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Genetics

High-frequency T-DNA-mediated gene tagging in plants

(insertional mutagenesis in plants/gene fusion/plasmid rescue/dicistronic transcript)

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ABSTRACT **An insertion element [transferred DNA (T-DNA)], transferred by soil agrobacteria into the nuclear genome of plants, was used for induction of gene fusions in *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Nicotiana plumbaginifolia*. A promoterless *aph*(3′)II (aminoglycoside phosphotransferase II) reporter gene was linked to the right end of the T-DNA and transformed into plants along with a plasmid replicon and a selectable hygromycin-resistance gene. Transcriptional and translational reporter gene fusions were identified by screening for APH(3′)II enzyme activity in diverse tissues of transgenic plants. The frequency of gene fusions, estimated by determination of the copy number of T-DNA insertions, showed that on average 30% of T-DNA inserts induced gene fusions in *Arabidopsis* and *Nicotiana*. Gene fusions were rescued from plants by transformation of the T-DNA-linked plasmid and flanking plant DNA into *Escherichia coli*. By dissection of gene fusions and construction of chimeric genes, callus- and root-specific promoters were identified that showed an altered tissue specificity in the presence of a 3′-downstream-located 35S promoter. Transcript mapping of a gene fusion and expression of a non-frame transcriptional fusion of bacterial luciferase *luxA* and *luxB* genes demonstrated that dicistronic transcripts are translated in tobacco.**

Defined phenolic compounds from plants activate the virulence genes of Ti plasmids in *Agrobacterium* (1, 2). In turn, these genes direct the processing and transfer of any DNA sequence flanked by specific 25-base-pair (bp) direct repeats into plants where the transferred DNA (T-DNA) is integrated into the nuclear genome. T-DNA can therefore be considered as a mutator element that can cause insertional inactivation of plant genes (3, 4).

Mutator T-DNAs carrying a selectable hygromycin-resistance gene, a plasmid replicon, and a promoterless *aph*(3′)II reporter gene (aminoglycoside phosphotransferase II gene of transposon Tn*5*; ref. 5) linked to the right end of the T-DNA were transformed to *Arabidopsis thaliana*, *Nicotiana plumbaginifolia*, and *Nicotiana tabacum* by *Agrobacterium tumefaciens*. To ascertain the proportion of T-DNA insertions leading to gene fusions in transgenic plants, callus, leaf, stem, and root tissues were screened for expression of the *aph*(3′)II reporter gene, and the number of such transformants was related to the total number of T-DNA insertions resulting from these experiments.

T-DNA mutated genes were rescued from transgenic plants in order to demonstrate that the T-DNA insertion was actually responsible for the observed tissue-specific expression of induced gene fusions. The analysis of gene fusions furthermore demonstrated that dicistronic transcripts are translated in plants.

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MATERIALS AND METHODS

Construction of Plant Gene Fusion and Promoter Test Vectors. A schematic map and construction of T-DNA vectors are outlined in Fig. 1. DNA manipulations, ligation of synthetic oligonucleotide linkers, and isolation of plasmid DNAs were as described (11).

Plant Transformation and Determination of the Copy Number of T-DNA Insertions. Protoplasts and leaf-discs prepared from *Nicotiana tabacum* SR1 and *Nicotiana plumbaginifolia* Viviani were cocultivated with agrobacteria and, following selection of transformed calli and shoots, regenerated to fertile transgenic plants as described (12). *Arabidopsis thaliana* var. Columbia stem, leaf, and root explants were transformed and regenerated as described (13). One hundred to 300 seeds, collected after self-pollination from each transgenic *Arabidopsis* and *Nicotiana* plant, were germinated in the presence of hygromycin (15 mg/liter) or kanamycin (100 mg/liter) to follow the segregation of the T-DNA-encoded antibiotic-resistance marker. From antibiotic-resistant T2 progenies DNA was purified and the copy number of T-DNA insertions was determined by Southern DNA hybridization using *ocs*, *aph*(3′)II, and pBR322 sequences of the T-DNA as probes (6, 11).

