Reports

Transient and Stable Expression of the Firefly Luciferase Gene in Plant Cells and Transgenic Plants

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The luciferase gene from the firefly, *Photinus pyralis,* was used as a reporter of gene expression by light production in transfected plant cells and transgenic plants. A complementary DNA clone of the firefly luciferase gene under the control of a plant virus promoter (cauliflower mosaic virus 35*S* RNA promoter) was introduced into plant protoplast cells (*Daucus carota*) by electroporation and into plants (*Nicotiana tabacum*) by use of the *Agrobacterium tumefaciens* tumor-inducing plasmid. Extracts from electroporated cells (24 hours after the introduction of DNA) and from transgenic plants produce light when mixed with the substrates luciferin and adenosine triphosphate. Light produced by the action of luciferase was also detected in undisrupted leaves or cells in culture from transgenic plants incubated in luciferin and in whole transgenic plants "watered" with luciferin. Although light was detected in most organs in intact, transgenic plants (leaves, stems, and roots), the pattern of luminescence appeared to reflect both the organ-specific distribution of luciferase and the pathway for uptake of luciferin through the vasculature of the plant.

THE FIREFLY LUCIFERASE GENE, which encodes an enzyme that catalyzes the light-producing, adenosine triphosphate (ATP)–dependent oxidation of luciferin (*1*), is a powerful reporter gene for assessing gene expression in eukaryotic organisms. We report that light emitted from firefly luciferase was detected in plants and plant cells in culture transformed with a luciferase complementary DNA (cDNA) construct driven by a plant virus promoter. The firefly luciferase gene has been expressed in bacteria (*2*) but is ideal for use as a reporter gene in eukaryotic organisms (*3*), because unlike the bacterial *lux* gene system (*4*), only one protein is needed for light production if the substrates ATP, O_2, and luciferin are available (*1*).

The introduction of DNA into plants for testing the expression of gene constructs has been made possible by the development of new methods for transforming plants and plant cells. Several procedures, including electroporation, have been developed to introduce naked DNA into plant cells for testing the transient expression of gene constructs (*5*). The production of transgenic plants, transformed with the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* (*6*), has permitted investigations into the regulation of organ-specific gene expression in plants, the development of pathogen or herbicide resistance, and the control of gene expression linked to plant physiological processes including responses to light (*7*).

To test the activity of the firefly luciferase gene in plant cells in a transient expression assay, we used electroporation (*5*) to introduce a luciferase cDNA construct into protoplasts of cultured carrot cells (*8*). The basic plasmid pDO432 (Fig. 1) contains an "intronless" luciferase gene created by de Wet *et al.* (*3*) from a partial cDNA clone linked at an Xba I site (129 bp downstream from the start of translation) to the 5′ end of the firefly gene. The fusion was created because a full-length cDNA covering all of the translated region was not obtained in the initial cDNA cloning effort (*2*). The luciferase gene was spliced upstream, via a Bam HI site, to the cauliflower mosaic virus (CaMV) 35*S* RNA promoter, making the expected start of transcription 81 bp upstream from the predicted start of translation of the luciferase gene (Fig. 1). The luciferase gene was spliced on its downstream side, via a Bam HI site, to a segment at the 3′ end of the nopaline synthase (*nos*) gene (*9*).

The plasmid pDO432 was introduced into carrot protoplasts by electroporation,

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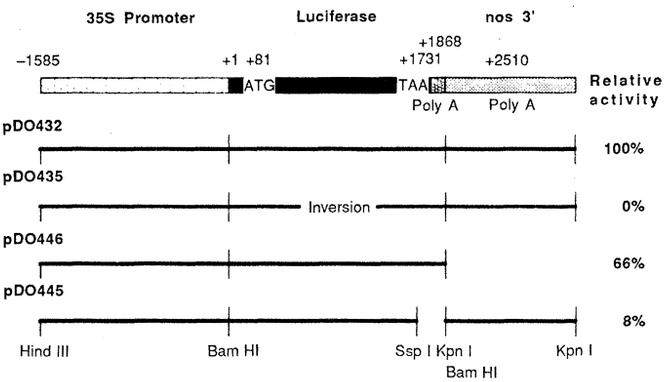

