Using the VersaFluor™ Fluorometer to Quantitate GUS Expression in Plant Tissues Bombarded with Biolistic® PDS-1000/He Particle Gun

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Introduction

In 1987 Jefferson *et al*. described the use of β-glucuronidase (GUS) as a marker to study gene expression in plants. Expression of GUS activity in plant tissue can be quantified by fluorometry or assayed qualitatively by histochemistry. The GUS enzyme cleaves the commercially available substrate 4-methylumbelliferyl-β-D-glucuronic acid (MUG) to produce fluorescent methylumbelliferone (MU). The VersaFluor fluorometer, when equipped with the simple-to-install 360 excitation and 460 emission filters, has the necessary sensitivity to accurately measure MU in plant tissues. Quantification of GUS activity using the VersaFluor fluorometer is relatively inexpensive and reliable. We have used the instrument to quantify transient expression of a CaMV 35S-GUS construct in bean abscission zones. Abscission is the process by which plants shed organs. The bean abscission cellulase (BAC) accumulates tissue specifically in induced abscission zones. GUS expression is used as an internal control in experiments designed to identify regulatory regions in the BAC gene promoter. An example of the approach used and results obtained is described below. In addition to quantification of GUS activity, we use the VersaFluor fluorometer equipped with the same set of filters to quantify the DNA preparations. The fluorescent dye for DNA quantification is Hoechst 33258 from Bio-Rad.

Materials and Methods

The promoter to be tested for hormonal or tissue-specific expression is fused to the luciferase gene taken from the pDO432 plasmid (Ow *et al*., 1986; see Koehler *et al*., 1996, for details of promoter construction). The GUS gene is fused to an enhanced 35S promoter (Kay *et al*., 1987). An accurate measure of the concentration of the gene constructs is determined with the VersaFluor fluorometer using the Hoechst 33258 dye from Bio-Rad. The two constructs are then co-precipitated at constant and equal-molar concentrations onto gold particles (Figure 1A). Plant material is bombarded with the gold particles using the Biolistic PDS-1000/He particle gun (Bio-Rad) as depicted in Figure 1B.

Following particle bombardment and a 48-hour incubation period in ethylene (a plant hormone that induces abscission), approximately 1 mm of tissue is harvested from the upper surface of the bombarded explants (abscission zones, petioles and stems), and frozen separately in liquid nitrogen (Figure 1B). The protein extraction procedure is modified from the original protocol described by Jefferson *et al*. (1987) to allow the assay of cellulase, luciferase, and GUS activity from the same tissue extract. Approximately 150 mg of plant tissue is ground in liquid nitrogen, thawed in 450 µl of extraction buffer (0.1 M sodium phosphate, pH 7.8, 1% Triton® X-100, 2 mM EDTA, 1 mM DTT and 0.1% BSA), vortexed and then centrifuged at 10,000 rpm in a microcentrifuge for 4 minutes.

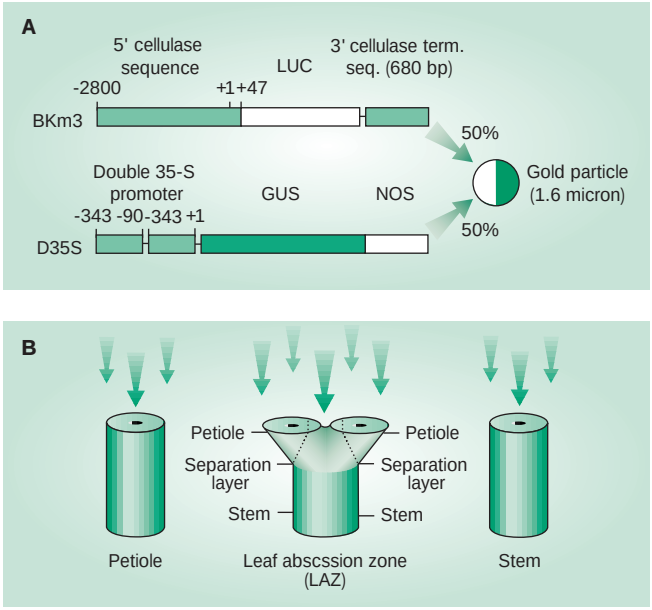

Fig. 1. Construct design and preparation of explants for transient expression assay. A. Chimeric gene constructs used for transient expression experiments. The BKm3 construct contains 2.8 kbp of 5' BAC sequence fused to the luciferase (luc) open reading frame. In addition, after the stop codon for luciferase, BKm3 is terminated by 680 bp of 3' BAC sequence. The D35S construct includes a double CaMV 35S promoter fused to a GUS reporter gene and NOS termination sequence. The D35S plasmid and BKm3 were co-precipitated at equal molar concentration onto 1.6 micron gold particles. B. Diagram showing how explants were prepared for particle gun bombardment.

