

Chemical-inducible systems for regulated expression of plant genes

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Chemical regulation of transgene expression presents a powerful tool for basic research in plant biology and biotechnological applications. Various chemical-inducible systems based on de-repression, activation and inactivation of the target gene have been described. The utility of inducible promoters has been successfully demonstrated by the development of a marker-free transformation system and large-scale gene profiling. In addition, field applications appear to be promising through the use of registered agrochemicals (e.g. RH5992) as inducers.

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Abbreviations

DBD	DNA-binding domain
dex	dexamethasone
ER	estrogen receptor
GR	glucocorticoid receptor
rtTA	reverse tTA
TetR	tetracycline repressor
tTA	tetracycline transactivator
VP16	herpes simplex virus protein 16

Introduction

Genetic manipulations by transgenic technology invariably involve the introduction of one or more transgenes that can turn on or turn off desired traits in plants. In many cases, constitutive promoters (e.g. the CaMV35S) [1] are used to transcribe a gene of interest. A major limitation of constitutive promoters, however, is that they cannot be used to investigate genes whose constant over- or under-expression has deleterious effects on the plant. In the more severe cases, expression of a sense or anti-sense transgene in transformed cells may be toxic, thereby blocking plant regeneration.

To a certain extent, the lethality problems can be partially overcome by using tissue-specific promoters. On the other hand, chemical-inducible systems for regulated gene expression offer a more general and flexible solution. In contrast to constitutive promoters, chemical-inducible systems are quiescent in the absence of inducers and therefore will not inhibit plant regeneration. By the judicious application of inducers, it is possible to regulate gene expression in transgenic plants at a particular developmental stage and for a specific duration. Furthermore, 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.

Chemical-inducible systems for regulated gene expression are extremely useful for basic plant biology research and biotechnology applications. For example, the ability to activate a specific gene trait in the field by using chemicals might circumvent the problem of saving of transgenic seeds by growers. In response to basic research interests as well as commercial needs, many chemical-inducible systems have been developed in the past decade. Because this subject was intensively surveyed 2–3 years ago [2,3,4] our review will focus primarily on results published in the past three years. We compare the relative merits of the various systems, describe their uses thus far, consider strategies for future development and discuss their potential applications.

Desired properties of an ideal chemical-inducible system

Table 1 lists the desired properties of an ideal chemical-inducible system. Stringent chemical regulation of the system requires many different properties. To prevent uncontrollable expression, it is important that the chemical inducer should not be a plant metabolite. Chemicals that elicit physiological responses in plants or are otherwise toxic should be avoided for obvious reasons.

Taking these properties into consideration, it is clear that promoters of plant genes whose expression is triggered by chemicals (e.g. salicylic acid or benzothiadiazole) [2] are probably not suitable. These promoters are likely to have some basal level expression, and because of the various *cis* elements embedded in their 5' upstream region, they are likely to respond to a number of physiological and environmental signals, in addition to the chemical in question. For these reasons, several laboratories have been engaged in the development of chemical-inducible systems using components derived from non-plant sources.

Artificial chemical-inducible systems

Chemical-inducible expression systems in plants, in general, are based on de-repression, inactivation, and activation of transcription of the target gene (Table 2). All these systems contain two transcription units. Whereas the first unit employs a constitutive (e.g. 35S) 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 plant promoter (a truncated 35S promoter in all reported systems), which is used to express the target gene. The properties of these systems are briefly summarized below.

De-repression system

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,

Table 1**Desired properties of an ideal chemical-inducible system in plants.**

Very low basal expression level
High inducibility
High specificity with respect to inducers
High dynamic range of response with respect to inducer concentrations
Fast response upon induction
Rapid switch-off following inducer withdrawal
Inducer not toxic and has no physiologic effects plants
Inducer not found in target plants

presumably due to the conversion of the dimeric TetR (DNA-binding form) to the monomeric form. Based on these observations, Gatz *et al.* [5,6] 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. In the absence of tetracycline, overexpressed 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 but it did not work in *Arabidopsis*, which presumably requires a higher but intolerable repressor concentration for efficient repression ([2,3^{*}] and references therein). Another disadvantage is that fresh tetracycline has to be supplied every other day due to the short half-life of the inducer in plants, making the system less convenient to use.

