Chapter 5

Inducible Gene Expression Systems for Plants

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Abstract

Several systems for induction of transgene expression in plants have been described recently. Inducible systems were used mainly in tobacco, rice, Arabidopsis, tomato, and maize. Inducible systems offer researchers the possibility to deregulate gene expression levels at particular stages of plant development and in particular tissues of interest. The more precise temporal and spatial control, obtained by providing the transgenic plant with the appropriate chemical compound or treatment, permits to analyze also the function of those genes required for plant viability. In addition, inducible systems allow promoting local changes in gene expression levels without causing gross alterations to the whole plant development. Here, protocols will be presented to work with five different inducible systems: AlcR/AlcA (ethanol inducible); GR fusions, GVG, and pOp/LhGR (dexamethasone inducible); XVE/OlexA (β-estradiol inducible); and heat shock induction.

Key words: Dexamethasone, β-estradiol, ethanol, heat shock, inducible system, GVG, XVE, OlexA, pOp, LhGR, AlcA, AlcR, GR fusion.

1. Introduction

Inducible systems are usually based on two components. The first component is typically a chimeric transcription factor that can specifically bind to tightly controlled promoters. The chimeric transcription factor can activate the promoter only after induction, and it is commonly called *driver* or *activator*. The second component contains the binding sites for the driver to control the expression of the gene of interest (the *target*) and is commonly referred to as *reporter* or *effector*.

The driver can be cloned under control of the *CaMV35S* promoter to obtain strong and ubiquitous expression. Alterna-

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tively, tissue-specific promoters can be used for local induction experiments. The reporter is usually flanked by a minimal 35S promoter, therefore allowing the binding of the basal transcription machinery but minimizing its activation by endogenous transcriptional activators. Driver and reporter can be part of the same cassette or split onto two different vectors for plant transformation (*see* **Fig. 5.1**).

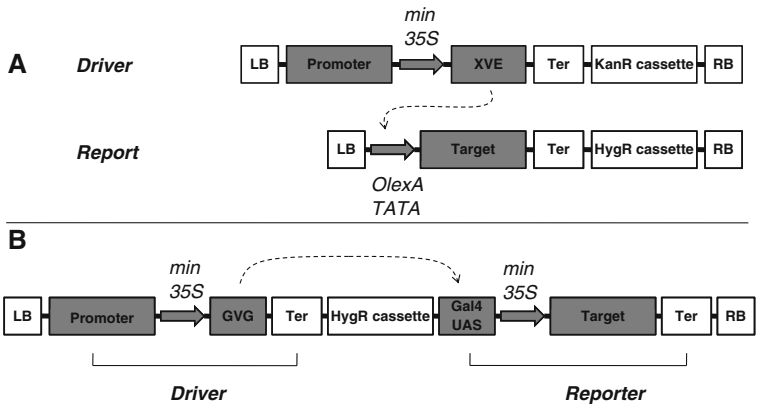

Fig. 5.1. **Inducible transgene expression.** (**A**) Two components system for β-estradiol-inducible expression. The chimeric transcription factor XVE cloned on the *driver* vector binds, after β-estradiol treatment, to the *OlexA* promoter placed on the reporter vector, thus activating the transcription of the gene of interest (33). (**B**) Single T-DNA vector for dexamethasone-inducible expression. The chimeric transcription factor GVG binds, after dexamethasone treatment, to six copies of the *Gal4 UAS*, thus activating the transcription of the gene of interest (20).

Single cassette systems speed up transformation procedures. However, binary systems give the possibility to better check for system functionality after integration in the plant genome. In fact, constitutive and ubiquitous expression of the driver can occasionally cause the so-called *squelching* (i.e., sequestration of general transcriptional factors) (1). Position effects can alter the activity of the driver as well the reporter (2). High transcriptional levels of the driver can lead to posttranscriptional gene silencing (PTGS) (3) or nonspecific binding, thus promoting the inactivation of the system or causing unpredictable side effects on plant development, as shown in (4–7). The choice of a binary system permits to select for functional driver and reporter lines without undesired side effects. Subsequently, these lines can be crossed to obtain the complete inducible system. In addition, binary systems assure great versatility by combining different driver and reporter lines, thus making it easy to generate lines with inducible expression confined to various specific tissues or bearing multiple reporters. It is always advisable to carry out experiments with several (at least two) driver and target lines, thus to isolate and discard those lines showing any side effects as previously mentioned.

Activation of inducible systems can be carried out by pouring, spraying, or adding the activator compound respectively to soil, plant surfaces, or plant growth media. Ways of actions, preparations, and treatments, being widely various depending on each case, will be described for each of the explained method in the following sections.

