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# Direct Determination of Estrogenic and Antiestrogenic Activities Using an Enhanced Plant Two-Hybrid System

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This paper reports a simple, low-cost, and extremely sensitive reporter-gene assay system for comprehensive analysis of estrogenic activity using transgenic *Arabidopsis thaliana*: the EPTH system. It had the capability to detect 17$\beta$-estradiol at a concentration of 10 pM. The system was rendered 5 times more sensitive than a previous system [Tojo, T.; Tsuda, K.; Wada, T.; Yamazaki, K. *Ecotoxicol. Environ. Saf.* **2006**, *64*, 106−114) (*1*)] by increasing the copy number of the transactivation domain fused to a nuclear receptor co-activator. The system can efficiently detect other estrogenic and antiestrogenic substances. Estrogenic activities were determined in treated sewage samples from four distinct sewage farms using the system. Results showed that the system can detect estrogenic activity directly and more efficiently than a yeast two-hybrid system without any manipulation for extraction and condensation of hydrophobic compounds and aseptic treatment. Furthermore, the system also is useful as a powerful tool for discovery of a new category of natural estrogenic substances that are undetectable by previous plant and yeast systems.

**KEYWORDS:** ***Arabidopsis thaliana*; bioassay; co-activator; endocrine disrupting chemicals (EDCs); 17$\beta$-estradiol; estrogen receptor (ER); $\beta$-glucuronidase (GUS); reporter-gene assay; transcriptional intermediary factor 2 (TIF2); enhanced plant two-hybrid (EPTH) system; transgenic plant**

## INTRODUCTION

Some endocrine-disrupting chemicals (EDCs) remain stable in the environment over a prolonged period and damage ecological systems and human health, particularly reproductive functions, mainly through their interaction with nuclear hormone receptors (NRs) (*2*). For these reasons, the development of convenient technologies for detecting EDCs in the environment is required to avoid unexpected exposure of organisms to EDCs. Because EDC detection methods that are based on analytical chemistry require expensive equipment, such as gas chromatography−mass spectrometry, and require extraction and purification processes of EDCs with organic solvents, such methods drive up costs for monitoring EDCs. Furthermore, it is impossible to evaluate their negative effects on organisms using such a method. For these reasons, a low-cost biological assay method for the detection of EDC activity has been anticipated.

The existence of EDCs that possess estrogenic activity in the environment is a major issue. Effluents after sewage treatment frequently contain high levels of estrogenic substances. Moreover, some industrially produced chemicals and agricultural chemicals show estrogenic activity (*3*). These estrogenic substances might affect physiological pathways by binding to estrogen receptors (ERs) in animals. As one explanation, ERs belong to NRs: they are one element of a large superfamily of ligand-dependent transcription factors (*4*). Furthermore, ERs are divisible into several functional regions (A/B, C, D, and E regions) (*5*). The E region contains the activation function 2 (AF-2) domain, a ligand-dependent transcriptional activation domain. Conformational change of AF-2 triggered by ligand binding to the E region (*6*−*8*) permits interaction of the AF-2 domain with transcriptional intermediary factor 2 (TIF2), a member of the p160 family co-activator (*9*), thereby inducing transcriptional activation of their target genes (*10*).

Several detection methods for estrogenic substances that employ reporter-gene assay systems have been reported using human cells (*11*), yeast cells (*11*−*13*), and fish (*14*). However, methods using human and yeast cells require complex aseptic manipulations. Those using fish require constant efforts to keep them alive because they cannot be preserved at any growth stage. Simpler techniques were greatly anticipated. Recently, Tojo et al. (*1*) developed a novel bioassay system based on a two-hybrid system described above using a transgenic plant: a plant two-hybrid (PTH) system. Two effector genes controlled by $P_{35S}$, a strong constitutive promoter, are overexpressed constitutively

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in transgenic *Arabidopsis* plants. Both effector proteins contain a nuclear localization signal (NLS) to retain them in the nucleus. Estrogen binding to a ligand-binding domain of estrogen receptor α from human (hERα LBD) of the chimeric estrogen receptor (effector 1) permits interaction with a nuclear receptor interaction domain of transcriptional intermediary factor 2 from human (TIF2 NID) of the chimeric co-activator (effector 2). Through this interaction, the transcriptional activation signal of the acidic transactivation domain of VP16 (VP16 AD) can stimulate transcription of a β-glucuronidase (GUS) reporter gene in the PTH system. The minimum detection limit of 17β-estradiol by the PTH system was 50 pM (13 pg/mL) in an inductive medium for both histochemical GUS staining and measurement of GUS activity. Sensitivity of the system to 17β-estradiol was comparable to the value recorded for a yeast two-hybrid system (*12*, *13*). Furthermore, seeds can be stored for a few years or more in an appropriate container. More than 2000 seeds were obtainable from a plant in 2 months. Therefore, the PTH system was more convenient and offered a much lower cost than any other reported assay method.