Inactivation system

In the tetracycline-inactivation system, the TetR repressor was converted into an activator (tetracycline transactivator [τ TA]) by fusing it to the acidic activation sequence of herpes simplex virus protein 16 (VP16). The target expression cassette contains multiple copies of the *tet* operator sequence. The expression of the target gene is therefore dependent upon binding of τ TA to the *tet* operator, which occurs in the absence of tetracycline. Introduction of the latter results in the release of the τ TA–tetracycline complex from the operator, thus turning off target gene transcription [7]. 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 [8]. Upon turning off the transcription of a transgene by applying the inducer, the turnover of the transgene product can be assessed. 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. An additional complication is that the promoter

Table 2**A list of chemical-inducible systems in plants.**

System	Transcription factor	Inducer	Reference
De-repressible	TetR	Tetracycline	[6]
Inactivatable	τ TA	Tetracycline	[7]
Activatable	GVG	Dexamethasone (dex)	[10]
	AlcR	Ethanol	[14]
Dual-control	GVGEc	RH5992	[12 ^{**}]
	ER-C1	β -estradiol	[11 [*]]
Dual-control	XVE	β -estradiol	(a)
	TGV	dex & tetracycline	[9 ^{**}]

(a) Zuo *et al.*, unpublished data.

containing *tet* operator sequences becomes silenced over time, presumably as a result of methylation of the *tet* operator as originally observed in bacteria cells ([3^{*}] and references therein; see also [9^{**}]).

Activation systems

Most inducible expression systems described in plants are based on transcriptional activation. The most common strategy is to constitutively or conditionally express an inactive chimeric transcription activator, which contains a heterologous DNA-binding domain (DBD), an activation domain, a nuclear localization signal (NLS) and, most critically, the regulatory domain of an animal steroid nuclear receptor. Regulation of steroid nuclear receptors has been well documented, and the molecular mechanism appears to be highly conserved from insects to mammals. 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. Association of a ligand with the hormone-binding domain leads to the release of HSP90 from the receptor. The receptor subsequently dimerizes, translocates into the nucleus, and binds to the target DNA. 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 [4]. The regulatory domains of the mammalian glucocorticoid receptor (GR; the GVG system) [10], estrogen receptor (ER) ([11^{*}]; J Zuo, Q-W Niu, N-H Chua, unpublished data) and an insect ecdysone receptor [12^{**}] 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 VP16 (V), and the regulatory region of the rat GR (G). 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 (dex), a synthetic GR ligand. The system has been successfully used to express a number of genes in

different studies (see below). Major shortcomings of the GVG system appear to be dex-dependent toxic effects in some cases, and the induction of defense-related genes ([13*]; N-H Chua *et al.*, unpublished data). The effects appear to occur in transgenic lines with high expression levels of the GVG transcription factor (T Aoyama, N-H Chua, unpublished data). Although the cause of these effects is still under investigation it might result from binding of the GVG transcription factor at high concentrations to plant *cis*-elements with sequence homology to the GAL4 recognition site. One solution to this problem is to select for experimental lines that express moderate or low levels of GVG, and use as negative controls empty vector transgenic lines expressing the same level of GVG as experimental lines.

An ER-based inducible system has been developed by Bruce *et al.* [11*]. The transactivation domain of the maize activator C1 was inserted in the activation domain of the human ER, and this *ER-C1* fusion gene was controlled by a modified 35S promoter. The target expression promoter contains four copies of ER element (ERE) fused to a minimal 35S promoter. In stably transformed maize BMS (Black Mexican Sweet) cell lines, the activity of a luciferase reporter gene ranges from undetectable in uninduced cells to 14,000 relative light units upon a 48 hour induction with estradiol (see also below). 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) (J Zuo *et al.*, unpublished data). The target promoter consists of eight copies of LexA-binding sites upstream from a 35S minimal promoter. The expression of a reporter gene can be readily induced by estradiol 3–5-fold over that of the 35S promoter without detectable background expression. 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 (T Klein, J Zuo, N-H Chua, unpublished data), presumably due to the presence of phyto-estrogen in soybean tissues.

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.* [12**], 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.

In addition to the examples described before, the intact *Aspergillus nidulans* AlcR activator was used to control the expression of target genes in plants using ethanol. In transgenic tobacco plants AlcR stimulated the expression of a chloramphenicol acetyltransferase reporter gene upon induction by ethanol to a level corresponding to 50% activity of the 35S promoter, whereas the background was nearly undetectable [14]. Pending development of non-volatile inducers, this system appears to hold promise for field application.

