REVIEW

The Use of Gene Vectors in Plant Molecular Biology

RICHARD WALDEN,*[1] CSABA KONCZ,[1,2] and JEFF SCHELL[1]
Max-Planck-Institut für Züchtungsforschung, Carl von Linne Weg 10, D-5000 Köln 30, Federal Republic of Germany,[1] and Institute of Plant Physiology, Biological Research Center of the Hungarian Academy of Sciences, M-6701 Szeged P.O. Box 521, Hungary[2]

Received 13 December 1989/Accepted 13 December 1989

Recent developments in plant molecular genetics have received much attention because of the potential applications of transgenic plants in agriculture and biotechnology. Genetic techniques that have emerged from the study of the natural transfer of genes from soil *Agrobacterium* to plants are now widely employed in both basic and applied research. How gene vectors used in *Agrobacterium* mediated transformation can be exploited to gain further insight into the molecular biology of plants is the focus of this review.

INTRODUCTION

Plant transformation is now a routine procedure and can be used to define DNA sequence motifs important for determining tissue specific and developmentally regulated expression of plant promoters, achieve the expression of foreign genes, elucidate the mechanisms of replication of viral genomes, and investigate transposition in heterologous systems. In addition, transformation vectors can be developed to carry out mutagenesis of the plant genome (for reviews, see Benfey and Chua, 1989; Döring and Starlinger, 1986; Klee et al., 1987a; Schell, 1987; Schell and Vasil, 1989; Weising et al., 1988, and Walden, 1988).

Many techniques have been used to introduce foreign DNA into plant cells. DNA mediated gene transfer (DMGT) has been achieved by fusion of plant protoplasts with bacterial spheroplasts (Hain et al., 1984; Okada et al., 1985) or liposomes containing foreign DNA (Deshayes et al., 1985), microinjection (Crossway et al., 1986; Neuhaus et al., 1987; Reich et al., 1986), macroinjection (De la Pena et al., 1987), treatment of protoplasts with DNA in the presence of polyvalent cations (Hain et al., 1985; Paszkowski et al., 1984), electroporation (Fromm et al., 1986; Pröls et al., 1989), or microbombardment of plant tissue with particle-linked DNA (Klein et al., 1987). Although DMGT techniques are widely used to assay transient expression of chimeric gene constructs, due to frequent rearrangement and multimerisation of the transforming DNA during insertion into the genome they are less favored for the engineering of stably transformed transgenic plants. Techniques that exploit the natural DNA transfer carried out by the soil bacterium *Agrobacterium tumefaciens* provide a solution to these problems, because in this case usually unrearranged sequences of DNA can be transferred to the plant genome.

BIOLOGY OF THE AGROBACTERIUM—*PLANT INTERACTION*

During the interaction of wounded plants with *Agrobacterium* a region of the bacterial Ti, or Ri plasmid, termed the T-DNA is transferred to the plant cell and stably integrated into the genome. This process is initiated by the wound induced release of plant cell wall compounds which provides a chemical signal triggering a cascade of

Methods in Molecular and Cellular Biology. 1990, pp. 175–194

*To whom correspondence should be addressed.

events, including chemotaxis and the binding of the bacteria to the plant cell. The production of plant growth hormones by the bacterium triggers the plant wound response, while phenolic plant metabolites, such as acetosyringone, activate the molecular mechanism of DNA transfer (for detailed reviews, see Draper et al., 1989; Schell, 1987; Walden, 1988, and Zambryski, 1988, 1989).

Induction of the *virulence (vir)* genes of the Ti plasmid (Fig. 1) results in the production of *vir* encoded gene products which recognize the 25 bp boundary sequences of the T-DNA and produce a single stranded DNA copy of the T-DNA, the T-strand (Albright et al., 1987; Close et al., 1987a, 1987b; Leroux et al., 1987; Stachel et al., 1987, and Stachel and Zambryski, 1986). In common with conjugative intermediates of other bacterial plasmids, the T-strand is protected against nuclease attack by a single stranded DNA binding protein (the *vir* E2 product, Christie et al., 1988; Gietl et al., 1987) and carries at its 5′ end a covalently bound mixed function topoisomerase (the *vir* D2 product, Yanofsky et al., 1986). It is believed, but not proven, that the T-strand is transferred to the plant cell by a conjugational process. Recent reports demonstrating *Agrobacterium* mediated transfer of non-Ti related plasmids into plants as well as the conjugative transfer of *E. coli* plasmids into yeast suggest that the capability of interspecies gene transfer has not evolved uniquely in *Agrobacterium* (Buchanan-Wollaston et al., 1987; Heinemann and Sprague, 1989). The process of integration of the T-DNA into the plant genome is not clear, but single or low copy inserts can be obtained with the ends delimited by the 25 bp repeats. In one type of *Agrobacterium* strain, C58, tandem copies of the T-DNA can be preferentially inserted into the genome (Jorgenson et al., 1987) and this might result from the *Agrobacterium* transferring more than one copy of the T-DNA into the plant cell or its replication prior to integration (Depicker et al., 1985). Using T-DNA vectors as gene tags it was found recently that integration takes place preferentially into sequences that potentially can be transcribed (Koncz et al., 1989). The process of integration is likely to involve both host encoded proteins as well as those bound to the T-strand. Hence this is likely to make the T-strand the ideal model of transforming DNA and this in itself is an intriguing question.

T-DNA of the Ti and Ri plasmids encode a variety of genes the expression of which cause major alterations in the differentiation and development of transformed plant tissue (Fig. 1). The conserved core regions of the T-DNAs of tumor inducing Ti plasmids encode genes for the synthesis of the plant hormones, auxin (gene 1 or *iaaM* and gene 2 or *iaaH*) and cytokinin (gene 4 or *ipt*), which incite continuous division of transformed cells (for review, see Schell, 1986). Genes 5 and 6b probably deregulate auxin and cytokinin levels (Körber and Koncz, unpublished; Spanier et al., 1989; Tinland et al., 1989). Other genes confer the production and secretion of opines which are specifically recognized and metabolized by the plant colonizing *Agrobacterium* (Kahl and Schell, 1982; Messens et al., 1985). The *rolA, B,* and *C* genes of the Ri plasmid T-DNAs induce root differentiation (hairy root formation) and dramatically modify both the cellular response to auxins and the development of transgenic plants (Shen et al., 1988; Spena et al., 1987; Schmülling et al., 1988). Promoters of all T-DNA genes are recognized by plant transcriptional factors which modulate their activity in a tissue specific fashion (Koncz and Schell, 1986; Ellis et al., 1987; An et al., 1988; Langridge et al., 1989). Analysis of the function and regulation of the expression of the T-DNA encoded genes therefore can give insight to the regulation of transcription and hormone signal transduction in plants. Moreover the promoters of the genes contained on the T-DNA can be used to direct the expression of foreign genes in transgenic tissue.

VECTORS FOR USE IN AGROBACTERIUM

A variety of plant transformation vectors which exploit the process of tumor formation, yet have the sequences responsible for the establishment of the tumor phenotype deleted, have been developed. Each of them capitalize on the experimental observations that the DNA transferred to the plant cell is defined by the 25 bp T-DNA border repeats, that transfer and integration into the plant genome does not require the presence of any of the genes encoded by the T-DNA and that the *vir* region of the Ti plasmid works in *trans*.

There are two types of vector used with *Agrobacterium,* cointegrative or binary vectors. While the many transformation protocols utilise *A. tumefaciens,* analogous cointegrative vectors have been developed for use in *A. rhizogenes* (Stougaard et al., 1987), whereas binary vectors can be used in both types of bacteria (Simpson et al., 1986).

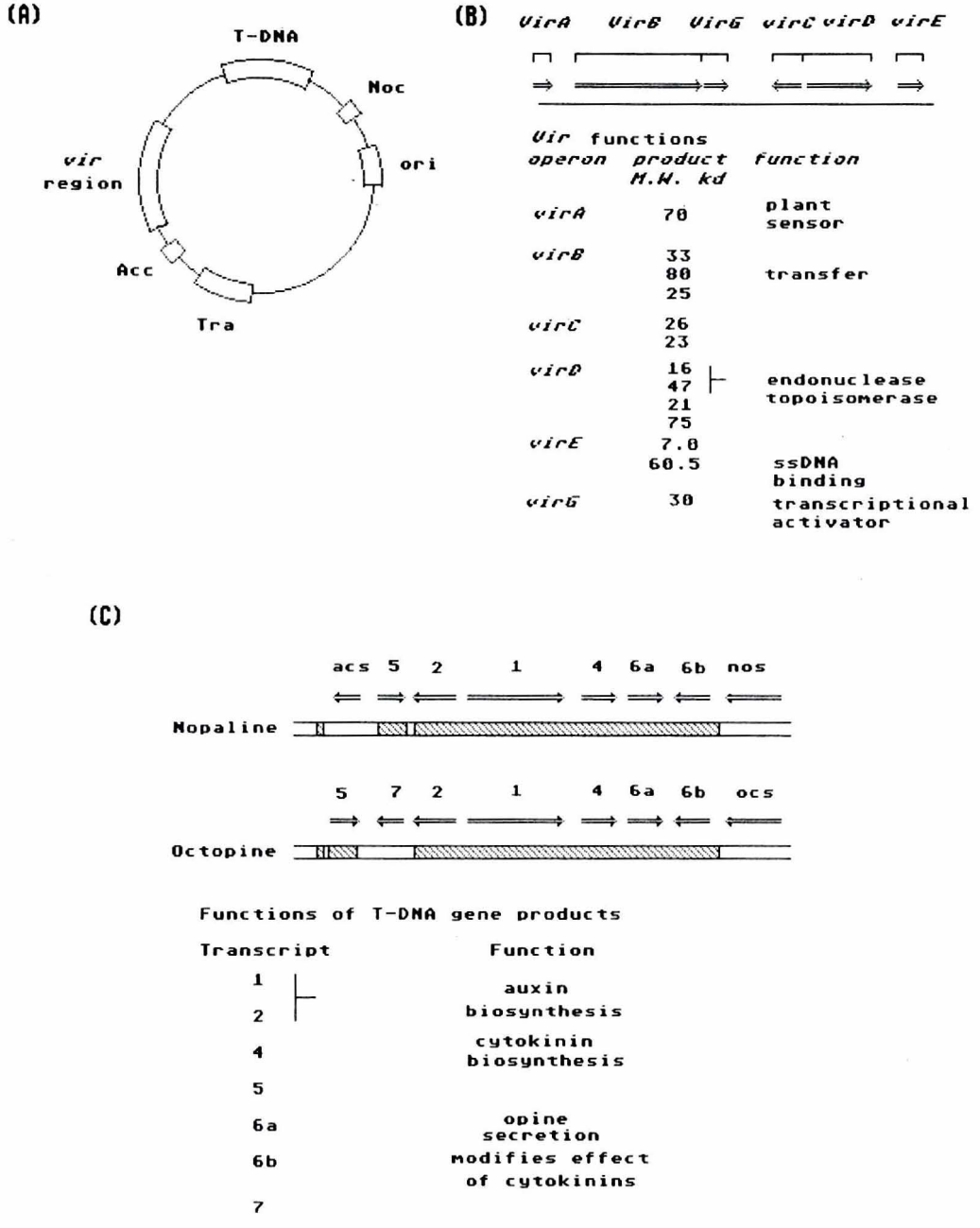

Figure 1. Functional organization of the Ti plasmid. (A) Map of a nopaline type pTiC58 plasmid. Major functional regions: ori, origin of replication; Tra, region important in the conjugative transfer of the plasmid; Noc, nopaline catabolism; Acc, agrocinophine catabolism. (B) Physical and structural function of the virulence region of an octopine Ti plasmid. Arrows indicate the orientation of transcription of the genetically determined *vir* loci. (C) Physical and structural function of the T-DNA. The regions of homology between the T-DNA of a nopaline and an octopine T-DNA are shown. Arrows indicate the orientation of transcription of the different genes. The genes involved in opine biosynthesis are: nos, nopaline synthase; acs, agrocinopine synthase; ocs, octopine synthase.

