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Variable patterns of expression of luciferase in transgenic tobacco leaves

(cauliflower mosaic virus 35S promoter/neomycin phosphotransferase/gene fusion/tomato protease inhibitor I/3′ end)

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ABSTRACT **A carboxyl-terminally modified firefly luciferase, encoded as a gene fusion to the neomycin phosphotransferase gene (which confers kanamycin resistance), was found to be enzymatically active for both enzymes when expressed in bacteria and in transgenic plants. A military-type starlight vision system was used to conveniently analyze the pattern of gene expression in transgenic tobacco plant leaves. Transgenic tobacco plants which expressed luciferase uniformly in all areas of the leaf, and assays for luciferin, demonstrated that luciferin rapidly penetrates all regions of a tobacco leaf in at least two dimensions. Depending on the test gene structure or, presumably, on the transferred DNA (T-DNA) insertional context, other transgenic plants were obtained that expressed luciferase with a wide range of nonuniform patterns from nominally the same cauliflower mosaic virus 35S promoter. For instance, the veins can be dark, while only the interveinal regions of the leaf lamina glow, or only the small capillary veins glow, or only the major veins glow. Local and/or systemic induction in response to wounding was also demonstrated.**

Transgenic plants can be constructed for many dicotyledenous species by making use of the natural ability of *Agrobacterium* to transfer DNA (T-DNA) to plant genomes. The artificial genes so introduced are used to study gene expression or for the practical purposes of expression of new phenotypes valuable to agriculture, such as herbicide resistance (1), virus resistance (2), and insect resistance (3, 4). The pattern of expression of natural or artificial resistance genes could be important in order to direct the expression to the sites of virus replication or transmission or to the sites of tissue ingestion by insects. It is becoming apparent that artificial genes introduced into plants are not expressed at uniform levels from cell to cell or from part to part of the plant, even when genetic control signals of no previously known organ specificity are employed (5, 6). In no case was this more graphically shown than for the expression of firefly luciferase as a marker gene, where the veins of a transgenic tobacco leaf glowed much more brightly than the interveinal regions (ref. 7; see GP19 in Fig. 3). That study did not address the question of whether the luciferin did in fact reach the interveinal regions of the leaf or whether there was some other metabolic reason for the lack of light production from nonconductive tissues.

I have constructed an artificial T-DNA that expresses a translational fusion gene that is a combination of the firefly luciferase gene (*luc*) and the neomycin phosphotransferase (NPTII) gene [also referred to as the kanamycin-resistance gene (*kan*)]; and both the amino-terminal luciferase and the carboxyl-terminal NPTII are expressed in an active form in both plants (tobacco) and bacteria (*Agrobacterium*). Two-dimensional analysis of the expression pattern in transgenic tobacco demonstrates a broad range of patterns from plant to plant. I further show that if a 3′ end from a wound-inducible tomato gene is present, expression favoring the interveinal regions of a leaf is sometimes obtained from the cauliflower mosaic virus (CaMV) 35S promoter, a promoter that otherwise favors the veins or expresses uniformly.

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MATERIALS AND METHODS

T-DNA Plasmids. Fig. 1 summarizes the structures of the artificial T-DNAs expressed in transgenic plants in this paper. In more detail, from left to right on the map, and except for various minor linkers joining the sections, the pWB146 insert [5573 base pairs (bp)] into pRK252 consists of the following spans of DNA: (*i*) pTiT37 DNA [the right border region, nopaline synthase (NOS) promoter, and 7 bp of the transcribed region of the NOS gene (nucleotides 2596–2096 as numbered in ref. 10)]; (*ii*) CaMV1841 DNA [987 bp as an artificial *Bgl* II fragment (6492–7478 of the CaMV sequence), but this DNA derives from an independent clone of CaMV1841 from T. Guilfoyle (University of Missouri, Columbia), and its sequence may vary slightly from that published (11)]; (*iii*) two in-frame start codons separated by a bacterial ribosome binding site; (*iv*) luciferase cDNA from firefly (the 4th–549th (next-to-last) codon; ref. 12); (*v*) NPTII gene (kanamycin resistance) from Tn*5* (codons 6 through the end, nucleotides 166–945; ref. 13), including the stop codon; (*vi*) DNA from the 3′ half of the tomato protease inhibitor I (TomPI) gene (716 bp from the *Hin*dIII site at nucleotide 1743 through the *Bgl* II site at nucleotide 2458; ref. 9); and (*vii*) NOS 3′ DNA [position 685 (10) through the *Hin*dIII site at position 2]. The complete known portion of the DNA sequence of pWB146 has been deposited in the GenBank data base (accession no. M38354). pWB142 was constructed by inserting the entirety of pD0432 (7) into pWV2 (unpublished results) as a *Hin*dIII restriction fragment.