1.1. Ethanol-Inducible Expression with the AlcR/AlcA System

The AlcR transcription factor (the activator) and the *AlcA* promoter (driving the target) were isolated from *Aspergillum nidulans* (8–10). AlcR is activated by acetaldehyde, which is produced in plants by ethanol metabolism. The *AlcA* promoter is not activated by Arabidopsis endogenous transcription factors (9) even under anoxic conditions (i.e., excessive watering, growth on agar plates), which are known to promote ethanol production. Driver and promoter are split in two different plant transformation vectors. This system is very suitable for pulse and local inductions (11), as AlcR expression can be restricted to precise patterns using specific promoters and ethanol quickly evaporates (12, 13). However, due to ethanol toxicity, pulse inductions have to be carefully calibrated to assure the maximum effect in the minimal time.

1.2. Dexamethasone-Inducible Expression with – GR Fusions, GVG/UAS or pOp/LhGR Systems

All these three systems are based on the interaction properties of the ligand-binding domain of the glucocorticoid receptor (GR) from rat (14). This domain has a size of 93 amino acids. In the absence of steroids, GR interacts with cytosolic complexes containing heat shock proteins 90 (HSP90). Transcription factors eventually fused to GR (the drivers) are inactive, because they bind to HSP90 anchored in the cytosol and thus cannot enter the cell nucleus (15). After treatment with the synthetic steroid hormone dexamethasone, the GR–HSP90 interaction is disrupted and the GR transcription factor fusion-protein is free to enter the cell nucleus where it can bind and activate its target(s). The GR domain can be directly combined with the transcription factor of interest (16, 17). If the gene under analysis is not a transcription factor, chimeric transcription factors containing the GR domain are available, as described below.

The GVG driver consists of a yeast Gal4 DNA-binding domain fused to the strong transcription activator VP16 (from the *Herpes virus*) and to the GR receptor (18). The target is cloned under control of an Upstream Activation Sequence (UAS). This system has been successfully used (18–20), but results need to be interpreted with great care because high GVG expression levels were reported to cause strong developmental defects in Arabidopsis and several other plants (4–7). The choice of the binary GVG version (instead of the single cassette containing both driver and target) is highly recommended, as strict controls must be conducted on the driver line: high GVG expression levels can even lead to plant death, as experienced in our group.

The driver LhGR consists of a high-affinity mutant of the *Escherichia coli* lac repressor fused to the yeast Gal4 transactivation domain and GR (21). The target can be cloned downstream of six lac operator copies. The system, both available as single and binary cassette, is strongly and quickly inducible and no side effects have been reported to date (22–25). Versions of this system optimized for tobacco are available (25). Several constitutive tissue-specific driver lines, which lack the GR domain, are also available (2, 21, 26, 27).

1.3. β-estradiol-Inducible Expression with the XVE/OlexA System

The XVE driver consists of a *lexA* repressor domain fused to the VP16 transcription activation domain and the human estrogen receptor ER (28). In the presence of estrogens like 17-β-estradiol, XVE binds to the eight copies of the *lexA* domain, thus activating the transcription of the downstream target. Phytoestrogens are unable to activate the system in Arabidopsis, while in soy bean their high concentrations are reported to cause unspecific activation (29). High levels of exogenous β-estradiol up to 25 μM do affect neither Arabidopsis development nor endogenous gene expression (28, 30). The laboratory of Dr. Mark Curtis generated a GATEWAY®-compatible β-estradiol two-components system for both drivers and reporters (31).

1.4. Heat Shock-Inducible System

The promoter of the gene for heat shock protein 18.2 (*HSP18.2*) from Arabidopsis (32) has been successfully used in several plants to induce gene expression after heat shock (33–35). The cis-activation is carried out by cloning the gene of interest downstream of the *HSP18.2* promoter and by inducing the transgenic plant with heat shocks at 37°C. In absence of heat stress, the *HSP18.2* promoter is known to be repressed (36).

2. Materials

2.1. Ethanol-Inducible Expression with the AlcR/AlcA System

1. Microcentrifuge tubes.
2. Closed containers for plant growth. Very suitable are trays of pots where the trays can be well closed with transparent hoods.
3. Ethanol. Pure ethanol (99.8%) is used at different dilutions, depending on the strength of activation required (*see* **Note 1**). Ethanol is diluted in water when poured on soils or vaporized. Most published studies use ethanol concentration between 0.5 and 1% (*see* **Note 2**), both for soil drenches and agar plates (9). 70–100% ethanol concentrations can be used to induce the system by ethanol vapors.

