Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2015) **16**(1), 83–91 DOI: 10.1111/mpp.12161

Technical advance

Development of a bipartite ecdysone-responsive gene switch for the oomycete *Phytophthora infestans* and its use to manipulate transcription during axenic culture and plant infection

HEBER GAMBOA-MELÉNDEZ AND HOWARD S. JUDELSON*

Department of Plant Pathology and Microbiology, University of California, Riverside, CA 92521, USA

SUMMARY

Conditional expression systems have been proven to be useful tools for the elucidation of gene function in many taxa. Here, we report the development of the first useful inducible promoter system for an oomycete, based on an ecdysone receptor (EcR) and the ecdysone analogue methoxyfenozide. In *Phytophthora infestans*, the potato late blight pathogen, a monopartite transactivator containing the VP16 activation domain from herpes simplex virus, the GAL4 DNA-binding domain from yeast and the EcR receptor domain from the spruce budworm enabled high levels of expression of a β-glucuronidase (GUS) reporter gene, but unacceptable basal activity in the absence of the methoxyfenozide inducer. Greatly improved performance was obtained using a bipartite system in which transcription is activated by a heterodimer between a chimera of VP16 and the migratory locust retinoid X receptor, and a separate EcR-DNA-binding domain chimera. Transformants were obtained that exhibited >100-fold activation of the reporter by methoxyfenozide, with low basal levels of expression and induced activity approaching that of the strong *ham34* promoter. Performance varied between transformants, probably as a result of position effects. The addition of methoxyfenozide enabled strong induction during hyphal growth, zoosporogenesis and colonization of tomato. No significant effects of the inducer or transactivators on growth, development or pathogenicity were observed. The technology should therefore be a useful addition to the arsenal of methods for the study of oomycete plant pathogens.

Keywords: biotechnology, functional genomics, inducible promoter, late blight, transformation.

INTRODUCTION

Technologies for the expression of genes at will are useful additions to the genetics toolkit of a species, with potential applications including the testing of the function of genes in development or pathogenesis. Such systems can be particularly helpful for the study of transgenes that may be lethal when expressed constitutively, or for the measurement of effects of graded expression. Several systems for the activation of a target gene have been developed for animal cells, plants, fungi and bacteria, using chemicals as inducers (Auslander and Fussenegger, 2013; Baneyx, 1999; Celik and Calik, 2012; Corrado and Karali, 2009). Only a few reports exist of conditional expression systems for filamentous fungal pathogens, however (Carneiro *et al*., 2013; Willyerd *et al*., 2009). An effective inducible transcription system has not yet been reported for any oomycete, a group which includes many significant plant pathogens.

Chemically inducible expression systems typically place a promoter motif upstream of a target gene, which binds a transcription factor that is activated by the chemical. The transcription factor may be native to the organism of interest or heterologous, and may combine DNA-binding, ligand-binding and transcriptional activation domains from several sources. Examples of methods used in eukaryotes include tetracycline-inducible machinery based on TetR of *Escherichia coli* (Stebbins *et al*., 2001), dexamethasone-activated systems using mammalian glucocorticoid receptors (Ouwerkerk *et al*., 2001), those based on ecdysone hormone receptors of insects (Tavva *et al*., 2006) and alcohol- or copper-induced systems that employ fungal regulators (Caddick *et al*., 1998; Saijo and Nagasawa, 2014). Several activation domains have been used in such systems, in addition to repression domains.

Challenges in the development of these systems include obtaining low basal expression with high induction ratios and avoiding deleterious effects of the inducer or transactivator. For example, when a dexamethasone-regulated system was adapted to plants, severe growth defects were observed in some species (Kang *et al*., 1999). Problems were also observed in our previous test of a tetracycline-based system in the oomycete *Phytophthora infestans*, the potato blight pathogen (Judelson *et al*., 2007). That effort employed a chimera of the TetR DNA-binding and ligand-binding domains of *E. coli* and the VP16 activation domain of herpes simplex virus. Chemically induced transcription was achieved, but performance was poor because of the toxicity of the chimeric transcription factor.

**Correspondence*: Email: howard.judelson@ucr.edu

In this study, we report the development of a superior chemically induced gene switch for *P. infestans* based on the ecdysone receptor (EcR). In insects, EcR regulates moulting and normally forms a heterodimer with a second nuclear receptor, Ultraspiracle (USP), which is an orthologue of the retinoid X receptor (RXR) of vertebrates (Kozlova and Thummel, 2000). Although EcR can bind DNA without USP, the natural ligand 20-hydroxyecdysone promotes heterodimerization, nuclear localization and DNA binding (Braun *et al*., 2009). Forms of EcR have been designed in recent years which induce transcription in animal and plant cells without USP in the presence of methoxyfenozide, an agonist of the natural hormone (Carlson *et al*., 2001; Esengil *et al*., 2007; Suhr *et al*., 1998; Tavva *et al*., 2006). These have been deployed as monopartite or bipartite systems (Fig. 1). The common monopartite system uses a transcription factor comprising VP16 activation, GAL4 DNA-binding and EcR domains to bind GAL4 response elements upstream of the target gene. The bipartite (two-hybrid) system places the DNA-binding and activation domains on separate proteins, which form a heterodimer, and was developed to reduce basal levels of expression in the absence of inducer (Tavva *et al*., 2006). Later studies achieved similar improvements in the monopartite system through judicious selection of EcR domains and mutagenesis of key residues (Tavva *et al*., 2008).

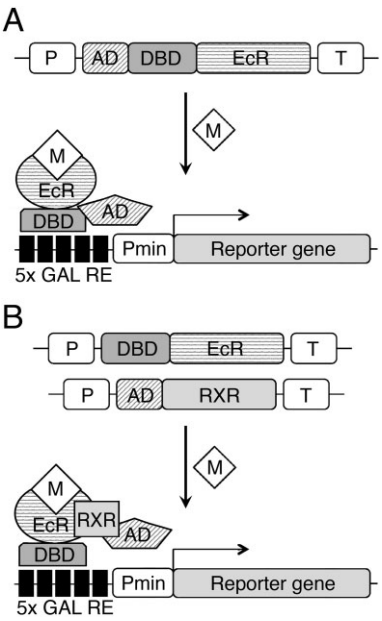

Fig. 1 Methoxyfenozide-inducible expression systems. (A) Monopartite system. This employs a transcription factor comprising a herpes simplex virus-derived VP16 activation domain (AD), the DNA-binding domain from *Saccharomyces cerevisiae* GAL4 (DBD) and an ecdysone receptor domain from *Christoneura fumiferana* (EcR), which are expressed from a promoter (P) and terminator (T). After binding methoxyfenozide (M), the chimeric transcription factor translocates into the nucleus, where it binds GAL4 response elements (5x GAL4 RE) upstream of a minimal promoter (Pmin) to activate a reporter gene. (B) Bipartite or two-hybrid system. An active transcription factor results when methoxyfenozide binds to a DBD-EcR chimera, which binds a chimera of AD and the retinoid X-receptor from *Locusta migratoria* (RXR).