Fig. 1. The structure of the CaMV 35*S* RNA promoter–luciferase–*nos* 3′ end construct in pUC19 (pDO432). The 35*S* RNA promoter (35*S* p) was obtained by creating a Bam HI site with oligonucleotide-directed mutagenesis of pCaMV10 (from CaMV isolate CM1841) (*14*) at the start of 35*S* RNA transcription (*15*) (position 7431 as underlined, GGACAC→GGATCC). The 35*S* RNA promoter (1586 bases) extends from this newly created Bam HI site upstream from the Hind III site at position 5849 on the map of CaMV-CM1841 (*14*). The luciferase gene was obtained as a Hind III–Bam HI fragment (1886 bases) from pJD201 (*3*). The upstream Hind III site was filled in and converted to a Bam HI site by the addition of Bam HI linkers. The luciferase gene in pJD201 is an intronless gene created by a fusion between a partial cDNA clone (Luc23) and the 5′ end of the corresponding firefly luciferase gene, joined in the coding region at an Xba I site (*3*). The presumed start of translation is 81 bases downstream from the newly created Bam HI site, the point of fusion of the luciferase gene and the 35*S* RNA promoter. A 1028-bp fragment containing polyadenylation sites from the 3′ end of the *A. tumefaciens nos* gene (*9*) was inserted downstream from the luciferase gene. The 3′ end of the luciferase cDNA, in particular, the 139 bp Ssp I–Bam HI fragment, carries a consensus polyadenylation signal (AATAAA) 28 bases upstream from its 3′ end. Plasmid constructs were tested for activity in a transient expression assay in which 10 μg of plasmid DNA and 500 μg of carrier calf thymus DNA were introduced by electroporation (*5*) into protoplasts prepared from carrot cells growing in suspension (*8*). Extracts were prepared 24 hours after electroporation by repeated cycles of freezing and thawing of 0.5×10^7 to 1×10^7 carrot cells in luciferase extraction buffer, 100 m*M* potassium phosphate buffer (*p*H 7.5), and 1 m*M* dithiothreitol, followed by centrifugation for 5 minutes in a microcentrifuge (Eppendorf) at 4°C. For luciferase assays, 1/10 volume of the supernatant fluid was diluted into 400 μl of assay buffer [14 m*M* glycylglycine buffer (*p*H 7.8), 14 m*M* $MgCl_2$, and 6 m*M* ATP], to which 100 μl of 1 m*M* luciferin was added by injection. The peak intensity of the resulting light flash was measured in a luminometer (LKB, model 1250), and the data from a single representative experiment are expressed in light units relative to that produced by pDO432.

and extracts were assayed for activity, usually 24 hours later. Extracts produced a light flash detected in a luminometer in the presence of the substrates luciferin and ATP. In the presence of excess substrates, light production, measured as peak height, was proportional to the amount of plant extract containing luciferase activity. No light above machine background [<5 light units (LU)] could be detected in the absence of luciferin, but low levels of light were produced with excess luciferin in the absence of added ATP, presumably as a result of ATP in the plant cell extracts. With pDO432, 1000 to 3000 LU per 10^6 cells or 60 to 90 LU per microgram of protein were obtained in transient expression assays corresponding, on average, to the activity of 300 to 900 molecules of luciferase per cell (*3*).

The expression of the luciferase gene in transient expression assays depended on certain critical features of the DNA construct. For example, transcription was directed by the 35*S* RNA promoter, and not by a promoter within the 5′ untranslated segment of the luciferase gene. This was indicated by the absence of luciferase expression from pDO435 in which the luciferase gene was inverted with respect to the promoter (Fig. 1). Removal of the downstream plant polyadenylation site (the *nos* 3′ segment), as in pDO446, reduced luciferase activity to 66% of full activity. However, when the 3′ end of the firefly luciferase gene, which contains the polyadenylation signal AATAAA that is a consensus sequence for both animal and certain plant genes (*10*), was deleted by removing the Ssp I–Bam HI DNA segment, as in pDO445, luciferase activity in extracts dropped to 8% of full activity. The explanation for this loss in activity is not clear, but may indicate that luciferase expression in pDO432 is more dependent on the proximal polyadenylation signal in the luciferase gene than on a distal signal in plant DNA.

