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GENETIC TOOLS FOR THE ANALYSIS OF GENE EXPRESSION IN PLANTS

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INTRODUCTION

The analysis of gene expression in plants is often based on correlations between data obtained by a variety of means: biochemical, physiological, and molecular genetic studies on the one hand, and classical genetic tools on the other hand. Among the techniques applied to the study of plant genes are screening of various genomic and complementary DNA (cDNA) libraries by synthetic oligonucleotides, gene- or organ-specific cDNAs, or antibody probes, in vitro translation of hybrid-released mRNAs, two-dimensional protein gel electrophoresis, immunoblotting, and transcript mapping (7,10). However, for an in depth understanding of how certain genes are regulated, alternative approaches are needed.

The method of transposon gene tagging provides a straightforward approach to molecular studies (27). Nevertheless, for the vast majority of isolated genes the usual method for studying gene regulation has been the identification of cis-regulatory sequences involved in the modulation of transcription of genes introduced in transgenic (host or nonhost) plants. To achieve this, chimeric genes containing an appropriate reporter gene, the expression of which is regulated by sequences under study, have to be reintroduced into plant cells. Two general transformation methods are currently used for this purpose.

For transient gene expression assays, the methods mainly used have been direct DNA transformation and electroporation (11,29). When organ-specific gene expression has to be studied, however, the Agrobacterium-mediated gene transfer system has been found to be the method of choice (9,28). Since the DNA transfer from agrobacteria to plant cells occurs in a regulated fashion, this system allows the use of techniques of bacterial genetics for the analysis of gene expression in plants. In this chapter we intend to explore some of these possibilities.

NEW DEVELOPMENTS IN AGROBACTERIUM-PLANT GENE TRANSFER SYSTEMS

Agrobacterium Ti plasmids represent a naturally evolved plant gene transfer system. A defined segment of such plasmids, called transfer DNA (T-DNA), which is bordered by 25-bp repeated sequences, is transferred from agrobacteria to the plant cell genome as a result of an interaction between these organisms (36). Bacterial genes, induced by specific metabolites of plant cell wall biosynthesis, are located in the virulence (Vir) region of Ti plasmids and govern this T-DNA transfer process (32). The products of some of these Vir genes (e.g., VirD) specifically recognize the 25-bp T-DNA border repeats, independently of whether these border sequences are linked or unlinked to the Vir loci. As a result of this specific interaction, a T-DNA intermediate is formed which is transferred to plant cells and integrated in their nuclear DNA by an as yet unknown mechanism (31).

Agrobacterium Ti plasmids were adapted for gene transfer purposes. Some of the more recent Ti plasmid-derived plant vector systems, referred to as binary systems, have two elements: a T-DNA-deficient helper Ti plasmid, providing virulence functions, and a broad host range vector, carrying cloning sites and marker genes for identification of transformed plant cells. The position of the T-DNA segment in these vectors is determined by the location and polarity of the 25-bp repeated sequences (1,5,19,20,33).

We developed such a binary vector system having a number of novel features: the different elements involved in plant gene transfer vectors, such as plant selectable or screenable marker genes flanked by 25-bp T-DNA border repeats, cloning sites, appropriate bacterial markers, and broad host range plasmid replication and mobilization functions, were all united in a single, small plant vector cassette (Fig. 1). The basic element of this cassette is a conditional mini-RK2 replicon which is maintained and mobilized by trans-acting functions derived from plasmid RK2. The trans-acting functions were introduced into both Escherichia coli and Agrobacterium hosts. This vector cassette can be inserted easily in various plasmids, in transposons, and in phage derivatives, which all thereby acquire plant gene vector functions. Genetic elements introduced between the 25-bp repeats of the cassette become part of the T-DNA unit, while others inserted outside of this segment provide other relevant functions (e.g., as carrier replicons).

