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ORIGINAL PAPER

Development of a tightly regulated and highly inducible ecdysone receptor gene switch for plants through the use of retinoid X receptor chimeras

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Abstract Chemical inducible gene regulation systems provide essential tools for the precise regulation of transgene expression in plants and animals. Recent development of a two-hybrid ecdysone receptor (EcR) gene regulation system has solved some of the drawbacks that were associated with the monopartate gene switch. To further improve the versatility of the two-hybrid EcR gene switch for wide spread use in plants, chimeras between *Homo sapiens* retinoid X receptor (HsRXR) and insect, *Locusta migratoria* RXR (LmRXR) were tested in tobacco protoplasts as partners with *Choristoneura fumiferana* EcR (CfEcR) in inducing expression of the luciferase reporter gene. The RXR chimera 9 (CH9) along with CfEcR, in a two-hybrid format gave the best results in terms of low-background expression levels in the absence of ligand and high-induced expression levels of the reporter gene in the presence of nanomolar concentrations of the methoxyfenozide ligand. The performance of CH9 was further tested in corn and soybean protoplasts and the data obtained was compared with the other EcR switches that contained the wild-type LmRXR or HsRXR as EcR partners. In both transient expression studies and stable transformation experiments, the fold induction values obtained with the CH9 switch were several times higher than the values obtained with the other EcR switches containing LmRXR or HsRXR. The new CfEcR two-hybrid gene switch that uses the RXR CH9 as a partner in inducing reporter gene expression provides an efficient, ligand-sensitive and tightly regulated gene switch for plants.

Keywords Ecdysone receptor · Methoxyfenozide · Retinoid X receptor · RXR chimera · Transgenic plants · Two-hybrid gene switch

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Introduction

Regulation of transgene expression is of particular importance in genetic engineering of plants. In order to achieve an optimal expression of transgenes with minimum undesirable effects, it is

highly desirable to regulate the expression of transgenes in a controllable fashion. A number of chemical inducible gene regulation systems, or gene switches, have been developed based on a diverse collection of non-plant regulatory elements that respond to a variety of chemicals (Gatz et al. 1992; Wilde et al. 1992; Williams et al. 1992; Mett et al. 1993; Rieping et al. 1994; Weinmann et al. 1994; Aoyama and Chua 1997; Caddick et al. 1998; Bohner et al. 1999; Martinez et al. 1999a, b; Bruce et al. 2000; Zuo et al. 2000; Padidam et al. 2003). A chemical inducible gene regulation system that specifically regulates transgene expression in response to an exogenous inducer at a particular stage of plant development or in a specific organ is very valuable when using transgenes whose constitutive over expression is detrimental or lethal to the plant. In addition, gene switches are also useful in reducing the environmental concerns such as gene pollution and antibiotic resistance development associated with genetically modified crops (Hare and Chua 2002; Palli 2005; Palli et al. 2005a).

An optimal chemical inducible gene switch should support undetectable levels of transgene expression in the absence of a chemical ligand followed by rapid and robust induction of transgene expression in the presence of low concentrations (nanomolar) of a chemical ligand. In an effort to find an optimal gene switch, several approaches have been tried (Ainley and Key 1990; Schena et al. 1991; Gatz et al. 1992; Mett et al. 1993; Lloyd et al. 1994; Weinmann et al. 1994; Aoyama and Chua 1997; Bruce et al. 2000; Zuo et al. 2000). However, most of the gene switches developed to date for use in plants are not suitable for field applications because of the impractical or incompatible characteristics of the chemical ligands. Furthermore, testing of these gene switches has been limited to one or two plant species such as Arabidopsis, tobacco or maize (Faiss et al. 1997; Bruce et al. 2000; Zuo et al. 2001; Unger et al. 2002; Chen et al. 2003; Laufs et al. 2003). None of the gene switches developed to date have most of the desirable properties (high specificity, low activity in the absence of ligand, robust and rapid response in the presence of ligand and functions in a wide variety of plant species) of an optimal gene regulation system (Lloyd et al. 1994; Corlett et al. 1996; Gatz 1996; Kang et al. 1999; Zuo and Chua 2000; Ouwerkerk et al. 2001; Andersen et al. 2003; Tang and Newton 2004).

Gene switches based on the insect ecdysone receptor (EcR) and its non-steroidal ligand are particularly attractive since commercially available non-steroidal ecdysone agonists, such as tubefenozide and methoxyfenozide have been approved for field use (see Palli et al. 2005a for a recent review). Ligand-binding domains from EcRs have been used to develop EcR gene switches for use in plants. Despite the demonstrated utility of EcR gene switches in diverse applications, it is apparent that tight gene regulation without background expression levels was not generally achieved with these constructs (Martinez et al. 1999a, b; Padidam et al. 2003). In addition, EcR gene switches developed to date require micromolar concentration of ligand for activation of the target gene (Padidam et al. 2003; Koo et al. 2004) and this has limited the use of this gene switch for large scale field application.

An EcR gene switch with a potential for use in large-scale field applications and its applicability to a variety of plant species has been developed by adopting a two-hybrid format (Tavva et al. 2006). In a two-hybrid switch format, the GAL4 DNA binding domain (GAL4 DBD) was fused to the ligand binding domain (LBD) of the *Choristoneura fumiferana* ecdysone receptor (CfEcR); and, the VP16 activation domain (VP16 AD) was fused to LBD of *Locust migratoria* retinoid X receptor (LmRXR) or *Homo sapiens* retinoid X receptor (HsRXR). Upon application of methoxyfenozide, the heterodimer of these two fusion proteins transactivates the luciferase reporter gene placed under the control of multiple copies of cis acting elements and a minimal 35S promoter. The sensitivity of the CfEcR gene switch was improved from micromolar to nanomolar concentrations of ligand by using the CfEcR : LmRXR two-hybrid combination and a reduction in the background expression levels was achieved by using the CfEcR : HsRXR two-hybrid combination (Tavva et al. 2006). However, the ideal switch should be an EcR switch that uses a RXR partner with sensitivity achieved by LmRXR and low background expression achieved by HsRXR.

