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Review

Ethanol-inducible gene expression system and its applications in plant functional genomics

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Abstract

External control of gene expression in plants is critical to both characterization of gene function and plant biotechnology. Ethanol-inducible gene expression system ('*alc* switch') is such a system suitable for regulated expression of transgene or native gene in plants temporally, spatially and quantitatively. The *alc* switch consists of two expression cassettes:*p35S*:*alcR* and *palcA*:*target gene*. The system has high specificity and efficiency. The induction exhibits a rapid, reversible and dose-dependent manner. Ethanol, the chemical inducer, is non-toxic, inexpensive, safe and easy to apply both in lab and field. The *alc* switch has been successfully used in *Arabidopsis*, tobacco, potato, oilseed rape and other plant species. It is believed that *alc* switch will have broad utility in plant functional genomics and molecular farming.

Keywords: Ethanol; *alc* switch; Inducible gene expression; Functional genomics

Contents

1. Introduction

The completion of genome sequencing of *Arabidopsis* and rice [1–3] and the abundant sequence data from other plants have provided a vast new resource to define gene function at morphological, biochemical and physiological level. For exploiting these genomic resources, we need to develop novel tools and approaches to evaluate gene

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functions [4]. One of the effective ways is to establish the regulated expression systems of either transgenes or native genes in plants. A number of gene expression systems have been developed including constitutive, tissue-specific and chemical-induced expression [5,6]. CaMV35S promoter has been successfully used to express a spectrum of target genes in plants. However, constitutive expression of certain genes in all tissues throughout development could result in highly deleterious or lethal effects. Additionally, this constitutive system is unsuitable for studies where expression of a transgene is desired to be restricted to specific tissues or at a specific stage of plant development [7,8], such as the conditional expression of pesticide or herbicide resistance, the induction of synchronous flowering of plants and the production of a conditional male sterility system.

To control gene expression in time and space, several approaches have been employed, including driving target genes with tissue-specific promoters [9-11] or promoters responding to environmental cues such as heat shock [12], light [13] and wounding [14]. Although these expression systems seem to work well in some plants, they are far from satisfactory either for the identification of gene function or for plant genetic improvement. The limited number of tissue-specific promoters restricts analysis to a few cell types or tissues. In addition, these promoters often appear to be active during the process of regeneration, causing unwanted pleiotropic effects [7]. Promoters responding to environmental cues also lead to unexpected effects. It is difficult to regulate the expression of foreign target gene because of the changeability and complexity of environmental factors [8].

The optimal system would employ an inexpensive, nontoxic inducer or inhibitor whose application can be fully controlled, and would lead to a reversibly dose-dependent expression with the potential to achieve high levels of gene expression at need but with negligible basal activity. In other word, this system should allow the expression of interested genes to be precisely regulated temporally, spatially and quantitatively. Toward these ends, a series of chemically regulated expression systems have been developed to enable target gene expression to be switched on or off, by adding or removing specific chemicals [5,6]. Chemical-dependent gene expression systems include those controlled by steroilds [15–17], ecdyson [18,19], estrogen [20], tetracycline [21], pristinamycin [22], copper [23] and ethanol [24]. Of them, the ethanol-inducible gene expression (‘*alc* switch’) has been considered as one of the most promising systems for both laboratory and field use because other chemical-dependent systems are unlikely to receive approval for large-scale field use [24–26]. The inducer, ethanol, is a cheap and simple biodegradable organic molecule, making *alc* switch great potential for investigating gene function in vivo [27,28].

Not only does *alc* switch work well in the regulated expression of a number of target transgenes in *Arabidopsis*, but it also functions effectively in several crop species including tobacco, oilseed rape, potato, tomato and rice [24–29]. Using the *alc* switch, we can precisely regulate the expression of target gene so as to accumulate the target compounds at specific tissues and developing stages, to make resistance mechanism functioned only under biotic and abiotic stresses, and to optimize plant architecture with higher yields. Here, we focus on describing the construction, properties, advantages and limitations of the *alc* switch as well as its applications in plant functional genomics.