Helper Plasmid and Transgenic Plant Construction. The helper Ti plasmid in a binary system was pTiunT92A in the bacterial strain designated WAg11, a disarmed strain of *Agrobacterium tumefaciens*. It was derived from strain A208 (14). The Ti plasmid T37 was disarmed by replacing the T-DNA and its borders (DNA from the *Kpn* I site to the left of T-DNA through to the *Kpn* I site to the right of T-DNA) with two tandem copies of the gene conferring ampicillin resistance from pBR322. Since this strain is resistant to β-lactam antibiotics such as carbenicillin, the dual formulation antibiotic Augmentin (Beecham Laboratories) is used to cure it from plant tissue cultures.

Transgenic tobacco (*Nicotiana tabacum* var. Xanthi) plants whose genomes carry and express the T-DNA of pWB146,

Abbreviations: NOS, nopaline synthase; TomPI, tomato protease inhibitor I gene; CaMV, cauliflower mosaic virus; NPTII, neomycin phosphotransferase; T-DNA, DNA transferred to plants by *Agrobacterium*; R0, original regenerated transgenic plants; R1, offspring of self-fertilized R0; MS, Murashige and Skoog.

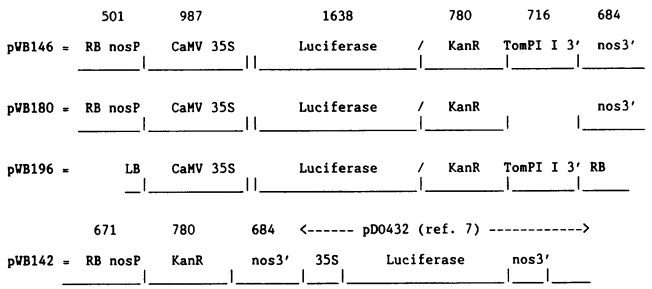

FIG. 1. Scaled linear maps of the T-DNA portions of the binary vector plasmids used in this paper. Vertical bars on the map separate DNA from different organisms; the size of each segment is given in base pairs above the pWB146 and pWB142 maps. The broad host range bacterial replicon for all of them is pRK252, 10.2 kilobases in size and resistant to tetracycline (8). LB and RB, left and right borders of T-DNA (Note that among these plasmids only pWB196 has both borders.); /, point of translational fusion between firefly luciferase and NPTII (KanR) genes; nosP and nos3', promoter and 3' regions of the NOS gene; 35S, 35S promoter from CaMV; Tom PI I 3', 3' region of the tomato protease inhibitor gene I (9).

pWB180, pWB196, or pWB142 were generated by a minor modification of the leaf disk method (15). Leaflets from aseptically grown tobacco were infected on M9 medium (16) adjusted to pH 5.4 and containing 40 μM acetosyringone (17). Regenerated shoots were selected on standard Murashige and Skoog (MS) salts medium (18) containing 3.5 μM zeatin, 2 μM indole acetic acid, kanamycin (250 μg/ml), and Augmentin (250 μg/ml). They were rooted in MS medium with Augmentin but without kanamycin and then transferred to soil.

