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Chemical-inducible, ecdysone receptor-based gene expression system for plants

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Abstract

We have developed an inducible gene expression system with potential for field application using the ecdysone receptor (EcR) from the spruce budworm and the non-steroidal EcR agonist, methoxyfenozide. Chimeric transcription activators were constructed with EcR ligand binding domain, GAL4 and LexA DNA binding domains, and VP16 activation domain. In the presence of methoxyfenozide, the transcription activators induced expression of the luciferase reporter gene cloned downstream of a promoter containing GAL4- or LexA-response element and a minimal 35S promoter. Low basal and high induced luciferase expression was optimized by cloning the activator and the reporter genes in different tandem orientations. Many transgenic *Arabidopsis* and tobacco plants were obtained with little or no basal expression in the absence of methoxyfenozide and inducible expression that was several fold higher than that observed with the constitutive 35S promoter. Moreover, gene expression was controlled over a wide range of methoxyfenozide concentration. Our results demonstrate that the inducible gene expression system based on the spruce budworm EcR ligand binding domain with methoxyfenozide as a ligand is very effective in regulating transgenes in plants. It is suitable for field applications because methoxyfenozide is commercially available and has an exceptional health and environmental safety profile.

Introduction

An inducible system to activate or inactivate plant gene expression has many potential applications in the basic understanding of gene function, in manipulating complex developmental pathways, and in plant biotechnology. Earlier attempts to inducibly regulate transgene expression involved endogenous promoters that respond to chemicals, hormones, light, pathogens, or salt (reviewed by Gatz, 1997; Jepson et al., 1998; Boetti et al., 1999). High basal (uninduced) expression and pleiotropic effects from the inducing agent are known limitations of endogenous promoter-based systems. Several labs have developed chimeric transcription activators and promoters (Guyer et al., 1998; Moore et al., 1998; Schwechheimer et al., 1998) and chemically regulated systems (reviewed by Gatz, 1997; Gatz & Lenk, 1998; Jepson et al., 1998; Zuo & Chua, 2000) that combine functional domains from non-plant sources. The chemical-inducible gene expression systems contain two transcription units. The product of the first transcription unit is a transcription factor that responds to a chemical. The second transcription unit contains a response element to which the transcription factor binds, a minimal promoter, and the gene of interest. Transgenic plants containing the chemical-inducible system should only express the gene of interest following chemical treatment.

The chemical-inducible systems developed for plants so far include: Tet repressor-based, tetracycline de-repressible (Gatz et al., 1992; Love et al., 2000); tTA-based, tetracycline inactivatable (Weinmann et al., 1994); glucocorticoid receptor-based, dexamethasone inducible (Aoyama & Chua, 1997); AlcR-based, ethanol inducible (Caddick et al., 1998; Roslan et al., 2001); Ecdysone receptor

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(EcR)-based, EcR agonist inducible (Martinez et al., 1999); estrogen receptor-based, β-estradiol inducible (Bruce et al., 2000; Zuo et al., 2000); and Tet and glucocorticoid receptors-based, dual control (Bohner et al., 1999). An ideal inducible gene expression system should have the following desirable properties (Zuo & Chua, 2000): low basal expression levels, high inducibility, specificity to inducer, high dynamic range to inducer concentrations, fast response, switch-off after removal of inducer, and low toxicity. Each of the chemical-inducible systems developed thus far satisfy only some of the criteria in an ideal system. Some systems have high basal expression, or are non-functional in some plants. Other systems may cause growth defects, or are not suitable for field application (Moore et al., 1998; Kang et al. 1999; Zuo et al., 2000).

Here we describe the characterization of a gene regulation system suitable for large scale field application using the EcR from spruce budworm (*Choristoneura fumiferana*) and the EcR agonist methoxyfenozide. EcR is a member of the steroid receptor family that is characterized by signature DNA binding, ligand binding, and activation domains (Thummel, 1995; Riddiford et al., 2000). EcR regulates growth, molting, and development in insects. Methoxyfenozide, a non-steroidal agonist of EcR, binds to EcR with nanomolar affinity and is safe to many organisms (Dhadialla et al., 1998). We optimized the system for low basal and high induced gene expression. Inducible expression in transgenic *Arabidopsis* and tobacco plants was responsive to a wide range of methoxyfenozide concentrations and was several fold higher than that of the constitutive 35S promoter.