The dual-control inducible system

The drawbacks of the de-repression and inactivation systems prompted Gatz and co-workers [9**] to develop a dual-control inducible system by combining the advantages of these two systems and eliminating most of the disadvantages. A chimeric transcription activator TGV was made by fusing the TetR DBD (T) to the regulatory region of the rat GR (G) and the VP16 transactivating sequence (V), and the resulting factor is therefore subjected to dual regulation by tetracycline and dex. In a dex-dependent fashion, TGV activates the expression of a reporter gene driven by a synthetic promoter consisting of multiple copies of the modified *tet* operator sequences placed upstream of a 35S minimal promoter. This dex-inducible activation is similar to the previously reported GVG system [10]. When dex is removed and tetracycline is applied, the system is promptly switched off as association of tetracycline renders the chimeric factor incapable of binding DNA. The redesigned target promoter, modified by eliminating putative CG methylation sites, resolved the problems caused by methylation of the *tet* operator over generations, but the cost is a higher background expression. In addition, because structurally similar activators and the same inducer (dex) were used, the TGV system may have similar side effects as the GVG system.

Present and potential applications

Conditional overexpression studies

An example is the conditional expression of the bacterial *avrRpt2* avirulence gene under the control of the GVG system in transgenic *Arabidopsis* plants carrying the *RPS2* disease-resistance gene [15]. 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.

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. Estelle and co-workers [16] found that transgenic plants expressing a *35S-TIR1* transgene (thus

being constitutively expressed) had no apparent phenotype. On the other hand, transgenic plants carrying a *GVG-TIR1* construct produced more lateral roots in the presence of dex, similar to wild-type plants treated with auxin. These results confirm the role of TIR1 in the auxin response pathway.

Co-suppression studies

Yoshizumi *et al.* [17] used the GVG system to express the antisense strand of the *Arabidopsis CDC2b* gene. Inhibition of *CDC2b* expression upon dex induction resulted in short hypocotyls and open cotyledons of the transgenic plants grown in the dark, and these phenotypes were fairly correlated to the level of the antisense gene expression. Their results revealed an important aspect of the *CDC2b* function in the light- and hormone-regulated seedling growth and development.

Conditional genetic complementation

Plant genes that affect development at an early stage (e.g. embryogenesis) may also play a role in later stages of development. Mutations in such genes arrest early development, thus precluding investigations on their possible late functions. Such a mutant can be transformed with the appropriate coding sequence under the control of a chemical-inducible system. In the presence of the inducer, transgenic 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.

Identification of downstream target genes

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. 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. 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. 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. An example of this is the identification of a *NAM*-like gene as an immediate downstream target of *AP3* [18]. More recently, Bruce *et al.* [11•] used an ER-C1 chimeric factor to conditionally overexpress 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.

Marker-free transformation

A novel use of chemical-inducible systems has been reported by Kunkel *et al.* [19••], who were able to select

transgenic plants without using an antibiotic resistance marker. The *ipt* gene of *Agrobacterium* Ti plasmid is known to cause cytokinin production in transformed cells leading to shoot regeneration. The uncontrolled production of cytokinins, however, causes developmental abnormalities, and the transgenic shoots were unable to produce roots and their flowers were infertile. Recognizing the shoot-regenerating potential of the *ipt* gene, Kunkel *et al.* [19••] placed it under the control of the GVG system [10]. *Agrobacterium* carrying the *GVG-ipt* construct were used to inoculate tobacco leaf disc in a medium without auxin and cytokinin. No shoot regeneration was observed in the absence of dex. By contrast, in the presence of the inducer many shoots regenerated, which were developmentally abnormal, for example, inhibition of root growth, the loss of apical dominance and sterility. These shoots were transferred to a medium without the inducer, and after several weeks normal plants developed, most of which were found to be transgenic. This work demonstrated the feasibility and potential of using a chemical-inducible system to regulate expression of genes that promote plant development.

DNA manipulations

Chemical-inducible systems can be used to activate specific recombinases (e.g. cre and flipase) for nuclear DNA remodeling in transgenic plants (see [20] for more comprehensive discussions). Depending on the configuration of the recombinase binding sites, the target DNA can be inverted or evicted resulting in activation or inactivation of transgenes (S Moller, N-H Chua, unpublished data). Organelle DNA can be similarly manipulated with a recombinase appended with the appropriate transit sequences.