T-DNA Rescue, Isolation, and Characterization of Gene Fusions. Callus, leaf, stem, and root tissues of transgenic plants were screened for expression of gene fusions by APH(3′)II enzyme gel assay as described (14). Gene fusions were isolated from plants carrying single T-DNA inserts. Five micrograms of *Arabidopsis* or 25 μg of *Nicotiana* nuclear DNA was digested with *Hin*dIII or *Xba* I, self-ligated at a concentration of 20 μg/ml, and transformed into *Escherichia coli* DH1 competent cells (15). From plasmids containing the rescued T-DNA and circular permutation of flanking plant DNA sequences, fragments were subcloned in M13mp18, as well as in pUC18 and pUC19 vectors to determine their nucleotide sequence (16), and used as probes for physical mapping of T-DNA mutated loci by Southern DNA hybridization.

Putative promoter sequences located 5′ upstream of the *aph*(3′)II reporter gene of rescued gene fusions were cloned upstream of a new *aph*(3′)II gene in promoter test vectors pPCV801 and pPCV811 and transformed into tobacco and *Arabidopsis*. The expression of chimeric genes was tested by APH(3′)II assays in at least five transgenic plants, from which poly(A)$^+$ RNA was purified as described (6). The initiation site of transcription of chimeric genes was determined by primer extension using an oligonucleotide (5′-

Abbreviations: *ocs*, octopine synthase; *nos*, nopaline synthase; *mas*, mannopine synthase; *ipt*, isopentenyltransferase; CaMV, cauliflower mosaic virus; APH(3′)II, aminoglycoside (kanamycin) phosphotransferase; T-DNA, transferred DNA; T_L-DNA, T-DNA from Ti plasmid Ach5.

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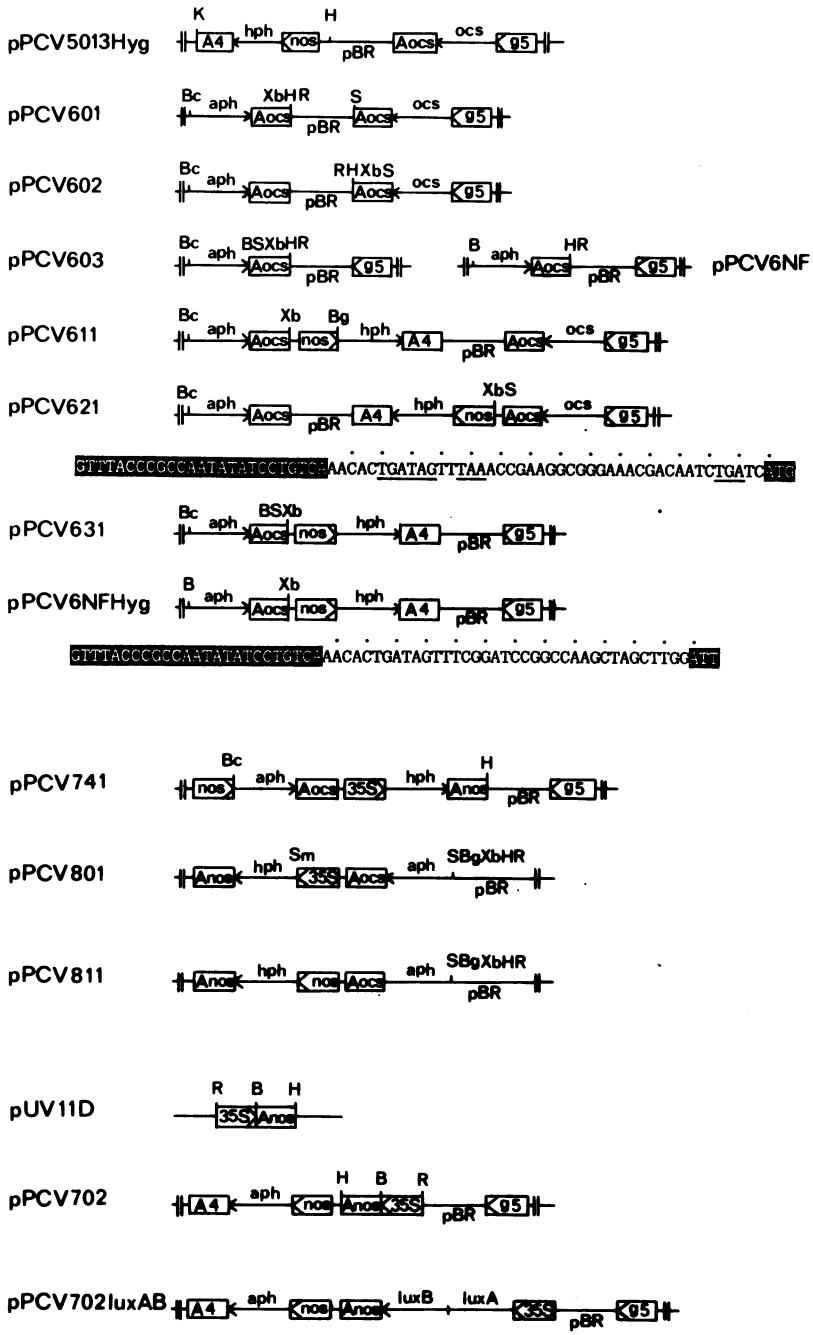