Such combinations allow for the easy construction of plant gene vectors with different properties. The genes to be analyzed in plants are first introduced in the appropriate vectors, which can subsequently be mobilized from E. coli to various Agrobacterium hosts that provide RK2 helper functions for vector maintenance. A further practical advantage provided by this system is that the vectors can also be mobilized back from Agrobacterium to E. coli, due to the presence of RK2 plasmid mobilization functions in Agrobacterium hosts. This facilitates the analysis of

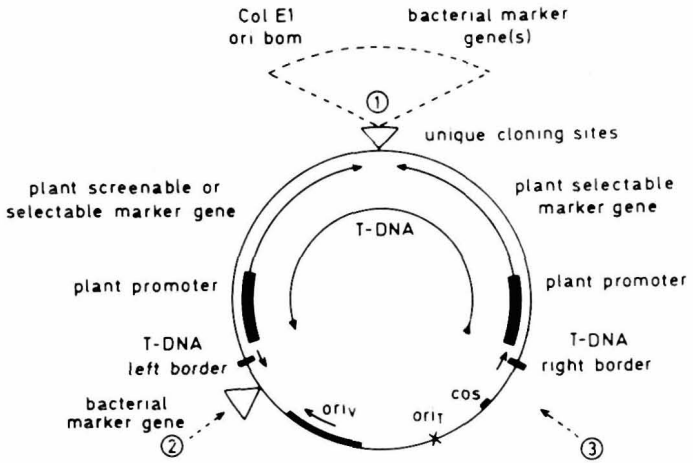

Fig. 1. Schematic design of plant vector cassettes. Position of the T-DNA unit, polarity of T-DNA border sequences, and direction of replication initiated at RK2 ori_V sequences are labeled by arrows inside the circle. Arrows 1, 2, and 3 show unique restriction endonuclease cleavage sites which can be used to introduce new elements into the cassettes. Notation "cos" designates the assembly recognition sequence for bacteriophage λ; ori_T is required for conjugative transfer of the plasmid under control of RK2.

the structural integrity of recombinant vectors prior to plant transformation. The detailed use of this system was recently described (20).

PLASMID VECTORS

A series of plasmid vectors was constructed by combining various pBR322 derivatives with a plant vector cassette containing a chimeric neomycin phosphotransferase (NPTII) gene as a selectable marker. These vectors were functionally characterized by transformation of Nicotiana tabacum, N. plumbaginifolia, Medicago varia, and Arabidopsis thaliana plants. The majority of transformed plants, obtained using different vectors and selected on kanamycin-containing media after protoplast co-cultivation and tissue infection experiments, were shown to carry only single T-DNA insertions. In plants containing multiple insertions the different T-DNA segments were not found to be in a tandem array. No alteration of the internal structure of the T-DNAs was detected in the transformed plants, indicating that the T-DNA units of the vectors were transferred and integrated without rearrangements.

To illustrate the application of these vectors, the expression of two T-DNA-derived genes, the nopaline synthetase (NOS) gene and gene 5, was compared in transgenic plants. The analysis of gene 5 expression was of interest because the function of this T_L-DNA-encoded gene of pTiAch5 is not yet known. This gene is thought to be expressed at a low level because its transcript is barely detectable in tumors (37). The promoter of both genes was fused to an enzymatic promoter probe and vectors harboring fusions with both promoters were constructed. The promoter probe genes were similar in size and coupled to identical 3' polyadenylation sequences. The analysis

of a large number of transformants showed that the amount of steady-state transcripts derived from the nopaline synthetase promoter is only twice as much as that for the promoter of gene 5. This observation indicated that the low steady-state level of gene 5 transcript in octopine crown gall tumors is not due to weak promoter activity. In nopaline crown gall tumors, it was previously shown that the level of gene 5 transcript is similar to that of the nopaline synthetase gene (37). In view of the fact that the overall homology between the DNA sequences of gene 5 in octopine and nopaline T-DNA regions is very high in the coding regions as well as in the 5' upstream regions, but breaks down 42 bp before the first polyadenylation site, it is conceivable that the 3' end of gene 5 may influence the steady-state level of its transcript in octopine-type tumors. Transcript analysis and comparison of the expression of marker enzymes indicated that the promoter of gene 5 is active in a tissue-specific fashion, while the nopaline synthetase promoter is constitutive and active in all tissues.

The gene 5 promoter is callus/stem-specific. The expression of gene 5 promoter fusions is barely detectable in fully developed leaves; however, it can be fully restored by treating leaf tissue with high auxin/low cytokinin hormone combinations. Conversely, a high cytokinin/low auxin treatment diminishes the gene 5 promoter activity in callus and stem tissues. This observation encouraged a more detailed analysis of the gene 5 promoter and provided a documented instance in which the expression of a gene derived from a prokaryotic host (Agrobacterium Ti plasmid) apparently was influenced by plant growth factors.