Here, we report the testing and optimization of a methoxyfenozide-inducible gene switch for *P. infestans*. A bipartite system outperformed the monopartite system, yielding transformants with low basal levels of expression and induction ratios in excess of 100-fold, although not all transformants performed equally well. Induction was achieved in hyphae grown on artificial media and *in planta*, and in spores, suggesting that the technology will be useful for the study of genes involved in growth and pathogenesis.

RESULTS

Methoxyfenozide has little effect on *P. infestans*

Before adapting the ecdysone-inducible system to *P. infestans*, we tested whether growth or development was affected by the synthetic inducer, methoxyfenozide. The chemical had negligible effects on the growth rate at concentrations up to 1 mM (10^6 nM; Fig. 2A), with no significant differences at $P < 0.1$ by Student's *t*-test. No significant differences were observed in the intensity of asexual sporulation (Fig. 2B). Whether the inducer affected zoospore release, which occurs when sporangia are chilled in water, was also tested. A possible effect ($P = 0.11$) was observed only at the highest concentration, which, as shown later, is much higher than that required to turn on the gene switch (Fig. 2C).

A monopartite system is inducible but has high background

Two plasmids were constructed to test the monopartite version of the gene switch (Fig. 3A). The first plasmid contains the β-glucuronidase (GUS) reporter gene driven by five GAL response elements and a minimal *P. infestans* promoter. The second was designed to express a protein containing the VP16 activation domain, GAL4 DNA-binding domain and DEF domains of the EcR nuclear receptor behind the constitutive *ham34* promoter. For the latter plasmid, the open reading frame was engineered on the basis of *P. infestans* codon usage, and includes the DEF domains of EcR with V395I and Y415E mutations, which are reported to increase the response to methoxyfenozide and to reduce background (Tavva *et al*., 2008). Both plasmids also contain the *neomycin phosphotransferase II* (*nptII*) gene for the selection of *P. infestans* transformants using G418.

The plasmids were co-transformed into *P. infestans*, and strains bearing both were identified by polymerase chain reaction (PCR). These and controls obtained using the GUS plasmid alone were then treated with 0, 10 μM and 1 mM of methoxyfenozide for 24 h,

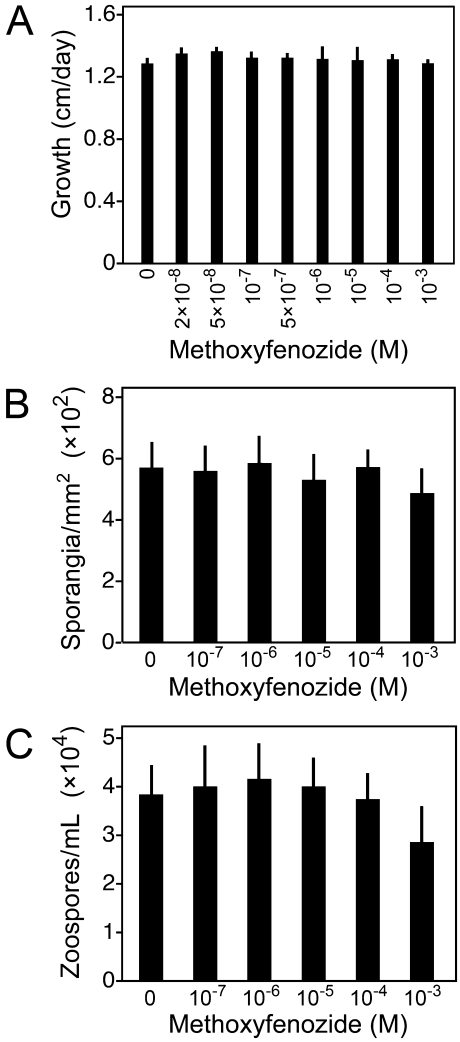

Fig. 2 Effects of methoxyfenozide on *Phytophthora infestans*. (A) Growth on rye–sucrose agar containing 0–1 mM methoxyfenozide. (B) Sporulation on rye–sucrose agar containing 0–1 mM methoxyfenozide, measured in cultures 8 days after inoculation. (C) Zoospore release from sporangia incubated in 0–1 mM methoxyfenozide. In each panel, values represent averages and standard deviations from three biological replicates.