However, it was slightly less sensitive than the system using human cells (*15*). Here we report a 5-fold enhancement of the PTH system sensitivity by increasing a copy number of the VP16 activation domain of a chimeric transcriptional co-activator; the novel system is an enhanced PTH (EPTH) system. This is the first paper to describe direct determination of estrogenic activities in samples without any pretreatment of samples or any aseptic treatment using the system.

## MATERIALS AND METHODS

**Plasmid Constructions.** Construction of the plasmid used in a prototype (the PTH system) was described in a previous paper (*1*). The plasmid DNA used in the present study comprises two effector genes (encoding effector 1 and modified effector 2) and a reporter gene. A DNA fragment encoding the acidic transactivation domain (VP16 AD, positions from 413 to 490 amino acid) of herpes simplex virus VP16 in effector 2 was replaced with a DNA fragment encoding a tandem repeat of five copies of VP16 AD. A DNA fragment encoding a single VP16 AD was amplified by PCR using herpes simplex virus VP16 gene (*16*) as a template and primer 1 (5′-GGCCATGGGCCCCCCCGACCGATGTCAGCCTGGGGGACGAGCTGCACTTAGACGGCGAGGACGTGGCGATGGCGCACGCCGACGCGCT-3′) and primer 2 (5′-GGTCATGACCCACCGTACTCGTCAAT-3′) as a primer set. The amplified DNA fragment of VP16 AD was digested by restriction enzymes, *Nco*I and *Bsp*HI. The gene encoding modified effector 2 was constructed by inserting DNA fragments corresponding to four tandem copies of VP16 AD between the *Nco*I and *Bsp*HI sites of the plasmid DNA used for the PTH system.

**Plant Materials, Plant Transformation.** The Columbia ecotype of *Arabidopsis thaliana* (L.) Heynh. was used in all experiments. Transformation of *A. thaliana* was carried out using the floral dipping method (*17*). Seeds derived from the original transformant, T1 seeds, were plated on MS medium (*18*) solidified with 0.8% phytoagar (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 1% sucrose, 50 μg/mL kanamycin, and 100 μg/mL Claforan. All reporter-gene assays were performed using seeds derived from the third generation (T4 seeds that contain an introduced T-DNA fragment on one locus of the *Arabidopsis* chromosome) after plant transformation.

**Chemicals.** Dimethyl sulfoxide (DMSO), 17β-estradiol, *p*-*n*-nonylphenol (NP), bisphenol A (BPA), 4-methylumbelliferyl-β-D-glucuronide (4-MUG), tamoxifen, and ICI 182780 were purchased from Wako Pure Chemical Industries, Ltd. Diethylstilbestrol (DES) was purchased from ICN Biomedicals Inc. Genistein was purchased from Extrasynthese, S.A. (Genay, France). 4-Methylumbelliferone (4-MU) and 4-hydroxytamoxifen were purchased from Sigma-Aldrich Japan K. K. (Tokyo, Japan). 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) was purchased from Inalco SPA (Milan, Italy).

**Histochemical Staining of Plants by β-Glucuronidase Activity.** Whole seedlings were stained, as described by Tsuda and Yamazaki (*19*). *Arabidopsis* seedlings were soaked in a GUS assay solution that included 50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA (pH 8.0), 2 mM X-gluc, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 0.1% (v/v) Triton X-100. After incubation in a vacuum desiccator for 15 min at room temperature, the seedlings were incubated at 37 °C for 1 h. After staining, the tissues were soaked in several changes of 70% ethanol to stop the enzyme reaction and remove chlorophyll.

**Determination of GUS Activity.** The GUS activity was determined as described by Jefferson et al. (*20*). In all, 20 seedlings that had been treated with each chemical were frozen in liquid nitrogen and powdered in a mortar. Proteins were extracted in 0.2 mL of extraction buffer [50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, 10 mM β-mercaptoethanol]. The extract was centrifuged twice at 10000*g* for 15 min at 4 °C to remove insoluble cell debris. A fluorometric assay was conducted using 4-MUG as a substrate for measurement of the activity in the soluble protein fraction. The reaction was performed at 37 °C in a reaction buffer (0.1 mL) containing 1 mM 4-MUG; it was terminated with the addition of 0.9 mL of 0.2 M $Na_2CO_3$. The activity was determined using a fluorescence spectrophotometer (F-4500; Hitachi High-Technologies Corp., Tokyo, Japan). The 4-MU released by the reaction was determined by reading the emission at 455 nm (excitation at 365 nm). The total soluble protein was determined as described by Bradford (*21*) to standardize each value of emission.

