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Bacterial and Firefly Luciferase Genes in Transgenic Plants: Advantages and Disadvantages of a Reporter Gene

CSABA KONCZ, WILLIAM H.R. LANGRIDGE, OLOF OLSSON, JEFF SCHELL, AND ALADAR A. SZALAY

Institute of Plant Physiology (C.K.), Biological Research Center, Hungarian Acadamy of Sciences, H-6701, Hungary; Max-Planck-Institut für Züchtungsforschung (C.K., J.S.), Köln 30, Federal Republic of Germany; Plant Molecular Genetics and Plant Biotechnology Centre and Department of Cell Biology (W.H.R.L., A.A.S.), Medical Sciences Building, University of Alberta, Edmonton, Alberta, Canada; and Department of Plant Physiology (O.O.), University of Umea, Umea, Sweden

ABSTRACT Genes encoding light-emitting luciferase were recently isolated from luminous marine bacteria and fireflies. Expression of luciferase genes in diverse organisms is a unique way for studying gene expression by simple and sensitive measurement of light. Recent advances in application of luciferase reporter genes are reviewed and documented by examples of in vivo visualization of their expression in transgenic plants.

Key words: *lux* and *luc* reporter genes, light emission, gene expression, single photon imaging in vivo

INTRODUCTION

Light-emitting organisms attracted the attention of scientists throughout history from Caius Plinius (23–79 A.D.) to E.N. Harvey [Harvey, 1957]. The terms of luciferase and luciferin were first applied by Dubois in 1885 to describe light-emitting proteins and their substrates extracted from jelly fishes. Luciferases and luciferins were purified and characterized from diverse species of fireflies, beetles, marine bacteria, molluscs, medusas, fishes, and earthworms. A schematic classification of luciferins shows that most eukaryotic luciferases use heterocyclic compounds as substrates while in marine bacteria and earthworms, photogenic substrates are aliphatic aldehydes [DeLuca, 1978; DeLuca and McElroy, 1986].

FIREFLY LUCIFERASE

Luciferase isolated from the North American firefly, *Photinus pyralis* (*Photinus*, luciferin:oxygen 4-oxydoreductase, EC. 1.13.12.7; 62 kD) catalyzes the oxydative decarboxylation of luciferin, a 6-hydroxybenzothiazole, to oxyluciferin in the presence of ATP, Mg^{2+} and O_2. The enzyme is specific for ATP and therefore has been used widely as a bioluminescent indicator for metabolic assays (see below). The catalytic reaction is initiated by the formation of an enzyme-bound luciferin-adenylate. This is followed by a change in protein conformation which provides a hydrophobic active site for deprotonation and hydroperoxide addition at the C4 position of luciferin. Subsequent decarboxylation and splitting of the linear peroxide leads to formation of CO_2, H_2O, AMP, and an excited, dianionic form of oxyluciferin. In excess of substrate the reaction produces a quick flash of light proportional to the quantity of the enzyme. After the flash, an extended low-light emission occurs indicating a slow-rate dissociation of the product. The quantum yield of firefly luciferase is 0.88, the highest among known luciferases.

All fireflies use the same substrates but diverse species emit different colours of light varying from yellow (582 nm) to green (522 nm). Low pH and divalent cations shift the light emission to red, indicating that the conformational change of the enzyme and alterations in its structure play an important role in determining the energy of the excited product and thus the colour of emitted light [DeLuca, 1976, 1978]. Recently, luciferase cDNAs were cloned from the Japanese firely [Masuda *et al.*, 1989] and from beetles [Wood *et al.*, 1989] which produce different colours of light when expressed in *E. coli*. The identification of amino acid exchanges between these enzymes should pinpoint peptide domains involved in enzyme-substrate interaction, as well as open the way to engineer novel luciferases.

From an evolutionary point of view it is intriguing that the reaction leading to luciferin adenylation is analogous to those involved in the activation of amino

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Address reprint requests to Csaba Koncz, Max-Planck-Institut für Züchtungsforschung, D-5000 Köln 30, Federal Republic of Germany.

acids and fatty acids. Furthermore, luciferase can also catalyze the addition of adenylated dihydroluciferin to CoA, in a reaction similar to that catalyzed by fatty acyl CoA synthases. Since this reaction is CoA-specific, it is probably not a coincidence that the enzyme shows a significant homology to other CoA-specific enzymes, such as plant 4-coumarate-CoA ligase [Schroeder, 1989].

