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Expression and assembly of functional bacterial luciferase in plants

(plant transformation/coordinate gene expression/*luxAB* genes)

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ABSTRACT **The *luxA* and *luxB* structural genes of *Vibrio harveyi* luciferase [alkanal,reduced FMN:oxygen oxidoreductase (1-hydroxylating, luminescing), EC 1.14.14.3] were introduced into a plant expression vector and transferred into tobacco and carrot cells by *Agrobacterium*-mediated or direct DNA transformation. Simultaneous expression of the *luxA* and *luxB* genes was monitored by protein immunoblot analysis. Luciferase-mediated light emission provided evidence for the assembly of the two protein subunits into a functional dimeric enzyme in plant protoplasts, in transformed calli, and in leaves of transformed plants. Bacterial luciferase may provide a useful marker-gene system for the quantitative assay of coordinate gene expression in transgenic plants.**

Genes involved in bioluminescence have recently been isolated and expressed in *Escherichia coli*. The best-characterized genes are the related luciferase *luxA* and *luxB* genes from *Vibrio harveyi* and *V. fisheri* (1, 2) and a nonhomologous gene from the firefly *Photinus pyralis* (3).

The *V. harveyi* luciferase [alkanal monooxygenase; alkanal,reduced-FMN:oxygen oxidoreductase (1-hydroxylating, luminescing), EC 1.14.14.3] is a heterodimer, composed of α (LuxA) and β (LuxB) polypeptide subunits (4), that catalyzes the oxidation of long-chain fatty aldehydes. The reaction requires reduced flavin mononucleotide and molecular oxygen and results in the emission of blue-green (490 nm) light (5). The expression of *luxA* and *luxB* genes is sufficient to produce the α and β subunits of functional luciferase in bacteria. *E. coli* cultures expressing *luxA* and *luxB* genes are bioluminescent when an aldehyde substrate (e.g., decanal) is supplied, indicating that viable cells take up the aldehyde (1, 6).

Previous work has demonstrated that a number of bacterial enzymes, such as neomycin phosphotransferase (7–9), chloramphenicol acetyltransferase (7), β-galactosidase (10), and hygromycin phosphotransferase (11), are expressed and can be used as selectable or screenable markers in transgenic plants.

All of the above enzymes require relatively complex assay procedures. The results of chloramphenicol acetyltransferase or β-galactosidase assays are not easily quantified, because of nonspecific reactions or the presence of endogenous enzyme activities in plant cells. To overcome these limitations, we chose light-emitting bacterial luciferase as a marker for plant-cell transformation.

To our knowledge, all the bacterial enzymes shown to be expressed in plants thus far are of a single-subunit type. The heterodimeric *V. harveyi* luciferase appeared to be a suitable system to test for the assembly of a complex bacterial enzyme in plant cells, thus opening the way for expression of multicomponent heterologous enzyme systems in higher plants.

In this paper we describe the construction of "cassettes" containing bacterial *luxA* and *luxB* genes that can be correctly and independently expressed in plant cells.

MATERIALS AND METHODS

Cloning Methods. Bacterial culture media; conditions for transformation of *E. coli* competent cells; and procedures for DNA-fragment isolation, for filling in or eliminating the protruding 3′ and 5′ ends of DNA fragments by use of *E. coli* DNA polymerase I Klenow fragment, bacteriophage T4 DNA polymerase, or mung bean nuclease, and for phosphatase treatment of DNAs, ligations, and addition of synthetic oligonucleotide linkers were as described (12, 13).