FIG. 1. Construction of T-DNA vectors. All T-DNA constructs are carried by a replicator cassette of plant cloning vectors as described (6). Maps show schematically the structure of T-DNAs. Vertical bars represent the right (at the left) and left (at the right) 25-bp direct repeats at the T-DNA ends. Junction sequences between the right 25-bp repeat and the coding region of *aph*(3')II reporter gene are shown below the maps of pPCV621 and pPCV6NFHyg T-DNAs. Dots above the sequence mark the *aph*(3')II reading frame. The first *aph*(3')II codon and the 25-bp repeat are printed in black; stop codons are underlined. Open boxes show transcription terminator sequences derived from the octopine synthase (Aocs), nopaline synthase (Anos), and *ipt* (A4) genes. "Arrowheaded" boxes correspond to promoters of the nopaline synthase gene (*nos*), T_L-DNA gene 5 (where T_L-DNA is T-DNA from Ti plasmid Ach5) (*g5*), and cauliflower mosaic virus (CaMV) 35S transcript (35S). Single restriction endonuclease cleavage sites within the T-DNA and sites used in constructions are as follows: *Bcl* I, Bc; *Bam*HI, B; *Bgl* II, Bg; *Hin*dIII, H; *Kpn* I, K; *Eco*RI, R; *Sal* I, S; *Sma* I, Sm; and *Xba* I, Xb. The position of pBR322 replicon is shown as pBR. *luxA* and *luxB* are bacterial luciferase genes. Arrows indicate the polarity of transcription. Gene fusion vectors pPCV611, pPCV621, and pPCV631 were constructed by insertion of a *hph* gene cassette from pPCV5013Hyg as a *Hin*dIII–*Kpn* I fragment into the *Hin*dIII site of plasmids pPCV601, pPCV602, and pPCV603, respectively. pPCV6NFHyg was obtained by replacing the *Xba* I–*Xho* I fragment of pPCV631 with the *Hin*dIII–*Xho* I fragment of plasmid pPCV6NF. To construct promoter test vector pPCV801, the CaMV 35S promoter-*hph* gene-*nos* terminator and joint *aph*(3')II gene-*ocs* terminator cassettes were excised from pPCV741 and inserted into plasmid pPCV002 (6), from which the promoter of T_L-DNA gene 5 was previously removed. Plasmid pPCV811 was obtained by replacing the CaMV 35S promoter-*aph*(3')II cassette of pPCV801 with the *Bcl* I–*Bgl* II fragment of pPCV611 carrying *aph*(3')II and *nos* promoter sequences. Expression vector pPCV702 was constructed by insertion of the CaMV 35S promoter-*nos* terminator cassette from pUV11D (kindly provided by P. Van den Elzen) into the *Eco*RI–*Hin*dIII sites of plasmid pPCV701 (7). *luxAB* operon was assembled by ligation of the *Sal* I–*Eco*RI *luxA* fragment of pLX109a DNA (8) and *Hin*dIII–*Eco*RI *luxB* fragment of pTB7 DNA (9) into *Bam*HI–*Hin*dIII sites of vector pUC19 (10). To construct pPCV702luxAB, the *luxAB* operon, in which 26 bp separate the *luxA* and *luxB* coding sequences, was inserted into the *Bam*HI site of pPCV702.