We hypothesized that the differences in ligand sensitivity and background expression level of the reporter gene in the absence of ligand supported by CfEcR : LmRXR and CfEcR : HsRXR switches was most likely due to the differences in the ability of Lm or HsRXR to heterodimerize with CfEcR in the absence of ligand. To test this hypothesis and to develop an EcR gene switch that is highly sensitive and exhibited low-background activity in the absence of ligand, we developed and tested chimeras between HsRXR and LmRXR as a partner for CfEcR (Fig. 1). The data presented here show that a gene switch containing the GAL4 DBD fusion protein of CfEcR in combination with a VP16 AD fusion protein of RXR chimera containing helices 1–8 from HsRXR and helices 9–12 from LmRXR (Chimera 9, CH9) supported low-basal expression of the reporter gene in the absence of ligand and high-induced expression of the reporter gene in the presence of nanomolar concentrations of ligand. The performance of this new switch was compared to the performance of EcR gene switches containing LmRXR and HsRXR as partners of CfEcR in inducing the luciferase reporter gene in tobacco, soybean and corn protoplasts. The results observed in protoplasts were confirmed in Arabidopsis transgenic plants.

Materials and methods

DNA manipulations

The LBD of HsRXR and LmRXR consists of 12 helices and the heterodimerization between EcR and RXR is dictated by the amino acid sequences present in the 9, 10, and 11 helices. In order to develop a tight two-hybrid EcR gene switch for plants, RXR chimeras were generated as described in Palli et al. (2005b), by fusing different helices from the LBD of HsRXR with different helices from the LBD of LmRXR (Fig. 1).

For transient expression studies, the receptor (EcR and RXR chimeras) and reporter (–46 35S:Luc) gene expression cassettes were cloned into the pKYLX80 vector (Tavva et al. 2006). Chimeras containing helices 1–5 from HsRXR and helices 6–12 from LmRXR were designated as CH6 (Fig. 1). Similarly, chimeras that contain helices 1–7, 1–8, and 1–10 of HsRXR fused to helices 8–12, 9–12, and 11–12, respectively, from LmRXR were designated as CH8, CH9, and CH11 (Fig. 1). The RXR chimeras were cloned downstream of the VP16 AD (*Herpes simplex* transcription AD) sequence in pVP16 vectors (BD Biosciences Clontech, San Jose, CA, USA). DNA sequences coding for the fusion proteins of VP16 AD and RXR chimeras were transferred from

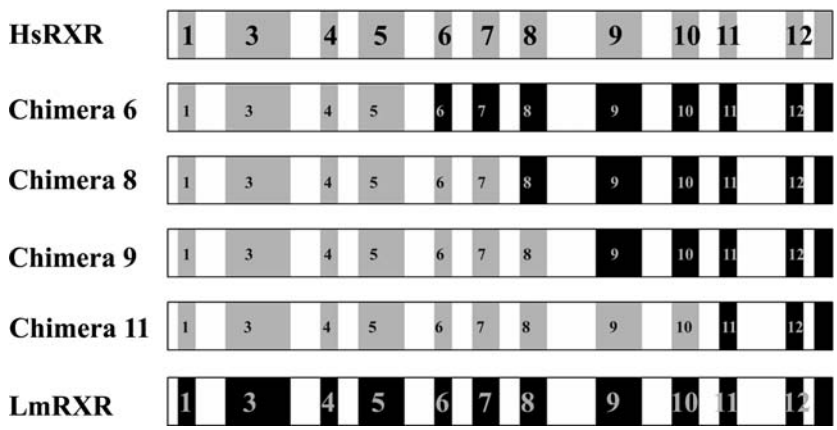

Fig. 1 Structure of Hs-LmRXR chimeras. Chimeric receptors were generated by fusing different helices in the ligand binding domains (EF) of *Homo sapiens* retinoid X receptor (HsRXR) with *Locust migratoria* retinoid X receptor (LmRXR). Chimera 6 1–5 helices of HsRXR fused with 6–12 helices of LmRXR, Chimera 8 1–7 helices of HsRXR fused with 8–12 helices of LmRXR, Chimera 9 1–8 helices of HsRXR fused with 9–12 helices of LmRXR, Chimera 11 1–10 helices of HsRXR fused with 11–12 helices of LmRXR

pVP16RXR chimera constructs (Palli et al. 2005b) using *Nhe*I and *Xba*I restriction endonucleases and cloned into the *Xba*I restricted pKYLX80 vector (Fig. 2). The resulting constructs were designated as pK80VCH6; pK80VCH8; pK80VCH9 and pK80VCH11. A detailed description of the construction of pK80GCfE and pK80–46 35S:Luc was reported in our previous publication (Tavva et al. 2006). Briefly, the D, E, and F domains of CfEcR were cloned downstream of the GAL4 DBD sequence in the pM vector (BD Biosciences Clontech). The fusion gene, GAL4 DBD : CfEcR was excised from the pM vector and cloned into the pKYLX80 vector (pK80GCfE). The reporter construct (pK80–46 35S:Luc) was generated by cloning the luciferase gene under the control of a CaMV 35S minimal promoter (–46 to +5 bp) and five copies of GAL4 response elements (Fig. 2c).

