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ORIGINAL ARTICLE

Aryl hydrocarbon receptor (AhR)-mediated reporter gene expression systems in transgenic tobacco plants

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Abstract In mammals, the aryl hydrocarbon receptor (AhR) mediates expression of certain genes, including *CYP1A1*, in response to exposure to dioxins and related compounds. We have constructed a mouse AhR-mediated gene expression systems for a *β-glucuronidase (GUS)* reporter gene consisting of an AhR, an AhR nuclear translocator (Arnt), and a xenobiotic response element (XRE)-driven promoter in transgenic tobacco plants. On treatment with the AhR ligands 3-methylcholanthrene (MC), *β*-naphthoflavone (*β*NF), and indigo, the transgenic tobacco plants exhibited enhanced GUS activity, presumably by inducible expression of the reporter gene. The recombinant AhR (AhRV), with the activation domain replaced by that of the *Herpes simplex virus* protein VP16, induced GUS activity much more than the wild-type AhR in the transgenic tobacco plants. Plants carrying AhRV expressed the *GUS* reporter gene in a dose- and time-dependent manner when treated with MC; GUS activity was detected at 5 nM MC on solid medium and at 12 h after soaking in 25 μM MC. Histochemical GUS staining showed that this system was active mainly in leaf and stem. These results suggest that the AhR-mediated reporter gene expression system has potential for the bioassay of dioxins in the environment and as a novel gene expression system in plants.

Keywords Aryl hydrocarbon receptor · Bioassay · Chemical-inducible · Dioxins · Gene expression · Transgenic plant

Abbreviations

AhR	Aryl hydrocarbon receptor
Arnt	AhR nuclear translocator
CYP1A1	Cytochrome P450 1A1
GUS	*β*-Glucuronidase
Hsp90	90-kDa Heat shock protein
IMM	Immunophilin-like protein
MC	3-Methylcholanthrene
MS	Murashige and Skoog
4MU	4-Methyl-umbelliferone
4MUG	4-Methyl-umbelliferyl-*β*-D-glucuronide
*β*NF	*β*-Naphthoflavone
Nos-T	Nos terminator
PCB	Polychlorinated biphenyl
PCDF	Polychlorinated dibenzofuran
PCDD	Polychlorinated dibenzo-*p*-dioxin

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POP	Persistent organic pollutant
35-S	CaMV35S promoter
35Sm-P	−60/+8 minimal CaMV35S promoter
TAD	Transactivation domain
X-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronide
XRE	Xenobiotic respons element
XRE-P	XRE-driven promoter

Introduction

In mammals, aryl hydrocarbon receptors (AhRs)—ligand-dependent transcription factors—mediate the toxic and biological effects of dioxins such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and related compounds. Dioxins specifically bind to AhRs as ligands below nanomolar order (Ema et al. 1994; Whitlock 1999). Expression of the cytochrome P450 1A1 (*CYP1A1*) gene via AhR is a common phenomenon caused by these compounds.

In the absence of ligands, AhR is found in the cytosol in complex with 90-kDa heat shock protein (hsp90) and the immunophilin-like proteins (IMMs) XAP2/AIP/ARA9 (Hollingshead et al. 2004) and p23 (Kazlauskas et al. 1999). Upon ligand binding, AhR translocates into the nucleus, dissociates from hsp90 and the other cofactors, and dimerizes with AhR nuclear translocator (Arnt; Kazlauskas et al. 2001). This heterodimer binds to the xenobiotic response element (XRE) in the promoter of *CYP1A1* (Ko et al. 1996; Lees and Whitelaw 1999), resulting in gene expression. CYP1A1 produced on microsomes metabolizes certain dioxins and related compounds except for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Sakaki et al. 2002). In this process, hsp90 and IMMs have been reported to play important roles in maintaining AhR in a high-affinity ligand-binding state and in repressed conformation (Whitelaw et al. 1995; Kazlauskas et al. 2001).

There are no reports of such an AhR-mediated gene expression system in higher plants, although hsp90 and IMMs are conserved (Harrell et al. 2002). Mammalian receptor-mediated gene expression systems such as the steroid receptor superfamily, which has analogy with the AhR's, have been established in transgenic plants (Aoyama and Chua 1997; Martinez et al. 1999a, b; Zuo et al. 2000; Tojo et al. 2006). On the basis of the interaction with their plant homologues, these systems should work in higher plants (Stancato et al. 1996; Harrell et al. 2002). Responding to binding to receptor-specific ligands, the transgenic plants mentioned above exhibited inducible gene expression.