Luciferase Assay and Photography. When the plants were 15–40 cm tall (total 8–18 nodes), unless otherwise noted, young leaves (from nodes 3–6 down from the apex) were excised with a sharp blade. For the luciferase analysis of fresh leaves, each leaf petiole was immersed into a well 8 mm in diameter and 8 mm deep containing 150 μl of 1 mM potassium luciferin (Analytical Luminescence Laboratories, La Jolla, CA)/67 mM sodium Mes, pH 5.4.

For photography, a Nikon f1.2 objective lens, a starlight scope with relay lens (light amplifier NVS100; Optic-Electronic, Dallas), a Nikon N8008 camera with MF-21 data back, and Ektachrome color slide film (EPT160, Kodak) were used. Kodak Tri-X film (ASA 400) was found to be too grainy to capture the resolution available from the NVS100; much improved resolution from slower black and white films (ASA 32 or 50) is still inferior to the color slide film. The green coloring obtained is false—it is the color of the image on the back of the starlight scope. The reproduction process introduces some other false color with increasing intensity in the order green, light blue, and white. A range of exposures (3.25 sec to 4 min) in an otherwise completely dark room was taken automatically every 10 min with the aid of an IBM PC-compatible computer running program CAMERA (available from the author). For most of the figures shown, an exposure time of 3.25 or 7.5 sec was used 20 min after administration of luciferin.

Table 1. Luciferase patterns with and without the TomPI 3' end

T-DNA plasmid	Total no. of transgenic plants photographed	Number of plants with phenotype				
		V+	V	C	IV	Other
pWB146 (TomPI 3' end)	31	12	1	1	7	10
pWB180 (only NOS 3' end)	29	18	5	0	0	6

This table summarizes the phenotypes exhibited by young leaves of R0 plants. V, veins only (such as WP158 in Fig. 2); V+, uniform expression including both veins and interveins (such as WP138-140 in Fig. 3). (Narrow, relatively dark veins were observed in 3 of the pWB180 plants and 5 of the pWB146 plants, but after examining many leaves this was determined to be a variation of the V+ pattern.) C, small capillaries only (see WP176 in Fig. 2); IV, wide dark veins, glowing interveinal areas (see WP61.1 and WP62.36 in Figs. 3A and 2, respectively); Other, very faint (and thus difficult to categorize) or midrib or petiole only.

The delayed light emission (19) from wild-type leaves was detectable for 1–2 min after lights out with this arrangement, so photography was always started after these plant photosystem emissions had decayed at least 10-fold. This decay takes at least 1 min, since the half-life is about 15 sec. [This 15-sec half-life is a rough average of a constantly changing rate, since the apparent order of the kinetics is 1.6, as measured for algae (19).] Use of the delayed luminescence effect for leaf imaging is called phytoluminography (20). The very low-light chemiluminescence of all biological materials (21) was not detectable with this system.

Luciferin Assay. As described above, leaf petiols were immersed in luciferin solution and, if transgenic, photographed. Weighed midrib sections (10–13 mm) were cut into 1–2 mm slices and freeze–thawed. Leaf disks (40 mm^2) from between the major veins were frozen with liquid nitrogen and then crushed to a coarse powder in a 1.5-ml reaction tube. After equilibration to room temperature, luciferase (1 μg of recombinant; AmGen) was then added in 83 μl of luciferase buffer L2A (30 mM Tris SO_4, pH 7.8/10 mM $MgSO_4$/10 mM dithiothreitol/1 mM EDTA/1 mM ATP), and the light output between 5 and 15 sec was immediately measured with a