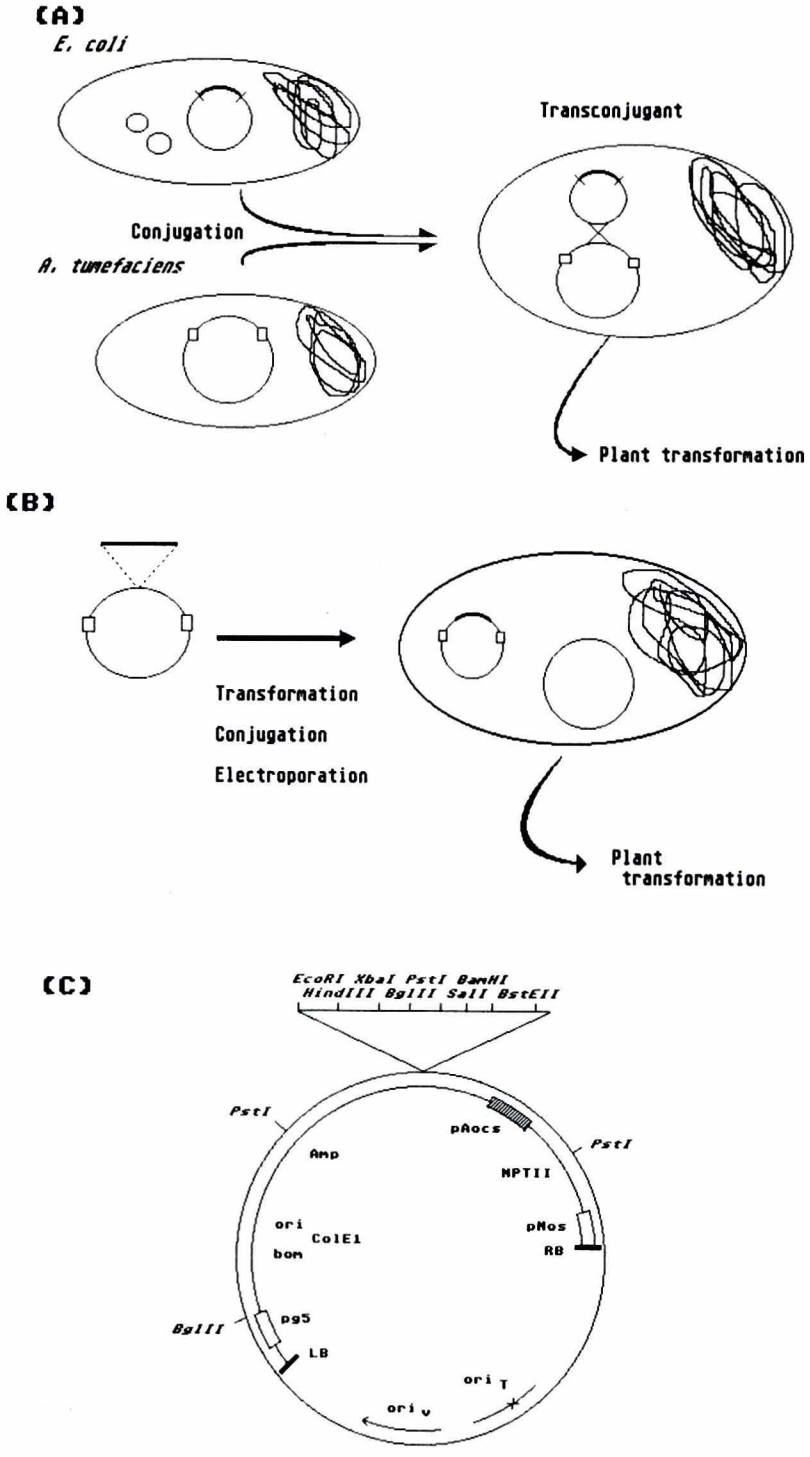

Figure 2. Introduction of plant transformation vectors into *Agrobacterium*. (A) Insertion of a defined sequence of DNA into a cointegrative vector. DNA to be transferred to the plant cell is cloned into an intermediate vector stable in *E. coli*. The intermediate vector is transferred into *E. coli* containing helper plasmids which allow conjugational transfer into *Agrobacterium* which contains the cointegrative Ti plasmid vector. Following transfer to *Agrobacterium* the *E. coli* plasmids are lost because they are unable to replicate and the foreign DNA is rescued by recombination with the cointegrative vector. Transconjugants can then be used in plant transformation. Open boxes refer to the border sequences of the T-DNA and the thick line to the DNA to be transferred to the plant cell. (B) Use of binary vectors in plant transformation. DNA to be transferred to the plant cell is inserted into a binary

Cointegrative vectors are derivatives of the Ti plasmid from which the majority of the T-DNA between the border repeats has been replaced by a defined sequence of DNA (Zambryski et al., 1983; Fraley et al., 1985). Foreign DNA which is to be transferred to the plant genome is cloned into an intermediate vector, which contains selectable markers and a sequence homologous to that present between the border repeats of the cointegrative vector. The intermediate vector containing the foreign DNA is introduced into the bacterium harboring the cointegrative vector by conjugation (Ditta et al., 1980; van Haute et al., 1983). Due to a lack of the appropriate origins of replication the intermediate vector is unable to be stably maintained within *Agrobacterium*. Therefore, selection for a marker carried by the intermediate vector will select for those bacteria in which homologous recombination with cointegrative vector has resulted in the foreign DNA being integrated between the border repeats of the T-DNA (see Fig. 2).

Binary vectors are plasmids containing origins of replication that are active in *E. coli* and *A. tumefaciens* as well as selectable markers which are functional in both types of bacteria. In addition they contain the 25 bp border repeats which define the DNA transferred to the plant genome and between which are located appropriate cloning sites for the insertion of foreign DNA, as well as markers that allow selection of transgenic tissue (An et al., 1985; Bevan 1984; Hoekema et al., 1983; Koncz and Schell, 1986) (Fig. 2). Binary vectors can be introduced by either conjugation, transformation (Ebert et al., 1987) or electroporation (Mattanovich et al., 1989) into the host *A. tumefaciens* which harbors a plasmid containing a functional *vir* region.

While cointegrative vectors have the advantage that they are generally very stable in *Agrobacterium*, binary vectors have proven to be versatile and simple to use.

THE USE OF AGROBACTERIUM TO PRODUCE TRANSGENIC PLANTS

The production of transgenic plants using *Agrobacterium* relies on the ability of the bacteria to transfer the DNA to an individual plant cell which in turn can regenerate into a whole plant. Protoplast co-cultivation (Depicker et al., 1985; Hain et al., 1985; Marton et al., 1979) involves isolating protoplasts, allowing them to initiate cell wall formation and then inoculating with *Agrobacterium* carrying an appropriate transformation vector. After the co-cultivation the majority of the bacteria are removed by washing and the protoplasts are cultured further so that they begin to divide and form callus. Antibiotics are used to prevent the growth of remaining bacteria and allow selection of transgenic tissue. Subsequent shoot and root growth is initiated by subculturing the callus on appropriate media. Protoplast co-cultivation allows the generation of a large number of independent transformants and biochemical selection for a particular phenotype. However, co-cultivation is currently limited to solanaceous species where high frequencies of cell division and subsequent growth can be achieved. Moreover, as a result of the culture process a proportion of the resultant plant population may exhibit somaclonal variation.

Explant inoculation is the most convenient method to produce transgenic plants and its general application has proven useful in an ever increasing number of plant species (for reviews, see Gasser and Fraley, 1989; Walden, 1988; and Weising et al., 1988). Explants are incubated with *Agrobacterium* and then placed on media which allows the induction of the growth of callus and the subsequent induction of growth of shoots. The shoots can then be subcultured to form roots. Selection for transformants can be applied at the stage of callus formation and at the point of transfer of the shoots to rooting media. This

vector by conventional cloning methods and introduced into *Agrobacterium* containing a Ti plasmid which contains an active *vir* region but from which the T-DNA has been removed. The resulting *Agrobacterium* is used in plant transformation. (C) Features of a binary vector. The structure of pPCV002 (Koncz and Schell, 1986) is shown. LB and RB, the left and right T-DNA border sequences respectively; ori_V and ori_T origins of replication functional in *Agrobacterium*; ori, bom, ColE1, origin of replication and mobilisation sequences functional in *E. coli*; Amp, the ampicillin resistance gene; pnos, nopaline synthase promoter; NPTII, neomycin phosphotransferase gene; pAocs, octopine synthase poly A addition site. Important restriction sites are indicated.

approach has proved successful with leaf (Rogers et al., 1986), stem (An et al., 1986), roots (Valvekens et al., 1988), tubers (Ishida et al., 1989), and epidermal strips derived from flowering branches (Trinh et al., 1987). Moreover, explant inoculation can be used with *A. rhizogenes* (for review, see Tepfer et al., 1989). In this case hairy roots are induced at the site of inoculation and shoot formation from the roots can be obtained by manipulation of the culture conditions. While explant inoculation can be used to generate transgenic tissue containing a specific insert, it is not a feasible approach when the aim of the experiment is to regenerate large numbers of individual transformants, for example, in insertional mutagenesis or in attempting to isolate uncharacterized plant genes from a shotgun library by complementation.

An alternative way of generating large numbers of individual transformants and avoiding the potential difficulty of somaclonal variation is seed inoculation of arabidopsis (Feldmann and Marks, 1987). Here arabidopsis seeds are imbibed for 12 h and then incubated with *Agrobacterium* containing an appropriate transformation vector. Resulting plants are selfed and the seeds obtained are germinated in the presence of selection to obtain transgenic plants. The mechanism by which tranformation takes place is not known although following selfing the selective marker can be inherited in a mendelian manner. This procedure has been used successfully to generate T-DNA insertional mutants (Feldmann et al., 1989, see later).

GENETIC MARKERS FOR USE IN PLANT CELLS

Genetic markers set the criteria by which a plant cell is judged to be transformed by providing novel nucleic acid sequences that can be detected by Southern analysis and whose enzyme products can be assayed. In general, the genetic markers that have been developed for use in plant cells are derived from either bacterial or plant sources although their expression is directed by plant-specific promoters (see Table 1). Dominant selectable markers allow for the direct selection of transgenic tissue, whereas screenable markers can be used for assays in the detailed analysis of gene expression.

The choice of marker for a particular experiment depends very much on its characteristics and the response of the plant cell. For example, one of the earliest developed selectable markers, kanamycin resistance, encoded by the bacterial NPTII gene, is used extensively (Herrera-Estrella et al., 1983). In tobacco, kanamycin selection can be used with isolated protoplasts, callus, tissue explants, and whole plants (De Block et al., 1984). Nevertheless, kanamycin might not be ef-

TABLE 1. Genetic Markers for Use in Plant Cells.

