

Review

# Regulated gene expression with promoters responding to inducers

Wei Tang<sup>a,\*</sup>, Xiaoyan Luo<sup>b</sup>, Vanessa Samuels<sup>c</sup>

<sup>a</sup> Department of Biology, Howell Science Complex, East Carolina University, Greenville, NC 27858-4353, USA

<sup>b</sup> Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC 27599, USA

<sup>c</sup> Department of Biology, Shaw University, Raleigh, NC 27601, USA

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## Abstract

Genetically engineered transgenic animals and plants have proven to be extremely useful for analyzing biochemical and developmental processes. Promoters responding to chemical inducers will be powerful tools for basic research in molecular biology and biotechnological applications. Various chemical-inducible systems based on activation and inactivation of the target gene had been developed. The transfer of regulatory elements from prokaryotes, insects, and mammals has opened new avenues to construct chemically-inducible promoters that differ in their ability to regulate the temporal and spatial expression patterns, and this will dramatically increase the application of transgenic technology. This review provides an overview on promoter activating systems, promoter inactivation systems, inducible gene over-expression, and inducible anti-suppression.

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## 1. Introduction

Chemically-inducible systems that activate or inactivate gene expression have many potential applications in the determination of gene function and in plant biotechnology [1–3]. Transgenic technology involving the introduction of one or more transgenes can turn on or turn off desired traits in animals and plants [4,5]. Although constitutive promoters are used to transcribe a gene of interest, a major limitation of constitutive promoters is that they cannot be used to investigate genes whose constant over- or under-expression has deleterious effects on the animals and plants where expression of a sense or anti-sense transgene in transformed cells may be toxic [4–6]. Chemical-inducible systems for regulated gene expression offer a more general and flexible solution because chemical-inducible systems are quiescent in the presence or absence of inducers, and therefore, will not inhibit physiological activities [5,6]. In addition, the use of an appropriate promoter to express the chemical-responsive transcription factor can further restrict the target transgene expression to specific organs, tissues, or even cell types

[4,5]. Chemicals that are used to regulate transgene expression include the antibiotic tetracycline (tc), the steroids dexamethasone (dex), and estradiol, copper, ethanol, the inducer of pathogen-related proteins benzothiadiazol, herbicide safeners, and the insecticide methoxyfenozide [1–3].

Chemical-inducible systems for regulated gene expression are very useful for basic biology research and biotechnology applications [1]. In *Escherichia coli*, an example of a promoter that provides such fidelity is the isopropyl β-D-thiogalactoside (IPTG) inducible *lac* promoter, which is routinely used whenever expression of the recombinant protein is going to interfere with growth [7]. Similarly, if a foreign gene product expressed in animals and plants is going to interfere with regeneration, growth or reproduction, an inducible promoter is required [5]. With such a tool, plants can be regenerated while the promoter is inactive. Further analysis can then be performed after activating expression of the transgene [8]. Several systems without disturbing endogenous plant gene expression have been developed to control transgene expression in plants using chemicals [3].

In addition to being a valuable means of elucidating gene function, chemically-inducible promoters are increasingly relevant to the improvement of crops by genetic engineering [9,10]. If only very low amounts of a gene product are required for a cellular process, then the expression level of

\* Corresponding author. Tel.: +1-2523282021; fax: +1-2523284178.  
E-mail address: [tangw@mail.ecu.edu](mailto:tangw@mail.ecu.edu) (W. Tang).

the promoter should be close to zero in the absence of the inducer [4,11]. In this case, high expression levels in the presence of the inducer are not an essential requirement. In contrast, if only high amounts of a gene product are effective, residual activity in the absence of the inducer can be tolerated, but it should be inducible to high levels in the presence of the inducer [12]. Ideally, both features very low expression levels in the absence of the inducer, and high expression levels in the presence of the inducer should be combined in one system as it is more broadly applicable [13,14]. A further advance in use of a chemically-inducible promoter is its use in combination with tissue-specific promoters, so that gene expression can be restricted to a given tissue at a specific time [15]. It is important that the chemical used is highly specific for the target promoter and it should neither influence the expression of other genes nor affect other cell functions [4–6]. Transgene expression levels and expression patterns can be adjusted by combining the protein coding region with a suitable promoter. These chimeric promoters offer a range of options for transgene design for experimental and field use [16].

