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Non-radioactive detection of β-glucuronidase and chloramphenicol acetyltransferase activities in co-transformed protoplasts by HPLC

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SUMMARY

The use of transient gene expression assays for the study of natural or engineered plant promoters is affected by a considerable degree of inter-experiment variability. As a means of obtaining interpretable data from a limited number of experiments, we worked out conditions for the simultaneous determi nation of the activity of two reporter genes, a "sample" and a "reference", on a single extract of co-transformed protoplasts. ß-glucuronidase (GUS) and chloramphenicol acetyl transferase (CAT) genes, both under the control of the CaMV 35S promoter, were transferred into tobacco (*Nicotiana tabacum* L.) protoplasts on two independent plasmids. The parallel expression of the two reporter genes in several independent co-transformation experiments was verified. Conditions for the use of a single protoplast extraction buffer and for the simultaneous assay of both reporter gene activities were set up. A HPLC method for the non-radioactive determination of both enzyme activities on a single aliquot of the reaction mixture was developed. The resulting procedure was tested using the GUS gene as "reference" and the CAT gene, under the control of either wild type or upstream-deleted (-90) CaMV 35S promoter, as "sample". The protocol is simple and allows the fast analysis of plant promoters in the presence of a true internal standard under conditions in which assay manipulations are reduced to a minimum and both reporter gene activities are subjected to the same experimental treatments.

Key Words. CAT, GUS, HPLC, Co-transformation, Internal Standard, Tobacco Protoplasts.

Abbreviations. CaMV- cauliflower mosaic virus, CAT- chloramphenicol acetyl transferase, EDTA- ethylenediaminetetraacetic acid, GUS- ß-glucuronidase, HPLC- high performance liquid chromatography, MES-2-morpholinoethanesulphonic acid, MS- medium after Murashige and Skoog (1962), MUG- 4-methyl umbelliferyl glucuronide, MU- methylumbelliferone, NOS- nopaline synthase, PEG- polyethylene glycol, TRIS- tris-hydroxymethyl aminomethane, UV- ultraviolet.

INTRODUCTION

Protoplast transformation with chimeric DNA constructions followed by transient gene expression assay of the activity of a reporter gene allows a fast analysis of natural or engineered plant promoters (Paszkowski et al. 1984; Junker et al. 1987; Ebert et al. 1987). A generally recognized limit of such analysis derives from the inherent variability of the multistep experimental procedure. As a consequence, a correct interpretation of experimental data usually requires a large number of assays. In an effort to control such variability, several potential sources of variation have recently been considered (Sala et al. 1989). Among them, the physical state of the DNA in the transformation medium (Mass and Werr 1989) as well as subtle differences between various preparations of plasmid DNA have been shown to be important (Lepetit et al. 1991). However, the most critical parameter in determining the final level of reproducibility of transient gene expression assays appears to be related to the physiological state of donor plants (Lepetit et al. 1991). Several attempts to reduce such a major source of variability, for example by utilizing well standardized environmental conditions of plant growth, have met, so far, with a limited degree of success. A possible solution to this problem has recently been proposed (Lepetit et al.

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1991). It involves the use of a specifically constructed vector containing two different reporter genes, a sample and a reference, which are then separately assayed. We have further pursued this approach by setting up experimental conditions in which a single extract from tobacco (*Nicotiana tabacum* L.) protoplasts that have been co-transformed with two independent plasmids is simultaneously assayed for two reporter genes. In this way, an additional source of variability is taken into account, namely, all the manipulations that intervene between transformation and the actual determination of reporter gene activities. Specifically, our simplified procedure is based on the use of two independent plasmids, a "sample" and a "reference", a single protoplast extraction buffer, and a single tube reaction for the simultaneous determination of GUS and CAT activities by HPLC.

