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A New Vector Set for GAL4-Dependent Transactivation Assay in Plants

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Abstract

To study transcriptional regulators, an *in vivo* functional assay is indispensable. Here we report a new convenient vector set for a transactivation assay in plants. The system consists of a luciferase reporter controlled by a synthetic promoter with GAL4-binding sites and an effector to express any fusion protein with the GAL4 DNA binding domain. Co-transfection of the two plasmids causes transactivation of the reporter by the expressed GAL4-effector fusion if the effector exhibits transcriptional activation activity. A multicloning site was introduced into the effector vector which should facilitate the construction of GAL4 -effector fusions. In tobacco transient assay by microprojectile bombardment, a 27-fold transactivation for the GAL4-B17 fusion as an effector was demonstrated with this system.

The yeast transcription factor, GAL4, contains separable functional modules. For example, it contains a DNA-binding domain (residues 1-147) and two necessary regions for transcriptional activation (residues 148-196 and residues 768-881) [1]. The transcriptional "activation domain" has been demonstrated to function independently from its protein context by a chimeric fusion protein between the activation domain of GAL4 and an *E. coli* DNA-binding protein, LexA. While LexA does not activate transcription by itself, LexA-GAL activation domain fusion activated transcription through the LexA-operator [2]. Likewise, the DNA-binding domain of GAL4 can activate the transcription of a reporter gene driven by the GAL4-binding site when fused to a transcriptional activation domain derived from herpesvirus VP16 [3, 4]. These early findings provided the basis for experimental systems called chimeric transactivation assays or "one-hybrid assays" that are being used in dissecting transcription factors for the identification of transcriptional activation or DNA-binding domains. Those systems are also used to identify co-activators which do not bind DNA by themselves but nevertheless exhibit transactivation activities.

Using the chimeric transactivation assay, several putative transcription factors from plants have been shown to be able to activate transcription in yeast [5, 6]. However, not every transcription factor can function in both yeast and plants. For example, the full-length GAL4 protein, which is a yeast transcriptional activator, fails to activate transcription mediated by the GAL4-binding site in tobacco [7]. In another case, a transcriptional activation domain of wheat HALF-1, which was identified by a transactivation assay in tobacco BY-2 cells, did not show transactivation activity in yeast [8]. Therefore, for the analysis of plant transcription factors, a plant assay system is essential in addition to the heterologous assay system. The utilization of a plant system can also provide an opportunity to examine the physiological regulation of the transcription regulators.

There are several reports about GAL4-based transactivation assays in plants utilizing reporter genes such as the chloramphenicol acetyl transferase gene or the β-glucronidase gene (GUS) [5, 7, 9, 10]. In this paper, we report a new and more sensitive luciferase reporter construct and a new convenient GAL4 (1 -147) transactivator vector with a multicloning site. Experimental conditions were surveyed in a transient assay of tobacco leaves using a microprojectile bombardment method, because there had been no report describing detailed experimental conditions for the assay. In our condition, a positive effector, GAL4-B17, was shown to activate the reporter gene expression up to 27-fold over the GAL4 (1-147) without the B17 transactivation domain.

Construction of plasmids for plant transactivation assay

In a transient assay using tobacco leaves, a luciferase reporter (LUC) under the control of cauliflower mosaic virus (CaMV) 35S promoter (CaMV35S:: LUC) displayed a two orders of magnitude higher

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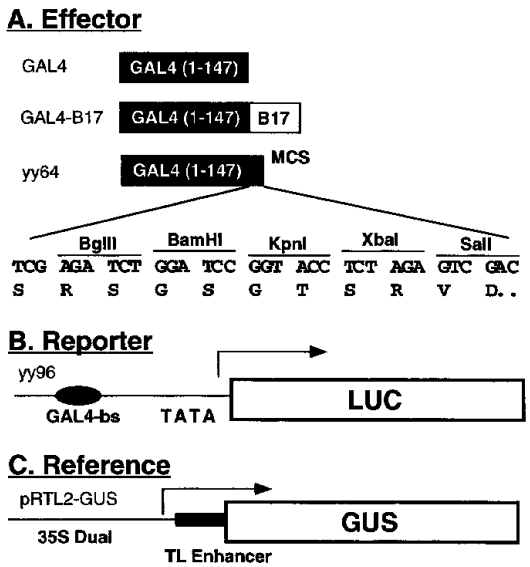