Applications of chemically-inducible promoters included: (1) expression of gene products that interfere with regeneration, growth or reproduction; (2) expression of gene products at different stages of development; (3) differentiation between primary and secondary effects; (4) analysis of primary effects before homeostatic mechanisms start to counteract; (5) clear correlation between induction of the transgene and occurrence of an altered phenotype, conditional expression of resistance genes as a means of pest management to delay adaptive processes of the pathogen [1–6,17,18]. The field of inducible gene expression in plants has been subjected to several recent reviews and a book [1,2,4–6,17–19]. This article provides an overview on promoter activating systems, promoter inactivation systems, inducible gene over-expression, and inducible anti-suppression.

## 2. Promoter activating systems

The most common strategy based on transcriptional activation is to constitutively or conditionally express an inactive chimeric transcription activator, which contains a heterologous DNA-binding domain (DBD), an activation domain (AD), a nuclear localization signal (NLS) and, most critically, the regulatory domain of an animal steroid nuclear receptor [13,14]. Steroids such as the glucocorticoid dexamethasone are attractive options as chemical inducers because they exhibit high specificity for the transcriptional activator, the glucocorticoid receptor (GR) [9,13]. Glucocorticoid-dependent transcription is based on the inhibitory interaction between the heat shock protein 90 (HSP90) and the ligand-binding domain of the receptor that occurs in the absence of the ligand [6,9] (Fig. 1). Binding of the ligand leads to dissociation of the receptor from HSP90 and thus to release of a transcriptional activa-

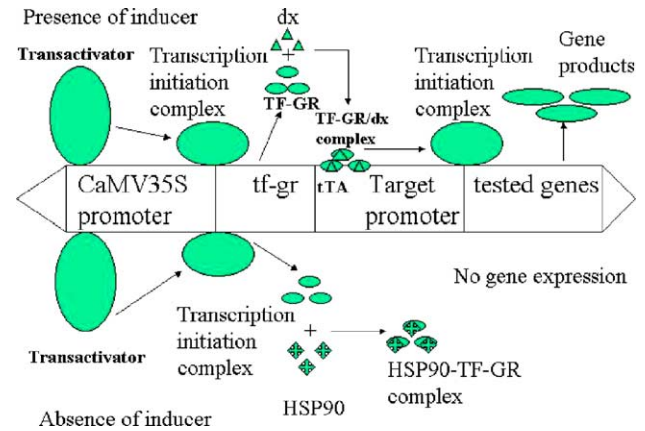


Fig. 1. The dexamethasone-inducible promoter activating system. The fusion protein TF-GR, consisting of a transcription factor (DNA binding domain and transcriptional activation domain) and the glucocorticoid binding domain, is expressed under the control of a strong CaMV 35S promoter. In the absence of dexamethasone the activator is trapped by the formation of an inactive complex with HSP90. The binding of dexamethasone mediates dissociation from HSP90 and allows binding of the activator to a target promoter that contains multiple TF-GR binding sites upstream of a short DNA fragment that encodes the TATA box. Transcription from the target promoter is induced. TF can be any transcription factor that contains a DNA-binding domain and an activation domain.

tor [6,9]. Although the complete receptor protein does not work efficiently in transgenic plants, the ligand-binding domain has been shown to confer ligand-dependent activation of *cis*-located protein domains [17–19].