In *Photinus* the luciferase is encoded by a transcript of about 1,800 nt, which is synthesized from a single copy *luc* gene containing six introns [deWet *et al.*, 1985, 1987]. Localization of luciferase protein in photocytes of firefly lantern, as well as in animal and plant cells, indicated that the enzyme is targeted to peroxisomes by a peptide signal (PTS) consisting of the last three C-terminal amino acids Lys-Ser-Leu [Keller *et al.*, 1987; Gould *et al.*, 1987, 1989; Gould and Subramani, 1988].

ANALOGIES BETWEEN EUKARYOTIC LUMINESCENCE SYSTEMS

Formation of a linear peroxide at various positions of eukaryotic luciferins appears to be a common event in most luciferase reactions producing CO_2 and H_2O. Although the excited oxyluciferin is always the primary emitter it is common that the energy is transmitted to a secondary emitter, i.e., protein-bound flavin chromophore. Luminescence systems are frequently regulated by the nerve net of organisms. When disturbed, cypridinas produce a blue luminescence by ejecting luciferase and luciferin from separate glands into seawater. A more complicated network, evolved in the anthozoan coelenterate *Renilla,* involves four proteins. A luciferin sulfokinase catalyzes the synthesis of luciferin from luciferyl sulfate and 3′,5′-diphosphoadenosine. Luciferin is oxydized to oxyluciferin and CO_2 by luciferase. The energy is transmitted to a luciferase-associated green fluorescent protein (GFP). The chromophore is enclosed in vesicles and emits green light only when a luciferin-binding protein (BP-LH_2) is activated by binding of Ca^{2+} upon excitation of nerves [Cormier, 1978]. In the hydromedusa *Aequorea* both luciferin and Ca^{2+} are bound by a single protein, aequorin, that catalyzes the formation of oxyluciferin. cDNA of blue light emitting aequorin has been isolated from *Aequorea victoria* and expressed in *E. coli* [Inouye *et al.*, 1986; Prasher *et al.*, 1986].

BACTERIAL LUCIFERASES

Luminous marine bacteria are ubiquitous and occur either in planktonic forms or as symbionts within light organs of fishes and squids. Luciferases from *Vibrio harveyi, V. fisheri,* and *Photobacterium phosphoreum* were extensively characterized [Ziegler and Baldwin, 1981; Hastings and Nealson, 1977].

All bacterial luciferases are heterodimeric ($\alpha\beta$), mixed function oxidases which catalyze the oxidation of reduced FMN and long-chain aldehydes with molecular oxygen to yield FMN, H_2O, corresponding carboxylic acids, and blue-green light (490 nm). The reaction can be considered as a branch of an electron-transport pathway which shunts electrons to oxygen at the level of flavin. The enzymatic reaction is unusual because it results in long-life intermediates. A key intermediate is an enzyme-bound 4a-hydroperoxide (FMN-OOH) whose reaction with the aldehyde probably leads to formation of a hydroxy-flavin emitter in its singulet excited state. Due to quick oxidation of free $FMNH_2$ and to long-time relaxation of the enzyme from its altered conformational state, only one catalytic cycle is possible. Therefore the light production is strictly proportional to the amount of enzyme in excess of $FMNH_2$ and aldehyde substrate. The quantum yield of bacterial luciferase is 0.1, equivalent with 60 ATP per photon.

The aldehyde-binding site of the luciferase α-subunit contains an essential sulfhydryl group close to the $\alpha\beta$-subunit interphase. The non-catalytic β-subunit is required for proper folding and conformational change of the α-subunit during interaction with the flavin molecule. Structural mutations in both enzyme subunits, as well as various flavin analogs, can alter the emission spectra between 490 and 535 nm [Hastings, 1978; Kurfürst *et al.*, 1984; Lee *et al.*, 1988; Aboukhair *et al.*, 1985; Paquette *et al.*, 1988; Chen and Baldwin, 1989].