As plants form the basis of the food chain and the centre of chemical circulation in the environment, they would be useful for on-site monitoring and remediation of persistent organic pollutants (POPs), including dioxins and organochlorine insecticides. Some plant species are covering the ground, have well-developed root systems and are able to absorb chemicals from a wide soil area. In addition, although dioxins and related compounds are extremely hydrophobic and immobile in soil, members of *Cucurbitaceae* can absorb and accumulate certain PCDDs and PCDFs through their roots and subsequently translocate them to the aerial tissues (Hülster et al. 1994; Harvey et al. 2002).

Here we demonstrate that the mouse AhR-mediated *GUS* reporter gene expression system consisting of mouse AhR, Arnt and a synthetic XRE-driven promoter responds to AhR ligands in transgenic tobacco plants. We discuss the potential of these plants for on-site monitoring of dioxins and related compounds in the environment.

Materials and methods

Chemicals and biochemicals

3-Methylcholanthrene (MC), β-naphthoflavone (βNF), indigo, 4-methyl-umbelliferyl-β-D-glucuronide (4MUG), 4-methyl-umbelliferone (4MU), and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) were purchased from Nacalai Tesque (Kyoto, Japan). Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA, USA). Other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), Nacalai Tesque, and Sigma (St Louis, MO, USA).

Plant culture and transformation

Nicotiana tabacum cv. Samsun NN, kindly provided by Dr. Yuko Ohashi (National Institute of Agrobiological Sciences, Japan) was grown as a shoot culture on sterile Murashige and Skoog (MS) medium in a plant box in a growth chamber at 25°C under a 16-h light and 8-h dark cycle. Tobacco leaf discs were transformed with *Agrobacterium tumefaciens* strain LBA4404 as previously described (Horsch et al. 1985). Regenerated plants were selected twice on MS medium supplemented with 100 mg l^{-1} kanamycin. Primary transformants (R_0 plants) were further selected by genomic PCR analysis.

Construction of expression plasmids

Mouse *AhR* (Ema et al. 1992) and *Arnt* (Reisz-Porszasz et al. 1994) cDNAs were obtained from the mouse Uni-ZAP XR cDNA library of C57BL/6 liver cells (Stratagene, La Jolla, CA, USA) by the use of an Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. *Xba*I and *Hin*dIII restriction enzyme sites were added at the 5′ and 3′ termini, respectively, of both cDNAs. The *AhR* cDNA and the

*Xba*I/*Hin*dIII-digested *Arnt* cDNA were subcloned into pT7Blue (Novagen, Madison, WI, USA) and *Xba*I/*Hin*dIII-digested pBluescript KS+ (Stratagene) to yield pT/AhR and pBlue/Arnt, respectively. The cDNA of the *Herpes simplex* protein VP16 transactivation domain (TAD; amino acids 413–490; Dalrymple et al. 1985) was primed from pER1 generously provided by Dr. Nam-Hai Chua (Rockefeller University, New York, USA). The *Xho*I and *Sal*I restriction enzyme sites were added at the 5′ and 3′ termini, respectively, of the cDNA. The resulting cDNA was subcloned into pT7blue to yield pT/VP16$_{stop}$. Each cDNA was confirmed by sequence analysis. *Sal*I/*Not*I-digested *AhR* cDNA from pT/AhR was subcloned into the *Xho*I/*Not*I-digested sites of pBluescript KS+ to yield pBlue/AhR494, and the *Sal*I site was introduced into the *Not*I-digested site of pBlue/AhR494 to yield pBlue/AhR494$_{NotI\text{-}linker}$ by insertion of the complementary oligonucleotides containing the *Sal*I site. *Xho*I/*Sal*I-digested *VP16* cDNA from pT/VP16$_{stop}$ was subcloned into the *Sal*I-digested site of pBlueAhR494$_{NotI\text{-}linker}$ to yield pBlue/AhRV. The synthetic oligonucleotide *Hin*dIII-linker was inserted into the *Sal*I-digested site of pBlue/AhRV to yield pBlueAhRV-$_{HindIII}$. The *Xba*I/*Hin*dIII-digested *AhR* cDNA from pT/AhR was subcloned into the *Xba*I/*Hin*dIII-digested sites of pSXA1 (Inui et al. 2000) to yield pSX/AhR. The *Sal*I/*Xho*I-digested fragment of pSX/AhR encompassing the *AhR* cDNA expression cassette under the control of the CaMV35S promoter (35S-P)/Nos terminator (Nos-T) was subcloned into *Sal*I/*Xho*I-digested pBlue$_{SalI/XhoI}$ to yield pBlueSX/AhR. *Xba*I/*Hin*dIII-digested *AhRV* and *Arnt* cDNAs from pBlue/AhRV-$_{HindIII}$ and pBlue/Arnt were each subcloned into the *Xba*I/*Hin*dIII-digested sites of pBlueSX/AhR to yield pBlueSX/AhRV and pBueSX/Arnt, respectively. The *Sal*I/*Xho*I-digested expression cassette fragments of pBlueSX/AhR and pBlueSX/AhRV were each subcloned into the *Sal*I/*Xho*I-digested sites of pSNTLX (Inui et al. 2000) to yield pSK/AhR and pSK/AhRV, respectively. The *Sal*I/*Xho*I-digested expression cassette fragment of pBlueSX/Arnt was subcloned into both pSK/AhR and pSK/AhRV to yield pSK/A2 and pSK/AVAt, respectively. To construct the XRE-driven promoter (XRE-P), we inserted complementary oligonucleotides containing six tandem repeated XRE sequences (Lusska et al. 1993) (6 × XRE, 5′-TCGA*CCTCGAG*TCT TCTCACGCAACTCCGTCTTCTCACGCAACTCCGTCT TCTCACGCAACTCCGTCTTCTCACGCAACTCCGTC TTCTCACGCAACTCCGTCTTCTCACGCAACTCCGC -3′ and 5′-TCGAGCGGAGTTGCGTGAGAAGACGGA GTTGCGTGAGAAGACGGAGTTGCGTGAGAAGACG GAGTTGCGTGAGAAGACGGAGTTGCGTGAGGAAG ACGGAGTTGCGTGAGAAGA*CTCGAG*G-3′) into the *Sal*I-digested site of pUC/35SmGUS containing the GUS expression cassette under the control of the −60/+8 minimal CaMV35S promoter (35Sm-P)/Nos-T to yield pUC/XREGUS. The overhanging ends of *Sal*I site are underlined, and *Xho*I sites are italicized. The *Sal*I/*Xba*I-digested fragment of pUC/XREGUS containing XRE-P was subcloned into the *Sal*I/*Xba*I-digested site of pBlueSX/AhR to yield pBlueSX/XREAhR with substitution of the *Sal*I/*Xba*I-digested fragment of 35S-P. The *Xba*I/*Eco*RI-digested fragment of pUC/35SGUS containing *GUS* cDNA and a partial sequence of Nos-T was subcloned into the *Xba*I/*Eco*RI-digested site of pBlueSX/XREAhR to yield pBlueSX/XREGUS with substitution of the *AhR* cDNA. The *Xho*I-digested fragment of pBlueSX/XREGUS encompassing the *GUS* cDNA expression cassette was cloned into the *Sal*I-digested site of pSNTLX, pSK/A2, and pSK/AVAt to yield pSX/XREGUS, pSK/A2G, and pSK/AVAtG. A schematic diagram of the expression plasmids is shown in Fig. 1.