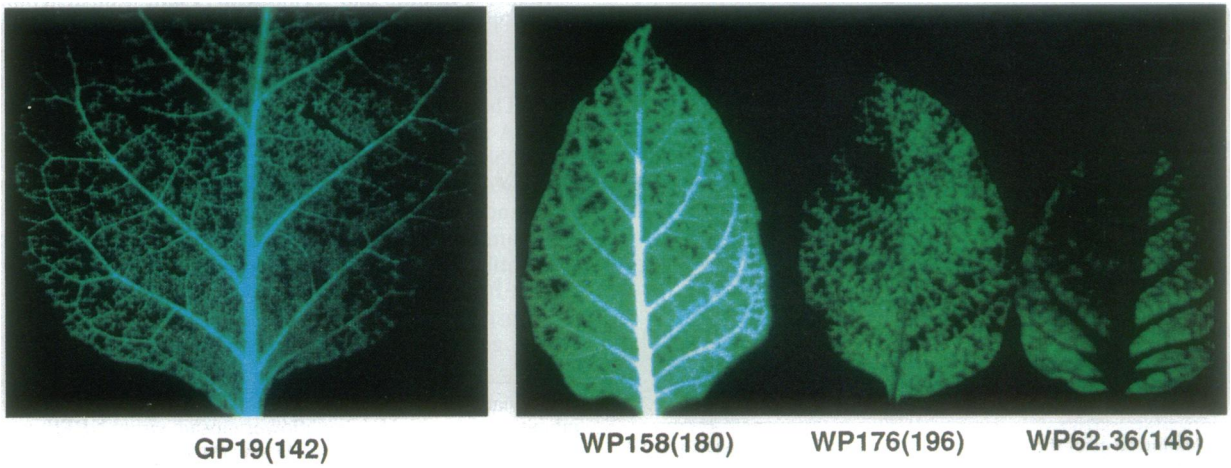

FIG. 2. Phenotypes of veins only, capillaries only, and interveinal at less reduction to show the capillaries.

Turner model 20 luminometer. A standard luciferin curve demonstrated that this assay is approximately linear in the range of 1–300 pmol of luciferin, which brackets the light yields obtained from explant assays. The efficiency of luciferin extraction was not determined, but it was probably lower for midrib sections than leaf disks.

RESULTS

Only a single gene (the *luc–kan* fusion gene) is carried on the T-DNA of pWB146, pWB180, and pWB196. The promoter region of the pWB146 T-DNA consists of 1 kilobase of CaMV DNA containing the 35S promoter, inserted at the start region of a previously existing NOS transcript, thus displacing to far upstream, but not deleting, the relatively weak NOS promoter. At the 3′ end of the luciferase–NPTII gene, immediately after the coding region stop codon, 700 bp of DNA from the 3′ end of TomPI have been inserted just upstream of the NOS 3′ DNA sequences. The gene expression presumably uses the CaMV 35S promoter and the TomPI poly(A) site, but data from experiments to directly establish whether the flanking NOS promoter and 3′ sequences have any role is not yet available except for the study in Table 1. Compared to pWB146, pWB180 is a simple deletion of the 716-bp 3′ end from TomPI, and it still has the NOS 3′ end. Compared to pWB146, pWB196 lacks all flanking NOS DNA and carries only the DNA from the CaMV 35S promoter, the *luc–kan* marker gene, and the TomPI 3′ end. pWB142 carries the previously published 35S-promoted luciferase gene with the NOS gene 3′ end as the entirety of pDO432 (7) on a binary vector pWV2 (W.M.B., unpublished data), which includes a selectable *kan* gene in a NOS expression cassette.

The luciferase codons were taken from pDO432 (7) by the use of the polymerase chain reaction (22), using primers that introduced a *Bam*HI site at the amino terminus and an *Xho* I site in place of the stop codon to create a translational fusion to the sixth codon of NPTII of the Bt (insecticide of *Bacillus thuringiensis*)–*kan* T-DNA plasmid pWB139 (W.M.B., unpublished results). The activity of each gene product of the resulting luciferase–NPTII fusion is not obviously diminished or enhanced in either *Agrobacterium* or tobacco, according to comparative bioassays of similar but separate genes expressing only one cistron. Since the level of expression observed in each of these bioassays is above the range of linear response, a change of less than an order of magnitude might not be detectable.