Fig. 1 Constructs for plant transactivation assay. A. The negative control (GAL4) and positive control (GAL4-B17) are exactly the same as pMA560 and pMA564, respectively [7]. yy64 is the effector plasmid with a polycloning site to facilitate translational fusions with GAL4(1-147). B. The Luciferase reporter (LUC) is placed under the control of the GAL4 -binding site (GAL4-bs). C. pRTL2-GUS Is described by Restrepo *et al.* [9]. All the plasmids have ampicilin resistance in *E. coli*.

sensitivity over that of CaMV35S:: GUS (in plasmid pBI221; data not shown). Because of this higher sensitivity of LUC reporter, we chose LUC for our new reporter construct for the GAL4 transactivation assay in plants (**Fig. 1B**). A DNA fragment with 13 copies of the GAL4-binding site, called "17mers", followed by a basal 35S promoter (-60/+7) was amplified by polymerase chain reaction (PCR) from a reported plasmid pMA558 [7]. The primers used were a M13 reverse primer with P*st*I site [5'-GGGCTGCAGGATAACAATTTCACACAGGAAAC -3'] and a CaMV35S-specific primer with *Xba*I site [5'-GCGTCTAGAAGCGTGTCCTCTCCAAAT-GAAATG-3']. The amplified fragment was first inserted into the *Pst*I/*Xba*I site of pBI221 to make yy76. The 17mers/35S basal promoter (TATA) fragment was isolated by *Hin*dIII and *Bam*H I partial digestion and was then inserted into the *Hin*dIII/*Bam*H I site of pBIL221, where GUS was replaced to *Bam*H I/*Sac* I fragment containing LUC from pGEM -LUC (Namamura and Obokata, personal communication). The resulting final construct was named GAL4op-LUC (yy96).

To produce a convenient effector vector for fusion, a DNA fragment from the GAL4 DNA binding domain (1-147) in pMA560 effector plasmid [7] was amplified by PCR with GAL4-specific primers: [5'-GAACAGCTATTTCTACTGATTTTT-3'] and [5'-GCGGTCGACTCTAGAGGTACCGGATC-CAGATCTCGATACAGTCAACTGTCTTTGA - 3']. The PCR product was digested with *Xho* I and *Sal* I, and was used to replace the *Xho*I/*Sal*I fragment of pMA560. The resultant plasmid, yy64, was confirmed by sequencing analysis (**Fig. 1A**). Both the negative control, GAL4 (1-147) itself and the positive control, GAL4-B17, which has the B17 fused to GAL4 (1-147) protein, are exactly the same plasmids as pMA560 and pMA564, respectively [7]. As a result of plasmid construction, every effector construct contains a non-functional fragment of the chloramphenicol acetyltransferase gene between NOS terminator and the *Sal* I site [7].

In order to monitor the transformation efficiency in the plant transient assay, a reference reporter gene was introduced into plants together with the testing reporter. We selected a reported CaMV35S:: GUS construct (plasmid pRTL2-GUS) [11], because of its high expression level and its largely constitutive expression in plants (**Fig. 1C**).

Tobacco transient assay with microprojectile bombardment

To test the suitability of our GAL4 system, the transient assay was conducted using tobacco leaves. Tobacco leaves grown in a greenhouse were briefly rinsed in 70% ethanol, soaked in 10% commercial bleach solution for 5 minutes, and then rinsed with sterile water five times. After this surface sterilization, leaves were cut to 2 cm square and placed upside down on GM medium [12] supplemented with 3% sucrose and 0.8% agarose (GIBCO BRL, MD, USA). Three plasmids, an effector, a reference (pRTL2-GUS), and a reporter (yy96) were simultaneously introduced into the leaf cells. The plasmids were prepared from *E. coli* (strain GM2163 for the reporter [13]), mixed, coated on tungsten particles of a 1.1 μm median size (M-17, BioRad, CA, USA), and shot twice at tobacco leaves by a Biolistic transformation system (BioRad, CA, USA). The bombardment was performed according to the manufacture's instructions with 1,100 psi rupture disks. After transfection, the leaf disks were cultured for one or two days at 25°C under continuous light condition. It should be noted that constant condition of light during posttransfectional culture should be kept for obtaining a reproducible expression level, because GUS mRNA level appears to be modulated by light in tobacco leaves (Yamamoto and Obokata, unpublished results). After the culture, all procedures were carried out at 4°C. The leaves were ground with a mortar and pestle in GUS/LUC buffer (0.1 M KPO_4, 2 mM Na_2 EDTA, 5% glycerol, 20 mM dithiothreitol, pH 7.8) in