MATERIALS AND METHODS

Plasmid Construction and Purification

All restriction and modification enzymes were from Promega and were used according to manufacturer's instructions. Plasmid pBI221, a derivative of plasmid pBI121 (Jefferson et al. 1987) containing 800 bp of the CaMV 35S promoter, the GUS gene and the NOS polyadenylation site, was obtained from Clontech Lab. Inc.. Plasmids pMB14 and pMB14-90 were constructed as follows. A 1.5 kb XbaI fragment of pCaMVCN (Pharmacia) carrying 430 bp of the CaMV 35S promoter, the CAT gene and the NOS polyadenylation site (Fromm et al., 1986), was inserted at the XbaI site of pUC18. Clones obtained from the transformation of ligation products were screened by restriction analysis (EcoRV, SphI). Constructions in which the asymmetrically located EcoRV site of the 35S promoter was inserted closer to the SphI site of pUC18, thus yielding a 354 bp instead of a 1188 bp restriction fragment, were selected and designated pMB14. The vector containing the fragment obtained from the digestion of pMB14 with EcoRV and SphI was blunt ended with mung bean nuclease before being self ligated to construct plasmid pMB14-90.

Plasmids for protoplast transformation experiments were purified by precipitation with polyethylene glycol (PEG) after alkaline lysis of the bacteria according to Sambrook et al. (1989). The concentration of plasmid DNA was determined by the fluorometric method of Labarca and Paigen (1980).

Protoplast Preparation

Seeds of *Nicotiana tabacum* L. SR1 were cultured *in vitro* on hormone-free MS medium (Murashige and Skoog 1962) supplemented with 30 g/l sucrose and 0.8% agar agar (pH 5.8) at 26°C, under a 16 h photoperiod with 27 $\mu mol\ m^{-2}s^{-1}$ light intensity. Plants were micropropagated by transferring the meristematic apex onto the same medium for no more than three passages (see below).

Protoplasts were prepared from 21 day-old micropropagated plants. Leaves were cut into narrow strips and digested in a solution containing 0.25% Driselase (Fluka) in K3 medium (Nagy and Maliga 1976) plus 0.4 M sucrose (520 mOsmol kg^{-1}). No growth regulator was added. After an overnight digestion at 26°C in the dark, debris was removed by passage through nylon sieves (110 µm). The protoplast suspension was then distributed into 10 ml test tubes and centrifuged at 800 rpm for 5 min (Beckman JS-7.5). Protoplasts were resuspended in 3 volumes of washing medium (WM) containing 0.5% (w/v) 2-morpholinoethanesulphonic acid (MES), 80 mM MgCl2, 80 mM $CaCl_2$ and 37.5 mM mannitol, pH 6 (490 mOsmol kg^{-1}) and centrifuged at 800 rpm for 5 min. The resulting pellet was resuspended in a small volume of the same medium and protoplast concentration and viability were assessed as follows. 50 µl of protoplast suspension were added to 50 µl of a 0.5% fluorescein diacetate solution and the number of viable protoplasts was counted in a double chamber haemocytometer (Power and Chapman 1985) with a Zeiss Axioskop microscope equipped with a fluorescence attachment. Using the above described procedure, viable protoplasts were usually more than 80%. Protoplast preparations exhibiting lower values of viability were discarded. Media osmolarity turned out to be an important parameter determining protoplast viability and it was routinely checked by a Digital Micro-Osmometer (Roebling).

Co-transformation

500,000 protoplasts in 100 µl of WM medium were transferred into a 4 ml sterile tube. Carrier DNA (16.7 µg calf thymus DNA type 1, Sigma, sonicated to an average size of 4-40 kb) followed by 20 µg of each plasmid DNA were added. In every experiment, 56.7 µg of carrier DNA were added to an identical aliquot of protoplasts to be used for background determination. Four 50 µl aliquots of a PEG solution (25% PEG 6000 Sigma, 0.5% w/v MES, 0.1 M $Ca(NO_3)_2$, 0.3 M mannitol, pH 6) were added sequentially, gently shaking the protoplasts after each addition. The final PEG concentration was 16%. After 20 minutes, 4 ml of WM Medium were added, protoplasts were sedimented (100xg, 5 min), plated in K3 medium (0.4 M glucose, 5.4 µM naphthalene acetic acid, 4.4 µM benzylaminopurine; pH 5.6, 510 mOs Kg^{-1}) at a density of $1x10^5$ /ml and incubated for 20 hours at 26°C in the dark.

