Plant Genetic Transformation and Gene Expression

A Laboratory Manual

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All of
Botany Department
University of Leicester
Leicester LE1 7RH

RICHARD WALDEN B.Sc., Ph.D

Leicester Biocentre
University of Leicester
Leicester LE1 7RH

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Contributors

JOHN DRAPER
RODERICK SCOTT
PHILIP ARMITAGE
GRAHAM DURY
LAWRENCE JACOB

Botany Department
University of Leicester
University Road
Leicester LE1 7RH
UK

RICHARD WALDEN

Leicester Biocentre
University of Leicester

AMAR KUMAR

Department of Genetics
University of Leeds
Leeds LS2 9JT
UK

RICHARD JEFFERSON

Department of Molecular Genetics
Plant Breeding Institute
Maris Lane
Trumpington
Cambridge CB2 2LQ
UK

JOHN HAMIL

Institute of Food Research
Colney Lane
Norwich NR4 7UA
UK

Preface

This manual is based on the proceedings of a course run in December 1986 at the University of Leicester. The course was the fifth relating to genetic engineering which has been offered by the University. Each successive year in previous courses on gene cloning, a larger proportion of the participants wished to learn more than basic gene cloning techniques and were particularly interested in eukaryotic molecular biology and genetic engineering. Thus, the Plant Genetic Transformation and Gene Expression course represents our first attempt to organize an organism-specific programme to meet this perceived need. Both the course and this manual would not have materialized but for the dedication and enthusiasm of the instructors and the invaluble organizational skills of the technical managers and administrators. Thanks are also due to the many postdoctoral research associates, research assistants, graduate students and technicians who worked hard, not only to establish many of the techniques to be demonstrated on the course, but who have also given their time and energy to help organize the scientific programme. The support of Professor Harry Smith (Head of the Botany Department) should also not go unmentioned, as much of the stimulus to run the course came from his direction.

Finally, we would like to acknowledge the financial support of the Biotechnology Directorate of the Science and Engineering Research Council of the UK and the Biotechnology Unit of the Department of Trade and Industry. We are also grateful for the generosity of many of the companies listed at the back of the manual who supplied materials and equipment for use on the course.

The manual is aimed at the researcher with some experience in plant tissue culture, molecular biology or recombinant DNA technology who wishes to apply their experience to fundamental or commercially applied studies involving transgenic plants. Overall we have attempted to present a balanced view of the disciplines involved in plant genetic engineering; namely plant cell and tissue culture, vector manipulations and some basic recombinant DNA procedures, gene transfer techniques and modern molecular analytical methods for the study of proteins and nucleic acids. For each field we have endeavoured to provide enough theoretical background to aid the inexperienced researcher, but the manual remains essentially a practical guide.

We have tried to provide as comprehensive a view of modern plant genetic transformation technology and approaches to the analysis of gene expression as possible. Inevitably, given the vast number of plant species and wide range of molecular techniques, some important methods will have been omitted. It should

also be noted that, for clarity, a number of specific plant systems have been used to describe different transformation techniques and various molecular procedures. It should be realized that most of the protocols are intended as a guide to the development of similar procedures for other types of plant material.

Plant genetic engineering has emerged from obscurity a decade ago to become a major discipline in both fundamental eukaryote molecular genetics and commerical biotechnology. The possibilities for the future exploitation of this technology are enormous and the next decade promises to be an exciting one.

JOHN DRAPER

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1 Vectors for the transformation of plant cells using *Agrobacterium*