In *Photobacterium* strains a lumazine protein forms a complex with luciferase and, by energy transfer from the flavin to the secondary emitter 6,7-dimethyl-8-(1′-D-ribityl)-lumazine, blue light (475 nm) is emitted [Lee *et al.*, 1989]. In *Vibrio fischeri* strain Y-1 energy transfer occurs from excited flavin intermediates to a flavin-containing secondary emitter protein, termed yellow fluorescent protein (YFP), causes a yellow shift (534 nm) in the emission of light. In the presence of NAD(P)H-FMN oxydoreductase, an enzyme supplying reduced FMN in luminous bacteria, the addition of YFP does not only shift the colour but also increases the intensity of total light emitted three- to four-fold [Daubner *et al.*, 1987]. A further accessory enzyme of bacterial bioluminescence systems is a fatty acid reductase which is co-induced with the luciferase and recycles fatty acid products to substrate aldehydes (usually converts tetradecanonic acid to tetradecanal).

Bacterial luciferase genes were identified by transposon insertional mutagenesis and by hybridization to mixed oligonucleotide probes of known peptide sequences [Belas *et al.*, 1982; Cohn *et al.*, 1983; Baldwin *et al.*, 1984; Engebrecht and Silverman, 1984; Engebrecht *et al.*, 1983; Delong *et al.*, 1987]. *Lux*CDABE genes are located in a single operon (R) in *Vibrio harveyi, V. fischeri,* and *Photobacterium phosphoreum. Lux*C, D, and E encode fatty acid reductase (54 kD), acyl-transferase (34 kD), and acyl-protein synthase (42 kD) subunits of fatty acid reductase, respectively, while the α- and β-subunits of luciferase (40 and 37 kD) are synthesized from genes *lux*A and B [Cohn *et al.*,

1985; Johnston *et al.*, 1986; Haygood and Cohn, 1986; Miyamoto *et al.*, 1985, 1987, 1988; Foran and Brown, 1988; Illarionov *et al.*, 1988; Mancini *et al.*, 1988]. In *Photobacterium phosphoreum* an additional gene, *lux*F, was identified between *lux*B and E and shown to result from a duplication of the *lux*B gene [Soly *et al.*, 1988]. Homology between *lux*A and *lux*B suggests that they also evolved by an earlier gene duplication event [Baldwin *et al.*, 1979]. Although overall homology exists between *lux* genes of diverse species, protein complementation studies between luciferase subunits of *Vibrio* and *Photobacterium* species demonstrated significant differences [Meighen and Bartlet, 1980]. In *Vibrio fischeri* gene *lux*I, located immediately upstream of *lux*C, belongs to the *lux*CDABE operon. A gene located further upstream, *lux*R, constitutes an independent operon (L) which is transcribed in the opposite direction. Expression of *lux* operons is regulated at the levels of both transcription and translation. *lux*R encodes a positive regulatory protein while *lux*I is responsible for the synthesis of an autoinducer, N-(β-ketocaproyl)-homoserine lactone. *lux* gene expression correlates with the density of cell cultures. Initially the *lux*R protein is constitutively produced and the *lux*CDABE operon is expressed only at a low level. At higher cell densities an increased concentration of *lux*R protein, bound to autoinducer, leads to further activation of the *lux*ICDABE operon and to a burst of autoinducer synthesis and light production. Later on the concentration of *lux*R protein becomes limiting because the *lux*R protein-autoinducer complex inhibits the translation of *lux*R transcript. *lux* operons are also regulated by catabolic repression because their promoters contain cAMP/CRP binding sites [Dunlap and Greenberg, 1985; Dunlap, 1989; Engebrecht and Silverman, 1987; Devine *et al.*, 1988]. At low O_2 concentrations the synthesis of luciferase is limited in *Vibrio harveyi* and *Photobacterium leiognathi*. In contrast, the synthesis of luciferase is not influenced by oxygen in *Vibrio fischeri* and *Photobacterium phosphoreum* which results in accumulation of luciferase at low O_2 tensions. Low osmolarity stimulates, whereas high iron concentrations repress bacterial luminescence [Dunlap, 1985; Haygood and Nealson, 1985].