The glucocorticoid receptor binding domain has been fused to different transcription factors: the flowering-time gene *CONSTANS* [20] and the MADS box factor AP-3 [21]. In these examples, the protein product to be studied was directly controlled by the ligand-binding domain of the receptor [4]. Regulation of steroid nuclear receptors has been well documented, and the molecular mechanism appears to be highly conserved from insects to mammals [13]. In the absence of the hormone ligand, the receptor associates with cellular regulatory proteins, including HSP90, and becomes anchored in the cytosol as a monomer [6]. Association of a ligand with the hormone-binding domain (HBD) leads to the release of HSP90 from the receptor [4,6]. The receptor subsequently dimerizes, translocates into the nucleus, and binds to the target DNA [4,6]. As the hormone inducibility appears to be transferable when the regulatory domain is fused to a heterologous DBD, and also because plants do not have an analogous hormonal system, steroid-based transactivation systems have been used in a number of studies [5]. The regulatory domains of the mammalian glucocorticoid receptor (the GVG system) [13], estrogen receptor (ER) [10] and an insect ecdysone receptor [22] have all been shown to give relatively tight control and high inducibility. The GVG chimeric factor contains the DBD of the yeast GAL4 transcription factor (G), the activating sequence of *Herpes simplex* virion protein VP16 (V), and the regulatory region of the rat GR (G) [5,9,13].

In transgenic tobacco plants, the expression of a luciferase reporter gene driven by the target promoter is stimulated over 100-fold by treatment with dexamethasone, a synthetic GR ligand [13]. The mammalian glucocorticoid receptor, which activates eukaryotic transcription only in the presence of steroids such as dexamethasone, has been used previously to establish a regulatory system in *Schizosaccharomyces pombe* [1,23]. In transiently transformed tobacco cells, transcription of a target promoter containing GR-binding sites upstream of a TATA-box strictly depended on the presence of GR and dexamethasone [5,24]. The hormone-binding domain of GR and other steroid receptors can be used as a molecular switch to regulate heterologous proteins in *cis* [1,25]. The unliganded HBDs of all vertebrate steroid receptors are known to assemble into a protein complex containing HSP90 [1,4]. This complex, which is released upon hormone binding, most likely effects inactivation of protein function by steric hindrance [1,4]. By fusing HBD to maize transcription factor R, a steroid-inducible transcriptional activator (R-GR) was generated [26]. The R-GR fusion protein was transformed into an *Arabidopsis* mutant (*ttg*) that can be complemented with maize transcription factor R. Using a similar approach, a fusion protein consisting of the *Arabidopsis* transcriptional activator *Athb-1* and the activation domain of *H. simplex* virion protein 16 (VP16) was fused to HBD of GR [27]. *Athb-1*-VP16-GR displayed the same function as *Athb-1*-VP16 only in the presence of 10 mM dexamethasone [27]. Similar to the situation in plants, TC and steroid derivatives are the most promising regulators in mammalian cells and transgenic mice. Because many experiments are of potential applicability to medical problems, regulated systems are of high economic interest, which is certainly a factor that pushes further refinements. Moreover, these inducible systems might be used 1 day for a safer application of gene therapy [1,4,6].

A promoter activation system, which allows a gene of interest to be activated in specific plant tissues after a cross between defined transgenic lines, has been developed by Moore et al. [28]. The promoter, *pOp*, consists of *lac* operators cloned upstream of a minimal promoter [28]. Transcription from the promoter was activated by crossing reporter plants with activator lines that expressed a chimeric transcription factor, LhG4, which comprised transcription-activation domain-II from Gal4 of *Saccharomyces cerevisiae* fused to a mutant *lac*-repressor that binds its operator with increased affinity [28]. The LhG4 system offers spatially regulated gene expression in the tissues of whole plants growing under normal conditions without the need for external intervention. It complements inducible expression systems that offer temporal control of gene expression in tissues that can be treated with inducing chemicals [28].