Construction of Plant Expression Vector pPCV701. Expression vector pPCV701 is an *Agrobacterium* binary plant cloning vector derived by a series of modifications from the plant vector pPCV002 described previously (14). Part of the vector pPCV701, extending from the ori_V and ori_T regions to the right 25-base-pair (bp) border sequence (B_R), remained identical to that of pPCV002. The plant selectable-marker "cassette" from pPCV002, however, was modified by coupling the neomycin phosphotransferase coding sequence from the *Bcl* I–*Sma* I fragment of plasmid pKm9 (15) to the promoter sequences of the nopaline synthase gene (8) and by adding the 3′-polyadenylylation sequence of the T_L-DNA gene *4* (ref. 16; nucleotides 8840–9240 of the T_L-DNA). This selectable-marker cassette was inserted between the *Hin*dIII and *Bcl* I sites of pPCV002 to yield plasmid pPCV002NKMA. An expression unit was assembled as follows: the *Bam*HI–*Hin*dIII fragment of plasmid pAP2034 (17) was replaced by that of plasmid pOP44392 (18) to obtain plasmid pAPTR1′2′, in which the promoter of gene *2′* is linked to the polyadenylylation sequence of the T-DNA gene *7*, derived from plasmid pAR2034 (18). After opening pAPTR1′2′ DNA by cleavage with *Sal* I, filling in the ends with T4 DNA polymerase, and *Hin*dIII digestion, the polyadenylylation sequence of the T-DNA octopine synthase gene was added from plasmid pAGV40 (8, 16) as a *Pvu* II–*Hin*dIII fragment downstream from the promoter of gene *1′*. This resulted in the regeneration of a single *Sal* I site. The expression cassette was thereafter isolated as an *Eco*RI–*Hin*dIII fragment and inserted into pPCV002NKMA to give the expression vector pPCV701.

Construction and Cloning of *luxA* and *luxB* Gene Cassettes in Expression Vector pPCV701. (See Fig. 1.) Plasmid pTB7, carrying the *luxAB* transcriptional unit, was linearized with *Sal* I and treated with various amounts of BAL-31 exonuclease. *luxA* fragments isolated from gels after *Eco*RI diges-

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Abbreviation: bp, base pair(s).

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tion were subcloned into the *Dra* I–*Eco*RI sites of pBR322 (19). The ATG codon preceding the translational initiation codon of *luxA* is part of an AAA triplet (20). Regeneration of *Dra* I sites indicated that the endpoints of deletions were AAA triplets. The *Dra* I–*Eco*RI fragment pool obtained from pBR322 recombinants was cloned into *Sma* I–*Eco*RI sites of the M13 vector mp18 (21), and the exact endpoints of deletions were determined for 196 independent clones by DNA sequencing (22). One of the isolated deletion endpoints was located 7 bp upstream from the ATG initiation codon of *luxA*. This plasmid was opened at its *Bam*HI site, treated with BAL-31 to make further deletions, and then religated. Out of 98 clones sequenced, the extra ATG was removed from 8, and of these, 4 retained the *Sal* I site of mp18. M13 replicative form DNA was isolated from one of these clones, digested with *Eco*RI, treated with mung bean nuclease, and ligated to *Sal* I linkers. The resulting *luxA* gene *Sal* I fragment, with all extra ATG codons removed, was sequenced in both directions.

The *luxB* gene was isolated as an *Ssp* I–*Pvu* II DNA fragment from the plasmid pTB7; following addition of *Bam*HI linkers to the 5′ and the 3′ ends, the fragment was inserted in both orientations into the *Bam*HI site of M13 mp18 and partially sequenced. The *Sal* I *luxA* cassette and the *Bam*HI *luxB* cassette were inserted in two steps into single *Sal* I and *Bam*HI sites of pPCV701, respectively. This resulted in plasmid pPCV701luxA&B and rendered *luxA* under gene *1′* and *luxB* under gene *2′* promoter control. pPCV701luxA&B was transformed into the *E. coli* strain SM10 and mobilized into *Agrobacterium* strain GV3101-(pMP90RK) as described (14).

Plant Transformation and Tissue Culture. *Agrobacterium* strain GV3101(pMP90RK) carrying plasmid pPCV701luxA&B was used in protoplast cocultivation (23, 24) and plant tissue infection experiments (25) to transfer the *luxA* and *luxB* genes, as well as the linked neomycin phosphotransferase selectable-marker gene, into tobacco and carrot cells. Conditions of tissue culture and tobacco (*Nicotiana tabacum*) plant regeneration were as described (14, 23, 24, 26). Protoplasts were isolated from the carrot (*Daucus carota*) cell line W001C. The protoplasts were purified and then suspended (10^7 per ml) in 0.37 M glucose/1.5 mM $CaCl_2$/10 mM 2-(*N*-morpholino)ethanesulfonate (pH 6.5), and 1.0-ml aliquots were transferred to a multiwell culture plate (27). Fifty micrograms of pPCV701luxA&B DNA was added to each well, and the protoplast/DNA mixture was subjected to electroporation (electric field-mediated DNA transfer) as described (28). The transformed protoplasts were cultured in K-3 medium at 26°C in the absence of light (27).