PHILIP ARMITAGE, RICHARD WALDEN and JOHN DRAPER

1.1 GENERAL INTRODUCTION

1.1.1 Tumour induction by *Agrobacterium*

Agrobacterium tumefaciens and *A. rhizogenes* are soil bacteria which induce crown gall and hairy root disease respectively at wound sites on dicotyledonous plants. Only a very few monocotyledonous plants in the families *Liliaceae* and *Amaryllidaceae* have been reported to be weakly susceptible to crown gall induction (see reviews below). The reason for this limit in host range is not currently understood. Once initiated, tumorous growth can continue in the absence of the bacterium and tumour tissue can grow axenically in tissue culture in media lacking exogenous supplies of auxins and cytokinins, which are normally required to promote growth of plant tissues *in vitro*. Tumour tissues synthesize novel amino acid and sugar derivatives known collectively as opines. The type of opine synthesized in the tumour (for example, nopaline, octopine, agrocinopine, mannopine and agropine) is dependent on the strain of *Agrobacterium* that initiated tumour formation. Octopine and nopaline are two types of opines derived from arginine and among the easiest to detect in crown gall tissue. Consequently, many common *Agrobacterium tumefaciens* strains are designated as octopine or nopaline types. Agropine, a sugar derivative, is commonly found in hairy root tumours induced by *A. rhizogenes*. The *Agrobacterium* responsible for tumour formation selectively catabolizes the opine whose synthesis it has induced, using it as a source of carbon and nitrogen.

Both tumour induction and opine synthesis are associated with the presence within the bacteria of a megaplasmid, the Ti (*Tumour inducing*) plasmid in the case of *A. tumefaciens* and the Ri (*Root inducing*) plasmid in *A. rhizogenes*. The molecular biology of tumour induction is reviewed in the following: Kahl and Schell (1982), Caplan *et al.* (1983), Gheysen *et al.* (1985) and Stachel and Zambryski (1986).

1.1.2 Ti plasmids of *Agrobacterium tumefaciens*

Ti plasmids (Fig. 1.1A), found in all virulent strains of *A. tumefaciens*, are around 200–250 kilobases (kb) in size and are stably maintained in the *Agrobacterium* at temperatures below 30 °C. Ti plasmids found in different strains of *Agrobacterium* have four regions of homology, as judged by DNA–DNA hybridization and heteroduplex mapping. Genetic analysis has shown that two regions, the T- (*Transferred*) DNA and the *vir* (*virulence*) region are associated with tumour formation, whereas the other two are involved with conjugative transfer and the replicative maintenance of the plasmid within the *Agrobacterium*.

During tumour formation a defined sequence of Ti plasmid, the T-DNA, is transferred to the plant cell and integrated into the plant nuclear genome. The T-DNA is stable within the plant genome and hybridization of a Ti plasmid-specific probe to tumour DNA has shown that the T-DNA found in the plant cell is