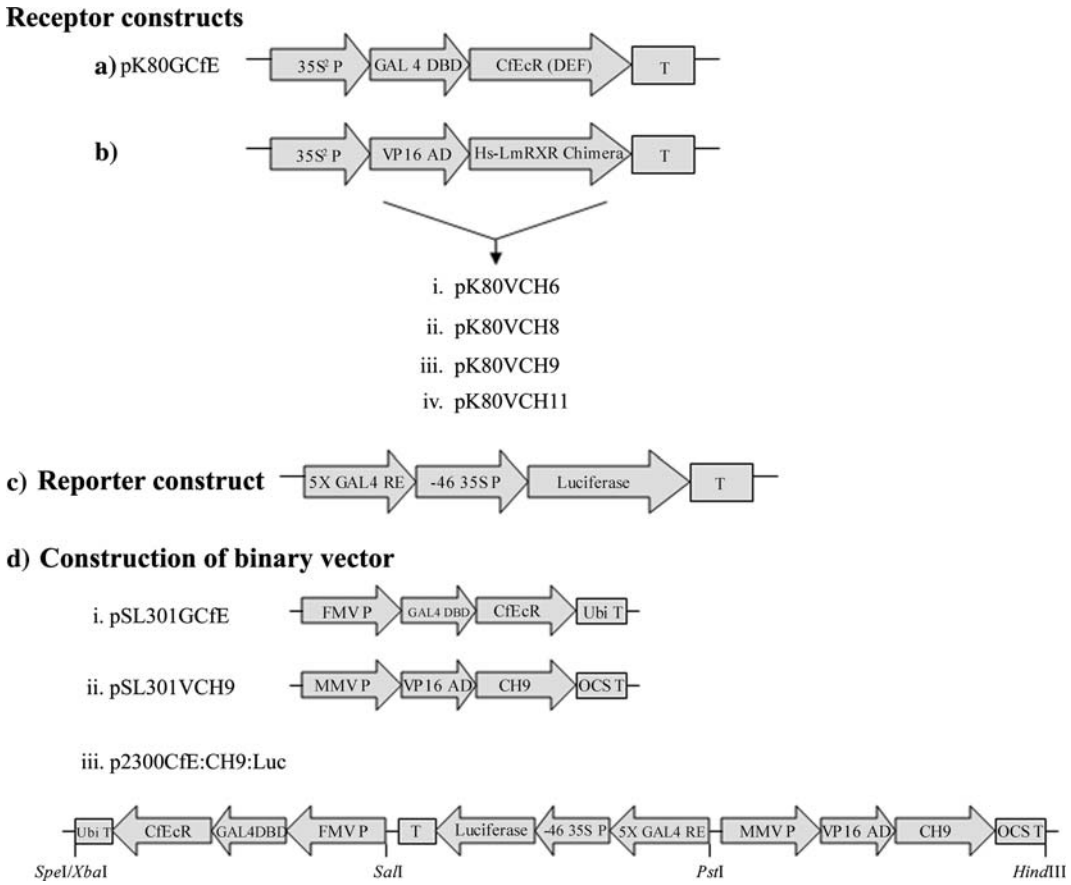

Fig. 2 Schematic representation of the receptor and reporter gene constructs. a pK80GCfE: GAL4 DBD fusion of CfEcR was cloned into pKYLX80 (pK80) vector. b VP16 AD fusions of Hs-LmRXR chimeras were cloned into pK80 vector. pK80VCH6, pK80VCH8, pK80VCH9, and pK80VCH11: receptor constructs, where the VP16 activation domain is fused to RXR chimera 6 or 8 or 9 or 11. c Reporter construct (pK80–46 35S:Luc): Reporter gene expression cassette was constructed by cloning the luciferase reporter gene under the control of –46 35S minimal promoter (–46 35S P) and five copies of GAL4 response elements (5X GAL4 RE). d Construction of the binary vector for plant transformation: (i) pSL301GCfE: pSL301 vector consists of an FMV promoter driven CfEcR expression cassette (FMV P : GAL4 DBD : CfEcR : Ubi T); (ii) pSL301VCH9: pSL301 vector consists of an MMV promoter driven RXR chimera 9 expression cassette (MMV P : VP16 AD : CH9 : OCS T); (iii) p2300CfE : -CH9 : Luc: T-DNA region of the pCAMBIA2300 binary vector showing the assembly of receptor and reporter expression cassettes. $35S^2$ P CaMV 35S promoter with double enhancer sequence, T rbcS Poly A sequence, FMV P figwort mosaic virus promoter, Ubi T ubiquitin 3 terminator, MMV P mirabilis mosaic virus promoter, OCS T *Agrobacterium tumefaciens* octopine synthase poly A

For the construction of a binary vector for plant transformation the GAL4 DBD : CfEcR fusion gene was cloned under the figwort mosaic virus (FMV) promoter and ubiquitin 3 (Ubi) terminator sequence and the VP16 AD : CH9 fusion gene was cloned under the mirabilis mosaic virus (MMV) promoter and *Agrobacterium tumefaciens* octopine synthase (OCS) poly A sequences. The FMV and MMV promoter driven expression cassettes were assembled into pSL301 vectors (Fig. 2c). The reporter and receptor expression cassettes were excised with appropriate restriction enzymes and assembled into the pCAMBIA2300 vector (CAMBIA, Canberra, ACT, Australia) for plant transformation. The resulting binary vector was designated p2300CfE : CH9 : Luc.

Transient expression studies

Transient expression studies were carried out by isolating protoplasts from cell suspension cultures of tobacco (*Nicotiana tabacum* cv. Xanthi-Brad), corn [*Zea mays* cv. Black Mexican Sweet (BMS)], and soybean [*Glycine max* cv. Williams 82 (W82)]. A detailed description of the isolation and electroporation of protoplasts was described earlier (Tavva et al. 2006).

Dose response study with tobacco protoplasts

The performance of different RXR chimeras in inducing luciferase reporter gene activity in a two-hybrid format was tested by co-electroporating pK80–46 35:Luc, pK80GCfE, and RXR chimera (pK80VCH6 or pK80VCH8 or pK80VCH9 or pK80VCH11) constructs. Electroporated protoplasts were resuspended in 1 ml of growth medium containing different concentrations of methoxyfenozide, 0 (DMSO control), 0.64, 3.2, 16, 80, 400, 2,000, and 10,000 nM. Twenty-four hours after addition of ligand, the protoplasts were assayed for luciferase reporter gene activity using a Fluoroscan FL plate reader (Fluoroscan Ascent FL, Thermo labsystems, Milford, MA, USA) as described earlier (Tavva et al. 2006).

Dose response study with corn and soybean protoplasts

The most efficient combination of receptor constructs (pK80GCfE + pK80VCH9) in inducing luciferase reporter gene activity was further tested in corn and soybean protoplasts with a methoxyfenozide dose-response study. Electroporated protoplasts were incubated in the growth medium containing 0, 3.2, 16, 80, 400, 2,000, and 10,000 nM concentrations of methoxyfenozide. Luciferase activity was measured 24 h after addition of ligand.

Plant tissue culture

Arabidopsis thaliana (L.) Heynth. ecotype Columbia ER was used for plant transformation experiments. The binary vector (p2300CfE : -CH9 : Luc) constructed for plant transformation was mobilized into *Agrobacterium tumefaciens*, strain GV3850 by the freeze-thaw method. Arabidopsis plants were transformed using the whole plant-dip method (Clough and Bent 1998). Transgenic Arabidopsis plants were selected by germinating the seed collected from the infiltrated plants on a medium containing 50 mg/l kanamycin. The analysis of transgenic plants for luciferase induction levels was carried out on T_2 and T_3 generations. Segregation pattern of the T_2 and T_3 seed plated on kanamycin containing medium indicated that the Arabidopsis lines, At1, At2, At3, At4, At5, and At6 were heterozygous at the transgene locus. For this reason the transgenic lines used in all the experiments were screened on kanamycin containing medium.