Enzyme Activity	Dominant Selection	Assay	Reference
Neomycin phosphotransferase	Yes	Yes	Reiss et al., 1984
Hygromycin phosphotransferase	Yes	Yes	Waldron et al., 1985
Dihydrofolate reductase	Yes	Yes	Herrera-Estrella et al., 1983
Chloramphenicol acetyl transferase	Yes	Yes	Herrera-Estrella et al., 1983
Gentamycin acetyl transferase	Yes	Yes	Hayford et al., 1988
Nopaline synthase	No	Yes	Otten and Schilperoort, 1978
Octopine synthase	No	Yes	Otten and Schilperoort, 1978
β-galactosidase	No	Yes	Helmer et al., 1984
β-glucuronidase	No	Yes	Jefferson et al., 1984
Streptomycin phosphotransferase	Yes	Yes	Jones et al., 1987
Bleomycin resistance	Yes	No	Hille et al, 1986
Firefly luciferase	No	Yes	Ow et al., 1986
Bacterial luciferase	No	Yes	Koncz et al., 1987
Threonine dehydratase	Yes	Yes	Colau et al., 1987
Metallothionein II	Yes	Yes	Lefebvre et al., 1987
EPSP synthase	Yes	No	Shah et al., 1986
Phosphinothricin acetyl transferase	Yes	Yes	Deblock et al., 1987
Acetolactate synthase	Yes	No	Haugh et al., 1988
Bromoxynil nitrilase	Yes	No	Stalker et al., 1988

fective in all species, hence before attempting to use a dominant selectable marker it is advisable to test its effectiveness in a given tissue.

Significant advances have been made in developing markers which allow screening for enzymatic activity in individual cells. β-glucuronidase (Jefferson et al., 1987), firefly luciferase (Ow et al., 1986), bacterial luciferase (Koncz et al., 1987; Olsson et al., 1989), and β-galactosidase (Terri et al., 1989) have been used extensively to study both cell specific and developmentally regulated expression of promoters. Luciferase has the advantage of a nondestructive assay, so enzymatic activity can be visualized in living tissue (Langridge et al., 1989).

PROMOTERS AND EXPRESSION CASSETTES FOR USE IN PLANT CELLS

Routinely, T-DNA mediated transformation is used to express foreign genes in plants. The genes that have been transferred to plants include genes from other plants and eucaryotes, procaryotic genes, viral genes, and genomes as well as plant transposable elements (for an exhaustive review, see Weising et al., 1988). In addition, T-DNA vectors have been used to assess the potential of antisense RNA in modifying plant phenotypes (Rothstein et al., 1987).

With the exception of those of the T-DNA, promoters from nonplant sources work poorly, if at all, in plants (Koncz et al., 1984). Hence, in order to obtain correct expression the genes to be transferred to the plant genome need to be linked to plant-specific promoters. Plant promoters comprise of sequence elements, which may or may not overlap, interacting to direct the correct qualitative and quantitative gene expression. These elements can be interchanged so as to confer a specific pattern of expression on the gene to which they are linked (for review, see Benfey and Chua, 1989). In order to express foreign DNA in plants, the most frequently used promoters have been derived from plant pathogens, i.e., the T-DNA itself or Cauliflower Mosaic Virus. These promoters have been well characterized and although initially they were judged to be constitutive in their mode of expression, it has become increasingly clear that no promoter can be considered as being truly constitutive and that the expression that they direct might be affected by environmental, developmental, and/or subtle changes in the levels of growth substances present within the plant.

The approximately 200 bp nopaline synthase promoter from T-DNA has been one of the most extensively studied (An et al., 1986; Ebert et al., 1987; Mitra and An, 1989). It contains several sequence motifs which are likely to be important in directing expression as well as an upstream element (spanning −130 to −97) which, when duplicated, increases the level of expression of the promoter threefold suggesting that this region has the properties of an enhancer (Ebert et al., 1987). Although expressed in most plant tissues, the *nos* promoter is most active in the basal region of transgenic tobacco suggesting that it may respond to differing levels of auxin. Once flowering has taken place, the activity of the promoter in the vegetative organs decreases dramatically although in the flower there is a general increase in its activity (An et al., 1988).

The gene 5 promoter of T-DNA is most active in callus, stems, and roots while its activity is barely detectable in mature leaf tissue. Moreover, it it can be induced by the addition of auxins (Koncz and Schell, 1986).

The divergent dual promoters of the mannopine synthase *(mas)* genes 1 and 2 of the T-DNA are located on a 467 bp fragment (Velten et al., 1984) and direct the synthesis of the two most abundant transcripts of the T-DNA. The expression directed by the promoters is developmentally regulated and they appear to be most active in the basal region of the plant (Langridge et al., 1989). Moreover, expression is induced by wounding. Being closely linked, the relative levels of expression directed by the two promoters remain constant between individual transformants allowing the development of a selection-expression cassette where selection for high levels of expression of a marker gene driven by one promoter ensures high levels of expression of a foreign gene linked to the other promoter (Vaeck et al., 1987; Velten and Schell, 1985).

The 35S RNA promoter of Cauliflower Mosaic Virus has been used extensively and has been found to direct high levels of gene expression in a wide variety of plants. The promoter fragment generally used is 343 bp long and contains a strong transcriptional enhancer (Fang et al., 1989; Fluhr et al., 1986; Odell et al., 1985) which upon duplication results in a 10-fold increase in expression (Kay et al., 1987). The enhancement in expression appears to be able to take place over distances of up to 2 kb. The expression of

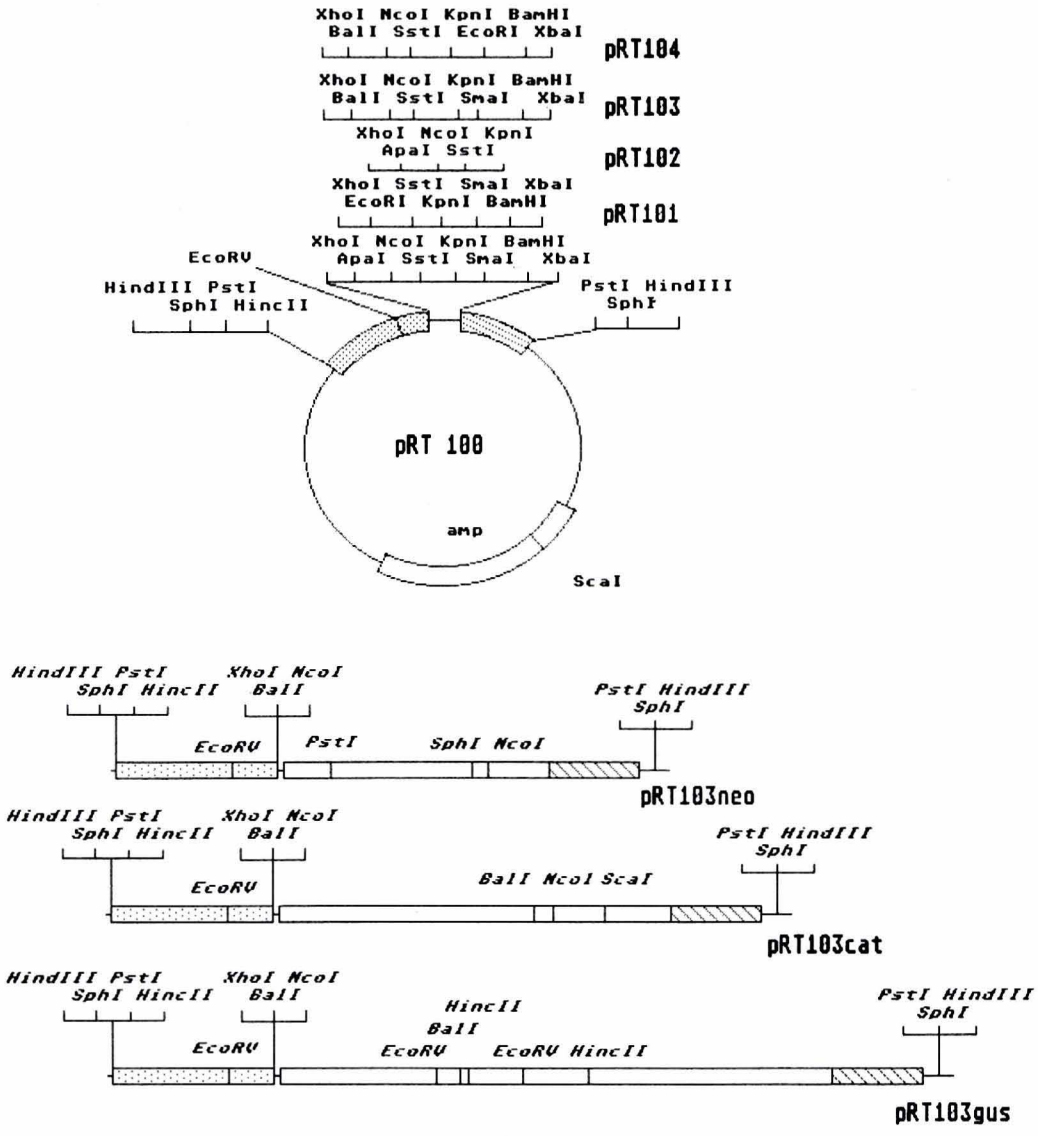

Figure 3. Expression cassettes functional in plant cells. (A) the pRT series of expression cassettes. Based on pUC18/19, the pRT series of plasmids contain a variety of polylinker sequences flanked by the promoter and poly A addition sequences of the 35S RNA of CaMV (Töpfer et al., 1987). Transcriptional fusions can be made with pRT101, whereas translational fusions can be made with pRT100, 102, 103 and 104 where the translation initiation codon is located at the Nco I site. (B) Genetic marker cassettes. Based on pRT103, these cassettes consist of the NPTII (pRT103neo), CAT (pRT103cat), or GUS (pRT103gus) gene inserted at the polylinker (Töpfer et al., 1988). Stipled boxes, promoter sequences; hatched boxes poly A addition sites. Important restriction sites are shown.

the 35S promoter, although generally considered to be constitutive, appears to be highest in leaf tissue (Jefferson et al., 1987). Recently, it has been shown that the constitutive mode of expression is a result of the interaction of different domains in the promoter which individually act to direct tissue specific expression (Benfey et al., 1989).

A number of expression cassettes have been constructed which allow the insertion of foreign genes, either as transcriptional or translational fusions behind plant specific promoters, and the pRT plasmids are typical [Fig. 3(A), Töpfer et al., 1987]. A variety of marker genes have been inserted into these cassettes [Fig. 3(B)], and these have proven to be active in both transient assays in protoplasts following DMGT and in stable transgenic tissue (Töpfer et al., 1988).

PLANT TRANSFORMATION VECTORS

As described previously, a large number of plant transformation vectors have been developed. Those that will be described here in detail are binary vectors based on the pPCV (plant cloning vector) series of plasmids (Fig. 2, Koncz and Schell, 1986; Koncz et al., 1987). These vectors contain a conditional mini-RK2 replicon which is maintained and mobilized by trans-acting functions derived from the plasmid RK2. The trans-acting functions are introduced into both *E. coli* and *Agrobacterium* so that the plasmid can be maintained and transferred between both. Between the T-DNA border sequences a variety of cloning sites and marker genes have been arranged, in addition to the origin of replication and a gene encoding ampicillin resistance which are both functional in *E. coli*. This not only allows the vectors to be transferred between *Agrobacterium* and *E. coli*, but also rescue of the T-DNA from the plant genome following transformation simply by cutting the plant nuclear DNA with a restriction enzyme that cuts outside of the T-DNA, religating and returning it to *E. coli* by transformation or electroporation. The *Agrobacterium* host, GV3101, contains a Ti plasmid (pMP90RK) from which the T-DNA has been deleted. The pPCV vectors have been used to transform a variety of plant species. Following integration into the plant genome the T-DNA can be present in one, two, or more copies in a roughly equal distribution between individual transgenics (Koncz et al., 1989).