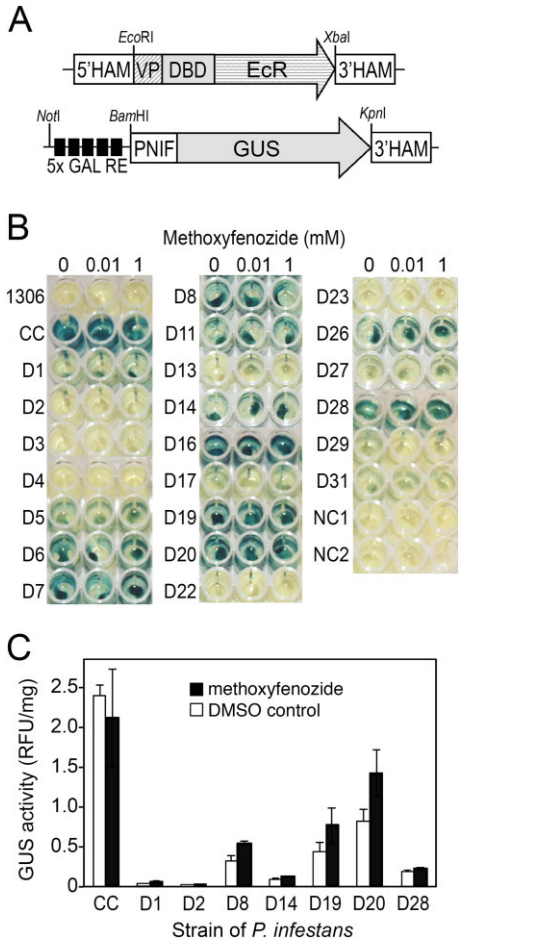

Fig. 3 Performance of monopartite gene switch in *Phytophthora infestans*. (A) Linearized maps of plasmids expressing the VP16-DBD-EcR chimera (top) and β-glucuronidase (GUS) reporter (bottom). Each also bears a *neomycin phosphotransferase II* (*nptII*) gene for selection of transformants (not shown). Transcription units employ the constitutive *Ham34* promoter from *Bremia lactucae* (5'HAM), the minimal *NifS* promoter from *P. infestans* (PNIF) and the *Ham34* transcriptional terminator (3'HAM). (B) Histochemical staining for GUS in hyphal plugs incubated for 24 h in rye–sucrose broth containing 0, 0.01 or 1 mM methoxyfenozide. Strains D1–D31 were obtained by co-transformation of the two plasmids shown in (A); NC1 and NC2 are negative control transformants obtained using the GUS plasmid alone; CC is a positive control that expresses GUS from the *Ham34* promoter. (C) Specific activity of GUS in transformants grown for 24 h in rye–sucrose broth containing 10 μM methoxyfenozide or the dimethylsulphoxide (DMSO) solvent alone, expressed as relative fluorescence units (RFU) per microgram. Values are from two biological replicates.

and stained histochemically for GUS. None of the 25 transformants obtained with the GUS plasmid alone showed activity. In contrast, 21 of the 67 co-transformants (31%) exhibited staining (Fig. 3B). In most cases, activity was observed even in the absence of inducer. A quantitative assay showed that methoxyfenozide caused, at best, a two-fold increase in reporter activity (Fig. 3C).

Although these results indicate that a monopartite methoxyfenozide-inducible gene switch may not be useful for *P. infestans*, the data have value. They indicate that the VP16:DBD:EcR chimera is needed to activate the reporter in *P. infestans*, and that VP16 is an effective transcriptional activator. The latter was unclear from a previous study of a tetracycline-inducible system in *P. infestans*, which performed poorly because of toxicity of the transactivator and/or weak performance of VP16 (Judelson *et al.*, 2007). Here, expression of the VP16:DBD:EcR protein did not impair growth or sporulation (not shown).

A two-hybrid system improves induction ratios

As a result of limitations in the function of the monopartite system, a bipartite (two-hybrid) version was tested. This was

evaluated initially using three separate plasmids containing GAL4 DBD:EcR, VP16:RXRCH9 and GUS reporter cassettes (Fig. 4A). In the presence of methoxyfenozide, heterodimerization of the EcR and RXRCH9 chimeras would be expected to activate the reporter, as portrayed in Fig. 1B.

Based on histochemical staining, about 6% of 200 transformants expressed GUS, and much higher levels of reporter expression were observed in the presence of methoxyfenozide (Fig. 4B). This indicated that the two-hybrid approach would be useful for *P. infestans*, in contrast with the monopartite system. Moreover, quantitative assays indicated low backgrounds and induction ratios up to 74-fold (Fig. 4C).

Although transformants exhibiting good performance of the methoxyfenozide-activated gene switch were obtained, the fraction of useful transformants was very low. This was probably a result of the failure of most to acquire or express each of the three plasmids. Unlike the experiment in Fig. 3, where co-transformation rates were enhanced using plasmids linearized with the same restriction enzyme (Judelson, 1993), this was not possible here because of a dearth of useful sites shared by the three plasmids.

'All-in-one' plasmid two-hybrid system

To increase the fraction of useful transformants, a single-plasmid system was developed. This involved combining the GAL4 DBD:EcR, VP16:RXRCH9 and GUS reporter cassettes into a single backbone (Fig. 5A). Of 122 transformants obtained with this plasmid, 25 (21%) exhibited methoxyfenozide-induced expression of GUS based on histochemical staining (Fig. 5B). Backgrounds in the absence of inducer were usually low. Occasionally, transformants were obtained that exhibited similar levels of expression with and without inducer, such as transformant S24 (Fig. 5B); in such cases, we assume that the reporter gene is being influenced by a native sequence near its chromosomal integration site.

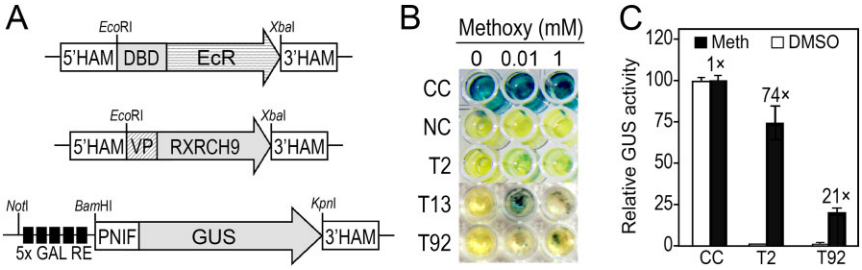

Fig. 4 Initial tests of two-hybrid switch in *Phytophthora infestans*. (A) Shown from top to bottom are the linearized maps of plasmids expressing the GAL4 DBD-EcR chimera, VP16-RXR fusion and β-glucuronidase (GUS) reporter. Not shown are the *neomycin phosphotransferase II* (*nptII*) cassettes. (B) Histochemical staining of GUS in transformants (T2, T13, T92) obtained using the three plasmids, a negative control transformant containing the GUS reporter alone (NC) and a strain expressing GUS from the *Ham34* promoter (CC). Cultures were grown as described in Fig. 3 with 0, 0.01 or 1 mM inducer. (C) Specific activity of GUS in transformants grown for 24 h in rye–sucrose broth containing 10 μM methoxyfenozide or the dimethylsulphoxide (DMSO) solvent alone. Numbers above the bars represent the fold induction by methoxyfenozide.