Protoplasts prepared from leaves obtained from seed-derived plantlets without any micropropagation were found to be extremely fragile, whereas protoplasts

from plants micropropagated for more than three passages were poorly transformable. None of these plants was used for protoplast preparation.

Simultaneous determination of GUS and CAT activities

Conditions for the simultaneous determination of GUS and CAT activities were set up using commercial enzymes (Fluka and Promega). All reactions were carried out at 37°C for 3 hours and stopped by the addition of 10% v/v acetonitrile. Each enzyme (0.2 units) was added to 400 µl of assay solution and reaction products were quantitated by HPLC analysis (see below). The following assay conditions were tested: i) GUS activity in GUS extraction buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl, 10mM ß-mercaptoethanol) containing 1 mM 4-methyl umbelliferyl glucuronide (MUG) (Jefferson et al. 1987); ii) CAT activity in CAT extraction buffer (156 mM Tris-HCl, pH 7.8, 310 µM leupeptin) plus 2.6 mM acetyl-CoA and 2.4 mM chloramphenicol (Gorman et al. 1982); iii) GUS activity in CAT extraction buffer containing 1mM MUG; iv) CAT and GUS activities in CAT extraction buffer plus 1mM MUG, 2.6 mM acetyl-CoA and 2.4 mM chloramphenicol. Simultaneous assay conditions as described above (iv) were applied to co-transformed protoplasts in the following way. $1.5x10^6$ protoplasts (more than 40% viable) were sedimented (100xg, 5 min), resuspended in 700 µl of CAT extraction buffer containing 100 µM phenylmethanesulphonyl fluoride and disrupted by freezing in liquid nitrogen, thawing at 37°C and sonication for 20 sec with a LabSonic 1510 Braun Sonifier equipped with a microtip (100 Watt setting). The resulting extract was then centrifuged for 5 minutes at room temperature and 20 µl of supernatant were used for protein determinations (Bradford 1976).

Extract (50 µg total protein) was added to CAT extraction buffer containing 1 mM MUG, 2.6 mM acetyl-CoA, 2.4 mM chloramphenicol in a final volume of 800 µl and incubated at 37°C for 16 hours. Prior to HPLC analysis, transformation efficiency was routinely monitored by direct visualization of microaliquots of the reaction mixtures (25 µl of sample mixture plus 25 µl of 1 M sodium carbonate) on a UV-transilluminator and comparison to known amounts of a 4-methylumbellyferone (MU) standard (Scott et al. 1988). Reactions were terminated by the addition of 10% v/v acetonitrile. No background activity could be detected in mock-transformed protoplasts. In some cases, GUS and CAT activities were determined by standard assays (Jefferson et al. 1987; Gorman et al. 1982).

HPLC analysis

Reaction products (4-methylumbelliferone, 1-acetyl chloramphenicol and 3-acetyl chloramphenicol) were simultaneously quantitated on a 50 µl aliquot of the reaction mixture. HPLC analyses were carried out in triplicate using a RP 18, 10 µm column (25x0.4 cm, Merck) connected to a Varian 9010 ternary gradient chromatograph equipped with a 50 µl loop injector (Rheodyne) and a two-wavelength PU 4021 detector operating at λ_1=320 nm (maximum absorbance of 4-methylumbelliferone), and at λ_2=278 nm (maximum absorbance of chloramphenicol and its derivatives). The HPLC mobile phase (solution A = 10 mM sodium acetate buffer (pH 6.0) containing 5% v/v acetonitrile; solution B = acetonitrile) was delivered as a 10% to 38% B gradient in 14 minutes, at a flow rate of 1 ml/minute.

Similar results, albeit with a slight loss of resolution, could be obtained in 6 minutes under isocratic conditions using a Spherisorb 5 µm column (25x0.4 cm) and acetonitrile : 10 mM sodium acetate buffer (pH 6.0) containing 5% v/v acetonitrile (53:47) as mobile phase.

4-methylumbelliferone and chloramphenicol (Fluka), 1-acetyl-, 3-acetyl and 1,3-diacetyl chloramphenicol, synthesized as described by Young et al. (1985), were used as standards. Reaction products were quantitated through the ratio of related peak areas, by comparison with known amounts of chloramphenicol and 4-methylumbelliferone standards. For all reaction products the response was linear from 80 µg/ml to 0.8 µg/ml. The lower limit of detection was 0.8 µg/ml.