Southern and Northern blot analyses

Southern and Northern blot analyses on transgenic Arabidopsis plants were carried out to verify the transgene integration pattern and its expression levels. Genomic DNA was isolated from transgenic and untransformed (Columbia ER) Arabidopsis plants as per the manufacturer's instructions by homogenizing 100 mg of seedling tissue in 300 µl of Plant DNAzol® reagent (Invitrogen™, Life technologies, Carlsbad, CA, USA). In order to eliminate RNA contamination in the

samples, 100 μg of RNase per ml of Plant DNAzol was added at the beginning of the isolation procedure. Ten micrograms of total genomic DNA from each plant was digested overnight with *Hin*dIII; separated on a 0.7% agarose gel; and, blotted onto a Zeta-probe® GT blotting membrane (Bio-Rad Laboratories, Hercules, CA, USA). Hybridization was done overnight at 65°C in a phosphate buffered solution (0.12 M sodium phosphate buffer, pH 7.2; 0.25 M sodium chloride; 1 mM EDTA; 7% sodium dodecyl sulfate) with GAL4 DBD : CfEcR fusion gene random primed with α^{32}P dCTP using the Prime-It® II Random Primer Labeling Kit (Stratagene®, Cedar Creek, TX, USA). After completion of the hybridization, the membrane was rinsed briefly with 2 × SSC and then washed successively by vigorous agitation at room temperature for 15 min in 2 × SSC + 0.1% SDS followed by 15 min in 0.5 × SSC + 0.1% SDS. Finally, the membrane was given a high-stringency wash with 0.1 × SSC and 0.1% SDS at 65°C and exposed in a phosphorimager cassette (Molecular Dynamics, Sunnyvale, CA, USA). The intensities of the bands were detected using the ImageQuanta™ software (Molecular Dynamics).

Total RNA was isolated from the transgenic and untransformed Arabidopsis plants by homogenizing 100 mg of seedling tissue in 1 ml of TRIzol® reagent (Invitrogen, Life technologies). Ten micrograms of total RNA was separated on 1.2% agarose gels containing formaldehyde and transferred onto a Zeta probe membrane. Hybridization was done as described above for Southern blot hybridization with either GAL4 DBD : CfEcR or α-tubulin gene (Gene bank accession # M17189).

Dose-response study with T_2 Arabidopsis plants

Seed collected from six T_1 Arabidopsis lines were plated on agar medium containing 50 mg/l kanamycin and different concentrations of methoxyfenozide (0, 0.64, 3.2, 16, 80, 400, 2,000, and 10,000 nM). Seed were allowed to germinate and grow on this medium for 20 days at 25°C, 16 h light/8 h dark. Three seedlings from each plate were collected separately and ground in a volume of 100 μl of 1 × passive lysis buffer (Promega Corporation, Madison, WI, USA) and luciferase activity was measured.

Time-course study with T_3 Arabidopsis plants

Seed collected from the T_2 Arabidopsis line, At2 was plated on agar medium supplemented with 50 mg/l kanamycin, without methoxyfenozide. After 10 days, the seedlings were transferred to induction medium containing 50 mg/l kanamycin and different concentrations of methoxyfenozide (0, 16, 80, 400, 2,000, and 10,000 nM). Luciferase activity was measured in seedlings collected from the induction medium for 7 days with a 24-h interval.

Results

Transient expression studies with tobacco protoplasts

In our previous studies (Tavva et al. 2006), we showed that the LmRXR and HsRXR in combination of CfEcR supported methoxyfenozide inducible reporter gene activity. However, CfEcR : LmRXR two-hybrid combination showed higher ligand sensitivity and CfEcR : HsRXR two-hybrid combination showed lower background expression of the reporter gene in the absence of ligand. In order to develop a better EcR gene switch, we screened a number of HsRXR : LmRXR chimeras (CH6, CH8, CH9, and CH11) as partners of CfEcR in inducing the luciferase reporter gene activity in plant protoplasts isolated from tobacco cell suspension cultures. As shown in Fig. 3a, the background luciferase activity observed in the absence of ligand was lowest in CH9. When compared to the EcR gene switch that contained CH9, the switches that contained CH11, CH8, and CH6 showed higher levels of luciferase activity in the absence of ligand. The differences in luciferase activity in the absence of ligand were reflected in fold increase in reporter gene activity observed in protoplasts electroporated with EcR gene switches containing chimeras. As shown in Fig. 3b, the luciferase reporter gene regulated by the EcR gene switch containing CH9 showed