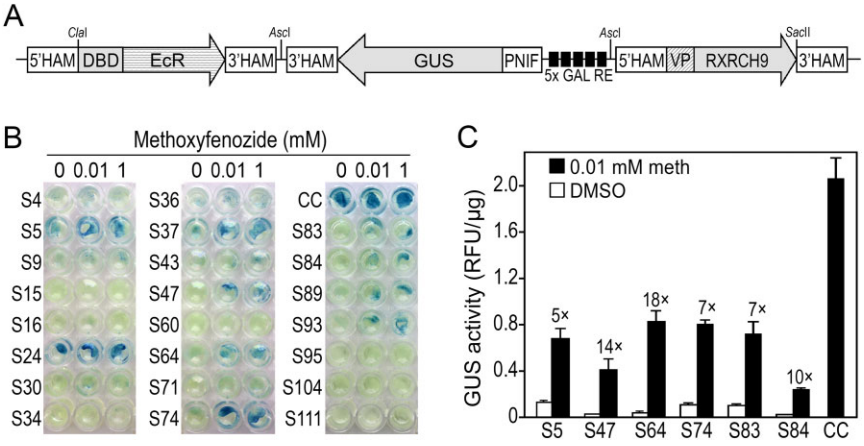

Fig. 5 Tests of two-hybrid switch expressed from a single plasmid. (A) Linearized map of plasmid encoding the GAL4 DBD-EcR chimera, VP16-RXR fusion and β-glucuronidase (GUS) reporter. Not shown is *neomycin phosphotransferase II* (*nptII*). (B) Histochemical staining of GUS in transformants (S4–S111) containing the two-hybrid plasmid and a strain expressing GUS from the *Ham34* promoter (CC). Cultures were grown as in Fig. 3 with 0, 0.01 or 1 mM inducer. (C) Specific activity of GUS in transformants grown in broth containing 10 μM methoxyfenozide or the dimethylsulphoxide (DMSO) solvent alone, expressed as relative fluorescence units (RFU) per microgram of protein. Numbers above the bars represent the fold induction by methoxyfenozide.

Transformants displaying inducible expression in the histochemical assays were assayed quantitatively for GUS (Fig. 5C). Although parameters for methoxyfenozide treatment may not have been optimal in the experiment, the ability of the inducer to up-regulate expression was confirmed. Approximately six-fold variation in basal activity and three-fold variation in induction ratio were observed among these transformants. Their average GUS specific activity after induction was 32% of the constitutive control (lane CC, Fig. 5C) which, on the basis of data from a previous study, ranks in the upper 20% of transformants expressing GUS from the *ham34* promoter (Judelson *et al*., 1993). As *ham34* is one of the strongest known oomycete promoters, it can be concluded that the EcR switch can drive high levels of transcription.

Colony growth rates on rye–sucrose agar, sporulation intensity and the efficiency of zoospore release were similar between the GUS-expressing 'all-in-one' transformants, the 1306 progenitor strain and transformants expressing GUS behind the *ham34* promoter (not shown). Similar results were observed in both the presence and absence of methoxyfenozide. Expression of the transactivator chimeras in *P. infestans* thus appears not to be toxic.

Optimization of induction conditions

Two transformants expressing the all-in-one plasmid were used to test parameters for activation of the reporter. These were transformants S47 and S64 (S for 'single-vector'). The ability to induce GUS was assessed using broth cultures of hyphae, hyphae grown on agar plates and sporangia treated to induce zoosporogenesis.

To test the effect of methoxyfenozide concentration, hyphal mats were transferred from unamended rye–sucrose broth to media containing 0.6 nM to 1 mM methoxyfenozide, and assayed for GUS after 24 h. Reporter levels reached a plateau with inducer in the 2–10 μM range (Fig. 6A). A similar experiment examined cultures exposed to 10 μM methoxyfenozide for varying times (Fig. 6B). Increases were detected by 6 h, and continued until termination of the experiment at 48 h. For transformant S64, for example, 10- and 92-fold increases in GUS activity were observed after 6 and 48 h, respectively.

Whilst performing these and other tests, it seemed that the results often varied between biological replicates. With transformant S64, for example, 24-h treatments with 10 μM methoxyfenozide resulted in induction ratios of 21 and 69 in the experiments shown in Fig. 6A and Fig. 6B, respectively. We postulated that such variation might be caused by our use of submerged broth cultures. Such cultures facilitate the addition of inducer, but *P. infestans* grows poorly and often unevenly when submerged. We therefore tested the approach of growing *P. infestans* on a polycarbonate membrane laid on rye–sucrose agar, and then

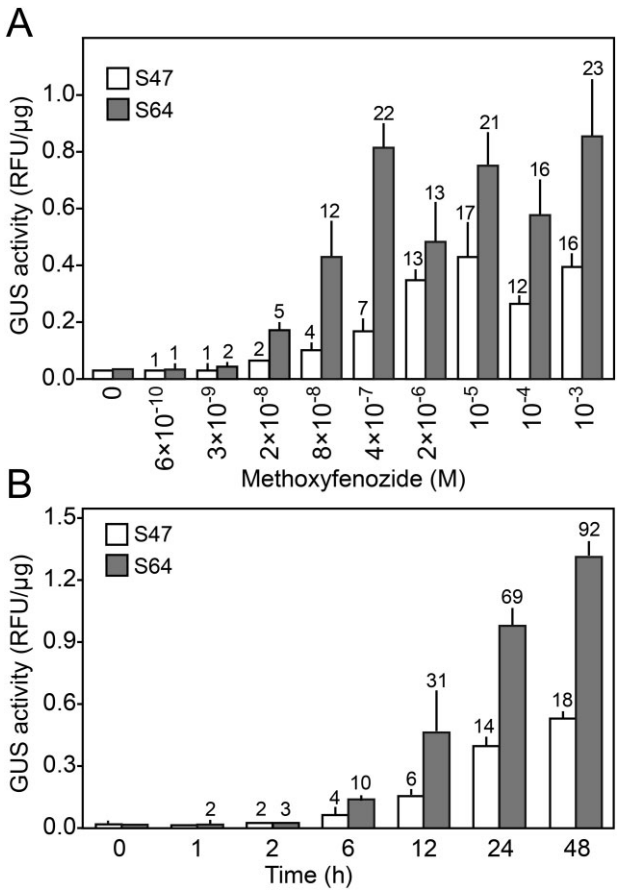

Fig. 6 Effect of methoxyfenozide concentration and incubation time on induction of the two-hybrid system. (A) Specific activity of β-glucuronidase (GUS) in transformants S47 and S64 (from Fig. 5) grown in broth and treated for 24 h with the indicated amounts of inducer. (B) Activities after treatment with 10 μM methoxyfenozide for 0–48 h. Numbers above the bars represent the fold induction by methoxyfenozide. RFU, relative fluorescence units.

transferring of the membrane to methoxyfenozide-containing rye–sucrose agar. This resulted in strong induction of the reporter (Fig. 7A). In the case of S64, for example, induction ratios of 115 and 144 were observed in agar cultures after 24 h of treatment with 10 and 30 μM methoxyfenozide, respectively, compared with 65- and 76-fold in the broth cultures. A histochemical assay also demonstrated strong induction of the reporter in agar cultures of S64 and S47 (Fig. 7B).