TRANSPOSON Tn5-DERIVED GENE TRANSFER AND PROMOTER PROBE VECTORS

Transposon Tn5 is a powerful tool for bacterial genetics. It is efficiently used for generating promoter and gene fusions as well as for mapping and cloning of gene mutations (3,4,6,24). In order to develop a similar technique for plant gene analysis, modified Tn5 transposons were constructed. A plant vector cassette was inserted in Tn5 in a position that does not affect its transposition properties to create Tn5-PV. Tn5-PV shares the properties of both Tn5 and plant gene vectors. It carries the replication and conjugational transfer origin sequences of plasmid RK2 linked to plant selectable and screenable marker genes (NOS promoter-NPTII, octopine synthetase) and to the 25-bp border repeats of Ti plasmid T-DNA. Tn5-PV was inserted into the chromosome of E. coli. Random Tn5-PV insertions were isolated easily in plasmids transformed into such a transposon donor E. coli strain by using "gene dosage" selection on neomycin gradient plates (30). After mapping, plasmids carrying Tn5-PV inserts were mobilized from E. coli to Agrobacterium with the help of plant vector cassette functions. Due to the orientation of the T-DNA 25-bp repeats toward the ends (IS50L and IS50R) of Tn5-PV, insertion of Tn5-PV in a plasmid resulted in a plant vector construction which transferred the target plasmid in its entirety to plant cells. As a consequence, any DNA sequence cloned in any E. coli plasmid vector can be randomly mutagenized in E. coli by Tn5-PV insertion, and the mutagenized DNA segments can be transferred to plant cells for functional analysis after mobilization to Agrobacterium. Coding regions in a large, cloned DNA sequence can be mapped by the analysis of resulting transcripts in transformed plants. Simultaneously, genes not affected by transposon insertions can be identified and their level of expression can be compared to that of co-transferred chimeric plant marker genes carried by Tn5-PV. The general scheme of Tn5-PV mutagenesis is outlined in Fig. 2.