**Exposure of the Transgenic Plant to Estrogenic Substances.** In experiments for **Figures 2**−**4**, and the experiment indicated "AP" in **Table 1**, T4 seeds were sown on a medium plate (1 × MS salt, 1% sucrose, and 0.8% phytoagar) and incubated for 3 days at 4 °C under dark conditions. Then seeds were germinated and grown at 22 °C in light conditions for a week in the medium containing estrogenic substances dissolved into DMSO. We added 0.01% DMSO to the control medium. Germination of seeds generally starts 24 h after seeds are placed in the light conditions. In the experiment indicated "D" in **Table 1**, T4 seeds were sown on processed rockwool (3 cm diameter, 7 mm thickness; Nitto Boseki Co. Ltd., Tokyo, Japan) soaked in 4 mL of 0.1% Hyponex (Hyponex Co. Ltd., Marysville, OH) and incubated for 3 days at 4 °C under dark conditions. Seeds were germinated and grown at 22 °C in light conditions for 4 days; then seedlings were exposed to treated sewage for 2 days after removal of 0.1% Hyponex by pipetting without aseptic treatment.

**Preparation of Hydrophobic Compounds from Treated Sewage.** Treated sewage samples for the yeast two-hybrid (YTH) assay were stored in solvent-rinsed glass bottles with Teflon-lined capping. Subsequently, the samples were filtered through a GF/C glass fiber filter (Whatman International Ltd., Maidstone, U.K.) and extracted with solid-phase extraction (SPE) cartridges (Aqusis PLS-3; GL Sciences Inc., Tokyo, Japan) within 12 h of collection. The cartridges were stored at −20 °C until analysis. The frozen SPE cartridges were thawed at room temperature. Then the trapped substances were eluted with dichloromethane. The eluate was purged to dryness with nitrogen gas and then dissolved immediately in DMSO.

**Determination of Estrogenic Activities in Treated Sewage Using the YTH System.** Estrogenic activities (dissolved in DMSO) recovered from treated sewage were determined using the YTH system, as described previously (*22*). The estrogenic activity was determined as the equivalent concentration of 17β-estradiol.

**Preparation of Soluble Fractions of Medicinal Herbs.** Soluble fractions extracted with acetone (A), *n*-butanol (B), dichloromethane (D), hexane (H), methanol (M), or boiling water (W) from 41 species of medicinal herbs were dissolved into DMSO (final concentration of each sample = 100 mg/mL).

**Exposure of the Transgenic Plant to Soluble Fractions of Medicinal Herbs.** Five seeds preincubated for 3 days at 4 °C in distilled water under dark conditions were sown on processed rockwool (14 mm diameter, 7 mm thickness) soaked in 1 mL of 0.1% Hyponex containing 100 μg/mL of each soluble fraction for each test and incubated for 5 days under light conditions at 22 °C without aseptic treatment.