To obtain stable transformants, we introduced pDO432 into plants via the *A. tumefaciens* Ti plasmid. The plasmid pDO432 was inserted at the Hind III site in the Ti plasmid binary vector Bin-19 (*11*) and transferred by conjugation into the *A. tumefaciens* strain GV3111 bearing the helper plasmid pTiB6S3SE (*6*). *Nicotiana tabacum* plants (Wisconsin-38 cultivar) were inoculated with the bacterial vector by the leaf disk inoculation method (*6*), and kanamycin-resistant transformants were selected. Primary transformants were screened for luciferase production by grinding a small, frozen leaf disk (diameter, 8 mm) in extraction buffer (see legend to Fig. 1) to prepare an extract for the luciferase assay. Screening can also be performed in a semiquantitative manner by dropping a small leaf cutting (~5 mm^2) in a reaction tube containing substrates and reading the light output in a luminometer.

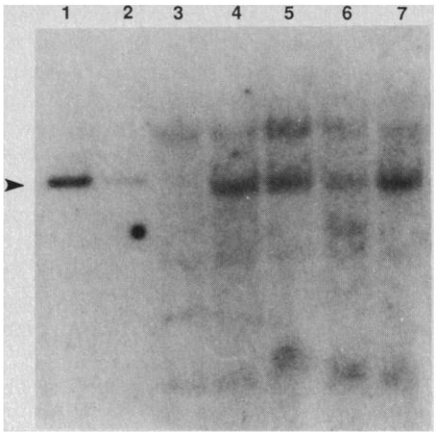

Fig. 2. Blot of genomic DNA from transgenic tobacco plants. DNA was extracted from the leaves of primary transformants as indicated, cleaved with Hind III, fractionated on a 1% agarose gel, transferred to a nitrocellulose filter, and hybridized with the nick-translated 1.6-kb Hind III–Bam HI fragment from pDO432 containing the CaMV 35*S* promoter (*16*). Hind III cleavage of integrated pDO432 DNA releases a linear 7-kb fragment. Gel migration position of the 7-kb fragment is indicated by the arrow on the left. (Lanes 1 and 2) Copy number standards. Hind III–cut pDO432 DNA in amounts (110 and 22 pg) equivalent to ten and two copies per tetraploid tobacco genome; (lanes 3 to 7) 10 μg of Hind III–cut DNA from an untransformed tobacco plant (lane 3) and transformed plants B10 (lane 4), B15 (lane 5), B22 (lane 6), and B28 (lane 7).

Several primary transformants (transgenic plants B10, B15, B22, and B28) with demonstrated luciferase activity were chosen for further study. DNA blots indicated the presence of pDO432 in the plant genome, which appeared as a 7-kb band in Hind III–cleaved DNA fragments from transformed plants but not from untransformed plants. The number of copies of pDO432 was quite similar in the selected transformants and was equivalent in reconstructions to one to several copies per tobacco genome (Fig. 2). The transgenic plants also produced a protein species of a size expected for firefly luciferase (*12*). Protein blots of leaf extracts from plant B15 showed the presence of a 62-kD protein band that reacts with antiserum and migrates with firefly luciferase.