PCR analysis

Genomic DNA was isolated from leaf tissues by using an ISOPLANT reagent (Nippon Gene, Tokyo, Japan), and PCR was performed with 50 ng of genomic DNA by using DNA

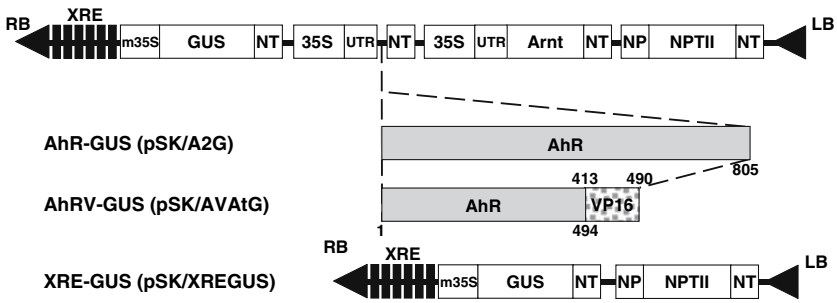

Fig. 1 Schematic diagram of the plant expression plasmids. AhR, mouse *AhR* cDNA; AhRV, recombinant *AhR* cDNA; Arnt, mouse *Arnt* cDNA; GUS, *β-glucuronidase* gene; NPTII, *neomycin phosphotransferase II* gene; 35S, CaMV35S promoter; m35S, minimal -60 CaMV35S promoter; NP, nopaline synthase promoter; NT, nopaline synthase terminator; XRE, mouse xenobiotic response element; UTR, alfalfa mosaic virus 5′-untranslated leader sequence; LB, left border; RB, right border; VP16, gene for the acidic transactivation domain of the *Herpes simplex* protein VP16. *Numbers* above and below the coding regions indicate the number of amino acid residues as counted from the N-terminus of the corresponding proteins