Strong induction ratios were also observed during zoosporogenesis, which is induced by cold treatment of sporangia. When sporangia were incubated for 90 min at 12 °C in the presence of methoxyfenozide, induction of GUS was observed in both quantitative and histochemical assays (Fig. 7A, B). Induction ratios were about half of those observed in hyphae, even though the time of exposure to methoxyfenozide was much shorter (90 min versus 24 h). This indicates that methoxyfenozide readily enters sporangia. Specific activities of GUS were several times higher in

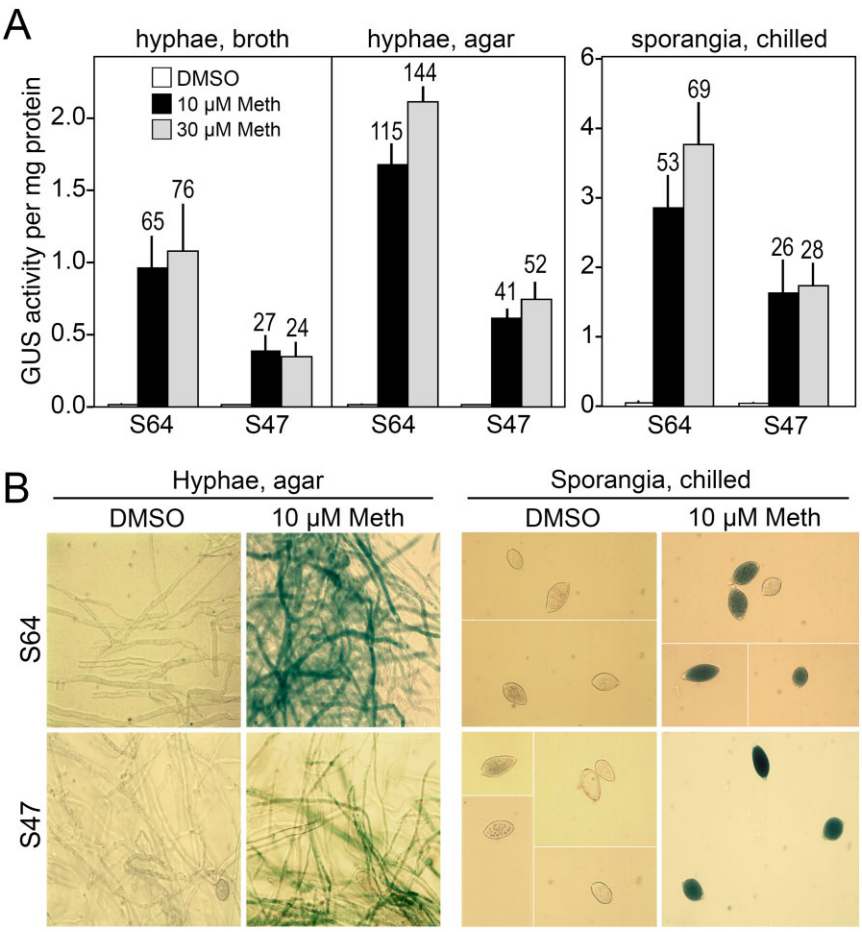

Fig. 7 Induction of two-hybrid reporter in two-hybrid transformants S64 and S47 in hyphae and germinating sporangia. (A) β-Glucuronidase (GUS) specific activity after treatment with 10 and 30 µM of inducer, or the dimethylsulphoxide (DMSO) solvent control. Left: the inducer was added to hyphae submerged in rye–sucrose broth, and tissue was analysed after 24 h. Centre: surface-grown hyphae from agar cultures were transferred to medium containing inducer and assayed after 24 h. Right: sporangia were placed in 0.25 mM $CaCl_2$ containing inducer, incubated for 90 min at 12 °C and assayed. Specific activity is expressed as picomoles of substrate cleaved per second per milligram of protein. (B) Histochemical staining of hyphae induced on agar medium, or chilled sporangia.

spores than in hyphae, probably as spores contain more active, non-vacuolated cytoplasm. Probably for the same reason, faint histochemical staining was sometimes observed in sporangia of S47 and S64, but not in hyphae, that were not treated with methoxyfenozide.

In planta induction

Three methods for the induction of the reporters during plant infection were tested with success, using several transformants from the bipartite system tested in Fig. 5. In the first, tomato leaf discs were inoculated with zoospores and incubated for 3 days. They were then floated on water solutions containing 10 µM methoxyfenozide or the dimethylsulphoxide (DMSO) solvent only. As shown in Fig. 8A for transformants 'all-in-one' S64 and S47, GUS was easily detected after 24 h of exposure to the inducer, but not in the DMSO solvent control.

In the second approach, tomato plants were inoculated by spraying with zoospores and then, at varying times, the plants were misted with 10 µM methoxyfenozide or the DMSO control. Figure 8B shows plants that were sprayed 4 days after being infected with transformants S64, S47 and S74, and then assayed for GUS after an additional 24 h [i.e. 5 days post-infection (dpi)]. Sporulation occurred between the 4- and 5-dpi time points. For each transformant, strong GUS expression was detected in hyphae and sporangia on the plant surface in samples treated with inducer. Although difficult to discern in the photograph, hyphae within the leaves also exhibited GUS staining. This is seen more easily in Fig. 8C, which shows S47-infected plants that had been sprayed with methoxyfenozide at 3 dpi, and stained for GUS at 4 dpi before sporulation occurred.

In the third approach for the induction of the reporter *in planta*, tomato plants were infected with transformants S64 and S74, watered with a solution containing 100 µM methoxyfenozide or the DMSO solvent alone, and stained for GUS after 24 or 48 h. Staining was observed only in the inducer-treated plants (Fig. 8D), with a higher signal observed at 48 h relative to 24 h (not shown). Stronger staining tended to be observed at the edges of the leaflets. As a similar pattern predominated in plants infected with the constitutive control transformant (GUS driven by the *ham34* promoter), this probably reflects the distribution of *P. infestans*, rather than suggesting that methoxyfenozide levels are lower in leaflet centres. No staining was observed in uninfected plants (not shown).