Luciferase Assay. The activity of luciferase was measured, by a luminometer, as the total light produced during the first 10 sec of enzymatic reaction (29). To calibrate each series of measurements, a titration curve showing the relationship between light emission and luciferase activity was established by measuring known amounts of commercially available *V. harveyi* luciferase (Sigma L-1637). Aliquots of 1:100 and 1:1000 dilutions of luciferase enzyme stock solution (1 mg/ml) were diluted with 0.5 ml of assay buffer (50 mM sodium phosphate, pH 7.0/50 mM 2-mercaptoethanol/0.2% bovine serum albumin), and the reaction was started by injection of a mixture of 0.5 ml of 100 μM reduced FMN and 10 μl of decanal substrate through the septum of the luminometer sample chamber. The FMN solution was prepared in 25 mM EDTA (pH 7.0) or in 200 mM tricine buffer (pH 7.0) and reduced by light (30). The substrate, 10% (vol/vol) decanal was prepared in 50 mM sodium phosphate (pH 7.0) buffer and used immediately after sonication (31). Linear titration plots were obtained in a concentration range of 1–50 ng of luciferase per ml of assay mixture. Due to the impurity of the commercially available luciferase preparation, the specific activity was about 1% that reported for purified *V. harveyi* luciferase (29).

Transformed protoplasts and cells were homogenized in Eppendorf tubes in assay buffer and centrifuged for 5 min. Aliquots of the cleared extracts were assayed for luciferase activity as described above.

The two FMN reduction methods (EDTA and tricine) gave comparable luciferase activities. Pretreatment of plant protoplasts, cells, and leaves showed that luciferase activity remained unchanged when incubated in tricine buffer but rapidly declined in the presence of EDTA. Therefore, to measure luciferase activities in extracts prepared from intact plant tissues, we used tricine to keep flavin in the reduced state.

Immunoblotting. The presence of luciferase α and β polypeptides (LuxA and LuxB) in transformed carrot protoplasts was detected by immunoblot analysis. Protoplasts were collected by centrifugation after electroporation and resuspended in 1.0 ml of luciferase assay buffer. Luciferase activity in the samples was determined as described above, and then the protein extracts were precipitated with ethanol at −20°C for 1 hr. The precipitated proteins were separated by electrophoresis in $NaDodSO_4$/10% polyacrylamide gels (32). The separated proteins were transferred to a nitrocellulose filter by electrophoresis, as described (33), and the blot was incubated at 26°C for 12 hr with anti-LuxA and anti-LuxB IgG. Unbound IgG was removed by washing the filter in 10 mM Tris·HCl, pH 7.4/0.9% NaCl, and the immunoblot was incubated for 6 hr with goat anti-rabbit IgG conjugated to alkaline phosphatase. The filter was washed to remove excess second antibody, and the luciferase α and β polypeptides were identified by incubating the blot in 10 mM Tris·HCl (pH 7.4) containing hydrogen peroxide and 4-chloro-1-naphthol.

RESULTS

Conversion of *luxAB* Transcriptional Unit into Separate Cassettes. In the *V. harveyi* genome, the *luxA* and *luxB* structural genes are part of a single transcriptional unit (1). In order to obtain expression and to permit correct translation of these genes in plant cells, it was necessary to separate the two genes and to remove possible translational initiation codons located in their 5′ untranslated leader sequences. Two separate "transcription–translation cassettes" were therefore constructed (Fig. 1). In the reconstruction of the *luxA* gene, 133 nucleotides, containing three nonessential ATG codons, were deleted from the 5′ leader sequence. The final construct resulted in a *luxA* cassette bordered by synthetic *Sal* I sites. The *Sal* I site on the 5′ end originated from the M13 mp18 polylinker sequence and is separated by 2 bp from the correct initiation codon. The added 3′ *Sal* I linker is located 58 bp downstream from the translational stop codon of the *luxA* gene. The *luxB* cassette has a 5′ *Bam*HI site separated by 23 bp from the first native ATG triplet and a 3′ *Bam*HI site 197 bp downstream from the stop codon. Alternative *luxA* and *luxB* cassettes were also constructed by ligating synthetic *Sal* I, *Hin*dIII, or *Bam*HI linkers at both the 5′ and the 3′ ends of *luxA* and *luxB* genes (data not shown).