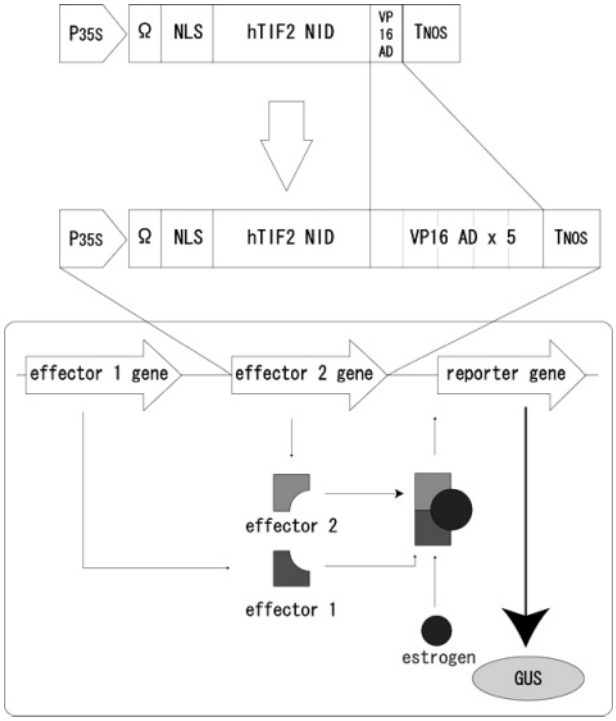


**Figure 1.** Schematic representation of the structure of a gene introduced into *A. thaliana* encoding a chimeric transcriptional coactivator for detecting estrogenic activity in the EPTH system. The detailed structures of effector 1 and reporter genes have already been described in a previous paper (*1*). $P_{35S}$, promoter of cauliflower mosaic virus 35S RNA; Ω, polynucleotide DNA sequence for translational activation; NLS, nuclear localization signal of T antigen from SV40 virus; hTIF2 NID, nuclear receptor interaction domain of transcriptional intermediary factor 2 from human; VP16 AD X 5, five copies of acidic transactivation domain of VP16 from herpes simplex virus; $T_{NOS}$, terminator of nopaline synthase gene from *Agrobacterium tumefaciens*; GUS, β-glucuronidase from *Escherichia coli*.

## RESULTS

**Molecular Mechanism for Detecting Estrogenic Activity Using the EPTH System.** Multimerization of the VP16 AD in a chimeric transcriptional co-activator has been reported to increase the activation potential of the transcription factor in tobacco mesophyll cells. Saturated levels of activation were observed when five to eight copies of the VP16 AD were fused to the DNA binding domain in the report (*23*). Therefore, we replaced a single copy of VP16 AD of the chimeric co-activator by five copies of VP16 AD to emphasize the capability of the co-activator for transactivation of the target reporter gene in the EPTH system, as shown in **Figure 1**.

**17β-Estradiol-Dependent Expression of GUS Reporter Gene in the EPTH System.** Seeds were germinated and cultured for a week on agar culture medium containing various concentrations of 17β-estradiol for GUS staining (**Figure 2A**) and determination of GUS activity (**Figure 2B**) to test the sensitivity of GUS expression to 17β-estradiol in transgenic *Arabidopsis*. The GUS staining was detectable by emergence of blue coloration associated with the production of indigo blue by degradation of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in the root of the transgenic plant grown on an agar culture medium containing concentrations of 17β-estradiol higher than 10 pM. The GUS staining was restricted to the boundary region between hypocotyls and roots when the transgenic *Arabidopsis* was grown on an agar culture medium with 10 pM 17β-estradiol. The GUS staining expanded to the whole root at concentrations higher than 50 pM. The response of the GUS reporter gene to

