ g^{-1}) for dioxins and dioxin-like compounds in soils in Japan was possibly detected in the transgenic plant XD4V-26 within 5 weeks of planting. However, the GUS activity of plants grown in 5100 pg-TEQ g^{-1} was not 10 times higher than that of plants grown in 510 pg-TEQ g^{-1}. This suggests that the tobacco plants may not be able to accumulate very high concentrations of these congeners and may reach the plateau between 510 and 5100 pg-TEQ g^{-1}. Moreover, the different levels of uptake of the

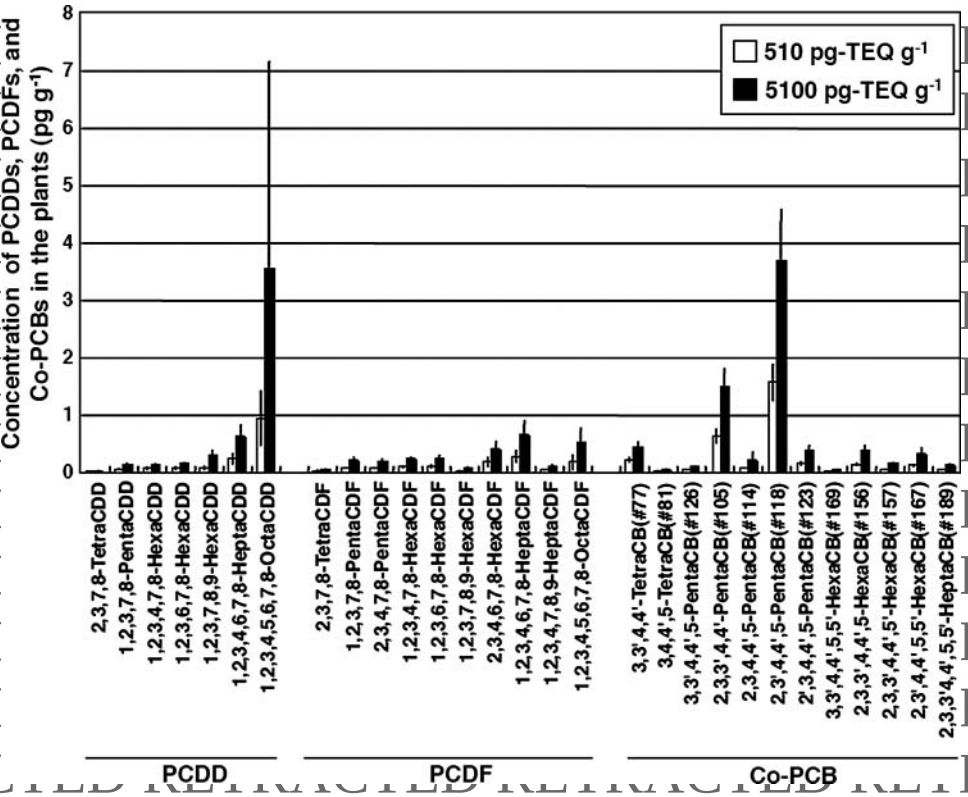

Fig. 3. Concentrations of PCDD, PCDF, and Co-PCB congeners in the transgenic tobacco plant XD4Y-26 cultured on the contaminated soils containing 510 and 5100 pg-TEQ g^{-1} for 33 days. Values are means ± SDs (510 pg-TEQ g^{-1}, $n = 8$; 5100 pg-TEQ g^{-1}, $n = 7$).

congeners in the plants may be due to the mass and growth stages of the plants.

The amounts of uptake of dioxins and dioxin-like compounds in the tobacco plants by passive diffusion were not so large as compared with that of zucchini plants.[6,14] It was reported that root exudates such as low-molecular-weight organic acids [15,16] and biosurfactants [17,18] may be involved in the uptake of hydrophobic compounds such as POPs. By the use of these compounds, the transgenic tobacco plants seem to increase the uptake of dioxins and dioxin-like compounds in soils.

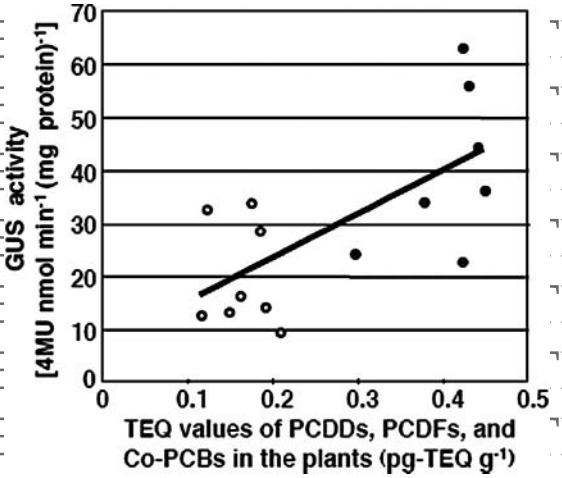

Fig. 4. Correlation between GUS activity and TEQ value in the transgenic tobacco plant XD4V-26 cultured on the actually contaminated soils containing 510 (open circle) and 5100 (closed circle) pg-TEQ g^{-1} for 33 days. Pearson's correlation coefficient: $r = 0.681$.

In this study, transgenic tobacco plant XD4V-26 carrying genes encoding a recombinant mouse AhR and the GUS reporter successfully detected residues of PCDD, PCDF, and Co-PCB congeners in the contaminated soils within 5 weeks. These results suggested that the assay using the plant seems to be useful for on-site assays of these residues. In contrast, cultivation of the transgenic plants on samples of the contaminated soils in the closed laboratory may provide some advantages such as public acceptance of the use of transgenic plants, and possible control of environmental conditions, including temperature, drought, and day length, all of which may influence on the stable GUS assay.

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Authors H. Inui and K. Gion contributed equally to this work.

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