Expression of photosynthesis-related gene fusions is restricted by cell type in transgenic plants and in transfected protoplasts

(Flow cytometry/gene expression/β-glucuronidase/Ptcotiana tabacum)

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ABSTRACT We have analyzed the expression of chimeric genes in populations of protoplasts isolated from the photosynthetic and nonphotosynthetic tissues within leaves of transgenic tobacco plants and separated by fluorescence-activated cell sorting. Expression of transcriptional gene fusions controlled by promoters from photosynthesis-associated genes showed a striking dependence on cell type. These patterns of expression were preserved when the gene fusions were transfected into normal (nontransgenic) tobacco leaf protoplasts.

Considerable advances in our understanding of gene regulation can come from the construction of chimeric genes, the design of methods for transfer of these constructs into target organisms, and the study of their expression (1). However, this approach is limited by difficulties presented by the cellular complexity of the organism under study. To accommodate this complexity, it is important to be able to recognize different cell types within organisms, according to appropriate biological criteria, and then to correlate differential gene expression with these criteria. A good example is found in the study of photosynthesis in higher plants. A fundamental characteristic of this process is the differentiation of photosynthetic and nonphotosynthetic cells. In tobacco leaves, photosynthesis occurs in cells containing mature chloroplasts, predominantly within the tissues of the spongy parenchyma and palisade mesophyll. Surrounding these tissues are a variety of nonphotosynthetic tissues, including the cells of the epidermis, the vascular system, and the perivascular parenchyma (2). A full understanding of the regulation of photosynthetic genes requires not only the development of methods for the quantitative analysis of gene expression but also an ability to accurately distinguish between the different cells within the leaf prior to this analysis. Histological techniques have provided some insight concerning cell type-specific gene expression (3), although these techniques are limited in terms of reproducibility and sensitivity. Microdissection can also be used to separate epidermal and mesophyll tissues prior to analysis (4, 5). However, it is impossible to completely separate the vascular and mesophyll tissues, and interpretation of data is complicated by the presence of guard cells within the epidermis.

An alternative to these approaches involves the reduction of the leaf tissue to populations of cells by production of protoplasts. The protoplasts are then separated according to different physical or biological criteria, prior to analysis of gene expression. We have used flow cytometry to characterize leaf protoplasts according to size and chlorophyll content and to isolate populations of viable protoplasts (6, 7). The combination of marker gene techniques (3, 8) with those of flow sorting should provide a precise analysis of cell type-specific gene expression. One potential drawback of this approach is that genes within protoplasts may not be regulated in the same manner as within the cells of the intact plant. This can be addressed by transfecting chimeric genes into protoplasts and comparing the activities of these genes with the activities observed in the corresponding transgenic plants. For example, parsley protoplasts exhibit patterns of gene expression in response to elicitors and ultraviolet illumination that are similar to those seen within intact parsley cells (9).

By the analysis of photosynthetic and nonphotosynthetic protoplasts purified by fluorescence-activated sorting, we show that light-regulated and constitutive chimeric genes in transformed tobacco (Ptcotiana tabacum) plants are differentially expressed within these two different tissues of the leaf. These patterns of gene expression are faithfully maintained when the chimeric genes are transduced into the isolated protoplast populations. A similar inhibition of gene expression is observed in these two experimental situations within cells containing chloroplasts whose function has been impaired by Norflurazon [4-chloro-5-methylamino-2-(a,a,a-trifluoro-m-tolyl)-3-(2H)-pyridazine; Sandoz].

MATERIALS AND METHODS

Plants. Plants were grown axenically in MS medium (10) under 2000 lux of continuous white light at 25°C. Transformed plants were maintained on medium containing kanamycin (100 μg/ml) and carbenicillin (500 μg/ml). When included, Norflurazon was at a concentration of 4 μg/ml.