Materials and methods

Plasmid constructs

The plasmid constructs for transient expression in protoplasts and stable expression in transgenic plants used in this study are shown in Figure 1. The combination of promoter, poly linker, and terminator sequences were first made in plasmid vector pBluescript SK$^-$ (Stratagene, La Jolla, CA, USA). Promoter and terminator sequences were amplified from the source plasmids (see below) by 15 cycles of PCR amplification using the *Pfu* polymerase (HF PCR kit from Clontech, Palo Alto, CA, USA), digested with the appropriate restriction enzymes, and cloned in to pBluescript SK$^-$. Subsequently, PCR amplified

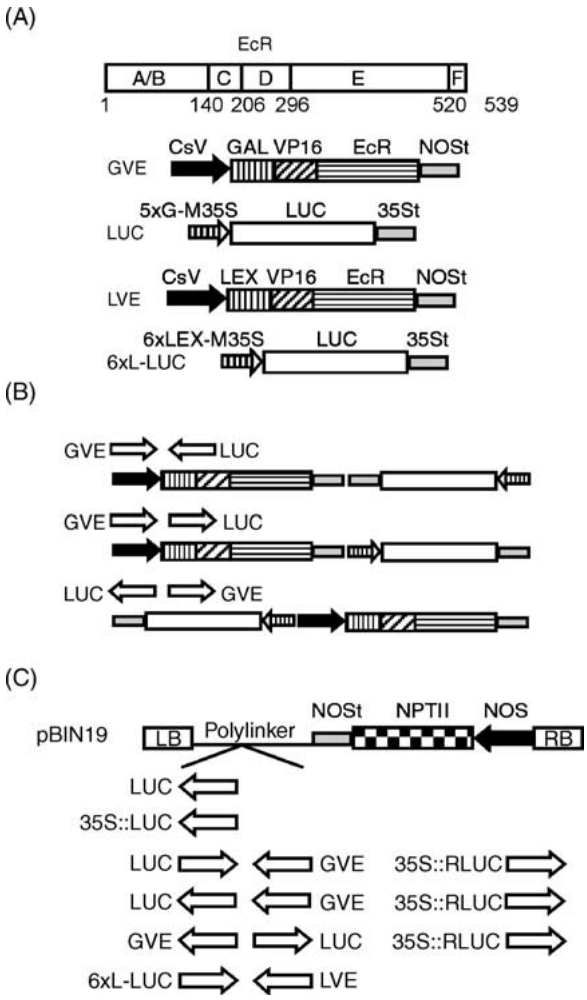

Figure 1. Schematic representation of the gene constructs used in this study. (A) Domains of spruce budworm ecdysone receptor (EcR) and chimeric activator and reporter genes. The numbers below EcR show the amino acid residues. Chimeric activator gene cassette GVE contains the EcR ligand binding region DEF transcriptionally fused to the DNA binding domain from GAL4 protein and activation domain from herpes simplex virus VP16 protein cloned between constitutive cassava mosaic virus promoter (CsV) and nopaline synthase terminator sequence (NOSt). The GAL4-responsive luciferase reporter gene (LUC) contains five copies of a 17 bp GAL4 response element (5 × GAL) and a 53 bp minimal promoter from the cauliflower mosaic virus 35S promoter (M35S) at the 5′ position, luciferase gene, and 35S terminator (35St) at the 3′ position. Activator gene cassette LVE is similar to GVE except that GAL4 domain is replaced with LexA DNA binding domain. LexA-responsive luciferase gene (6 × L-LUC) contains six copies of 18 bp LexA-response element and the M35S at the 5′ position of the luciferase gene. (B) Orientations of GVE and LUC genes in plasmids used in tobacco protoplasts. (C) T-DNA region of binary vectors. Activators (GVE or LVE), inducible reporters (LUC or 6 × L-LUC), and constitutive reporters (35S::LUC or 35S::RLUC) were inserted into polylinker region of pBIN19 as shown. The constitutive luciferase (35S::LUC) *Renilla* luciferase (35S::RLUC) expression cassettes have 35S promoter and 35St at the 5′ and 3′, respectively. The direction of arrow represents the direction of transcription. LB, left border; RB, right border.