AGAACCTGCGTGCAATCCAT-3') that hybridized to the 5' end of the *aph*(3')II coding region (17).

Luciferase activities were determined in diverse tissues of pPCV702luxAB-transformed tobacco plants as described (7) using a homogeneous enzyme as standard.

RESULTS

Induction of Gene Fusions by T-DNA Insertions in Plants. To detect T-DNA insertions in transcribed genomic loci, a promoterless *aph*(3')II reporter gene was fused to the right end of the T-DNA of plant gene fusion vectors pPCV621 and pPCV6NFHyg (Fig. 1). Both T-DNAs were provided with a chimeric hygromycin-resistance gene and with a pBR322 plasmid replicon to facilitate the selection of transformed plant cells and the recovery of T-DNA tagged genes in *E. coli*.

In pPCV6NFHyg the ATG codon of the *aph*(3')II reporter gene was deleted, whereas in pPCV621 two in-frame stop codons were inserted upstream of the ATG triplet. The synthesis of an enzymatically active APH(3')II fusion protein in pPCV6NFHyg-transformed plants thus implied that the T-DNA induced a translational gene fusion by integrating into the protein coding region of a host gene. In pPCV621-transformed plants, on the other hand, the APH(3')II activity was indicative of transcriptional gene fusions resulting from insertion either in a coding region or in nontranslated sequences of transcribed loci. Both T-DNAs were transferred from *Agrobacterium tumefaciens* to *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Nicotiana plumbaginifolia* plants. From independent hygromycin-resistant transformants selected as calli in tissue culture, 510 *Arabidopsis*, 200 tobacco, and 200 *Nicotiana plumbaginifolia* seed-bearing plants were regenerated.

High Frequency of T-DNA-Induced Gene Fusions. Gene fusions were identified by screening for APH(3')II activity in callus, as well as in leaf, stem, and root tissues, of hygromycin-resistant T2 progenies of transformed plants. The copy number of T-DNA insertions was determined either by

following the segregation of the T-DNA-encoded hygromycin-resistance marker in T2 progenies of self-pollinated plants or by Southern hybridization of plant DNAs using probes derived from the left and right ends of T-DNAs. Data summarized in Table 1 show that gene fusions expressed in calli, as well as in one or more vegetative organs of transgenic plants, were detected in about 25% of all transformants in both *Arabidopsis* and *Nicotiana*. Transgenic plants carrying 1, 2, or more T-DNA inserts showed a roughly equal (1:1:1) distribution. The ratio of gene fusions to total T-DNA inserts decreased when plants carrying 1, 2, or more insertions were compared. This indicated that the APH(3')II enzyme assay cannot resolve single from multiple gene fusions. Nevertheless, the high proportion of transcriptional and translational gene fusions indicated that the T-DNA frequently integrated into transcribed regions of *Arabidopsis* and *Nicotiana* genomes. In 9 of 18 tobacco transformants, which did not express the *aph*(3')II gene in callus and vegetative organs, APH(3')II activity was detected in diverse flower organs. This suggested that an ongoing transcription of the target locus is probably not required for T-DNA integration and that the actual proportion of gene fusions might even be higher since our assay focused on few organs at certain developmental stages only.

Isolation of Gene Fusions by T-DNA Rescue. Gene fusions induced by single pPCV621 T-DNA insertions were isolated from transgenic plants by plasmid rescue. Nuclear DNAs prepared from hygromycin-resistant T2 progenies of *Arabidopsis* and tobacco transformants were digested with an endonuclease that had no or only a single recognition site within the T-DNA. Following ligation, the self-circularized DNA fragments containing the pBR322 replicon of the T-DNA, as well as circular permutation of flanking plant DNA sequences, were rescued by transformation in *Escherichia coli*.

From an *Arabidopsis* transformant, Ath621-37, which displayed reporter gene activity in callus and root, the T-DNA was rescued as a tag in a genomic *Hin*dIII DNA fragment of 1.9 kilobases (kb). The nucleotide sequence of a 0.9-kb *Hin*dIII–*Bcl* I fragment and of a 1.1-kb *Cla* I fragment flanking the T-DNA in the rescued plasmid was determined and showed that the integration of the T-DNA occurred by retaining 15 bp from the left and 1 bp from the right 25-bp direct repeats.