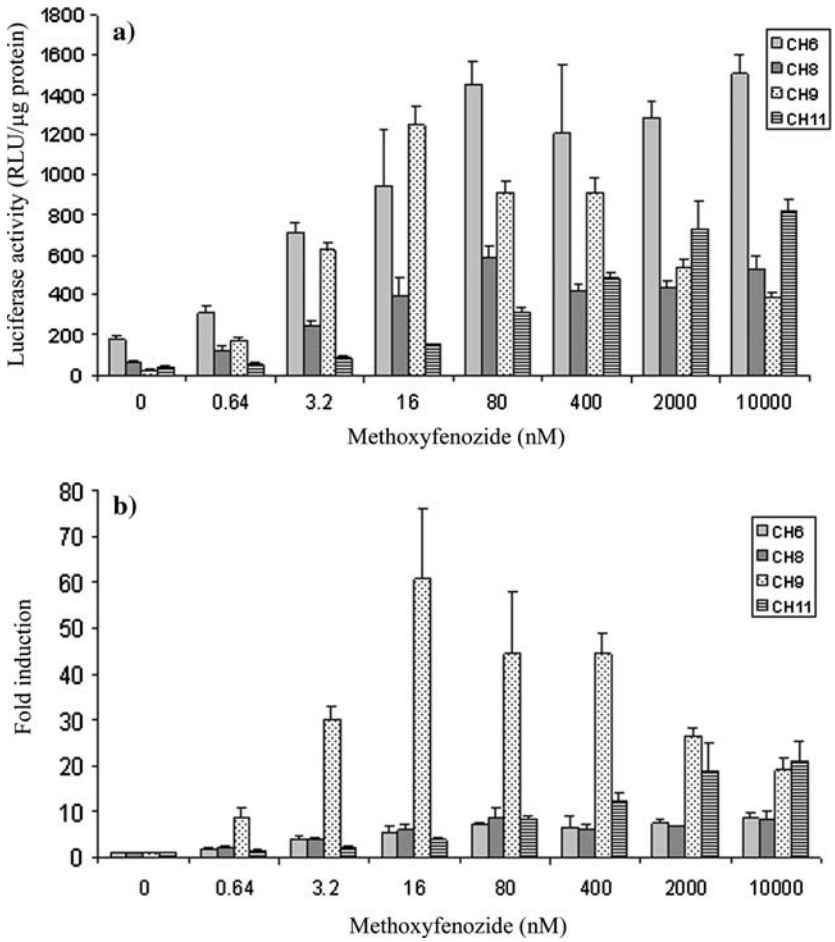

Fig. 3 Dose-dependent induction of the luciferase gene by EcR gene switches. Tobacco protoplasts were electroporated with pK80GCfE + pK80VCH6 or pK80VCH8 or pK80VCH9 or pK80VCH11 and reporter construct and the electroporated protoplasts were exposed to varying concentrations of methoxyfenozide. Luciferase activity was measured after 24 h of incubation and the values were expressed as relative light units per microgram protein (a). Fold induction values (b) shown were calculated by dividing RLU/µg protein in the presence of ligand with RLU/µg protein in the absence of ligand. The data represent the average of three replicates ±SD

higher fold induction when compared to the switches containing the other three chimeras. The two-hybrid gene switches containing RXR chimeras showed dose-response induction of luciferase activity in the presence of methoxyfenozide. An increase in luciferase activity was observed at the lowest concentration of methoxyfenozide tested (0.64 nM) in protoplasts electroporated with switch plasmids containing CH6, CH8, and CH9 (Fig. 3a). In the case of the switch containing CH11, an increase in luciferase activity was initiated at a concentration of 16 nM methoxyfenozide (Fig. 3b). Of the four chimeras tested, CH9 appears to be the most sensitive since the maximum induction of luciferase activity supported by the EcR gene switch containing this chimera was reached with 16 nM methoxyfenozide. In contrast, switches containing CH6 and CH8 showed maximum luciferase activity at 80 nM and CH11 showed maximum luciferase activity at 10,000 nM methoxyfenozide (Fig. 3a). Thus, the EcR gene switch containing CH9 supported low background activity in the absence of ligand as well as high-ligand sensitivity and was chosen for further testing in soybean and corn.

Comparison of luciferase induction levels by GCfE + VCH9, GCfE + VHsR, and GCfE + VLmR switches in tobacco, corn and soybean protoplasts

To compare ligand sensitivity and background expression levels of the luciferase reporter gene in the absence of ligand, combinations of receptor constructs, pK80GCfE + pK80VCH9, pK80GCfE + pK80VHsR, and pK80GCfE + pK80VLmR were electroporated into tobacco, corn, and soybean protoplasts. The electroporat-

ed protoplasts were exposed to various concentrations of methoxyfenozide and luciferase activity was quantified at 24 h after the addition of ligand. As shown in Table 1, dose-response induction of luciferase was supported by all three switches in all three plant species tested. In tobacco, soybean, and corn protoplasts the background activity of the luciferase gene in the absence of ligand was lower in the case of the EcR gene switch containing CH9 when compared to EcR gene switches containing LmRXR or HsRXR (Table 1). This lower background activity of the EcR gene switch containing CH9 was reflected in the fold induction of the luciferase. As shown in Table 1, in all three plant species, the EcR gene switch containing CH9 showed higher fold induction of the reporter gene activity when compared to the fold induction values observed with EcR gene switches containing LmRXR and HsRXR.

In tobacco protoplasts, luciferase activity supported by the EcR gene switch containing CH9 reached maximum levels at 16 nM methoxyfenozide (Fig. 3; Table 1). Alternatively, the luciferase activity supported by the EcR gene switch containing LmRXR and HsRXR reached maximum levels at 80 and 10,000 nM methoxyfenozide, respectively (Table 1). A similar pattern of a higher sensitivity of the EcR gene switch containing CH9 when compared to the EcR gene switches containing LmRXR and HsRXR was observed in corn protoplasts (Table 1). In soybean protoplasts, the EcR gene switch containing CH9 induced luciferase activity that reached a peak level (64.1) at 2,000 nM methoxyfenozide. However, it showed a 50.1-fold increase at 80 nM and 57.6-fold increase at 400 nM methoxyfenozide (Table 1). In all three plant species tested, the EcR switch containing CH9 appears to be most sensitive switch compared to the other two-hybrid gene switches containing LmRXR or HsRXR.

Stable transformation and analysis of transgenic plants

To further confirm the characteristics of the GCfE + VCH9 two-hybrid gene switch, stable transgenic Arabidopsis plants were recovered and analyzed. Southern and Northern blot analyses were carried out on six T_2 Arabidopsis lines (At1, At2, At3, At4, At5, and At6) to verify the transgene integration pattern and its expression levels. Total genomic DNA was isolated from these six lines, and the Southern blot hybridization of the *Hin*dIII digested DNA with the GAL4 DBD : CfEcR gene probe indicated that all the lines tested were positive and showed different transgene integration pattern (Fig. 4a). The results of the Southern blot hybridization analysis shown in the Fig. 4a revealed the presence of multiple gene insertions except in At4 line. The expression pattern of GAL4 DBD : CfEcR fusion gene was verified in all the six transgenic Arabidopsis plants by Northern blot hybridization (Fig. 4b). The level of GAL4DBD : CfEcR gene expression is slightly higher in lines At2 and At6 compared to the other lines tested. However, no significant differences were observed in the receptor gene expression levels as compared to the variation in the transgene copy numbers observed. Northern blot hybridization of total RNA was also carried out to verify the α-tubulin gene expression (Fig. 4b).

