Simple monitoring of endocrine-disrupting chemicals using transgenic Arabidopsis plants expressing medaka estrogen receptor

Stoykova, Petya

Ohkawa, Hideo

Inui, Hideyuki

(Citation)
Chemosphere,286(1):131633

(Issue Date)
2021-07-27

(Resource Type)
journal article

(Version)
Accepted Manuscript

Title: Simple monitoring of endocrine-disrupting chemicals using transgenic *Arabidopsis* plants expressing medaka estrogen receptor

Authors: Petya Stoykova[a,b], Hideo Ohkawa[a,c], Hideyuki Inui[a,c*]

[a] Biosignal Research Center, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe, Hyogo 657-8501, Japan

[b] AgroBioInstitute, 8 "Dragan Tsankov" Blvd, 1164 Sofia, Bulgaria

[c] Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe, Hyogo 657-8501, Japan

[*] Corresponding author: Hideyuki Inui

Address: Biosignal Research Center, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe, Hyogo 657-8501, Japan

Tel & Fax: +81-78-803-5863

E-mail: hinui@kobe-u.ac.jp

[1] Abbreviations

[1] AhR, aryl hydrocarbon receptor; DMSO, dimethyl sulfoxide; EDC, endocrine-disrupting chemical; mER, medaka estrogen receptor; hER, human estrogen receptor; GFP, green fluorescent protein; NP, nonylphenol; OP, 4-*t*-octylphenol; PFAS, per- and polyfluoroalkyl substances; PFOS, perfluorooctanesulfonic acid

Highlights

- A simple plant-based monitoring of endocrine-disrupting chemicals was developed.
- Transgenic *Arabidopsis* plants were produced using medaka estrogen receptor.
- Transgenic plants detected 0.1 ng/mL 4-*t*-octylphenol and 1 pg/mL 17β-estradiol.
- Imidacloprid, fipronil, and perfluorooctanesulfonic acid were detected by the plants.
- This system does not require extraction and concentration steps for detection.

Abstract

Endocrine-disrupting chemicals (EDCs) are widespread contaminants that severely affect the endocrine systems of living organisms. In addition to the conventional instrument-based approaches for quantifying organic pollutants, a monitoring method using transgenic plants has also been proposed. Plants carrying a recombinant receptor gene combined with a reporter gene represent a system for the easy detection of ligands that specifically bind to the receptor molecule. Here, the EDC detection sensitivity of transgenic *Arabidopsis* plants expressing the medaka (*Oryzias latipes*) *estrogen receptor* (*mER*) and *green fluorescent protein* (*GFP*) genes, was assessed. Four transgenic *Arabidopsis* lines, obtained by transformation with expression plasmids constructed using combinations of two types of the ligand-binding domains of mER, the DNA-binding domain of LexA and the transactivation domain of VP16 in the chimeric receptors, showed significant induction of *GFP* when germinated on a medium contaminated with 1 ng/mL 4-*t*-octylphenol (OP). The most sensitive XmEV19-2 plants detected 0.1 ng/mL OP and 1 pg/mL 17β-estradiol. *GFP* expression was suppressed by the insecticides imidacloprid and fipronil, whereas perfluorooctanesulfonic acid induced it at 0.1 ng/mL. Experiments with river water-based medium showed that XmEV19-2 can be used for monitoring polluted waters, detecting OP at concentrations as low as 5 ng/mL. Notably, XmEV19-2 showed a significant decrease in root length when grown on 0.1 ng/mL OP. mER transgenic plants can be a promising tool for simple monitoring of EDCs, without the need for extraction and concentration steps in sample preparation.

Keywords

Biomonitoring, endocrine disruptors, environmental contamination, estrogen receptor, 4-*t*-Octylphenol, transgenic plants

1. Introduction

Environmental pollution is a major concern worldwide; hence, its prevention, remediation, and monitoring are important. Organic pollutants are widespread contaminants in soil, water, and air. They cause significant health impacts on humans and wildlife. Endocrine-disrupting chemicals (EDCs) are hormone-mimicking compounds that resemble the structure of natural hormones such as estrogen, testosterone, thyroid hormone, and others. EDCs alter the functions of the endocrine system and affect the health of living organisms and their progeny. EDCs include phenolic compounds, such as bisphenol A, 4-*t*-octylphenol (OP), nonylphenol (NP), and their ethoxylates (Laws et al., 2000); pesticides, such as 2,4-dichlorophenoxyacetic acid, aldrin, acetochlor, chlordane, endosulfan, fipronil, and imidacloprid (Mnif et al., 2011); polychlorinated biphenyls and polychlorinated dibenzo-*p*-dioxins (Wang et al., 2004); per- and polyfluoroalkyl substances (PFASs) (Blake and Fenton, 2020). Various personal care products and pharmaceuticals also harbor endocrine-disrupting activities (Witorsch and Thomas, 2010). EDCs are released into the environment as a result of agricultural and industrial activities, incomplete combustion of fossil fuels, and daily activities, leading to contamination of soil, groundwater, and river water, including drinking water.

EDCs can seriously impair the health of humans and wildlife. Consequences in humans include reproductive problems and infertility, transgenerational effects, insulin and other metabolite disorders, neurodevelopmental toxicity, and different types of cancers (see reviews by Mnif et al., 2011; Li et al., 2017). EDCs can also influence plant homeostasis and induce stress responses that include changes in gene expression (Chen and Yen, 2013), metabolic effects caused by disorders in the synthesis of endogenous plant hormones, and decrease in biomass production (Chen and Yen, 2013; de Bruin et al., 2019; Kim et al., 2019).

OP ethoxylates and their degraded products are organic contaminants that, owing to their varied applications, can pollute diverse aquatic environments. OP is used industrially for the production of phenolic resins, and its ethoxylates are used for the manufacture of textiles, paints, pesticides, and other products. OP commonly enters the environment through wastewater. Insecticides, such as fipronil and imidacloprid, may have harmful endocrine-disrupting effects on different classes of animals and humans (Baines et al., 2017; Kim et al., 2019). PFASs are industrially produced chemicals that are still

not completely banned. Perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid have been studied most intensively because of their pronounced environmental persistence and harmful effects on humans and wildlife (Li et al., 2017). One of the exposure routes for PFAS is the consumption of contaminated crop plants that accumulate these compounds in their edible parts (Ghisi et al., 2019). Therefore, rigorous monitoring of environmental contamination and development of various methods for the detection of pollutants are important.

In addition to well-developed instrumental methods for the measurement of soil and water pollution with organic pollutants, other procedures utilize living organisms, such as microorganisms (García-Reyero et al., 2001), mammalian cells (Wagner and Oehlmann, 2011), and whole multicellular organisms (Kodama et al., 2009; Lee et al., 2012). These alternatives offer a holistic way of assessing the influence of a mixture of toxic compounds to reveal their combined toxic effect (Eichbaum et al., 2014). In this regard, bioassays could provide additional information on pollutants. However, in the case of *in vivo* assessments, whole plants or animals, if sufficiently sensitive, are extremely appropriate for direct monitoring of soil and water pollution.

To our knowledge, only a few reports on the successful development of organic pollutant monitoring plants have been published (Tojo et al., 2006; Inui et al., 2009; Kodama et al., 2009). Plants as monitors of organic pollution have been developed using a combination of a recombinant receptor gene and an inducible reporter gene, for easy detection (Kodama et al., 2007; Inui et al., 2009). Such expression systems allow fast and accurate quantification of low concentrations of pollutants *in vitro*. Our group is intensively and gradually working on development and improvement of monitoring plant lines expressing receptor genes for different types of ligand molecules: transgenic *Arabidopsis* lines carrying the recombinant human *estrogen receptor* (*hER*) and *green fluorescent protein* (*GFP*) reporter genes could reportedly detect as little as 1 pg/mL 17β-estradiol and 100 ng/mL bisphenol A, NP, or OP (Inui et al., 2009). Transgenic tobacco plants harboring the recombinant mouse *aryl hydrocarbon receptor* (*AhR*) gene, combined with the *β-glucuronidase* (*GUS*) reporter gene, were able to detect 5 nM 3-methylcholanthrene (Kodama et al., 2009). The sensitivity of these plants was further improved by introduction of each three major latex-like proteins cloned from zucchini plants (Stoykova and Inui, 2021). Transgenic *Arabidopsis* plants with a guinea pig AhR could detect 1 ng/mL

dieldrin and 100 ng/g 3,3',4,4',5-pentachlorobiphenyl in soil (Gion et al., 2012). The concept of transgenic plant-based monitoring systems is improving its potential and allows the evaluation of soil and/or water pollution in a rapid, simplified, and cost-efficient way by eliminating the need for time- and labor-consuming procedures such as extraction, purification, and concentration of pollutants from environmental samples with complex matrices for instrumental analyses.