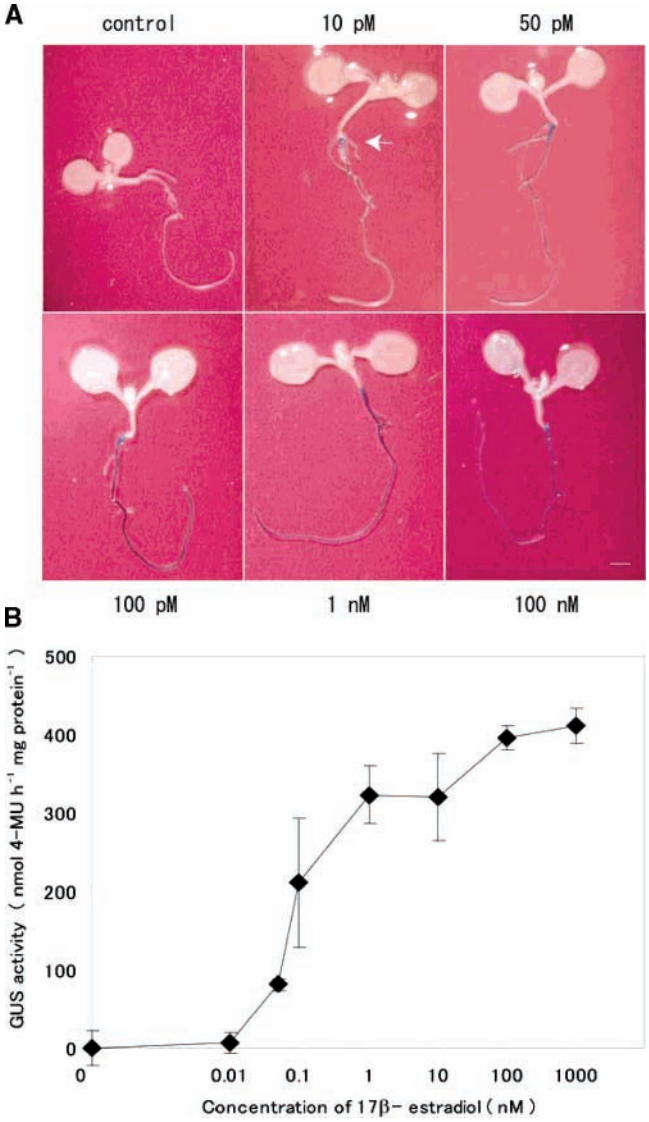


**Figure 2.** Estrogen-dependent induction of GUS expression in the EPTH system: (**A**) Seedlings were grown for a week, as described under Materials and Methods, on inductive media containing different concentrations of 17β-estradiol (indicated above or below each photograph). Ten seedlings were used for histochemical analyses of GUS expression; 1 of the 10 seedlings is shown as an example. Bar = 1 mm. (**B**) For measurement of GUS activity as described under Materials and Methods, 20 seedlings were grown as described above and then used. The pattern of GUS expression was reproduced in three independent experiments.

17β-estradiol was also determined by measurement of specific GUS activity in all soluble proteins. The GUS activity was detected at concentrations of 17β-estradiol higher than 10 pM, reached an almost saturated level at 1 nM 17β-estradiol, and increased gradually at concentrations of 17β-estradiol higher than 1 nM. The maximum GUS activity was about 450 nmol of 4-MU/h/mg of protein. The level was 6-fold higher than that observed in the PTH system (*1*). The GUS activity was not affected by the addition of 1 nM testosterone, 1 nM progesterone, or 1 nM dexamethasone to the agar culture medium (data not shown). The growth of *Arabidopsis* seedlings appeared to be normal at any concentration of 17β-estradiol (data not shown).

**Responsiveness of GUS Gene Expression to Estrogenic Substances in the EPTH System.** To determine estrogenic activities induced by other estrogenic substances, seeds were

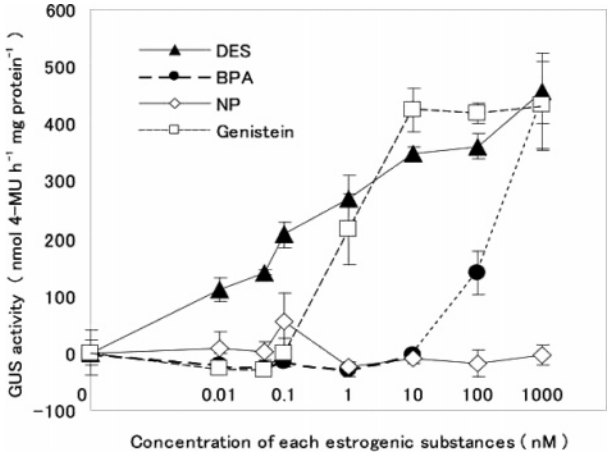


**Figure 3.** Dose–response curves of GUS activity induced by various estrogenic substances. DES (▲), NP (◇), BPA (●), genistein (□) were used as estrogenic substances. As described under Materials and Methods, 20 seedlings were exposed to media containing various concentrations of chemicals for a week. Each value of GUS activity was measured as described under Materials and Methods. The GUS expression was reproduced in three independent experiments.

germinated and grown on agar culture medium containing various concentrations of diethylstilbestrol (DES), *p*-*n*-nonylphenol (NP), bisphenol A (BPA), and genistein for a week, followed by determination of GUS activity (**Figure 3**). The profile of the GUS expression pattern in those transgenic plants grown on an agar culture medium containing DES closely resembled that observed in plants grown on the medium with 17$\beta$-estradiol. Using transgenic *Arabidopsis* plants, 1 nM genistein and 100 nM BPA on the agar culture medium were detectable; the GUS activity retained a maximum level at concentrations higher than 10 nM genistein and 1 $\mu$M BPA, respectively. GUS activities in the plants grown on agar culture medium containing NP were not detectable at any concentration tested.

**Responsiveness of GUS Gene Expression to Antagonistic Substances of 17$\beta$-Estradiol in the EPTH System.** To determine estrogenic activity associated with antagonistic substances of 17$\beta$-estradiol, seeds were germinated and grown on agar culture medium containing various concentrations of tamoxifen, 4-hydroxytamoxifen, or ICI 182780 for a week followed by determination of GUS activity (**Figure 4A**). We observed no estrogenic activity at any concentration of 4-hydroxytamoxifen or ICI 182780. However, we did observe the activity at concentrations of tamoxifen of higher than 100 nM. The existence of the weak estrogenic activity associated with tamoxifen at higher concentration has also been reported by Fent et al. (*24*) using a yeast reporter-gene system. To determine antagonistic activity associated with 4-hydroxytamoxifen and ICI 182780, seeds were germinated and grown on agar culture medium containing various concentrations of 17$\beta$-estradiol for a week in the presence of 1 $\mu$M 4-hydroxytamoxifen and 1 $\mu$M ICI 182780 or in the absence of these chemicals as control (**Figure 4B**). The GUS activity retained a maximum level at concentrations of 17$\beta$-estradiol higher than 10 nM in the presence of each antagonist. However, the levels were less than one-third of that observed in the control experiment at any concentration of 17$\beta$-estradiol higher than 10 nM.