activator or reporter sequences were inserted in the poly linker region to obtain the plant expression cassettes (i.e., combinations of promoter, gene, and terminator). Expression cassettes cloned in pBluescript SK^- were used for transient expression assays in tobacco protoplasts. For plant transformation, the combination of expression cassettes initially assembled in SK^- were subsequently transferred into *Agrobacterium* binary plasmid pBIN19 (Frisch et al., 1995). The binary plasmids were transformed into *Agrobacterium* strain LBA4404. The sources of sequences used in this study were the 423 bp cauliflower mosaic virus 35S promoter (35S; −415 to +8; Odell et al., 1985), 515 bp cassava vein mosaic virus promoter (CsV; −443 to +72, Verdaguer et al., 1996), 264 bp 35S transcription termination (polyadenylation) sequence (35St; Odell et al., 1985), 264 bp NOS termination sequence (NOSt; Frisch et al., 1995), 53 bp minimal 35S promoter containing the TATA box (M35S, −45 to +8), five copies of 17 bp GAL4 response element (Giniger et al., 1985), 441 bp GAL4 DNA binding domain (amino acids 1–147; Laughon & Gesteland, 1984), six copies of 18 bp LexA-response element (Estojak et al., 1995); 606 bp LexA DNA binding domain (amino acids 1–202; Horii et al., 1981), 264 bp VP16 acidic activation domain (amino acids 413–490; Dalrymple et al., 1985), D to F regions of spruce budworm EcR (amino acids 206–539; Perera et al., 1999), 1416 bp USP (amino acids 1–472; Perera et al., 1998), 1653 bp firefly luciferase gene (Ow et al., 1986), and 936 bp *Renilla* luciferase gene (Lorenz et al., 1991). All gene sequences were confirmed by DNA sequencing.

Transient expression in protoplasts

Cell suspension cultures of *Nicotiana tobacum* BY2 were maintained in MS medium in the dark and subcultured weekly (Nagata et al., 1992). Protoplasts prepared from 3-day-old cultures were resuspended in 0.4 M mannitol (Watanabe et al., 1987) and distributed into 35 mm petri dishes in 1 ml aliquots ($\sim 5 \times 10^5$ cells). Protoplasts were mixed with plasmid DNA and electroporated at 0.56 kV for 80 μs using a square wave electroporation system with Petripulser electrode (BTX, San Diego, CA, USA). The amount of DNA added to protoplasts was 10 μg for the LUC reporter plasmid and 20 μg for the activator plasmid. Initial experiments with varying amount of plasmid DNA showed that 10 μg activator and 20 μg reporter was optimum. Fifteen micrograms of DNA was added when both the activator and reporter genes were on the same plasmid. This corresponds to molar equivalent of 10 μg reporter plasmid DNA. Salmon sperm DNA was added to bring the total amount of DNA to 50 μg. Two micrograms of 35S::RLUC plasmid DNA was included in each transformation as an internal control to normalize the luciferase enzyme activity. Following electroporation, protoplasts were diluted with 1 ml of 2× protoplast culture medium (Watanabe et al., 1987), aliquoted as two 1 ml cultures, and incubated at 27°C for 17 h. One aliquot received 10 μM methoxyfenozide (induced) and the other did not (basal). Protoplasts were lysed by freeze thawing and addition of 250 μl 5× passive lysis buffer (Promega, Madison, WI, USA). Twenty microliters of cell extract was assayed for luciferase and *Renilla* luciferase activity using the Dual Luciferase Assay Kit reagents (Promega) in a Microtiter Plate Luminometer (Dynex, Chantilly, VA, USA) equipped with injectors. Initial testing of different concentrations of methoxyfenozide (0.01–30 μM) showed that 5–10 μM ligand was optimum for luciferase induction. Methoxyfenozide (Rohm and Haas, Spring House, PA, USA) stock solutions were made in DMSO and diluted 100-fold in protoplast culture medium.

Plant transformation and luciferase assays

Arabidopsis thaliana ecotype Columbia was transformed using the vacuum infiltration method (Bechtold et al., 1993). Transgenic *Arabidopsis* plants were selected by germinating the seed collected from the infiltrated plants on medium containing 250 mg/l carbenicillin and 100 mg/l kanamycin. Resistant T_1 seedlings were transferred to soil to set seed. Tobacco plants of the cultivar Xanthi were transformed by employing the standard leaf discs transformation method (Fisher & Guiltinam, 1995). T_1 plants in this study are defined as kanamycin resistant *Arabidopsis* plants derived from the seed collected from the vacuum infiltrated plants and tobacco plants derived from the leaf discs. Both *Arabidopsis* and tobacco plants in this generation are expected to be heterozygous at the transgene locus and are similar to F_1 in a sexual cross.

Leaf disks collected from the T_1 plants were floated on half strength MS medium with or without 10 μM methoxyfenozide in 24-well microtiter plates (four discs/plant, one disc/well). Plates were incubated for 24 h at a day/night cycle of 16 h, 25°C/8 h, 23°C. Leaf discs were ground in 100 μl of 1 × passive lysis

buffer (Promega) using Kontes Pellet Pestles (Kontes, Vineland, NJ, USA). Twenty microliters of supernatant was assayed for luciferase and *Renilla* luciferase activities using Dual Luciferase Assay reagents (Promega) in a Microtiter Plate Luminometer (Dynex). Protein was measured using BCA reagent (Pierce, Rockford, IL, USA). The *Renilla* luciferase activity in plants did not interfere with the luciferase assay as the assay uses different substrates for *Renilla* and firefly luciferases.