Selection/expression vectors contain the dual *mas* 1′ 2′ promoters of the T-DNA and are designed to direct high levels of expression of foreign genes (Fig. 4). For example, in the case of pCV730, cloning a gene at the Sal I site and selecting for resistance to high levels of hygromy-

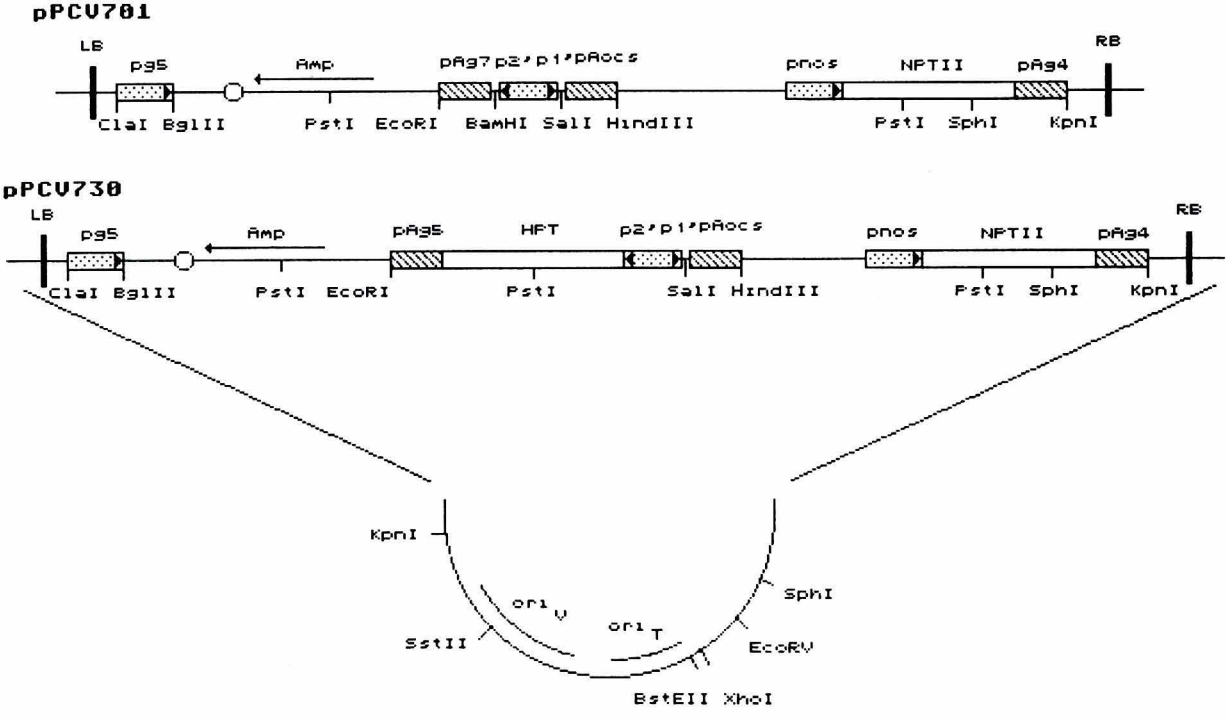

Figure 4. Selection/expression vectors for plant transformation. The plasmids are based on the pPCV series of transformation vectors (Koncz and Schell, 1986, see Fig. 2) which contain the ori_V and ori_T regions of pRK2 allowing maintenance in *Agrobacterium* pGV3101 harboring pMP90RK, a mutated Ti plasmid lacking the T-DNA but retaining an active *vir* region. The T-DNA regions are shown in detail. Stipled boxes containing arrows, promoters and direction of transcription; hatched boxes, poly A addition sequences, open boxes, marker genes; open circle, pBR322 origin of replication; Amp, bacterial ampicillin resistance gene; pg5, p2′, p1′ pnos, promoters of gene 5, 2′, 1′, and nopaline synthase, respectively; pAg7, pAocs, pAg4, poly A addition sites of gene 7, octopine synthase, and gene 4, respectively, LB, RB, the left and right T-DNA border sequences; NPT, neomycin phosphotransferase; HPT, hygromycin phosphotransferase. Important restriction sites are shown.

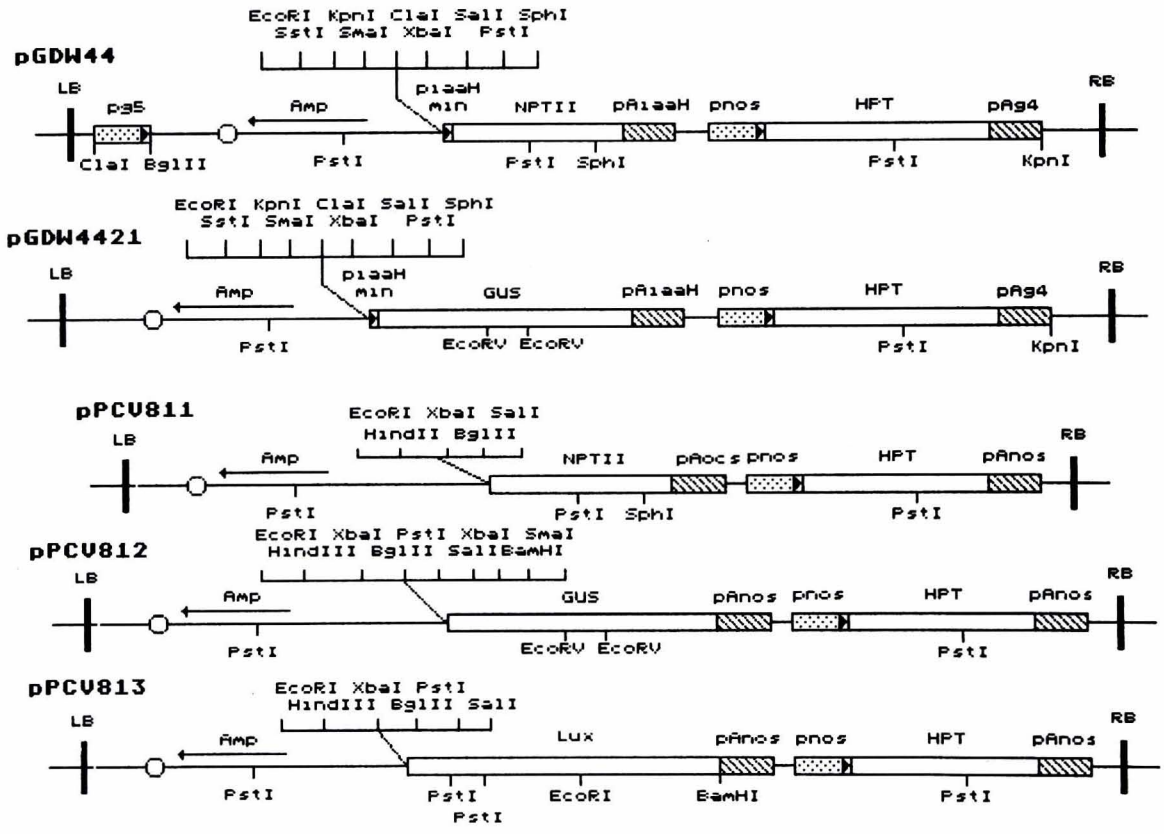

Figure 5. Promoter test plant transformation vectors. Vectors based on the pPCV series of plasmids are shown (Wing et al., 1989; Koncz et al., 1989). piaaH min and pAiaaH, minimal promoter and poly A addition site, respectively, of gene 2; pAnos, poly A addition site of nopaline synthase; p35S, 35S RNA promoter of CaMV; GUS, β-glucuronidase marker gene; Lux, monocistronic bacterial luciferase (Olsson et al., 1989). For further details see Figure 4 legend.

cin resistance will yield transgenic tissue in which foreign genes are also transcribed at high levels.

The ability of a specific plant sequence to direct gene expression can be investigated using promoter test vectors (Fig. 5). One type of vector contains a promoter sequence from which regulatory control sequences have been removed, i.e., a minimal promoter, linked to a marker gene. Insertion of fragments of DNA into this vector allows a test for the ability of a fragment to confer a specific pattern of gene expression (Wing et al., 1989). The other types of vector contain a marker gene with no promoter, in this case fragments of DNA being able to direct gene expression can be tested (Koncz et al., 1989).

The general purpose vectors shown (Fig. 6) contain different combinations of marker gene cassettes inserted into the T-DNA of the pPCV plasmids. Each allows dominant selection on kanamycin and provides the opportunity of using either a second dominant marker or Lux genes from bacteria as a screenable marker.

USE OF T-DNA IN SEQUENCE TAGGING

By virtue of its insertion into the plant genome, T-DNA in its own right can be considered as an insertional mutagen and if engineered appropriately can be used to tag specific genomic sequences. The advantages offered by the T-DNA as a plant insertional mutagen when compared with other potential mutagens, for example, EMS or transposons, are that single, stable insertions can be obtained. Moreover, if the T-DNA is appropriately engineered, it can be used to tag sequences from the genome which carry out a specific function. In addition, using a vector such as those based on the pPCV series, allows rescue of the plant sequences which flank the T-DNA in *E. coli*.

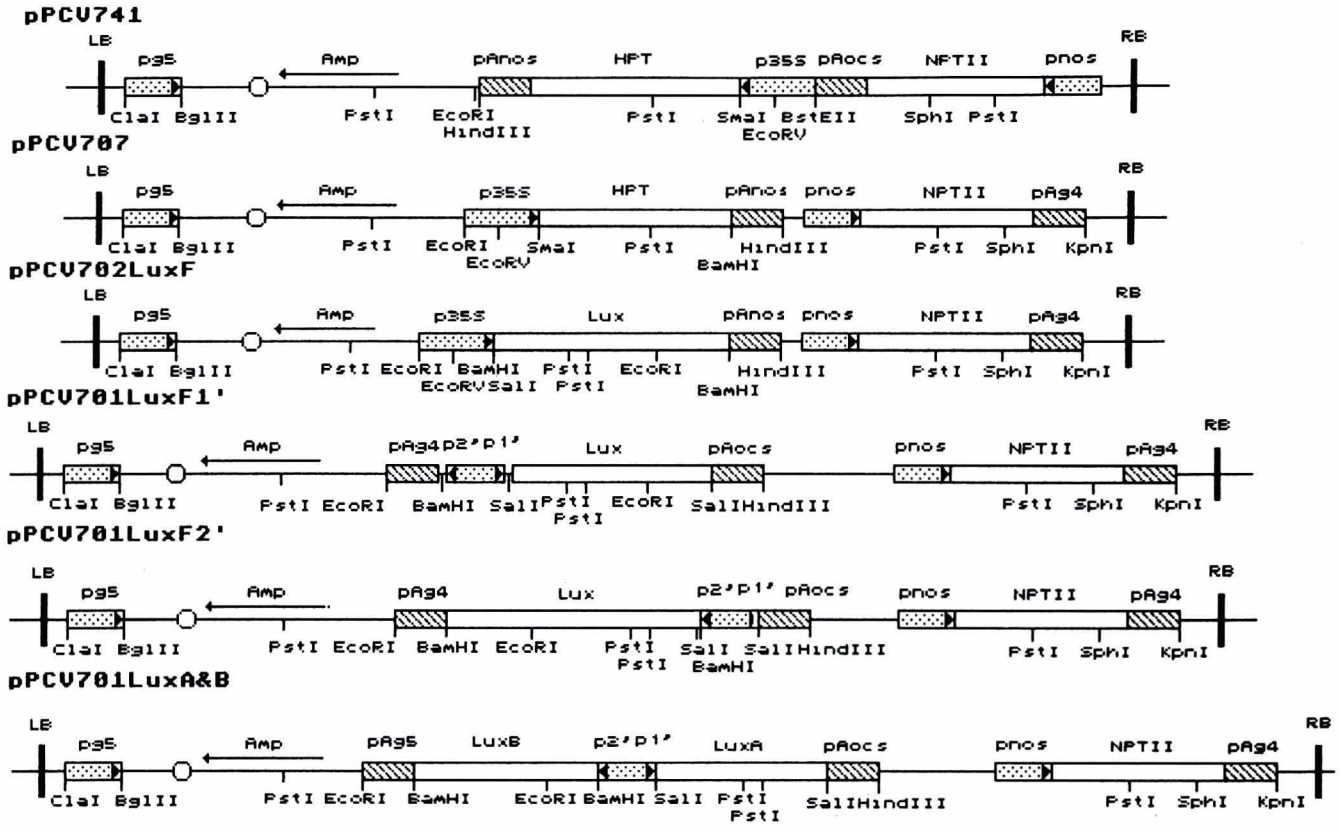

Figure 6. General purpose plant transformation vectors. Vectors based on the pPCV series of plasmids are shown. Lux A and Lux B, the α and β subunits of bacterial luciferase, respectively (Koncz et al., 1987). For further details see Figure 4 and 5 legends.