Here, we report the selection of sensitive transgenic *Arabidopsis* plants transformed with both medaka *ER* (*mER*) and inducible expression system of the *GFP* gene, and assessment of the monitoring sensitivity of transgenic *Arabidopsis* plants for different EDCs. mER has different sensitivity toward EDCs compared with mammalian receptors and relatively high sensitivity among fish ERs (Miyagawa et al. 2014).

2. Materials and Methods

2.1. Construction of expression plasmids containing the mER gene

2.1.1. Cloning of ligand binding domain gene in medaka ERα

Mature female medaka were kept in tap water containing 1 μg/mL 17β-estradiol and 0.1% dimethyl sulfoxide (DMSO) as a solvent for 17β-estradiol, for 1 d to induce transcription of ERs. Treated medaka were frozen in liquid nitrogen and total RNA was extracted using Sepasol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan). After DNase I treatment, total RNA was used to synthesize cDNA using a ThermoScript RT-PCR system (Thermo Fisher Scientific Inc., Waltham, MA, USA). The primers XVmE-5′s, XVmE-3′s, XmEV-5′NLSx, and XmEV-3′x for cloning two lengths of the mER ligand-binding domain were designated based on the cDNA sequence of the *mER* gene (Accession number, D28954), with restriction sites at the 5′ and 3′ ends (Supplementary Table 1). XmEV-5′NLSx contains a nuclear localization signal (NLS) sequence from the SV40 T-antigen (Ylikomi et al., 1992). The regions of mER for XVmE and XmEV consisted of amino acids 282-620 and 301-582, respectively. The annealing temperatures for amplification were 67°C and 70°C, respectively. The two lengths of *mER* genes were cloned into the pT7Blue T-Vector (Merck KGaA, Darmstadt, Germany).

Sequences of the *mER* genes in the resultant plasmids pxmEx and psmEs were confirmed by DNA sequencing.

2.1.2. Construction of recombinant transcription factors XmEV and XVmE

The pxVa plasmid, containing a transactivating domain of *Herpes simplex* VP16 (V), and pxmEx were digested with *Xho* I. The mE fragment was ligated to generate pxmEVa containing the mEV fragment. The plasmid was digested with *Xho* I and *Asc* I, and the fragment was ligated into *Sal* I- and *Asc* I-digested ppXsa containing a DNA-binding domain of *Escherichia coli* LexA (X). The resulting plasmid, ppXmEVa, contained the gene encoding the recombinant transcription factor XmEV. In contrast, plasmid ppXVsa containing the gene for XV was digested with *Sal* I, and the fragment of *mER* from *Sal* I-digested psmEs was inserted into ppXVsa to generate ppXVmEa containing the gene for XVmE. The fragments of *Pac* I- and *Asc* I-digested ppXmEVa and ppXVmEa were ligated into the plant expression vectors pER8PAS and pX6PAS containing *Pac* I and *Asc* I sites instead of the gene encoding the recombinant transcription factor containing a human ER derived from pER8-GFP (Accession number, AF309825.2) and pX6-GFP (Accession number, AF330636.1), respectively (Zuo et al., 2000; Zuo et al., 2001). Finally, pER8-XmEV, pER8-XVmE, pX6-XmEV, and pX6-XVmE were constructed (Supplementary Fig. 1). The genes for XmEV and XVmE were expressed under the control of a constitutive promoter, while *GFP* was regulated by an inducible promoter containing the LexA DNA-binding domain.

2.2. Production of transgenic Arabidopsis plants expressing the recombinant transcription factor gene containing mER genes

Each expression plasmid was transferred into *Rhizobium radiobacter* using electroporation. *A. thaliana* ecotype Columbia was infected with *Rhizobium* using vacuum infiltration (Inplanta Innovations Inc., Kanagawa, Japan) (Bechtold and Pelletier, 1998). Seeds from transformed *Arabidopsis* plants were selected on MS agar medium containing 40 μg/mL hygromycin for pER8-based plasmids and 50 μg/mL kanamycin for pX6-based plasmids. The fitness of the segregation ratio of antibiotic-resistant versus -sensitive T_2 plants to theoretical segregation ratio 3:1 was determined using a Chi-square test.

Seeds were harvested from the lines suspected to be homozygous. T_3 plants were grown on MS medium with appropriate antibiotics to confirm the homozygosity of the lines.

2.3. Inducible expression of GFP gene in transgenic Arabidopsis plants

2.3.1. Treatments

The sterilized seeds of the homozygous T_4 progeny were germinated on a solid MSB5 medium prepared from MS macro- and microelements (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Gamborg B5 vitamin mixture from individual ingredients, containing different concentrations of OP, 17β-estradiol, imidacloprid, or fipronil dissolved in DMSO, for 2 weeks. As a control, the same *Arabidopsis* plants were germinated on MSB5 medium containing only DMSO at the same concentration as in the treatment media (0.1, 0.02, or 0.001% depending on the experiments). PFOS was dissolved in water, and MSB5 medium was used as the control. For the experiments with river water, transgenic *Arabidopsis* seeds were sterilized and germinated on solid MSB5 medium. After 1 week of growth, the seedlings were transferred to a 5 cm petri dish containing liquid syringe-sterilized half-strength MS medium prepared with river water and spiked with OP. After an additional 1 week of incubation, the above-ground parts of the plants were collected for analysis.

2.3.2. Quantification of gene expression in transgenic Arabidopsis plants

Total RNA was isolated from the green part (six plants per sample) of 2-week-old *Arabidopsis* plants using the Plant Total RNA Extraction Miniprep System (Viogene-Biotek Corp., New Taipei City, Taiwan). One microgram RNA was used to synthesize cDNA using the ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. Quantitative reverse transcription-PCR (RT-PCR) (Light Cycler 480 II, Roche Applied Science, Indianapolis, IN, USA) of *mER* and *GFP* was carried out using a Thunderbird SYBR qPCR Mix (Toyobo) with the primers listed in Supplementary Table 1. PCR was conducted under the following conditions: 95 °C for 1 min, 40 cycles of 95 °C for 15 s and 60 °C for 30 s, and 95 °C for 5 s and 65 °C for 1 min. The β-tubulin gene was amplified as an internal standard under the same conditions. After confirmation of

specific amplification of the genes with melting curves, the relative expression levels were calculated using the $\Delta\Delta C_T$ method.

2.4. Measurement of root length in the transgenic Arabidopsis plants treated with OP

The transgenic *Arabidopsis* line XmEV19-2 and wild-type *Arabidopsis* plants were germinated and incubated on MSB5 medium containing different concentrations of OP, for 2 weeks at 25 °C, photo-period of 16 h fluorescent light/ 8 h dark. The plants were removed from the medium and the root lengths of 10–15 individual plants from each treatment group were measured.

2.5. Statistical analysis

Statistical analysis for determination of significance was performed using the Microsoft Excel *t*-test. Grubb's test was performed to identify outliers from normal distribution.

3. Results and discussion

3.1 Background

Small fish, such as zebrafish and medaka, have proven to be appropriate to detect contamination with EDCs and to identify chemicals with endocrine-disrupting activities (Scholz and Mayer, 2008). Our previous studies, using transgenic plants expressing mammalian receptors for estrogens and dioxins, clearly showed that plants can serve as a monitoring tool for the detection of contaminants in soil and water environments (Kodama et al., 2007; Inui et al., 2009). However, to our knowledge, there are no reports on the functional expression of fish *ER* genes in plants and the development of environmental monitoring systems, particularly using mER, which possesses relatively high sensitivity toward EDCs, such as alkylphenols, among fish ERs (Miyagawa et al., 2014). It was assumed that mER could be successfully utilized for development of monitoring systems *in planta*. Also, it was hypothesized that the relative binding affinity of recombinant mER and hER expressed in *Arabidopsis* toward different EDCs would vary based on the data published by the Ministry of the Environment of Japan (2001) regarding values of the relative binding activity. Human ER (IC_{50}, 2.1×10^{-9} M) has 2 times

higher affinity to 17β-estradiol than mER (IC_{50}, 4.8×10^{-9} M), and OP has 200 times higher binding affinity toward mER (IC_{50}, 3.2×10^{-8} M) compared to hER (IC_{50}, 6.6×10^{-6} M) (MEJ, 2001). Therefore, we attempted to establish a monitoring system using *mER*s as it was expected that plants expressing *mER* would demonstrate increased sensitivity toward OP compared to *hER*-expressing *Arabidopsis* lines (Inui et al., 2009).