Seed collected from T_2 and T_3 plants was surface sterilized and plated on half strength MS medium. Petri dishes were incubated at 4°C for 2 days and transferred to a growth chamber (16 h, 25°C day and 8 h and 23°C night). Selection for the resistant seedlings was achieved by including 100 mg/l kanamycin in the germination medium. Two and half weeks after plating, seedlings (without roots) from each plate were ground separately in 100 µl of 1× passive lysis buffer. Twenty microliters of supernatant was then assayed for luciferase and *Renilla* luciferase activities.

Application of methoxyfenozide to pots

Eight T_2 plants derived from a transgenic tobacco plant were grown in pots until pod set (∼ 4-month-old, 1.5–2.0 m tall, and with 20–30 leaves) in a greenhouse. Methoxyfenozide at 0.2, 1, 5, or 20 µM concentrations were made by diluting 50 mM stock solution (in dimethylsulfoxide) in water. One liter of the above solutions was applied to pots (two plants per concentration) on days 0, 3, and 6. Subsequently, plants were watered normally. Leaf discs were collected from two middle and two top leaves before the ligand application (day 0) and on days 1, 3, 7, and 16. Luciferase activities in leaf discs were assayed as described earlier except that leaf discs were not floated on the MS medium with methoxyfenozide.

Results

Chimeric transcription activators with spruce budworm EcR

In insect cells, EcR and ultraspiracle protein (USP) dimerize in the presence of 20-hydroxyecdysone and activate many genes (Riddiford et al., 2000). EcR from spruce budworm contains five functional regions: A/B and F regions for transactivation, C region for DNA binding, hinge region D, and E region for ligand binding (Figure 1(A); Perera et al., 1999). Initial experiments in tobacco BY2 protoplasts using full length EcR and USP showed that the activation domain of EcR is weak in plant cells and USP was required for ligand binding and activation (data not shown). Subsequently, we made a chimeric transcription activator GVE (Figure 1(A)) comprising cassava vein mosaic virus (CsV) promoter, GAL4 DNA binding domain (amino acids 1–147), VP16 activation domain (amino acids 410–490), and EcR ligand binding domain (amino acids 206–539). GVE induced a GAL4-responsive luciferase gene (LUC) 12-fold (Figure 2). Similarly, chimeric activator LVE (Figure 1(A)) containing LexA DNA binding domain (amino acids 1–202) in place of GAL4 induced LexA-responsive luciferase gene (6 × L-LUC) 7-fold (Figure 2). Inclusion of USP in the transfection had no effect on activation (data not shown). Replacing the DNA binding domain of EcR with GAL4 or LexA DNA binding domain, which consist of both the DNA binding and dimerization regions, obviated the need for the EcR–USP heterodimer.

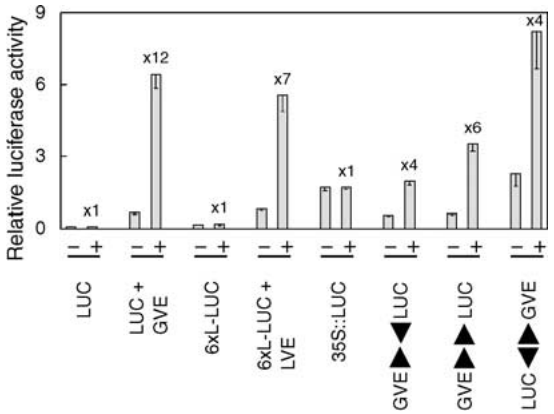

Figure 2. Induction of luciferase activity in tobacco protoplasts. Tobacco BY2 protoplasts were transformed with GAL4-responsive luciferase gene (LUC) alone or in combination with GVE activator, LexA-responsive luciferase gene (6 × L-LUC) alone or in combination with LVE activator, constitutively expressed luciferase gene (35S::LUC), or plasmids containing both LUC and GVE genes (GVE →← LUC, GVE →→ LUC, LUC ←→ GVE). Protoplasts were incubated in the absence (−) or presence (+) of 10 µM methoxyfenozide. Relative luciferase activities represent normalized luciferase activity, shown as ratios of luciferase to *Renilla* luciferase enzyme activity. Fold-inductions relative to luciferase activity in the absence of methoxyfenozide are indicated above each column. Each data point is the result of 8–16 transformations and the bars represent standard error. The direction of arrowhead in GVE and LUC constructs depict the direction of transcription.