Since the *luxAB* genes in *V. harveyi* are linked in one transcriptional unit, it was important to determine whether a functional luciferase could also be assembled when the individual subunits were translated from two separate transcriptional units. To answer this question, the *luxA* cassette was inserted into a pBR322-derived expression vector and transcribed under control of an upstream T7 promoter (34). Similarly, the *luxB* cassette was inserted into a pACYC184 derivative and transcribed by an identical T7 promoter. *E. coli* colonies containing both plasmids in the same cell

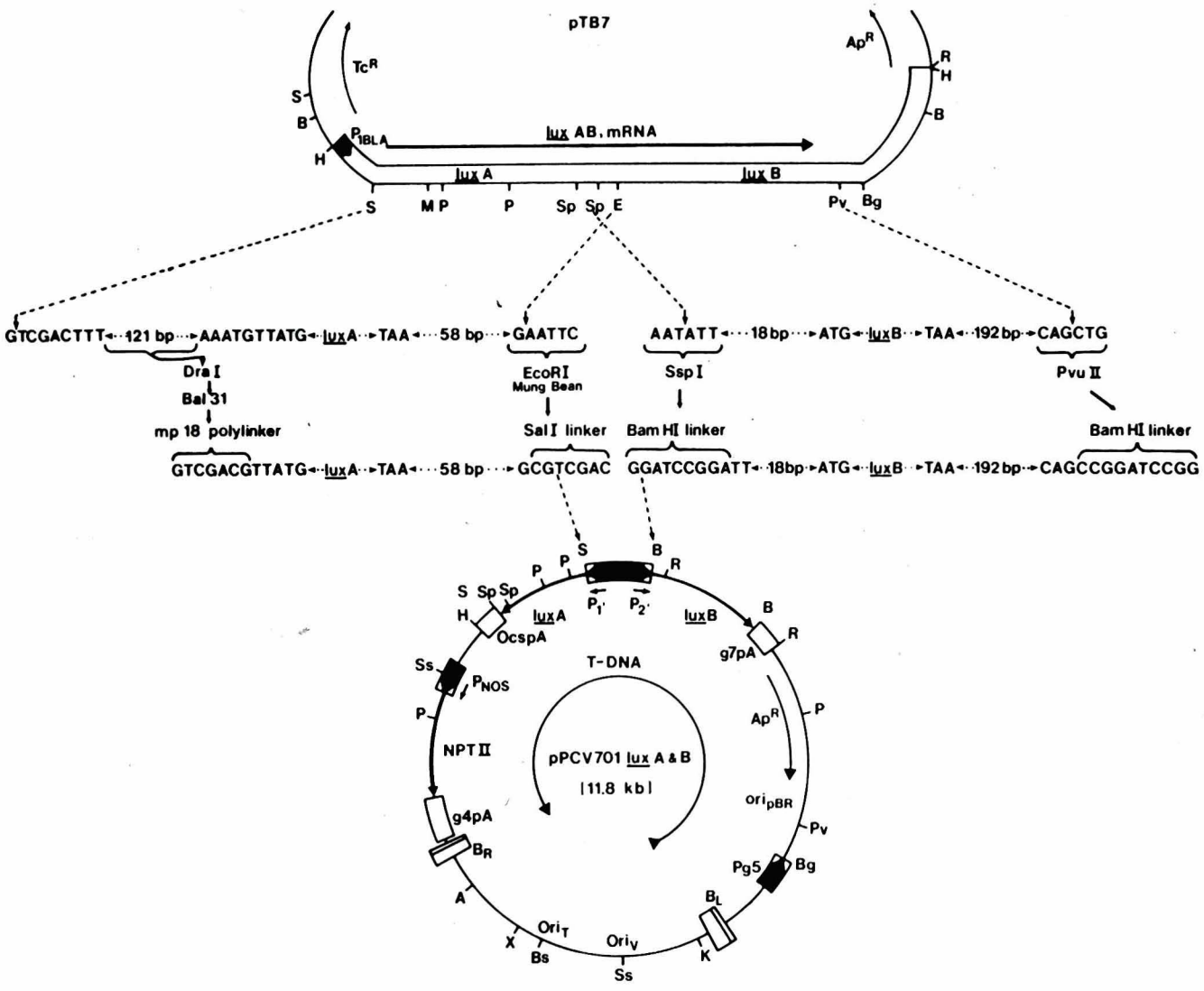

FIG. 1. Construction and cloning of *V. harveyi luxA* and *luxB* gene cassettes in plant expression vector pPCV701. Construction of plant vector pPCV701luxA&B, carrying *luxA* and *luxB* genes under transcriptional control of T_R-DNA promoters *1'* and *2'*, is described in *Materials and Methods*. Arrows indicate direction of transcription; P_{1BLA}, *P1* promoter of β-lactamase gene; $P_{1'}$ and $P_{2'}$, promoters of T_R-DNA genes *1'* and *2'*; P_{NOS}, nopaline synthase promoter; Pg5, promoter of T_L-DNA gene *5*; g4pA, OcspA, and g7pA, polyadenylylation sequences derived from T_L-DNA gene *4*, the octopine synthase gene, and gene *7*; NPTII, neomycin phosphotransferase gene; B_L and B_R, 25-bp left- and right-border repeats of T-DNA; ori_T and ori_V, replication and conjugational-transfer origin sequences derived from plasmid pRK2; ori_{pBR}, replication origin of pBR322; Ap^R and Tc^R, genes conferring resistance to ampicillin and tetracycline; T-DNA, transferred DNA; kb, kilobases. Restriction sites: A, *Apa* I; B, *Bam*HI; Bg, *Bgl* II; Bs, *Bst*EII; H, *Hin*dIII; K, *Kpn* I; M, *Mae* I; P, *Pst* I; Pv, *Pvu* II; R, *Eco*RI; S, *Sal* I; Ss, *Sst* II; Sp, *Ssp* I; X, *Xho* I.

exhibited high luciferase activity (unpublished data). Thus, when the α and β subunits of luciferase are translated from two different mRNAs, they can assemble to form a functional luciferase enzyme in *E. coli*.