2. Ethanol-inducible gene expression system (*alc* switch)

Ethanol-inducible system is derived from the filamentous fungus *Aspergillus nidulans* [30,31]. In *A. nidulans*, the gene *alcR* encodes transcription factor (ALCR). ALCR controls the activation of several structural genes, such as *alcA* and *aldA*, which encode alcohol dehydrogenase I (ADHI) and aldehyde dehydrogenase (AldDH) respectively. In the absence of ethanol, the ALCR protein is inactive. When ethanol is added, ALCR and ethanol interact to form an activated ALCR. This activated ALCR then binds to the promoter of the target gene such as *alcA*, inducing the expression of the gene [32–35].

The ethanol-inducible gene expression system consists of two transcription units (Fig. 1). The first one is *alcR* expression cassette (*p35S*:*alcR*), which is constructed by cloning a constitutive CaMV 35S promoter with an *A. nidulans alcR* gene placed downstream. CaMV 35S promoter can be replaced by cell/tissue/developmental-stage-specific promoter, providing additional control. The other one is target gene expression cassette (*palcA* + *mini-p35S*:*target gene*), which is composed of a minimal 35S promoter with the upstream activator region of the *alcA* promoter (*palcA*) and a target gene. When ethanol is absent, ALCR protein expressed from the first unit has no activity and can not bind to the *palcA* promoter located in the second unit. Therefore, no target gene expresses. Upon ethanol adding, ALCR interacts with ethanol, leading to its conformational change and becoming active. The activated ALCR then binds to the specific *cis*-element in *palcA* promoter, and directs the transcription of the downstream target gene. When ethanol is removed, ALCR lost its activity and released from *alcA* promoter region, resulting in termination of target gene expression [24].

3. The advantages and limitations of the *alc* switch system

Compared with other gene expression systems, the *alc* switch has the following merits:

(1) The construct of the system is relatively simple. It is composed of two main elements: the *alcR* gene and the *alcA* promoter.

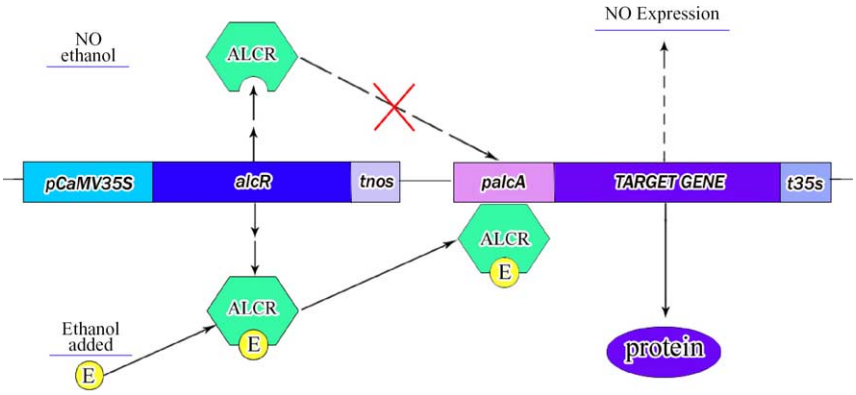

Fig. 1. The ethanol-inducible gene expression system. The construct includes the full CaMV35S promoter (p35S) driving the expression of transcription factor ALCR which was positioned upstream of the *NOS* terminator (t *NOS*). The chimaeric promoter *alcA-35S* contains two ALCR binding sites of the *Aspergillus nidulans alcA* promoter and a *35S* minimal promoter. The target gene is under the control of the inducible *alcA* promoter and terminated by the *35S* terminator.