In the above experiment, protoplasts were co-transfected with activator and reporter genes on separate plasmids. We subsequently made plasmids with three orientations of the GVE activator and LUC

reporter gene in tandem (Figure 1(B)) and tested them in protoplasts. The results presented in Figure 2 show that basal as well as induced levels of luciferase activity were low when GVE and LUC were in the tail-to-tail (GVE $\rightarrow\leftarrow$ LUC) or head-to-tail (GVE $\rightarrow\rightarrow$ LUC) orientations and high when they were in the head-to-head orientation (LUC $\leftarrow\rightarrow$ GVE). These results indicate that orientations of activator and reporter genes can affect the basal and induced levels of gene expression.

Activation of transgene expression in Arabidopsis and tobacco

Binary plasmids for plant transformation were made with GVE and LUC in three orientations and LVE and 6 × L-LUC in one orientation. The T-DNA regions of the pBIN19-based constructs are shown in Figure 1(C). A constitutively expressed *Renilla* luciferase gene (35S::RLUC) was also cloned into the binary plasmid containing GVE and LUC as an internal control for luciferase activity determination. Binary plasmids containing the GAL4-responsive luciferase gene (LUC) or constitutively expressed luciferase gene (35S::LUC) were used as controls (Figure 1(C)). The orientation of NPTII and 35S::RLUC genes were the same in each binary plasmid containing activator and reporter genes.

The six binary constructs shown in Figure 1(C) were transformed into *Arabidopsis* and tobacco, and inducer-mediated induction of luciferase was assessed. The luciferase activity was assayed by incubating leaf discs from T_1 plants in the absence or presence of 10 μM methoxyfenozide for 24 h. The luciferase activities observed in plants transgenic for LUC alone were similar to activities observed in wild type plants (0.1–0.2 relative light units (RLU)/μg protein), suggesting that the 5 × GAL4 response element and minimal 35S promoter has no or low levels of detectable activity in *Arabidopsis* and tobacco plants (data not shown). The results from 56 *Arabidopsis* and 56 tobacco plants transgenic for GVE and LUC in three orientations and 22 *Arabidopsis* plants transgenic for LVE $\rightarrow\leftarrow$ 6 × L-LUC are summarized in Table 1. The following general observations can be made from the data obtained. (1) Plants transgenic for GVE $\rightarrow\leftarrow$ LUC, GVE $\rightarrow\rightarrow$ LUC, and LVE $\rightarrow\leftarrow$ 6 × L-LUC showed lower basal and induced luciferase expression levels than plants transgenic for LUC $\leftarrow\rightarrow$ GVE. (2) Many transgenic plants had low basal activities with induced luciferase activities that were many times higher than constitutive 35S::LUC plants. (3) Some plants had little or no luciferase activity both under induced and uninduced conditions while some plants showed the same levels of luciferase activity in both uninduced and induced conditions. (4) Fold-inductions up to 7649 were observed among transgenic plants.

We propagated four T_1 *Arabidopsis* plants transgenic for GVE $\rightarrow\leftarrow$ LUC and GVE $\rightarrow\rightarrow$ LUC to T_2 and collected seed. T_2 plants were not assayed for luciferase induction. These plants were selected to represent plants with low basal and high induced expression levels. T_3 seed from each T_2 plant was germinated on two agar plates, one with 10 μM methoxyfenozide and one without. Seventeen-day-old seedlings from each plate were assayed for luciferase activity. Average basal and induced activities of T_3 progeny derived from each T_2 plant is shown in Figure 3. The average luciferase activity varied from 777 to 3811 RLU/μg protein while fold-inductions varied from 347 to 32,300. Progeny derived from these T_3 plants were assayed again in the T_4 generation. The average luciferase activities in T_4 among the four lines varied from 877 to 12,629 RLU/μg protein with the fold induction ranging from 1605 to 4872 (Figure 3). These results show that induction of luciferase activity is stable over four generations. The variation observed between the T_1, T_3, and T_4 generations may be due to the assay methodology (leaf disc assays in T_1 and seedling assays in T_3 and T_4), heterozygosity, sample size, and others.