Plasmid Constructions and Plant Transformation. The transcriptional gene fusions (pBI111.1, pBI121.1, and pBI131.1) and their means of introduction into tobacco plants have been described (3, 11). The plasmids employed for transfection comprised the 3.8-kilobase (kb) EcoRI–Sal I fragment (from pBI111.1), or the 2.85-kb and 3.05-kb HindIII–EcoRI fragments (from pBI121.1 and pBI131.1), subcloned into pUC8 or pUC19; these plasmids are termed pBI211DG, pBI221DG, and pBI231DG, respectively.

Protoplast Preparation. Protoplasts were prepared from young leaves (fresh weight ~150 mg) that were sliced into 1 × 10-mm pieces and placed in 10 ml of 0.5 M mannitol/10 mM CaCl2/3 mM Mes, pH 5.7, containing 0.1% (wt/vol) Cellulase, 0.1% (wt/vol) Macerase (Calbiochem), and 0.1% (wt/ vol) Driselase (Sigma). After incubation for 15–18 hr, the protoplasts were filtered, diluted with 2 volumes of solution W5 (12), and collected by centrifugation at 60 × g for 8 min.

Abbreviations: CaMV, cauliflower mosaic virus; GUS, β-glucuronidase.
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In some of the experiments, mesophyll and achlorophyllous protoplasts were partially separated by resuspension of the protoplasts in 15 ml of 25% (wt/vol) sucrose. The protoplast suspension was overlaid with 5 ml of 10% sucrose dissolved in a solution comprising equal volumes of medium T0 (13) and medium W5. This was overlaid with 5 ml of medium W5 and centrifuged at 60 × g for 10 min. Mesophyll protoplasts were enriched at the lower interface, whereas the achlorophyllous protoplasts were found at the upper interface. They were collected by centrifugation at 60 × g for 5 min, after dilution with 2 volumes of medium W5.

Flow Sorting. Sterile sorting employed a Coulter Epics V flow cytometer–cell sorter, as described (6, 7). The sort decision was gated on fluorochromas of fluorescein diacetate (14) so as to identify only viable protoplasts for analysis. Sorting of achlorophyllous protoplasts was based on time-of-flight analysis of the fluorescence signals of fluorescein diacetate; sorting of mesophyll protoplasts was based on time-of-flight analysis of chlorophyll autofluorescence (6).

Protoplast Transfection. Wild-type protoplasts were transfected by a modification of published procedures (12). The protoplasts were suspended at 10⁶ per ml in medium MaMg (12) or, for some of the experiments of Fig. 3, in medium W5, containing sheared calf thymus DNA (50 μg/ml) and the appropriate plasmid DNA (20 μg/ml). After addition of 1.5 volumes of PEG 4000 CMS solution (12), followed by incubation at room temperature for 25 min, the protoplasts were diluted with 8 volumes of culture medium T0 (13) modified by addition of 0.38 M glucose, 0.154 M mannitol, and 75 μg of ampicillin per ml and were incubated at 25°C in darkness. For experiments involving flow sorting, the protoplasts were partially purified into mesophyll and achlorophyllous subpopulations by gradient centrifugation, prior to transfection. Sorted protoplasts were cultured in 0.15 ml of the modified culture medium in 96-well microtiter wells for 24 hr at 25°C in darkness.

Analytical Procedures. Kinetic assays of β-glucuronidase (GUS) activity (8) were performed in the wells of 96-well culture plates in lysis buffer in a final volume of 0.3 ml.

Microscopy. Protoplasts were examined with an Olympus inverted microscope equipped with a Zeiss epifluorescence illuminator. Protoplast viability was determined after incubation with fluorescein diacetate (14).

RESULTS
Cell Type-Specific Gene Expression in Protoplasts from Transformed Plants. Protoplasts prepared from single leaves were analyzed according to diameter and chlorophyll content (6), revealing two protoplast populations (Fig. 1A). Sorting based on these parameters led to the recovery of two homogeneous preparations of viable protoplasts. One set, derived from the photosynthetic mesophyll tissue of the leaf, consisted of cells containing many chloroplasts (Fig. 1B). The second set, derived from the nonphotosynthetic epidermis and perivascular parenchyma (2), contained no chlorophyll and lacked chloroplasts (Fig. 1C). When large populations (20,000) of sorted protoplasts were reanalyzed, the purities of the mesophyll and achlorophyllous populations were found to be 98.2% and 99.6%, respectively.