**Determination of Estrogenic Activities in Treated Sewages Using the EPTH System.** We determined estrogenic activities in four treated sewages (samples 1−4) from four distinct sewage farms in Japan using the EPTH system with (AP in **Table 1**)

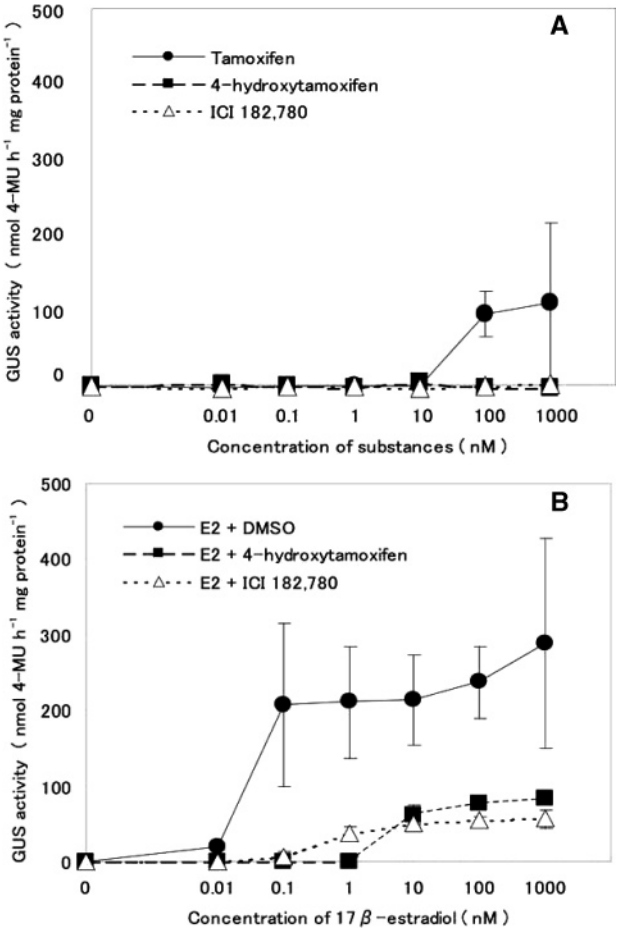


**Figure 4.** Responsiveness of GUS gene expression to antagonistic substances of 17$\beta$-estradiol in the EPTH system. (**A**) As described under Materials and Methods, 20 seedlings were grown for a week on agar culture medium containing various concentrations of antiestrogenic chemical (●, tamoxifen; ■, 4-hydroxytamoxifen; △, ICI 182780) for measurement of GUS activity. (**B**) Seeds were germinated and grown on agar culture medium containing various concentrations of 17$\beta$-estradiol in the presence of (●) 0.01% DMSO, (■), 1 $\mu$M 4-hydroxytamoxifen, or (△) or 1 $\mu$M ICI 182780 for a week for determination of GUS activity. Each value of GUS activity was measured as described under Materials and Methods. The GUS expression was reproduced in three independent experiments.

or without (D in **Table 1**) extraction and purification steps of hydrophobic compounds using hydrophobic affinity column chromatography from treated sewage. We found no significant discrepancy of values obtained using these two distinct methods for preparing samples in the same source of the sample.

**Comparison of the EPTH System with the YTH System.** We also determined estrogenic activities in affinity-purified samples from these same treated sewages using the YTH system (*22*), as shown in a row indicated as "YTH" at the top of the row, and compared these activities with the values obtained by employing the EPTH system (AP in **Table 1**). Estrogenic activities determined by the EPTH system were 6−24-fold higher than those determined by the YTH system (**Table 1**). This observation strongly suggests that the EPTH system can detect estrogenic activity more efficiently than the YTH system.