T-DNA has been used to tag promoter sequences (Andre et al., 1988; Koncz et al., 1989; Teeri et al., 1986), as well as produce novel phenotypes (Feldmann et al., 1989; Koncz et al., in press). Using T-DNA to construct transcriptional and translational fusions with plant genes it was found that a high frequency of transformants (25%) contained fusions in both arabidopsis and tobacco (Koncz et al., 1989). Bearing in mind that the transgenics contained one, two or more inserts in a 1 : 1 : 1 ratio, the difference in size (1.6×10^9 bp and 7×10^7 bp for tobacco and arabidopsis, respectively) and the relative amount of repeated sequences of the two genomes (low in arabidopsis and 60% in tobacco) this result is surprising. It suggests that the T-DNA preferentially integrates into regions of the genome that potentially can be transcribed. Hence it would seem that T-DNA is ideally suited for gene tagging.

Use of the T-DNA as a specific tag relies on the ability to engineer it to contain either genetic markers, or sequences of DNA affecting gene expression, so that following plant transformation a desired phenotype can either be screened or selected for. Without this, creation of a particular phenotype relies solely on luck.

Promoter Sniffing

In these types of experiment the T-DNA is engineered so that a marker gene lacking a promoter sequence is located near the right border sequence of the T-DNA and a separate selectable marker gene is placed in another region of the T-DNA (Fig. 7). Following insertion into the plant genome, transformants are selected and the activity of the marker gene can be screened. The vectors can be used to investigate the activity of the tagged promoter in different plant tissues, but care needs to be exercised in screening marker activity as only those promoters active at the stage of development when screening takes place will be observed. Such vectors have been used

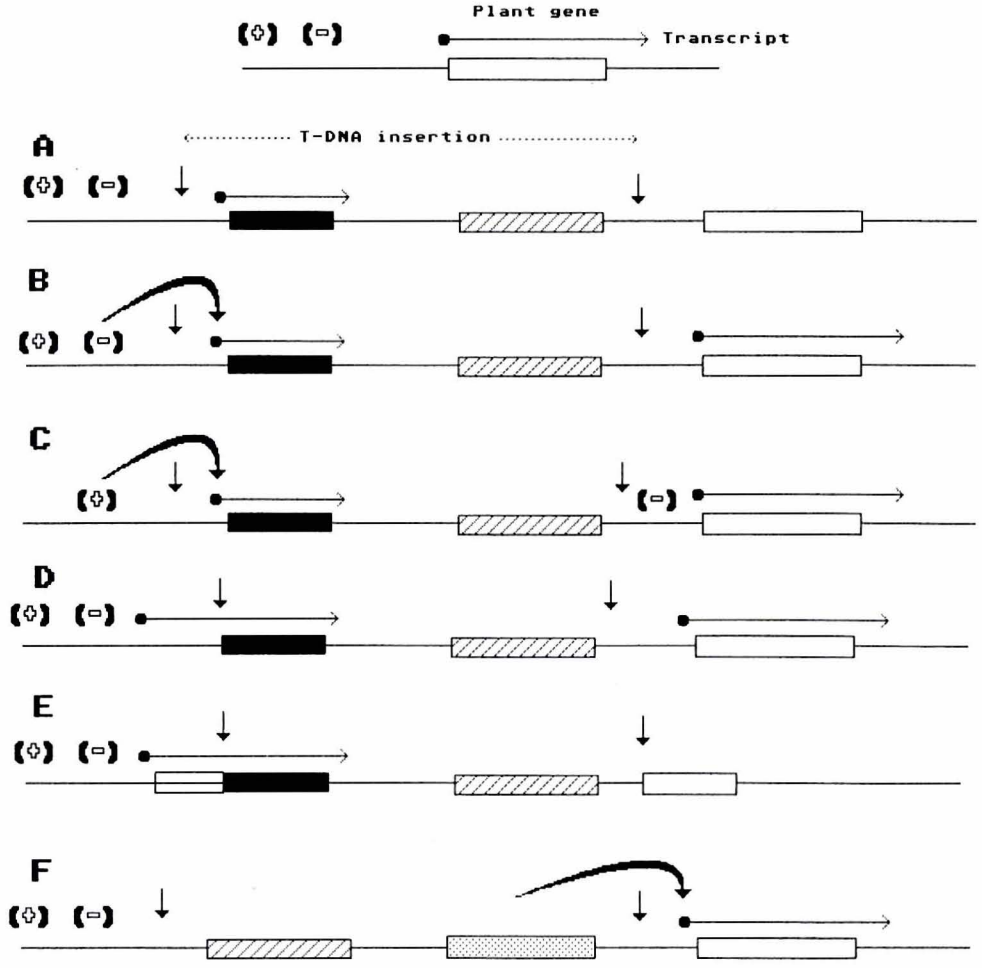

Figure 7. Using T-DNA as a gene tag. Schematic representation of the use of T-DNA to tag plant genes. A plant gene is depicted by an open box. 5′ regions controlling expression in a positive (+) or negative (−) manner are shown. T-DNA insertion into the 5′ leader sequence (or the coding region) will produce a mutation (A). Insertion of a T-DNA containing a marker gene linked to a minimal promoter can be used to detect the expression of the marker gene under the influence of either a negative (B) or positive transcriptional control element (C) located in the 5′ region of the plant gene. A promoterless marker gene fused to the border of the T-DNA can be used to produce transcriptional (D) or translational fusions (E). A T-DNA engineered to contain an enhancer sequence can influence the expression of flanking plant genes following insertion (F). Open box, plant gene; horizontal arrows, transcripts; vertical arrows, T-DNA border sequences; filled box, screenable marker gene; hatched box, selectable marker; stipled box, transcriptional enhancer.

successfully to tag promoters in both tobacco and arabidopsis (Andre et al., 1988 Koncz et al., 1989). Vectors developed for promoter sniffing are shown in Figure 8.

Enhancer/Silencer Fishing

These vectors are similar to those used in promoter sniffing except that the marker gene is linked either to a minimal promoter able only to direct low levels of gene expression or a normal complete promoter. Transgenic tissue is then screened for activity of the marker gene. Tagging an enhancer will increase levels of expression of the marker gene in the former case, whereas in the latter a silencer will reduce gene expression (Fig. 7). A variation in the strategy to isolate silencers is to use a marker gene, such as gene 2 of T-DNA, which allows negative selection (Depicker et al., 1988).

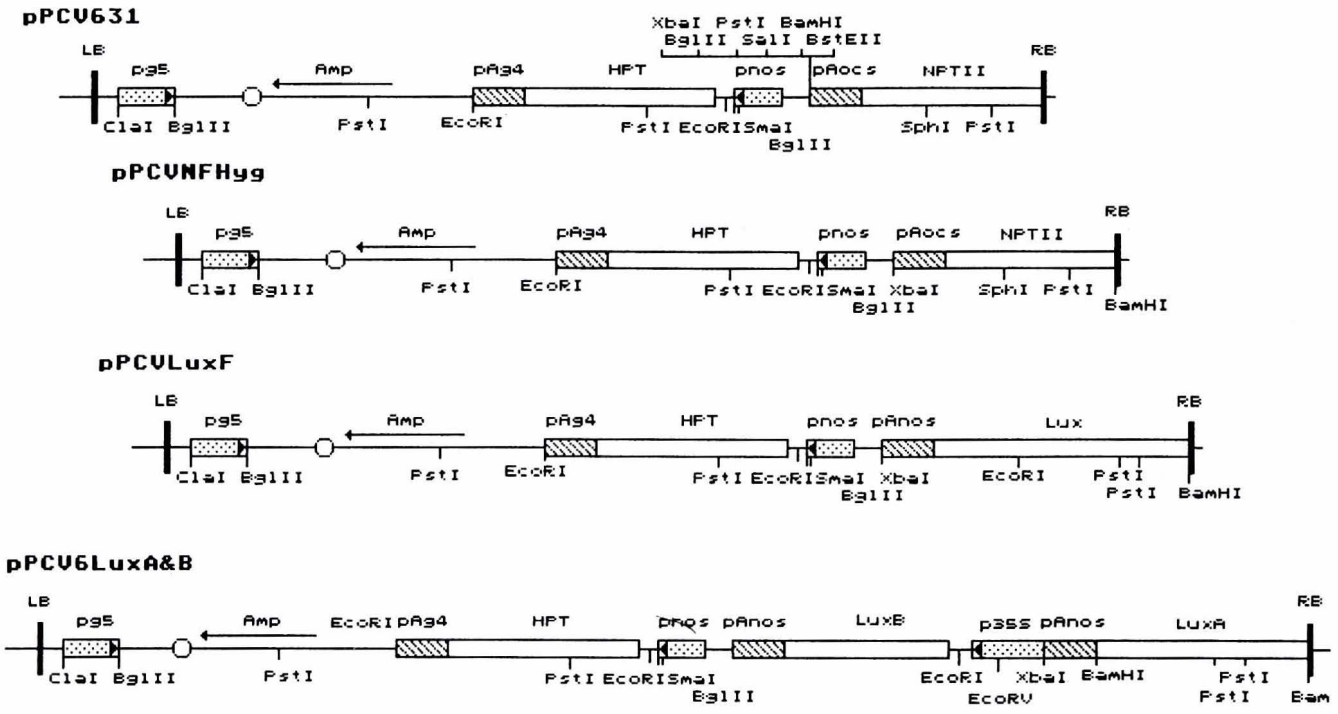

Figure 8. T-DNA vectors for gene tagging. All vectors are based on the pPCV series of vectors; for details see Figures 4, 5, and 6. pPCV6NFHyg is engineered to form translational fusions, whereas pPCV631, pPCV6LUXF, and pPCV6LUXA&B form transcriptional fusions.

Production of Over-expression Mutants

Insertional mutagenesis will disrupt gene expression so that in the homozygous state it may prove lethal. An alternative is to produce over-expression mutants in which the gene in question is not disrupted and a dominant phenotype is obtained. In order to do this, the T-DNA can be engineered so that it contains enhancer sequences which act to activate the expression of flanking plant DNA sequences following insertion into the plant genome. In this way it may be possible to obtain mutants which express genes in a developmentally independent manner (Fig. 7).