(2) The *alcA* promoter exhibits high induction, has a rapid but reversible onset of action, and gives sufficient levels of expression to result in phenotypic effects [24,26].
(3) The system responds in an approximately dose-dependent manner, although high levels of ethanol are deleterious. The level and duration of expression can be adjusted to suit the gene product expression. Both short- and long-term expression could be achieved through adjustment of concentration or duration of contact of ethanol with the plant [24–28].
(4) ALCR is derived from non-plant organisms, *A. nidulans*, which is distant from higher plants in evolution. So far, no report showed that ALCR activation affected the expression of other endogenous genes in plants.
(5) Ethanol is an ideal inducer. It is inexpensive, biodegradable and environmentally safe. At the levels used, ethanol gives no visible phytotoxic symptoms in the treated plants.
(6) The system appears to be compatible with both experimental and field use. Ethanol can be applied to plants using different methods, such as foliar sprays, root drenches, liquid growth media and ethanol vapor [24–28].
(7) The risk of accidental induction under anaerobic conditions appears small although extreme anoxia can induce the *alc* switch. The system shows negligible induction in plants subjected to wound, cold or drought stress, or following treatment with either salicylic acid or methyl jasmonate [24–28].

Although extremely useful, this system is not without drawbacks. For example, basal expression of reporter genes under control of *alc* switch was observed in seedlings grown on agar in the absence of exogenous ethanol [24]. Such background activity could be a problem if the transgene inhibits plant growth. Another potential disadvantage is that exogenous application of ethanol might affect other endogenous gene expressions inadvertently although no detailed data have been reported so far. External adding of ethanol might also alternate the endogenous ethanol level, which might result in unexpected effects on other pathways. However, it was argued that the levels of natural ethanol in plants under normal growth conditions were extremely low. In addition, it is not known if the DNA binding activity is directly or indirectly affected by ethanol.

4. Applications of the *alc* switch system

4.1. External control of transgenic expression in plants for both experimental and field utilization

External control of gene expression is a very effective approach for dissection of gene functions in higher plants, which avoids the deleterious effects of constitutive mis-expression of a given gene and makes it easier to get more precise and reliable results. Thus, one can distinguish between primary effects of gene expression and long-term secondary effects. *Alc* switch proved to be such a system suitable for regulating the expression of transgene in plants.

Salter et al. (1998) [25] demonstrated the efficiency and applicability of *alc* switch in tobacco (*Nicotiana tabacum*). The *alc* switch construct contained a chloramphenicol acetyl transferase (CAT) reporter gene which was inserted to the downstream of *palcA* + *mini-p35S* promoter. In the complete absence of ethanol, there is no detectable expression of *CAT* in transgenic plants. CAT activity, however, was initiated at 0.01% (1.7 mM) ethanol in liquid growth media within 4 h, with maximal expression occurring after 4 days, suggesting that this *alc* switch is very sensitive and the induction is rapid. The level of CAT activity remained constant between 0.02 and 0.08% ethanol, but increased to a maximum at 0.1% ethanol, after which it declined, indicating the dose-response of induction. Leaf spraying and root drenching with ethanol also activated the *CAT* expression in transgenic lines, reflecting accessibility of inducer to the target tissue. At the maximal induction of *alc* switch, no detrimental

effects were observed. In addition, wounding, drought, cold stress and treatment with salicylic acid and methyl jasmonate did not cause induction of this system, showing specificity. The similar results were also obtained in greenhouse and field experiments.

This research group successfully manipulated carbon metabolism in transgenic tobacco plants using *alc* switch to conditionally express cytosolic invertase [24]. Upon rapid induction of yeast cytosolic invertase by ethanol, a marked phenotype appears in developing leaves that is absent from leaves that developed before induction or after it has ceased. Roslan et al. (2001) [26] further developed optimal strategies for utilizing the *alc* switch in *Arabidopsis thaliana* under a variety of regimes. By linking the *alcA* promoter to β-lucuronidase (GUS), luciferase (LUC) and green-fluorescent protein (GFP) genes, they indicated that these reporter genes were induced to express throughout the plant in a highly responsive manner. Induction occurred within 1 h of addition of ethanol and was dose-dependent, with negligible activity in the absence of the exogenous inducer for soil-grown plants.