**Rapid Screening of Medicinal Herbs Rich in Estrogenic Substances Using the EPTH System.** In general, plant crude extract consists of a major amount of primary metabolites, such as oils and saccharides, together with a minor amount of secondary metabolites. Estrogenic substances are often found

**Table 1.** In Estrogenic Activity of Treated Sewage, the Difference between the Value Determined Using the EPTH System and That Determined Using the YTH System[a]

| | EPTH system | | YTH system |
|---|---|---|---|
| sample | D (pM) | AP (pM) | AP (pM) |
| 1 | 150.9 ± 17.3 | 133.3 ± 79.1 | 20.6 |
| 2 | 119.7 ± 29.0 | 163.4 ± 36.5 | 11.0 |
| 3 | 278.6 ± 26.3 | 516.5 ± 60.7 | 25.3 |
| 4 | 274.0 ± 5.4 | 580.8 ± 36.8 | 24.2 |

[a] Four treated sewage samples were from four distinct sewage farms in Japan. Estrogenic activities were determined by direct exposure (D) of the transgenic plant to treated sewage and by exposure of the plant to solutions containing affinity-purified (AP) hydrophobic compounds from treated sewage. Each value of GUS activity was measured as described under Materials and Methods. GUS expression induced by each sample was reproducible in three independent experiments. Estrogenic activities estimated by the YTH system were determined as described under Materials and Methods.

as such secondary metabolites in plant crude extracts. Estrogenic activities in extracts from 41 species of medicinal herbs were determined roughly by visual evaluation, as described in the footnote for **Table 2** to test if the EPTH system is useful for a rapid first screening of medicinal herbs that are rich in estrogenic substances. It has been impossible to detect any estrogenic activity directly in those extracts by employing a transgenic *Arabidopsis* with an estrogen-based transactivator, XVE, system (*25*) or by the YTH system, because of their low sensitivity to estrogenic substances (data not shown). However, we might detect the activity in extracts from 9 of 41 species by employing the EPTH system (**Table 2**). Furthermore, we found that two species were extremely rich in estrogenic substances.

## DISCUSSION

The minimum detection limit of 17$\beta$-estradiol by the EPTH system was 10 pM (3 pg/mL) in an inductive medium for both visual evaluation by histochemical GUS staining of the transgenic *Arabidopsis* and direct measurement of GUS activity of the crude extract from the transgenic plant (**Figure 2**). The sensitivity of the EPTH system to 17$\beta$-estradiol was 1000 times that of the XVE system (*25*) and 5 times those of the PTH system (*1*) and the YTH system (*12*, *13*). In fact, it was comparable to that of the system employing human cells (*15*). The GUS activity reached an almost saturated level at 1 nM 17$\beta$-estradiol; thereafter, it increased between concentrations of 17$\beta$-estradiol higher than 1 nM depending on the increase of concentration of 17$\beta$-estradiol. However, some important aspects remain unknown: Why is the expression of the reporter gene in PTH system decreased at higher ranges of concentrations of 17$\beta$-estradiol? Why does that decrease not occur in the EPTH system? In any event, the increase of copy number of transactivation domains in a chimeric co-activator led to the increase of both sensitivity and total GUS activity induced by 17$\beta$-estradiol in the transgenic plant.

Regarding the results obtained by histochemical GUS staining of transgenic plants, higher reporter activity was observed in the roots and boundary region between hypocotyls and roots than in cotyledons (**Figure 2A**). Only weak GUS staining was observed in whole cotyledons (**Figure 2A**). These results were consistent with those obtained using a PTH system (*1*). However, weak GUS staining was observed in hypocotyls and cotyledons of the transgenic plant by extending the time period of GUS staining (data not shown). Therefore, the appearance of a weak GUS staining in these tissues might be caused by a low transport rate of 17$\beta$-estradiol to these tissues during seedling growth.