Taq polymerase (Takara Shuzo, Kyoto, Japan). Temperatures were cycled as follows: 94°C for 2 min; followed by 30 cycles of 94°C for 30 s, 56°C (for *AhR/AhRV*) or 60°C (for *Arnt* and *GUS*) for 30 s and 72°C for 1 min; and 1 cycle of 72°C for 5 min. PCR products were visualized in 1.5% (w/v) agarose gels under UV. Total RNA was isolated from leaf tissues by using an RNeasy Plant Mini Kit combined with RNase-free DNase (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RT-PCR was performed with 1 μg of total RNA in a One Step RNA PCR Kit (AMV; Takara Shuzo), according to the manufacturer's instructions. Temperatures were cycled as follows: 50°C for 30 min, then 94°C for 2 min; followed by 30 cycles of 94°C for 30 s, 56°C (for *AhR/AhRV*) or 60°C (for *Arnt* and *GUS*) for 30 s and 72°C for 90 s; and 1 cycle of 72°C for 5 min. The following primer pairs were used: for *AhR* and *AhRV*, AhR-n (5′-GCACGCTTGATTTACA GAAA-3′) and AhR-c (5′-GGCACTCATAAGAGAACT G-3′); for *Arnt*, Arnt-n (5′-CAGAGTTCATCTCCCGAC AC-3′) and Arnt-c (5′-ACAGGCTGGACAGAACCTG-3′); for *GUS*, GUS-S (5′-CAGGCAGTTTTAACAGTCAGT TC-3′) and GUS-AS (5′-CACGCGCTATCAGCTCTTTA ATC-3′); for *actin*, actin-S (5′-CAGGGTTTGCTGGAGA TGATGC-3′) and actin-AS (5′-GGCCACTGGCATAAA GGGATAG-3′) (Thangavelu et al. 1993).

Treatment of plants with AhR ligands

Two-week-old axillary buds of tobacco plants grown on sterile MS medium were assayed for induction of GUS activity. Aerial parts were transplanted and grown on MS medium containing an AhR ligand for 2 weeks. The induction medium contained DMSO as a solvent for AhR ligands and Tween20 at final concentrations of 0.1 and 0.01% (v/v), respectively. Each chemical had no effect on plant growth at these concentrations. For time-dependent GUS activity, we soaked 6-week-old axillary buds grown on sterile MS medium in 1/50 MS solution containing MC or DMSO and Tween 20, and three leaves from the top of intact plants were subjected to GUS assay.

GUS assay

Aerial parts of tobacco plants were harvested and immediately frozen in liquid nitrogen. Samples were homogenized in 1.5 volumes of extraction buffer (50 mM sodium phosphate buffer (NaPB, pH 7.0), 10 mM EDTA, 0.1% (v/v) TritonX-100, 0.1% (v/v) *N*-lauroylsarcosine sodium salt, 10 mM 2-mercaptoethanol) and centrifuged at $1.7 \times 10^4 g$ for 5 min. Supernatants were subjected to a fluorometric GUS assay, being incubated with the substrate 1 mM 4MUG. Reaction was stopped by addition of nine volumes of 0.2 M Na_2CO_3. Fluorescence at 360 nM excitation and 450 nM emission was quantified by the use of a microplate reader (MTP-100F; CORONA, Katsuta, Japan), referring to the fluorescence of 4MU. The protein concentration of supernatants was determined by a Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA, USA). For GUS staining analysis, plant samples were soaked in a histochemical staining solution (0.1 M NaPB (pH 7.0), 1.9 mM X-Gluc, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, 0.3% (v/v) Triton X-100, 20% (v/v) methanol) and vacuum-infiltrated for 20 min. Samples were incubated at 37°C for 16 h then incubated with 70% (v/v) ethanol at room temperature for at least 10 h.

Results

Construction of recombinant plasmids for AhR-mediated gene expression systems

To develop a novel dioxin-inducible gene expression system in tobacco plants, we constructed recombinant plasmids for the minimal components of a mammalian AhR-mediated reporter gene expression system. Three expression plasmids—pSK/A2G, pSK/AVAtG, and pSK/XREGUS—are shown in Fig. 1. pSK/A2G and pSK/AVAtG consist of four gene expression cassettes. The first cassette contains the *GUS* reporter gene regulated by a synthetic XRE-driven promoter containing six copies of XRE and the minimal 35S promoter. The second and third are constitutive expression cassettes for the cDNA clones of mouse AhR and Arnt. To enhance the transactivation activity of AhR, we replaced its C-terminal TAD with the *Herpes simplex* protein VP16 TAD to construct the AhRV in pSK/AVAtG. The fourth is the NPT II expression cassette for the selection of transformed tobacco plants. pSK/XREGUS was constructed as a control, because it contains neither the *AhR* nor the *Arnt* cDNA expression cassettes.

Transgenic tobacco plants with AhR-mediated reporter gene expression systems

Tobacco plants were transformed with the three expression plasmids. Regenerated tobacco plants selected twice by kanamycin-resistance test were further selected by genomic PCR analysis using primers specific to the cDNA clones of AhR, Arnt, or GUS. PCR-positive regenerated plants were termed AhR-GUS (pSK/A2G), AhRV-GUS (pSK/AvAtG), or XRE-GUS (pSK/XREGUS). We selected 11 AhR-GUS, 53 AhRV-GUS, and 28 XRE-GUS plants. Twenty-eight independent 35S:GUS plants expressing the *GUS* gene under the control of the 35S promoter were also produced (Supplementary Fig. 1) and two independent plants

expressing at average levels were used as the positive GUS control.