Table 1. Proportion of transgenic plants expressing T-DNA-induced *aph*(3')II gene fusions

	Arabidopsis thaliana		*Nicotiana tabacum*		*Nicotiana plumbaginifolia*	
Type of gene fusion	TF	PF	TF	PF	TF	PF
Callus sample	179	20	35	20	50	20
aph$^+$	48	5	8	4	10	4
Plant sample aph$^+$	77	123	50	25	50	—
L	4	5	1	1	2	—
S	1	10	1	2	2	—
R	—	1	1	—	—	—
LS	2	4	—	—	1	—
SR	4	4	3	1	2	—
LR	2	3	2	—	3	—
LSR	12	11	4	3	4	—
Total aph$^+$	25	38	12	7	14	—
aph$^+$/T-DNA copy						
1 copy	5/9	8/30	3/6	1/5	ND	—
2 copies	2/8	10/27	2/5	3/5	ND	—
3 or more copies	4/9	13/42	3/6	3/5	ND	—

TF, transcriptional gene fusion induced by pPCV621 T-DNA; PF, translational gene fusion generated by pPCV6NFHyg T-DNA; aph$^+$, APH(3')II enzyme activity in a given tissue or organ; L, leaf; S, stem; R, root; LS, leaf and stem; SR, stem and root; LR, shoot and root apices; LSR, organs with vascular tissues; aph$^+$/T-DNA copy, proportion of aph$^+$ transformants among plants carrying 1, 2, or more T-DNA insertions; ND, not determined.

To demonstrate that the rescued clone carries a functional plant promoter, overlapping fragments located 5' upstream of the reporter *aph*(3')II gene were inserted in promoter test vectors pPCV801 and pPCV811. New chimeric *aph*(3')II genes carrying either a 0.38-kb *Xba* I–*Bcl* I fragment (Fig. 2*a*) or a 0.9-kb *Hin*dIII–*Bcl* I fragment were coupled 5' to a selectable hygromycin gene driven by the *nos* promoter in pPCV811 or by the CaMV 35S promoter in pPCV801 and transferred into *Arabidopsis* and tobacco.

Distribution of the APH(3')II activity in plants transformed by pPCV811 constructs showed that the DNA sequence extending 0.9 kb upstream of the right T-DNA border of the rescued gene fusion contains a promoter and all signals necessary for callus–root-specific expression of chimeric genes in *Arabidopsis* and tobacco (Fig. 2*b*). Chimeric genes carrying the shorter 0.38-kb fragment also showed expression in callus and root but were also active in stem and petioles. Neither of these genes was expressed in leaves. In contrast, pPCV801 constructs conferred a high level of expression in leaf and stem, as well as in callus and root tissues (Fig. 2*c*). This indicated that the CaMV 35S promoter located 3' downstream altered the expression of chimeric genes in a cis-dominant fashion.

Dicistronic Gene Fusions Are Translated in Tobacco. From a tobacco transformant, Nt621-9, that expressed APH(3')II activity during late development of root, a plasmid containing the right arm of the T-DNA and linked plant DNA was rescued. The nucleotide sequence of the plant DNA–T-DNA junction revealed that the last nucleotide of the right 25-bp repeat was retained by the T-DNA insertion. Plant DNA sequences located upstream of the *aph*(3')II gene were isolated from the rescued clone as a 1.8-kb *Eco*RI–*Bcl* I fragment and inserted into promoter test vectors pPCV801 and pPCV811 to identify transcriptional regulatory sequences. Plants transformed by the pPCV811 construct displayed APH(3')II activity only in roots, whereas the analogous pPCV801 construct carrying the CaMV 35S promoter conferred *aph*(3')II expression in all vegetative organs. Mapping of the 5' end of the fusion *aph*(3')II transcript indicated that the original gene fusion and the constructed chimeric genes synthesized a dicistronic transcript (data not shown, Fig. 3*a*). The first cistron encoded a truncated plant protein and terminated in the T-DNA 33 bp upstream of the *aph*(3')II gene, which represented the second cistron.