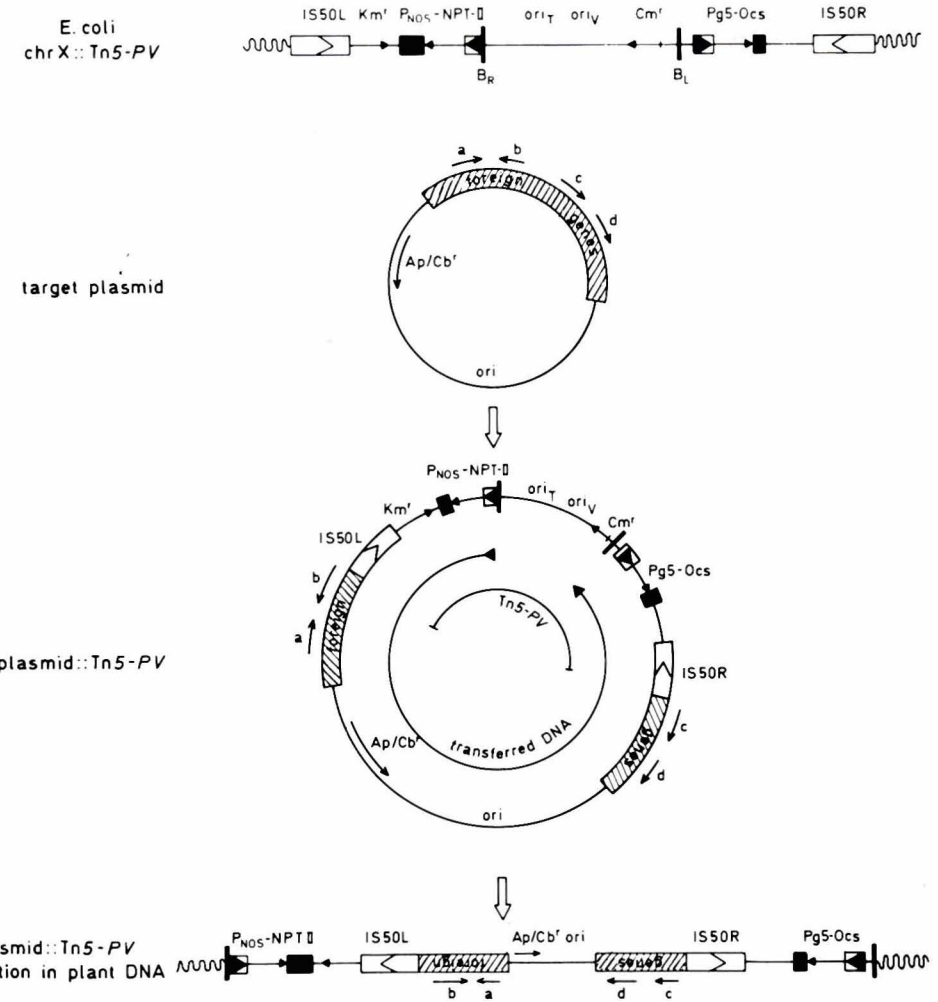

Fig. 2. General scheme of Tn5-PV-mediated mutagenesis and gene transfer. The Tn5-PV insertions are isolated in a target plasmid by using an E. coli donor strain (chrX::Tn5-PV). A structure similar to Agrobacterium binary plant vectors is formed by Tn5-PV insertion. The target plasmid linked to Tn5-PV is transferred into the plant cell and integrated into the nuclear genome. Notations a, b, c, and d designate putative coding sequences in a foreign DNA cloned in the target plasmid. Inversion of the a, b, c, and d sequences occurs during the integration of plasmid::Tn5-PV derivatives into plant DNA. Km^r, Cm^r: bacterial kanamycin and chloramphenicol resistance genes; ori_V, ori_T: replication and transfer origin sequences of plasmid RK2; B_R, B_L: right and left border sequences of Ti plasmid T-DNAs; P_{NOS}-NPTII: chimeric neomycin phosphotransferase gene transcribed by the promoter of the nopaline synthetase gene; Ocs: octopine synthetase gene; Pg5: the promoter of T_L-DNA-encoded gene 5.

To demonstrate the applicability of this approach, a known DNA segment, i.e., the T-DNA of pTiC58 carried by the plasmid pGV354, was subjected to mutational analysis by Tn5-PV. The function and structure of tumor-inducing T-DNA genes located on pGV354 were analyzed in detail previously (18,37). It was demonstrated with the help of this model system that Tn5-PV can both mutagenize and elicit Agrobacterium-mediated transfer of the mutagenized T-DNA segment to different plants. When the structure of pGV354::Tn5-PV derivatives, as present in E. coli, was compared to the structure of the transferred DNA in transformed plant cells, the data fit the model for the mechanism of transfer and integration as depicted in Fig. 2. No rearrangements of pGV354::Tn5-PV sequences occurred during transfer from E. coli to the plant nucleus via Agrobacterium. There also was a strict correlation between the precise site of Tn5-PV insertions in pGV354, the absence or presence of T-DNA-encoded transcripts, and the expected organogenic properties of the transformed plant tissues. These results, described in detail elsewhere (21), demonstrated that Tn5-PV can provide a direct and simple method for the transfer and the transcriptional and functional analysis of large, cloned DNA segments in plants.

In bacteria, Tn5 derivatives have been successfully used to identify and characterize promoter and protein export signal sequences (3,24). To follow this analogy, Tn5-related plant vectors were constructed for isolation of transcriptional or translational fusions in plant genes. The left IS50 sequence of Tn5 was shortened to retain only the 35-bp sequence necessary for transposition. This sequence was fused to a eukaryotic promoter probe gene, such as the NPTII gene supplemented with a 3' polyadenylation signal. Finally, a plant vector cassette, containing 25-bp T-DNA repeats, conditional plasmid replication and mobilization functions, and a bacterial Km^r gene from Tn903, was placed in the central position of this modified Tn5. In certain derivatives the plant vector cassette also carries a dominant plant selectable marker gene (e.g., a hygromycin phosphotransferase chimeric gene driven by the NOS promoter). The scheme of the mutagenesis and gene transfer for these Tn5 derivatives is identical to that shown for Tn5-PV. Insertion of such a promoter probe transposon (e.g., Tn5-PR2) in a plant DNA sequence cloned in a plasmid can result in the formation of transcriptional or translational plant gene-reporter gene fusions. These fusions will confer a dominant antibiotic resistance phenotype to plants and can be either directly selected after the transfer of plasmids with promoter probe Tn5 inserts to plant cells via Agrobacterium, or can be screened in different organs of the transformed and regenerated transgenic plants by enzymatic assay.