An estrogen receptor-based inducible system has been developed by Bruce et al. [10]. The transactivation domain of the maize activator C1 was inserted in the activation domain of the human ER, and the *ER-C1* fusion gene was controlled by a modified 35S promoter [10]. The target expression pro-

motor contains four copies of ER element (ERE) fused to a minimal 35S promoter [10]. In stably transformed maize Black Mexican Sweet (BMS) cell lines, the activity of a luciferase reporter gene ranges from undetectable in uninduced cells to 14,000 relative light units upon a 48 h induction with estradiol [10]. Another ER-based inducible system, designated the XVE system, was recently developed by using the DBD of the bacterial repressor LexA (X) and the transactivation domain of VP16 (V) [29,30]. The target promoter consists of eight copies of LexA-binding sites upstream from a 35S minimal promoter [29]. The expression of a reporter gene can be readily induced by estradiol three- to five-fold over that of the 35S promoter without detectable background expression [29]. The GVG-like toxic effects have not been found in the XVE system. This system, however, appears to be deregulated in transiently transformed soybean cells, presumably due to the presence of phyto-estrogen in soybean tissues [31,32].

By exploiting the same principle, but using domains from different proteins, a steroid-inducible promoter has been established for transgenic maize [33]. In this system, the DNA-binding domain and the ligand-binding domain were taken from the estrogen receptor, whereas the activation domain was taken from the maize transcription factor C1. By conditionally expressing the male fertility gene *MS45* in a male-sterile background, male fertility was chemically controlled [33]. All of the systems discussed above employ chemical inducers that are not suitable for field applications because of the toxicity of dex, estradiol, and tetracycline to the ecosystem. This restriction, however, appears to have been partially overcome by the efforts of Martinez et al. [22] who have developed a non-steroidal agrochemical-inducible system. In this new system, the hybrid activator contains transactivating sequences from GR and VP16, the DBD of GR and the hormone regulatory domain of the *Heliothis virescens* ecdysone receptor. In transgenic tobacco plants, the activator induced the expression of a reporter gene over 400-fold, corresponding to 150% of the activity of a 35S promoter. The system is highly responsive to RH5992, a non-steroidal ecdysone agonist that lacks phytotoxicity and is currently used as a lepidopteran control agent on a range of crops. A main drawback of this system is the relatively high background expression.

Recently, an inducible RNAi system has been developed in transgenic *Arabidopsis thaliana* and *Nicotiana benthamiana* plants by Guo et al. [34]. In this system, a chemical-inducible *Cre/loxP* (CLX) recombination system was used to trigger the expression of an intron-containing inverted-repeat RNA, and expression of target gene was stringently controlled [34]. Moreover, it can be used to induce silencing of both transgenes and endogenous genes at different developmental stages and at high efficiency. This system could be a useful tool to study signaling mechanisms of gene silencing and to identify gene function in higher plants [34–38]. The major limits of the GR system are the intermittent toxic effects of dexamethasone and its

induction of defense related genes [14,37]. Some of the negative effects may be overcome by selecting plants that express low-to-medium levels of activator and by developing dexamethasone analogs that are less toxic to plants. The advantage of this system is that many plants do not synthesize steroid compounds that activate the target gene in the absence of external steroid application [9,13].

### 3. Promoter inactivation systems

Chemical-inducible expression systems in animals and plants are based on inactivation and activation of transcription of the target gene. All these systems contain two transcription units [1,5]. Whereas the first unit employs a constitutive promoter to express a chemical-responsive transcription factor, the second unit consists of multiple copies of the transcription factor binding site linked to a minimal animal or plant promoter (a truncated 35S promoter in all reported systems), which is used to express the target gene [4,6]. Regulatory sequences responsive to chemical treatment are attractive because their use requires only the cloning of the responsive promoter upstream of the coding region of the gene of interest [9]. It is important to choose an inducer that affects a set of genes that does not interfere with normal growth and development [9]. In this context, promoters responding to the five classical phytohormones or to other plant growth regulators can be excluded [1,5].