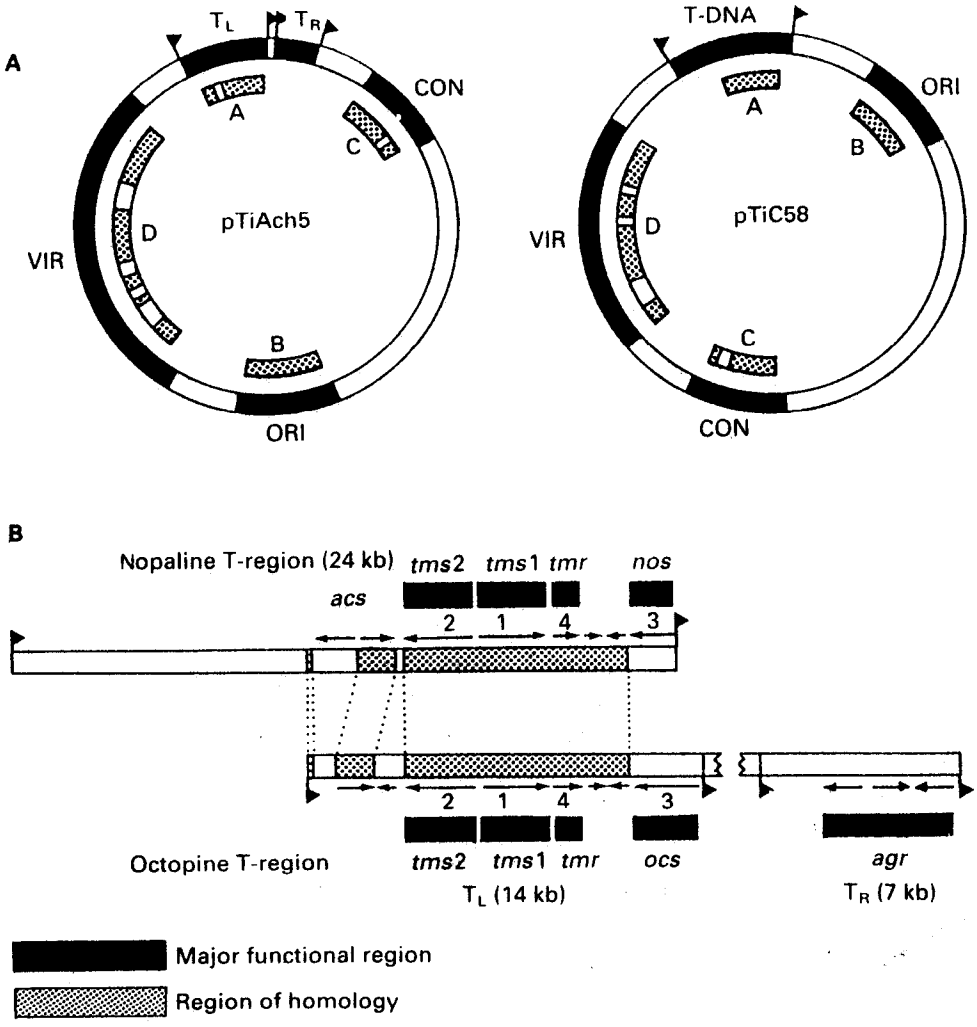

Fig. 1.1 *General organization and expression of octopine- and nopaline-type Ti plasmids.*
A Map of an octopine-type (pTiAch5) and a nopaline-type (pTiC58) Ti plasmid showing the relative positions and sizes of the major functional regions. Shaded areas indicate the regions of extensive homology between the two plasmids. ‖ = 25 bp direct repeats; CON = regions encoding conjugation functions; ORI = region encoding replication functions and origin of replication; VIR = virulence region; T-DNA, T_L, T_R = regions containing T (Transferred)-DNA.
B Maps of a nopaline- and octopine-type T region showing the major functional domains, the regions of homology and transcripts. Products of transcription which have been identified to date are: 3 (*nos*) = nopaline synthase; 3 (*ocs*) = octopine synthase; *acs* = agrocinopine synthase; 1 (*tms*1) = tryptophan mono-oxygenase; 2 (*tms*2) = indole-3-acetamide hydrolase; 4 (*tmr*) = DMA transferase; *agr* = 3 transcripts required for agropine synthesis.

co-linear with the T-DNA found in the Ti plasmid of the *Agrobacterium*, indicating that no major rearrangements of the sequence take place during establishment of the tumour. One or more copies of the T-DNA can be present in the plant DNA and, although multiple T-DNA copies can occur in tandem repeats, they can also be separate and linked to different regions of plant DNA. The site of integration of T-DNA into plant DNA is apparently random. Regions homologous to the T-DNA are found on different Ti plasmids (Fig. 1.1B). In commonly used nopaline strains of *A. tumefaciens* the T-DNA region is around 24 kb. In some octopine-type crown galls two non-contiguous segments are found, the T_L and T_R. T_L (14 kb) is present in all transformed cell lines and is functionally equivalent to the T-DNA found in nopaline cell lines. T_R (7 kb), which originates from the right of the T_L-DNA on the Ti plasmid, is not found in all tumour lines but when it is its copy number can differ from that of T_L, suggesting an independent transfer process.