Use of a Dual-Promoter Expression Vector Allowing Simultaneous Expression of *luxA* and *luxB* in Transgenic Plants. In order to transfer both *luxA* and *luxB* genes simultaneously into plant cells and to allow LuxA and LuxB proteins to be expressed, a plant expression vector was constructed from elements of available expression and binary cloning vectors.

The *luxA* cassette was inserted into the single *Sal* I site of the expression vector, and the *luxB* cassette was inserted into the *Bam*HI site; *luxA* and *luxB* thus were placed under the transcriptional control of the T_R-DNA *1'* and *2'* gene promoters, respectively (17). The resulting plasmid, designated pPCV701luxA&B, was mobilized from *E. coli* to *Agrobacterium* and transferred into tobacco and carrot cells by using protoplast cocultivation and leaf disk-infection methods (23–25). In addition, plasmid pPCV701luxA&B DNA was also used for transformation of tobacco and carrot protoplasts by electroporation (28).

Quantitative Assay of Luciferase in Plant Extracts. To determine whether functional luciferase can be quantitatively assayed in plant extracts, known amounts of commercially available *V. harveyi* luciferase were mixed with carrot and tobacco cell extracts and bioluminescence was measured. The light-emission values obtained were proportional to known amounts of luciferase in the presence or absence of plant extracts (data not shown). Furthermore, as little as 0.5 ng of the commercially available luciferase was clearly detectable in the assay. To check for the occurrence of proteolytic degradation of luciferase enzyme in plant extracts, selected amounts of commercially available luciferase were incubated with extracts prepared from tobacco and carrot cells for various times. No proteolysis of luciferase was detected. Therefore, the values obtained for luciferase activity represent an accurate estimate of the amount of luciferase protein present in the plant extracts. When a

standardized procedure is applied, it is possible to use bioluminescence as a quantitative and sensitive assay of luciferase activity in different plant extracts.