To examine cell type-specific gene expression within the tissues from which these protoplast populations were derived, we analyzed six transgenic plants containing three different chimeric constructions (3, 11) in which GUS expression was directed by the cauliflower mosaic virus (CaMV) 35S promoter or by the promoters of tobacco genes encoding the small subunit of ribulose-bisphosphate carboxylase/oxygenase (rbcS) or the chlorophyll a/b-binding protein (Cab). We prepared protoplasts from single leaves of these plants, sorted them according to the presence or absence of chlorophyll (Fig. 1), and assayed them for GUS activity in vitro. Plants containing the CaMV–GUS gene fusions showed similar levels of GUS activity in protoplasts derived either from photosynthetic or from nonphotosynthetic tissues (Fig. 2). In contrast, plants containing the rbcS–GUS and Cab–GUS gene fusions exhibited substantial GUS activity only in protoplasts derived from photosynthetic tissues. Protoplasts derived from nonphotosynthetic tissues of the same leaf had GUS activities that were greatly reduced or were undetectable. To examine the possibility that a subpopulation of the CaMV–GUS-transformed protoplasts was responsible for the GUS activity, we sorted defined numbers (1–100) of the different protoplast types. The kinetics of methylumbelliferyl glucuronide (Sigma) hydrolysis were linear with respect to time and with respect to the numbers of protoplasts that were sorted (data not shown); this was true even over the range of 1–5 sorted protoplasts, for which the coefficient of determination (r²) between total GUS activity and numbers of protoplasts sorted was 0.97. These results indicate that the different cell types were homogeneous with respect to CaMV–GUS expression and also highlight the sensitivity of this combination of flow cytometry and gene fusion analysis. Inclusion of cycloheximide (50 μg/ml) during protoplast preparation did not affect the amounts of the GUS activities

![Protoplast Size](image_url)
Plant rbcS-GUS 21: 0.80 (nonphotosynthetic), 0.51 (photosynthetic).

Plant CaMV-GUS 21: 0.80 (photosynthetic), 0.51 (nonphotosynthetic).

Plant CaMV-GUS 30: 0.36, 0.31. Plant rbcS-GUS 2: 3.82, 0.09. Plant rbcS-GUS 5: 1.00, 0.10. Plant Cab-GUS 26: 1.14, <0.03. Plant Cab-GUS 27: 0.93, 0.06.

measured in the sorted protoplasts or the patterns of cell-specific expression. When protoplasts were cultured for 4 days in the presence of cycloheximide, the in vivo half-life of GUS activity was about 45 hr. Thus, over the time scale in which the protoplasts were prepared and used, the measured GUS activities were representative of the amounts of the chimeric gene products within the cells of the intact leaf.

Cell Type-Specific Gene Expression in Transfected Protoplasts. Experiments using published procedures for the transfection of DNA into plant protoplasts (12) revealed a variety of factors that affected the level of GUS activity. First, we found that the use of medium MaMg, reported to increase the numbers of transformed cell lines derived from transfected protoplasts (12), resulted in increased GUS expression in the transient assay (Fig. 3). Second, the form in which the plasmids were presented also affected GUS expression. For pBI211DG, expression increased when the plasmid was presented in the closed circular form. In contrast, for pBI221DG and pBI231DG, 5' linearization resulted in increased expression over that observed with the closed circular form. In all cases, however, expression was decreased by linearization of the plasmids at the 3' end of the gene (Fig. 3).

After transfection of the different chimeric genes into nontransgenic Nicotiana tabacum leaf protoplasts, the degree of cell-type dependence of gene expression was determined after flow sorting of the protoplasts derived from photosynthetic and nonphotosynthetic tissues (Fig. 4). In several replicated experiments, the patterns of expression of the three chimeric genes paralleled those observed in the permanently transformed plants (Fig. 2). Expression of the CaMV–GUS gene fusion was observed in both protoplast populations (Fig. 4). In contrast, expression from the rbcS–GUS and Cab–GUS fusions was restricted to the mesophyll protoplasts (Fig. 4). For the CaMV–GUS fusion, the GUS activities per cell were greater than those measured in protoplasts isolated from the corresponding transgenic plants (Fig. 2), whereas for the rbcS–GUS and Cab–GUS fusions, the activities were comparable to those values seen in the transformed plants.