Rapid and Complete Penetration by Luciferin. Luciferase activity became visible at the top of the main leaf vein (midrib) in as little as 2 min, and, for leaves that expressed luciferase appropriately, I observed that only a further 20 min was required for the luciferin to penetrate essentially all interveinal areas and small capillaries (time course data not shown). The small dark area in the WP176(196) leaf in Fig. 2 was filled in within 5 min after the photograph was taken. The glow pattern remained stable over the time between 20 and 60 min after addition of luciferin.

To investigate whether differing access of luciferin could be responsible for the different patterns obtained below, crushed or diced explants were assayed for gross luciferin access by carrying out an *in vitro* light reaction with excess luciferase and ATP. No significant difference was found between the amount of luciferin extractable from dark versus bright midribs (both about 50 pmol/100 mg) or from the interveinal regions of young versus older, partly senescent leaves (both about 100 pmol/100 mg). Very senescent (yellow) leaves did show a decrease in luciferin uptake (9 pmol/100 mg).

A Broad Spectrum of Luciferase Patterns Is Obtainable. Figs. 2 and 3*A* show examples of the types of patterns observed in whole leaves of transgenic tobacco plants. The luciferase gene constructed by Ow *et al.* (7) was observed by them and by me to give rise to the pattern shown by the pWB142 transformant [GP19(142)] shown in Fig. 2—where only the conductive tissues exhibit luciferase activity, albeit down to the smallest capillary. I have not yet categorized a