Dose response study with T_2 Arabidopsis plants

Six Arabidopsis lines (At1, At2, At3, At4, At5, and At6) were analyzed for methoxyfenozide dose response. T_2 seed were plated on agar medium supplemented with 50 mg/l kanamycin and 0 (DMSO), 0.64, 3.2, 16, 80, 400, 2,000, and 10,000 nM methoxyfenozide. After 20 days, three seedlings from each plate were collected and assayed separately for luciferase activity. In the six lines tested, the level of luciferase gene expression in the absence of methoxyfenozide was indistinguishable from the background readings detected using untransformed Arabidopsis seedlings (data not shown). In all six lines tested, luciferase activity began to increase at the lowest concentration (0.64 nM) of methoxyfenozide and reached maximum levels at 3.2–400 nM methoxyfenozide (Fig. 5). Some variability in the level of luciferase induced by methoxyfenozide was observed among the six lines tested. The results obtained with the stable transgenic plants also confirm the high-ligand sensitivity and low-back-

Table 1 Comparison of luciferase induction levels of the GCfE + VCH9 gene switch with other two-hybrid gene switches in different plant species

Methoxyfenozide (nM)	Luciferase activity (RLU/μg protein)a,b								
	Tobacco			Corn			Soybean		
	LmRXR	HsRXR	CH9	LmRXR	HsRXR	CH9	LmRXR	HsRXR	CH9
0	128.4 ± 32.5	44.0 ± 6.9	20.6 ± 4.5	5.2 ± 0.9	2.5 ± 0.8	2.1 ± 0.5	8.8 ± 2.9	4.0 ± 2.5	2.5 ± 0.6
3.2	426.4 ± 18.4 (3.3)c	70.9 ± 3.7 (1.6)	622.8 ± 39.5 (30.2)	28.6 ± 6.9 (5.5)	9.6 ± 2.9 (3.8)	47.0 ± 9.9 (22.4)	27.3 ± 9.3 (3.1)	11.3 ± 2.6 (2.8)	19.1 ± 6.4 (7.6)
16	698.8 ± 25.0 (5.4)	121.0 ± 21.2 (2.7)	1,250.4 ± 92.9 (60.7)	51.3 ± 11.3 (9.9)	20.7 ± 3.6 (8.3)	251.2 ± 73.2 (119.6)	117.7 ± 29.1 (13.4)	13. 6 ± 2.4 (3.4)	73.1 ± 23.5 (29.2)
80	1,024.8 ± 70.2 (8.0)	276.6 ± 21.9 (6.3)	915.4 ± 60.4 (44.4)	67.6 ± 8.1 (13.0)	33.2 ± 3.6 (13.3)	217.6 ± 25.7 (103.6)	96.8 ± 18.2 (11.0)	35.8 ± 12.9 (8.9)	125.2 ± 39.3 (50.1)
400	883.9 ± 2.2 (6.9)	377.4 ± 10.8 (8.6)	915.1 ± 74.9 (44.4)	112.0 ± 26.9 (21.5)	90.1 ± 12.9 (36.0)	159.2 ± 31.9 (75.8)	78.0 ± 25.8 (8.9)	71.2 ± 20.2 (17.8)	149.0 ± 38.3 (57.6)
2,000	891.2 ± 109.1 (6.9)	479.2 ± 47.4 (10.9)	540.7 ± 38.5 (26.2)	103.5 ± 26.1 (19.9)	171.0 ± 23.4 (68.4)	155.1 ± 23.6 (73.8)	79.8 ± 15.5 (9.1)	150.3 ± 37.5 (37.6)	160.2 ± 19.6 (64.1)
10,000	741.8 ± 109.6 (5.8)	561.4 ± 69.4 (12.8)	389.9 ± 24.8 (18.9)	105.6 ± 29.6 (20.3)	284.3 ± 46.7 (113.7)	114.2 ± 11.8 (54.4)	47.9 ± 16.0 (5.4)	219.5 ± 38.3 (54.9)	66.2 ± 13.8 (27.8)

[a] Luciferase activity was measured in protoplasts that were electroporarted with a mixture of plasmid DNA (LmRXR: pK80GCfE + pK80VLmR + pK80–46 35S:Luc; HsRXR: pK80GCfE + pK80VHsR + pK80–46 35S:Luc; CH9: pK80GCfE + pK80VCH9+ pK80–46 35S:Luc) and incubated in growth media containing different concentrations of methoxyfenozide

[b] The data represent the average of three replicates ±SD

[c] Values in parentheses are the fold-induction values calculated by dividing RLU/μg protein in the presence of ligand with RLU/μg protein in the absence of ligand

ground activity of the GCfE + VCH9 switch observed in protoplasts.

Time course study

In order to evaluate the regulation of the GCfE + VCH9 two-hybrid switch over time, T_3 seed collected from the Arabidopsis transgenic line, At2 were germinated on agar medium containing 50 mg/l kanamycin without methoxyfenozide. After 10 days the seedlings were transferred to new agar plates containing different concentrations of methoxyfenozide. Luciferase reporter gene expression was monitored at 24-h intervals over 7 days (168 h). As shown in Fig. 6, luciferase activity began to increase 24 h after transfer to ligand plates, and continued to increase throughout the experiment (7 days). At most of the time points tested, both the quantity of luciferase and the fold induction values observed were higher in the seedlings exposed to 16 and 80 nM methoxyfenozide (Fig. 6a, b). A 695.9-fold increase in luciferase activity was observed at 7 days after transfer onto the 80 nM methoxyfenozide concentration (Fig. 6b).