3.2. Quantification of mER expression in transgenic Arabidopsis plants

The monitoring sensitivity of the transgenic plants toward EDCs is hypothesized to be directly correlated with the expression of *mER* in plants, accounting for its role in activation of the reporter system. High amounts of mER molecules contribute to high induction of the *GFP* gene by loading the mER-EDC complex on the promoter sequence. The relative expression of *mER* in the homozygous transgenic *Arabidopsis* plants obtained by the introduction of one of the four different constructs, pER8XmEV, pER8XVmE, pX6XmEV, and pX6XVmE (Supplementary Fig. 1), was evaluated. We employed two different types of inducible expression vectors, pER8 and pX6, conferring transient and constitutive expression of the reporter gene after treatment with EDCs, respectively (Zuo et al., 2000; Zuo et al., 2001; Inui et al., 2009). The reporter gene in pER8-based transgenic plants was expressed in the presence of EDCs. In contrast, the reporter gene in pX6-based transgenic plants was constitutively expressed through excision of a part of the *lox* site in the vector sequence by Cre recombinase after treatment with EDCs. In the pER8 and pX6 vector groups of transgenic plants, expression of the recombinant *XmEV* and *XVmE* genes was shown in lines XmEV30-5 and XVmE5-4, and XmEV19-2 and XVmE15-2, respectively (Fig. 1A). Lines XVmE5-4 and XVmE15-2 showed higher expression levels than the others. In contrast, non-transgenic *Arabidopsis* plants did not exhibit *mER* expression.

3.3. Inducible expression of GFP in transgenic Arabidopsis upon treatment with OP

A significant fold induction of *GFP* expression was observed in all of the tested transgenic plants after treatment with 1 ng/mL OP (Fig. 1B). As the lowest concentration of OP detected by the *Arabidopsis* plants expressing recombinant *hER* genes was 100 ng/mL OP, it was found that the use of mER resulted in a 100-times higher sensitivity toward OP (Inui et al., 2009). It was also reported that

pX6-based transgenic plants showed higher sensitivity toward OP than pER8-based transgenic plants. A similar tendency was observed in this study.

Although the plants showed varying expression levels of the recombinant *XmEV* and *XVmE* genes, the *GFP* expression levels were not significantly different among the lines. It was suggested that high amounts of recombinant XmEV and XVmE did not always contribute to high induction of the reporter gene, indicating that the expression levels of both XmEV and XVmE were sufficient to induce the expression of reporter gene. The high expression probably resulted from a high background derived from endogenous ligand binding to the mERs.

The order of the ligand-binding domain of ER, the DNA-binding domain of LexA, and the transactivation domain of VP16 in the chimeric receptor also influenced the level of induction of the reporter gene. In one study, LexA-AhR-VP16 showed higher induction levels of a reporter gene in recombinant yeast than LexA-VP16-AhR (Kodama et al., 2009). The authors suggested that the spatial arrangement of each domain in the recombinant receptor is important for its function as a sensitive receptor. Transgenic *Arabidopsis* plants with recombinant XEV showed higher sensitivity toward 17β-estradiol and OP (Inui et al., 2009). However, this study did not show a clear high induction in XmEV plants as compared to that with XVmE. The XmEV19-2 line was used for further experiments, as it showed the highest fold induction of *GFP* in the presence of OP.

Organic compounds, including those with endocrine-disrupting activities, are absorbed by the root cells and translocated to the aerial part of the plant. In the case of transgenic *Arabidopsis* plants, OP that accumulated in the aboveground parts bound to the ER. The resulting ER-OP complex binds to the inducible promoter to induce the *GFP* reporter system. Therefore, the level of induction of *GFP* would be positively correlated with the concentration of compounds with agonistic effects on the ER in the medium. Physicochemical properties of EDCs, such as hydrophobicity, are responsible for their uptake pattern, since highly hydrophobic compounds mainly accumulate in plant roots and are resistant to transport to the upper parts of plants (Collins et al., 2006).

3.4. Inducible expression of GFP in transgenic Arabidopsis plants treated with different concentrations of 17β-estradiol and OP

The selected *Arabidopsis* XmEV19-2 response to OP was further assessed for induction, on a medium with lower concentrations of 17β-estradiol (0.001-1 ng/mL) or OP (0.1-100 ng/mL). Significant and dose-dependent induction of *GFP* expression was evident for all the applied concentrations of both 17β-estradiol and OP (Fig. 2). The limit of detection of XmEV19-2 for OP and 17β-estradiol was 0.1 ng/mL and 1 pg/mL, respectively. These data indicated the comparable sensitivity of XmEV19-2 plants toward 17β-estradiol, as *Arabidopsis* plants express *hER* against 17β-estradiol (Inui et al., 2009). Surprisingly, the OP detection sensitivity of XmEV19-2 was 1,000 times higher than plants expressing *hER*. Compounds with endocrine-disrupting activities are common contaminants of fresh-water basins, such as lakes, ponds, and rivers, since some EDCs are discharged to water environments by daily human activities. The concentrations of some EDCs in water sources, such as OP (Olaniyan et al., 2020), bisphenol A and NP (Careghini et al., 2015), can reach ng/mL levels, and such as benzo-phenones (Careghini et al., 2015) and 17β-estradiol (Hashimoto et al., 2005), can reach pg/mL levels. Therefore, the development of conceptual methods capable of detecting very low amounts is im-portant to monitor EDCs. XmEV19-2 appeared to be potentially appropriate for the detection of al-kylphenol compounds in highly polluted sites.

3.5. Inducible expression of GFP in transgenic Arabidopsis plants treated with pesticides and PFOS with EDC activity

Environmental pollution with pesticides is problematic because of their widespread use to improve crop yields. Although some pesticides were banned years ago, active compounds and their metabo-lites are very stable and remain detectable in soil, groundwater, and drinking water sources. Substan-tial monitoring of contamination is necessary because these contaminants can be transferred through food chains to humans and wildlife. The harmful EDC potential of the main compounds and the me-tabolites of the insecticides imidacloprid (Baines et al., 2017) and fipronil (Lu et al., 2015) has been demonstrated in various animal species. Imidacloprid is a widely applied neonicotinoid insecticide that is highly and selectively toxic to insects. Imidacloprid can affect non-target organisms (Malev et

al., 2012) and can stably accumulate in the human body (Zhang et al., 2019). Fipronil is a phenylpyrazole insecticide. Its increased use has led to its detection in urban runoff as a contaminant that can have harmful effects on non-target aquatic or insect organisms (Gunasekara et al., 2007).

Pesticides can be absorbed from the roots and accumulate in the aboveground tissues of plants. Sensitivity of XmEV19-2 plants to imidacloprid and fipronil was evident by the significant decrease and dose-dependency of *GFP* expression (Fig. 3A). Imidacloprid has the potential to induce reporter gene expression by binding to human ER (Zhang et al., 2020). In contrast, fipronil showed an antagonistic effect on hamster ER, evident by the decrease in reporter activity during 17β-estradiol treatment (Lu et al., 2015). The binding activities of mER toward these pesticides were different from those reported for ERs. Imidacloprid is metabolized in plants (Li et al., 2019), which might result in changes in the affinity of the newly derived molecule to mER in the transgenic *Arabidopsis* plants. Several factors related to the binding of ER to these pesticides and their metabolites lead to the suppression of *GFP* expression.

Numerous studies have reported on the EDC potential of PFASs (Li et al., 2017; Li et al., 2017) and their ability to bind to the ER (Qiu et al., 2020). Lower concentrations of PFOS stimulated *GFP* expression; however, a decrease in induction was detected at increasing concentrations (Fig. 3B). These findings suggest that the uptake amount of PFOS can be decreased and/or plants can be stressed due to the amphipathic property of PFOS as a detergent.