It was recently reported that low concentration of ethanol vapor efficiently induced the *alc* gene expression system in tobacco, potato (*Solanum tuberosum*) and oilseed rape (*Brasica napus*) [27]. Although induction was seen with less than 0.4 μM ethanol vapor, maximal induction of *CAT* gene was achieved after 48 h in leaves of tobacco plants enclosed with 4.5 μM ethanol vapor. Treatment of potato tubers with ethanol vapor resulted in uniform GUS expression. Vapor treatment of a single oilseed rape leaf lead to induction of GUS in the treated leaf only and ^{14}C-ethanol labeling in tobacco confirmed that the inducer was not translocated. Therefore, by exposing different parts of a transgenic plant to ethanol vapor, it is possible to target expression of the *alc* switch to particular organs. This information shows that ethanol vapor may be particularly useful in controlling gene expression in post-harvest crops such as potato, cut flowers, or fruits. For example, in potato, expression of a pyrophosphatase leads to non-sprouting tubers [36], a phenotype that could be used to improve tuber storability. Coupling expression of an inhibitor of pyrophosphatase (e.g. its antisense gene) to the *alc* switch and treatment with ethanol vapor could be used to restore sprouting.

4.2. Fine temporal and spatial analysis of gene functions

The *alc* switch permits both temporal and spatial regulation of gene expression, which has been used for the analysis of gene function during plant development. Deveaux et al. (2003) [28] successfully employed *alc* switch to mediate the target gene expression at defined time during development and in specific tissue or organs of *Arabidopsis* plants. In the expression constructs, *alcR was* driven by tissue-specific promoter such as aintegumenta (ANT), leafy (LFY), unusual floral organs (UFO) and clavata3 (CLV3). These promoters only express at specific domains of vegetative apices and inflorescences. It was found that spatially limited expression of reporter gene (GUS/GFP) was precisely controlled in transgenic plants by exogenous ethanol. The kinetics of reporter gene activation and inactivation appeared as a pulse of ethanol induction, showing this system is dynamic and suitable for precise temporal control of target gene expression. They also obtained the transgenic plants with simultaneous expression of two genes in a given domain or the expression of a gene in two separate domains.

To extend these findings, Deveaux et al. further used this system to study the interested gene functions during development. Shoot meristemless (STM) and cyclin D3; 1(CYCD3; 1) are two of key developmental genes. Their expressions are normally within the entire meristem, indicating a function in cell division [37,38]. *CYCD3;1* is also expressed during the proliferation phase of the developing leaf [39]. cDNAs of *STM* and *CYCD3;1* were cloned downstream of the *alcA* promoter respectively (*alcA*::STM/*alcA*::CYCD*3;1*) and were mis-expressed in the young leaf primodia using ANT::*alcR*. Ethanol induction led to all the tested lines of STM expression to yield twisted and undifferentiated leaves at a very early stage compared to the wild type *Arabidopsis* plants. When the ethanol was removed after 4-day induction, normal plant development was restored, showing that the effects of STM over-expression were transient. The similar inhibition of organ differentiation was also observed when STM ectopic expression during flower development. Clearly, this changed floral phenotype is related to STM expression during flower formation and not to a secondary effect of expression during vegetative development. Similar findings were achieved for the ethanol-induced CYCD3;1 ectopic-expression under the ANT::*alcR*. The same strategy of using *alc* switch was also successfully employed to identify *KNAT6* gene functions in lateral root formation of *Arabidopsis* [40] and functions of the trehalose pathway for carbohydrate utilization and growth in *Arabidopsis thaliana* [41]. All these studies demonstrate that *alc* switch is a very useful tool for a detailed analysis of gene function during different developmental windows, and can simplify the analysis as one is able to study the same line or the same plant at different developmental stages with the same genetic background and control.