Plant gene fusions identified by the simple tool of transposon mutagenesis can be readily isolated from the mutagenized plasmids and further characterized. This method can help the analysis of the expression of known genes and should also facilitate the isolation of new plant promoters active in a tissue-specific fashion.

VECTORS FOR THE USE OF T-DNA INSERTS AS GENE TAGS

Agrobacterium-mediated gene transfer results in a T-DNA insertion in the plant nuclear genome. The T-DNA can therefore be considered as a mutator element which can cause insertional inactivation of plant genes. The integration of the T-DNA appears to be random with regard to the chromosomal location of T-DNA insertions. Whether or not the integration of the T-DNA has a defined sequence specificity is not yet known. Assuming

that T-DNA integration is not specific for particular sequences, it can be used in plants to isolate in situ gene fusions. A number of vectors were therefore constructed in which the coding sequence of a selectable marker gene lacking transcription initiation sequences was fused to the 25-bp border sequence of the T-DNAs. The integration of these T-DNAs in transcribed plant chromosomal loci can be readily identified because the neomycin (NPTII) or the hygromycin B phosphotransferase (HPT) genes will give a dominant antibiotic resistance phenotype to plant cells carrying an insertionally activated selectable marker gene.

Two sets of vectors with NPTII marker genes were constructed (Fig. 3). In type I vectors the initiation codon of the NPTII coding sequence was retained, while in type II vectors it was removed. In type II vectors the NPTII sequence was fused in all three reading frames to the 25-bp border sequence (26). These constructions were also provided with a constitutively expressed hygromycin selectable marker gene. This allows the selection for transformed plants by hygromycin resistance. These plants can subsequently be screened for the organ- or tissue-specific expression of the NPTII marker gene. In order to facilitate the rescue by cloning of independent T-DNA insertions, the vectors also carry a plasmid replicon and a bacterial marker gene in their T-DNA segment.

The T-DNA insertions flanked by the mutated plant gene sequences can readily be reisolated in E. coli by digestion, religation, and transformation of plant DNAs from different transformed plants (20,22). By genetic crosses it is possible to ascertain that a given mutant phenotype is linked to the locus carrying the T-DNA insert.

Transformed diploid plants will be hemizygotic for the mutations caused by the T-DNA insertions. Therefore, insertions in diploids can only be correlated with mutant phenotypes in homozygote progeny of the transformed plants. Alternatively, haploid plants can be used for easier detection of mutations.

In order to show the applicability of the T-DNA-mediated gene tagging approach, gene fusions were isolated in allotetraploid N. tabacum (SR1) and

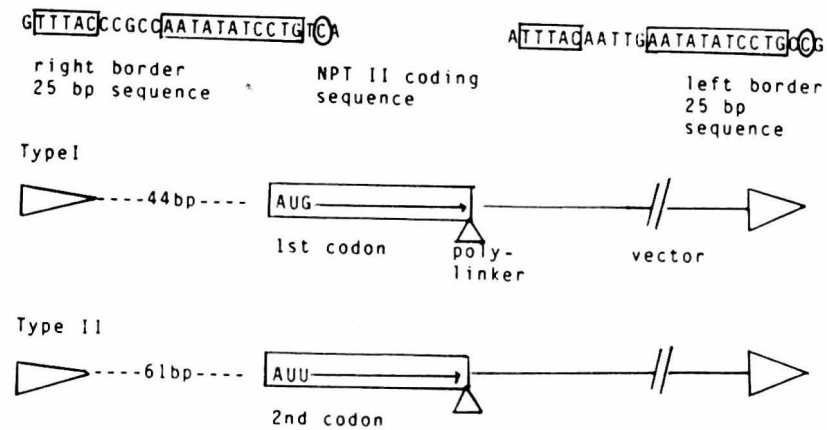

Fig. 3. Schematic representation of type I and type II vectors used for T-DNA-mediated gene tagging.

haploid N. plumbaginifolia plants by using either protoplast co-cultivation (25) or leaf-disc infection (17) methods followed by a complex screening scheme. In experiments involving direct selection for kanamycin-resistant transformed plant cells, the frequency of transformation was 5% to 10% of that obtained with control vectors carrying NPTII genes transcriptionally driven by known promoters (P_{NOS}, $T_R P1'$). This frequency varied according to the hormone combination and the level of kanamycin concentrations used in the different transformation experiments. When the transformed plants were first selected on hygromycin and then screened for the expression of the promoter probe marker gene, NPTII activity was detected in one-third of all transformed and hygromycin-resistant calli.