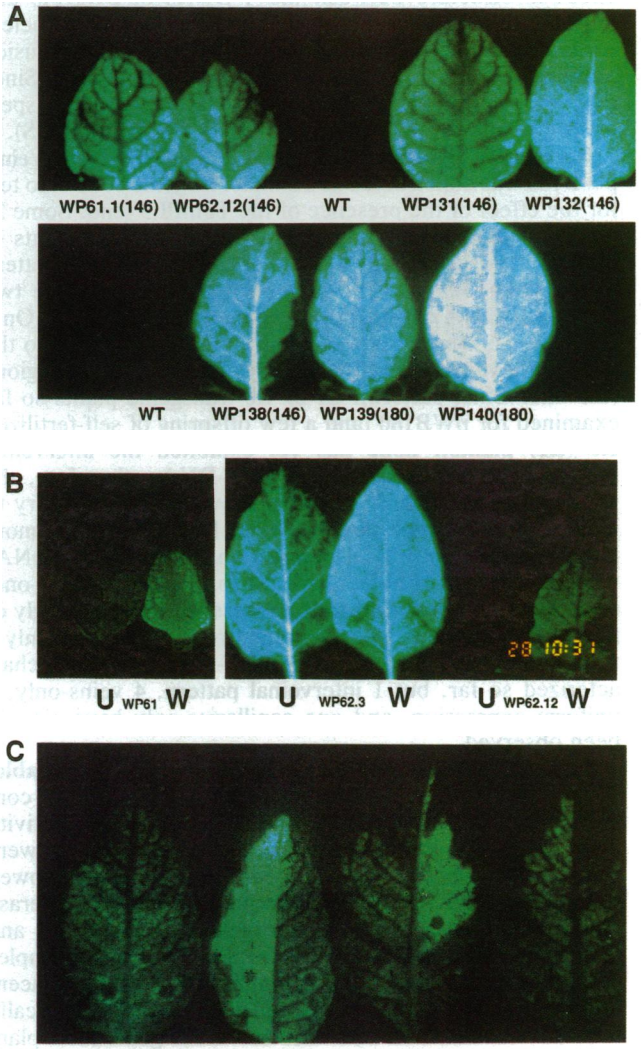

FIG. 3. (*A*) Examples of patterns of luciferase expression. Everything visible in the images is light from luciferase. The complete darkness of untransformed, wild-type leaves is illustrated by the invisible leaves marked WT. The top row shows three interveinal (dark veins) and one uniform (veins + interveinal regions) phenotypes. The second row shows three uniform (veins + interveinal) phenotype leaves, one of which (WP138) has a chimeric, apparently nontransgenic edge region exhibited by every leaf from this plant. Numbers in parentheses refer to T-DNAs in Fig. 1. Plant serial numbers with decimals refer to R1 generation individuals, and the others are leaves from R0 transgenic plants. (*B*) Wound induction. Two leaves of similar size were excised from adjacent nodes of young R0 (WP61) or R1 (WP62.) transgenic plants (pWB146 T-DNA). One leaf of each pair was wounded (W) by mashing it in three places (total about 1 cm^2) with ridged forceps; the other leaf (U) served as an unwounded control. The sites of wounding are visible as slanted dark lines in the wounded leaf of WP62.3. For 22 hr after the time of wounding, the petiole of each leaf was immersed in water or one-fifth-strength MS major salts (18), and the leaves were incubated under constant low light. (*C*) Luciferase photoassay of leaves from R0 pWB146 transgenic plant WP62 that has lost the systemic wound response (the marker gene is highly expressed without recent wounding) but that exhibits chimeric sectoring and a local wound response. Four holes (7 mm) were punched in each leaf, two each at 20 hr and 2 hr before this photoassay. Note the local wound response around most of these holes.

statistically significant number of my own transgenic plants for pDO432, but 4 out of 4 exhibit this same veins-only pattern.

My noteworthy finding is that pWB146 T-DNA can in some transgenic contexts be expressed with the *opposite pattern*—dark veins and bright interveinal regions. The many differences in structure between the T-DNAs of pWB146 and pDO432 (pWB142 in this paper) and the lack of sufficient examples of pDO432 insertions preclude any firm conclusion at this time about why they exhibit different patterns. Since they use approximately the same promoter, however, speculation points to their different 3′ ends (TomPI or NOS).

Correlation Between the Tomato 3′ End and Dark Veins. pWB180 was constructed as a simple, isogenic control to test for the effect of the presence of the TomPI 3′ end. Some 30 independent original regenerated (R0) transgenic plants of both pWB146 and pWB180 were characterized as to pattern in the young leaves. The patterns exhibited by these two isogenic T-DNA constructs are enumerated in Table 1. Only pWB146 has been observed (7 out of 31) to give rise to the wide, dark veins, with glowing only in the interveinal regions (the interveinal pattern). Of 29 R0 transgenic plants so far examined for pWB180 [and a few offspring of self-fertilized R0 (R1) plants], none has yet exhibited the interveinal pattern. For this isogenic pair of T-DNAs, therefore, the presence of the TomPI 3′ end is shown to be necessary to make this phenotype possible or at least to make it more likely amongst many T-DNA insertion events. Both T-DNAs were observed to be able to give rise to at least one veins-only plant and to several uniformly expressing (either strongly or faintly in both veins and interveinal regions) plants. Only 6 transgenic plants carrying pWB196 T-DNA have been characterized so far, but 1 interveinal pattern, 4 veins-only, 1 uniform expression, and one capillaries-only have already been observed.

Systemic Wound Induction Is Sometimes Observable. Wound inducibility was anticipated for the pWB146 construct because previous attempts to assay NPTII activity from the Bt–*kan* construct pWB139 (data not shown) were unsuccessful unless a leaf was wounded slightly and allowed to incubate for 22 hr. Initial attempts to visualize luciferase activity from early pWB146 transgenics (plants WP61 and WP62) therefore included wounding. Fig. 3*B* shows examples of this result, for which a leaf of similar size from the adjacent node of the same plant was excised and incubated identically but not wounded. In one case for a R1 generation plant (WP62.3), only veins were expressing luciferase without wounding, but the interveinal regions increased to full brightness in the wounded leaf. The entire leaves, not just the wound sites, exhibit the response, and therefore this is a systemic effect similar to the wound response reported for tomato and potato protease inhibitors I and II (23, 24). This is not merely local wound stimulation, such as that reported for the mannopine synthase promoter (6) and the NOS promoter (6, 25), although such a local effect is occasionally superimposable on pWB146 T-DNA expression patterns (see below, Fig. 3*C*).