The three binary vectors with GVE activator and LUC reporter combination also contained the constitutive *Renilla* luciferase gene (35S::RLUC; Figure 1(C)). *Renilla* luciferase activity, measured simultaneously with the luciferase enzyme in the same assay, was used as an internal assay control as well as to confirm the transgenic nature of plants. We also computed the relative luciferase activities of transgenic plants as a ratio of luciferase to *Renilla* luciferase activities. There was a general agreement between fold-inductions based on relative luciferase activity and luciferase activity (RLU/μg protein). For example, the average basal and induced LUC/RLUC ratios for 34 *Arabidopsis* plants transgenic for GVE $\rightarrow\leftarrow$ LUC were 0.025 (range 0.001–0.19) and 5.13 (range 0.001–34.2), respectively. The average fold induction based on LUC/RLUC ratios was 1627 (range 1–7556) compared to 1324 (range 1–7647) based on relative light units per microgram of protein.

Table 1. Summary of luciferase gene activation in transgenic *Arabidopsis* and tobacco plants

Construct	Plants tested	Positive events[a]	Luciferase activity (RLU/μg)[b]		Fold-induction[c]
			Basal	Induced	
Arabidopsis					
GVE →← LUC	34	32	15 (0.2–89)	918 (1–3227)	1324 (1–7649)
GVE →→ LUC	10	10	21 (0.5–189)	417 (6–1753)	867 (1–4777)
LUC ←→ GVE	12	12	777 (5–2665)	5143 (117–18183)	10 (2–23)
LVE →← 6 × L-LUC	22	19	0.2 (0.1–2.2)	64 (3–413)	643 (1–1583)
35S::LUC[d]	12	11	82 (1–247)	95 (3–429)	1 (1–3)
Tobacco					
GVE →← LUC	15	11	1 (0.1–5)	42 (0.1–220)	69 (1–267)
GVE →→ LUC	21	16	8 (0.1–51)	43 (0.1–315)	22 (1–341)
LUC ←→ GVE	20	17	34 (1–299)	160 (1–562)	10 (1–38)
35S::LUC[d]	15	10	52 (0.1–269)	48 (0.1–214)	1 (1–2)

[a] Positive events are transgenic T_1 plants with induced luciferase activity higher than the non-transgenic plants.
[b] Luciferase activities (relative light units/μg protein) given are mean (range) for all the plants tested.
[c] Fold-induction is the inducer (10 μM) dependent luciferase activity over the basal (i.e., uninduced) activity. Values given are mean (range) of all the plants tested.
[d] 35S::LUC plants are expected to have similar basal and induced luciferase activities.

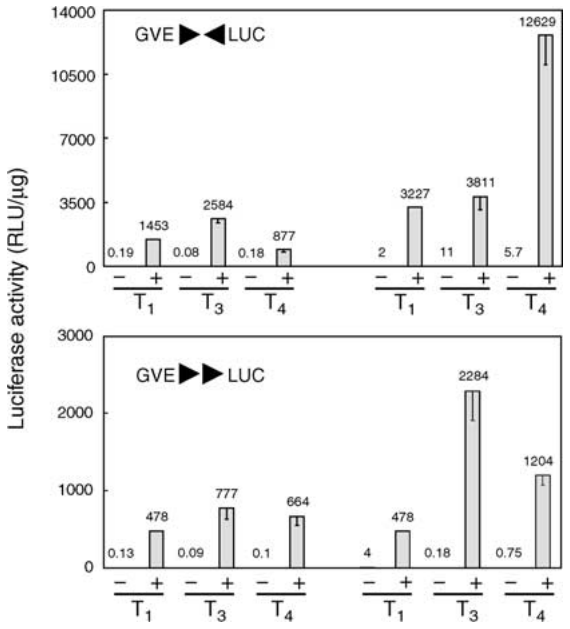

Figure 3. Induction of luciferase activity in *Arabidopsis* lines transgenic for GVE →← LUC and GVE →→ LUC. T_1 plants were assayed by incubating leaf discs in the absence (−) or presence (+) of 10 μM methoxyfenozide and values shown are average of two leaf discs. T_3 and T_4 seedlings were germinated in the presence or absence of 10 μM methoxyfenozide and 17-day-old plants were assayed for luciferase activity. Each T_3 and T_4 column shows the average luciferase activity in four T_3 plants and 27 T_4 plants derived from a single T_2 plant, respectively. Bars represent standard error. RLU/μg, relative light units per microgram of protein.