In tobacco, culture media will determine whether transformed cells will grow as calli or regenerate shoots and roots (18,34). Accordingly, certain gene fusions, e.g., those expressed only in leaves or only in roots, will not be selected in callus cultures. Therefore, appropriate hormonal conditions should be used to isolate gene fusions expressed in a tissue-specific fashion. In haploid N. plumbaginifolia, any insertion resulting in an auxotrophic mutation may cause lethality. Therefore culture media should also be supplemented with amino acids, vitamins, etc. Figure 4 shows the tissue specificity of some gene fusions isolated with type I vectors in N. tabacum SR1 plants by selecting for callus formation in the presence of kanamycin. Calli containing gene fusions and expressing the NPTII enzyme were regenerated to plants in the absence of selection. These plants were recurrently tested in the presence of kanamycin. The tissue specificity of the NPTII gene fusions was easily detected because the plants showed a rapid death and degeneration of tissues in which the NPTII selectable marker was not expressed (e.g., plants with stem-specific fusions lost their leaves; the leaves of plants containing root/stem-specific NPTII fusions were bleached, etc.). In haploid N. plumbaginifolia, where the appearance of a mutant phenotype should be apparent immediately after transformation, calli and transformed plants carrying 800 independent gene fusions were screened for possible deficiencies in amino acid metabolism, photosynthesis, differentiation, and morphological traits. No auxotrophic mutants turned up in these screens, but lines altered in leaf and flower morphology and several albinos were obtained. Their characterization will be described elsewhere (C. Koncz et al., ms. in prep.).

In seed germination assays, the isolated gene fusions showed dominant Mendelian, single- and, in a few cases, double-factor inheritance of the NPTII marker. This test is reliable only for gene fusions expressed in seedlings. Additional tests and hybridization data indicated that in some plants showing single-factor inheritance of the NPTII marker, additional silent copies of vector T-DNAs were nevertheless present.

In transformants obtained by type II vectors (Fig. 3), allowing formation of translational gene fusions only, NPTII fusion proteins of different sizes were indeed detected, while most transformants selected by type I vectors, providing both transcriptional and translational fusions, synthesized normal-size NPTII enzyme proteins. This observation was not expected, since the statistical probability for the integration of the vector T-DNA into coding sequences should be higher than that for the integration of the T-DNA in transcriptional leader sequences.

Many other intriguing questions remain to be answered, such as: (a) can one select for insertions in introns?, (b) is the high frequency appearance of transformants carrying putative gene fusions due to the