T-DNA VECTORS AS DELIVERY SYSTEMS FOR TRANSPOSON TAGGING

In addition to T-DNA itself as a tag, plant transposons, introduced into the plant genome on T-DNA can also be used to tag genes. Using T-DNA vectors it has been shown that transposable elements can transpose in heterologous host plants (Baker et al., 1986; Van Sluys et al., 1987 Knapp et al., 1988; Yoder et al., 1988; Masterson et al., 1989). The *Ac/Ds* system of maize has been studied in most detail and this system comprises the autonomous *Ac* (Activator) element which is able to transpose by itself and *Ds* (Dissociator) which is nonautonomous and can transpose only when *Ac* is present in the genome (for review, see Döring and Starlinger, 1986). With an increasing clarification of the molecular mechanism of transposition of these elements, assays have been devised so that not only excision but also reinsertion of them in a heterologous plant genome can be investigated. This can be achieved using a transposon tag vector depicted in Figure 9. The intermediate vector pKU4 contains a *Ds* element inserted into the leader sequence of a chimeric NPTII gene (Baker et al., 1986, 1987). When this is introduced into tobacco cells using a cointegrative vector, the resultant tissue is sensitive to kanamycin due to the presence of the *Ds* element within the construct. On the other hand, when the construct is transferred to cells that contain *Ac*, a proportion of them aquire resistance to kanamycin which is correlated with the transposition of the *Ds* element. Hence, this provides a simple assay of transposition. Furthermore, the *Ds* element itself can be

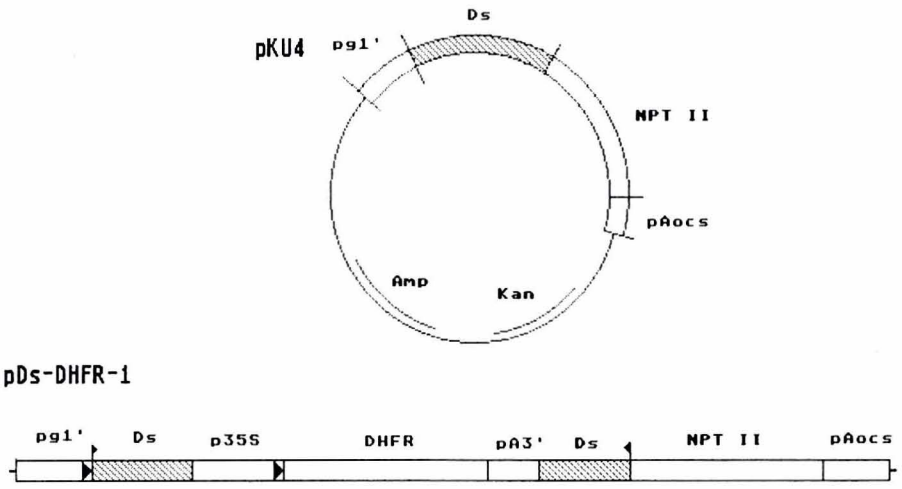

Figure 9. Vectors for the study of the transposition of transposable elements and gene tagging. pKU4 is based on pBR322, and contains a chimeric NPT II gene with a *Ds* element inserted into the 5' untranslated region so that normal transcription is destroyed (Baker et al., 1987). pDs-DHFR-1 is based on pKU4 (only the region of the chimeric gene is depicted here) and contains a functional chimeric DHFR gene inserted into the *Ds* element. pg1', p35S, the promoters of gene 1', and the 35S RNA of CaMV, respectively; pAocs, pA3' the poly A addition site of octopine synthase, and gene 3, respectively. NPT II, neomycin phosphotransferase; DHFR, dihydrofolate reductase, Amp, Kan, ampicillin, and kanamycin resistance, respectively; hatched box, the *Ds* element with the ends of the element depicted by flags.

engineered to contain a selectable marker, such as dihydrofolate reductase. Following transfer of this construct into an *Ac* containing cell, selection of kanamycin resistant cells on methotrexate can be used to assay the frequency of reinsertion of the element into the genome (Masterson et al., 1989). This forms the basis of a gene tagging system because tagged genes will be associated with a dominant selectable marker that can be screened for co-segregation with any novel phenotype.

DISCUSSION

The properties of T-DNA insertion into the plant genome make it the system of choice for the engineering of stably transformed plants. Moreover, the versatility of gene vectors that exploit this natural process of genetic engineering is such that T-DNA can be used for a variety of purposes other than simple gene transfer.

T-DNA, carrying viral genomes or transposable elements may allow the development of further novel gene delivery systems. As yet, work on viral vectors remains relatively limited (for reviews, see Gronenborn and Matzeit, 1989; Walden, 1988) although they have already proven their worth in being able to transfer foreign DNA to plants and replicating to high copy number so that high levels of expression of the foreign DNA can be detected (Brisson et al., 1984; Lefebvre et al., Hayes et al., 1988).

While the model systems used with *Agrobacterium* mediated gene transfer remain the dicotyledonous species, it is becoming increasingly clear that *Agrobacterium* can also transfer DNA to monocotyledonous plants (Bytebier et al., 1989; Grimsley et al., 1989; Hernalsteens et al., 1984; Hooykaas-Van Slogteren et al., 1984; Schäfer et al., 1987). This observation, coupled with the advances in the plant regeneration from monocotyledonous cells (Lörz et al., 1989), suggests that in the future the techniques described in this review will be extended from the dicotyledonous species.

Finally, as we have discussed, T-DNA vectors can be used to probe the structure and organization of the plant genome. While the mechanism of insertion of the T-DNA into the genome remains to be established, it is likely that the continued study of this process will yield information concerning chromatin structure, the control of transcription as well as DNA replication. Over the years the unique ability of *Agrobacterium* to transfer its DNA to the plant cell has provided us

with a means to study plant/bacterial interactions, plant development, gene transfer, and the control of gene expression. With the vectors and the marker genes that are currently available to us it is clear that the power of this intriguing natural phenomena to investigate plant biology is far from being exhausted.

ACKNOWLEDGMENTS

Our thanks to Reinhard Töpfer, Norbert Martini, and David Wing for providing maps of the plasmids which they have constructed. We also thank our co-workers in Abteilung Schell for valuable discussions and Desy Rosahl, Bob Masterson, and Ellen Buschfeld for their comments on the manuscript.

LITERATURE CITED

Albright, L.M., Yanofsky, M.F., Leroux, B., Ma, D., and Nester, E.W. (1987), Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single stranded T-DNA. *J. Bact.* **169:**1046–1055.

An, G., Costa, M.A., Mitra, A., Ha, S-B., and Marton, L. (1988), Organ-specific and developmental regulation of the nopaline synthase promoter in transgenic tobacco plants. *Plant Physiol.* **88:**547–552.

An, G., Ebert, P.R., Yi, B-Y., and Choi, C-H. (1986), Both TATA box and upstream regions are required for nopaline synthase promoter activity in transformed tobacco cells. *Mol. Gen. Genet.* **203:**245–250.

An, G., Watson, B.D., and Chiang, C.C. (1986), Transformation of tobacco, tomato, potato and *Arabidopsis thaliana* using a binary Ti vector system. *Plant Physiol.* **81:**301–305.

An, G., Watson, B.D., Stachel, S., Gordon, M.P., and Nester, E.W. (1985), New cloning vectors for the transformation of higher plants. *EMBO J.* **4:**277–284.

Andre, D., Colau, D., Schell, J., Van Montagu, M., and Hernalsteens, J.P. (1988), Gene tagging in plants by a T-DNA insertion that generates APH (3′)II plant gene fusions. *Mol. Gen. Genet.* **204:** 512–518.

Baker, B., Coupland, G., Federoff, N., Starlinger, P., and Schell, J. (1987), Phenotypic assay for excision of maize controlling element Ac in tobacco. *EMBO J.* **6:**1547–1554.

Baker, B., Schell, J., Lörz, H., and Federoff, N. (1986), Transposition of the maize controlling element *activator* in tobacco. *Proc. Nat. Acad. Sci. U.S.A.* **83:**4844–4848.

Benfey, P.N., and Chua, N-H. (1989), Regulated genes in transgenic plants. *Science.* **244:**174–181.

Benfey, P.N., Ren, L., and Chua N-H. (1989), The 35S enhancer contains at least two domains which can confer different developmental and tissue specific expression patterns. *EMBO J.* **8:**2195–2202.

Bevan, M. (1984), Binary *Agrobacterium* vectors for plant transformation. *Nuc. Acids Res.* **12:**8711–8721.

Brisson, N., Paszkowski, J., Penswick, J., Gronenborn B., Potrykus, I., and Hohn, T. (1985), Expression of a bacterial gene in plant cells using a viral vector. *Nature.* **310:**511–514.

Bytebier, B., Deboeck, F., Greve, H.D., van Montagu, M., and Hernalsteens, J.P. (1987), T-DNA organisation in tumour cultures and transgenic plants of the monocotyledon *Asparagus officinalis*. *Proc. Nat. Acad. Sci. U.S.A.* **84:**5345–5349.

Buchanan-Wollaston, V., Passiatore, J.E., and Canon, F. (1987), The *mob* and *oriT* mobilisation functions of a bacterial plasmid promote its transfer to plants. *Nature.* **328:**172–175.

Christie, T.J., Ward, J.E., Winans, S.C., and Nester, E.W. (1988), The *Agrobacterium tumefaciens virE2* gene product is a single-stranded-DNA-binding protein that associates with T-DNA. *J. Bacteriol.* **170:**2653–2667.

Close, T.J., Rogowsky, P.H., Kado, C.I., Winans, S.C., Yanofsky, M.F., and Nester, E.W. (1987a), Dual control of *Agrobacterium tumefaciens* Ti plasmid virulence genes. *J. Bact.* **169:**5113–5118.

Close, T.J., Tait, R.C., Rempel, H.C., Hiroaka, T. Kim, L., and Kado, C.I. (1987b), Molecular characterisation of the *virC* genes of the Ti plasmid. *J. Bact.* **169:**2336–2344.

Czernilofsky, A.P., Hain, R., Herrera-Estrella, L., Lörz, H., Goyvaerts, E., Baker, B., and Schell, J. (1986), Fate of selectable marker DNA integrated into the genome of *Nicotiana tabacum*. *DNA* **5:**101–113.

Colau, D., Negrutiu. I., Van Montagu, M., and Hernalsteens, J-P. (1987), Complementation of a threonine dehydratase-deficient *Nicotiana plumbaginifolia* mutant after *Agrobacterium tumefaciens* mediated transfer of *Saccharomyces cerevisiae* ILV1 gene. *Mol. Cell. Biol.* **7:**2552–2557.

Crossway, A., Oakes, J.V., Irvine, J.M., Ward, B., Knauf, V.C., and Shewmaker, C.K. (1986), Integration of foreign DNA following microinjection into tobacco mesophyll protoplasts. *Mol. Gen. Genet.* **203:**179–185.

Damm, B., and Willmitzer, L. (1988), Regeneration of fertile plants from protoplasts of different *Arabidopsis thaliana* genotypes. *Mol. Gen. Genet.* **213:** 15–20.

Dean, C., Favreau, M., Tamaki, S., Bond-Nutter, D., Dunsmiur, P., and Bedbrook, J. (1988), Expression of tandem gene fusions in transgenic tobacco plants. *Nuc. Acids Res.* **16:**7601–7617.

Dean, C., Jones, J., Favreau, M., Dunsmiur, P., and Bedbrook, J. (1988), Influence of flanking sequences on variability in expression levels of introduced genes in transgenic tobacco plants. *Nuc. Acids Res.* **16:**9267–9283.

DeBlock, M., Batterman, J., Vandewiele, M., Dockx, M., Theon, C., Gassele, V., Rao Movva, N., Thompson, C., Van Montagu, M., and Leemans, J. (1987), Engineering herbicide resistance into plants by expression of a detoxifying enzyme. *EMBO J.* **6:**2513–2518.

DeBlock, M., Herrera-Estrella, L., Van Montagu, M., Schell, J., and Zambryski, P. (1984), Expression of foreign genes in regenerated in plants and their progeny. *EMBO J.* **3:**1681–1689.

De La Pena, A., Lörz, H., and Schell, J. (1987), Transgenic rye plants by injecting DNA into young floral leaf tillers. *Nature.* **325:**274–276.

Depicker, A.G., Jacobs, A.M., and Van Montagu, M. (1988), A negative selection scheme for tobacco protoplast-derived cells expressing the T-DNA gene 2. *Plant Cell Rep.* **7:**63–66.