We assayed these transgenic plants for AhR-ligand-dependent inducible GUS activity. Six of the 11 AhR-GUS plants and 19 of the 53 AhRV-GUS plants showed MC-dependent inducible GUS activities with varying intensities in their aerial tissues (Table 1). None of the XRE-GUS plants showed GUS activity. The AhRV-GUS plants showed higher GUS activity than the AhR-GUS plants (data not shown).

Although the AhRV-GUS plant line 114 showed the highest GUS activity (up to 240% of that of the 35S-GUS), the induction level was approximately sixfold (Fig. 2). The AhRV-GUS plant line 4 showed higher inducible GUS activity on the treatment with MC, whereon its induction and induced levels were approximately 46 times and 93% of those of 35S-GUS plants. In contrast, none of the XRE-GUS plants exhibited any GUS activity in the presence or absence of MC (data not shown).

Table 1 Numbers of the transgenic tobacco plants selected

Transgenic plant (expression plasmid)	Number of transformants	
	Genome PCR positive	Induced GUS activity positive
AhR-GUS (pSK/A2G)	11	6
AhRV-GUS (pSK/AVAtG)	53	19
XRE-GUS (pSK/XREGUS)	28	0

After double seletion for kanamycin resistance, regenerated plants were subjected to genome PCR analysis for *AhR*, *Arnt*, and *GUS*. PCR positive plants were grown on MS medium with or without 25 μm MC to select plant with inducible GUS activity

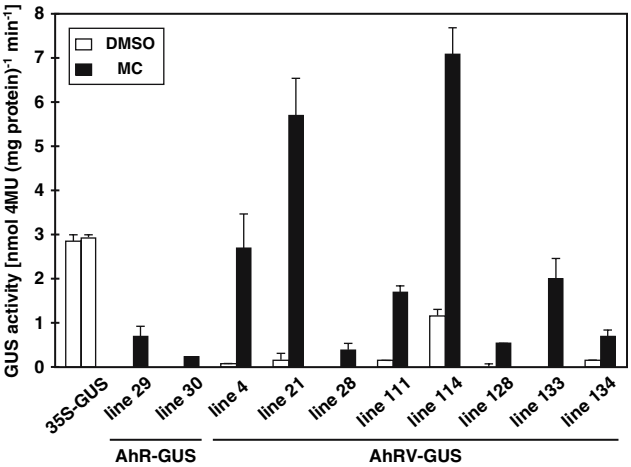

Fig. 2 Induced GUS activities in the transgenic tobacco plants. Two-week-old axillary buds of tobacco plants grown on sterile MS medium were transplanted and grown on inductive MS medium containing 25 μM MC for 2 weeks, then aerial parts were subjected to fluorometric GUS assay. Each value is the mean of three independent measurements of individual plant lines ± SD. 35S-GUS, transgenic tobacco plants expressing *GUS* gene under the control of the CaMV35S promoter

Eight independent lines of transgenic tobacco plants with MC-dependent GUS activity were analyzed by RT-PCR. The transcripts specific to each of the cDNAs were amplified in all the lines examined, as shown in Fig. 3.

The AhRV-GUS plant lines 21 and 114 showed severe leaf atrophy (data not shown). Furthermore, the degree of phenotypic changes intensified in parallel with the increase of GUS activity when these two lines were grown on MS medium containing MC at 0.25–25 μM (data not shown).

Induced GUS activity responded to three AhR ligands in the transgenic tobacco plants

Two types of AhR ligands other than MC have been reported to act as agonists of AhR in both mammalian and yeast cells: βNF and indigo (Fig. 4a). All transgenic plants showed similar responses to these three compounds, although with varying intensities of inducible GUS activity: both MC and βNF induced GUS activity highly and indigo to a lesser degree (Fig. 4b). Lines 21 and 114, in which GUS activities were highly induced on treatment with both MC and βNF, showed leaf atrophy when they were grown in the presence of MC and βNF but not indigo (data not shown). On the other hand, line 4 exhibited highly induced GUS activity almost equal to that of 35S-GUS plants with tight regulation and no phenotypic changes (Fig. 2). These results clearly demonstrate that on the whole, the AhR-mediated *GUS* reporter gene expression systems seem to function similarly in mammalian and plant cells. We selected line 4 for further analyses on account of its tight and strong response to AhR ligands without phenotypic changes.