3.6. Monitoring of OP in liquid medium using transgenic Arabidopsis plants

To assess the monitoring capacity of XmEV19-2 toward OP under practical conditions, half-strength MSB5 liquid medium was prepared with river water and spiked with OP in concentrations ranging from 5–100 ng/mL. A significant dose-dependent increase in the fold induction of *GFP* expression was observed, revealing the potential of XmEV19-2 to detect 5 ng/mL OP in contaminated water samples (Fig. 4). OP pollution of some freshwater sites was reported to reach 0.18 ng/mL in the Tokyo area (Isobe et al., 2001). In other locations, OP concentrations of 0.5 ng/mL (Cheng et al., 2018) or >15 ng/mL (Olaniyan et al., 2020) have been reported. These findings indicate the applicability of XmEV19-2 plants for monitoring of highly polluted sites.

In vivo biomonitoring of environmental contamination with EDCs has been performed mainly using animal species by assaying reproduction-related parameters. In one study, transgenic medaka embryos with a *GFP* reporter system were used to detect EDC-polluted river water. The detection sensitivity for low concentrations of contaminants was evident (Lee et al., 2012). We proposed the possible utilization of transgenic plants as a monitoring system for some pollutants with endocrine-disrupting activities. Utilizing plants as a monitoring tool can reduce the cost of monitoring.

3.7. Changes in root length of transgenic Arabidopsis plants due to OP treatment

Average root length of the XmEV19-2 plants incubated with increasing concentrations of OP significantly decreased at 1 ng/mL and higher OP concentrations (Fig. 5A). However, no such change was observed in non-transgenic *Arabidopsis* plants (Fig. 5B). The molecular mechanisms of root growth inhibition might result from the suppressive effects of the mER-OP complex on genes involved in the regulation of root physiology and development. The non-transgenic plants did not show inhibited root growth, suggesting that OP itself does not regulate genes related to root growth. Although not as popular as toxicological studies on animals, plant-based investigations on evaluation of the phytotoxic effects of some EDCs have been performed. Shoot and root development are initiated during the seed germination phase and are characterized by higher root sensitivity toward contaminants than shoots. Germination of rice plants on fipronil-containing medium was reported to lead to the enhancement of radicle growth by 31% (Moore and Kröger, 2010). Another study described a significant decrease in the root dry weight and length in *Arabidopsis* when treated with OP at higher concentrations (1–50 μg/mL) for 10 days (Chen et al., 2013). Our experiment with non-transgenic plants showed that 100 ng/mL OP was not sufficient to influence root growth (Fig. 5B). Notably, based on our observations during the treatment experiments, we suggest that the transgenic *Arabidopsis* plants expressing *mER* responded to treatment with OP owing to the expression of *GFP*. Furthermore, a difference in root length between transgenic and non-transgenic *Arabidopsis* plants was also detected. Phenotypic responses of XmEV19-2 plants toward OP appear to be a feasible marker for the detection of contamination with OP and possibly various EDCs.

3.8. Effects of EDCs on plant physiology and phenotype

Similar to that of humans and animals, plant physiology is also influenced by EDCs (Chen et al., 2013; de Bruin et al., 2019; Kim et al., 2019). EDCs vary widely in their chemical structures and properties. These compounds affect the functions of tissues and organs of different plant species in a specific manner by disrupting microtubules during mitotic division (Adamakis et al., 2016), thereby altering the ultrastructure of organelles (de Bruin et al., 2019) and resulting in decreased shoot and/or root length (Chen and Yen, 2013; Chen et al., 2013; Adamakis et al., 2016). Some studies have pursued the phytotoxic effects of EDCs in more depth by assessing the mechanisms of their action. Assessment of the influence of NP on plant physiology revealed that low concentrations of NP affect the mitochondrial activity and DNA content in *Polystichum setiferum* in response to acute and subchronic toxic effects, whereas higher concentrations do not affect these parameters, suggesting a hormetic response to low doses of the pollutant (Esteban et al., 2016). Similarly, a strong effect of the lower doses of OP (0.1 µg/mL) on some plant growth parameters (e.g., shoot and root fresh weight, chlorophyll content), compared to higher OP concentrations (≥1 µg/mL), was also observed in *Arabidopsis* (Chen et al., 2013), implying that the overall estimation of the specific effect of the xenobiotic ligand on the treated plant, as well as the susceptibility of plant species toward the EDC itself and the plant response at low and high doses, are of significant importance in order to accurately interpret the changes observed in plants as a result of EDC uptake.

4. Conclusion

Environmental contamination of fresh waters with compounds exhibiting endocrine-disrupting activity is a serious concern worldwide. In this study the monitoring capacity of transgenic *Arabidopsis* plants expressing *mER* was assessed. A positive correlation between the presence of OP at concentrations exceeding 0.1 ng/mL and the expression of *GFP,* was detected in XmEV19-2 plants. The ability of XmEV19-2 to detect OP was 1000 times higher in transgenic *Arabidopsis* plants expressing *hER*. Induction of *GFP* in XmEV19-2 was detected at ≥5 ng/mL OP when incubated in liquid medium prepared with river water. These results show that XmEV19-2 is capable of detecting OP in river water.

Additionally, this plant line can detect other EDCs, including 17β-estradiol, imidacloprid, fipronil, and PFOS. Further studies on whether XmEV19-2 plants can respond to a mixture of EDCs, are needed. Interestingly, OP pollution can be monitored by observing the root length of XmEV19-2 plants, which is a simple way to monitor EDCs. Thus, the procedures for extraction, purification, and concentration of pollutants are unnecessary for detection.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

We deeply thank Mr. Tadashi Shimomura (Graduate School of Agricultural Science at Kobe University) for helpful assistance and fruitful discussion.

Author contributions

PS and HI performed the experiments, collected and analyzed the data. PS, HO, and HI wrote the manuscript. HO and HI conceptualized the overall work.

Funding

This work was funded in part by the Bio-oriented Technology Research Advancement Institution (BRAIN) and the Japan Society for the Promotion of Science (JSPS), KAKENHI 17F17748, and Postdoctoral Fellowships for Research in Japan (Standard, P17748).

406 **References**

407

408 Adamakis, I.D.S., Panteris, E., Eleftheriou, E.P., 2016. Bisphenol A disrupts microtubules and in-
409 duces multipolar spindles in dividing root tip cells of the gymnosperm *Abies cephalonica*. Chem-
410 osphere 149, 202–210. https://doi.org/10.1016/j.chemosphere.2016.01.082.

411 Baines, D., Wilton, E., Pawluk, A., De Gorter, M., Chomistek, N., 2017. Neonicotinoids act like en-
412 docrine disrupting chemicals in newly-emerged bees and winter bees. Sci. Rep. 7, 1–18.
413 https://doi.org/10.1038/s41598-017-10489-6.

414 Bechtold, N., Pelletier, G., 1998. In Planta AgrobacteriumMediated Transformation of Adult *Ara-*
415 *bidopsis thaliana* Plants by Vacuum Infiltration, in: Martinez-Zapater, J.M., Salinas, J. (Eds.),
416 Arabidopsis Protocols. Humana Press, Totowa, NJ, pp. 259–266. https://doi.org/10.1385/0-
417 89603-391-0:259.

418 Blake, B.E., Fenton, S.E., 2020. Early life exposure to per- and polyfluoroalkyl substances (PFAS)
419 and latent health outcomes: A review including the placenta as a target tissue and possible driver
420 of peri- and postnatal effects. Toxicology 443. https://doi.org/10.1016/j.tox.2020.152565.

421 Careghini, A., Mastorgio, A.F., Saponaro, S., Sezenna, E., 2015. Bisphenol A, nonylphenols, benzo-
422 phenones, and benzotriazoles in soils, groundwater, surface water, sediments, and food: a review.
423 Environ. Sci. Pollut. Res. 22, 5711–5741. https://doi.org/10.1007/s11356-014-3974-5.

424 Chen, B.S., Hsiao, Y.L., Yen, J.H., 2013. Effect of octylphenol on physiologic features during growth
425 in *Arabidopsis thaliana*. Chemosphere 93, 2264–2268. https://doi.org/10.1016/j.chemo-
426 sphere.2013.08.002.

427 Chen, B.S., Yen, J.H., 2013. Effect of endocrine disruptor nonylphenol on physiologic features and
428 proteome during growth in *Arabidopsis thaliana*. Chemosphere 91, 468–474.
429 https://doi.org/10.1016/j.chemosphere.2012.11.072.

430 Cheng, J. rui, Wang, K., Yu, J., Yu, Z. xun, Yu, X. biao, Zhang, Z. zhao, 2018. Distribution and fate
431 modeling of 4-nonylphenol, 4-*t*-octylphenol, and bisphenol A in the Yong River of China. Chem-
432 osphere 195, 594–605. https://doi.org/10.1016/j.chemosphere.2017.12.085.