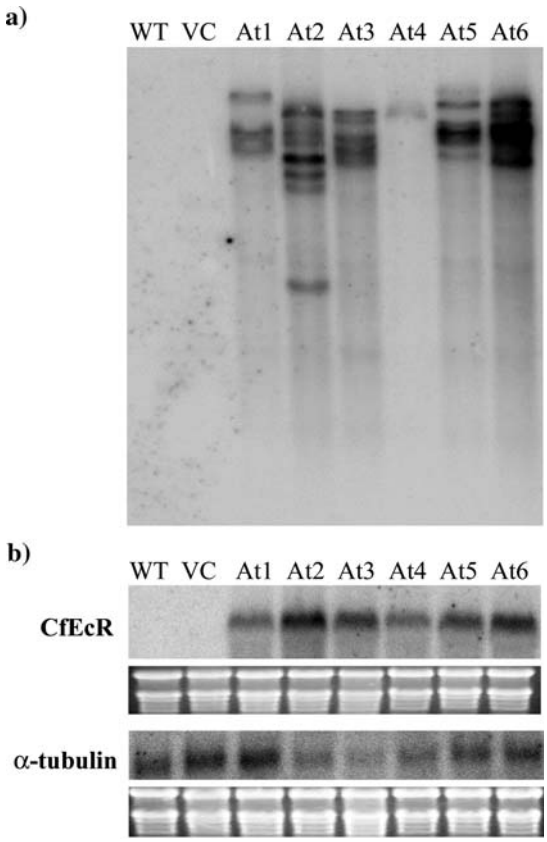

Fig. 4 Southern and Northern blot analyses of transgenic Arabidopsis plants transformed with p2300CfE : -CH9 : Luc construct. a Southern blot analysis of HindIII restricted total genomic DNA isolated from the T2 Arabidopsis seedlings and hybridized with GAL4 DBD : CfEcR fusion gene probe. b Northern blot analysis of the total RNA isolated from the seedlings collected from six T2 Arabidopsis lines and probed with either GAL4 DBD : CfEcR or α-tubulin gene. WT wild-type untransformed controls, VC: vector control plants, where the transgenic Arabidopsis plants developed for the pCAMBIA2300 vector; Lanes At1 to At6 represent the samples isolated from the seedlings of transgenic Arabidopsis lines generated for the p2300CfE : CH9 : Luc construct

Discussion

In this study, we have shown that the two-hybrid format switch containing the RXR chimera (CH9) as a partner of CfEcR supported high-ligand sensitivity and low levels of reporter gene activity in the absence of ligand. These two features are probably the two most desirable characteristics that have limited the wide-spread use of the EcR gene switch. Several versions of EcR gene switches have been developed for use in plants (Martinez et al. 1999a, b; Unger et al. 2002; Padidam et al. 2003). The EcR gene switches described to date are monopartate and these versions require high concentrations of ligand for induction of reporter genes and the background activity of the reporter gene supported by these versions of EcR gene switches are high (Martinez et al. 1999a, b; Unger et al. 2002; Padidam et al. 2003).

Experiments aimed at developing an EcR gene switch that has lower background and higher induced expression levels were initiated by adopting a two-hybrid format switch (Tavva et al. 2006). The CfEcR + LmRXR two-hybrid gene switch showed higher ligand sensitivity (increased from micromolar to nanomolar concentration of methoxyfenozide) but the reporter gene supported by this gene switch showed higher levels of activity in the absence of ligand. On the other hand, the CfEcR + HsRXR gene switch supported lower levels of reporter activity in the absence of ligand but the ligand sensitivity of this

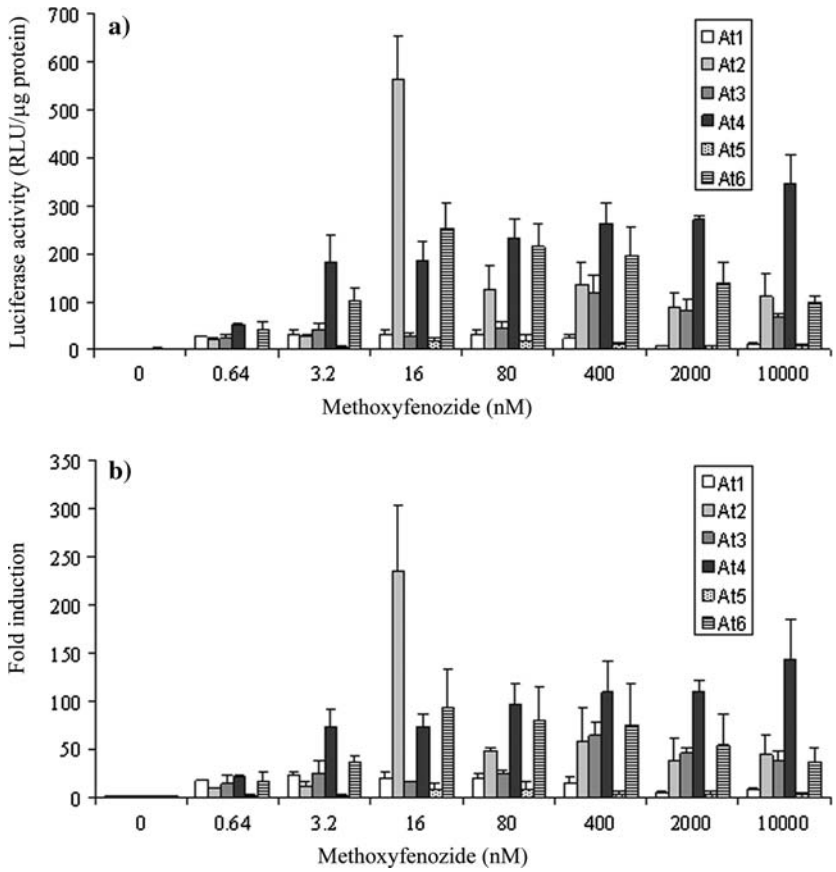

Fig. 5 Dose response study with T2 Arabidopsis plants containing CfEcR + CH9 gene switch. Seed collected from six transgenic lines (At1, At2, At3, At4, At5, and At6) were plated on agar medium containing different concentrations of methoxyfenozide. Luciferase activity was measured in the seedlings collected at 20 days after plating the seed on the induction medium. Luciferase activity in terms of RLU/µg protein (a) and fold induction values (b) shown are the average of three replicates ±SD

switch is not as high as that of the CfEcR + LmRXR switch. To combine the desirable properties of these two versions of the EcR gene switches into one version, we tested the Lm-HsRXR chimera as a partner of CfEcR. The GCfE + VCH9 switch showed low-basal reporter gene expression in the absence of chemical ligand and high-fold induction values in the presence of nanomolar concentration of methoxyfenozide in all three plant species tested (Table 1). The ligand sensitivity of the GCfE + VCH9 switch is similar to the ligand sensitivity observed with the GCfE + VLmR switch. Moreover, when compared to the GCfE + VLmR switch, the background expression levels of the luciferase reporter gene were greatly reduced with the GCfE + VCH9 switch.