Four different groups of chemicals that elicit changes in plant physiology are considered potential inducers of gene expression: chemicals inducing genes required for systemic acquired resistance (SAR), elicitors, safeners, and wound signals [1,4,6]. In the tetracycline-inactivation system, the Tet repressor (TetR) was converted into an activator (tetracycline transactivator, tTA) by fusing it to the acidic activation sequence of *H. simplex* virus protein 16 (VP16) [39–41]. The target expression cassette contains multiple copies of the *tet* operator sequence. The expression of the target gene, is therefore, dependent upon binding of tTA to the *tet* operator, which occurs in the absence of tetracycline [40]. Introduction of the tTA results in the release of the tTA–tetracycline complex from the operator, thus turning off target gene transcription (Fig. 2) [39–43]. This transcription–inactivation system has been used in both tobacco and *Arabidopsis*, and appears to be very useful for the study of gene product stability [44]. Upon turning off the transcription of a transgene by applying the inducer, the turnover of the transgene product can be assessed [43]. A negative control by the inducer, however, makes the system less practical to use than a positively controlled system because plants have to be maintained in the presence of tetracycline in order to turn off transcription [42,43]. An additional complication is that the promoter containing *tet* operator sequences becomes silenced over time, presumably as a result of methylation of the *tet* operator as originally

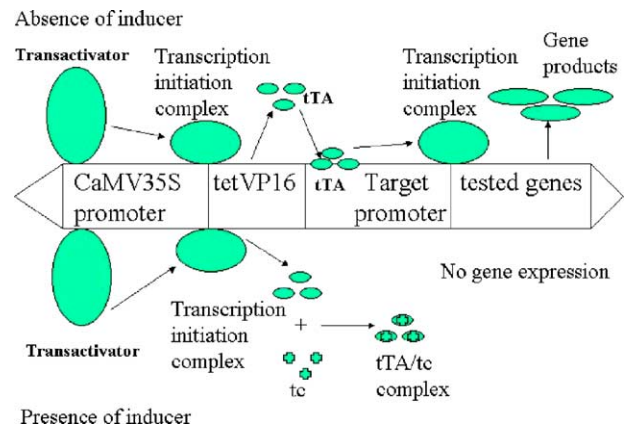


Fig. 2. The tetracycline-inducible promoter inactivation system. A fusion protein consisting of TetR and the acid activation domain of *H. simplex* protein 16 (VP16) is expressed under the control of a strong CaMV 35S promoter. The target promoter contains several *tet* operators upstream of a short DNA fragment encoding the TATA box. Multiple binding sites guarantee strong activation owing to the synergistic effects of multiple tTA proteins. In contrast to TetR, tTA does not compete with endogenous transcription factors for access to the binding sites. Thus, considerably less amounts of tTA are needed compared with TetR. The antibiotic tc binds to the TetR moiety with high affinity, abolishing its DNA-binding ability. Under these conditions, expression is efficiently turned off. The system has been shown to work in tobacco and *Arabidopsis*.

observed in bacteria cells [4,43]. The repression principle is based on sterical interference of a repressor protein with proteins important for transcription (Fig. 2) [39–43].

It is a common mechanism in bacteria but it is found infrequently in higher eukaryotes, where protein–protein interactions are the primary mechanism to mediate stimulating or inhibitory effects on the transcription machinery [40–42]. The TetR encoded by the *E. coli* transposon Tn10 regulates expression of the tetracycline resistance enhancer module TATA-box enhancer module TATA-box [1,39]. The DNA-binding activity of this protein is abolished by very low amounts of the antibiotic tc [15]. Because tc readily enters eukaryotic cells, it is a favorable chemical inducer for laboratory experiments. In tobacco, expression of the tc inducible promoter can be modulated up to 500-fold [45]. In tomato, high levels of TetR cause reduced shoot dry weight and leaf chlorophyll content, reduced leaf size, and an altered photosynthetic physiology when grown between July and September [42]. This phenotype was almost completely reversed by the application of tc. Many laboratories have tried to establish the TetR system in *Arabidopsis* [19]. Repressor concentrations sufficient for transcriptional control cannot be tolerated in *Arabidopsis*, a phenomenon that has also been reported for mammalian cells [46]. In addition, the *Drosophila ecdysone* receptor has been shown to mediate ecdysone-specific gene expression when co-expressed with the retinoid X receptor [1,4,6]. These newly developed systems are worth being tested in animals and plants, in order to extend the tools available for regulated expression of transgenes [1,5,19].