Depicker, A.G., Herman, L., Jacobs, A., Schell, J., and Van Montagu, M. (1987), Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. *Mol. Gen. Genet.* **201:**477–484.

Deshayes, A., Herrera-Estrella, L., and Caboche, M. (1985), Liposome-medated transformation of tobacco mesophyll protoplasts by an *Escherichia coli* plasmid. *EMBO J.* **4:**231–2737.

Dhaese, P., De Greve, H., Decraemer, H., Schell, J., and Van Montagu, M. (1979), Rapid mapping of transposon insertion and deletion mutations in the large Ti plasmids of *A. tumefaciens. Nuc. Acids Res.* **7:**1837–1849.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (1980), Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti. Proc. Natl. Acad. Sci. U.S.A.* **77:**7347–7351.

Draper, J., Scott, R., Armitage, P., and Walden, R. (1988), *Plant Genetic Transformation and Gene Expression, A Laboratory Manual.* Blackwell Scientific, Oxford.

Döring, H-P., and Starlinger, P. (1986), Molecular aspects of transposable elements in plants. *Ann. Rev. Genet.* **20:**175–200.

Ebert, P.R., Ha, S.B., and An, G. (1987), Identification of an essential upstream element in the nopaline synthase promoter by stable and transient assays. *Proc. Nat. Acad. Sci. U.S.A.* **84:**5745–5749.

Fang, R-X., Nagy, F., Sivasubramaniam, S., and Chua, N-H. (1989), Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. *The Plant Cell.* **1:**141–150.

Feldmann K.A., and Marks, M.D. (1981), *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana;* a non tissue culture approach. *Mol. Gen. Genet.* **208:**1–9.

Feldmann, K.A., Marks, M.D., Christianson, M.L., and Quatrano, R.S. (1989), A dwarf mutant of *Arabidopsis* generated by T-DNA insertion mutagenesis. *Science.* **243:**1351–1354.

Fluhr, R., Kuhlemeyer, C., Nagy, T.F., and Chua, N-H. (1986), Organ-specific and light induced expression of plant genes. *Science.* **232:**1106–1112.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Eichholtz, D.A., Flick, C.A., Hoffman, N.L., and Saunders, P.R. (1985), The SEV system: A new disarmed Ti plasmid vector system for plant transformation. *Bio/Technology.* **3:**629–635.

Fromm, M., Taylor, L.P., and Walbot, V. (1985), Expression of genes transferred to monocot and dicot plant cells by electroporation. *Proc. Nat. Acad. Sci. U.S.A.* **82:**5824–5828.

Gasser, C.S., and Fraley, R.T. (1989), Genetically engineering plants for crop improvement. *Science.* **244:**1293–1299.

Gietl, Ch., Koukolikova-Nicola, Z., and Hohn, B. (1987), Mobilization of T-DNA from *Agrobacterium* to plant cells involves a protein that binds single-stranded DNA. *Proc. Nat. Acad. Sci. U.S.A.* **84:**900–910.

Grimsley, N., Hohn, B., Ramos, G., Kado, C., and Radowky, P. (1989), DNA transfer from *Agrobacterium* to *Zea mays* or *Brassica* by agroinfection is dependent on bacterial virulence functions. *Mol. Gen. Genet.* **216:**309–316.

Gronenborn, B., and Matzeit, V. Plant gene vectors and genetic transformation: Plant viruses as vectors, in *Cell Culture and Somatic Genetics of Plants: Molecular Biology of Plant Nuclear Genes,* eds. J. Schell and I.K. Vasil, Academic Press, New York (1989), pp. 69–101.

Hain, R., Stabel, P., Czernilofsky, A.P., Steinbiss, H.H., and Schell, J. (1985), Uptake, integration, expression and genetic transmission of a selectable chimeric gene by plant protoplasts. *Mol. Gen. Genet.* **199:**161–168.

Hain, R., Steinbiss, H.H., and Schell, J. (1985), Fusion of *Agrobacterium* and *E. coli* protoplasts with *Nicotiana tabacum* protoplasts—direct gene transfer from microorganisms to higher plants. *Plant Cell Rep.* **3:**60–64.

Haughn, G.W., Smith, J., Mazur, B., and Somerville, C. (1988), Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. *Mol. Gen. Genet.* **211:***266–271.*

Hayes, R.J., Petty, I.T.D., Coutts, R.H.A., and Buck, K.W. (1989), Gene amplification and expression in plants by a replicating geminivirus vector. *Nature.* **344:**179–182.

Hayford, M.B., Medford, J.I., Hoffman, N.L., Rogers, S.G., and Klee, H. (1988), Development of a plant transformation selection system based on

expression of genes encoding gentamycin resistance. *Plant Physiol.* **86:**1216–1222.

Heinemann, J.A., and Sprague, G.F., Jr. (1989), Bacterial conjugative plasmids mobilise DNA transfer between bacteria and yeast. *Nature.* **340:**205–209.

Helmer, G., Casadaban, M., Bean, M., Kayes, L., and Chilton, M-D. (1984), A new chimeric gene as a marker for plant transformation: The expression of *Escherichia coli* β-galactosidase in sunflower and tobacco cells. *Bio/Technology.* **2:**520–527.

Hernalsteens, J.P., Thia-Toong, L., Schell, J., and van Montagu, M. (1984), An *Agrobacterium*-transformed cell culture from the monocot *Asparagus officinalis*. *EMBO J.* **3:**3039–3041.

Herrera-Estrella, L., DeBlock, M., Messens, E., Hernalsteens, J-P., Van Montagu, M., and Schell, J. (1983), Chimeric genes as dominant selectable markers in plant cells. *EMBO J.* **4:**2987–2995.

Hille, J., Roelvink, A., Franssen, H., Van Kammen, A., and Zabel, P. (1986), Bleomycin resistance: a new dominant selectable marker for plant cell transformation. *Plant Mol. Biol.* **7:**171–176.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J., and Schilperoort, R.A. (1983), A binary plant vector strategy based on the separation of the *vir* and T-region of agrobacteria. *Nature.* **303:**79–181.

Hooykaas-Van Slogteren, G.M.S., Hooykaas, P.J.J., and Schilperoort, R.A. (1984), Expression of Ti plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens*. *Nature.* **311:**763–764.

Inze, D., Follin, A., Velten, J., Velten, L., Prinsen, E., Rudelsheim, P., Van Onckelen, H., Schell, J., and Van Montagu, M. (1987), The *Pseudomonas stavastonai* tryptophan-2-monooxygenase is biologically active in *Nicotiana tabacum*. *Planta.* **172:**555–562.

Ishida, B.K., Snyder, G.W., and Belknop, W.R. (1989), Use of in vitro grown microtuber discs in *Agrobacterium* mediated transformation of Russett Burbank and Lemhi Burbank potatoes. *Plant Cell Rep.* **8:**325–328.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987), GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in plants. *EMBO J.* **6:**3201–3207.

Jones, J., Gilbert, D., Grady, K., and Jorgenson, R. (1987), T-DNA structure and gene expression in petunia plants transformed by *Agrobacterium tumefaciens* C58 derivatives. *Mol. Gen. Genet.* **207:**478–485.

Jones, J.D.G., Svab, Z., Harper, E.C., Horwitz, C.D., and Maliga, P. (1987), A dominant nuclear streptomycin resistance marker for plant cell transformation. *Mol. Gen. Genet.* **210:**86–91.

Jorgensen, R., Snyder, C., and Jones, J.D.G. (1987), T-DNA is organised predominantly in repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. *Mol. Gen. Genet.* **207:**471–477.

Kahl, G., and Schell, J. (1982), *Molecular Biology of Plant Tumours*. Academic Press, New York.

Kay, R., Chan, A., Daly, M., and McPherson, J. (1987), Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science.* **236:**1299–1302.

Klee, H.J., Hayford, M.B., and Rogers S. (1987), Gene rescue in plants: A model system for "shotgun" cloning by retransformation. *Mol. Gen. Genet.* **210:**282–287.

Klee, H.J., Horsch, R., and Rogers, S. (1987), *Agrobacterium*-mediated plant transformation and its further applications to plant molecular biology. *Ann. Rev. Genet.* **20:**467–486.

Klein, T.M., Wolf, E.D., Wu, R., and Stanford, J.C. (1987), High velocity microprojectiles for delivering nucleic acids into living cells. *Nature.* **327:**70–73.

Knapp, S., Coupland, G., Starlinger, P., and Salamini, F. (1987), Transposition of the maize transposable element Ac in *Solanum tuberosum*. *Mol. Gen. Genet.* **213:**285–290.

Koncz, C., Kreuzaler, F., Kalman, Zs., and Schell, J. (1984), A simple method to transfer, integrate and study expression of foreign genes, such as chicken ovalbumin and α-actin in plant tumors. *EMBO J.* **3:**1029–1037.

Koncz, C., Martini, N., Koncz-Kalman, Zs., Olsson, O., Radermacher, A., Szalay, A.A., and Schell, J., Genetic tools for the analysis of gene expression in plants, in *Tailoring Gene for Crop Improvement*, eds. G. Bruening, J. Harada, T. Kosuge, and A. Hollaender, Plenum, New York (1987), pp. 197–209.

Koncz, C., Martini, N., Mayerhofer, R., Koncz-Kalman, Zs., Körger, H., Redei, G.P., and Schell, J. (1989), High-frequency T-DNA-mediated gene tagging in plants. *Proc. Nat. Acad. Sci. U.S.A.* **86:**8467–8471.

Koncz, C., Olsson, O., Langridge, W.H.R., Schell, J., and Szalay, A. (1987), Expression and assembly of functional bacterial luciferase in plants. *Proc. Nat. Acad. Sci. U.S.A.* **84:**131–135.

Koncz, C., and Schell, J. (1986), The promoter of the T_L-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* **204:**383–396.

Langridge, W.H.R., Fitzgerald, K.J., Koncz, C., Schell, J., and Szalay, A.A. (1989), Dual promoter of *Agobacterium tumefaciens* mannopine synthase genes is regulated by growth hormones. *Proc. Nat. Acad. Sci. U.S.A.* **86:**3219–3223.

Lefebvre, D.D., Miki, B.L., and Laliberte, J-F. (1987), Mammalian metallothionein functions in plants. *Bio/Technology.* **5:**1053–1056.

Leroux, B., Yanofsky, M.F., Winans, S.C., Ward, J.E., Ziegler, S.F., and Nester, E.W. (1987), Characterization of the *virA* locus of *Agrobacterium tu-*

mefaciens: a transcriptional regulator and host range determinant. *EMBO J.* **6**:849–856.

Lloyd, A.M., Barnason, A.R., Rogers, S.G., Byrne, M.C., Fraley, R.T., Horsch, R.B. (1986), Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. *Science*. **234**:464–466.

Lörz, H., Göbel, E., and Brown, P. (1988), Advances in tissue culture and progress towards genetic transformation of cereals. *Plant Breeding*. **100**:1–25.

Marton, L., Wullems, G.J., Molendijk, L., and Schilperoort, R.A. (1979), *In vitro* transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens*. *Nature*. **277**:129–131.

Masterson, R.V., Furtek, D.B., Grevelding C., and Schell, J. (1989), A maize Ds transposable element containing a dihydrofolate reductase gene transposes in *Nicotiana tabacum* and *Arabidopsis thaliana*. Mol. Gen. Genet. **219**:461–466.

Mattanovich, D., Rüker, F., da Camara Machado, A., Laimer, M., Regner, F., Steinkeliner, H., Himmler, G., and Katinger, H. (1989), Efficient transformation of *Agrobacterium* spp. by electroporation. *Nuc. Acids Res.* **17**:6747.