Dose-response and systemic induction

In all assays we used 10 μM methoxyfenozide for gene activation. Initial experiments in protoplasts showed that 5–10 μM inducer is optimal for activation. To know the dynamic range of gene activation in transgenic *Arabidopsis* and tobacco plants, seed collected from an *Arabidopsis* T_2 plant transgenic for GVE →→ LUC and a tobacco T_1 plant transgenic for GVE →← LUC was germinated on agar media containing various concentrations of methoxyfenozide (0.01–30 μM). Seedlings were assayed for luciferase activity 17 days later. The basal luciferase activity in transgenic plants was similar to the wild type plants and the luciferase activity was induced at the lowest concentration of methoxyfenozide (0.01 μM) tested (Figure 4). Luciferase activity increased with the increasing concentrations of methoxyfenozide and reached a peak at 10 μM and declined at 30 μM. The luciferase activity at 10 μM was ∼21,000 and ∼4370-fold higher than the basal activity for *Arabidopsis* and tobacco, respectively.

We also utilized mature tobacco plants grown in the greenhouse to test the systemic response in plants that are not actively growing. T_2 plants derived from a T_1 plant transgenic for LUC ←→ GVE were grown in pots until they developed pods and some of the bottom leaves senesced. Three root drench applications of methoxyfenozide at 0.2, 1, 5, or 20 μM in water were

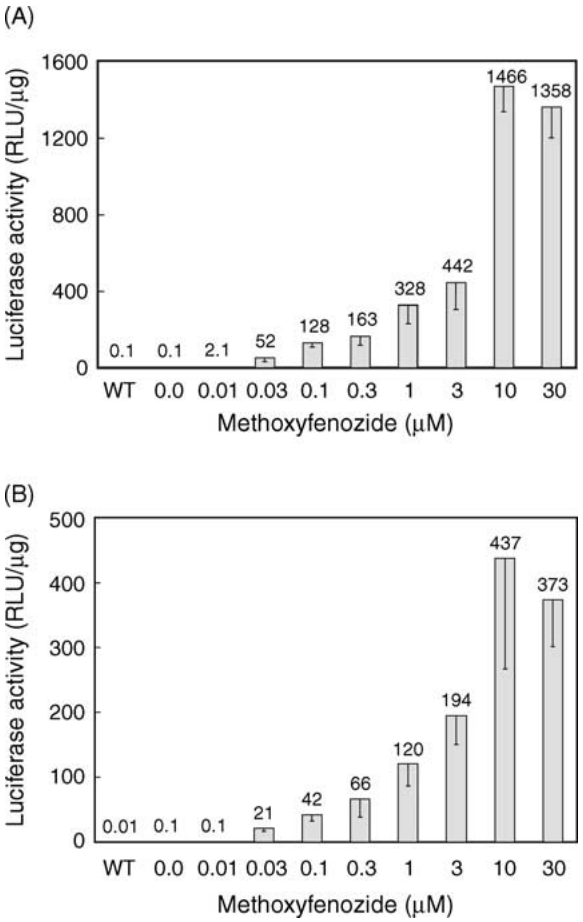

Figure 4. Methoxyfenozide dose-dependent induction of luciferase activity in *Arabidopsis* and tobacco plants. Seed collected from an *Arabidopsis* T_2 plant transgenic for GVE →→ LUC (A) and a tobacco T_1 plant transgenic for GVE →← LUC (B) were germinated on agar medium containing various concentrations of methoxyfenozide and 17-day-old seedlings were assayed for luciferase activity. Values indicated above each column are luciferase activities (given as relative light units (RLU)/µg protein) and error bar represent standard error based on eight plants. WT, wild type plants.

applied to pots on days 0, 3, and 6. Luciferase activity was assayed in leaf discs collected from two middle and two top leaves on days 0 (before the ligand application), 1, 3, 7, and 16. Luciferase induction was observed within a day for all the concentrations tested (Figure 5). The activity increased as the ligand concentration increased.

Discussion

In an effort to regulate gene expression in plants we developed a system based on EcR and a commercially

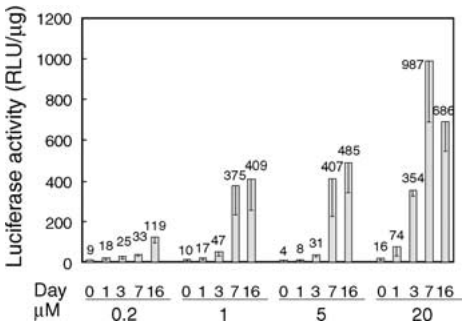

Figure 5. Systemic induction of luciferase activity in tobacco plants. T_2 plants transgenic for LUC ←→ GVE were grown in pots until maturity (4-month-old plants with flowers and pods). Methoxyfenozide (0.2, 1, 5, or 20 µM) in water was applied to pots on days 0, 3, and 6 (1 l per application). Leaf discs were collected and assayed on days 0, 1, 3, 7, and 16. Luciferase activity on day 0 is the basal activity, assayed just before the first application of the inducer. Values shown above each column are relative light units (RLU)/µg protein. The error bar represents standard error based on four leaf discs from two plants.

available inducer, methoxyfenozide. The system was optimized by constructing chimeric transcription activators and by cloning activator and reporter genes in different orientations. Our data show that basal luciferase activity in many transgenic plants was similar to wild type plants and induced levels reached several fold higher than the constitutive 35S::LUC levels. Further, the transgene induction responded proportionally to a dynamic range of methoxyfenozide concentrations. The luciferase induction was observed within 30 min of ligand application (data not shown).