The inactivation principle is based on the construction of fusion proteins between transcriptional transactivation domains and bacterial repressor proteins such as the Lac repressor or TetR [1,6]. The bacterial tetracycline repressor (TetR) binds to the *tet* operator in the absence of tetracycline. Upon association with tetracycline, TetR is released from its operator, presumably due to the conversion of the dimeric TetR (DNA-binding form) to the monomeric form [1,5,19]. As discussed by Gossen and Bujard [39] and Weinmann et al. [40], stringent control of transcription in higher eukaryotes is more likely to be achieved by promoter activation than by repression, most likely because transcriptional activators have free access to their target sites, whereas repressors compete with endogenous transcription factors for binding (Fig. 2). Thus, higher levels of a repressor protein are needed for the same degree of occupancy of target sites. In addition, 50% occupancy of binding sites can be sufficient for transcriptional activation but definitely not sufficient for stringent repression. Nonetheless, a regulation over a range of three orders of magnitude has been achieved in trypanosomes based on the repression principle, using again TetR [19,41]. In this case, a promoter driven by polymerase I was used, and repression was only efficient when the construct was integrated in the non-transcribed spacer of ribosomal DNAs. The tTA system is definitely intriguing because of its low background activity in the presence of tc [1,18]. However, plants must be permanently cultivated on tc to silence the transgene, a difficult task considering that plants require light and tc is unstable in the light. Furthermore, inactivation of tc is slower than uptake of tc for induction of gene expression [1,19,47].

A promising alternative is the use of a mutant TetR that shows a “reverse phenotype” [12]. This reverse repressor binds DNA only in the presence of tc. Gatz et al. [45] developed the first de-repression system in plants. The target promoter is a modified 35S promoter, in which one and two copies of the *tet* operator were placed upstream and downstream from the TATA-box, respectively [45]. In the absence of tetracycline, over-expressed TetR binds to the *tet* operator, thereby preventing target gene expression. Upon binding tetracycline, TetR is released from the operator, relieving the repression. The tetracycline de-repression system has been successfully used for expressing a number of genes in tobacco, tomato, and potato [1,19,47]. Advantages of this system include: (1) low amounts of inducer are sufficient for activation; (2) inducer was taken up by cells; (3) target promoter can be turned off and on efficiently. Limitation of this system is leaky expression and short half-life of inducer [2,6].

#### 4. Inducible gene over-expression

Chemical gene induction systems provide an essential tool for the temporal and quantitative control of transferred genes and have applications in many areas of basic and

applied biology, including the study of gene function, cell lineage ablation experiments, enhanced synthesis of recombinant proteins, and expression of commercially valuable traits [1,3,6]. The ideal regulatable expression system should have the following features: (1) expression levels should be very low in the absence of the chemical and should increase rapidly to high levels upon application of the inducer; (2) the ideal chemical should be non-toxic to the plant and all other organisms in the plant's ecosystem; (3) it should be easily applicable in the field and in the greenhouse by spraying, or under tissue culture conditions by adding it to the synthetic medium; (4) induction should be efficacious at a low use rate and a chemically-inducible system should also be combinable with tissue-specific expression [1,3,5,6]. An example is the conditional over-expression of the bacterial *avrRpt2* avirulence gene under the control of the GVG system in transgenic *Arabidopsis* plants carrying the *RPS2* disease-resistance gene [37]. Induction of the *avrRPT2* gene by dex led to a hypersensitive cell-death response. These transgenic plants offer the opportunity to investigate the molecular events surrounding *avrRPT2*–*RPS2* gene interaction. Because the latter leads to plant death, these transgenic lines can be used to isolate for mutants blocked in the signaling pathway leading from the *avrRPT2*–*RPS2* gene interaction to cell death, thereby identifying components in the pathway [37]. Other than lethality, expression of transgene in the sense or anti-sense orientation can lead to physiological adaptations of transgenic plants, thus masking the true gene functions. This problem is most powerfully illustrated by the recent example of the *TIR1* gene [48]. More recently, Bruce et al. [10] used an ER-C1 chimeric factor to conditionally over-express two transcription factors, CRC (a fusion factor between C1 and R) and P, which are believed to be involved in the flavonoid pathway, thereby identifying a large number of downstream target genes [10,19].