To demonstrate that similar dicistronic transcripts are translated in plants, bacterial luciferase *luxA* and *luxB* genes separated by 26 bp were transformed into tobacco plants under transcriptional control of the CaMV 35S promoter in vector pPCV702luxAB. Following the structural analysis of the T-DNA and detection of *luxAB* transcript in pPCV702-luxAB-transformed plants by DNA and RNA filter hybridization, the synthesis of luciferase enzyme was analyzed by luminometric measurement of enzyme activity and by immunoblot analysis of leaf proteins. Immunoblotting of fractionated leaf proteins indicated that both luciferase subunits were translated in plants from the dicistronic construct (Fig. 3*b*). In comparison to pPCV701luxA&B-transformed plants (7), in which separated *luxA* and *luxB* genes were transcribed from *mas* 1' and 2' promoters, pPCV702luxAB transformants expressed 50- to 100-fold lower luciferase activities in leaves.

DISCUSSION

The main goal of the present investigation was to develop a reliable genetic approach for efficient isolation of T-DNA-induced plant gene mutations. Recently we have ex-

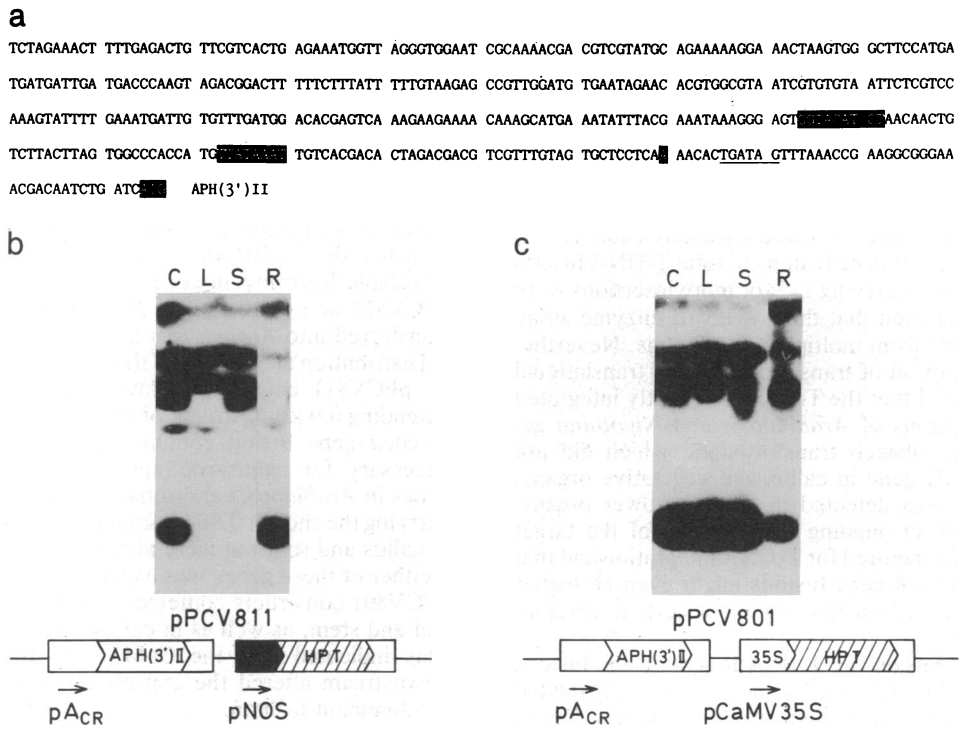

FIG. 2. Isolation of an *Arabidopsis* callus–root-specific gene fusion. (*a*) Nucleotide sequence of a 0.38-kb *Xba* I–*Bcl* I fragment carrying the junction between the right end of the T-DNA and plant DNA in a plasmid rescued from *Arabidopsis* transformant Ath621-37. The ATG codon of the *aph*(3′)II gene, the last nucleotide of a T-DNA border 25-bp repeat, and putative TATA and CAAT sequences are printed in black background. Stop codons in frame with the ATG codon of *aph*(3′)II gene are underlined. (*b*) A 0.9-kb *Hin*dIII–*Bcl* I fragment carrying a callus–root-specific promoter (pA_{CR}) from gene fusion Ath621-37 was cloned in the promoter test vector pPCV811 and transformed into tobacco and *Arabidopsis*. The autoradiograph shows an APH(3′)II gel assay of callus (C), leaf (L), stem (S), and root (R) extracts prepared from a transformed *Arabidopsis* plant. Double bands at the top correspond to protein kinases, whereas kanamycin phosphate spots closer to the bottom display APH(3′)II activities. (*c*) The pA_{CR} callus–root-specific promoter-driven *aph*(3′)II gene in promoter test vector pPCV801 shows expression in all vegetative organs. Abbreviations are as in *b*.

plored a gene fusion technique in order to select for T-DNA insertions in functional plant genes (20). A promoterless *aph*(3′)II gene was linked to the right end of the T-DNA and kanamycin-resistant transformants were selected in tobacco and *Nicotiana plumbaginifolia* tissue cultures. Characterization of these gene fusions demonstrated that selection for tissues expressing a promoterless reporter gene not only limits the type of detected gene fusions to those expressed in tissue culture but also results in aberrant and multiple T-DNA insertions that impede the genetic analysis (unpublished).