In order to determine how much, if any, luciferase activity could be contributed in various transformation experiments by the *Agrobacterium* strain harboring plasmid pPCV701luxA&B, cell cultures or sonicated cell extracts of this strain were assayed for luciferase activity (29). Luciferase activity was barely detectable, corresponding to less than 1.0 ng of luciferase per 10^6 cells. In comparison, an *E. coli* strain carrying plasmid pTB7 (1), in which the *luxAB* operon is controlled by the *P1* promoter of the pBR322 β-lactamase gene, produces 0.2–2.0 μg of luciferase per 10^6 cells (data not shown). The fact that luciferase expression was detected in *Agrobacterium* carrying plasmid pPCV701luxA&B was unexpected, as previous experiments did not demonstrate expression of T_R-DNA *1'* and *2'* promoters in *Agrobacterium*. This result emphasizes the great sensitivity of the luciferase assay. In spite of the low level of luciferase expression in *Agrobacterium*, particular care was taken to eliminate surviving *Agrobacterium* cells in transformed plant cultures prior to assay of luciferase activity.

Luciferase Activity in Transformed Plant Tissues. Luciferase activity was detected readily in the transformed plant tissues (Table 1). The great sensitivity of the luciferase assay allowed *luxA* and *-B* gene expression to be detected in carrot protoplasts as early as 8–24 hr after introduction of the DNA by electroporation (data not shown). As expected from the known properties of the bacterial enzyme, the activity of luciferase in plant extracts was also dependent on the addition of reduced FMN and the long-chain fatty aldehyde substrate decanal. Stably transformed carrot calli or tobacco plants emitted from 4000 to 26,000 light units per g (wet weight) of plant tissue (Table 1).

Expression of Luciferase in Transformed Plant Tissues Requires the Presence of Both *luxA* and *luxB* Products. Although the catalytic site for the luciferase activity is carried by the α subunit (LuxA), both LuxA and LuxB polypeptides must be properly assembled in order to obtain light emission by *E. coli* cells or extracts (5). It was conceivable, however, that LuxA might have independent luciferase activity in plants as a result of interaction(s) with unknown plant factor(s). To rule out this possibility, carrot protoplasts were transformed with plasmid pPCV701luxA, which carries the correct promoter-*luxA* gene fusion but not the *luxB* gene fusion. No luciferase activity was detected in transformed cells 24 hr or even 7 days after electroporation (Table 2). In the converse experiment, carrot protoplasts were transformed with plasmid pPCV701luxB, which carries only the correct promoter-*luxB* gene fusion. As expected, no luciferase activity was detected in the transformed cells (Table 2).

Table 2. Simultaneous expression of *luxA* and *luxB* is required for luciferase activity in plant cells

Addition(s) to extract	Activity, light units		
	pPCV701-luxA	pPCV701-luxB	pPCV701-luxA&B
None	5.2	3.1	17.0
Decanal	2.3	2.7	1.4
Decanal + $FMNH_2$	5.9	4.8	4280.0

Carrot W001C protoplasts were prepared and transformed as described in *Materials and Methods*. Values are expressed in light units per 10^7 protoplasts, extracted 24 hr after electroporation with 50 μg of the indicated plasmid DNA.

However, when both *luxA* and *luxB* genes were present on the same plasmid, luciferase activity was detected, indicating that LuxA and LuxB polypeptides can assemble to form a functional enzyme in plant cells. As further and definitive confirmation that both LuxA and LuxB polypeptides were present in transformed plant cells showing luciferase activity, extracts of carrot protoplasts were tested by immunoblot analysis 24 hr after transformation with pPCV701luxA&B DNA. Transformed carrot protoplasts were found to contain both luciferase subunits, in similar amounts (Fig. 2). In comparison, carrot protoplasts transformed only with the *luxA* or the *luxB* construct contained only LuxA or LuxB, respectively (data not shown). The amount of luciferase protein present in transformed protoplast extracts was estimated by comparison between measured light units and the light units emitted by a known amount of purified *V. harveyi* luciferase. It was reported (31) that 1.0 mg of purified luciferase emits $\approx 1.6 \times 10^{14}$ quanta of light per sec when decanal is used as substrate (31). Based on this value, the luminescence measured in an extract prepared from 10^7 transformed carrot protoplasts (Table 1, extract A) was 5.4×10^9 quanta per sec, which corresponds to 34 ng of luciferase. This amount of luciferase protein agrees well with the estimation from the intensity of the stained bands in the immunoblots.