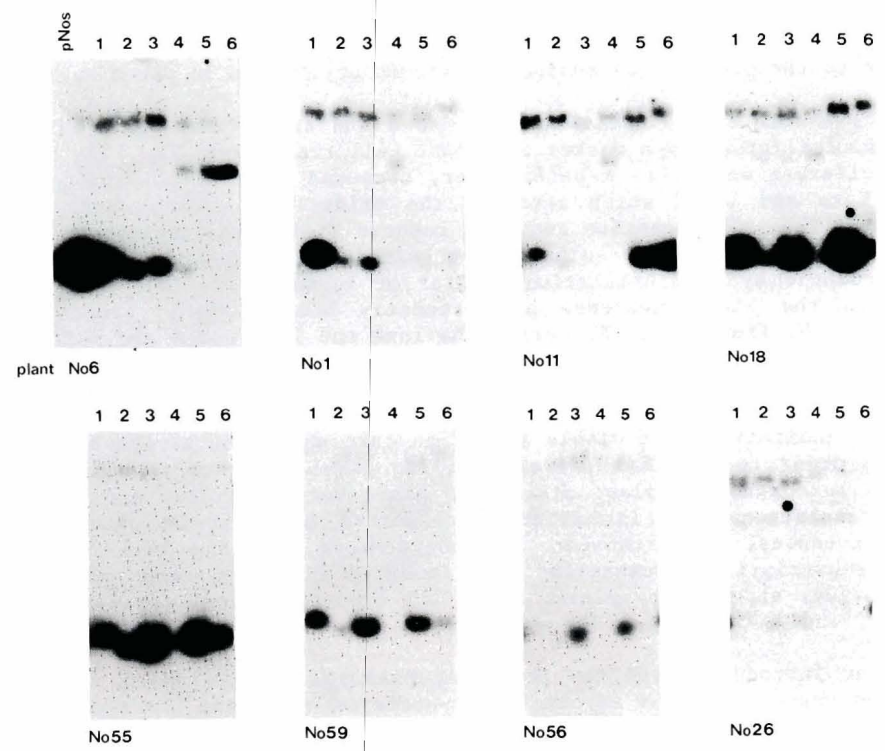

Fig. 4. Tissue-specific expression of a NPTII selectable marker in Nicotiana tabacum plants transformed by type I NPTII promoter probe vectors. NPTII activities in callus (lane 1), upper stem and leaf primordia (lane 2), upper stem (lane 3), leaves (2nd and 3rd) (lane 4), lower stem (lane 5), and root (lane 6). The sample was taken out of 600 independent lines carrying transcriptional and translational NPTII fusions. The NPTII assay is standardized for equal amount of protein in the extracts. pNO, control extract from callus tissue containing a chimeric pNOS-NPTII gene.

preferential integration of the vector T-DNA in transcriptionally active sequences which are moderately repeated?, and (c) does the selection for higher levels of antibiotic resistance select for the specific amplification of promoter probe sequences? All these questions should be answered by further molecular studies of reisolated gene fusions.

EXPRESSION VECTORS AND ALTERNATIVE REPORTER PROTEINS

The DNA sequences coding for reporter proteins have been used for the construction of plant chimeric genes. Previous work has demonstrated that a number of bacterial enzymes can be expressed and used as selectable and screenable markers in transgenic plants, e.g., neomycin phosphotransferase (NPTII) (15,16), chloramphenicol acetyl transferase (CAT) (16), β-galactosidase (14), and hygromycin phosphotransferase (33). All of these enzymes

require relatively complex assay procedures. The results of CAT or β-galactosidase assays are not easily quantified, due to nonspecific reactions or to the presence of endogenous enzyme activities in plant cells.

To overcome these limitations we chose and tested the light-emitting bacterial luciferase as a marker for plant cell transformation. The bacterial luciferase enzyme is a heterodimer, composed of two polypeptide subunits, Luxα and Luxβ, which catalyze the oxidation of long chain fatty aldehydes (12). The reaction requires reduced flavin mononucleotide (FMN) and molecular oxygen and results in the emission of blue-green light, easily measurable by a scintillation counter or luminometer (13). Genes involved in the bioluminescence have recently been isolated from Vibrio harveyi and V. fischeri in E. coli. The luxA and luxB genes are part of a single transcriptional unit in V. harveyi and encode the α and β subunits of luciferase (2,8). Escherichia coli cultures expressing luxA and luxB genes are bioluminescent when an aldehyde substrate (e.g., n-decanal) is supplied, indicating that viable cells can take up the substrate aldehydes (2). In order to obtain expression and to permit correct translation of luxA and luxB genes in plant cells, the genes were separated and possible extra translational initiation codons, located in their 5' untranslated leader sequences, were removed. The constructed luxA and luxB "translational-transcriptional" cassettes were inserted in a plant expression vector, pPCV701, and thereby placed under the transcriptional control of the T_R-DNA 1' and 2' promoters (35), as shown in Fig. 5.

After introduction of the resulting plasmid, pPCV701luxA&B, into tobacco and carrot cells by Agrobacterium-mediated or direct DNA transformation, it was possible to demonstrate the expression of luciferase genes by measuring bioluminescence and showing the presence of the α and β luciferase subunits by immunoblotting in the transformed cells. Both luxA and luxB genes were expressed simultaneously and to similar levels in transformed protoplasts, calli, and organs of regenerated plants. Due to the great sensitivity of the luciferase assay, the chimeric luxA&B genes could be used to demonstrate DNA uptake and gene expression in carrot protoplasts as early as 8 hr after electroporation.

In view of the fact that specific luciferase activity can easily and quantitatively be detected in plant cell extracts, this enzyme appears to be suited as a convenient reporter to monitor the transcriptional regulation of chimeric genes and the transcriptional activity of promoter 5' upstream sequences in transgenic plants as well as in transient gene expression assays. All bacterial enzymes shown to be expressed in plants thus far were of a single subunit type. The heterodimeric V. harveyi luciferase also provided a suitable system to test for the assembly of a complex bacterial enzyme in plant cells, thus opening the way for expression of a multicomponent, heterologous enzyme system in higher plants (23).