Dose- and time-dependent GUS activities in the transgenic tobacco plants

Because the maximum soluble concentration of MC is approximately 25 mM, and over 0.1% DMSO in the MS medium caused adverse effects on plant growth (data not shown), we used less than 25 μM MC in this experiment. Distinct inducible GUS activity was detectable at a concentration of 5 nM or higher and increased gradually up to 25 μM in a dose-dependent manner (Fig. 5a). The AhRV-GUS plant line 133 showed a similar result (data not shown).

GUS activity in line 4 increased in response to MC in a time-dependent manner (Fig. 5b). Inducible GUS activity was detectable after 0.25 day, increased linearly between 0.25 and 1 day after treatment, and appeared to reach a plateau. In contrast, treatment with DMSO did not induce GUS activity at any time.

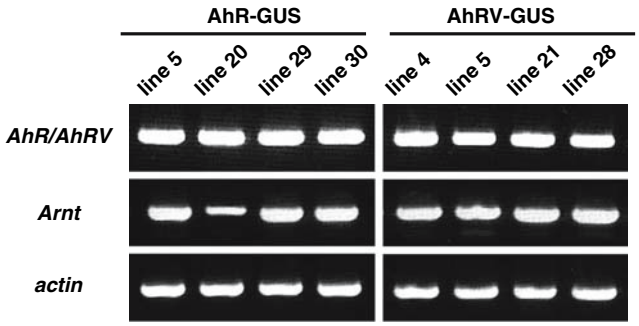

Fig. 3 RT-PCR analysis of transcription of *AhR/AhRV* and *Arnt* genes in the transgenic tobacco plants. Total RNA was extracted from leaves. 1 μg of total RNA was subjected to RT-PCR analysis with specific pairs of AhR, Arnt, and actin primers. Actin was used as an internal control

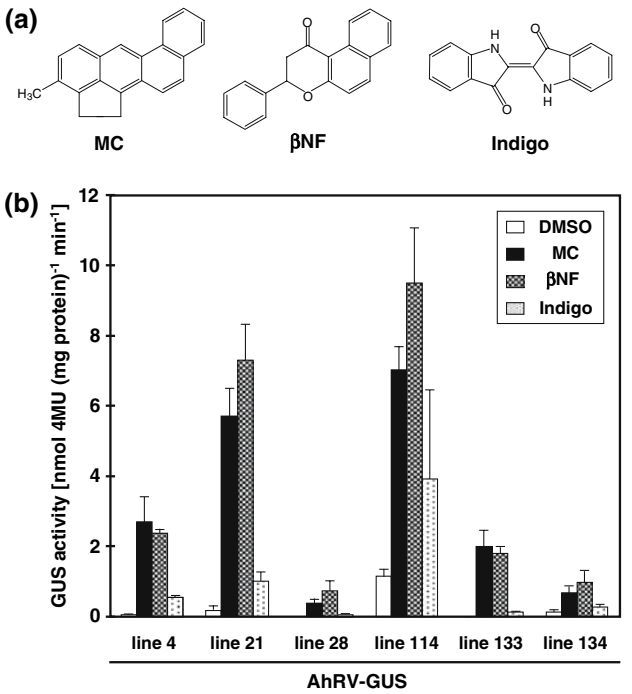

Fig. 4 **a**, **b** GUS activities respond to several AhR ligands in the transgenic tobacco plants. **a** Chemical structures of AhR ligands. **b** GUS activities in the transgenic tobacco plants. Two-week-old axillary buds of tobacco plants grown on sterile MS medium were transplanted and grown on inductive MS medium containing MC (25 μM), *β*NF (25 μM), or indigo (50 μM) for 2 weeks, then aerial parts were subjected to fluorometric GUS assay. Each value is the mean of three independent measurements of individual plant lines. The error bars represent standard deviations. DMSO was used as the control