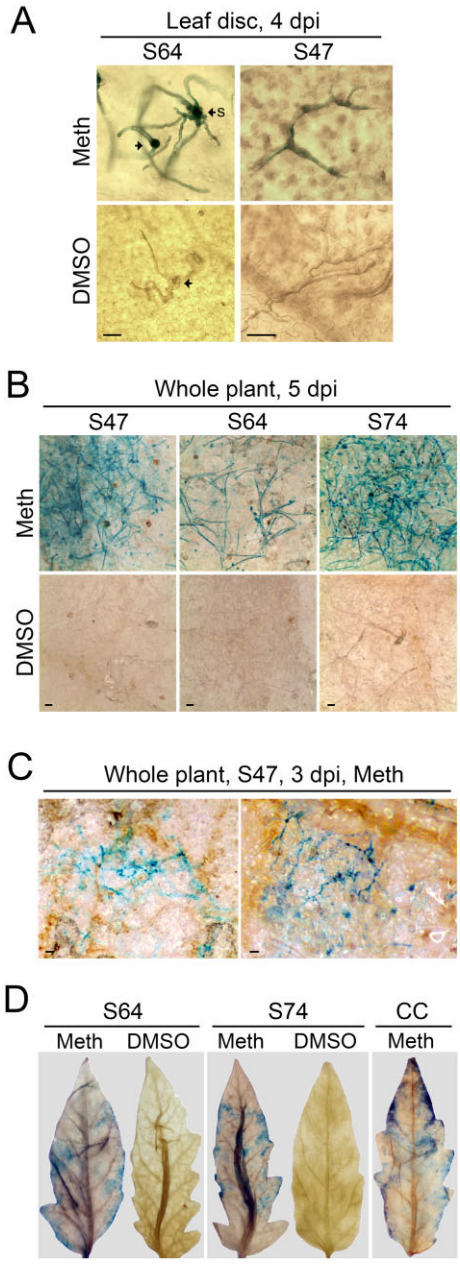

Fig. 8 Performance of two-hybrid system in tomato tissue infected with zoospores of *Phytophthora infestans* transformants, using the histochemical staining assay. (A) Leaf discs floated on water containing 10 μM methoxyfenozide (Meth) or the dimethylsulphoxide (DMSO) solvent control at 4 days post-infection (dpi). In the S64 panel, *P. infestans* hyphae are starting to emerge from stomata (S) onto the leaf surface. In the S47 panel, *P. infestans* is shown at a slightly earlier stage of the disease cycle, in the apoplastic space. (B) Leaflets at 5 dpi from infected whole tomato plants sprayed with 10 μM methoxyfenozide or the control. (C) Same as (B) but at 3 dpi, showing two representative leaflets. (D) Leaflets from whole plants induced by adding methoxyfenozide to the soil. Strains were all-in-one transformants (S64, S74) and the constitutive β-glucuronidase (GUS) strain (CC). Size bars in (A–C) are 40 μm.

DISCUSSION

Based on the low toxicity to *P. infestans* of methoxyfenozide, the lack of deleterious effects of the transactivator proteins and the ability to generate transformants with low basal expression and high levels of induction of the target gene, the bipartite EcR gene switch should be useful for the study of gene function in oomycetes. The utility of the system is enhanced by methoxyfenozide's ability to induce expression in hyphae, spores and *in planta*. This is an advantage of the EcR technology over most other conditional expression methods, such as the use of promoters sensitive to different carbon sources. In addition, methoxyfenozide is inexpensive as it is sold widely as an insecticide (Carlson *et al*., 2001).

The levels of GUS basal expression and induction varied between *P. infestans* transformants. We attribute this to position effects, which influence all transgene studies in the genus, and cannot be controlled, as methods do not currently exist for the targeting of transgenes by homologous recombination. Most *P. infestans* genes reside in tightly spaced clusters, with average intergenic distances of only 430 nucleotides (Roy *et al*., 2013). Our assumption is that most integration events that enable GUS expression occur within the gene-dense clusters; we assume that the rest of the genome, which is repeat rich, is mostly heterochromatic. Transgenes are thus likely to be influenced by native regulatory sequences that flank the integration site. The practical consequence is that, when using the EcR system, transformants need to be screened for those that will be most useful. This situation also occurs in plant and animal systems. For example, *Arabidopsis* lines expressing an EcR system displayed from five- to 150-fold induction at 10 μM methoxyfenozide, and maize lines exhibited from one- to 100-fold induction, which resembles the range observed here for *P. infestans* (Tavva *et al*., 2007; Unger *et al*., 2002). In mammalian cells stably expressing an EcR gene switch, different lines showed from one- to 16-fold induction (Suhr *et al*., 1998). Higher fold-change levels have been reported in transient expression systems, which are not subject to position effects (Palli *et al*., 2003).

Potential applications of the EcR promoter system in *P. infestans* include both ectopic expression and gene silencing. Some genes have been proven to be difficult to stably express in transformants, and may be more easily studied in an inducible system. For example, overexpression of the RNA polymerase subunit RPA1A by Randall *et al*. (2014) severely retarded growth. Transformants that overexpress some Myb transcription factors have also been found to grow slowly, and tend to inactivate the transgene (Xiang and Judelson, 2014). Gene silencing, which in *P. infestans* usually involves a DNA-directed RNAi approach, has also been proven to be difficult for some genes which are presumably lethal when stably inactivated.

It is notable that conditional expression systems have been developed for only a few filamentous plant pathogens. In the

ascomycete *Gibberella zeae*, a promoter induced by the mycotoxin zearalenone was identified that enabled 50-fold induction of a target gene, but it is unclear whether this could be employed *in planta* (Lee *et al*., 2010). Carbon catabolite-regulated systems have also been reported, such as that employing a glucose-regulated *alcA* promoter in *Ophiostoma novo-ulmi* (Carneiro *et al*., 2013). Nutrient conditions cannot be controlled *in planta*, however, and a potential issue with nutritional control is that colony development and morphology may be altered. In *Cryphonectria parasitica*, a copper-repressed promoter was successfully tested in artificial media (Willyerd *et al*., 2009); however, copper is toxic to many fungi (Lee *et al*., 2010) as well as oomycetes. Our success with the EcR system in *P. infestans* may encourage its further development in oomycete plant pathogens, and in fungal plant pathogens as well.