Generation of chimeric plants

Specific organs of transgenic plants carrying a chemical-inducible system can be treated with the appropriate inducer to activate gene expression only in the treated organ. In this case, however, the induced expression is only transient. On the other hand, 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 organs/tissues with an inducer, thereby permanently activating or inactivating the transgene in the treated organs/tissues. The resulting genetic chimera may provide new information on the mechanisms of long distance signaling in plants.

Ablation of specific cells

Cell ablation is an important tool for investigating cell fate specification, cell lineage and cell-cell interactions during plant development. 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 (reviewed in [21,22]). This technique, requiring an expensive and highly specialized facility, however, can be replaced by fusing a cell-specific promoter to a toxic gene (e.g. diphthera toxin) and the cells are killed at the onset of the specific promoter

activity. The incorporation of a chemical-inducible system will allow one to investigate cell fate and lineage at different developmental times.

Conclusions and future challenges

The development of chemical-inducible systems for tight control of plant gene expression is a challenging task and is the subject of considerable current activities. Because the components for such systems are usually derived from non-plant sources, progress in this area depends to a large extent on discoveries of chemical-responsive transcription factors in other organisms. Based on the published examples, some general rules have evolved that would be helpful to the future development of new inducible systems. 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. A longer minimal promoter (e.g. up to -60) [12**] will enhance the overall promoter strength but it will also lead to a higher basal expression level. On the other hand, a shorter minimal promoter (e.g. -31 to +1) [14] has the opposite effect. In most cases (e.g. the GVG, XVE and TGV systems), truncations to around -46 to -48 appear to be optimal for low background activity and high inducibility.

The transactivator tTA binds to *tet* constitutively but this interaction is disrupted by tetracycline or its derivatives. By mutagenesis, tTA can be converted into the so-called reverse tTA (rtTA), which now requires the association of tetracycline for DNA binding [23]. Baron *et al.* [24] developed a new system that co-expresses tTA and rtTA in HeLa cells; the DNA-binding domain of the latter was also modified in order to recognize a variant *tet* operator sequence upon association of doxycycline. This new system allows one to reversibly control the expression of two genes in a mutually exclusive manner. Unfortunately, rtTA did not work in transgenic tobacco or *Arabidopsis* plants for unknown reasons [2]. Thus far, the choice of the DBD is fairly restricted, confined to those derived from well-characterized transcription factors of bacteria (e.g. TetR and LexA), yeast (e.g. GAL4) and mammals (e.g. GR and ER). In the course of expanding this list, two properties have to be kept in mind: firstly, the dissociation constant between the DNA-binding protein and its cognate binding site will determine the specificity and inducibility of the system; secondly, the shorter the length of the binding site used the less likely it will be recognized by any endogenous plant factors. 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 [25]. If transferable to plants, this technology will allow the construction of chemical-inducible systems with very low basal expression level and high inducibility.

Several activation domains have been used with success in chemical-inducible systems, including VP16 [9**,10,12**], GR [12**], and those from plants [11*,18]. As no comparative studies have been conducted so far, their relative

merits are difficult to assess at this time. In general, VP16 appears to work well in a number of species.

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. 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. For example, the hormone-binding domain of the ER binds estradiol with a very high affinity of 0.05 nM [26] as compared to ~10 nM for binding of dex to the GR [27]. In theory, the regulatory domains of many mammalian nuclear receptors (e.g. thyroid hormone, androgen, vitamin D3, and peroxisome-proliferator-activated receptors) can be incorporated into chemical-inducible systems for plants. Several of them may not be suitable, however, because they bind to chemicals present in plants (e.g. peroxisome-proliferator-activated receptors bind to linolenic and linoleic acids, and androgen receptors may bind to intermediates of the brassinosteroid biosynthetic pathway) [28]. In some cases, an inducible system that works in one species may not function in others. It is therefore prudent to first test the activity of candidate regulatory domains in plant cells before using them as a component of the chimeric transcription factor.

As mentioned before, most of the systems reported thus far are unsuitable for field applications because of the chemical nature of the inducers. Further work should focus on systems suitable for applications with transgenic crop plants, with particular emphasis on agricultural chemicals (e.g. insecticides and safeners; the latter chemicals are used in agriculture to render crops tolerate to herbicides) that have already been registered for field usage. An additional interest would be to develop multiple-inducible systems to independently regulate several target genes.

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