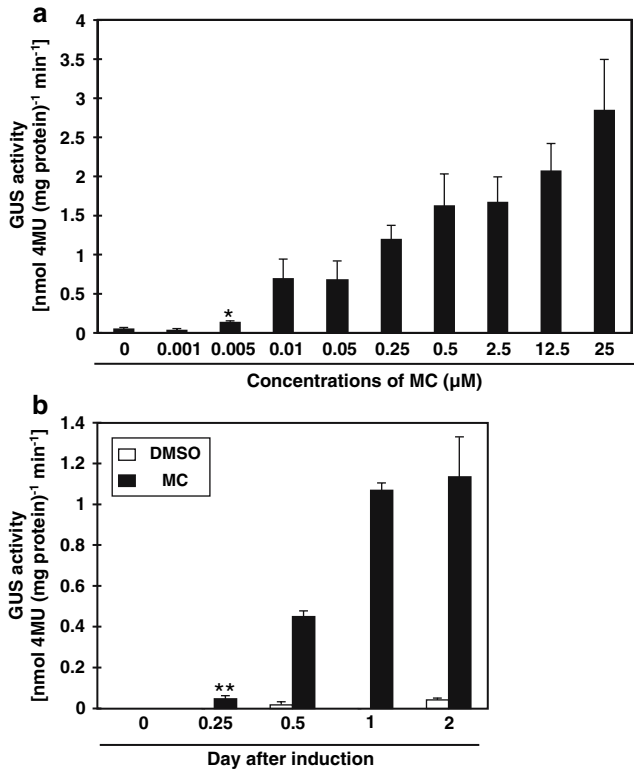

Fig. 5 **a**, **b** Dose- and time-dependent GUS activities in the transgenic tobacco plants. **a** Two-week-old axillary buds of the transgenic tobacco AhRV-GUS plant line 4 grown on sterile MS medium were transplanted and grown on inductive MS medium containing MC at a series of concentrations for 2 weeks, then aerial parts were subjected to fluorometric GUS assay. **b** Three leaves from the top of 6-week-old axillary buds of the transgenic tobacco AhRV-GUS plant line 4 grown on sterile MS medium were soaked in 1/50 MS solution containing 25 μM MC or DMSO (final concentration, 0.1%) and Tween 20 (final concentration, 0.01%) for the indicated times. Leaves were subjected to fluorometric GUS assay. Each value is the mean of three independent measurements ± SD. * $P < 0.05$ for plants grown in the presence of 0.005 μM MC vs. plants grown in the absence of MC; ** $P < 0.01$ for plants treated with MC vs. plants treated with DMSO

Histochemical GUS staining in the AhRV-GUS tobacco plants treated with MC

All examined plants exhibited significant GUS staining of varying intensities in leaves and stems when grown in the presence of MC (Fig. 6). The GUS staining was clearer in the lower aerial parts than in the upper parts. In line 4, distinct staining was observed in leaves and stems but not in roots. In stems, significant staining was observed in the xylem vessels and surface hydathodal hairs (Fig. 6h–j). Vertical sections of the stems showed significant GUS staining at the branching points of the xylem vessels. The diameter of stems was 3–5 mm (Fig. 6c, h). In the presence of MC, patchy and local GUS staining was observed in growing, mature and aged leaves (Fig. 6f, g). No visible GUS staining was observed when the axillary buds were grown in the absence of MC (Fig. 6a–e). Occasionally, slight GUS staining was detectable in both aged leaves and the stem-root transition zone even in the absence of MC (data not shown). Line 133 showed a similar GUS staining pattern to that in line 4 (data not shown).

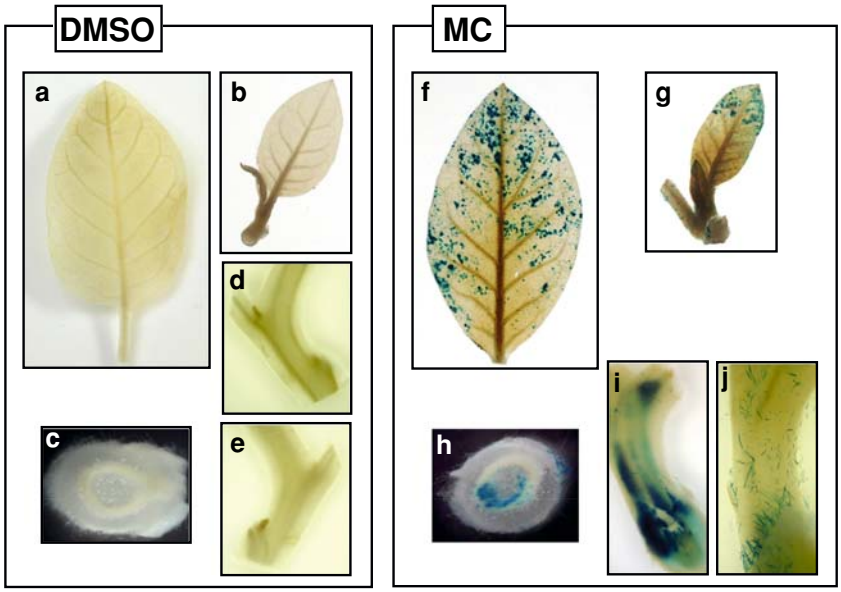

Fig. 6 **a**–**j** Histochemical GUS staining assay of the transgenic tobacco plants. Two-week-old axillary buds of the transgenic tobacco AhRV-GUS plant line 4 were transplanted and grown in the absence (0.1% DMSO) or presence of 25 μM MC for 2 weeks. Samples were subjected to histochemical GUS staining. **a**, **b** Whole leaf with DMSO. **f, g**, Whole leaf with MC. **c**–**e**, Stem with DMSO. **h**–**j**, Stem with MC