Within the tumour cells the T-DNA is transcribed (Fig. 1.1B) to produce a variety of polyadenylated mRNAs. The levels of the T-DNA transcripts which accumulate are relatively low compared with other plant mRNAs and the relative abundance of each can differ. Sequencing of the nopaline-type T-DNA has revealed 13 large open-reading frames, whilst there are eight and six large open-reading frames in octopine-type T_L- and T_R-DNA respectively. The transcripts on the right-hand side of the nopaline T-DNA are functionally equivalent to those on T_L-DNA (Fig. 1.1B). The overall organization of the T-DNA genes and their flanking regions are similar to those found in eukaryotic genomes, although they do not contain introns. Sequence comparisons, deletion and transposon mutagenesis as well as overproduction of the individual gene products in *E. coli* have been used to identify the functions of several of the gene products encoded by T-DNA. One gene in the octopine T_L region (transcript 3) encodes octopine synthase. In nopaline Ti plasmids, opine synthase genes include nopaline synthase (*nos*) and agrocinopine synthase (*acs*). In octopine Ti plasmids the T_R region encodes two proteins responsible for the synthesis of mannopine and one gene product responsible for the conversion of mannopine to agropine. The *tmr* locus (transcript 4) encodes an enzyme involved in the synthesis of cytokinin, and mutations here result in root proliferation from crown galls induced on some species (rooty mutants). The *tms*1 and *tms*2 loci (transcripts 1 and 2) are involved with the unregulated synthesis of auxins, and mutations in either of these result in shoot proliferation from crown galls on many types of plants (shooty mutants). Thus, the T-DNA contains genes (*tms*1, *tms*2 and *tmr*) whose products override the normal regulation of plant metabolic pathways involved in the synthesis of phytohormones and this results in the oncogenic phenotype. Hence, these genes (*tms*1, *tms*2 and *tmr*) can be considered to be *oncogenes* (see 2.1 for more detail). However, it must be noted that the genes encoded by the T-DNA are not required for the transfer of the T-DNA to the plant cell, nor its stable maintenance in the plant genome.

1.1.3 Ri plasmids of *Agrobacterium rhizogenes*

Initiation of hairy root disease by *A. rhizogenes* is analogous to transformation by *A. tumefaciens*. Under the control of a virulence region, two separate T-DNA regions of the Ri plasmid are transferred to the plant genome (Huffman *et al.*, 1984; De Paolis *et al.*, 1985). These are termed T_L (T-left T-DNA) and T_R (T-right T-DNA). The T_R T-DNA contains genes for opine production (mannopine or agropine) and strains are characterized by their particular opine genes (De Paolis *et al.*, 1985). In addition the T_R T-DNA contains two genes which code for auxin synthesis. These genes are highly homologous to the auxin genes of *A. tumefaciens* and have been shown to be capable of complementing *A. tumefaciens* strains carrying mutations in their auxin genes (Offringa *et al.*, 1986). The T_L T-DNA, on the other hand, does not have homology with the T-DNA of *A. tumefaciens* (Huffman *et al.*, 1984). The entire T_L T-DNA of *A. rhizogenes* has been sequenced and contains at least 11 open-reading frames, similar to those found in eukaryotic genomes, which have the necessary promoter and polyadenylation elements needed to function upon transfer into the plant genome (Simpson *et al.*, 1986). These genes are not homologous to any known genes; nor are there significant homologies with (octopine type) T-DNA from *A. tumefaciens*. T_R T-DNA is not absolutely required for the maintenance of the hairy root phenotype. However, strains of *Agrobacterium* possessing both T_L and T_R T-DNA are more virulent, on a wider range of plant species, than are strains possessing only one T-DNA (Vilaine and Casse-Delbart, 1987).

1.1.4 Mechanism of T-DNA transfer

T-DNA regions in both *A. tumefaciens* and *A. rhizogenes* are flanked by 25 base pair (bp) direct repeats (Fig. 1.1) and the endpoints of integrated T-DNA in the plant genome are found close to these sequences. The consensus sequence of the T-DNA border is $GGCAGGATATT^{C/G\ A/G}\ G^{T/G}\ TCTAA^{A/T\ T/C}$. Most studies of the mechanism by which T-DNA is transferred from the *Agrobacterium* to the plant cell have been carried out with *A. tumefaciens*. The removal of the right border of a nopaline-type Ti plasmid abolishes tumour formation; but when a 25 bp oligonucleotide homologous to the right border is cloned in the correct orientation into a Ti plasmid lacking the right border, tumour formation is restored (Wang *et al.*, 1984), suggesting that the 25 bp repeat sequences are both polar and *cis*-acting.