433 Collins, C., Fryer, M., Grosso, A., 2006. Plant uptake of non-ionic organic chemicals. Environ. Sci.
434 Technol. 40, 45–52. https://doi.org/10.1021/es0508166.
435 de Bruin, W., Kritzinger, Q., Bornman, R., Korsten, L., 2019. Occurrence, fate and toxic effects of the
436 industrial endocrine disrupter, nonylphenol, on plants - A review. Ecotoxicol. Environ. Saf. 181,
437 419–427. https://doi.org/10.1016/j.ecoenv.2019.06.009.
438 Eichbaum, K., Brinkmann, M., Buchinger, S., Reifferscheid, G., Hecker, M., Giesy, J.P., Engwall,
439 M., Van Bavel, B., Hollert, H., 2014. In vitro bioassays for detecting dioxin-like activity - Appli-
440 cation potentials and limits of detection, a review. Sci. Total Environ. 487, 37–48.
441 https://doi.org/10.1016/j.scitotenv.2014.03.057.
442 Esteban, S., Llamas, P.M., García-Cortés, H., Catalá, M., 2016. The endocrine disruptor nonylphenol
443 induces sublethal toxicity in vascular plant development at environmental concentrations: A risk
444 for riparian plants and irrigated crops? Environ. Pollut. 216, 480–486.
445 https://doi.org/10.1016/j.envpol.2016.05.086.
446 García-Reyero, N., Grau, E., Castillo, M., De Alda, M.J.L., Barceló, D., Piña, B., 2001. Monitoring of
447 endocrine disruptors in surface waters by the yeast recombinant assay. Environ. Toxicol. Chem.
448 20, 1152–1158. https://doi.org/10.1002/etc.5620200603.
449 Ghisi, R., Vamerali, T., Manzetti, S., 2019. Accumulation of perfluorinated alkyl substances (PFAS)
450 in agricultural plants: A review. Environ. Res. 169, 326–341. https://doi.org/10.1016/j.en-
451 vres.2018.10.023.
452 Gion, K., Inui, H., Sasaki, H., Utani, Y., Kodama, S., Ohkawa, H., 2012. Assays of PCB congeners
453 and organochlorine insecticides with the transgenic *Arabidopsis* and tobacco plants carrying re-
454 combinant guinea pig AhR and GUS reporter genes. J. Environ. Sci. Heal. - Part B Pestic. Food
455 Contam. Agric. Wastes 47, 599–607. https://doi.org/10.1080/03601234.2012.668453.
456 Gunasekara, A.S., Truong, T., Goh, K.S., Spurlock, F., Tjeerdema, R.S., 2007. Environmental fate
457 and toxicology of fipronil. J. Pestic. Sci. 32, 189–199. https://doi.org/10.1584/jpestics.R07-02.
458 Hashimoto, S., Horiuchi, A., Yoshimoto, T., Nakao, M., Omura, H., Kato, Y., Tanaka, H., Kannan,
459 K., Giesy, J.P., 2005. Horizontal and vertical distribution of estrogenic activities in sediments

460 and waters from Tokyo Bay, Japan. Arch. Environ. Contam. Toxicol. 48, 209–216.
461 https://doi.org/10.1007/s00244-003-0205-3.
462 Inui, H., Sasaki, H., Chua, N.-H., Ohkawa, H., 2009. Bioassay of estrogenic compounds in transgenic
463 *Arabidopsis* plants carrying a recombinant human estrogen receptor gene and a GFP reporter
464 gene. Transgenic Res. 18. https://doi.org/10.1007/s11248-009-9277-9.
465 Isobe, T., Nishiyama, H., Nakashima, A., Takada, H., 2001. Distribution and behavior of nonylphe-
466 nol, octylphenol, and nonylphenol monoethoxylate in Tokyo metropolitan area: Their association
467 with aquatic particles and sedimentary distributions. Environ. Sci. Technol. 35, 1041–1049.
468 https://doi.org/10.1021/es001250i.
469 Kim, D., Kwak, J. Il, An, Y.J., 2019. Physiological response of crop plants to the endocrine-disrupting
470 chemical nonylphenol in the soil environment. Environ. Pollut. 251, 573–580.
471 https://doi.org/10.1016/j.envpol.2019.04.101.
472 Kim, Y.A., Yoon, Y.S., Kim, H.S., Jeon, S.J., Cole, E., Lee, J., Kho, Y., Cho, Y.H., 2019. Distribu-
473 tion of fipronil in humans, and adverse health outcomes of in utero fipronil sulfone exposure in
474 newborns. Int. J. Hyg. Environ. Health 222, 524–532.
475 https://doi.org/10.1016/j.ijheh.2019.01.009.
476 Kodama, S., Okada, K., Akimoto, K., Inui, H., Ohkawa, H., 2009. Recombinant aryl hydrocarbon re-
477 ceptors for bioassay of aryl hydrocarbon receptor ligands in transgenic tobacco plants. Plant Bio-
478 technol. J. 7. https://doi.org/10.1111/j.1467-7652.2008.00378.x.
479 Kodama, S., Okada, K., Inui, H., Ohkawa, H., 2007. Aryl hydrocarbon receptor (AhR)-mediated re-
480 porter gene expression systems in transgenic tobacco plants. Planta 227.
481 https://doi.org/10.1007/s00425-007-0592-1.
482 Laws, S.C., Carey, S.A., Ferrell, J.M., Bodman, G.J., Cooper, R.L., 2000. Estrogenic activity of oc-
483 tylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicol. Sci. 54, 154–167.
484 https://doi.org/10.1093/toxsci/54.1.154.
485 Lee, W., Kang, C.W., Su, C.K., Okubo, K., Nagahama, Y., 2012. Screening estrogenic activity of en-
486 vironmental contaminants and water samples using a transgenic medaka embryo bioassay.
487 Chemosphere 88, 945–952. https://doi.org/10.1016/j.chemosphere.2012.03.024.

Li, K., Gao, P., Xiang, P., Zhang, X., Cui, X., Ma, L.Q., 2017. Molecular mechanisms of PFOA-induced toxicity in animals and humans: Implications for health risks. Environ. Int. 99, 43–54. https://doi.org/10.1016/j.envint.2016.11.014.

Li, X., Gao, Y., Wang, J., Ji, G., Lu, Y., Yang, D., Shen, H., Dong, Q., Pan, L., Xiao, H., Zhu, B., 2017. Exposure to environmental endocrine disruptors and human health. J. Public Heal. Emerg. 1, 8–8. https://doi.org/10.21037/jphe.2016.12.09.

Li, Y., Long, L., Ge, J., Li, H., Zhang, M., Wan, Q., Yu, X., 2019. Effect of Imidacloprid Uptake from Contaminated Soils on Vegetable Growth. J. Agric. Food Chem. 67, 7232–7242. https://doi.org/10.1021/acs.jafc.9b00747.

Lu, M., Du, J., Zhou, P., Chen, H., Lu, C., Zhang, Q., 2015. Endocrine disrupting potential of fipronil and its metabolite in reporter gene assays. Chemosphere 120, 246–251. https://doi.org/10.1016/j.chemosphere.2014.07.015.

Malev, O., Klobučar, R.S., Fabbretti, E., Trebše, P., 2012. Comparative toxicity of imidacloprid and its transformation product 6-chloronicotinic acid to non-target aquatic organisms: Microalgae *Desmodesmus subspicatus* and amphipod *Gammarus fossarum*. Pestic. Biochem. Physiol. 104, 178–186. https://doi.org/10.1016/j.pestbp.2012.07.008.

MEJ, 2001. Ministry of the Environment of Japan. Report of test results of endocrine disrupting effects of nonylphenol on fish. (in Japanese). https://www.env.go.jp/chemi/end/speed98/commi_98/kento1301/02.pdf

Miyagawa, S., Lange, A., Hirakawa, I., Tohyama, S., Ogino, Y., Mizutani, T., Kagami, Y., Kusano, T., Ihara, M., Tanaka, H., Tatarazako, N., Ohta, Y., Katsu, Y., Tyler, C.R., Iguchi, T., 2014. Differing species responsiveness of estrogenic contaminants in fish is conferred by the ligand binding domain of the estrogen receptor. Environ. Sci. Technol. 48, 5254–5263. https://doi.org/10.1021/es5002659.

Mnif, W., Hassine, A.I.H., Bouaziz, A., Bartegi, A., Thomas, O., Roig, B., 2011. Effect of endocrine disruptor pesticides: A review. Int. J. Environ. Res. Public Health 8, 2265–2303. https://doi.org/10.3390/ijerph8062265.