The selected transgenic plants (primary transformants), grown in sterile culture, were examined for the distribution of luciferase activity in various plant organs. We found that extracts from all organs—leaves, roots and stems—expressed activity to differing extents (Fig. 3). Considerable variation in activity was observed from leaf to leaf, with young, uppermost leaves generally showing higher specific activities than older, lower leaves. In general, roots and stems (upper stems were tested) had more lucifer-

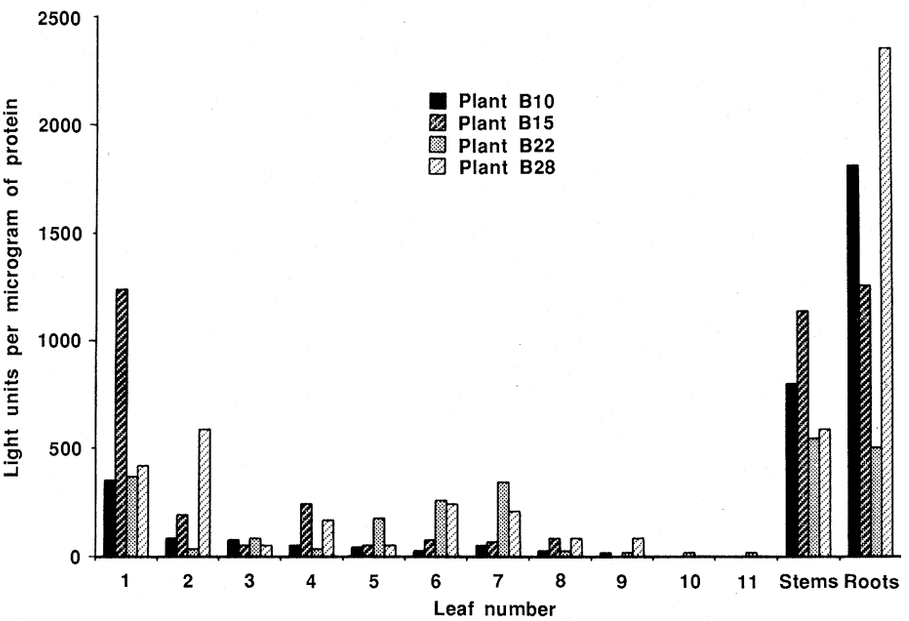

Fig. 3. Distribution of luciferase activity in the organs of transgenic plants. Plants were grown in glass jars under sterile conditions in a modified Murashige and Skoog agar medium (MS medium) (*17*) and extracts were prepared when plants reached a height of 12 to 15 cm. Extracts from various plant organs were prepared by grinding a small amount of tissue, frozen in liquid N_2 in a 1.5-ml microcentrifuge tube, with the extraction buffer described in Fig. 1. The soluble extract obtained after centrifuging for 5 minutes at 4°C in a microcentrifuge was assayed for luciferase activity and protein content (*18*). Leaf number is the position of the leaf on the plant, starting from the younger leaves at the top of the plant progressing to the older leaves on the bottom.

ase activity (usually more than 2000 LU per microgram of protein) than leaves. This finding is consistent with the observations of Fluhr *et al.* (*13*), who demonstrated a similar organ-specific distribution for chloramphenicol acetyltransferase (CAT) activity driven by the CaMV 35*S* promoter. Many factors may contribute to variation in the overall activity or the distribution of luciferase activity in plant organs, including differences in developmental stage, plant growth conditions, stability of the enzyme, and variation in the expression of the gene, possibly due to position effects of integration of the luciferase gene in the plant genome (*13*).

We also attempted to determine the distribution of luciferase activity in various plant organs by introducing luciferin into intact plant tissue. We found that by soaking a young leaf from a transgenic plant in a solution containing 1 m*M* luciferin, we could detect emitted light by exposing the leaf to x-ray film (Kodak OG) for 15 to 60 minutes (Fig. 4). Light emission was enhanced by adding dimethyl sulfoxide (DMSO) up to 20% and sodium citrate (*p*H 5) to the luciferin solution. These additions probably aided in the uptake of the substrate. No background light was detected on x-ray film from leaves of untransformed plants or from leaves of transformed plants that were not soaked in luciferin. Finally, luciferin was introduced through roots into whole plants by watering small, sterile plants with a luciferin solution. Plant B21 (a primary transformant with high luciferase activity) was watered for 6 hours with 5 ml of a 1 m*M* luciferin solution and exposed by contact to Kodak Ektachrome 200 film (4 by 5 inch sheet) for 24 hours (Fig. 5). The pattern of light emission appeared to reflect both the distribution of luciferase activity in the plant and the movement of luciferin through the vasculature of the plant. Light emission was most intense from roots, stems, and young, upper leaves. However, many factors may contribute to the pattern of light emission from intact plants, including possible organ differences in the accessibility of luciferase to luciferin, compartmentalization of luciferase, and absorption of emitted light.