Contact of the *Agrobacterium* with compounds released from wounded plant tissue results in the transcription of the *vir* region of the Ti plasmid (Fig. 1.2). One specific chemical highly active in this respect has been identified as acetosyringone (Stachel *et al.*, 1986). *Vir*A and *vir*G are expressed in vegetatively growing bacteria, although *vir*G is only transcribed at a low level. When *Agrobacterium* is exposed to wounded plant cell exudates, or pure acetosyringone, the *vir*A gene product (possibly membrane-associated) is thought to recognize and interact with acetosyringone and transmit this extracellular signal intracellularly, resulting in

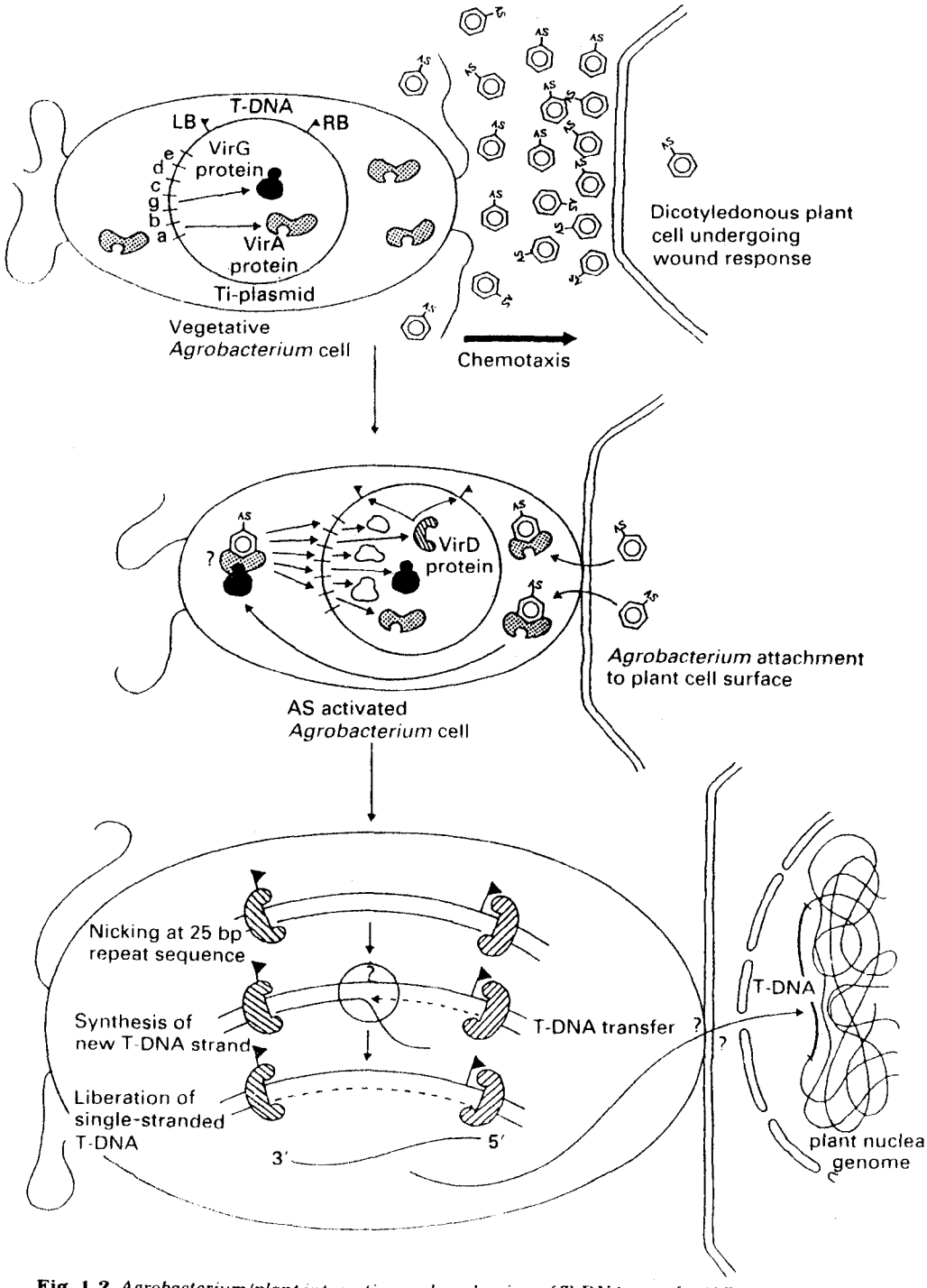

Fig. 1.2 *Agrobacterium/plant interaction and mechanism of T DNA transfer (AS = acetosyringone).*

the activation of the *vir*G gene product. The altered *vir*G protein then activates the rest of the virulence genes (*vir*B, C, D and E), as well as elevating transcription from the *vir*G locus.

