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 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 oriV and oriT regions to the right 25-base-pair (bp) border sequence (B9), 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′-polyadenylation sequence of the T1–DNA gene (4, ref. 16; nucleotides 8840–9240 of the T1–DNA). This selectable-marker cassette was inserted between the HindIII and Bcl I sites of pPCV002 to yield plasmid pPCV002NKMA. An expression unit was assembled as follows: the BamHI–HindIII fragment of plasmid pAP2034 (17) was replaced by that of plasmid pOP44392 (18) to obtain plasmid pAPTR1′, in which the promoter of gene 2′ is linked to the polyadenylation sequence of the T1–DNA gene 7, derived from plasmid pAR2034 (18). After opening pAPTR1′ DNA by cleavage with Sal I, filling in the ends with T4 DNA polymerase, and HindIII digestion, the polyadenylation sequence of the T-DNA synthase gene was added from plasmid pAGV40 (8, 16) as a Pvu II–HindIII 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 EcoRI–HindIII 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 EcoRI diges-

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tion were subcloned into the Dra I–EcoRI 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–EcoRI fragment pool obtained from pBR322 recombinants was cloned into Sma I–EcoRI 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 digested with BamHI 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 SalI site of mp18. M13 replicative form DNA was isolated from one of these clones, digested with EcoRI, treated with mung bean nuclease, and ligated to SalI linkers. The resulting luxA gene SalI 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 BamHI linkers to the 5' and the 3' ends, the fragment was inserted in both orientations into the BamHI site of M13 mp18 and partially sequenced. The SalI luxA cassette and the BamHI luxB cassette were inserted in two steps into single SalI and BamHI sites of pCV701, 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-(pMPP90RK) as described (14).

**Plant Transformation and Tissue Culture.** Agrobacterium strain GV3101(pMPP90RK) 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 (106 per ml) in 0.37 M glucose/1.5 mM CaCl2/10 mM 2-(N-morpholino)ethanesulfonate (pH 6.5), and 1.0-ml aliquots were transferred to a multiwell culture plate (27). Fifty micromillimolar 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-1657). 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 NaDodSO4/10% 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 SalI sites. The SalI 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' SalI linker is located 58 bp downstream from the translational stop codon of luxA gene. The luxB cassette has a 5' BamHI site separated by 23 bp from the first native ATG triplet and a 3' BamHI site 197 bp downstream from the stop codon. Alternative luxA and luxB cassettes were also constructed by ligating synthetic SalI, HindIII, or BamHI 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
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 SalI site of the expression vector, and the luxB cassette was inserted into the BamHI site; luxA and luxB thus were placed under the transcriptional control of the Tn5-DNA 1' and 2' 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.
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 Tn5-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 lucA 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).

**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-
genes were expressed simultaneously and at similar levels in gene activity during embryogenic or organogenic development of plant cells. An enzyme appears to be a suitable "reporter" to monitor transcriptional regulation of chimeric genes and their endogenous counterparts 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).

Introduction 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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