CONCLUSION

We have described examples for the application of techniques of bacterial genetics in plant gene analysis. In view of the largely methodological character of this chapter, we do not think that a sophisticated conclusion is required. Instead, we would like to encourage further development in the use of these and similar approaches. They allow plant molecular geneticists to work with the simple tools which have been used with such striking success in the analysis of gene regulation in bacteria and yeasts.

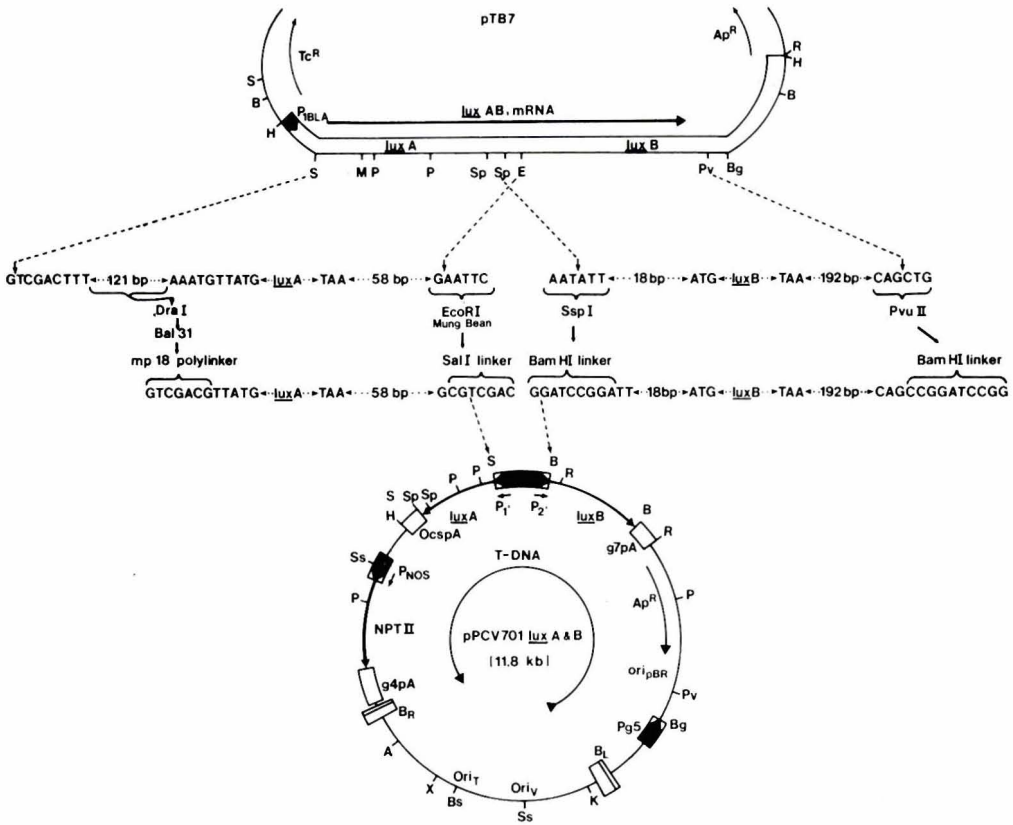

Fig. 5. Construction and cloning of Vibrio harveyi luciferase luxA and luxB gene cassettes in the plant expression vector pPCV701. $P1_{BLA}$: P1 promoter of β-lactamase gene used for expression of luxA&B transcriptional unit in E. coli plasmid pTB7 (2); P1' and P2': promoters of T_R-DNA-encoded genes 1' and 2'; Pg5 and P_{NOS}: promoters of gene 5 and nopaline synthetase genes; g4pA, OcspA, g7pA: polyadenylation sequences derived from T_L-DNA-encoded gene 4, the octopine synthetase gene, and gene 7; ori_{pBR}: replication origin of pBR322. A, ApaI; B, BamHI; Bg, BglII; Bs, BstEII; H, HindIII; K, KpnI; M, MaeI; P, PstI; Pv, PvuII; R, EcoRI; S, SalI; X, XhoI; Ss, SstII; Sp, SspI.

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