Discussion

Chemical-inducible reporter gene expression systems in higher plants were developed with steroid receptors that respond to oestrogen (Zuo et al. 2000), glucocorticoid (Aoyama and Chua 1997), or ecdysone (Martinez et al. 1999a, b). These systems provide a basic tool for plant physiology research, where the expression of genes of interest can be regulated by treatment with their specific inducers. Besides their success in basic research, such systems have potential for practical applications. Certain POPs such as dioxins and organochlorine insecticides in the environment have biological effects in animals, interacting with transcription factors such as AhR and steroid receptors (Rowlands and Gustafsson 1997; Hoivik et al. 1998; Whitlock 1999). These receptors have been used for bioassays for POPs (Anderson et al. 2005; Windal et al. 2005). On the other hand, because certain plant species with well-developed root systems are able to absorb and accumulate POPs from a wide area of soil, transgenic plants with these systems would be useful for detecting POPs on site. As mammalian AhR-mediated gene expression has been well studied, transgenic plants carrying the AhR-mediated systems are possible tools for on-site bioassays of dioxins and related compounds in the environment (Inui et al. 2005).

The transgenic tobacco plants carrying AhR and Arnt showed inducible GUS activity driven by the XRE promoter in response to MC, βNF, and indigo, where ligand binding appeared to trigger nuclear translocation of AhR, heterodimerization with Arnt and binding to XRE, resulting in inducible *GUS* reporter gene expression. The hsp90 chaperone system plays important roles in maintaining the high affinity of ligand binding, conformational repression and intercellular localization of AhR and steroid receptors in association with other factors (Kazlauskas et al. 2001; Picard 2006). Because the hsp90 chaperone system is conserved in yeast, plants, and mammals, the mammalian AhR-mediated systems should function in plant cells, interacting with the plant hsp90 chaperone system as in mammalian cells. The hsp90 chaperone system of yeast worked for introduced AhR and Arnt (Carver et al. 1994; Miller 1997). Steroid receptors such as glucocorticoid, oestrogen, and insect ecdysone superfamily receptors appear to function in higher plants by substituting the chaperone system of plant homologues (Aoyama and Chua 1997; Martinez et al. 1999a, b; Zuo et al. 2000).

The XRE sequence has been thought to be specific to the heterodimer of AhR/Arnt in mammals (Rowlands and Gustafsson 1997). Owing to its specificity, the heterodimers should also bind to XRE in plant cells, and the transgenic tobacco plants showed a relatively high induction of the reporter gene expression with tight regulation (Inui et al. 2005). It would be the same as the other reported systems such as the GVG and XVE systems in transgenic *Arabidopsis* and tobacco plants, respectively (Aoyama and Chua 1997; Zuo et al. 2000).

There are reports of large inter- and intra-species differences in susceptibility to dioxins and related compounds (Clark et al. 1992). These could be due to differences in the structure and capabilities of AhR, including affinity for ligands and transactivation (Ema et al. 1994; Rowlands and Gustafsson 1997; Korkalainen et al. 2001; Wong et al. 2001). In particular, comparison of amino acid sequences of AhRs shows significant differences in the C-terminal half, corresponding to the TAD, suggesting an association

between transactivation capability of AhR and species susceptibility (Bank et al. 1992; Korkalainen et al. 2001). In tobacco plants, the recombinant AhRV was more active than the original AhR (Fig. 2). Similar results have been reported in relation to recombinant transcription factors consisting of the ecdysone receptor-ligand binding domain, the GR TAD and its DNA-binding domain (Martinez et al. 1999a). The VP16 TAD can directly interact with general transcription factors evolutionarily conserved among eukaryotes (Lin et al. 1991; Shen et al. 1996) and was also potent in plant cells (Schwechheimer et al. 1998).

Both the absorption and accumulation of chemicals in plant cells are considerably dependent on the chemicals' $\log K_{ow}$ values as well as on metabolism by enzymes such as P450s. That is, chemicals with lower $\log K_{ow}$ and resistance to metabolism would be preferentially accumulated in plant cells. Most AhR ligands have extreme physico-chemical characteristics such as high $\log K_{ow}$ values, some of which with octanol–water partition coefficient values above 4 seemed to be immobile and partitioned preferentially into the humic fractions of soil rather than the aqueous phase in the environment. However, the bioavailability of such compounds is extremely dependent on the plant species and environmental conditions such as the constituents of soil (Harvey et al. 2002). Notably, Cucurbits could absorb PCDD and PCDF from the soil and translocate them to the aerial parts (Hülster et al. 1994). GUS staining was prominent in the leaves, vascular bundles, and stem surfaces, and more active in the lower aerial parts of the plants (Inui et al. 2005), suggesting that Cucurbits will be suitable as a host for this system.

In this study, we demonstrated inducible expression of the *GUS* reporter gene in transgenic tobacco plants carrying AhR and Arnt when in the presence of the AhR ligands. These transgenic plants are useful for not only bioassay of dioxins in the environment, but also for a novel time-dependent gene expression system for genes of interest.

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