2.2. Dexamethasone-Inducible Expression with – GR Fusions, GVG or pOp/LhGR Systems

1. Dexamethasone (Sigma) stock: 20 m*M* dexamethasone in DMSO or ethanol (*see* **Note 3**). Stocks can be stored at 4°C or at –20°C for 2 weeks. Avoid repeated freeze/thaw.
2. Silwet L-77 (GE Silicones).
3. Dexamethasone-containing lanolin paste. Place approximately 1 mL of lanolin into a 2-mL microcentrifuge tube and heat it to 60°C (e.g., in a water bath). Add dexamethasone to obtain a concentration of 1–2 μ*M*, vortex the tube and return to 60°C.
4. Dexamethasone-containing MS plates: 1×MS salts (Sigma,) 1 μ*M* dexamethasone, and 1% plant agar (Duchefa). Add dexamethasone to medium when temperature is around 40°C (i.e., when you can touch the bottle after autoclaving).
5. Spray nozzle.

2.3. β-estradiol-Inducible Expression with the XVE/OlexA System

1. 17-β-estradiol (Sigma) stock: 20–40 m*M* β-estradiol in DMSO or 70% ethanol (*see* **Note 3**). Stocks can be stored in the dark at 4°C or at –20°C for 1 week. Avoid repeated freeze/thaw. β-estradiol is light- and temperature-sensitive: stocks should be wrapped with aluminum foils.
2. Silwet L-77 (GE Silicones).
3. β-estradiol-containing lanolin paste. Place approximately 1 mL of lanolin into a tube and warm it at 60°C. Add the proper amount of β-estradiol to obtain a concentration of 10–25 μ*M*, vortex the tube and put it back at 60°C.
4. β-estradiol-containing MS plates: 1×MS salts (Sigma,) 5 μ*M* estradiol, and 1% plant agar (Duchefa). When preparing plates containing estradiol (*see* **Note 4**), add β-estradiol to 1% MS agar medium when temperature is around 40°C (i.e., when you can touch the bottle after autoclaving). Plates containing β-estradiol should be not older than 1 week.
5. Spray nozzle.

2.4. Heat Shock-Inducible System

Preinduction and induction can be carried out in incubators tightly set, respectively, at 38 and 37°C (*see* **Note 5**).

3. Methods

3.1. Ethanol-Inducible Expression with the AlcR/AlcA System

1. Grow the plants to be induced in trays that can be closed with transparent hoods (*see* **Note 6**).
2. For ethanol vapor inductions, fill 1.5- or 2-mL microcentrifuge tubes with 0.5–1 mL of 70–100% ethanol and force

them into the soil (8–16 tubes for 27×27-cm trays, 16–28 tubes for 30×50-cm trays). Distribute the open tubes in a way to allow ethanol vapors to evenly saturate the tray (*see* **Note 7**).

3. Keep the tray closed overnight or for shorter times (*see* **Note 8**), depending on the strength of induction needed.
4. Remove hoods and tubes. Keep the trays open for at least half a day and then repeat the procedure for further inductions.

Additionally or alternatively, the soil can be soaked with 0.5–1% ethanol. Also in this case, alternate inductions times with aeration times, the latter being not shorter than 12 h, thus avoiding growth of fungi and moulds. Repeat the procedure for prolonged inductions.

Ethanol induction on plates can be tricky. Even 0.1% ethanol may inhibit growth of Arabidopsis seedlings (22, 25). Anoxia (*see* **Note 9**) is also frequent in roots of seedlings grown on agar plates and cell culture (37).

3.2. Dexamethasone-Inducible Expression with – GR Fusions, GVG or pOp/LhGR Systems

GR fusions and GVG systems are fully induced with 1 μ*M* dexamethasone (18). pOp/LhGR systems are maximally induced with 2 μ*M* and are 50% induced with 0.2 μ*M* dexamethasone dilutions (38). Treatments should always be carried out under fume hoods or equivalent delimited zones.

1. Grow the plants to be induced on soil.
2. Spray plants with dexamethasone solution (*see* **Note 10**). Adding 0.01% Silwet L-77 supports dexamethasone penetration into plant tissues by lowering water surface tension. Avoid inducing neighboring plants: let sprayed droplets dry before returning plants into their growth chamber. Although single treatments are known to be sufficient for induction, repeated sprayings every 24 h increase expression levels of the target of interest (39).

Beads or lanolin soaked with dexamethasone can be used for local inductions (*see* **Note 11**). Prewarmed pipette tips can be used to transfer lanoline–dexamethasone drops from the tube to the plant to be induced.