To overcome this problem, the mutator T-DNAs were supplemented with a *nos* promoter-driven hygromycin-resistance gene. Hygromycin-resistant transgenic plants were obtained by transformation and gene fusions were identified by screening for the expression of a promoterless *aph*(3′)II reporter gene in vegetative organs. The results described above show that this modified approach led to gene fusions in at least 25% of all transformed plants, which carried intact T-DNA insertions at a low copy number.

Although the low sample size prevented a statistical evaluation, the results indicated that T-DNA insertions in *Arabidopsis* and tobacco induced transcriptional and translational gene fusions at similar frequencies. This is intriguing because the genome organization of these species is strikingly different. *Arabidopsis* has a small genome (7×10^7 bp) with a low content of repeated DNA, whereas the tobacco genome is large (1.5×10^9 bp) and consists of 60% repeated and 40% single-copy DNA (21, 22). The transcript complexity of tobacco is 7×10^7 nucleotides, which corresponds to 60,000 genes and to 11% of single-copy DNA (23). DNA of similar size, equivalent to the genome size in *Arabidopsis*, was calculated to encode 15,000 genes (24). Due to differences in the density and distributions of transcribed sequences, it is not conceivable that a similar frequency of gene fusions can be obtained in both species by random T-DNA insertions. Rather, this suggests that the T-DNA is preferentially integrated into sequences that potentially can be transcribed.

T-DNA-mutated plant genes were recovered from plants by a simple plasmid rescue. By construction of new chimeric genes, it was demonstrated that gene fusions linked the reporter gene to functional plant promoters, the activity of which showed tissue-specific regulation. In pairwise combination with the CaMV 35S promoter, the isolated plant promoters displayed an altered specificity. It has been reported that the CaMV 35S promoter contains a positive enhancer element that activates transcription in a distance-dependent manner when placed upstream of other promoters (25). Our data indicate that this also happens when the CaMV 35S promoter is located 3′ downstream of diverse genes, indicating that particular care has to be taken when constructing promoter test vectors.

The analysis of a tobacco gene fusion and the expression of joint *luxA* and *luxB* bacterial luciferase genes showed that dicistronic transcripts are translated in plants. Detection of Luxα and Luxβ subunit proteins argues against the notion that the measured luciferase expression results from synthesis of a fusion luciferase by combined frameshift and read-through translation. If such a fusion luciferase was synthesized, it is unlikely that a proteolytic degradation would lead to intact subunits. Fusion luciferases, obtained by deletion of the stop codon of *luxA* and altering of the reading frame, as well as individual Luxα and Luxβ subunits, were shown to be stable in tobacco (7, 26).