EXPERIMENTAL PROCEDURES

Plasmid construction

Sequences of the VP16 activation domain, GAL4 DNA-binding domain, DEF domains of the EcR nuclear receptor and RXRCH9 were identified from previous publications (Tavva *et al*., 2007, 2008). V395I and Y415E mutations were included in EcR as these reportedly improve performance (Tavva *et al*., 2008). Genes optimized for *P. infestans* codon usage were synthesized (Fig. S1, see Supporting Information) and cloned into vectors for transformation, which employ the *nptII* gene for G418 selection. For the monopartite system, the VP16:GAL4:EcR chimera was cloned into the *Eco*RI and *Xba*I sites of pTOR (GenBank accession EU257520.1), between the constitutive *ham34* promoter of *Bremia lactucae* and *ham34* terminator (*5′ham* and *3′ham*, respectively). For the reporter plasmid, five repeats of the GAL4 response element were cloned into *Not*I and *Bam*HI restriction sites of p74NIF, which contains the minimal promoter from the *NifS* gene of *P. infestans* upstream of the gene encoding GUS (Ah Fong *et al*., 2007). For single- and three-vector versions of the two-hybrid system, transactivator genes were expressed in pTOR. The three-vector system used the GUS plasmid described above, a plasmid encoding GAL4-DBD:EcR (cloned in the *Eco*RI-*Xba*I sites of pTOR) and a plasmid encoding VP16:RXRCH9 (also in *Eco*RI-*Xba*I-digested pTOR). The two-vector system used the GUS plasmid with a derivative of pTOR containing *5′ham*:GAL4-DBD:EcR:*3′ham* and *5′ham*:VP16:RXRCH9:*3′ham* cassettes cloned into its *Eco*RV and *Sac*II sites. The single-vector system contained a single plasmid in *5′ham*:GAL4-DBD:EcR:*3′ham*, *5′ham*:VP16:RXRCH9:*3′ham* and GUS reporter cassettes. Each fusion fragment was generated by double-joint PCR (Yu *et al*., 2004), cloned into pBS-SK+ and sequenced prior to insertion into pTOR.

Growth and transformation of *P. infestans*

Isolate 1306 (A1 mating type, CA, USA) was the wild-type strain and transformation progenitor for this study. Cultures were maintained on rye–sucrose agar at 18 °C. Transformants were obtained using the plasmids described above, or pTH209-35G (Cvitanich and Judelson, 2003), to obtain a strain expressing GUS behind a constitutive promoter. The transformants shown in Fig. 3 included those obtained by both the protoplast and zoospore electroporation methods (Ah-Fong and Judelson, 2011), whereas those in later figures were obtained by the electroporation technique. Developmental stages were obtained as described by Gamboa-Meléndez *et al*. (2013). Plant infections involved 4-week-old plants of tomato cultivar New Yorker, which were grown with a 14-h day (25 °C), 10-h night (18 °C) cycle, in soil containing sand, bark and peat moss as its principal components. After inoculation with *P. infestans,* plants were kept at 18 °C at high humidity. Leaf disc infections were performed using 10 μL of a zoospore suspension (10^5/mL). Whole-plant assays involved spraying plants to run off with the same zoospore suspension.

Methoxyfenozide treatments and GUS assays

Methoxyfenozide stocks were prepared in DMSO, and care was taken to ensure that all treatments, including controls, used the same amount of DMSO (typically 0.1%). For hyphal tissues destined for histochemical analysis, plugs from rye–sucrose agar cultures were placed in clarified rye–sucrose broth containing methoxyfenozide for 24 h at 18 °C, and the broth was then replaced with histochemical staining solution (Jefferson *et al*., 1987) and held overnight at 37 °C. As noted in the Results section, some experiments involved the growth of *P. infestans* for 3 days on the surface of 0.4-μm pore polycarbonate filters (Sterlitech, Kent, WA, USA) on rye–sucrose agar, transfer of the filter to rye–sucrose agar containing methoxyfenozide for 24 h and staining the tissue from the filter.

Methoxyfenozide treatments of plant samples involved the floating of infected leaf discs on water containing 10 μM of inducer, the spraying of plants with 10 μM of inducer to run off or the watering of each plant (in 10-cm pots) with 20 mL of 100 μM methoxyfenozide. In the case of the latter, the soil concentration of methoxyfenozide was estimated to be about 25 μM based on the total water content of the soil. After incubation for the times noted in the Results section, samples were vacuum infiltrated with staining solution, incubated overnight at 37 °C and decolorized in ethanol.

For quantitative assays, hyphae were prepared using either the filter transfer method described above or liquid cultures. The latter involved the inoculation of clarified rye–sucrose broth with sporangia (10^4/mL), followed by 3 days of incubation at 18 °C. Methoxyfenozide was then added for the times and concentrations noted in the Results section. Quantitative assays of hyphae or sporangia involved the grinding of samples in E buffer [0.25 M sodium phosphate, pH 7.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 50 mM β-mercaptoethanol, 0.5% sodium n-lauroylsarcosine, 0.5% Triton X-100], which were clarified by centrifugation and assayed for protein by the Bradford method. For most assays, 20 μg of protein was incubated in a total of 0.2 mL of E buffer with 1 mM 4-methylumbelliferyl-β-D-glucuronide (4-MUG) for 1 h at 37 °C. After stopping the reactions with 2 mL of 0.1 M Na_2CO_3, fluorescence was measured using a Vectra fluorometer (Perkin Elmer, Santa Clara, California, USA; 365 nm excitation, 455 nm emission), employing a 4-methylumbelliferone solution as a standard. Some assays used *p*-nitrophenyl-β-D-glucuronide as a substrate, with reaction products detected at 420 nm.

ACKNOWLEDGEMENTS

This work was supported by awards to H.S.J. from the National Institute of Food and Agriculture of the United States Department of Agriculture. H.G.-M. was supported in part by the Consejo Nacional de Ciencia y Tecnología (México).

REFERENCES

Ah Fong, A., Xiang, Q. and Judelson, H.S. (2007) Architecture of the sporulation-specific Cdc14 promoter from the oomycete *Phytophthora infestans*. *Eukaryot. Cell*, **6**, 2222–2230.

Ah-Fong, A.M. and Judelson, H.S. (2011) Vectors for fluorescent protein tagging in *Phytophthora*: tools for functional genomics and cell biology. *Fungal Biol.* **115**, 882–890.

Auslander, S. and Fussenegger, M. (2013) From gene switches to mammalian designer cells: present and future prospects. *Trends Biotechnol.* **31**, 155–168.

Baneyx, F. (1999) Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol.* **10**, 411–421.

Braun, S., Azoitei, A. and Spindler-Barth, M. (2009) DNA-binding properties of Drosophila ecdysone receptor isoforms and their modification by the heterodimerisation partner ultraspiracle. *Arch. Insect Biochem. Physiol.* **72**, 172–191.

Caddick, M.X., Greenland, A.J., Jepson, I., Krause, K.P., Qu, N., Riddell, K.V., Salter, M.G., Schuch, W., Sonnewald, U. and Tomsett, A.B. (1998) An ethanol inducible gene switch for plants used to manipulate carbon metabolism. *Nat. Biotechnol.* **16**, 177–180.