Effects of an Inhibitor of Chloroplast Function. Inclusion of Norflurazon in the medium used for plant subculture induced a bleaching of the newly emerging leaves. These leaves were morphologically normal although the plastids lacked thylakoids (Fig. 5C). Flow cytometry of protoplasts prepared from these leaves revealed a single population, devoid of cellular chlorophyll (Fig. 5A). Sorted protoplasts had well-defined cytoplasmic strands that exhibited active streaming (Fig. 5B). In transgenic plants, Norflurazon had no effect upon the expression of the CaMV–GUS gene fusion (Table 1). In striking contrast, Norflurazon treatment led to a complete inhibition of expression of the Cab–GUS gene fusion. Expression of the rbcS–GUS fusion was inhibited by Norflurazon to a lesser extent. When these chimeric genes were transfected into wild-type protoplasts isolated from white leaves of Norflurazon-treated plants, qualitatively similar effects were observed. Norflurazon did not inhibit CaMV–GUS expression but reduced by ≈60% the levels of Cab–GUS and rbcS–GUS expression.
DISCUSSION

Cell-Specific Gene Expression in Transformed Plants. The cell-specific patterns of expression measured in protoplasts are a clear function of the regulatory sequences 5'-cis to the GUS coding region. This work provides a precise, quantitative analysis of differential gene regulation at the single-cell level and supports the concept of differential expression of rbcS and Cab genes within photosynthetic tissues. It also confirms observations obtained from analyses of organ-specific and microdissected tissue-specific expression of light-regulated nuclear genes (4) and from in situ immunofluorescence analyses (5). However, slight differences are apparent; we observed low levels of expression of the rbcS-

![GUS expression in transfected protoplasts](image)

**Fig. 4.** Patterns of GUS expression in transfected protoplasts. After transfection, ~1000 protoplasts either containing (filled bars) or lacking (hatched bars) chlorophyll were sorted into wells of a microtiter culture plate; protoplast numbers were confirmed by light microscopy prior to assay for GUS activity. Three independent experiments were performed with each construct, and the mean values (pmol per hr per protoplast) were as follows. pBI221DG: 8.73 (photosynthetic), 5.97 (nonphotosynthetic). pBI231DG: 1.84, 0.11. pBI211DG: 0.96, 0.04.

<table>
<thead>
<tr>
<th>Transgenic plants</th>
<th>GUS activity, pmol per hr per protoplast</th>
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<tbody>
<tr>
<td>CaMV-GUS 21</td>
<td>Control: 1.11 ± 0.39, Norflurazon: 1.45 ± 0.70 (131%)</td>
</tr>
<tr>
<td>CaMV-GUS 30</td>
<td>Control: 0.70 ± 0.15, Norflurazon: 0.77 ± 0.40 (110%)</td>
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<tr>
<td>rbcS-GUS 2</td>
<td>Control: 2.79 ± 0.77, Norflurazon: 1.71 ± 0.44 (72%)</td>
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<tr>
<td>rbcS-GUS 5</td>
<td>Control: 0.39 ± 0.06, Norflurazon: 0.10 ± 0.10 (26%)</td>
</tr>
<tr>
<td>Cab-GUS 26</td>
<td>Control: 0.23 ± 0.14, Norflurazon: 0.00 ± 0.00 (0%)</td>
</tr>
<tr>
<td>Cab-GUS 27</td>
<td>Control: 0.07 ± 0.03, Norflurazon: 0.00 ± 0.00 (0%)</td>
</tr>
<tr>
<td>Transient genes</td>
<td>Control: 11.14 ± 3.27, Norflurazon: 22.02 ± 6.97 (198%)</td>
</tr>
<tr>
<td>rbcS-GUS</td>
<td>Control: 1.4 ± 0.06, Norflurazon: 0.55 ± 0.25 (39%)</td>
</tr>
<tr>
<td>Cab-GUS</td>
<td>Control: 0.72 ± 0.09, Norflurazon: 0.25 ± 0.10 (35%)</td>
</tr>
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Data are means ± SD derived from three independent experiments and are expressed in terms of the total number of viable protoplasts. Values in parentheses indicate activity relative to control.