The differences in the ligand sensitivity and background expression levels of the luciferase reporter gene activity between LmRXR and HsRXR containing EcR gene switches might be due to the differences in their ability to heterodimerize with CfEcR in the absence of ligand. Heterodimerization between EcR and RXR is dictated by the amino acid sequences of RXR in helices 9, 10, and 11. Comparison of amino acid sequences in helices 9, 10, and 11 between LmRXR and HsRXR showed that the amino acid residues in helix 11 are identical in HsRXR and LmRXR. There is only one amino acid residue that is different in helix 10 between HsRXR and LmRXR. Most of the differences in amino acids between HsRXR and LmRXR occur in and around helix 9. The RXR CH9 was generated by fusing 1–8 helices of HsRXR LBD with 9–12 helices of LmRXR LBD (Fig. 1). Therefore, replacing 9–12 helices of HsRXR LBD with that of LmRXR LBD in RXR CH9 was sufficient to reduce the background activity and maintain the ligand sensitivity of the CfEcR + LmRXR gene switch. These results are similar to previous reports on using HsRXR

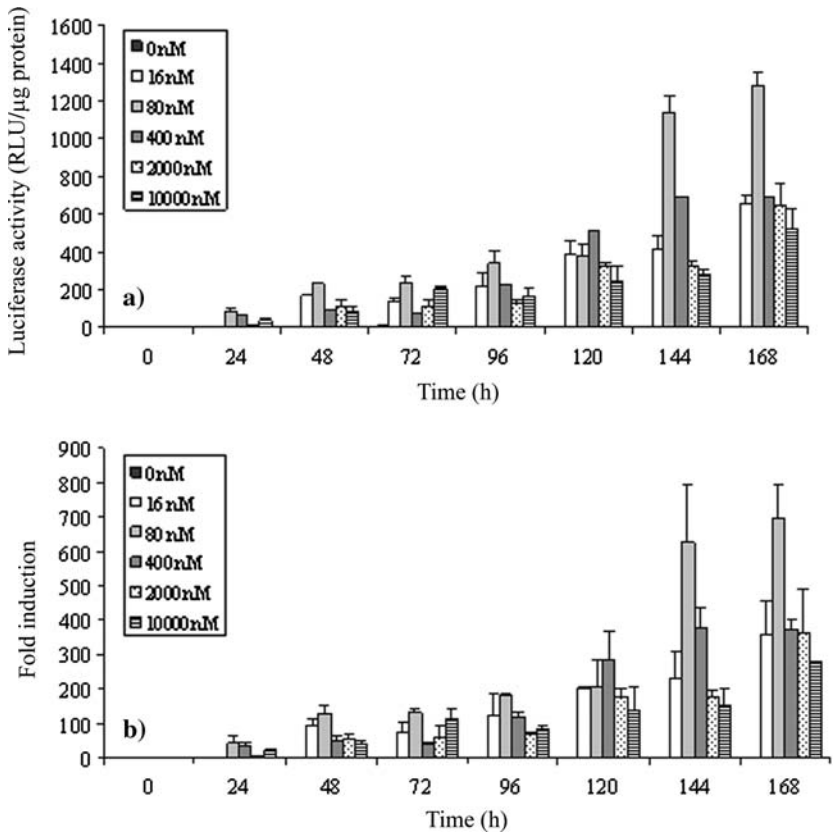

Fig. 6 Time-course induction of luciferase gene activity in T3 Arabidopsis plants containing CfEcR + CH9 gene switch. Seedlings grown on agar medium without any added methoxyfenozide were transferred to agar medium containing 0, 16, 80 400, 2,000, and 10,000 nM of methoxyfenozide. Samples were collected at 24-h interval up to 168 h and the luciferase activity was measured. The luciferase activity in terms of RLU/µg protein (a) and fold induction (b) shown are the averages of three samples ±SD. Fold-induction values were calculated by dividing RLU/µg protein collected at 24 or 48 or 72 or 96 or 120 or 144 or 168 h with RLU/µg protein collected at 0 h

and LmRXR chimeras for improving the performance of the EcR gene switch in mammalian cells (Palli et al. 2005b). In mammalian cells, studies aimed at the comparison of HsRXR and LmRXR chimeras as a partner for CfEcR in regulation of the luciferase reporter gene showed that a chimera that contains helices 1–8 from HsRXR and helices 9–12 from LmRXR is the best partner for CfEcR in achieving higher ligand sensitivity and lower background activity (Palli et al. 2005b). Studies in mammalian cells have shown that the amino acid residues that are present in and around helix 9 are sufficient to achieve this improved performance of the CfEcR gene switch (Palli et al. 2005b).

The efficiency of the RXR CH9 as a partner of CfEcR in inducing luciferase reporter gene activity in stable transgenic Arabidopsis plants was observed in the dose response and time-course experiments (Figs. 5, 6). The six transgenic Arabidopsis lines tested showed low levels of luciferase gene expression in the absence of the chemical inducer. This is an important attribute, which may be essential where low levels of inappropriate expression may prove lethal. There were some variations among the six transgenic lines tested in terms of their responses to the inducer concentrations applied. Except in line At4, all the transgenic lines analyzed showed maximum luciferase induction values in the presence of nanomolar concentrations (3.2–400 nM) of methoxyfenozide (Fig. 5). Luciferase induction kinetics in a time-course study provided further evidence that maximum luciferase induction levels can be achieved at nanomolar concentrations of methoxyfenozide (Fig. 6).

The data presented here clearly demonstrated that luciferase reporter gene expression is tightly regulated by the GCfE + VCH9 two-hybrid switch. The system is very sensitive, induction observed even with an application of only 3.2 nM methoxyfenozide (Fig. 5; Table 1). The GCfE + VCH9 switch showed negligible levels of background reporter gene activity in the

absence of ligand and the highest levels of induced reporter gene activity in the presence of nanomolar concentrations of ligand, resulted in a strikingly sensitive switch with high-fold induction values. At all methoxyfenozide concentrations tested, the fold induction values of luciferase reporter gene expression were higher with the GCfE + VCH9 switch compared to other two-hybrid or monopartate gene switches tested in all the plant species. The improvements made to the EcR gene switch in this and previous studies (Tavva et al. 2006) along with other desirable properties such as availability of safe and field registered ligand should provide wide-spread use for this system.

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