the presence of silica beads, and spun in a microfuge tube at 14 krpm for 30 min. The supernatant was frozen in liquid N_2 and stored at -80°C or used immediately. The protein concentration was determined by the Bradford method (Protein Assay Kit, BioRad, CA, USA). The GUS assay was carried out with 4-methylumbellyferyl β-D-glucuronide according to Jefferson *et al.* [14]. The luciferase activity was determined using luciferin as a substrate (Luciferase Assay System, Promega, WI, USA) [15]. The luciferase activities were divided with the GUS activities to normalize for transfection efficiencies.

Fig. 2A shows the LUC/GUS ratio 24 h after transfection. The LUC/GUS ratio without any effector plasmid was arbitrarily set at 1.0 units. The addition of GAL4(1-147) as a negative control had no significant effect on the LUC/GUS ratio (1.2). However, when the positive control, GAL4-B17, which has been shown to transactivate in tobacco protoplast assay [7], was introduced together with the reporter and reference plasmids, transactivation was observed. Within the range examined, a two fold higher concentration of this effector plasmid, relative to the reporter plasmid, gave the maximum response (20 fold activation).

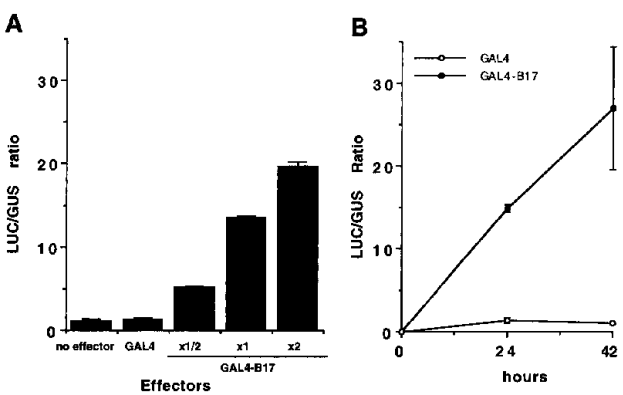

Fig. 2 Transactivation by GAL4-B17 in tobacco leaf cells.

A. Dose response of Effector plasmid. The ratio of the effector to the reporter plasmid (w/w) is shown. The LUC/GUS ratio of "no effector" is expressed as 1.0 and the relative values are presented. **B.** Time course of the transactivation with the effector/reporter ratio of 1.0. The LUC/GUS ratio the of GAL4 effector (GAL4) at 42 h is expressed as 1.0 unit.

Three μg of a reference plasmid (pRTL2-GUS), 6 μg of the reporter plasmid (yy96), and 3 to 12 μg of the effector plasmids (GAL4 or GAL4-B17) per shooting were coated on tungsten particles and introduced into tobacco leaves by shooting twice. After 24 or 42 h of post-transfectional culture, soluble protein was extracted. LUC and GUS activities of the extract were determined. The LUC activity was derived by the corresponding GUS activity to normalize transfection efficiency.

Fig. 2B shows a time course after transfection. The background expression with GAL4 itself was measurable after 24 h, but was then slightly reduced after 42 h. In contrast, transactivation by GAL4-B17 is still increasing at 42 h, reaching a impressive 27 fold activation.

The result in **Fig. 2B** tells that, for the optimal response, the amount of the effector plasmid for the transfection should be twice as much as of the reporter plasmid, and the post-transfectional culture be done for two days before harvest. However, the most effective ratio of effector to reporter plasmids depends on the effector proteins tested and must be determined empirically. For example, one effector gave the highest transactivation with an effector/reporter ratio of 4/1, whereas another effector we tested required 2/1 ratio for the maximum response ([16]; data not shown). This variation of optimal conditions by different effectors might be the result of different expression levels of the effectors, varied stabilities of the effector proteins, or different modes of transactivation.

In summary, we developed a new GAL4 system for a plant transactivation assay and tested its suitability in a tobacco transient assay with microprojectile bombardment. The bombardment technique can applied to a wide range of plant materials, and preparation of the materials is simple and thus easy to reproduce. We consider this method to be superior to the protoplast transfection method for DNA introduction. Although the transactivation analysis by *in vivo* transient assay does not reveal the biochemical mode of action for the protein examined, this method provides a convenient way to dissect the structure/function relationship of transcription factors as well as to identify co-activators which do not bind DNA by themselves.

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