Ethanol-inducible gene expression is a promising alternative to the over-expression systems [1,4,6]. The novel promoter in this system is based on the regulatory elements of the *Aspergillus nidulans alcA* promoter, which is strongly inducible by ethanol [1,6]. It is the most widely used promoter for over-expressing proteins in *A. nidulans* and other filamentous fungi, both for fundamental research and for applied biotechnology [1,19]. The transcriptional activator AlcR, a DNA binding protein belonging to the C6 zinc binuclear cluster family, binds to its target sequences within the *alcA* promoter when cells are grown in the presence of ethanol or other inducers such as ethyl methyl ketone [49]. The system was adapted for plants by placing the *alcR* coding region under the control of the CaMV 35S promoter [49]. The target promoter contains the TATA box as well as upstream sequences of the *alcA* promoter fused to position 223 of the CaMV 35S promoter [22]. When stably transformed into tobacco these constructs mediate ethanol-dependent expression of transgenes [49,50]. It is not known if the DNA-binding activity is directly or indirectly affected by ethanol, but the principle of the regulation is

similar to that of the copper-inducible promoter [16]. The promoter activity in the induced state is in the range of the CaMV 35S promoter. As this system has only recently been developed, some questions concerning the specificity of the inducer for the transgene, toxicity, background levels, and induction levels over a longer time course have yet to be addressed [6,19]. As the system is put to greater use, the extent of its usefulness will emerge. The system has already been used to analyze carbon metabolism by conditionally expressing cytosolic invertase in transgenic plants [22]. Constitutive expression of high levels of cytosolic invertase prevents plant maturation as chlorosis develops in the sink leaves; plants conditionally expressing invertase grew normally until induced with ethanol [51,52]. Within 4 days of induction, the phenotype of the youngest leaves was severely affected. Advantages of ethanol-inducible gene expression are inexpensive inducer and biodegradable, rapid reversible induction, suitable for field application. Limitation of this system is volatile inducer, inducer cannot be used for more than 2 days, and induction can be triggered inadvertently [2,4].

## 5. Inducible anti-suppression

Animal and plant genes that affect development at an early stage may also play a role in later stages of development [1,19]. Mutations in such genes arrest early development, thus precluding investigations on their possible late functions [1,3,6]. Such a mutant can be transformed with the appropriate coding sequence under the control of a chemical-inducible system based on the mechanism of anti-suppression [1,19]. In the presence of the inducer, transgenic animals and plants will be able to undergo early development. Withdrawal of the inducer at a later time will allow evaluation of the late functions of the gene product [5,19]. Constitutive expression triggers a highly aberrant phenotype, and induction with saturating amounts of tetracycline is lethal. By using lower amounts of tc (0.1 mg/l), it proved possible to study the metabolic fate of de novo-synthesized cytokinin in different parts of the plant [6,18]. In combination with grafting experiments this system allowed an assessment of the role of cytokinins as long-range root–shoot signals in the correlative control of apical dominance and sequential leaf senescence in tobacco. The experiments supported the hypothesis that cytokinin is involved in paracrine signaling [53]. The system was also used to obtain transgenic tobacco plants that express high levels of oat arginine decarboxylase in their leaves [4,19]. These experiments were carried out to investigate the action of specific polyamines in different developmental processes. A study with transgenic potato plants has demonstrated that successful anti-sense RNA experiments can also be performed using this promoter [4,6]. For expression of the target gene, the 35S minimal promoter was used in all cases, which ranges in length from 60 to –31 at the distal end of the promoter [22]. A longer minimal promoter in length from 60 to –60 will enhance the overall

promoter strength but it will also lead to a higher basal expression level [22]. On the other hand, a shorter minimal promoter in length from –31 to +1 has the opposite effect. In most cases such as the GVG, XVE, and TGV systems, truncations to around –46 to –48 appear to be optimal for low background activity and high inducibility [6,19].