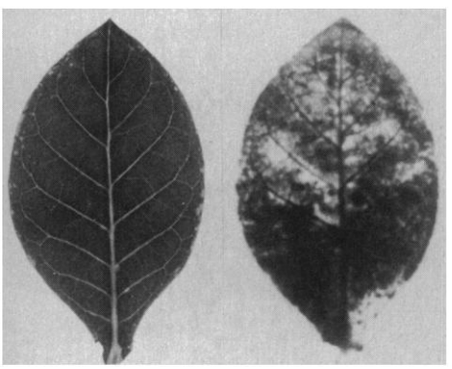

Fig. 4. Leaf luminescence recorded on x-ray film (autoluminograph). Small leaf (2.5 by 4.5 cm) from primary *N. tabacum* transformant B15 illuminated with ordinary white light (left). The leaf was soaked for 2.5 hours in 1 m*M* luciferin solution containing 100 m*M* sodium citrate (*p*H 5.0) and 20% dimethyl sulfoxide. The same leaf (right) was exposed by contact to x-ray film (Kodak OG).

Fig. 5. Luminescence from transgenic tobacco plant B21 bearing the firefly luciferase gene. The plant was watered with a 1 m*M* solution of luciferin, the luciferase substrate, and exposed by contact to Kodak Ektachrome 200 film.

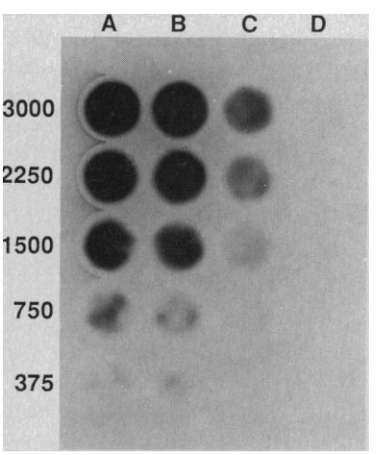

Fig. 6. Detection of luciferase activity in cells distributed in a 96-well microtiter dish (Falcon 3072, Becton Dickinson). Portions (200 μl) of a cell suspension culture derived from transgenic tobacco plant B15 (columns A to C) and an untransformed tobacco (column D) plant were distributed into microtiter dish wells as indicated. Numbers to the left indicate the number of cells per well in each row. Luciferin in MS medium was added to the wells (column A, 400 μ*M*; B, 80 μ*M*; C, 16 μ*M*; and D, 400 μ*M*) and the plate was exposed by contact with x-ray film (Kodak OG) for 1 hour. The edges of the microtiter dish were milled so that the bottoms of the wells were in direct contact with the film.

Because it would be useful for cell screening procedures to detect luciferase activity in undisrupted cultured cells, we tried to assay for the presence of luciferase activity in intact, living cells distributed in a microtiter plate. A cell line (B15-1) was derived from leaf protoplasts of the transgenic plant B15, and extracts from cells grown in suspension culture showed moderate levels of luciferase activity (210 LU per microgram of protein). Various numbers of cells were distributed into a microtiter dish, incubated in growth medium in the presence of different concentrations of luciferin and exposed for 1 hour to x-ray film (Kodak OG). Wells with luciferase-containing cells produced strong light signals (Fig. 6). After 1 hour of exposure, minimal signals were detected from 375 to 750 cells in 80 μM luciferin. Cells survived the lower concentrations of luciferin (80 μM or less) in long-term incubations (several weeks), but higher luciferin concentrations (400 μM and above) were apparently toxic and blocked further growth of the cells. Hence, at lower luciferin concentrations, this assay constitutes a simple, nondisruptive procedure for screening reasonably large numbers of independent cell lines for luciferase activity.

The luciferase gene is an important new tool for studying gene expression in both plant and animal cells. Recently, de Wet *et al.* (*3*) demonstrated the expression of an SV40 promoter–driven luciferase gene in mammalian cells (monkey CV-1 cells). For plant genetic engineering purposes, the luciferase gene can be particularly valuable as a reporter of gene expression, as a marker in genetic crosses, and as a probe for a variety of plant cell functions. The speed and ease by which the luciferase assay can be performed permit more rapid screening of large numbers of transgenic plants or transformed cell lines. The luciferase gene is a sensitive reporter gene, because the luciferase assay itself can detect the light output from 3×10^6 molecules of luciferase (10 LU on a LKB model 1250 luminometer), which is at least 100 times more sensitive than a standard CAT assay (*3*). Furthermore, luciferase activity can be detected by simple x-ray film exposure or by more elaborate devices such as luminometers or image-intensifying video equipment. Luciferase offers many new possibilities as a reporter gene since detection of activity can be noninvasive and nondestructive, and luciferase activity can be assessed periodically over time, such as through the course of plant development. In addition, luciferase can be used as a cell marker to identify cells and as a tag to follow the targeting of proteins to various destinations within and outside of cells. Finally, because luciferase activity is influenced by a variety of parameters (*1*), light production from luciferase-expressing cells potentially could be used to monitor such events as ATP production and *p*H changes.

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19. We thank M. Fromm for advice on electroporation procedures, S. C. Lee for assistance in plant tissue culture, Z. Renee Sung for W001C cells, J. Schell for plasmid pNCAT4, R. J. Shepherd for plasmid pCaMV10, M. Bevan for the Ti plasmid binary vector Bin-19, and S. Rogers for *A. tumefaciens GV3111* containing the helper Ti-plasmids pTiT37SE and pTiB6S3SE. Supported by grant 85-CRCR-1-1540 from the U.S. Department of Agriculture Competitive Research Grants Program (to S.H.H.), grant PCM83-05446 from the National Science Foundation (to M.D.), by the University of California Biotechnology Research and Education Program, and by an NSF postdoctoral fellowship in plant biology (to D.W.O.).

22 August 1986; accepted 7 October 1986

Novel Interleukin-2 Receptor Subunit Detected by Cross-Linking Under High-Affinity Conditions

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Interleukin-2 (IL-2) binds to both high- and low-affinity classes of IL-2 receptors on activated T lymphocytes. Only the high-affinity receptors are involved in receptor-mediated endocytosis and normally transduce the mitogenic signals of IL-2; however, the structural features distinguishing the high- and low-affinity receptors are unknown. When ^{125}I-labeled IL-2 was chemically cross-linked to activated human T lymphocytes, two major bands were identified. First, as predicted, a 68- to 72-kilodalton band, consisting of IL-2 (15.5 kilodaltons) cross-linked to the IL-2 receptor (55 kilodaltons), was observed. Second, an unpredicted 85- to 92-kilodalton moiety was detected. This band was not present when IL-2 was cross-linked to transfected C127 cells, which exclusively express low-affinity receptors. The data presented are most consistent with the existence of a 70- to 77-kilodalton glycoprotein subunit (p70) which, upon associating with the 55-kilodalton low-affinity receptor (p55), transforms it into a high-affinity site. It is proposed that p55 and p70 be referred to as the α and β subunits, respectively, of the high-affinity IL-2 receptor.

THE LYMPHOKINE INTERLEUKIN-2 (IL-2) is synthesized and secreted by activated T cells and plays a critical role in the proliferative expansion of T-lymphocyte effector cells during the immune response (*1*). Concomitant with IL-2 production, two distinct classes of receptors with radically different affinities for IL-2 are expressed by activated lymphocytes (*2*). The ratio of low-affinity sites [with a reported dissociation constant (K_d) of 1 to 30 nM] to high-affinity sites (reported K_d of 5 to 90 pM) is approximately 10:1 in both normal

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