In addition, *alc* switch can be easily modified for different research requirements. For example, Koroleva et al.(2004) [42] used a modified *alc* switch system to define the role of *CycD1*, a putative G1 cyclin from *Antirrhinum majus*, during the cell cycle. In this modified system, the inducible cassette *alcR*:*GR* is the translational fusion of alcR transcription factor to GR transactivator domain, allowing response to dexamethasone (Dex) rather than ethanol. The second cassette is *alcA*:*CycD1*. Their data indicate that CycD1 can accelerate the cell cycle in cultured tobacco BY-2 cells by enhancing both G1/S entry and progression

through S and G2 phases unlike mammalian D cyclins whose primary function is implemented at G1 phase.

The *alc* switch can also be used to direct temporal-spatial expression of target genes in the genetic background of mutants for dissecting a given gene's functions much effectively. Laufs et al. (2004) [43] successfully identified the separable roles of *UFO* by ubiquitously expressing it in *Arabidopsis ufo* loss-of-function flowers at different developmental stages and for various durations using *alc* switch. They also found that *UFO* has an additional role in petal outgrowth. Maizel and Weigel (2004) [44] developed a flower-specific expression construct of *alc* switch (LFY promoter::*alcR*/*alcA* promoter::LFY *cDNA*). Using this construct, they achieved complete, ethanol-dependent rescue of defective flower development in *Arabidopsis Ify* mutants, including restoration of fertility, without confounding effects of ectopic expression. This system of conditional rescue will permit new types of genetic modifier screens starting with mutations that confer lethality or sterility.

4.3. Temporal and spatial control of RNA silencing

RNA silencing including post-transcriptional gene silencing and virus-induced gene silencing plays an important regulation role in plant life activities, naturally providing a defense system against invasive nucleic acids such as viruses, transposons, and transgenes [45–49]. RNA interference (RNAi) is a conserved mechanism that recognizes double-stranded RNA (dsRNA) as a signal to trigger sequence-specific degradation of homologous mRNA [45–47]. The dsRNA-mediate RNA silencing technique has been used to investigate the functions of the interested genes. However, like the overexpression of a gene, constitutive dsRNA-mediated silencing of the gene also produces detrimental effects or even causes plant lethality, resulting in no recovery of transgenic plants if the target gene is required for basic cell function or development. Guo et al.(2003) [50] developed a chemical-regulated inducible RNAi system in plant, which can obviate these problems. Alternatively, *alc* switch with high efficiency and safety enables RNA silencing to be controlled temporally and spatially.

The *alc* switch-mediated dsRNA expression system can be used to investigate the mechanism of RNA silencing and the function of target gene by blocking the expression of endogenous genes, which is demonstrated by Chen et al. (2003) work [51]. Magnesium (Mg)-chelatase subunit I (*chl I*) and glutamate 1-semialdehyde aminotransferase (*GSA*) are two nuclear genes involved in chlorophyll (*chl*) biosynthesis. Transgenic tobacco plants with silencing of *chl I* and *GSA* were established by expressing dsRNA in the form of intron-spliced hairpin structures under the control of *alc* switch. Ethanol-inducible silencing of the target genes caused strong but transient phenotypic alternation, which persisted for about 7–9 days before newly growing leaves completely recovered. Local silencing of *chl I* could be achieved by confined ethanol treatment of a single tobacco leaf. Recently, Ketelaar et al. (2004) [52] analyzed the effect of the ethanol-inducible expression of actin-interacting protein AIP1 RNAi in *Arabidopsis* plants to assess AIP1's role in vivo. The result shows that AIP1 is essential for the normal functioning of the actin cytoskeleton in plant development. Therefore, *alc* switch-mediated RNAi system promises to extend the RNA silencing studies that can be carried out in transgenic plants, particularly, with respect to temporal and spatial resolution of silencing effects, rendering this system extremely useful for metabolic studies.