Although we obtained T_1 plants with high fold inductions, some transformants had little or no luciferase activity while others showed high levels of luciferase in both induced and uninduced conditions. Variable expression of transgenes in independent transgenic plants is a common phenomenon (reviewed by Gelvin, 1998). One of the hypotheses proposed to explain the variation is that the local chromatin domain determines the gene expression. Some of the variation may also be explained on the basis of T-DNA integration patterns and integration of partial T-DNA. Over all, plants with LUC ←→ GVE showed higher basal and induced activities compared to plants with GVE →← LUC or GVE →→ LUC. These results can be explained on the basis of enhancer activity of CsV promoter on the minimal 35S promoter (TATA box) 5′ to the luciferase gene (Figure 1(B)). CsV and 35S promoters are from viruses of *Caulimoviridae* family and have similar sequence structure (Verdaguer et al., 1996).

Recently it was shown that the GAL4-mediated expression in tobacco was susceptible to methylation-induced silencing (Galweiler et al., 2000). The GAL4 response element was extensively methylated at cytosine in transgenic tobacco and the methylation inhibited GAL4 binding to its response element. Our study was not designed to address the stability of GAL4-mediated gene expression. We also used LexA DNA binding domain (Horii et al., 1981) to induce gene expression. The 17 bp GAL4 response element used in this study has two 5′-CG-3′and one 5′-CNG-3′ methylatable sequences whereas the LexA operator we used (Estojak et al., 1995) has one 5′-CNG-3′ methylatable sequence. It is not know if LexA binding site is methylated or methylation of the LexA binding site affects LexA binding. Our results show that low basal and high induced luciferase activities can be obtained with LVE activator.

The ability to tightly regulate gene expression in plant cells is an effective tool for the elucidation of gene function. Chemical-inducible gene expression systems have advantages over other systems as heterologous transcription activators are used and transgene induction can be regulated in a spatial and temporal fashion using tissue or developmental specific promoters to express the activator, and by applying inducer at a desired time (reviewed by Gatz, 1997; Gatz & Lenk, 1998; Jepson et al., 1998; Zuo & Chua, 2000). Gene expression can also be restricted to a particular window by constructing transactivators that can be switched on with one chemical and switched off with another chemical (Bohner et al., 1999). In addition, transgenes can be expressed at physiologically relevant levels by varying the inducer concentration. The best use of chemical-inducible systems will be in modulating the endogenous gene expression by targeting a transactivator or repressor to native promoters. Progress in designing proteins that bind to any given unique DNA sequence in the genome (Beerli et al., 2000; Klug, 1999) will aid outgrowth of such applications.

While no single chemical-inducible system may have all the desirable properties, the type of system one can use will depend on the gene being expressed or repressed and the specific application. For example, an inducible system that has no basal and very low induced activity but without a dynamic range to inducer concentrations may still be ideal for expressing transcription factors where a few molecules can turn on or off a phenotype. Also, high basal levels can be tolerated if the application is for expression of large quantities of protein in transgenic plants. The system described here complements the existing systems and has potential for regulating gene expression under field conditions. Similar to our study, a system using non-steroidal ecdysone agonist tebufenozide and a chimeric activator containing *Heliothis virescens* EcR receptor, glucocorticoid receptor DNA binding domain, and VP16 activation domain was shown to exhibit high levels of induced gene expression (Martinez et al., 1999). Our study differs from the earlier study in that we used the GAL4 and LexA DNA binding domains, spruce budworm EcR ligand binding domain, and a new ligand Methoxyfenozide. Methoxyfenozide is 6–10 times more active than tebufenozide (unpublished results). Methoxyfenozide has an excellent safety profile – is safe to humans, animals, and plants (Dhadialla et al., 1998). We did not observe any unusual phenotypes in the plants expressing the activator. Further improvements to EcR-based, methoxyfenozide-inducible system may be possible by modifying the ligand binding domain and using different DNA binding and activation domains. Multiple-inducible systems to independently regulate several genes can be developed using EcRs from different classes of insects and synthesizing inducers specific to each EcR.

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