Carlson, G.R., Dhadialla, T.S., Hunter, R., Jansson, R.K., Jany, C.S., Lidert, Z. and Slawecki, R.A. (2001) The chemical and biological properties of methoxyfenozide, a new insecticidal ecdysteroid agonist. *Pest Manage. Sci.* **57**, 115–119.

Carneiro, J.S., Bastide, P.Y. and Hintz, W.E. (2013) Regulated gene silencing in the fungal pathogen *Ophiostoma novo-ulmi*. *Physiol. Mol. Plant Pathol.* **82**, 28–34.

Celik, E. and Calik, P. (2012) Production of recombinant proteins by yeast cells. *Biotech. Adv.* **30**, 1108–1118.

Corrado, G. and Karali, M. (2009) Inducible gene expression systems and plant biotechnology. *Biotech. Adv.* **27**, 733–743.

Cvitanich, C. and Judelson, H.S. (2003) Stable transformation of the oomycete, *Phytophthora infestans*, using microprojectile bombardment. *Curr. Genet.* **42**, 228–235.

Esengil, H., Chang, V., Mich, J.K. and Chen, J.K. (2007) Small-molecule regulation of zebrafish gene expression. *Nat. Chem. Biol.* **3**, 154–155.

Gamboa-Meléndez, H., Huerta, A.I. and Judelson, H.S. (2013) bZIP transcription factors in the oomycete *Phytophthora infestans* with novel DNA-binding domains are involved in defense against oxidative stress. *Eukaryot. Cell*, **12**, 1403–1412.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) Gus fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3908.

Judelson, H.S. (1993) Intermolecular ligation mediates efficient cotransformation in *Phytophthora infestans*. *Mol. Gen. Genet.* **239**, 241–250.

Judelson, H.S., Dudler, R., Pieterse, C.M.J., Unkles, S.E. and Michelmore, R.W. (1993) Expression and antisense inhibition of transgenes in *Phytophthora infestans* is modulated by choice of promoter and position effects. *Gene*, **133**, 63–69.

Judelson, H.S., Narayan, R., Fong, A.M., Tani, S. and Kim, K.S. (2007) Performance of a tetracycline-responsive transactivator system for regulating transgenes in the oomycete *Phytophthora infestans*. *Curr. Genet.* **51**, 297–307.

Kang, H.-G., Fang, Y. and Singh, K.B. (1999) A glucocorticoid-inducible transcription system causes severe growth defects in *Arabidopsis* and induces defense-related genes. *Plant J.* **20**, 127–133.

Kozlova, T. and Thummel, C.S. (2000) Steroid regulation of postembryonic development and reproduction in Drosophila. *Trends Endocrinol. Metab.* **11**, 276–280.

Lee, J., Son, H., Lee, S., Park, A.R. and Lee, Y.W. (2010) Development of a conditional gene expression system using a zearalenone-inducible promoter for the Ascomycete fungus *Gibberella zeae*. *Appl. Environ. Microbiol.* **76**, 3089–3096.

Ouwerkerk, P.B.F., de Kam, R.J., Hoge, J.H.C. and Meijer, A.H. (2001) Glucocorticoid-inducible gene expression in rice. *Planta*, **213**, 370–378.

Palli, S.R., Kapitskaya, M.Z., Kumar, M.B. and Cress, D.E. (2003) Improved ecdysone receptor-based inducible gene regulation system. *Eur. J. Biochem.* **270**, 1308–1315.

Randall, E., Young, V., Sierotzki, H., Scalliet, G., Birch, P.R.J., Cooke, D.E.L. *et al.* (2014) Sequence diversity in the large subunit of RNA polymerase I contributes to mefenoxam insensitivity in *Phytophthora infestans*. *Mol. Plant Pathol.* **15**, 664–676.

Roy, S., Poidevin, L., Jiang, T. and Judelson, H.S. (2013) Novel core promoter elements in the oomycete pathogen *Phytophthora infestans* and their influence on expression detected by genome-wide analysis. *BMC Genomics*, **14**, 106.

Saijo, T. and Nagasawa, A. (2014) Development of a tightly regulated and highly responsive copper-inducible gene expression system and its application to control of flowering time. *Plant Cell Rep.* **33**, 47–59.

Stebbins, M.J., Urlinger, S., Byrne, G., Bello, B., Hillen, W. and Yin, J.C.P. (2001) Tetracycline-inducible systems for *Drosophila*. *Proc. Natl. Acad. Sci. USA*, **98**, 10 775–10 780.

Suhr, S.T., Gil, E.B., Senut, M.C. and Gage, F.H. (1998) High level transactivation by a modified Bombyx ecdysone receptor in mammalian cells without exogenous retinoid X receptor. *Proc. Natl. Acad. Sci. USA*, **95**, 7999–8004.

Tavva, V.S., Dinkins, R.D., Palli, S.R. and Collins, G.B. (2006) Development of a methoxyfenozide-responsive gene switch for applications in plants. *Plant J.* **45**, 457–469.

Tavva, V.S., Dinkins, R.D., Palli, S.R. and Collins, G.B. (2007) Development of a tightly regulated and highly inducible ecdysone receptor gene switch for plants through the use of retinoid X receptor chimeras. *Transgenic Res.* **16**, 599–612.

Tavva, V.S., Palli, S.R., Dinkins, R.D. and Collins, G.B. (2008) Improvement of a monopartite ecdysone receptor gene switch and demonstration of its utility in regulation of transgene expression in plants. *FEBS J.* **275**, 2161–2176.

Unger, E., Cigan, A.M., Trimnell, M., Xu, R.J., Kendall, T., Roth, B. and Albertsen, M. (2002) A chimeric ecdysone receptor facilitates methoxyfenozide-dependent restoration of male fertility in ms45 maize. *Transgenic Res.* **11**, 455–465.

Willyerd, K.L., Kemp, A.M. and Dawe, A.L. (2009) Controlled gene expression in the plant pathogen *Cryphonectria parasitica* by use of a copper-responsive element. *Appl. Environ. Microbiol.* **75**, 5417–5420.

Xiang, Q. and Judelson, H.S. (2014) Myb transcription factors and light regulate sporulation in the oomycete *Phytophthora infestans*. *PLoS ONE*, **9**, e92086.

Yu, J.H., Hamari, Z., Han, K.H., Seo, J.A., Reyes-Dominguez, Y. and Scazzocchio, C. (2004) Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* **41**, 973–981.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Transactivator sequences used in 'all-in-one' plasmid (optimized for *Phytophthora infestans* codon usage).