515 Moore, M.T., Kröger, R., 2010. Effect of three insecticides and two herbicides on rice (*Oryza sativa*)
516 seedling germination and growth. Arch. Environ. Contam. Toxicol. 59, 574–581.
517 https://doi.org/10.1007/s00244-010-9519-0.
518 Olaniyan, L.W.B., Okoh, O.O., Mkwetshana, N.T., Okoh, A.I., 2020. Environmental Water Pollution,
519 Endocrine Interference and Ecotoxicity of 4-*tert*-Octylphenol: A Review. Rev. Environ. Con-
520 tam. Toxicol. 248, 81–109. https://doi.org/10.1007/398_2018_20.
521 Qiu, Z., Qu, K., Luan, F., Liu, Y., Zhu, Y., Yuan, Y., Li, H., Zhang, H., Hai, Y., Zhao, C., 2020.
522 Binding specificities of estrogen receptor with perfluorinated compounds: A cross species com-
523 parison. Environ. Int. 134, 105284. https://doi.org/10.1016/j.envint.2019.105284.
524 Scholz, S., Mayer, I., 2008. Molecular biomarkers of endocrine disruption in small model fish. Mol.
525 Cell. Endocrinol. 293, 57–70. https://doi.org/10.1016/j.mce.2008.06.008.
526 Stoykova, P., Inui, H., 2021. Transport enhancement of hydrophobic pollutants by the expression of
527 zucchini major latex-like protein genes in tobacco plants. J. Plant Physiol. 263, 153464.
528 https://doi.org/10.1016/j.jplph.2021.153464.
529 Tojo, T., Tsuda, K., Wada, T.S., Yamazaki, K. ichi, 2006. A simple and extremely sensitive system
530 for detecting estrogenic activity using transgenic *Arabidopsis thaliana*. Ecotoxicol. Environ. Saf.
531 64, 106–114. https://doi.org/10.1016/j.ecoenv.2005.03.014.
532 Wagner, M., Oehlmann, J., 2011. Endocrine disruptors in bottled mineral water: Estrogenic activity in
533 the E-Screen. J. Steroid Biochem. Mol. Biol. 127, 128–135.
534 https://doi.org/10.1016/j.jsbmb.2010.10.007.
535 Wang, X., White, J.C., Gent, M.P.N., Iannucci-Berger, W., Eitzer, B.D., Mattina, M.I., 2004. Phyto-
536 extraction of weathered *p,p'*-DDE by zucchini (*Cucurbita pepo*) and cucumber (Cucumis sativus)
537 under different cultivation conditions. Int. J. Phytoremediation 6, 363–385.
538 https://doi.org/10.1080/16226510490888910.
539 Witorsch, R.J., Thomas, J.A., 2010. Personal care products and endocrine disruption: A critical re-
540 view of the literature. Crit. Rev. Toxicol. 40, 1–30.
541 https://doi.org/10.3109/10408444.2010.515563.

Ylikomi, T., Bocquel, M.T., Berry, M., Gronemeyer, Y., Chambon, P., 1992. Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. EMBO J. 11, 3681–3694. https://doi.org/10.1002/j.1460-2075.1992.tb05453.x.

Zhang, C., Schilirò, T., Gea, M., Bianchi, S., Spinello, A., Magistrato, A., Gilardi, G., Di Nardo, G., 2020. Molecular basis for endocrine disruption by pesticides targeting aromatase and estrogen receptor. Int. J. Environ. Res. Public Health 17, 1–18. https://doi.org/10.3390/ijerph17165664.

Zhang, Q., Lu, Z., Chang, C.H., Yu, C., Wang, X., Lu, C., 2019. Dietary risk of neonicotinoid insecticides through fruit and vegetable consumption in school-age children. Environ. Int. 126, 672–681. https://doi.org/10.1016/j.envint.2019.02.051.

Zuo, J., Niu, Q., Møller, S.G., Chua, N., 2001. Chemical-regulated, site-specific DNA excision in transgenic plants. Nat. Biotechnol. 157–161. https://doi.org/10.1038/84428.

Zuo, J., Niu, Q.W., Chua, N.H., 2000. An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J. 24, 265–273. https://doi.org/10.1046/j.1365-313X.2000.00868.x.

557 **Figures**

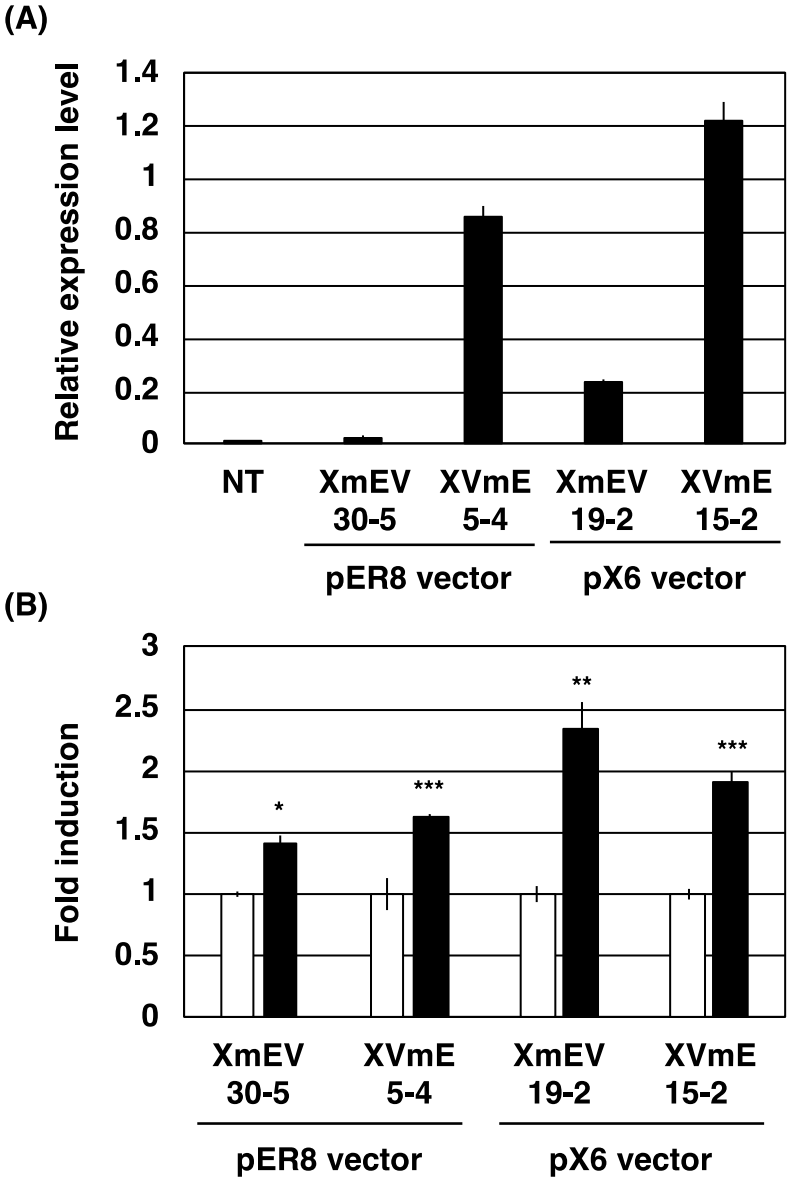


Figure 1

558

559 **Figure 1** Selection of transgenic *Arabidopsis* plants expressing the medaka *estrogen receptor* (*mE*)

560 gene with inducible expression of the *green fluorescent protein* (*GFP*) gene.