The systemic wound induction was reproducible for at least the first five occasions, but since then some unknown variable, believed to be environmental, has intervened to allow observation of wound-induction behavior in only some of R0 plants and in only 25% (3/12) of the R1 offspring. I show it here to demonstrate the facility of the luciferase assay, but the explanation for the variability in the response of the plants to wounding and the firm identification of the DNA sequences responsible await further experiments. In particular, the parallel remains to be established between the presence of the TomPI 3′ end in pWB146 and the reported requirement of the potato protease inhibitor II 3′ end for systemic induction from its cognate (potato protease inhibitor II) promoter (24).

Fig. 3*C* shows leaves from plant WP62 when it was about half grown and after leaves from it had twice been harvested and found to be systemically wound-induced by the assay in Fig. 3*B*. In this experiment disks 7 mm in diameter were punched from these four leaves *in planta* before the two on the left were wounded even more with forceps. A drop of luciferin was added to the disks, and they were counted in a scintillation counter. This count determined that the leaves were already expressing luciferase at a high level without wounding, and on several further occasions, leaves from this plant never failed to glow when unwounded, right up to and including the time of flowering. Of course, the constant harvesting of leaves may well have constituted wounding. I show these leaves because they exhibit two other interesting patterns of luciferase expression, regardless of their lack of systemic wound response. One of these features is the sectors of different brightness. These sectors are analogous in shape and position to developmental clones of leaf cells that can be demonstrated by x-ray marking of axillary leaf buds (26). Since none of 24 luciferase-positive plants from the R1 generation exhibit this chimericity, it may be concluded that this plant was merely an R0 chimera.

Local Wound Induction Is Sometimes Observable. The other interesting feature in Fig. 2*C* is a local wound response, visible as a noticeable increase in brightness around most of the punched holes. This is similar to the millimeter-range wound response seen for the NOS promoter (6, 25) and the mannopine synthase promoter (6), although in the latter case the response was limited to the veins near the wound. One pWB146 transgenic (designated JP50) always and only responded to wounding in this local way, exhibiting increased brightness directly coincident with wound sites (three out of three trials, data not shown). The DNA signals on pWB146 that may be associated with the local wound response remain to be identified by careful comparison of isogenic test T-DNAs.

The Expression Pattern and Level Are Constant from Leaf to Leaf for Young Leaves. The variation shown is from plant to plant, not leaf to leaf. By stripping off many unwounded leaves for analysis at once, I have determined that at least leaves 2–8 from the top of the plants exhibit a constant, reproducible pattern and comparable expression levels. The apparent wound induction and the different expression levels are therefore not merely random variation. Several pWB146 transformants whose older leaves were tested showed a change from dark veins to a veins-only pattern in the older leaves (data not shown), at the same time that younger leaves on the same plant (those in Fig. 3*C*) continued to exhibit the interveinal pattern with wide, dark veins. The patterns shown in all of the figures and in Table 1 are from the constant-pattern region of young leaves near the top of the plants.

DISCUSSION

A Fusion Gene with One Selectable and Two Assayable Activities. The *luc–kan* fusion gene described here represents a penalty-free addition of a convenient, sensitively detectable marker to a selection for NPTII or the addition of a selectable marker to the luciferase gene, allowing one gene to do double duty. Although there is yet no evidence for it, one may speculate that selection for NPTII activity could bias the type of genetic insertion events obtained toward those chromosomal locations or configurations that support expression at the time of the selection for kanamycin-resistant transgenic shoots. If there were such a bias, this selection might not include all of those insertions that are expressable later at the time of analysis, such as during seed formation or during stress response. For the case of *Agrobacterium*-mediated transformation and shoot regeneration, however, it has been demonstrated that the tightly controlled promoter for 7S seed

storage protein is constitutively expressed in transgenic shoots and young plants but reverts to normal control with time and in subsequent generations (27). The data presented here demonstrate that transgenic shoots selected as kanamycin-resistant can and do result in mature plants that exhibit nonconstitutive expression patterns for the kanamycin-resistance gene.