Chemical-inducible systems provide a very important tool for investigating the sequence of events that ensue upon transient perturbation of the gene under their control [1]. Based on theoretical considerations, we can expect two classes of downstream target genes: firstly, primary response genes, the expression levels of which are effected in the absence of new protein synthesis; and secondly, secondary response genes, which require new protein synthesis to change their transcription rates [4,6,19,28]. Identification of genes from the first class requires that the regulatory gene product itself (transcription factor, kinases, etc.) be directly placed under chemical control. In the case of a transcription factor, fusing a steroid regulatory domain to it will render the activity of the fusion protein dependent on the appropriate steroid [5,19]. In the presence of the inducer and a protein synthesis inhibitor, only the primary response genes are activated, which can be identified by methods such as differential display [4,6]. An example of this is the identification of a *NAM*-like gene as an immediate downstream target of *AP3* [21]. The chemical-inducible systems were also able to use to select transgenic plants without using an antibiotic resistance marker [31,34]. This work demonstrated the feasibility and potential of using a chemical-inducible system to regulate expression of genes that promote plant development [31]. Using a chemical-inducible cre/lox system one can create transgenic plants that are genetic chimera; this can be done by a confined treatment of tissues with an inducer, thereby permanently activating or inactivating the transgene in the treated tissues [31,34,38]. The resulting genetic chimera may provide new information on the mechanisms of long distance signaling in plants. Cell ablation is an important tool for investigating cell fate specification, cell lineage and cell–cell interactions during plant development [34]. Traditionally, this is carried out by laser ablation of a cell or a group of cells in question, followed by tracking the effects of ablation on development of other cells [54]. The incorporation of a chemical-inducible system will allow one to investigate cell fate and lineage at different developmental times. Advantages of the Cre/loxP DNA recombination system include tightly controlled gene expression, high-efficiency inducible DNA excision, suitable for generating marker-free transgenic plants and for efficient genetic modification of crops. Limitation of this system is not suitable for plants with phytosteroids such as soybean [2,31,34].

## 6. Conclusions and future prospects

The development of chemical-inducible systems for regulation of gene expression is the subject of considerable

current activities. Progress in this area depends to a large extent on discoveries of chemical-responsive transcription factors in other organisms [19,55–58]. Based on the published examples, some general rules have evolved that would be helpful to the future development of new inducible systems. Several activation domains have been used with success in chemical-inducible systems [19,55,56]. It is feasible that chemically-inducible promoters could be constructed using novel regulatory elements from organisms such as *E. coli*, mammals, fungi, and insects. VP16 appears to work well in a number of species [19]. The regulatory domain not only confers tight control and high inducibility to a system, but also provides the diversity of inducers that can be used [57,58]. A regulatory domain with a high binding affinity to its cognate inducer is preferable, as only low concentrations of inducer would be required for activation. In some cases, an inducible system that works in one species may not function in others. As mentioned before, most of the systems reported thus far are unsuitable for field applications because of the chemical nature of the inducers [19]. Further work should focus on systems suitable for applications with transgenic crop plants, with particular emphasis on agricultural chemicals that have already been registered for field usage. An additional interest would be to develop multiple-inducible systems to independently regulate several target genes. In this regard, the possibility of designing artificial zinc finger proteins that can specifically bind to any synthetic DNA sequences with high affinity is of special interest [4,6]. A comparative analysis of the different promoters under standardized experimental procedure will help in the evolution of transgene design [19,28]. Properties required for an ideal inducer include: (1) high specificity for the transgene and high environmental compatibility; (2) high efficiency at low concentrations and low use rates; (3) availability of different derivatives with different properties including inducing chemicals, inactivating chemicals, chemicals that move systemically, chemicals that are confined to the site of application, and chemicals with different half-lives in animals and plants. In contrast to the recently described activator LhG4 that consists of the Lac repressor and the GAL4 activation domain, regulated activators will allow gene expression to be activated or inactivated by chemical inducers [1,19,28]. Genomics will yield more sequences of interest and the function of these sequences might be analyzed by inducible expression.

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