Medford, J., and Klee, H ., in *The Molecular Basis of Plant Development,* ed. R. Goldberg, Alan R. Liss, New York (1989).

Mitra, A., and An, G.A. (1989), Three distinct regulatory elements comprise the upstream promoter region of the nopaline synthase gene. *Mol. Gen. Genet.* **215**:294–299.

Neuhaus, G., Spangenburg, G., Mittelsten-Seneid, O., and Schweiger, H.-G. (1987), Transgenic rape seed plants obtained by microinjection of DNA into microspore-derived embryoids. *Theor. Appli. Genet.* **75**:30–36.

Odell, J.T., Nagy, F., and Chua, N-H. (1985), Identification of DNA sequences required for the activity of the cauliflower mosaic virus 35S promoter. *Nature*. **313**:810–812.

Okada, K., Hasekawa, S., Syono, K., and Nagata, T. (1985), Further evidence for the transformation of *Vinca rosea* protoplasts by *Agrobacterium tumefaciens* spheroplasts. *Plant Science Rep.* **4**:133–136.

Otten, L.A.B., and Schilperoort, R.A. (1978), A rapid microscale method for the detection of lysopine and nopaline dehydrogenase activities. *Biochem. Biophys. Acta.* **527**:497–500.

Olsson, O., Escher, A., Sandberg, G., Schell, J., Koncz, C., and Szalay, A.A., (1989), Engineering of monomeric bacterial luciferases by fusion of *luxA* and *luxB* genes in *Vibrio harveyi*. *Gene* **81**:335–347.

Olsson, O., Koncz, C., and Szalay, A. (1988), The use of the luxA gene of the bacterial luciferase operon as a reporter gene. *Mol. Gen. Genet.*, **215**:1–9.

Ow, D., Jacobs, J.D., and Howell, S.H. (1987), Functional regions of the cauliflower mosaic virus 35SRNA promoter determined by the use of firefly luciferase gene as a reporter of promoter activity. *Proc. Nat. Acad. Sci. U.S.A.* **84**:4870–4874.

Ow, D., Wood, K.V., DeLuca, L., DeWet, J.R., Helinski, D., Howell, S.H. (1986), Transient and stable expression of firefly luciferase gene in plant cells and transgenic plants. *Science*. **234**:856–859.

Paszkowski, J., Saul, M., and Potrykus, I., Plant gene vectors and genetic transformation: DNA mediated direct gene transfer to plants, in *Cell Culture and Somatic Cell Genetics of Plants,* eds. J. Schell, and I.K. Vasil, Academic Press, New York (1989); pp. 51–68.

Paszkowski, J., Shillito, R.D., Saul, M., Mandak, V., Hohn, T., Hohn, B., and Potrykus, I. (1984), Direct gene transfer to plants. *EMBO J.* **3**:2717–2722.

Pröls, M., Schell, J., and Steinbiss, H-H., Critical evaluation of electromediated gene transfer and transient expression in plant cells, in *Electroporation and Electrofusion in Cell Biology,* eds. E. Neumann, E.A. Sowers, and C.A. Jordan, Plenum Press, New York (1989), pp. 367–375.

Reiss, B., Sprengel, R., Will, H., and Schaller, H. (1984), A new and sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. *Gene*. **30**:211–218.

Reich, T.J., Iyer, V.N., and Miki, B.L. (1986), Efficient transformation of alfalfa protoplasts by the intranuclear microinjection of Ti plasmids. *Biotechnology*. **4**:1001–1004.

Rogers, S.G., Horsch, R.B., and Fraley, R.T., Gene transfer to plants: production of transformed plants using Ti plasmid vectors, in *Methods in Enzymology 118,* eds. A. Weissbach and H. Weissbach, Academic Press, New York (1986), pp. 627–640.

Rothstein, S.J., Dimaio, J., Strand, M., and Rice, D. (1987), Stable and heritable inhibition of the expression of nopaline synthase in tobacco expressing antisense RNA. *Proc. Nat. Acad. Sci. U.S.A.* **84**: 8439–8443.

Schäfer, W., Görz, A., and Kahl, G. (1987), T-DNA integration and expression in a monocot crop plant after induction of *Agrobacterium*. *Nature*. **327**: 529–532.

Schell, J., The T-DNA genes of *Agrobacterium* plasmids appear to be a complex evolutionary origin. in *Genetics, Development and Evolution,* eds. P. Gustafson, L.G. Stebbins, and F.J. Agala, Plenum Press, New York (1986), pp. 343–353.

Schell, J. (1987), Transgenic plants as tools to study the molecular organisation of plant genes *Science*. **237**:1176–1183.

Schell, J., and Vasil, I.K. (1989), *Cell Culture and Somatic Genetics of Plants; Molecular Biology of Plant Nuclear Genes*. Academic Press, New York.

Schmülling, T., Schell, J., and Spena, A. (1988), Single genes from *Agrobacterium rhizogenes* influence plant development. *EMBO J.* **7**:2621–2629.

Shah, D., Horsch, R.B., Klee, H.J., Kisherer, G.M., Winter, J.A., Tumer, N., Hironaka, C.M., Sanders, P.R., Gasser, C.S., Aykent, S., Siegel, N.R., Rog-

ers, S.G., and Fraley, R.T. (1986), Engineering herbicide tolerance in transgenic plants. *Science.* **233:**478–481.

Shen, W.H., Petit, A., Guern, J., and Tempe, J. (1988), Hairy roots are more sensitive to auxin than normal roots. *Proc. Nat. Acad. Sci. U.S.A.* **85:**3417–3421.

Simpson, R.B., Spielmann, A., Margossian, L., and McKnight, T.D. (1986), A disarmed binary vector from *Agrobacterium tumefaciens* functions in *Agrobacterium rhizogenes. Plant Mol. Biol.* **6:**403–415.

Spanier, K., Schell, J., and Schreier, P.H. (1989), A functional analysis of T-DNA gene 6b: The fine tuning of cytokinin effects on shoot development. *Mol. Gen. Genet.* **219:**209–216.

Spena, A., Schmülling, T., Koncz, C., and Schell, J. (1987), Independent and synergistic activity of *rolA, B* and *C* loci in stimulating abnormal growth in plants. *EMBO J.* **6:**3891–3899.

Stachel, S.E., Timmermann, B., and Zambryski, P. (1987), Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5′ *virD* product. *EMBO J.* **6:**857–863.

Stachel, S.E., and Zambryski, P. (1986), *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *Agrobacterium tumefaciens. Cell.* **46:**325–333.

Stalker, D., McBride, K.E., and Malyj, L.D. (1988), Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. *Science.* **242:** 419–423.

Stougaard, J., Abildstein, D., and Marcker, K.A. (1987), The *Agrobacterium rhizogenes* pRi TL segment as a gene vector system for the transformation of plants. *Mol. Gen. Genet.* **207:**251–255.

Teeri, T.H., Herrera-Estrella, L., Depicker, A., van Montagu, M., and Palva, E.T. (1986), Identification of plant promoteres *in situ* by T-DNA mediated transcriptional fusions to the NPTII gene. *EMBO J.* **5:**1755–1760.

Teeri, T.H., Lehväslaiho, H., Franck, M., Uotila, J., Heino, P., Palva, E.T., Van Montagu, M., and Herrera-Estrella, L. (1989), Gene fusions with *lacZ* reveal new expression patterns in chimeric genes in transgenic plants. *EMBO J.* **8:**343–350.

Tepfer, D., Ri T-DNA from *Agrobacterium rhizogenes:* A source of genes having application in rhizosphere biology and plant development, ecology and evolution, in *Plant Microbe Interactions: Molecular and Genetic Perspectives,* Vol. 3, eds. T. Kosuge and E.W. Nester, McGraw-Hill New York (1989), pp. 294–342.

Tinland, B., Huss, B., Paulus, F., Bonnard, G., and Otten, L. (1989), *Agrobacterium tumefaciens* 6b genes are strain specific and affect the activity of auxin as well as cytokinin genes. *Mol. Gen. Genet.* **219:**217–224.

Töpfer, R., Pröls, M., Schell, J., and Steinbiss, H-H. (1988), Transient gene expression in tobacco protoplasts: II Comparison of reporter gene systems for CAT, NPTII and GUS. *Plant Cell Rep.* **7:**225–228.

Töpfer, R., Schell, J., and Steinbiss, H-H. (1988), Versatile cloning vectors for transient gene expression and direct gene transfer in plants. *Nuc. Acids Res.* **16:**8725.

Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J., and Steinbiss, H-H. (1987), A set of plant expression vectors for transcriptional fusions. *Nuc. Acids Res.* **15:**5890.

Trinh, T.H., Manto, S., Pua, E-C., and Chua, N-H. (1987), Rapid production of transgenic flowering shoots and F1 progeny from *Nicotiana plumbaginifolia* epidermal peels. *Bio/Technology.* **5:**1081–1084.

Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., DeBeukeleer, M., Dean, C., Zabeau, M., Van Montagu, M., and Leemans, J. (1987), Transgenic plants protected against insect attack. *Nature.* **328:**33–37.

Valvekens, D., Van Montagu, M., and Lijebettens, M. (1988), *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Nat. Acad. Sci. U.S.A.* **85:**5536–5540.

Van Haute, E., Joos, H., Maes, S., Warren, G., Van Montagu, M., and Schell, J. (1983), Intergenic transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for reversed genetics of the Ti plasmids of *Agrobacterium tumefaciens. EMBO J.* **2:**411–418.

Van Sluys, M.A., Tempe, J., and Federoff, N. (1987), Studies on the introduction and mobility of the maize activator element in *Arabidopsis thaliana* and *Daucus carota. EMBO J.* **6:**3881–3889.

Velten, J., and Schell, J. (1985), Selection-expression plasmid vectors for use in genetic transformation of higher plants. *Nuc. Acids Res.* **13:**6981–6998.

Velten, J., Velten, L., Hain, R., and Schell, J. (1984), Isolation of a dual promoter fragment from the Ti plasmid of *Agrobacterium tumefaciens. EMBO J.* **3:**2723–2730.

Walden, R. (1988), *Genetic Transformation in Plants.* Open University Press, Milton Keynes.

Waldron, C., Murphy, E.B., Roberts, J.L., Gustafson, G.D., Armour, S.L., and Malcolm, S.K. (1985), Resistance to hygromycin B. *Plant Mol. Biol.* **5:**103–108.

Weising, K., Schell, J., and Kahl, G. (1988), Foreign genes in plants: transfer, structure and expression. *Ann. Rev. Genet.* **22:**421–477.

Wing, D., Koncz, C., and Schell, J. (1989), Conserved function in *Nicotiana tabacum* of a single *Drosophila hsp70* promoter heat shock element when fused to a minimal T-DNA promoter. *Mol. Gen. Genet.* **219:**9–16.

Yanofsky, M.F., Porter, S.G., Young, C., Albright, L.M., Gordon, M.P., and Nester, E.W. (1986), The *virD* operon of *Agrobacterium tumefaciens* encodes a site specific endonuclease. *Cell.* **47**:471–477.

Yoder, J.I., Pals, J., Alpert, K., and Lassner, M. (1988), Ac transposition in transgenic tomato plants. *Mol. Gen. Genet.* **213**:291–296.

Zambryski, P. (1988), Basic processes underlying agrobacterium-mediated DNA transfer to plant cells. *Ann. Rev. Genet.* **22**:1–30.

Zambryski, P., Joos, H., Gentello, C., Leemans, J., Van Montagu, M., and Schell, J. (1983), Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J.* **2**:2143–2154.

Zambryski, P., Tempe, J., and Schell, J. (1989), Transfer and function of T-DNA genes from agrobacterium Ti and Ri plasmids in plants. *Cell.* **56**:193–201.