DISCUSSION

The results described above give positive answers to two major questions: are transgenic plant cells able to correctly assemble complex heterodimeric bacterial enzymes, and can the bacterial luciferase enzyme be used as a convenient assay to monitor the expression of chimeric genes in plants?

A dual-promoter expression vector was used to separate the A and B cistrons of the *V. harveyi* luciferase operon into two separate plant transcription–translation units. After in-

Table 1. Luciferase activity in transformed plant tissues

Addition(s) to extract	Activity, light units*						
	Carrot					Tobacco	
	A	B	C	D	E	F	G
None	34.0	1.4	3.9	50.0	10.0	16.6	5.6
Decanal	32.0	1.7	1.4	50.0	10.0	16.6	10.4
Decanal + $FMNH_2$	4522.0	5516.0	1.2	26,065.0	114.0	4152.0	9.6

A, extract prepared from 10^7 carrot protoplasts 24 hr after electroporation with 50 μg of plasmid pPCV701luxA&B DNA; B, extract from 10^7 carrot cells 8 days after protoplasts were electroporated with pPCV701luxA&B; C, extract of 10^7 untransformed carrot protoplasts; D, extract of carrot cells (1 g wet weight) 8 days after electroporation with plasmid pPCV701luxA&B; E, extract of untransformed carrot cells (1 g wet weight) supplemented with 100 ng of commercial luciferase; F, extract of leaf tissue (1 g wet weight) of a tobacco plant transformed by *Agrobacterium* carrying plasmid pPCV701luxA&B; G, extract of leaf tissue (1 g wet weight) of an untransformed tobacco plant.

*1 light unit = 1.2×10^6 quanta/sec. Values obtained when reduced FMN ($FMNH_2$) was not added indicate that the level of endogenous $FMNH_2$ in tissue culture cells is not sufficient for *in situ* detection of luciferase activity.

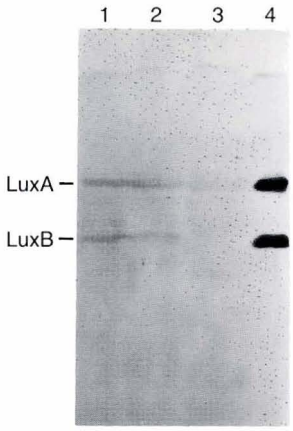

FIG. 2. Immunoblot analysis of LuxA and LuxB polypeptides in transformed carrot cells. Lanes 1 and 2: protein extracts equivalent to 2×10^6 and 4×10^6 carrot protoplasts transformed by electroporation with pPCV701luxA&B DNA. Lane 3: protein extract obtained from 4×10^6 untransformed (negative control) carrot protoplasts. Lane 4: 10 μg of commercial *V. harveyi* luciferase. Positions of LuxA and LuxB polypeptides in transformed carrot protoplasts (lanes 1 and 2) and in the positive control (lane 4) are indicated at left. No bands corresponding to LuxA and LuxB were detected in the untransformed carrot protoplast extract (lane 3).

troduction of the dual-promoter vector pPCV701luxA&B, into tobacco and carrot cells, both *luxA* and *luxB* were expressed: luciferase activity was readily detected by luminescence assay and the presence of the α and β subunits (LuxA and LuxB) was shown by immunoblotting. The two genes were expressed simultaneously and at similar levels in transformed plant cells. Luciferase activity was detected only in cells carrying genes for both subunits, thus excluding the possibility that the α subunit, which carries the catalytic site, could by itself be responsible for the observed luciferase activity. Our results therefore indicate that the α and β subunits of the bacterial luciferase enzyme were properly assembled in plant cells.

In view of the ease with which specific luciferase activity can be quantitatively detected in plant cell extracts, this enzyme appears to be a suitable "reporter" to monitor transcriptional regulation of chimeric genes and transcriptional activity of promoter 5'-upstream sequences in transgenic plants as well as in transient gene-expression assays. Establishment of accurate *in situ* measurements of gene activity during embryogenic or organogenic development should also be possible in intact plants, provided that their endogenous FMN synthesis can be induced and increased or that the reduced FMN cofactor can be exogenously supplied for the luciferase-mediated light reaction in plant cells.

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