RESULTS AND DISCUSSION

In an effort to reduce the generally acknowledged variability of the results obtained from the assay of the transient expression of a single reporter gene, we focussed initially on the standardization of environmental conditions of plant growth. The use of optimized experimental conditions, and especially a careful control of media osmolarity and of the age of donor plants, greatly improved the overall rate of successful transformations (data not shown, but see Experimental Procedures). However, inter-experiment variability of the activity of individual reporter genes was practically unaffected and could be as high as 8-fold in the case of either CAT or GUS (18 to 152 x 10^2 cpm and 16.3 to 161 arbitrary units of fluorescence, respectively). Such variability cannot be attributed solely to the physiological state of recipient cells. It is known to be influenced by the steps that intervene during transformation, but it might also be contributed by the manipulations that occur during protoplast extraction and determination of reporter gene activity. Co-transformation of two

distinct reporter genes, a "reference" and a "sample", followed by their assay under identical experimental conditions could thus represent an effective way of controlling these different sources of variability.

Similar to the experimental approach that is commonly used in the case of mammalian cells (Sambrook et al. 1989), we determined the levels of expression of two reporter genes (GUS and CAT) co-transfected into plant protoplasts on two independent plasmids. Under these conditions, using standard protoplast extraction and assay procedures (Jefferson et al. 1987, Gorman et al. 1982) we observed side by side variations of GUS and CAT over at least 9 independent transformation experiments (data not shown).

Using commercially available preparations of both enzymes, we then tested the feasibility of employing a single reaction mixture to simultaneously monitor the activity of ß-glucuronidase and chloramphenicol acetyl transferase. The products of the two reporter enzymes (acetylated chloramphenicol derivatives and 4-methylumbelliferone) were resolved and quantitated by a single HPLC analysis (see Experimental Procedures). A representative chromatogram obtained from a mixture of standard compounds is presented in Figure 1.

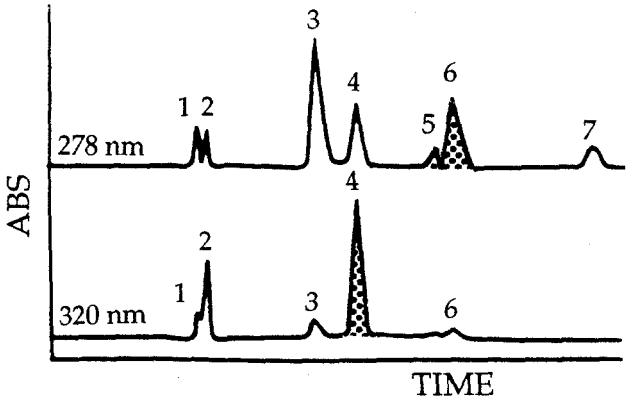

Figure 1. *HPLC separation of a mixture of standards containing chloramphenicol and its acetylated derivatives plus 4-methylumbelliferone.*
Time = elution time ; ABS = absorbance.
1 = acetyl-CoA (3.85 min)
2 = 4-methylumbelliferyl glucuronide (3.98 min)
3 = chloramphenicol (6.63 min)
4 = 4-methylumbelliferone (7.67 min)
5 = 1-acetylchloramphenicol (9.21 min)
6 = 3-acetylchloramphenicol (9.65 min)
7 =1,3-diacetylchloramphenicol (12.63 min).
The separation was carried out using a gradient elution system and monitored simultaneously at two wavelengths (278 and 320 nm) corresponding to the absorption maxima of chloramphenicol and 4-methylumbelliferone, respectively. Filled peaks represent the main reaction products that were quantitated to determine the activity of reporter genes in protoplast extracts. 1,3-diacetylchloramphenicol (peak 7) was detected only in reactions carried out in the presence of commercial preparations of CAT enzyme. Details about HPLC conditions are reported in Experimental Procedures.

Furthermore, GUS activity determinations carried out either in the presence of GUS-extraction buffer (Jefferson et al. 1987) or CAT-extraction buffer (Gorman et al. 1982) yielded nearly identical values, thus indicating the absence of any cross-interference between the two different substrates even when both enzymes are present in the same reaction mixture (data not shown).