LUCIFERASE ASSAYS

Light can be monitored visually, photographically, or electronically at different sensitivities. A great variety of methods for detection and measurement of bioluminescence have been described [Van Dyke, 1985]. Following pioneering work by several laboratories on the purification and immobilization of luminescent enzymes, luciferases found a wide range of applications in most areas of life sciences. A particular advantage of luciferase assays is their ease, sensitivity, and efficiency. Practically any reaction which can be linked to measurement of ATP, NAD(P), FMN, fatty acids, or aldehydes can be monitored by firefly and bacterial luciferases. Special features of other luminescent proteins, such as aequorin also allow one to measure Ca^{2+}-mediated reactions. The range of in vitro assays extends from clinical, microbial detection of pathogens to biochemical assays of enzymes, cofactors, and substrates, to mutagenicity tests, to detection of steroid hormones and insect pheromones, and to the measurement of membrane transport and organellar functions [Weinhausen and De Luca, 1982; Campbell *et al.*, 1985; Ulitzur, 1986; Kricka, 1988]. Recent advances in selective modification of a reactive sulfhydryl group of bacterial luciferase and in the synthesis of firefly luciferins derivatized at the 6-position led to the general application of luciferases in immunoassays, protein immuno-blotting, and non-radioactive nucleic acid hybridization [Baldwin *et al.*, 1986; Haubner and Geiger, 1988].

LUCIFERASE REPORTER GENES

It has been realized early that a number of sensitive assays, such as determination of the concentration of O_2, anaesthetics, antibiotics, mutagens, etc., can be carried out in vivo by expression of luciferases in living cells [Hastings and Nealson, 1977].

Cloning of luciferase genes opened the way to novel applications in molecular biology. Gene fusion is a general approach to study the temporal and spatial regulation of gene expression and to delineate regulatory DNA sequences both in procaryotic and eucaryotic organisms. Reporter genes, such as β-galactosidase (*lac*), β-glucuronidase (*gus*), chloramphenicol acetyltransferase (*cat*), and aminoglycoside phosphotransferase (*aph*(3′)II), are fused to transcriptional regulatory elements by construction of chimaeric genes which are then transformed into cells of target organisms. The expression of reporter gene fusions is followed in transient assays or in stable transformants either by in vitro enzyme assays or by histological staining. Alternatively, promoterless reporter genes are linked to the ends of transposable elements such that their insertion into genes will generate transcriptional or translational gene fusions.

Engebrecht *et al.* [1985] used the latter technique for isolation of gene fusions to the *lux*CDABE operon by mini-Mu transposon insertional mutagenesis in bacteria and demonstrated that light production provides a simple and sensitive in vivo indicator of gene expression. A systematic development of luciferase gene constructs and light assays followed this initial report. A similar transposon, Tn*4431*, and diverse plasmid constructs were designed to visualize gene expression in *Xanthomonas* during its pathogenic invasion of plant tissues [Shaw et al., 1986, 1987, 1988]. Expression of *lux*AB genes in *E. coli* was successfully visualized by exogenous addition of n-decanal demonstrating that volatile aldehyde substrates of bacterial luciferase are promptly taken up by living cells and that *lux*AB struc-

tural genes are as effective reporters as the full-length *lux* operon [Baldwin *et al.*, 1986]. Subsequently expression of *lux*AB genes was demonstrated in filamentous cyanobacteria [Schmetterer *et al.*, 1986], in *Bacillus subtilis* [Karp, 1989], and in *Pseudomonas, Agrobacterium,* and *Rhizobium* [Legocki *et al.*, 1986; Boivin *et al.*, 1988]. Various vectors were constructed for the study of promoters and transcription terminators in bacteria [Carmi *et al.*, 1987; Peabody *et al.*, 1989]. In order to monitor gene activation in symbiotic bacteroids of rhizobia, *lux*AB genes were fused to a regulated promoter of *nif*D [O'Kane *et al.*, 1988]. This offered a good model system to adjust several methods for visualization of light-emitting tissues and cells inside the plant body (Fig. 1).

Expression and stability of firefly luciferase in *E. coli* has been explored by the synthesis of N-terminal Cro-luciferase fusion proteins by using partial *luc* cDNA clones. However, attempts to monitor light-emission mediated by the firefly enzymes in vivo failed because bacteria did not take up the luciferin substrate [deWet *et al.*, 1985].

EXPRESSION OF LUCIFERASE GENES IN TRANSGENIC PLANTS

Application of luciferase genes in eucaryotes is still in its infancy, but will certainly lead to a burst of novel experimental systems soon. Among eukaryotic organisms the expression of luciferase genes was first demonstrated in plants. Linked to the promoter of the Cauliflower Mosaic Virus 35S transcript, the firefly luciferase gene was introduced into carrot protoplasts by electroporation and into tobacco by *Agrobacterium*-mediated transformation. In addition to in vitro measurements of luciferase activity in transient assays, the luciferase-mediated light emission was visualized by autoradiography of cell suspensions and by contact exposure of a transgenic plant to a photographic emulsion [Ow *et al.*, 1986].

To study the expression of bacterial luciferase genes in plants, *lux*A and *lux*B coding sequences were dissected from the *lux* operon and fused separately to 1′ and 2′ promoters of mannopine synthase genes. *lux* gene expression vectors were transformed into carrot and tobacco cells. Light emission and detection of luciferase subunits showed that assembly of functional luciferase occurred in the cytoplasm of transformed plant cells [Koncz *et al.*, 1987].

PROBLEMS

These initial reports incited much debate about favourable and disadvantageous traits of both luciferases. It was initially thought that the bacterial system would not be useful for application in the eukaryotic cells because expression of two genes is required for the synthesis of an active enzyme. Although the stability of individually synthesized Luxα and Luxβ subunits has been confirmed in plants, there was some question regarding protease sensitivity of the catalytic α-subunit. Furthermore, inefficient in vitro assembly of separately folded luciferase subunits led to the assumption that coordinate folding of subunits during translation in bacteria would be essential. Since transcription and translation are coupled in bacteria, this model predicted poor assembly of bacterial luciferase in eukaryotic systems. Other concerns predicted that the concentration of FMN would be limiting because FMN is enzyme-bound or enclosed in cell compartments in eucaryotes. In contrast, the firefly luciferase substrate ATP is ubiquitous in eukaryotic cells. Comparisons of kinetic parameters and quantum yields of luciferases also favoured the firefly enzyme which needs only one ATP per emitted photon in contrast to the requirement of 60 ATP for the bacterial enzyme. On the other hand, it is known that luciferin is taken up inefficiently by living cells. In order to enhance the uptake, cells have to be treated by DMSO, low pH, and high concentrations of luciferin, all of which reduce their viability. Furthermore, the transport of the firefly enzyme to peroxisomes further reduces the availability of the substrate.

ADVANCES

Recent developments in the use of both reporter genes provided some answers to the initial questions. The firefly luciferase gene has been used successfully for the analysis of promoters [Ow *et al.*, 1987], transcription terminator signals, and translational enhancer elements in plants [Gallie *et al.*, 1989]. The *luc* gene was also exploited to optimize transient assays and to confirm stable transformation in plants [Ballas et al., 1988; Gupta *et al.*, 1988; Ellis *et al.*, 1989; Komari, 1989], mammalian cells [deWet *et al.*, 1987; Nguyen *et al.*, 1988; Maxwell and Maxwell, 1988; Williams *et al.*, 1989], *Dictyostelium* [Howard *et al.*, 1988] and transgenic mice [DiLella *et al.*, 1988]. Insertion of the *luc* gene into a vaccinia virus genome illustrated possible applications of the *luc* reporter gene for the monitoring of viral gene expression and virus dissemination in cell cultures and in tissues of infected animals [Rodriguez *et al.*, 1988]. The sensitivity of the in vitro luciferase assay was estimated to be 100- to 1,000-fold higher than that of β-galactosidase or CAT. When luciferin was supplied in low pH buffer with DMSO, this sensitivity permitted photographic detection of light in bacterial colonies as well as in animal and in plant tissues. Computer-enhanced video imaging of individual cells was also achieved [Wood and DeLuca, 1987; Gallie *et al.*, 1989; Maly *et al.*, 1988].

A step forward in the systematic development of bacterial luciferase reporter genes involved a functional analysis of *lux*A and *lux*B coding sequences. It was demonstrated that, although the N-terminal domain of the α-subunit is required for enzyme activity, both 5′

and 3′ transcriptional and translational fusions to the *lux*A gene can be generated [Olsson *et al.*, 1989]. *lux*A and *lux*B genes introduced into bacteria on separate plasmids expressed active luciferase in an amount equal to that produced by cells carrying linked *lux*AB genes [Gupta *et al.*, 1985; Olsson *et al.*, 1988]. In cells in which the β-subunit is present in excess, light production is correlated with the limiting concentration of the α-subunit, indicating that *lux*A alone can be employed as a reporter gene. A similar analysis showed that transcriptional and translational gene fusions can also be generated by using both 5′ and 3′ ends of the *lux*B gene [Sugihara and Baldwin, 1988].

These observations led to the construction of *lux*A-B and *lux*B-A gene fusions encoding functional monomeric bacterial luciferases [Olsson *et al.*, 1989; Boylan *et al.*, 1989] which were expressed in bacterial, yeast, and plant cells. Analysis of the correlation between structure and activity of monomeric luciferases indicated that the length of the interconnecting peptide region plays an important role in determining proper folding of the fusion enzymes. Luciferases displaying different thermal stability and light emission properties were crystallized for comparison of their structure to that of the native enzyme [Swanson *et al.*, 1985; Escher *et al.*, unpublished].

It was demonstrated that both binary and fused *lux* genes can be used for precise and reproducible in vitro assays and for in vivo visualization of temporal and spatial regulation of gene expression in plants [Langridge *et al.*, 1989] (Fig. 2). Recently, due to a great interest in the identification and isolation of plant regulatory genes, we developed a technology to screen large numbers of plants expressing luciferase reporter gene fusions. T-DNA vectors carrying at their integration borders either fused *lux*AB or promoterless *lux*A and linked chimaeric *lux*B gene cassettes were introduced into tobacco and *Arabidopsis* by *Agrobacterium*-mediated transformation. Since the T-DNA is frequently integrated into transcribed loci of the plant genome [Koncz *et al.*, 1989], transcriptional *lux* gene fusions can thus be isolated at high frequencies. The expression of T-DNA-tagged genes can be followed continuously during plant development by in vivo visualization of the expression of *lux* gene fusions in diverse tissues of transgenic plants (Fig. 2) [Redei GP, Univ. Missouri, personal comm.].

PROSPECTIVES

The results described above clearly demonstrate that both luciferase reporter gene systems can contribute very significantly to various fields of molecular and cellular biology. Some trends for the improvement of luciferase reporter genes and for their application are already predictable. Deletion analysis of the firefly *luc* gene showed that the removal of C terminal amino acids does not influence enzyme activity but converts

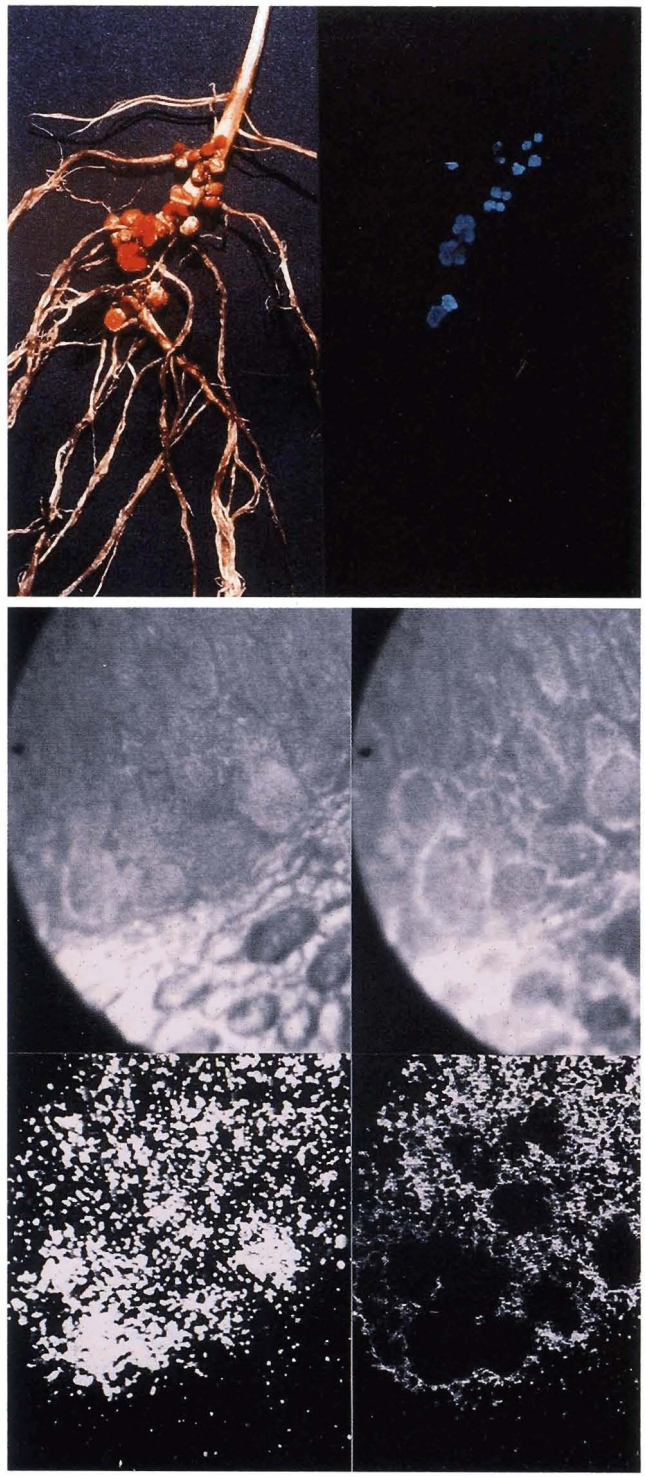

Fig. 1. Visualization of bioluminescence in soybean nodules and in single nodule cells infected by *Bradyrhizobium japonicum* containing a *nif*D-*lux*AB gene fusion. **Top (left):** Nodules under normal illumination. **Top (right):** Bioluminescence recorded on ISO 400 Ektachrome film. **Middle:** Nodule cells under normal illumination. **Bottom:** Visual image of light-emitting cells recorded by IDG low-light video microscope system [O'Kane *et al.*, 1988].