**Table 2.** Rapid Screening of Medicinal Herbs Rich in Estrogenic Substances Using the EPTH System

| medicinal herb | activity | solvent |
|---|---|---|
| *Bauhinia candicans* | – | A |
| *Bauhinia forficate* | – | A |
| *Brachychiton acerifolius* | – | A |
| *Caesalpina ferrea* | – | A |
| *Calotropis gigantea* | – | A |
| *Cadaba farinosa* | – | A |
| *Cissus sicyoides* | – | A |
| *Helicteres isora* | – | A |
| *Myrcia multiflora* | – | A |
| *Rubia cordifolia* | – | A |
| *Achilea millefolium* | – | B |
| *Sonchus asper* | – | D |
| *Maytenus emarginata* | – | H |
| *Ambrosia arborescens* | – | M |
| *Baccharis genistelloides* | – | M |
| *Bidens pilosa* | – | M |
| *Caesalpinia spinosa* | – | M |
| *Leonurus sibiricus* | – | M |
| *Maytenus ilicifolia* | – | M |
| *Mutisia acuminata* | – | M |
| *Notholaena nivea* | – | M |
| *Sauropus androgynus* | – | M |
| *Schinus molle* | – | M |
| *Schkuhria pinnata* | – | M |
| *Senecio culcitioides* | – | M |
| *Senecio nivalis* | – | M |
| *Stachys arvensis* | – | M |
| *Urtica urens* | – | M |
| *Vinca minor* | – | M |
| *Xanthium canadense* | – | M |
| *Anemopaegma arvense* | – | W |
| *Peumus boldus* | – | W |
| *Calophyllum inophyllum* | + | A |
| *Echinosophora koreensis* | + | A |
| *Garcinia mangostana* | + | A |
| *Phellodendron amurense* | + | A |
| *Poeciloneuron pauciflorum* | + | A |
| *Pongamia glabra* | + | A |
| *Solanum americanum* | + | A |
| *Sophora alopecuroides* | ++ | A |
| *Gentianella nitida* | ++ | M |

[a] Estrogenic activities in each fraction from medicinal herbs were determined roughly by visual evaluation by comparing the extent of GUS staining of transgenic *Arabidopsis* exposed to each fraction with that of transgenic *Arabidopsis* exposed to given concentrations of 17$\beta$-estradiol as standards. Estrogenic activities of each sample were classified into three classes: –, corresponding to exposure to DMSO as background level; +, corresponding to exposure to 1 nM 17$\beta$-estradiol; ++, corresponding to exposure to 10 nM 17$\beta$-estradiol. Determined estrogenic activities in each fraction were reproduced in three independent experiments.

This transgenic plant also recognized other estrogenic substances (**Figure 3**). Among those chemicals, genistein was recognized more efficiently by the transgenic plant than by yeast (*12*). Genistein is a phytoestrogen: it might be absorbed more efficiently into plant cells than into yeast cells. The profile of the GUS expression pattern obtained using the EPTH system when the transgenic plants were grown on agar culture medium containing estrogenic substances closely resembled that obtained using the PTH system. The sensitivities of the EPTH system against each estrogenic substance were about 5 times those of the PTH system. These results suggest that the EPTH system can detect estrogenic chemicals more efficiently than other bioassay systems. The GUS activity induced by the addition of 17$\beta$-estradiol to the EPTH system was lower with the addition of 4-hydroxytamoxifen or ICI 182780 to a marked extent (**Figure 4B**). These observations suggest that the EPTH system

is also very useful for determining antiestrogenic activities associated with various chemicals.

The levels of estrogenic activities in water from sewage-treatment facilities is reported to be below 400 pM for samples collected in Zurich, Switzerland (*26*). We directly determined estrogenic activities in treated sewages from four distinct sewage farms in Japan using the EPTH system without any manipulation for extraction and condensation of hydrophobic compounds from treated sewages and aseptic treatment. Results indicated that the estrogenic activities in these samples corresponded to 120−280 pM 17$\beta$-estradiol (**Table 1**). These values were comparable to the reported results and were not much increased or decreased by additional manipulation of extraction and condensation of samples. These observations suggest that the EPTH system can detect critical levels of estrogenic activities in original samples without any manipulation for extraction and condensation of samples and aseptic treatment. Consequently, the EPTH system can be a useful tool as an indicator for water pollution control. Such systems will also facilitate identification of highly contaminated areas in the environment as comprehensive analyses of estrogenic activity. Unexpectedly, estrogenic activities of these treated sewages determined by the YTH system were about $^1/_6$ to $^1/_{24}$ of that determined by the EPTH system (**Table 1**). These observations suggest that the EPTH system can detect estrogenic activity more efficiently than the YTH system.

Reduction of costs for first screening of medical drugs is an important issue to reduce costs of pharmaceutical products. Estrogenic and antiestrogenic chemicals are important candidate molecules to be medical drugs. Chang et al. (*27*) reported that the XVE systems are useful to detect estrogen agonists and antagonists. However, the system was not widely used for the first screening of estrogenic chemicals because of its low sensitivity to estrogen. The sensitivity of the YTH system was also insufficient for screening of estrogenic substances from plant crude extracts. Here we have described that the EPTH system is useful as a powerful tool in searching for natural estrogen agonists and antagonists. Consequently, this extremely sensitive and simple system was proven to be the most suitable system for the discovery of a new category of estrogenic substances that are undetectable by conventional and previously used plant and yeast systems.

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