Finally, we set out to exploit the availability of an internal standard and of an enzyme assay that allows the simultaneous determination of GUS and CAT for the quantitative comparison of two different promoters by a reduced number of transformation experiments. We, thus, compared the extractive efficiency of the two buffer systems that were previously found to be equally suitable for GUS activity determination. Total recovery of GUS activity with the two extraction systems was approximately the same, although extracts prepared with GUS buffer contained about twice as much protein than a corresponding extract prepared with CAT buffer. As indicated by SDS-PAGE analysis of the two types of extract, such difference is likely to be due to chloroplast lysis induced by the detergents which are present in GUS extraction buffer (data not shown).

The possible presence of interfering deacetylase activities, which can be inactivated by a brief heat treatment, has been reported both in animal and plant extracts (Sambrook et al. 1989; Scott et al. 1988). Since GUS is known to be heat-labile, the utilization of a single protoplast extract for the determination of both reporter enzymes required a preliminary assessment, under our experimental conditions, of the effect of heat treatment on the final yield of CAT activity. We found that heating the extract at 65° C for 10 minutes totally inactivates ß-glucuronidase without any appreciable variation of chloramphenicol acetyltransferase activity. In addition, using standard methods of detection we also verified that both enzymes are stable for at least 16 hours incubation.

Altogether, the above results indicate that it is possible to use a single protoplast extract for the simultaneous assay by HPLC of two distinct reporter genes.

We then wished to apply our simplified assay procedure to the analysis of two different promoters. A plasmid containing the GUS gene under the control of the 35S CaMV promoter was used as "reference". Two other plasmids, containing the CAT gene under the control of the intact CaMV 35S promoter and of a derivative of the same promoter from which the region

upstream of -90 had been deleted (-90CaMV 35S), were used as "sample ". Previous studies have shown that the activity of -90CaMV 35S is about 80% reduced compared to the activity of the intact promoter (Ow et al. 1987). Data reported in Table 1 indicate that using GUS as a co-transformed "reference" it is always possible to distinguish unambiguously between different promoters.

The sensitivity of the HPLC detection system we have developed is lower than that achievable with standard methods of detection. However, as shown in Table 1, even in the presence of -90CaMV 35S-CAT and relatively low efficiencies of transformation, the amount of products that are formed after a 16-hours reaction is always higher than the limit of sensitivity (0.8 µg/ml) of our HPLC detection system.

Table 1 *. Application of the single tube assay to the comparison of intact and 5' deleted (-90) CaMV 35S-CAT constructions in the presence of GUS as an internal standard. CAT and GUS activities are expressed as 1-acetyl plus 3-acetyl chloramphenicol (µg/ml) and 4-methylumbelliferone (µg/ml), respectively. Values are the average of three HPLC determinations which differed by no more than 10% of the mean.*

Exp. Number	35S-CAT	35S-GUS	CAT/GUS
1	11.36	2.79	4.07
2	16.59	4.31	3.85
3	16.17	4.09	3.95
4	13.45	4.22	3.19
5	6.34	2.09	3.03
6	20.81	5.47	3.81
7	24.08	6.45	3.84
	-90 35S-CAT	35S-GUS	-90CAT/GUS
8	1.23	3.58	0.34
9	1.44	5.27	0.27
10	1.67	5.13	0.32
11	2.59	6.03	0.43
12	6.39	14.03	0.45
13	7.52	16.01	0.47
14	1.94	2.37	0.81

In conclusion, our method differs from previously reported procedures with respect to the use of a single extraction buffer, a single tube reaction and the simultaneous assay by HPLC of two reporter genes. The detection method is fast (20 min/assay), it does not require either any manipulation of the reaction mixture or the use of radioactive substrates, it can be applied to large sample pools, and it can be carried out with commercially available plasmids containing either CAT or GUS genes as "sample", under conditions in which the co-transformed "reference" gene behaves as an *authentic* internal standard. Of course, the possibility of measuring the expression of GUS and CAT genes on a single protoplast extract can advantageously be extended to the determination of reporter enzyme activities with standard procedures.

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