The supernatant is then transferred to new tubes in 75 µl aliquots for measurement of cellulase and luciferase activity as described elsewhere (Koehler *et al*., 1996). For GUS assay, 75 µl of the supernatant is combined with 25 µl of a GUS adjustment buffer (0.4 M citrate buffer pH 5.5, 34 mM EDTA and 17 mM DTT) and the combined mixtures incubated at 55 ˚C for 30 minutes. The GUS marker enzyme, originally from *E. coli*, is resistant to heat inactivation at 55 ˚C; however, heating significantly reduces endogenous GUS-like activity in plant extracts. Following incubation, the mixture is combined with 20 µl of methanol, which further reduces background GUS-like activity (Kosugi *et al*., 1990), and 12.5 µl of 10 mM MUG. Immediately after addition of MUG, 10 µl of the mixture is removed and combined with 2 ml of 0.2 M sodium carbonate to stop GUS conversion of MUG to MU. These tubes are labeled T_0. The remaining mixture is incubated at 37 ˚C overnight and the T_0 tubes stored in the dark at room temperature. Following incubation at 37 ˚C, 10 µl of the mixture is combined with 2 ml of 0.2 M sodium carbonate and the tube labeled T_{12}.

MU standards are prepared at 50, 100 and 500 nM in 0.2 M sodium carbonate. The VersaFluor fluorometer is warmed for 15 minutes before conducting the assay. The instrument has low, medium and high gains. The range of MU standards described above is best measured at a medium gain.

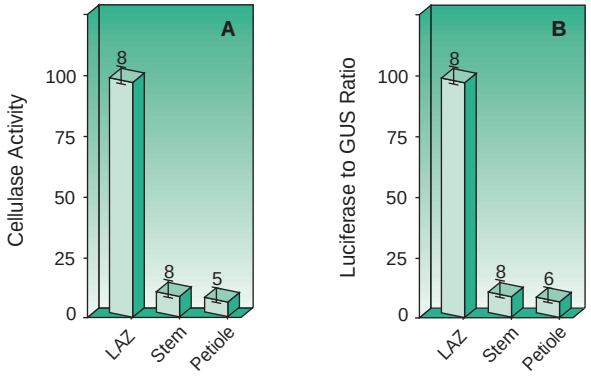

Fig. 2. Cellulase activity and transient expression of a chimeric BAC-luciferase construct in bean. Constructs were prepared and co-precipitated onto gold particles as shown in Figure 1A. Leaf abscission zones (LAZ), stems, and petioles were prepared as shown in Figure 1B and bombarded with gold particles. A and B. Explants were exposed to 1.0 µl/l ethylene for 48 hours and the bombarded surface (1 mm) harvested and assayed for cellulase, luciferase, and GUS activity. A. Cellulase activity per gram of tissue. B. The ratios of luciferase to GUS activity normalized to the mean ratio for BKm3 in this series of experiments.

The instrument is zeroed with a blank consisting of 0.2 M sodium carbonate. First, the highest standard, 500 mM, is measured and set to equal 5,000 relative units with the range option of the fluorometer. Next, the two remaining standards are measured to ascertain a linear response. Finally, the fluorescence of extracts in the T_0 and T_{12} tubes is measured and recorded.

Results and Discussion

Fusion of the enhanced CaMV 35S promoter (Kay *et al*., 1987) to the GUS reporter gene creates a gene construct (D35S) that expresses GUS constitutively in transformed plant tissues. Inclusion of this construct is used as an internal control to reduce variability in all our particle bombardment experiments (Figure 1A). The expression results (Figure 2B) are reported as the ratio of luciferase to GUS activity normalized to 100% for BKm3 expression in abscission zones. Expression of luciferase from the test promoter, the BKm3 construct (Figure 1A), relative to GUS expression from D35S (*i.e.*, luciferase to GUS ratio) in stems and petioles is approximately 10% of the expression in abscission zones (Figure 2B). This pattern of expression is very similar to that for native cellulase activity measured in the same extracts collected from particle bombarded stems, petioles and leaf abscission zones (LAZ) (Figure 2A).

The VersaFluor fluorometer is inexpensive and easy to use. It has the necessary sensitivity to reproducibly measure transient GUS activity in particle gun bombarded plant tissues. In addition, using the Hoechst 33258 dye to quantitate DNA by the VersaFluor fluorometer is reliable, simple, and accurate.

References

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