GUS and Cab-GUS fusions in nonphotosynthetic tissue. Similar observations have been made concerning the expression of gene fusions in nonphotosynthetic tobacco callus tissue (15). The observation of nonzero transcriptional activities of the rbcS–GUS and Cab–GUS chimeric genes in nonphotosynthetic tissues may reflect a higher sensitivity of the GUS system as compared to the other techniques (4, 5). It may also imply that posttranscriptional events are involved in the maintenance of cell-specific patterns of protein product accumulation, such as is observed with ribulose-bisphosphate carboxylase (4), perhaps achieved through modulation of mRNA stability or processing efficiency (16). The detection of CaMV–GUS expression in both photosynthetic and nonphotosynthetic cell types confirms previous results (3), in which histochemical analyses of stem sections revealed GUS activity within the nonphotosynthetic phloem and epidermal tissues, as well as within the photosynthetic cortical parenchyma.

Cell-Specific Gene Expression in Transfected Protoplasts. The cell-specific regulation of the rbcS and Cab promoters in chimeric GUS constructs introduced in transience parallels the patterns of expression seen in the transformed plants. There are no previous reports of regulated transient expression of heterologous genes under the control of Cab or rbcS promoters. This previous lack of success may reside in the physiological condition of the protoplasts. We used low levels of polysaccharidases for protoplast production; these polysaccharidases are crude cell-free preparations from bacterial or fungal filtrates, which may possess elicitor activity.

![Flow sorting of homogeneous populations of protoplasts from leaf tissues of N. tabacum treated with Norflurazon.](image)

**Fig. 5.** Flow sorting of homogeneous populations of protoplasts from leaf tissues of N. tabacum treated with Norflurazon. (A) Biparametric analysis of fluorescence emission (IRFL, chlorophyll content) and time-of-flight (TOF, protoplast size) revealed one population. (B) Viable protoplasts lacking mature chloroplasts were recovered after sorting when sort windows were placed around this population. (Bar = 35 μm.) (C) Plastids within Norflurazon-treated leaf cells lacking thylakoids. (x5000.)
The protoplasts. The both in fusion may be transfected as linearized DNA rather than Z-DNA formation, of rbcS genes in Nicotiana haps transfected as linearized DNA rather than Z-DNA formation, which contrasting in copy number. The transient system) could abrogate the effect of the decreased affinity of the regulator, through mass action.

Conclusions. The combination of the quantitative aspects of the GUS marker gene technology with those of cytomtery has several important consequences. It now should be feasible to relate the chlorophyll content of a cell to the level of transcription of a particular photosynthetic gene and to ascertain whether there exist within plant leaves subpopulations of mesophyll cells, differing in size and chlorophyll content, that differentially express photosynthetic genes. The methods should also be applicable for the characterization of the response of plant cells to external perturbations, the isolation of rare variants differing in size, chlorophyll content, or photosynthetic activities, and the identification of gene products expressed exclusively in nonphotosynthetic tissues. Our ability to measure in vitro the expression of chimeric genes within single plant cells opens the way for analysis and sorting of protoplasts based on their expression of GUS measured in vivo. Since plant regeneration from sorted protoplasts is routine for numerous species, this may permit the production of transformed plants without drug selection. Moreover, since these techniques allow rapid analysis and sorting of large numbers of protoplasts, their use should permit the isolation of rare events such as mutations affecting expression of GUS gene fusions. Finally, since expression of genes within transfected protoplasts appears to correlate with the expression patterns observed in permanently transformed cells, these techniques should accelerate the analysis of the molecular basis for cell type-specific gene expression.

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