561 (A) The seeds of homozygous transgenic *Arabidopsis* plants were sowed in a solid medium and incu-

562 bated for 2 weeks. Total RNA was extracted from the aerial parts of plants and subjected to quantita-

563 tive RT-PCR of the genes encoding mER and β-tubulin as a standard (n = 3). Relative expression lev-
564 els of the *mE* genes in transgenic *Arabidopsis* plants are indicated. NT, non-transgenic *Arabidopsis*
565 plants. (B) Seeds of the homozygous transgenic *Arabidopsis* plants were sowed and incubated in a
566 solid medium containing 0.1% dimethyl sulfoxide (DMSO) as a control or 1 ng/mL 4-*t*-octylphenol
567 (OP) for 2 weeks. Total RNA was extracted from the aerial parts of transgenic plants and subjected to
568 quantitative RT-PCR of the genes coding for GFP; β-tubulin was used as the standard (n = 3). Fold
569 induction was calculated by dividing the relative expression level at 1 ng/mL OP treatment by that at
570 0.1% DMSO treatment. DMSO treatment was set at 1. White and black bars indicate the treatments
571 with DMSO and OP, respectively. Asterisks indicate significant differences compared to the fold in-
572 duction in DMSO treatment (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Student's t-test).
573

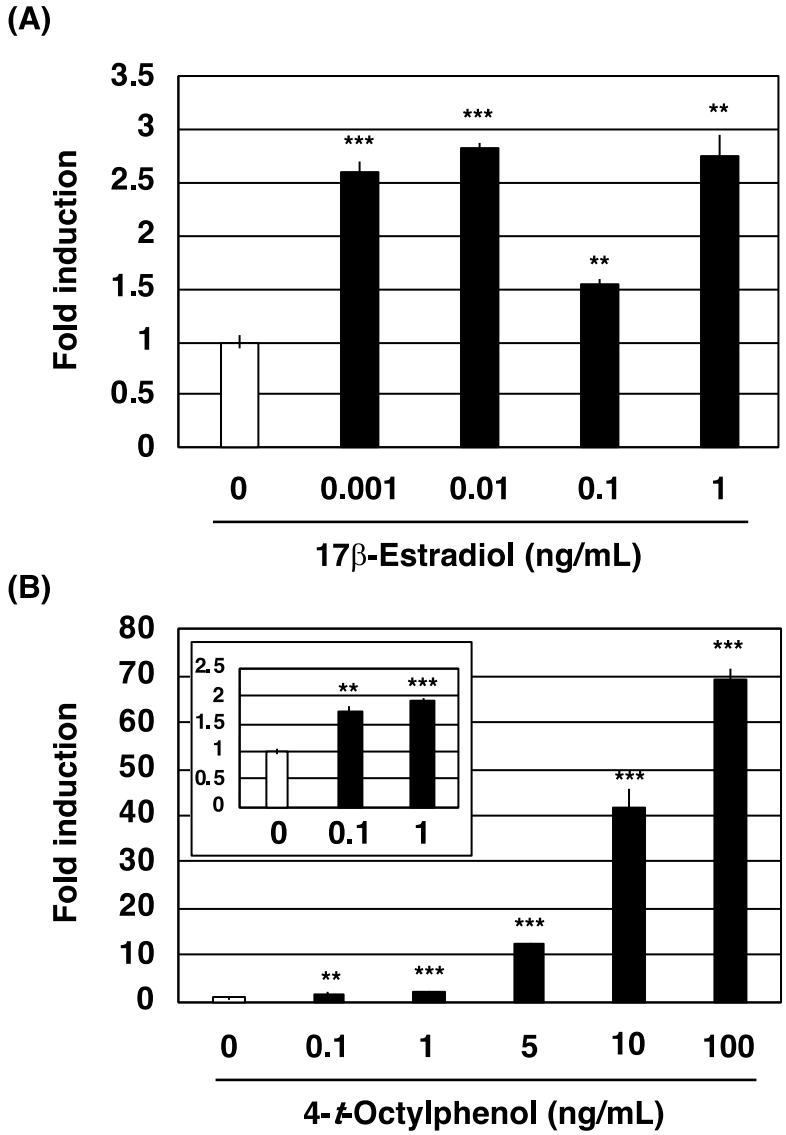


Figure 2

Figure 2 Inducible expression of the *green fluorescent protein* (*GFP*) gene in transgenic *Arabidopsis* plants expressing *medaka estrogen receptor* gene in the presence of 17β-estradiol (A) or 4-*t*-octylphenol (OP) (B) treatment.

579 Seeds of the homozygous transgenic *Arabidopsis* plant XmEV19-2 were sown and incubated in a
580 solid medium containing 0.001% dimethyl sulfoxide (DMSO) as the control or different concentra-
581 tions of 17β-estradiol or OP for 2 weeks. Total RNA was extracted from the aerial parts of transgenic
582 plants and subjected to quantitative RT-PCR *GFP*; β-tubulin was used as the standard ($n = 3$). Fold
583 induction was calculated by dividing the relative expression level upon each treatment by that upon
584 0.001% DMSO treatment. DMSO treatment was set at 1. White and black bars indicate treatments
585 with DMSO, 17β-estradiol, or OP, respectively. Asterisks indicate significant differences compared to
586 fold induction in DMSO treatment (** $p < 0.01$; *** $p < 0.001$; Student's *t*-test).
587

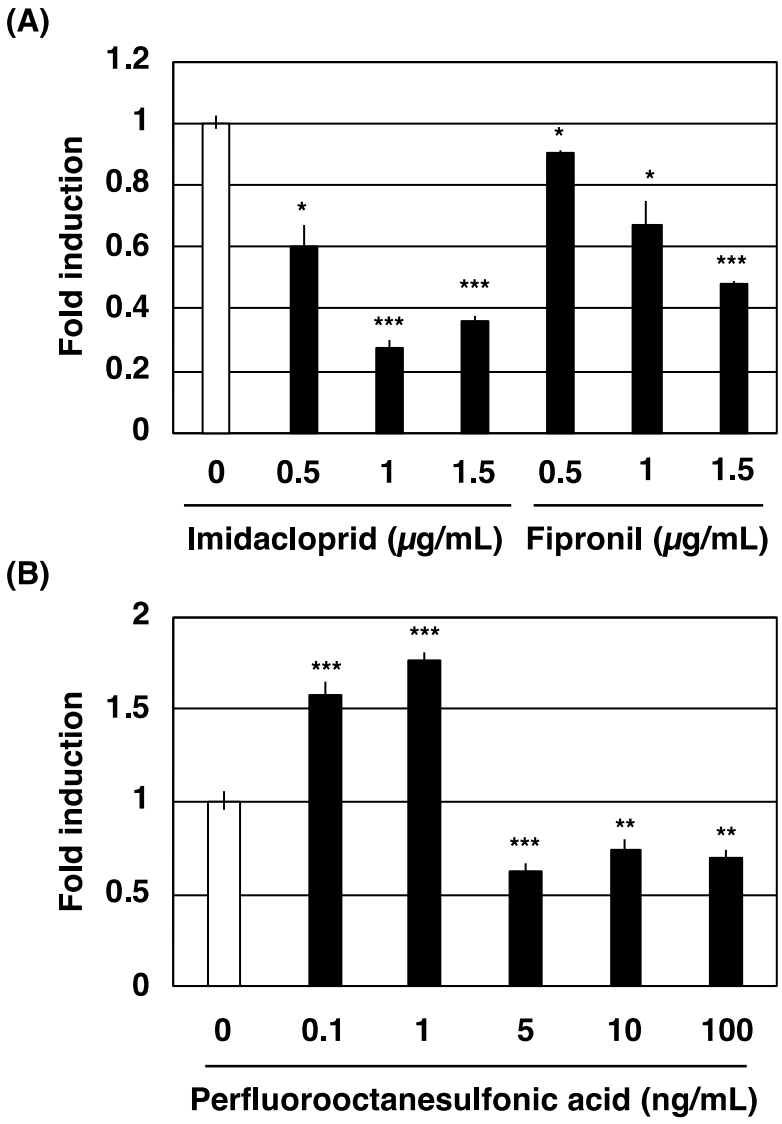


Figure 3

Figure 3 Inducible expression of the *green fluorescent protein* (*GFP*) gene in transgenic *Arabidopsis* plants expressing *medaka estrogen receptor* gene, upon treatment with the insecticides imidacloprid and fipronil (A) or perfluorooctanesulfonic acid (PFOS) (B).

Seeds of the homozygous transgenic *Arabidopsis* plant XmEV19-2 were sown and incubated in a solid medium containing 0.02% dimethyl sulfoxide (DMSO) for imidacloprid and fipronil treatments or water for PFOS treatment as a control, or different concentrations of imidacloprid, fipronil, or

PFOS, for 2 weeks. Total RNA was extracted from the aerial parts of transgenic plants and subjected to quantitative RT-PCR for amplifying *GFP*; β-tubulin was used as the standard ($n = 3$). Fold induction was calculated by dividing the relative expression level at each treatment by that in the 0.02% DMSO or water treatment. DMSO and water treatments were set at 1. White and black bars indicate the control and compound treatments, respectively. Asterisks indicate significant differences compared to the fold induction in DMSO treatment (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Student's *t*-test).