For induction on plates, seeds can be either germinated directly on dexamethasone-containing medium, or established seedlings can be transferred from noninducing to inducing plates at an age of 3–10 days.

3.3. β-estradiol-Inducible Expression with the XVE/OlexA System

β-estradiol-inducible systems are fully activated with 5 μ*M* β-estradiol (31), even if concentrations up to 25 μ*M* have been used without causing side effects to plant development (30). Exogenous estrogens are toxic for human health. Although β-estradiol does not evaporate (30), wear protective gloves (*see* **Note 12**) whenever working with it.

1. Grow the plants to be induced on soil.
2. Spray plants with β-estradiol solution (*see* **Note 10**) under a fume hood. Adding 0.01% Silwet L-77 supports β-estradiol penetration into plant tissues by lowering water surface tension (31). Avoid inducing neighboring plants: let sprayed droplets dry before returning plants into their growth chamber. Repeat sprayings daily to increase expression levels of the target of interest.

Local inductions (*see* **Note 13**) can be carried out by applying lanolin paste containing β-estradiol on the tissue of interest. Prewarmed pipette tips can be used to transfer drops of lanoline containing β-estradiol from the tube to the plant (*see* **Note 14**).

For induction on plates, seeds can be either germinated directly on β-estradiol-containing medium, or established seedlings can be transferred from noninducing to inducing plates at an age of 3–5 days. The physical contact between β-estradiol MS medium and the tissue of interest greatly enhances the system induction strength.

3.4. Heat Shock-Inducible System

1. If plants are grown on soil, water them properly before starting the induction procedure (*see* **Note 15**).
2. Preincubate at 38°C for 15 min to activate Arabidopsis thermotolerance (40) and return plants to 22°C for 1 h.
3. Induce the plants at 37°C every other hour for 3–5 times (*see* **Note 16**): during no-induction times return plants to 22°C (*see* **Note 17**).
   Local inductions can be obtained by incubating part of the plants in 37°C water for 5–15 min.

4. Notes

1. Ethanol dilutions can be freshly prepared before starting induction.
2. 2% ethanol v/v soil is the optimal concentration to obtain full system induction (9). However, as 5% ethanol v/v soil is toxic for plant development, lower ethanol concentrations are used for system induction.
3. DMSO is preferred to ethanol for dilutions, as 0.1% v/v ethanol is sufficient to alter root development in seedlings grown on Petri dishes (22). However, as DMSO tends to accumulate into soil, dilutions in ethanol are preferred to DMSO for soil drenches.
4. Submerging 3- to 5-day old seedlings on β-estradiol-containing plates with 2–5 mL of 5 μ*M* estradiol solution

for short times (3–6 h) permits to obtain high induction levels similar to spraying (unpublished observation).

5. The incubators have to be kept closed during the experiment, as temperature must be constant to obtain maximum induction.
6. Ethanol is toxic to plants: proper system calibrations are required to obtain the maximum induction at the lowest toxicity. Toxicity changes along with plant developmental stages: young seedlings are far more sensitive to ethanol than adult plants.
7. Ethanol vaporizes and penetrates also internal cells (9, 12). Strong precautions have to be kept to avoid inductions of neighboring plants (10, 41). Growth chambers/greenhouses where the experiment takes place must be 100% ethanol-free (cleaning products containing ethanol can be sufficient to activate the system).
8. A few hours are sufficient to induce the system, as reported in (9, 11, 12) and (42). Prolonged inductions can promote moulds and fungal growth in pots and can be detrimental to plant fertility.
9. Avoid anoxic conditions on soil as well on plates. Do not water in excess and use high concentrations of agar (around 1.5%) to prevent roots growing into media.
10. Use spray bottles with adjustable spraying nozzles to minimize droplet size, thus obtaining a more even distribution of the inductor.
11. Because dexamethasone is systematically transported throughout the plant (22), systems induced by β-estradiol are preferable for local induction experiments.
12. Properly clean the area where induction occurred with ethanol. Avoid breathing vapors from agar containing β-estradiol.
13. β-estradiol is not easily transported in adult plants (30): applications on leaves do not affect newly produced organs. Brushing plant surfaces with 5–10 μM estradiol solutions can be used to promote local inductions (31).
14. Prepare new lanolin β-estradiol mixes every time you need them.
15. Both seedlings in sealed petri dishes and plants on soil can be induced.
16. Three to five cycles of inductions of 1-h each promote stronger system activity in comparison to single inductions, without altering plant development (Y. Laizet, internal communication). The HSP promoter stays active for approximately 24 h after induction (36).

17. High temperatures are detrimental to pollen development: carry on parallel experiments with wild-type plants to recognize and discard false positive effects.

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