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Protocol

Firefly Luciferase as a Reporter of Regulated Gene Expression in Higher Plants

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Abstract: The firefly luciferase, assayed *in vivo* with a low-light video camera, acts as a non-invasive, real-time reporter of the temporal and spatial regulation of gene expression in single plants. Furthermore, the sensitivity of the luciferase assay in extracts of transformed plant tissue makes it a particularly useful marker in transient or stable transformation experiments.

Luciferases catalyze light-emitting reactions and have apparently evolved independently in several phyla (Hastings, 1983). Luciferases from the luminous beetles catalyze the ATP-dependent oxidative decarboxylation of beetle luciferin, a heterocyclic carboxylic acid. A photon is released in a large proportion of catalytic cycles: the quantum yield of the North American firefly (*Photinus pyralis*) luciferase approaches 90%, with maximal emission at 560 nm (Aflalo, 1991). Coenzyme A (CoA) may also participate in the reaction: the inclusion of CoA in the assay mixture transforms the "flash" of luminescence obtained by mixing luciferase with the other substrates into a plateau lasting 30

Abbreviations: GUS, β-glucuronidase; ddH_2O, double-distilled water; BSA, bovine serum albumin; LAR, luciferase assay reagent; CLR, cell lysis reagent.

seconds or more, followed by a gradual decrease in light emission (Wood, 1991; Ford et al., 1992). The native enzyme is targeted to the peroxisome in plants, animals and fungi (Gould et al., 1990), though it is also active in other cellular compartments (Schneider et al., 1990).

Luc , the gene encoding firefly luciferase, is proving to be a highly versatile reporter (Alam and Cook, 1990) for several reasons. Luciferase activity can report protein modifications and the concentrations of several biochemicals (cf. review of Aflalo, 1991). Only the reporting of gene expression levels will be considered here, although the enzyme assays will be fundamentally similar for many applications. Some advantages of *Luc* are shared by other commonly used marker genes, such as β-glucuronidase (GUS): the marker is active as a monomeric, primary translation product, which is readily extracted from plant material and easily assayed in crude extracts. The original, "flash" luciferase assay was non-isotopic and had negligible background in many organisms but required a luminometer equipped with an automatic injector to capture the peak of luminescence (DeLuca and McElroy, 1978; Luehrsen et al., 1992). The "stable" assay, incorporating CoA (Wood, 1991), retains the benefits of the original assay, though automatic injectors are not required and the luminometer may be replaced by a scintillation counter. Luciferase now surpasses the quantitative performance of other markers *in vitro*, as the "stable" assay is exceedingly sensitive, due to the extended period of light emission, and has a very wide linear range. As few as 2000 molecules of luciferase have been detected, with eight orders of magnitude in the linear range of the assay (Wood, 1991). The sensitivity of the luciferase assay has made it the marker of choice for studies of transient gene expression (Luehrsen et al., 1992), particularly following microprojectile bombardment (Goff et al., 1990), in which case only a small proportion of the sample expresses the test construct (Fig. 1). The effects of mRNA structure on gene expression, for example, have been characterized in plant cells using luciferase constructs containing a variety of cap structures, introns and untranslated regions (Callis et al., 1987; Gallie, 1991).

Luciferase activity is relatively unstable *in vivo* with a half-life of about 3 h in animal cells (Nguyen et al., 1989; Thompson et al., 1991), such that reductions in the abundance of luciferase mRNA are reflected in reduced luciferase activity over a time scale of several hours. Luciferase activity in transgenic tobacco plants is destabilized in the presence of the luciferin substrate by an unknown mechanism (cf. Fig. 2 below; Millar et al., 1992; Quandt et al., 1992), so the properties of the reporter may be modified to suit experimental requirements. Luciferase activity in the unstable form

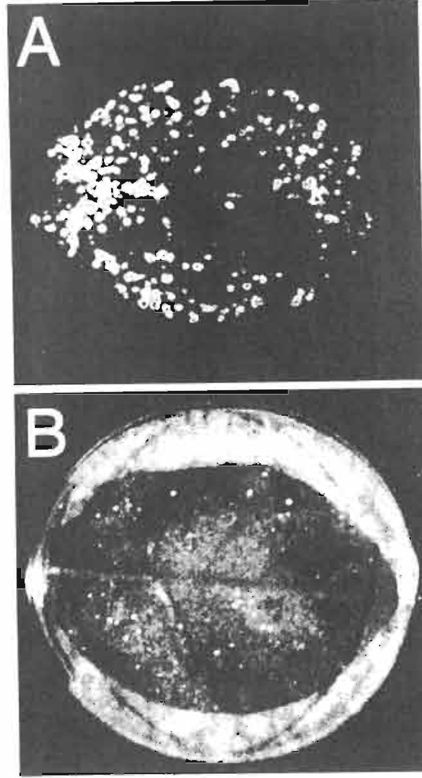

Fig. 1. Transient expression of luciferase following high velocity microprojectile bombardment. A young tobacco (var SR1) leaf was bombarded with BoxII::*Luc* DNA using a model PDS-1000/He particle-delivery system (Bio-Rad). Four days after bombardment, luminescence was collected in the low-light imaging system for 5 minutes, beginning 20 minutes after luciferin application. (A) luciferase bioluminescence from the leaf. (B) reference image of the tissue culture dish containing the leaf, in reflected light. The region of tissue damaged by the bombardment is visible as a lighter area in the center of the leaf (which has low luminescence in panel A). The bright spots on the leaf surface are water droplets.

may not respond as sensitively as more stable markers to increases in mRNA abundance, as instability is expected to delay the maximal accumulation of activity following an increase in mRNA abundance (Thompson et al., 1991; Pazzagli et al., 1992).

Luciferase is uniquely suited to the non-invasive reporting of regulated gene expression. Cells transformed with luciferase will luminesce when supplied with luciferin (if they are transparent to green light), because ATP, O_2 (and possibly CoA) are supplied by endogenous pools (Ow et al., 1986). The low intensity of light produced may be measured either using a photomultiplier tube, without spatial resolution (similar to the approach of [Knight et al., 1991]), or by low-light video imaging (Wick, 1989), in which case the spatial resolution of the assay is determined by the camera and by the optics employed. Given the instability of luciferase activity, the system functions as a real-time marker for gene expression in intact organisms. We have observed no damage or ill effects whatsoever in calli, regenerating shoots or whole seedlings, following the *in -vivo* imaging procedure (data not shown). Seedlings showing aberrant patterns of luciferase expression can be recovered,

such that genetic screens may isolate mutants in any regulatory pathway that controls the expression of the luciferase transgene, based on a phenotype at the molecular level.

We have used *Luc* as a bioluminescent reporter for transcription mediated by a fragment of the *Arabidopsis thaliana Cab2* promoter, in order to isolate mutants in genes controlling the activity of this promoter. *Cab2::Luc* mRNA accumulates in patterns that are very similar to those reported previously, reflecting phytochrome, circadian and spatial regulation (Mitra et al., 1989; Millar and Kay, 1991). We have used *in-vitro* luciferase assays and a low-light video imaging system to establish that the bioluminescence assay accurately reports the regulation of *Cab2* transcription in individual, intact seedlings (Millar et al., 1992).

Materials

Solutions required

MS medium (Murashige and Skoog, 1962), containing 3% sucrose and kanamycin, if necessary.

D-luciferin (potassium or sodium salt, Promega Corporation, Madison, WI), 150 mM stock solution in ddH_2O kept in 1-mL aliquots, in the dark at -70°C. The working solution (5 mM in 0.01% Triton X-100) is prepared at the beginning of the experiment and may be kept in darkness at room temperature for a few hours.

Promega luciferase assay kit, including luciferase assay reagent (LAR) and cell lysis reagent (CLR).

Bio-Rad (Richmond, CA) DC protein assay kit.

Purified or recombinant firefly luciferase, serial dilutions in buffer (*e.g.* , 1x CLR) containing 1mg/mL BSA.

DNA constructs

Cab2::Luc combines nucleotides -319 to -16 of the *Arabidopsis thaliana Cab2* promoter with an oligonucleotide that reconstructs the *Cab2* promoter to position +1, followed by the tobacco mosaic virus Ω sequence (Gallie et al., 1987). This promoter-leader fragment is fused to the firefly luciferase coding region from plasmid pJD261 (Luehrsen et al., 1992) and the poly(A) addition sequence from the pea *RbcS-E9* gene in the polylinker of the binary shuttle vector pMON721 (Monsanto Corporation, St. Louis, MO). The BoxII::*Luc* construct combines a tetramer of the light-responsive BoxII element from pea *RbcS-3A* with the -90 to +8 promoter fragment from the CaMV 35S promoter (Lam and Chua, 1990), the Ω-*Luc* fusion from pJD261 and the pea *RbcS-3A* poly(A)-addition region, in the

pGEM3 vector (Promega Corp.). The 35S::*Luc* construct is a fusion of the CaMV 35S promoter (positions -343 to +8) to the Ω-*Luc* fusion from pJD261 and the pea *RbcS-3A* poly(A) addition region, in the *Not* I site of pMON721. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis* root explants was performed according to established protocols (Valvekens et al., 1988).

Standard protocols

The "flash" reaction for *in vitro* assays and a procedure for luciferase imaging in intact plants using a contact exposure to X-ray film are described in Howell (1989). Briefly, for the *in-vitro* assay, frozen tissue powder is extracted in potassium phosphate buffer without detergents, an aliquot of the cleared extract is diluted in assay buffer containing ATP and the peak of luminescence following luciferin injection is measured in a luminometer. Pazzagli et al., (1992) and Luehrsen et al., (1992) have recently reported detailed comparisons of the "flash" and "stable" luciferase assays.

Protocol for the Promega ("stable") luciferase assay in tissue extracts[1]

- Harvest plant material in aluminum foil and immediately freeze in liquid nitrogen.[2]
- Chill a mortar and pestle in liquid nitrogen. Grind frozen tissue to a fine powder.[3,4]
- Use a chilled spatula to dispense approximately 100 µL powder into a microcentrifuge tube chilled in dry ice.
- Add two volumes ice-cold 1x cell lysis reagent (CLR).[5] Mix with a small spatula and allow to thaw at room temperature. As soon as the powder thaws, vortex to mix.
- Clear the extracts in a microcentrifuge at full speed, at 4°C, for 5 minutes. Transfer supernatant to a fresh microcentrifuge tube and repeat centrifugation.
- Transfer supernatant to a fresh microcentrifuge tube on ice.
- Bring sufficient LAR for all assays to room temperature. Dispense 50-µL aliquots into luminometer[6] cuvettes.
- Add 10 µL extract to the cuvette. Mix by swirling. Read luminescence at the plateau: we use the highest of ten successive 2-second counts, beginning about 10 seconds after swirling the reaction mixture.
- Perform protein assay[7] on 5 µL extract according to manufacturer's recommendations.
- Luminescence intensity may be converted to specific luciferase activity (moles or grams of luciferase per gram of total protein) using a standard curve[8] prepared with purified or recombinant firefly luciferase.

Notes

1. **Luciferin application and luciferase stability *in vivo*.** The presence of luciferin destabilizes luciferase activity in transgenic tobacco and *Arabidopsis* tissue. Stronger luminescence is obtained from untreated material (Fig. 2); plants must, however, be exposed to luciferin if luminescence is to reflect a reduction in luciferase synthesis. We typically spray seedlings three times at 6-h intervals before the start of a harvesting time course and again before each time point (see below for *in-vivo* imaging). A spraying device capable of producing a fine mist with a small volume of liquid (*e.g.*, pump-action cosmetic spray bottle) is used to apply approximately 150 µL of the working luciferin solution to a 10-cm square tissue culture dish of seedlings.
2. **Freezing agent.** Dry ice may be substituted for liquid nitrogen, so long as steps are taken to ensure the most rapid freezing possible.
3. **Tissue homogenization.** Transgenic leaf tissue bearing *Cab2::Luc* or 35S::*Luc* requires 20 to 30 seconds grinding; cell types with thicker cell walls or samples that include only small proportions of luciferase-positive cells may require considerably more grinding. N.B.: We have found that grinding efficiency can vary considerably between researchers, leading to variations in the specific enzyme activity of the extract, especially in transient expression experiments following microprojectile bombardment.
4. **Stability of luciferase activity during the extraction.** Luciferase activity is stable in tissue frozen at -70°C but has a half-life of approximately 2 h in extracts kept on ice. Samples should be extracted and assayed in batches small enough to limit this loss of activity. We typically assay fresh extracts, although activity appears to be stable in extracts frozen at -70°C.
5. **Extraction buffer.** As judged on western blots 1x CLR, containing 1% Triton X-100, extracts similar amounts of luciferase protein to boiling 2% SDS buffers (data not shown). CLR is slightly inhibitory to luciferase activity in our hands (Promega technical bulletin 101; data not shown): all assays and standards should contain the same volume of CLR. Other laboratories, however, report no such inhibition (Pazzagli et al., 1992; Luehrsen et al., 1992). Buffers without detergent (*e.g.*, 25 mM sodium citrate, pH 7.8) are less inhibitory to enzyme activity and can extract luciferase activity as efficiently as CLR; CLR extracts, however, give more reproducible results in our hands (data not shown). We have not analyzed the detergent-free extracts by western blotting. If the extracted activity exceeds the linear range of the assay, the extracts may be diluted in 1x CLR containing 1mg/mL BSA.
6. **Luminometer.** We routinely detect 50 fg purified luciferase using a model ILA911 luminometer from Tropix (Bedford, MA) and 3 fg in a Turner Designs (MT View, CA) model 20e (Graham Teakle, personal communication). Several other models are available (Stanley, 1992). A scintillation counter may be substituted for the luminometer, albeit with a possible loss of sensitivity (see Promega technical bulletin 101).
7. **Protein assay.** The DC protein assay, a modification of the Lowry assay (Lowry et al., 1951), is particularly convenient, as it is compatible with CLR extracts.
8. **Standard curve.** Serial dilutions of purified (Boehringer Mannheim Biochemicals, Indianapolis, IN) or recombinant (United States Biochemical, Cleveland, OH) luciferase should be prepared in 1x CLR containing 1 mg/mL BSA, to avoid a loss of activity associated with dilute solutions, and assayed as described. The addition of CLR extracts of untransformed tobacco and *Arabidopsis thaliana* plants has no effect on the activity of the purified enzyme (data not shown). The specific activity of luciferase synthesized in plants, however, has not been measured directly, so calibration with purified luciferase standards assumes that these are representative of the extracted enzyme.

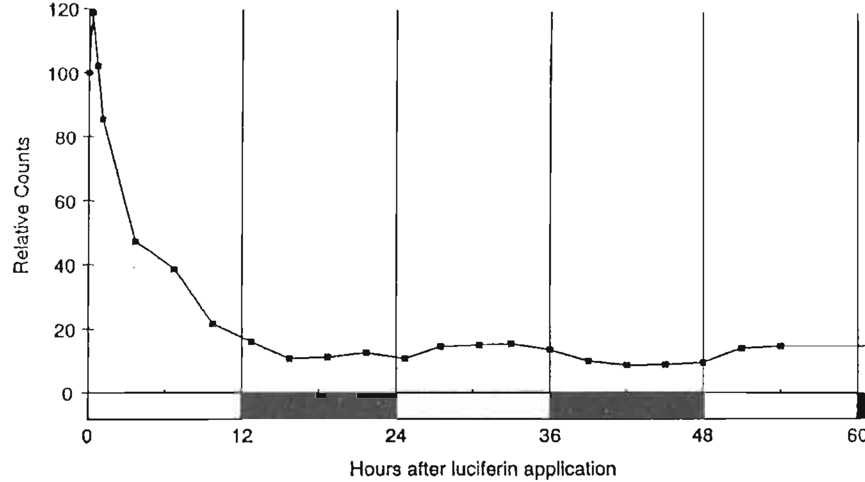

Fig. 2. Luciferin application dramatically reduces the intensity of luciferase bioluminescence *in vivo*. 35S::*Luc Arabidopsis* seedlings were grown on MS medium containing kanamycin, in a light-dark cycle of 12 h light:12 h dark (12L12D) for 5 days. Luciferin was applied at time 0 and luminescence was collected in the low-light imaging system for 20 minutes, first in 4 successive exposures, then every 3 hours, 20 minutes after luciferin application. No further luciferin was applied after 27 h. The data are presented as the total counts for a group of approximately 30 seedlings, relative to counts in the first image (= 100%). The two-fold diurnal cycle in luminescence (16-60h) is characteristic of 35S promoter fusions: GUS mRNA abundance shows a similar diurnal cycle in 35S::GUS tobacco seedlings (Millar et al., 1992). □ = light period; ■ = dark period.

Rapid protocol for luciferase extraction from bombarded tissue

- Grind fresh tissue (*ca.* 0.5 g) with 2 mL ice-cold 1x CLR, using a chilled mortar and pestle, on ice.
- Transfer extract to a microcentrifuge tube. Clear the extract once in a microcentrifuge at full speed, at 4°C, for 5 minutes.
- Transfer supernatant to a fresh tube, on ice, and perform luciferase assay immediately, as described above, using 5 µL of the extract.

Notes

Homogenizing bombarded tissue in liquid nitrogen may require as much as 5 minutes grinding per sample to extract maximal specific luciferase activity (data not shown). Grinding fresh tissue on ice allows a more rapid extraction than the standard protocol but may only be used with very small batches of samples, as luciferase activity is much less stable in ice-cold extraction buffer than in frozen tissue (see Note 3, above).

Protocol for imaging luciferase bioluminescence in intact tissue

- Estimate the sensitivity and linearity of the camera to luciferase bioluminescence using *in-vitro* assays of a dilution series of the purified luciferase imaged in a 96-well microtiter dish. The calibration should be repeated with the intensifier set at several different gain levels.
- Focus camera on position of tissue within a light-tight imaging chamber under reflected light.
- Spray a test batch of tissue with working luciferin solution and collect a series of exposures over a total of 60 to 90 minutes to determine the kinetics of light emission after spraying.
- Spray experimental material and transfer to the imaging chamber at the beginning of the plateau of luminescence, predicted from the test kinetics.
- Collect a luminescent image, if necessary followed by a reference image under reflected light.
- Quantify luminescence over the seedlings or tissues of interest, correcting for camera or incident light background if necessary.

General notes

1. **Luciferin application.** The same considerations of luciferase stability apply to *in-vivo* assays as described above for the *in-vitro* procedure (Note 1 and Fig. 2). The plateau of luminescence is typically reached 30 minutes after spraying in tobacco seedlings, or 20 minutes in *Arabidopsis thaliana* (Fig. 2). The luciferin working solution may be filter-sterilized if necessary, for example in assays of transformed calli or regenerating shoots.
2. **Endogenous luminescence.** A low level of luminescence is detectable in the leaves of untransformed, light-grown plants, probably due to chlorophyll chemiluminescence (Abeles, 1986). The intensity of this background emission is much lower than the luciferase luminescence from a *Cab2::Luc* plant and decreases to below the camera background level after about 5 minutes in darkness.
3. **Camera systems.** We use the VIM intensified CCD camera and Argus-50 photon-counting image processor from Hamamatsu Photonic Systems, Bridgewater, NJ. A variety of other CCD cameras are available (Stanley, 1992), though the VIM / Argus system has the advantage of being a turn-key package including all necessary components to collect and analyze low-light images. The imaging chamber, for example, is a critical component of the system, unless a completely light-tight dark room is available.

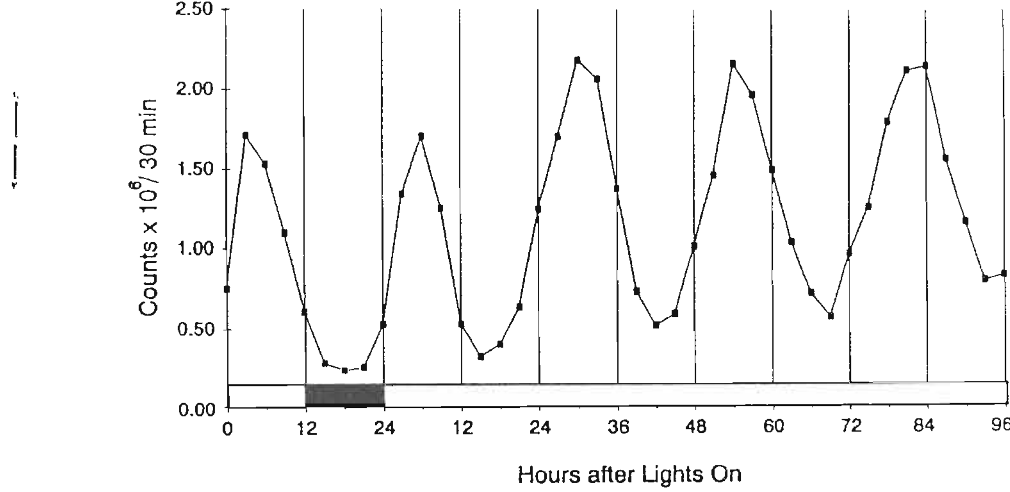

Fig. 3. Circadian-regulated luciferase activity in intact *Arabidopsis* under constant light. Approximately 500 *Cab2::Luc* seedlings were grown in a 15-cm tissue culture dish in light-dark cycles of 12h light:12h dark (12L12D) for 5 days and pre-treated with luciferin as described in the text. Luminescence was collected in the low-light imaging system for 30 minutes, every three hours, 20 minutes after luciferin application. Seedlings were transferred to constant light 24 h after the experiment began. ☐ = light period; ■ = dark period.

Results and Discussion

Several authors have cited the potential of luciferase genes as non-invasive reporters of regulated gene expression (Subramani and DeLuca, 1988; Howell et al., 1989; Koncz et al., 1990). The first report using luciferase as a genetic reporter appeared in 1986 (Ow et al., 1986), but neither animal nor plant systems have yet exploited the unique advantages of the luciferase system to the full. Studies of regulated bioluminescence in transgenic plant tissue have either used video imaging of excised organs or contact luminography of whole plants (pressed flat against film) to demonstrate the spatial regulation of transcription (Quandt et al., 1992; Ow et al., 1986; Schneider et al., 1990) in various conditions (Langridge et al., 1989). These assays are unsuitable for following luminescence over extensive time courses in individual plants. In contrast, we have used the imaging protocol described above to assay circadian-regulated expression of *Cab2::Luc* in transgenic *Arabidopsis*

thaliana at three-hour intervals for up to five days, without any apparent ill effects on the plants (Fig. 3). Moreover, video imaging allows bioluminescence measurements of single seedlings in populations of up to six hundred (data not shown), so rare events, such as the occurrence of mutant individuals, are detectable.

The major disadvantages of this protocol are the investment required in imaging equipment and also the high price of luciferin; these difficulties may ease as luciferase and imaging procedures are more widely used. Earlier publications (Subramani and DeLuca, 1988; Koncz et al., 1990) identified the access of luciferin to living tissues as a potential problem for the luciferase system, as luciferin is charged at neutral pH and also, because luciferase is targeted to the peroxisome (Gould et al., 1990). We found that the availability of substrates, exogenous luciferin and endogenous ATP and O_2, did not obscure the cyclic patterns of *Cab2::Luc*-mediated luminescence in 7- to 10-day-old tobacco seedlings (Millar et al., 1992). Schneider et al. (1990), using *35S::Luc* tobacco plants, suggested that differential substrate availability might be responsible for the lower luminescence *in vivo* that they observed in leaves compared to roots, relative to luciferase activity measured in extracts. This apparent discrepancy may be due to differences in the age of the tissue, the methods of luciferin application or the diversity of cell types expressing the promoters in question. Barnes (1990) found little differential luciferin accumulation between leaf blade and midrib tissue when luciferin was supplied to excised leaves in a solution bathing the cut petiole. These measurements, however, do not necessarily reflect the availability of luciferin to the enzyme *in vivo*. We did not find it necessary to vacuum-infiltrate luciferin solutions (Howell et al., 1989).

The stable luciferase assay improves on the "flash" assay, for studies in tissue extracts, in both convenience and sensitivity (Pazzagli et al., 1992; Luehrsen et al., 1992). In this respect, the firefly luciferase now provides a more sensitive and more linear assay than GUS, with the added advantage of conditional instability in intact plant tissue. Although non-invasive procedures for GUS assays *in vivo* have been reported (Martin et al., 1992), video imaging of luciferase bioluminescence offers far greater accuracy and sensitivity, with negligible tissue damage. The fluorescent assay for GUS activity in green tissue, in particular, seems to have a narrow window, in which the detection of GUS activity is compatible with seedling viability. The histochemical localization of GUS in plant tissue sections, however, is a common application (*e.g.*, Benfey et al., 1990), for which luciferase has not yet been

exploited. Patterns of luminescence very similar to those reported using other markers (Ludwig et al., 1990) are easily observed following microprojectile bombardment with luciferase gene fusions (Fig. 3), suggesting that small groups of cells can express sufficient luciferase to permit detection at higher magnification. Cellular resolution of luciferase activity has been reported in protoplasts following transient *Luc* expression (Gallie et al., 1989) but no results from tissues or sections have been described, in plants or animals. Light leakage and light piping between cells may be of concern in such studies, although the blue product of the histochemical GUS assay (using the X-gluc substrate) is also known to diffuse between cells (Ludwig et al., 1990).

Bacterial luciferase from *Vibrio harveyi* has been used as a reporter gene in transgenic plants, for several years (Langridge et al., 1989). This reporter system has the advantage that the aldehyde substrate (decanal) diffuses readily into plant tissue when supplied by evaporation from a wick (Koncz et al., 1990). We made transgenic tobacco lines carrying a bacterial *luxAB* fusion (Escher et al., 1989) in *Cab2::lux* and CaMV 35S::*lux* constructs identical to the firefly *Luc* constructs described in Millar et al., (1992) and tested these in the low-light camera system (A. J. Millar, S. R. Short, N.-H. Chua and S. A. Kay, unpublished results). While decanal substrate was indeed easily administered, high levels caused severe tissue damage to the seedlings; after titration of substrate concentration, we found that the decanal level required for seedling survival gave less than maximal luminescence. Even at high concentrations of decanal, however, luminescence was considerably less than from plants carrying equivalent firefly luciferase constructs. This observation is likely due, at least in part, to the lower quantum yield of bacterial luciferase (0.1 versus 0.9 for the firefly system; cf. Ziegler and Baldwin, 1981). In older plants, the bacterial system may have advantages over the firefly marker, as such tissue may be both more resistant to decanal toxicity and less uniformly accessible to beetle luciferin. The choice of reporter will then depend on the developmental stage and tissue of interest, and on the expression level of the promoter in question.

The future of the firefly luciferase system holds great promise. The uniformity of luciferin access to all cell types and the mechanisms of substrate-dependent instability of luciferase activity require further study in order to reach cellular resolution and to understand the *in-vivo* assay more thoroughly. The development of luciferase enzymes targeted to the cytoplasm alone and lacking the pH-sensitivity of the native enzyme will reduce the potential problems due to multiple enzyme pools

with differing characteristics *in vivo*. Finally, simultaneous labeling with multiple luciferases may soon become a reality, as enzymes with different emission maxima are cloned and characterized (Wood et al., 1989).

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