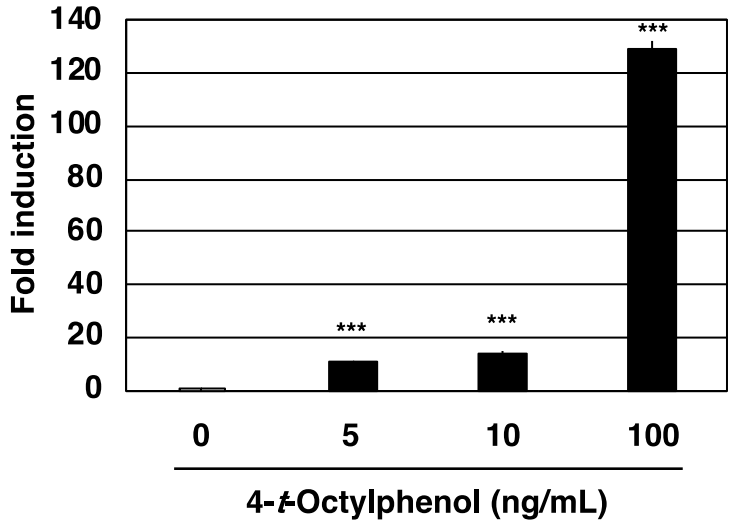


Figure 4

Figure 4 Inducible expression of the *green fluorescent protein* (*GFP*) gene in transgenic *Arabidopsis* plants expressing *medaka estrogen receptor* gene upon treatment with 4-*t*-octylphenol (OP) in a liquid medium.

Seeds of the homozygous transgenic *Arabidopsis* plant XmEV19-2 were sown and incubated in a solid medium for 1 week. The seedlings were then transferred to a liquid medium prepared with river water spiked with 0.001% dimethyl sulfoxide (DMSO) as a control and different concentrations of OP for another 1 week. Total RNA was extracted from the aerial parts of plants and subjected to quantitative RT-PCR of the genes encoding GFP; β-tubulin was used as the standard ($n = 3$). Fold induction was calculated by dividing the relative expression level in the OP treatment by that in the 0.001% DMSO treatment. DMSO treatment was set at 1. White and black bars indicate the control and OP treatments, respectively. Asterisks indicate significant differences compared to the fold induction in DMSO treatment (*** $p < 0.01$; Student's *t*-test).

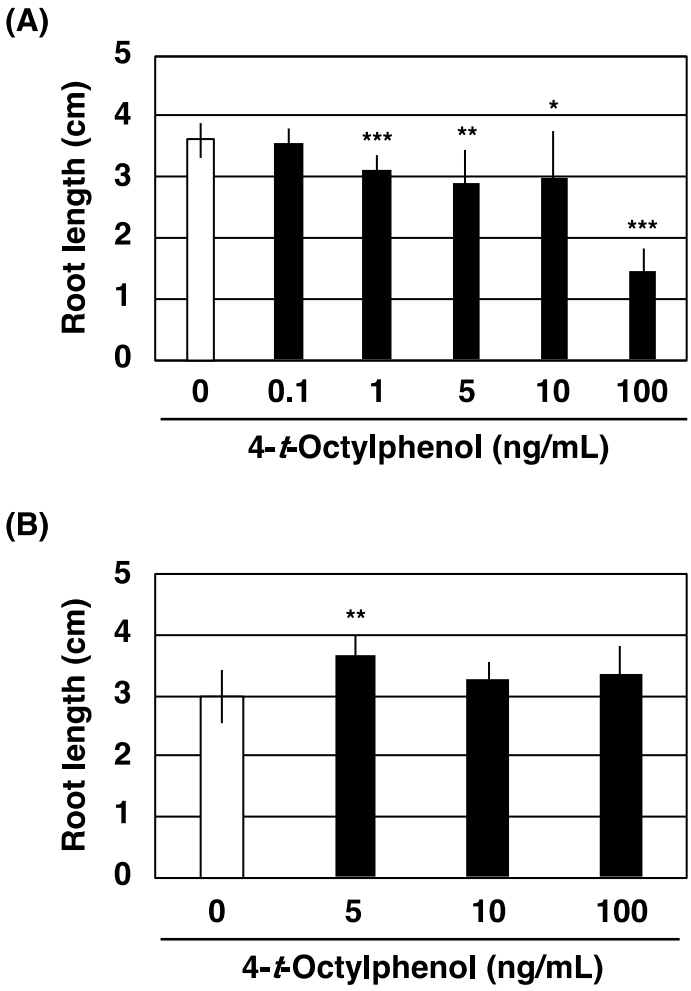
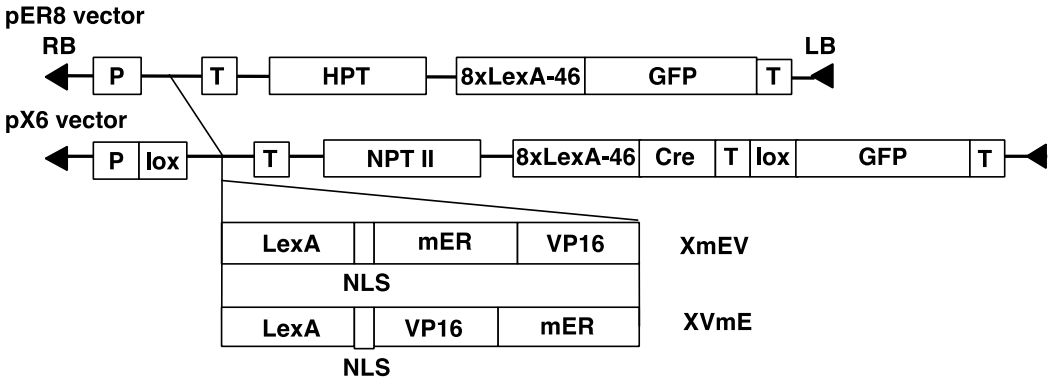


Figure 5

Figure 5 Root length of transgenic *Arabidopsis* plants expressing *medaka estrogen receptor* gene (A), and non-transgenic *Arabidopsis* plants (B) treated with 4-*t*-octylphenol (OP).

Seeds of the homozygous transgenic *Arabidopsis* line XmEV19-2 and non-transgenic *Arabidopsis* were sown and incubated in a solid medium containing 0.001% dimethyl sulfoxide (DMSO) as a control or different concentrations of OP for 2 weeks. Seedlings were taken from the medium, and root lengths were measured (n = 10-15). White and black bars indicate the control and OP treatments, respectively. Asterisks indicate significant differences compared to the root length under DMSO treatment (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Student's t-test).



Supplementary Figure 1

Supplementary Figure 1 Plasmids for inducible expression of the *green fluorescent protein* (*GFP*) gene in transgenic *Arabidopsis* plants expressing *medaka estrogen receptor* (*mER, mE*) gene upon treatment with estrogenic compounds.

Cre, Cre recombinase gene; HPT, expression unit for hygromycin resistance gene; LB, left border; LexA, DNA-binding domain of *Escherichia coli* LexA; 8xLexA-46, 8 copies of LexA binding domain combined with cauliflower mosaic virus 35S minimal promoter; lox, target site for truncation by Cre recombinase; NLS, SV40 large T-antigen nuclear localization signal; NPT II, expression unit for kanamycin resistance gene; P, promoter; RB, right border; T, terminator; VP16, transactivating domain of Herpes simplex VP16.

638 Supplementary Table 1 Primer sequences used in this study

Primer name	Sequence
XVmE-5′s	5′-GTCGACGGTCAGGAGCATAAAACGGT-3′
XVmE-3′s	5′-GTCGACCTAGTCTTGAAGGGCCGGGGTGC-3′
XmEV-5′NLSx	5′-CTCGAGCCAAAAAAGAAGAGAA--AGGTCGGAGGAGGAGGAGGAGGAGG-3′
XmEV-3′x	5′-CTCGAGGGGAGCGATTCCACCCCCGC-3′
mER-qPCR-s	5′-TGGACAGGAATGAGGGAGAC-3′
mER-qPCR-as	5′-CCAGCAGCATGTCGAAGAT-3′
GFP-qPCR-s	5′-CCAACGAAAAGAGAGACCACA-3′
GFP-qPCR-as	5′-ATAGTTCATCCATGCCATGTGTA-3′
tubulin-qPCR-s	5′-TGGAATGGATCCCAAACAAC-3′
tubulin-qPCR-as	5′-TTCAAACCCTTTGGTGCAAT-3′

639

640

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: