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Use of Low Light Image Microscopy to Monitor Genetically Engineered Bacterial Luciferase Gene Expression in Living Cells and Gene Activation Throughout the Development of a Transgenic Organism

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ABSTRACT

Procaryotic and eucaryotic expression vectors which contain a marker gene for selection of transformants linked to genes encoding bacterial luciferase for detection of promoter activated gene expression in vivo were used to transform the appropriate host organisms and drug resistant colonies, cells, or calli were obtained. Bacterial luciferase expression was measured by a luminescence assay for quantitative determination of promoter activation. The cellular localization of bacteria inside the host plant cell cytoplasm was achieved in a single infected plant cell based on the light emitting ability of the genetically engineered bacteria. In addition, the bacterial luciferase marker gene fusions were used to monitor cell type, tissue, and organ specific gene expression in transgenic plants in vivo. To monitor physiological changes during ontogeny of a transformed plant, low light video microscopy, aided by real time image processing techniques developed specifically to enhance extreme low light images, was successfully applied.

1. INTRODUCTION

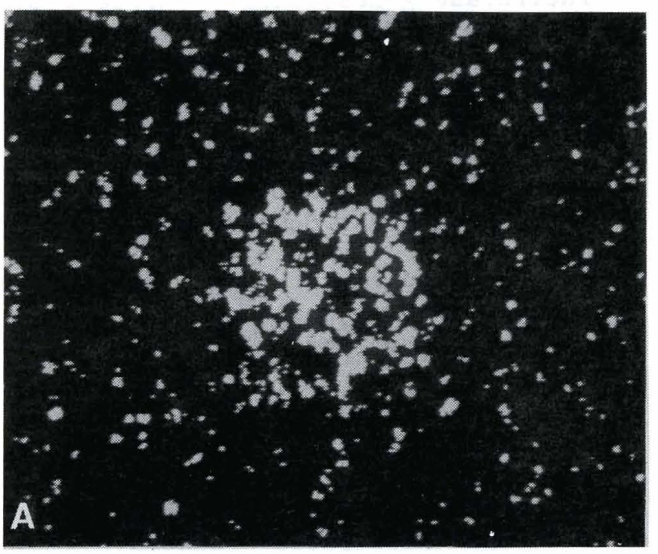
The morphological development of an organism and its adaptation to environmental stress is regulated to a large extent through coordinated gene expression. Development of selectable and detectable marker genes (1-8) has lead to the detection of spacial and temporal regulation of gene expression in eukaryotic organisms (9-10). Recently, a biological light emitting system based on the bacterial luciferase genes from Vibrio harveyi has been made available in which transformed tissue need not be destroyed during the measurement of gene expression (11-15). The bacterial luciferase low light imaging assay procedure permits, for the first time, continuous analysis of gene expression throughout ontogeny.

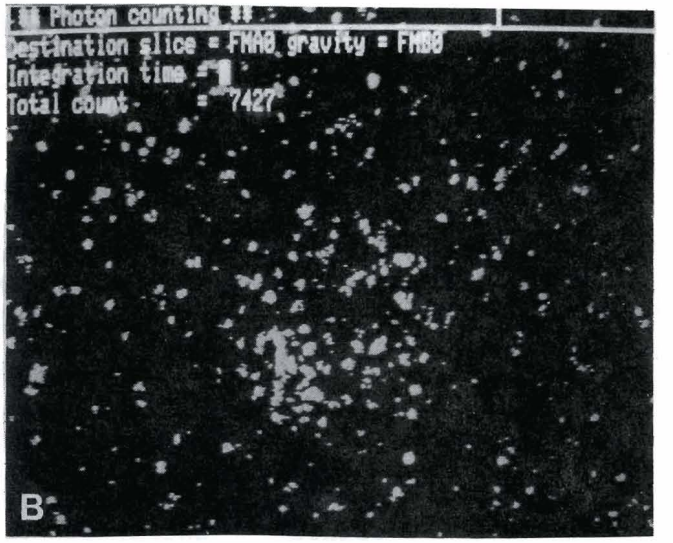
In these proceedings, we demonstrate how bacterial luciferase genes, when coupled with the available technology for low light photon collection and image enhancement permits both the detection and cytological localization of regulated gene expression in bacteria, yeast, and throughout plant development.

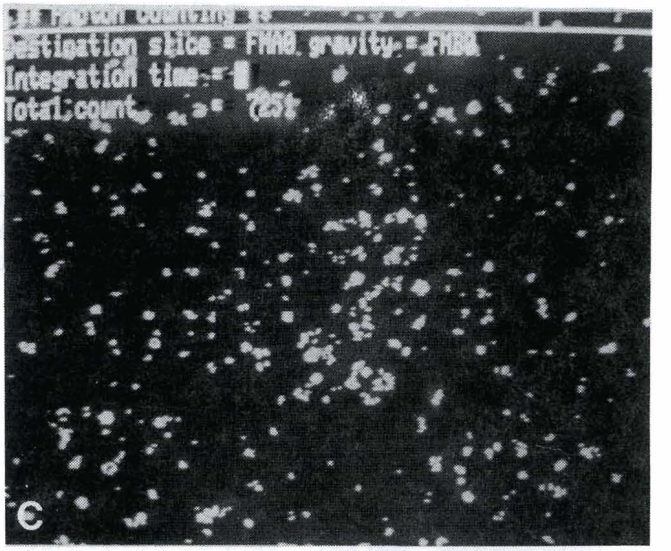
2. DETECTION OF BACTERIAL LUCIFERASE ACTIVITY BY A SINGLE PHOTON IMAGING METHOD.

Bacterial luciferase bioluminescence was assayed quantitatively in a luminometer by mixing purified enzyme or tissue homogenates from transformed plants with the substrates reduced flavin mononucleotide ($FMNH_2$) and the 10 carbon aldehyde decanal. To measure bacterial luciferase activity using ultra low light photon imaging equipment, reduced FMN must be continuously supplied throughout the

assay interval. Therefore, an FMN-NADH oxidoreductase and reduced NAD were included in the reaction mixture. A glass scintillation vial containing the reactants was transferred to the counting chamber of the single photon counting detection equipment containing a photographic lens attached to an image intensifier coupled to a saticon video tube, (Argus-100 Vim 3 camera, Hamamatsu Corp., Japan). Based on recorded bioluminescence, the lowest amount of luciferase detected over a 10 minute interval was in the 1-10 picogram range, Figure 1, panels B and C. The low levels of luciferase measurable by the bioluminescence assay provide a sensitive method for the identification of early gene expression in transformed organisms.







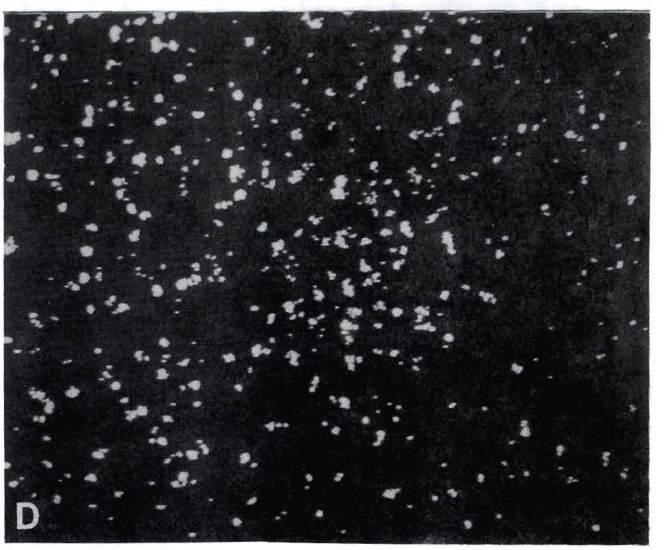


FIGURE 1 Photon counting and imaging of purified bacterial luciferase. All substrates were added to a scintillation vial, which was then placed in the counting chamber of an Argus Vim 3 camera. The photons emitted by the sample were collected for 10 minutes and stored in the central processing unit. The photon image displayed on the monitor was photographed on Kodacolor film ASA 100. Luciferase concentrations: panel A = 100 picograms, panel B = 10 picograms, panel C = 1 picogram, and panel D = no luciferase.

3. DETECTION OF LUCIFERASE GENE EXPRESSION IN BACTERIA AND YEAST BY LOW LIGHT IMAGE ANALYSIS.

The level of luciferase activity in transformed bacteria containing a promoter-bacterial luciferase gene fusion was quantitatively determined by low light imaging techniques. Figure 2, demonstrates the induction of luciferase in E. coli containing bacterial luciferase A and B genes fused to an inducible promoter. The addition of inducing compound in increasing concentrations from right to left, panel A, middle row of wells, results in a proportional increase in luciferase activity detected by single photon capture after addition of the luciferase aldehyde substrate as a drop inside the lid of the multiwell plate.

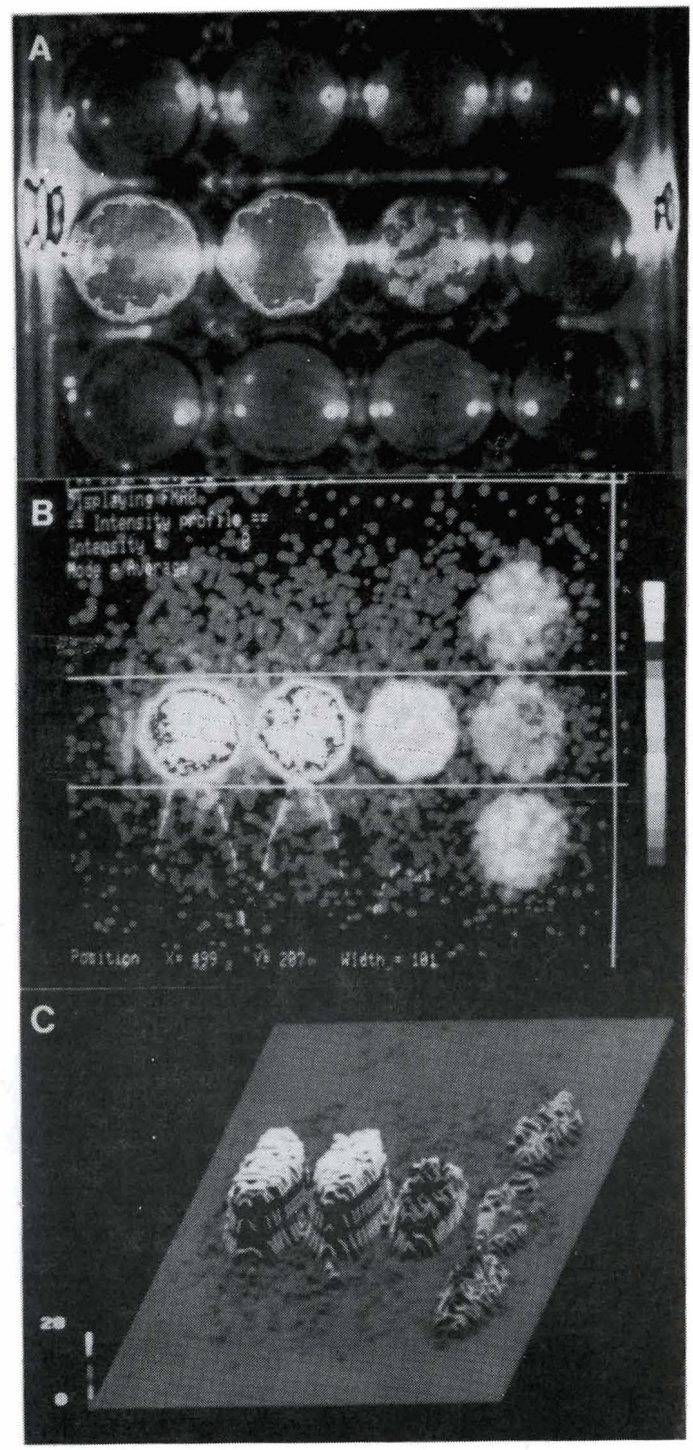


FIGURE 2 Induction of luciferase activity in E. coli cells transformed with a prokaryote expression vector containing bacterial luciferase A and B genes fused to an inducible promoter.

Panel A, is the collected photon image of luciferase bioluminescence in the bacterial cells, superimposed on the video image of the multiwell culture plate containing the bacteria. The middle row of wells in the multiwell plate contain 1.0 ml per well of the transformed E. coli cells at 1 x 10^7 cells/ml. To each well from right to left are added increasing amounts of the inducing substance, the top lane of wells contain the inducer only, the bottom lane of wells contains the transformed bacteria without the inducer. Panel B, is the stored photon image of the multiwell plate described in Panel A. Panel C is a computer generated graphic image of the photons stored in computer memory during the photon collection interval, increasing numbers of photons are represented by the intensity bar at lower left of the panel. The three dimensional image of emitted photons generated in panel C indicates the relationship between inducer concentration and bioluminescence.

In panel B, photons present in the region delineated between the two horizontal lines are depicted graphically at the base of the panel (black line), providing a quantitative correlation of luciferase activity with inducer concentration in the transformed E. coli cells. The light intensity bar at the right depicts the number of photons or multiples thereof present at any location in the image.

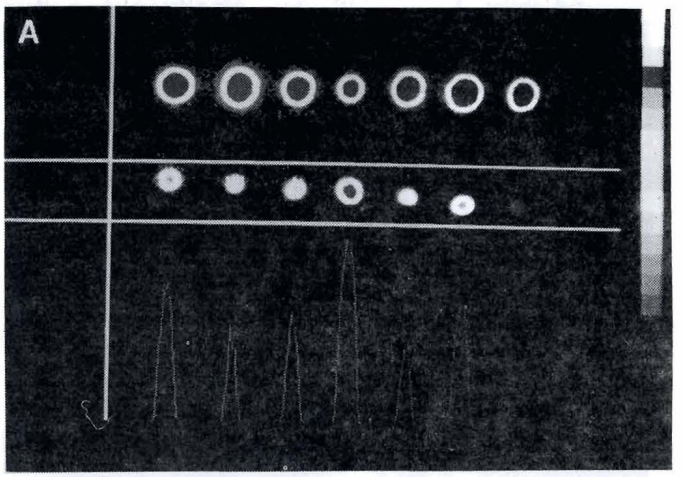
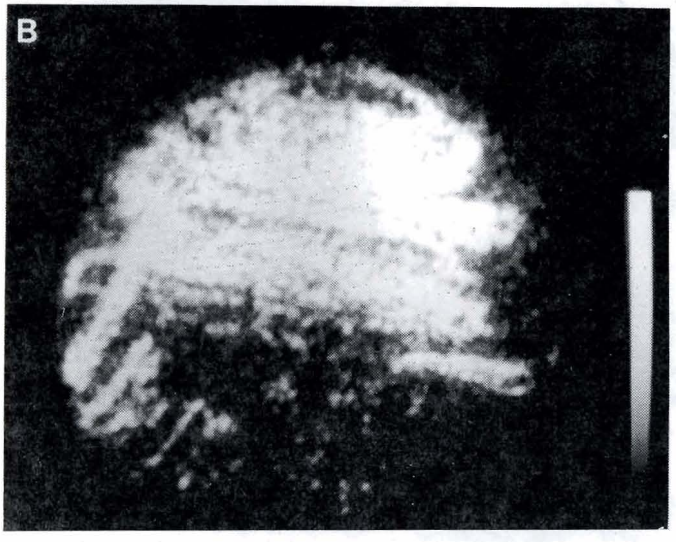


FIGURE 3 Expression of bacterial luciferase gene fusions in E. coli and yeast. Two rows of 7 bacterial colonies are present on the culture plate in panel A, each colony contains a different promoter-bacterial luciferase gene fusion. The intensity of photon emission from each colony is indicated by the light intensity bar at the right of the panel. An integrated photon measurement program permits the quantitative comparison of light emission among the colonies located between the 2 horizontal lines.

In Figure 3, low light image analysis is used to quantitatively measure bacterial luciferase gene expression in E. coli cells. In panel A, the seven bacterial colonies located between the horizontal lines contain different luciferase gene constructs. E. coli contains high levels of the luciferase substrate reduced FMN, thus the activity of gene constructs in transformed E. coli can be rapidly determined by measurement of the number of photons emitted during a 10 second interval following direct exposure of the colonies to vapors of the aldehyde substrate. Analysis of the numbers of photons emitted from each colony is performed in seconds

by a supporting data analysis program which is part of the imaging system, and the luciferase bioluminescence displayed as peaks below the colonies, clearly indicating the relative luciferase activity in the bacterial colonies containing the engineered constructs, panel A.

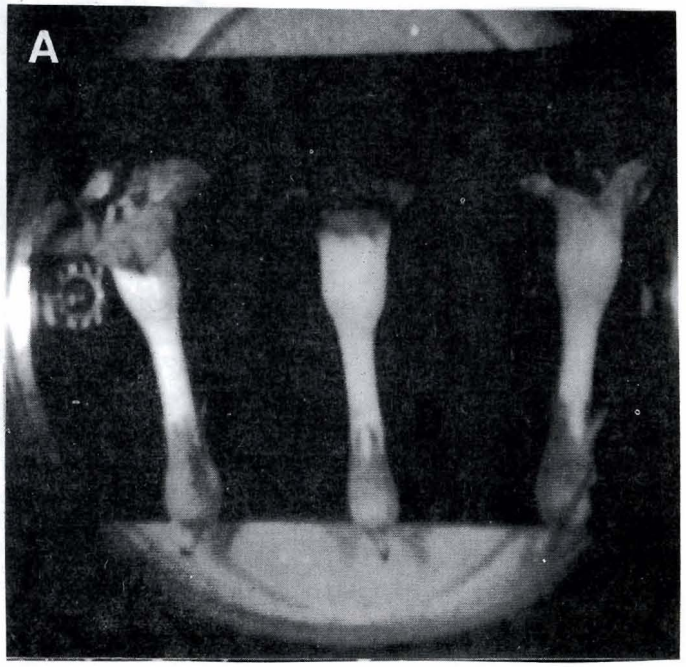


In panel B, bioluminescent transformed yeast colonies expressing bacterial luciferase are detected by photon counting on a culture plate upon addition of 10 µl of the volitile aldehyde substrate decanal to the lid of the culture plate. This photon image was generated during a 1 minute counting interval.

The application of low light imaging for detection of luciferase gene expression in the lower eukaryote yeast is shown in Figure 3, panel B. The bioluminescent colonies seen on the culture plate are from a yeast strain, transformed with an expression vector containing the bacterial luciferase A and B genes, driven by a yeast promoter. Photons were collected for 1 minute. Bioluminescence emitted from individual transformed colonies can be detected after 30 seconds exposure to decanal vapors. In each colony, the intensity of light emission, a measure of luciferase expression, correlates with the intensity bar at the right of panel B.

4. LOCALIZATION OF LUCIFERASE EXPRESSION IN THE ORGANS OF TRANSGENIC PLANTS.

Tobacco cells were transformed with a plant expression vector containing bacterial luciferase A, B genes fused to a bidirectional promoter from Agrobacterium tumefaciens (15). Transgenic plants were regenerated and plant organs assayed for luciferase activity by low light video image enhancement methods previously described (14). In Figure 4, panel B, the bioluminescent image of the flower from the transgenic plant (center) was generated during a 30 minute photon counting period. The bioluminescence from transformed flowers can be identified after 5-10 minutes of photon collection, which provides a rapid assay method for screening flowering transgenic plants.



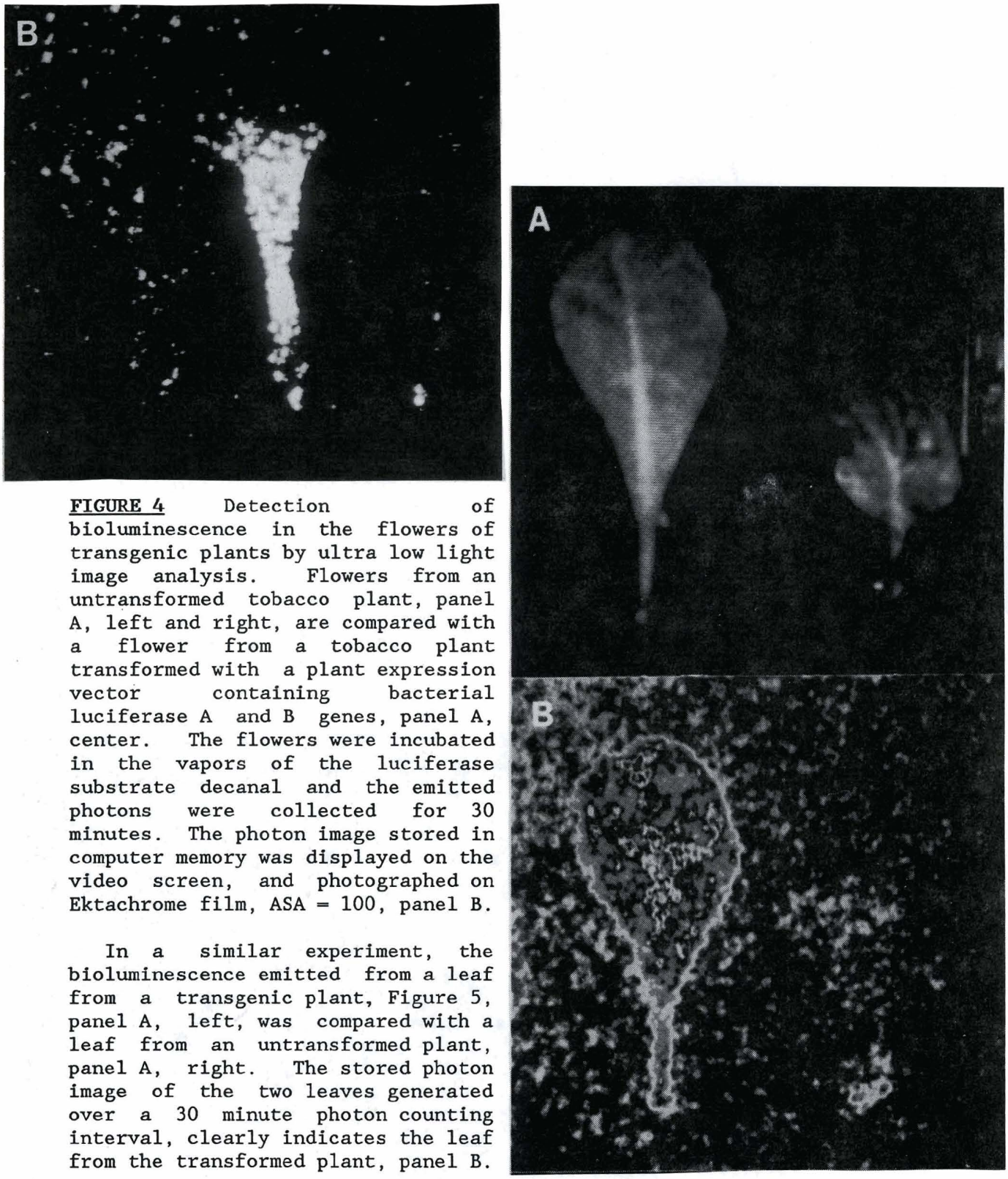


FIGURE 4 Detection of bioluminescence in the flowers of transgenic plants by ultra low light image analysis. Flowers from an untransformed tobacco plant, panel A, left and right, are compared with a flower from a tobacco plant transformed with a plant expression vector containing bacterial luciferase A and B genes, panel A, center. The flowers were incubated in the vapors of the luciferase substrate decanal and the emitted photons were collected for 30 minutes. The photon image stored in computer memory was displayed on the video screen, and photographed on Ektachrome film, ASA = 100, panel B.

In a similar experiment, the bioluminescence emitted from a leaf from a transgenic plant, Figure 5, panel A, left, was compared with a leaf from an untransformed plant, panel A, right. The stored photon image of the two leaves generated over a 30 minute photon counting interval, clearly indicates the leaf from the transformed plant, panel B.

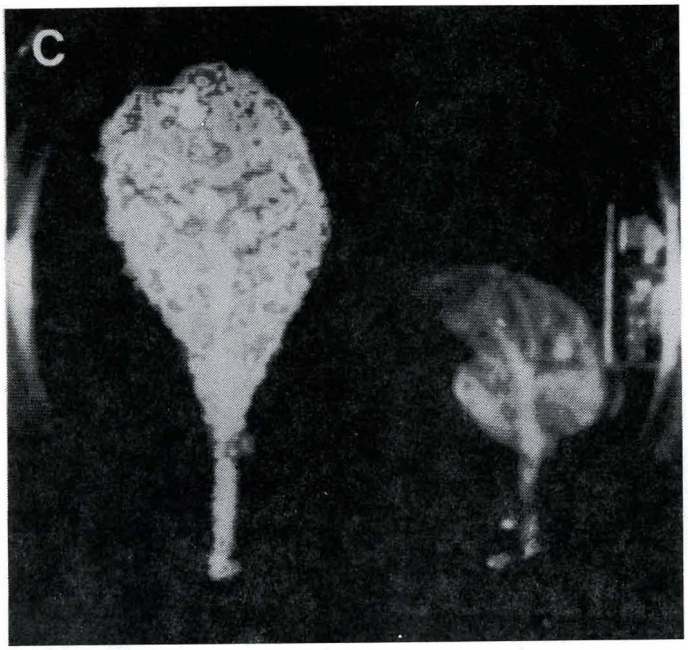
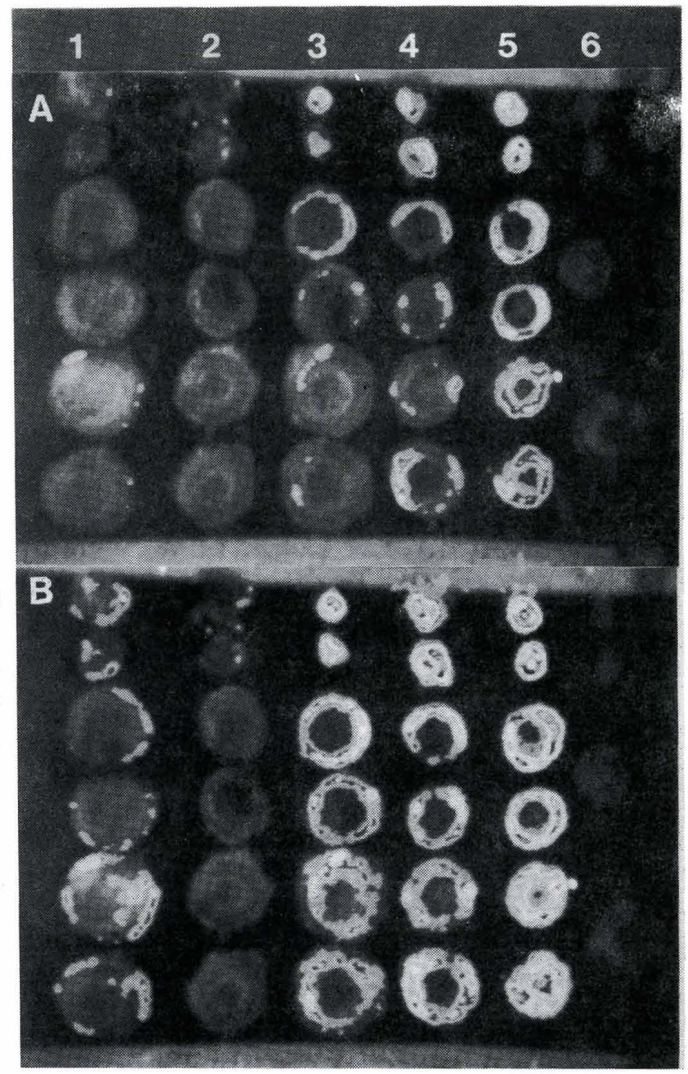


FIGURE 5 Bioluminescence in leaves of transgenic tobacco plants. Panel A, left, is the video image of a leaf from tobacco plant transformed with a plant expression vector containing luciferase A and B genes, panel A, right, is a leaf from untransformed tobacco plant. Panel B, photon image of the leaves in Panel A following 30 minutes of photon acquisition. Panel C, superimposition of the collected photon image of panel B on the video image presented in panel A.

Superimposition of the stored bioluminescent image upon the video image of the leaves indicates the exact location of luciferase activity in the transformed leaf tissue, panel C. When the aldehyde substrate is added directly to the base of the petiole, bioluminescence is detected predominantly in the vascular tissue of the transgenic leaf, panel C. The different shades of grey represent the distribution of luciferase activity in the leaf.

5. DETECTION OF VARIATION IN THE LEVELS OF LUCIFERASE GENE EXPRESSION DURING SCREENING OF TRANSGENIC PLANTS.

The point of insertion of a foreign gene within the plant chromosome may substantially influence the level of expression of the incoming gene. In addition to screening transformants for luciferase activity, low light imaging methods can discriminate between levels of luciferase expression in individual transformants, as seen in Figure 6.



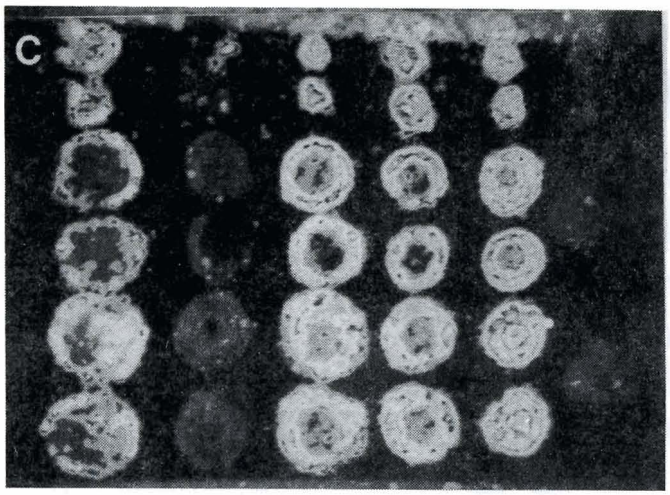
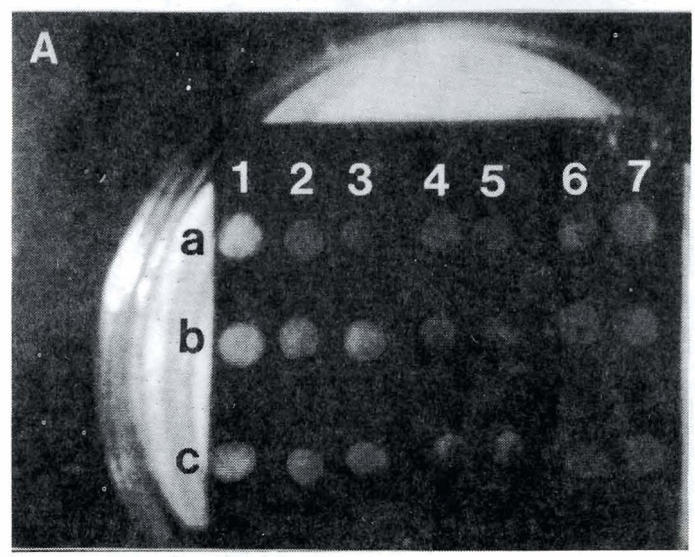


FIGURE 6 Use of low light imaging methods for detection and discrimination between levels of luciferase expression in transformed plants. Panel A shows stem discs from 5 putative transformants, stem discs are arranged from the shoot apex (top of panel) to the base of the plant, lanes 1-5. In lane 6, are stem sections from an untransformed plant. After 30 minutes of photon counting, the stored image was superimposed upon the video image. Panel A shows the location of photons stored at the highest memory level in which photons could be detected. Panels B and C are successively lower levels of memory storage containing increasing numbers of photons.

Panels A, B, and C depict successively increasing numbers of photons stored in the computer memory. In the putatively transformed plants, lanes 1 to 5, the expression of luciferase expression in transformants 3 and 4 is considerably less than in number 5. Luciferase expression in transformant 1 is substantially lower than detected in transformants 3 and 4. The expression of luciferase in transformant 2 is lower than any of the other transformants. These results demonstrate the capacity of the low light image analysis technique for discrimination between levels of gene expression in different transformants.

6. DISTRIBUTION OF LUCIFERASE GENE EXPRESSION THROUGHOUT THE PLANT.

Low light imaging techniques were used to detect luciferase gene expression in leaf discs excised from the top, middle and basal leaves of a tobacco plant, transformed with a plant expression vector containing the bacterial luciferase genes fused to a plant promoter. The distribution of luciferase activity, in leaves throughout the plant under control of this promoter, can be seen in Figure 7. The results indicate that luciferase expression is greatest in the tips of upper leaves of tobacco, panels B and C. In panel D, the exact distribution of luciferase activity throughout the transformed plant is indicated by superimposition of the stored photon image upon the video image of the leaf discs.



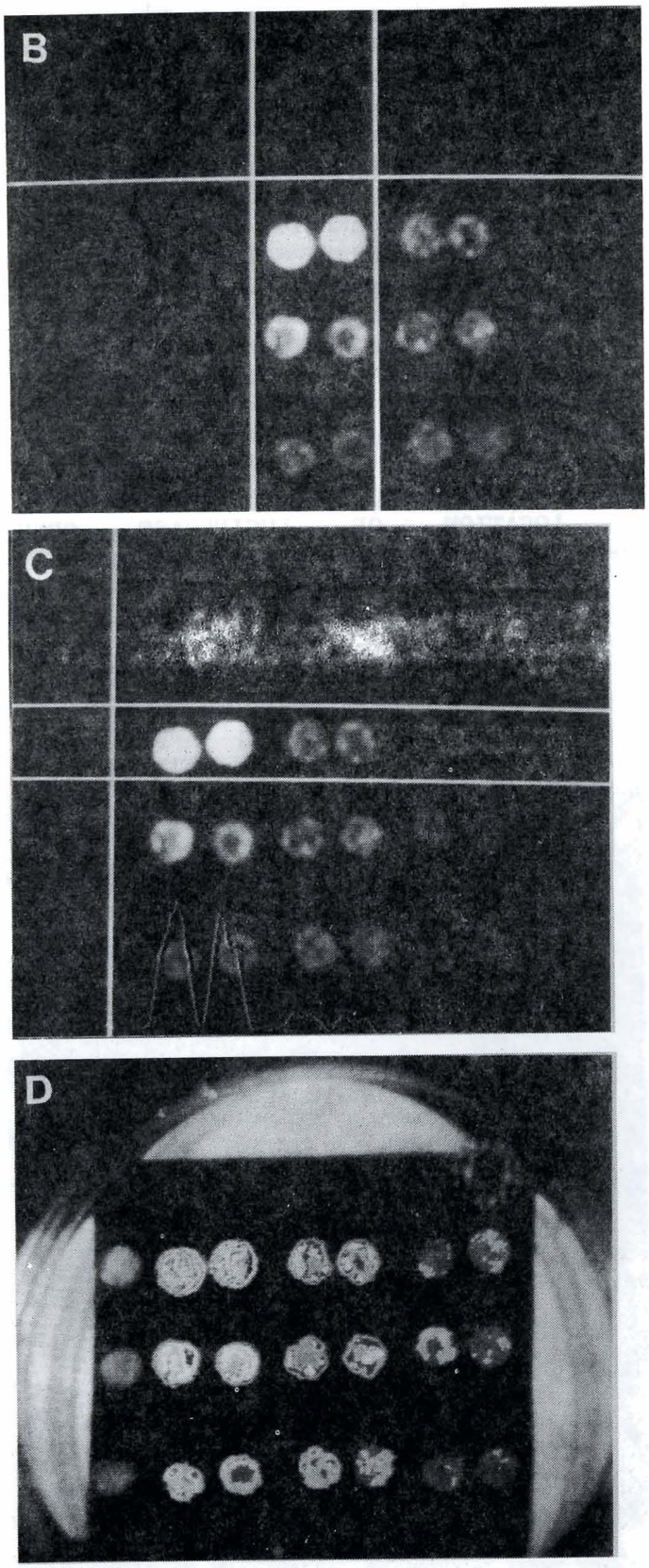


FIGURE 7 Location of luciferase activity in the leaves of transformed tobacco plants. Panel A. is the video image of duplicate leaf discs excised from: an untransformed plant, lane 1; leaf near the top of a 1 meter tall transgenic tobacco plant, lanes 2 and 3; tissue discs from a leaf halfway down the stem of transgenic plant lanes 4 and 5; and tissue discs from a leaf located half at the base of transgenic plant, lanes 6 and 7. In all leaves, the leaf discs at the top of the figure (a) are taken from the tip of the leaf, the second set of discs (b) are taken from the middle of the leaf at the edge, and the third set of discs (c) are taken from the base of the leaf adjacent to the petiole. Panels B and C are the stored photon images of the leaf discs in Panel A, collected over a period of 30 minutes. Stored photons present in the area between the vertical lines in Panel B or the horizontal lines in Panel C are graphically represented, and indicate the comparative luciferase activities in duplicate leaf discs from the apex to the base of the plant. Panel D is the collected photon image (bright white areas), superimposed upon the video image of the leaf discs.

7. GENE ACTIVATION IN RESPONSE TO WOUNDING STRESS DETECTED BY LOW LIGHT IMAGING METHODS.

The induction of luciferase activity in lateral buds due to removal of the apex, is seen in Figure 8. The large number of photons detected in the wound induced lateral buds, panel B, sections 3 and 4, confirms the usefulness of low light image detection methods for measurement of gene activation in plant organs in response to traumatic external stimuli.

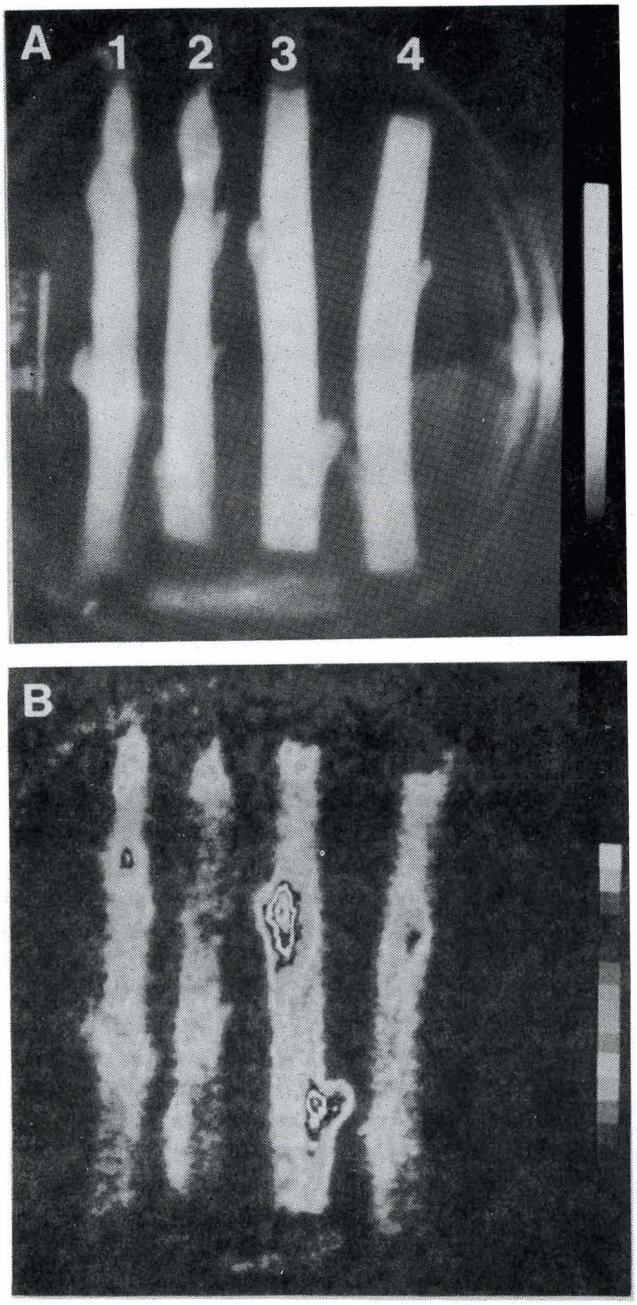


FIGURE 8 Detection of luciferase gene activation in plant organs in response to wounding. The video image of longitudinally bisected stems from 2 identical transgenic tobacco plants grown from vegetative cuttings of the same plant is shown in panel A. The stem on the right (sections 3 and 4), was wounded by excision of the apical meristem, 12 hours prior to removal of the stem from the plant. The stem on the left (sections 1 and 2), was not wounded prior to removal from the plant. A smaller stem section from an untransformed tobacco plant is placed horizontally at the base of the culture plate as a negative control. Panel B is the collected photon image after 30 minutes exposure of the stem sections to aldehyde substrate vapors. The bright intensity bar at the right of panel B indicates increasing numbers of photons from bottom to top.

8. DETECTION OF THE CELLULAR LOCATION OF LUCIFERASE GENE EXPRESSION IN TRANSGENIC PLANTS.

Low light imaging methods detect luciferase gene expression in individual root cells of soybean plants infected with nodulating rhizobia containing constitutively expressed bacterial luciferase genes, Figure 9.

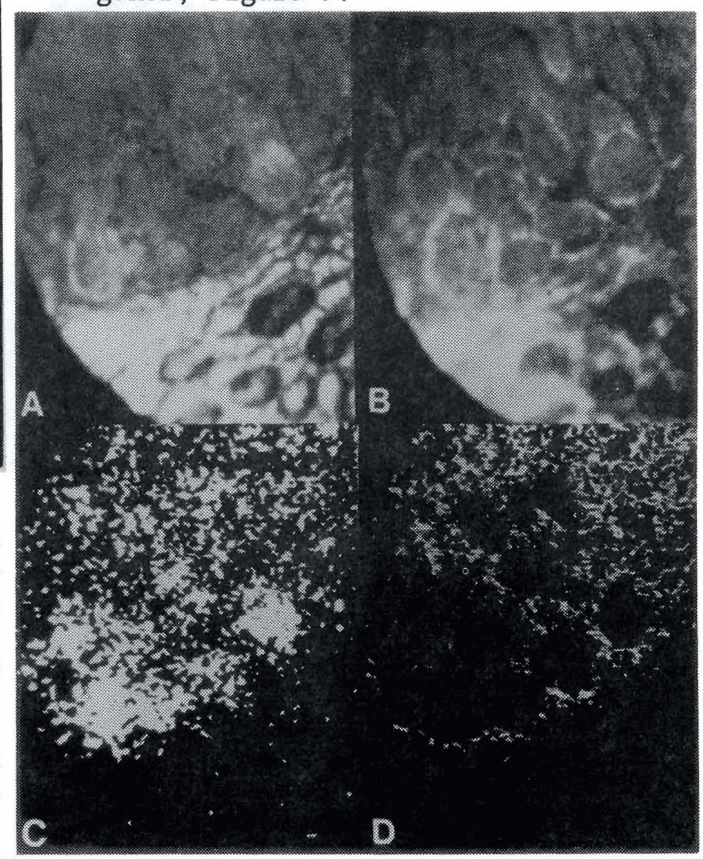
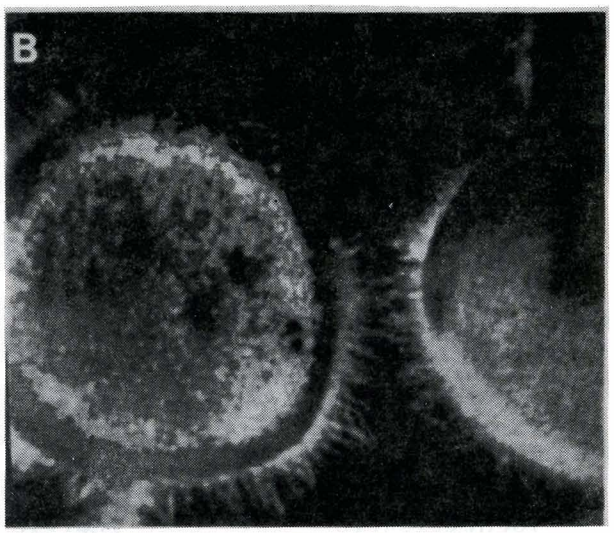
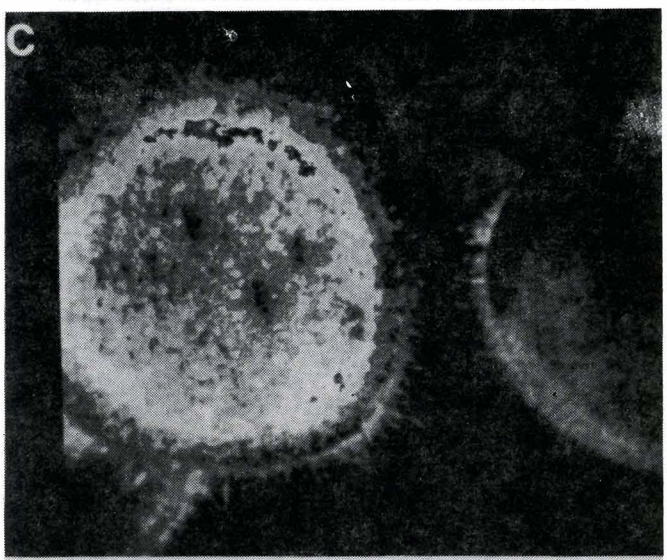
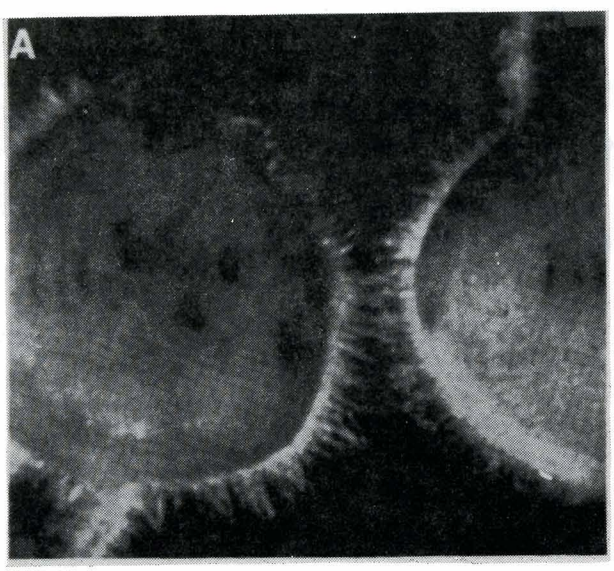


FIGURE 9 Visualization of the bioluminescence obtained from a single Rhizobium infected soybean plant cell. The soybean root nodule cells are filled with transformed Rhizobium bacteroids containing a P1 promoter - lux AB gene fusion. In panels A and B, are shown two transmitted light images of sections through soybean stem nodule tissue. Each section has a slightly different focal plane. Panel C is the bioluminescence images obtained when the section is exposed for 2 minutes to aldehyde substrate vapors. Panel D is the negative image of Panel C.

Thick sections through soybean root nodules show the arrangement of individual cells in the nodule tissue, panels A and B. The bioluminescent images in panels C and D specify the exact location within the root nodule of individual plant cells containing bacteroids expressing luciferase activity. The cellular location of luciferase gene expression in transgenic tobacco stem sections was detected by low light imaging methods, Figure 10.







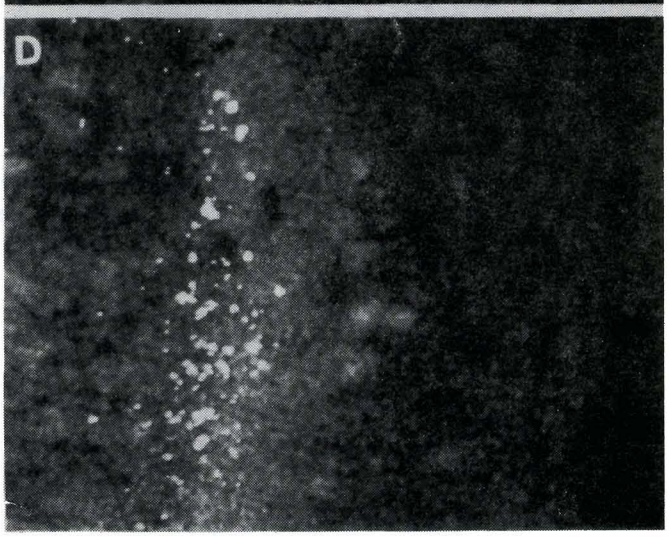


FIGURE 10 The cellular location of luciferase gene expression visualized in stem sections of transgenic plants by low light imaging methods.

Panel A (left) is the video image of stem section excised from the middle of a 1 meter tall transgenic tobacco plant transformed with a luciferase gene containing plant expression vector. Panel A (right) is an equivalent stem section cut from an untransformed plant. The sections are enlarged 10 x to permit identification of the individual tissues which constitute the stem. From the outside of the section toward the center, are found epidermal cells with secretory hairs, a clear band of cortical cells, a darker layer of cells comprising the vascular tissue, and finally, pith paranchymal cells extending toward the centre of the stem. Panel B is the photon image of luciferase bioluminescence after 10 minutes of photon collection superimposed upon the video image of the stem sections. Panel C is identical to panel B with the exception that a total of 20 minutes of photon collection elapsed prior to superimposition of the 2 images.

Panel D shows a stem section from the transgenic plant magnified 50 x, permitting visualization of individual cells (from right to left) of epidermis, cortex, vascular and pith tissue cells. The white dots are individual cells located in the vascular tissue which are emitting light visualized by superimposition of a photon image collected for 20 minutes upon the video image of the stem cross section.

After 10 minutes of photon imaging in the presence of substrate, luciferase gene expression is clearly identified in cells of the vascular tissue, panel B (light areas of bioluminescence). Upon increasing the photon collection time to 20 minutes, pith cells can be seen to contain low levels of luciferase activity, panel C. Superimposition of the collected photon image upon the video image, panel D, indicates the location of individual cells containing luciferase activity within the vascular tissue (white dots).

9. DISCUSSION

The experimental results we have presented emphasize the utility of bacterial luciferase genes for detection of regulated gene expression in plants. With a simple stereo or compound optical microscope, coupled to a photon amplification tube - linked to a video camera, it is possible to visualize expression of luciferase genes at the cellular level. The principal advantages of bacterial luciferase as a marker gene system for study of gene expression in eucaryotes are the ability to detect luciferase activity in tissues and cells at extremely low levels, the quick assay procedure, which permits detection of luciferase activity in 100 tissue samples in as little as 10 minutes, and most important, the ability to perform nondestructive analysis of gene expression in transformed cells which permits continuous analysis of gene expression in an organism during environmental stress and throughout morphogenesis.

ACKNOWLEDGEMENTS

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