The carboxyl-terminal three amino acids of firefly luciferase constitute the targeting signal for portage to the peroxisome, and changing of the last amino acid or addition of amino acids blocks this transport (28). The *luc–kan* fusion replaces the last amino acid with the entire enzyme NPTII, and thus the fusion gene product probably is not transported to the peroxisome. High expression levels of wild-type luciferase in transformed cells results in saturation of the peroxisomes, with the overflow of luciferase localized to the cytosol (28). Although the referenced study made no mention of luciferase activity for altered carboxyl termini, it is possible that the intracellular location of luciferase or altered numbers of peroxisomes in different tissues could influence its activity. If so, the *luc–kan* fusion may provide a simplification for the luciferase marker, since intracellular localization is now unlikely to be a complicating factor.

Firefly luciferase as a marker for gene expression in transgenic plants depends on the accessibility of luciferin to all parts of the plant. Previous studies with this marker (7) had demonstrated glowing only in the veins of a leaf, leaving open the question of whether this is the pattern of luciferin access or the pattern of luciferase gene expression or enzyme activity or stability. The presented examples of transgenic plants that glow uniformly demonstrate that luciferin is rapidly transported to all parts of a transgenic tobacco leaf whose cut petiole is dipped into a luciferin solution. Assays for extractable luciferin also demonstrated that it is transported by old and young leaves, and it is present in equal amounts in dark and bright transgenic midrib veins. Observation of marker gene luciferase activity is therefore possible in all areas of the leaf in at least two dimensions.

Expression Patterns Would Be Missed by Assay of Extracted Protein. Most assays of reporter genes such as chloramphenicol acetyl transferase (CAT) employ small explant assays or assays that involve dissection and removal of the midrib and destruction (grinding) of the tissue. These analyses can miss areas of very high or very low relative expression. Some short-range spatial information can be demonstrated if the marker genes coding for β-glucuronidase and β-galactosidase are used without grinding the leaf (6), but these assays nevertheless utilize killed leaf tissue. The photographic luciferase assay demonstrated here retains pattern information for whole leaves. In addition, the rapidity of luciferin uptake, the sensitivity of the equipment used, and the use of live plant parts open up possibilities of observing short-term variation of gene expression *in vivo* or the nondestructive screening of transgenic plant tissue *in vitro*.

The power of this assay has demonstrated a disconcerting range of gene expression patterns for each construct that was tested. This is a reminder that unknown, largely uncontrollable factors such as the chromosomal context can affect expression patterns, perhaps at least as much as the genetic control signals that are carried on the T-DNA. Only the analysis of multiple independent transgenic plants can distinguish and identify nonrandom genetic control patterns that can be attributed to the particular gene construct or mutation being studied. We have observed that our test gene on pWB146 T-DNA is subject to subtle plant growth environmental effects and/or to wounding stresses and that its pattern is even variable in the next generation. Despite the variability of the transgenic material studied here, I believe that I have observed enough transformants, with and without the TomPI 3′ end, to conclude that this 3′ end extends the range of phenotypes available to expression from the CaMV 35S promoter.

The marker gene in pWB146 T-DNA is expressed strongly, and sometimes only, in exactly those interveinal regions of a leaf that are the preferred sites of ingestion by insect larvae (29). This gene was constructed as a more convenient marker for the expression behavior of an analogous, effective, wound-inducible insecticide gene to be described fully elsewhere, but which contains *B. thuringiensis* HD73 crystal toxin codons in the place of the firefly luciferase cDNA codons of the exact same gene expression cassette. An expression pattern that includes or even favors these interveinal regions promises an efficient delivery of artificial defense proteins such as Bt, in the likely event that the Bt gene product can be expressed with the same pattern from this gene cassette.

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