An ultimate goal of T-DNA insertional mutagenesis is to isolate developmental or metabolic mutations and thereby genes that cannot be cloned by conventional methods. The

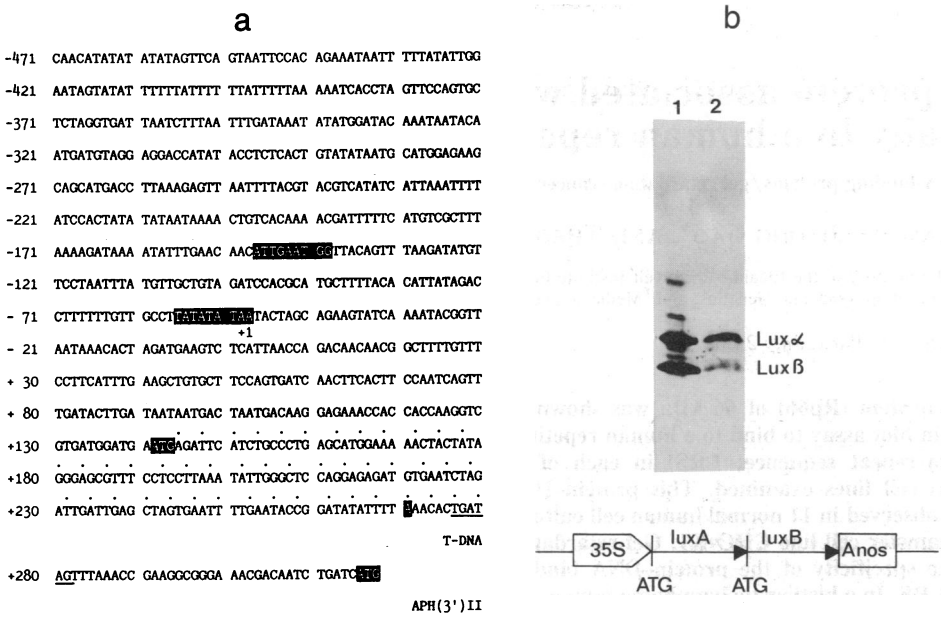

FIG. 3. Expression of dicistronic gene fusions in tobacco. (*a*) Nucleotide sequence of the T-DNA proximal part of a 1.8-kb *Eco*RI–*Bcl* I fragment carrying a root-specific promoter rescued from transgenic tobacco plant Nt621-9. The transcriptional start site determined by primer extension is indicated by +1. Dots above the sequence mark the reading frame of the first cistron that terminates at the underlined stop codons. ATG codons of both cistrons, the last nucleotide of the right 25-bp T-DNA border repeat, and putative TATA and CAAT sequences are printed in black background. (*b*) Expression of dicistronic *luxAB* genes in tobacco. Leaf extracts were prepared from pPCV702luxAB- and pPCV701luxA&B-transformed plants of similar age, and luciferase activities were determined luminometrically. Measured values, expressed in light units (LU)/mg of protein, were 0.5–2 × 10^6 LU/mg in pPCV701luxA&B plants and 0.5–4 × 10^4 LU/mg in pPCV702luxAB plants. Five LU was equivalent to 1 pg of luciferase, as determined by calibrations with a homogeneous enzyme. Leaves from pPCV702luxAB plants were homogenized in extraction buffer (0.2 M Tris·HCl, pH 8.0/0.1 M NaCl/0.4 M sucrose/0.01 M EDTA/0.014 M 2-mercaptoethanol/0.001 M phenylmethylsulfonyl fluoride) on ice, cleared by centrifugation at 100,000 × *g* (Beckman SW 50.1 rotor) for 30 min, and fractionated by 9% SDS/PAGE (18). Fractions containing the luciferase were eluted, concentrated by chloroform/methanol precipitation (19), and subjected to immunoblot analysis as described (7). Lane 1, 150 ng of purified bacterial luciferase; lane 2, protein extract purified from pPCV702luxAB-transformed tobacco plants.

frequency of observable gene mutations is however much dependent on the tissue culture steps involved in T-DNA transformation, which can themselves induce somatic mutations. The identification of T-DNA-induced mutations therefore must include a rigorous genetic linkage and complementation analysis. As an example of a morphologically visible mutation, one pale mutation was identified among the T2 progenies of 450 *Arabidopsis* transformants. This pale mutation cosegregated with the T-DNA-encoded hygromycin-resistance marker. Characterization of the gene that corresponds to the pale mutation and encodes a protein of 46.251 D will be reported elsewhere.

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