MicroRNAs (miRNAs) are small, single-strand RNA molecules of 19–25 nucleotides found in most eukaryotes that regulate the transcription and translation of the target genes. miRNA-mediated RNA silencing acts a key function in plant growth and development [53,54]. It is crucial to identify target genes downregulated by miRNAs for understanding the miRNAs' functions and their pathways. Using *alc* switch linked to tissue-specific promoters for precise controlling of the target gene expression, Laufs et al. (2004) [55] investigated the role of *miR164* regulation of CUC (CUP-SHAPED COTYLEDON) genes during *Arabidopsis* development. They indicates that *miR164* constrains the expansion of the boundary domain in *Arabidopsis* meristems by degrading *CUC1* and *CUC2* not *CUC3* mRNAs. This work shows that *alc* switch could be also used for identifying the miRNA-mediated RNA silencing pathway.

4.4. Optimizing plant architecture for crop and horticultural improvement

It is well known that the introduction and application of the semi-dwarf varieties contributed a lot to substantial improvements in world-wide cereal grain yields. A number of dwarfing genes have been cloned, such as wheat *Rh1*, rice *sd1*, maize *D8* and barley *Sln1* [56]. Transgenic expression of these genes can lead to dwarfism. However, this constitutive expression also has detrimental effects. For instance, rice plants expressing *Arabidopsis* gene *gai* constitutively has a lower ability to extrude panicles, reducing the number of seeds [29]. Conditional expression of these genes might be a good way to manipulate plant architecture and grain yields. Ait-ali et al.(2003) [57] demonstrated that *alc* switch-mediated expression of dwarfing genes could be successfully used for flexible control of plant architecture and yields.

GAI (GA insensitive) belongs to a family of proteins called DELLA, which are nuclear regulators whose activities are opposed by the growth-promoting hormone gibberellin (GA). The transgenic expression of *Arabidopsis* GAI confers dwarfism and a reduced GA response in rice [58,59]. Using ethanol-inducible *gai* construct (*35S*:*alcR*; *alcA*:*gai*), Ait-ali et al. (2003) have shown that no GAI

protein is detected in the absence of ethanol, but it is expressed within 6 h of 0.1% ethanol treatment. Compared to wild-type plants, no phenotypic change occurred for plants grown in the absence of ethanol while those grown in ethanol presence exhibited dwarfism similar to a transgenic line expressing *gai* constitutively. The degree of dwarfing can be controlled in a dose-dependent manner, and the dwarfing plants showed no yield loss. Their results also indicated that GAI protein can be induced to act as a growth repressor at various stages of the growth of *alcA*:*gai* plants, and that the effect of the induction on plant architecture depends on the developmental stage at which induction is effected. This means that plant architecture can be regulated effectively via *alc* switch in different ways at different developmental stages. With this ability, we can optimize agronomic traits and increase harvest index and harvestable yields. This *alc* switch-mediated modification of plant architecture also opens up the potential for horticultural improvement.

5. Conclusions and future perspectives

It is a challenging task for plant biologists to identify the function of all the individual genes and interactions among genes in the post-genome era. Understanding developmental and physiological processes in plants requires the conditional or targeting over-expression/suppression of either transgenes or native genes.

The ethanol-inducible *alc* gene-expression system provides researchers with a lot of flexibility such as time point of induction, expression level, spatial control, and duration of expression, and is applicable to a variety of plant species. The *alc* switch also responds to several non-toxic chemicals including acetaldehyde, threonine, ethylamine, propan-1-ol, and butan-2-ol and may be suitable for field uses [24,25,60]. These attributes greatly enhance the reproducibility of regulated gene-expression experiments, which is of particular importance in metabolic studies requiring a large population of uniformly over-expressed/silenced individuals. The system facilitates to dissect the primary and secondary effects of gene over-expression/silencing and to make precise prediction of gene functions.

Further characterization of the system is needed to examine induction in different plant species and the side effects of both exogenous ethanol and ALCR activation on the expression of other endogenous genes as mentioned above. Systematic approaches such as genome-wide transcriptome analysis and global profiling of protein expression, combined with computational methods, would help to address these important questions. Determining the mechanism of ALCR and ethanol interaction would benefit the further optimization of *alc* switch. We envisage the application of *alc* switch will contribute significantly to the characterization of gene functions, precisely genetic modification of agronomic traits and molecular farming.

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