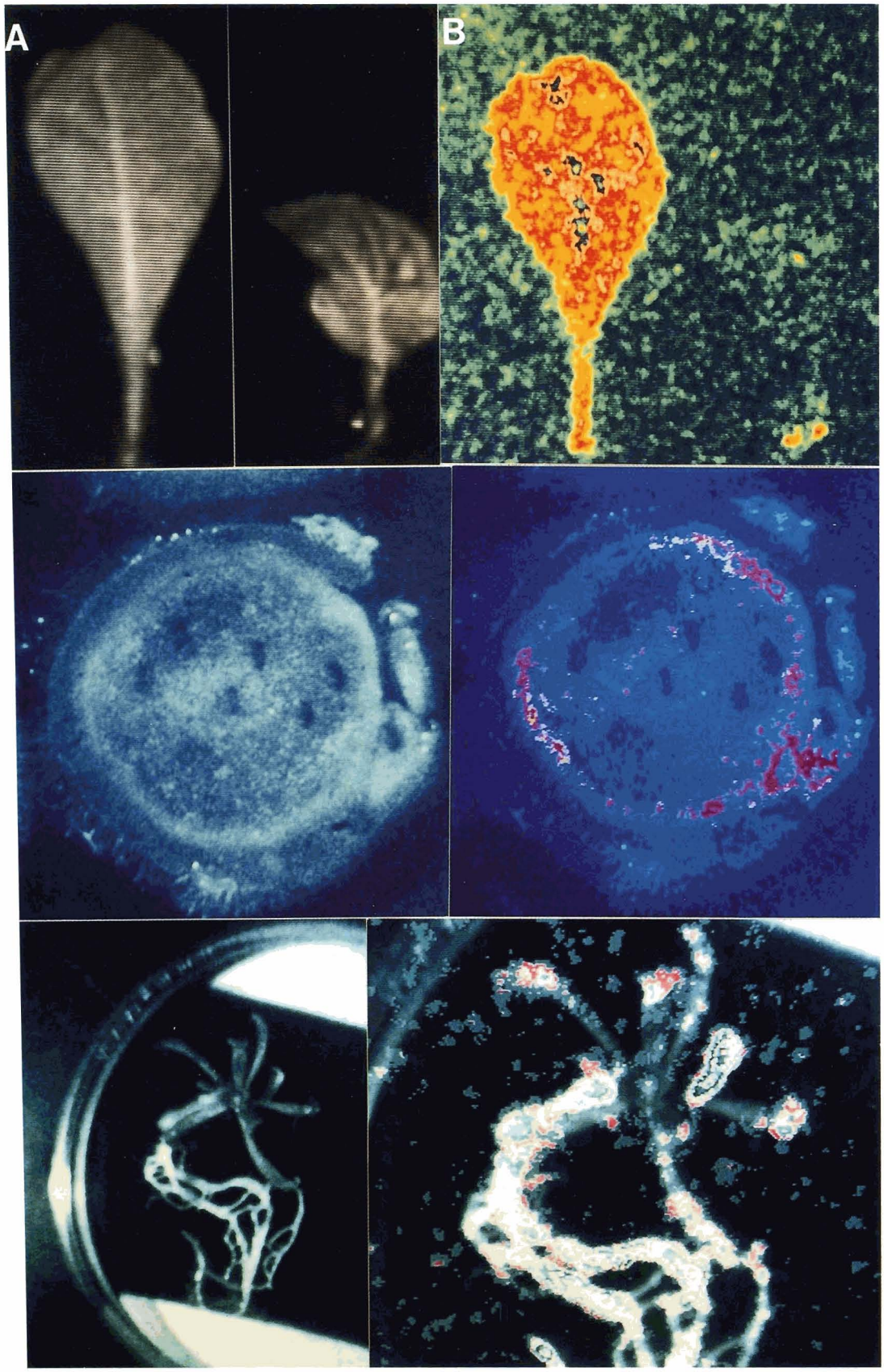

Fig. 2.

Fig. 2. Visualization of *lux* gene expression in transgenic plants, organs, and tissues by a photon-counting video camera–photomultiplier system. **Panel A:** Light image. **Panel B:** Recorded image of light emission. **Top:** Tobacco leaf expressing *lux*A and *lux*B genes driven by 1′ and 2′ promoters of mannopine synthase genes **(left)** and leaf of a non-transformed tobacco plant **(right). Middle:** Stem section of a tobacco plant expressing *mas* promoter–*lux* gene fusions. **Bottom:** Expression of a *lux* gene fusion in a transgenic *Arabidopsis thaliana* plant.

the peroxisomal luciferase to a cytoplasmic enzyme; thus the need for the transport of luciferin to peroxisomes can be overcome [Gould *et al.*, 1989]. The peroxisomal targeting signals identified in luciferases can moreover be employed to transport other proteins into peroxisomes. Genes for beetle luciferases responsible for the emission of light of different colours could provide tools for the simultaneous monitoring of the expression of different genes in a single cell. Inactive monomeric bacterial luciferases carrying recognition sites for specific proteases in between the fused subunits could be used for sensitive detection of proteases within cells or during fermentation in vivo. Both enzymes can be applied to measure thermal denaturation of proteins in diverse tissues during heat-shock. By addition of appropriate signal peptides, luciferases could probably be transported into cell organelles such as chloroplasts or mitochondria for monitoring ATP-consuming synthesis or electron transport. Production of luciferase-fused antibodies may play an important role for diverse aspects of immunology. Fusion of luciferases to chromosomal proteins can lead to unique approaches in cytology. The binary *lux*A-*lux*B system may find an immediate application for the visualization of cell fusion and fertilization events or help in hybrid seed production. Both binary and monocistronic luciferase genes can be employed to monitor virus-cell interactions and virus or virus-induced gene expression. Furthermore, luciferase gene fusions could be constructed to detect site-specific recombination or translocation events in a cell population. Expression of proteins associated with the bacterial luciferase may also provide in vivo assays for selection of fatty-acid- or riboflavin-overproducing organisms. This prospective list of applications can probably be extended, but our intention was simply to illustrate the potential of this reporter gene system.

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