Vir gene induction is followed by the appearance of single-stranded nicks within the 25 bp border sequences which flank the T-DNA (Stachel and Zambryski, 1986; Albright *et al.*, 1987) and the appearance of a single-stranded linear molecule which corresponds to the T-DNA (Fig. 1.2). The products of the *vir*D operon are thought to be responsible for this specific endonuclease activity (Yanofsky *et al.*, 1986). By a mechanism which remains unknown, although thought to be analogous to bacterial conjugation, the T-DNA is transferred to the plant cell and stably inserted into the nuclear DNA (Stachel and Zambryski, 1986), an event possibly involving proteins coded for by the *vir*E operon (Winans *et al.*, 1987). A model (after Lichtenstein, 1986) depicting the early molecular events in the interaction between plant and *Agrobacterium* during crown gall formation is shown in Fig. 1.2.

1.1.5 *Agrobacterium* plasmids as transformation vectors

The natural ability of *Agrobacterium* to transfer defined sequences of DNA into the plant genome has been exploited in the development of a variety of plant transformation vectors. These vectors capitalize on several inherent characteristics of the *Agrobacterium*-mediated transformation process. During the early 1980s several research groups engineered Ti plasmids to remove all the T-DNA *onc* genes. The first important discovery was that the *onc* genes encoded by the Ti plasmid are neither required for the transfer of the T-DNA to the plant cell, nor its integration into the nuclear DNA. Hence, these genes can be replaced, not only allowing the insertion of foreign DNA, but also removing the *onc* functions. However, it should be noted that *nos* or *ocs* are useful marker genes for transformation because the enzyme activity of their gene products can be detected by a simple assay. To date a limit on insert size has not been reported. The third important milestone was the discovery that the *vir* gene products can also function in *trans*. Finally, non-oncogenic T-DNAs present in regenerated whole plants are transmitted to progeny in a Mendelian fashion.

1.1.6 Basic components of non-oncogenic Ti plasmid vectors

Taking the characteristics of gene transfer mediated by *Agrobacterium* into account, any foreign DNA that has been cloned can be transferred into the genome of a dicotyledenous plant cell. The foreign DNA to be transferred must be flanked by the T-DNA border sequences and stably maintained in an *Agrobacterium* strain harbouring a full complement of *vir* genes, either in *cis* or in *trans* (located on a separate virulence 'helper' plasmid). It should be mentioned here that *Agrobacterium* chromosomal genes associated with virulence have also been

described and are thought to be involved with the bacterial recognition of a component of the plant cell surface and subsequent attachment.

The removal of *onc* functions means that transformed tissues are no longer recognizable as neoplastic outgrowths which can be selected by their ability to grow on a medium lacking phytohormones. To resolve the problem of identifying transformed cells, bacterial antibiotic-resistance genes have been placed under the control of T-DNA promoters and polyadenylation signals and inserted between the 25 bp repeat sequences. Such chimaeric antibiotic-resistance genes are efficiently expressed in plant cells in a dominant fashion in any genetic background. It is useful to have marker genes tightly linked to the foreign DNA for two reasons; firstly, so that direct selection of transformed plant tissue can be carried out to ensure that the foreign DNA is transferred, and secondly, as a guarantee that the flanking foreign DNA in the particular transformed clone selected has not been inserted into a region of the genome that is not transcribed.

1.1.7 General purpose non-oncogenic plant transformation vectors based on the Ti plasmid

Both *A. tumefaciens* and *A. rhizogenes* have been used to transfer foreign DNA to plants. Much less is known concerning the genetic functions of the Ri plasmid and so transformation vectors involving *A. rhizogenes* will be discussed separately (1.1.8). A wide variety of general purpose transformation vectors based on the Ti plasmid have been developed (see Tables 1.1 and 1.2 for examples). These do not contain any oncogenic sequences and hence normal plant growth can be obtained following the transfer of DNA into the nucleus of the plant cell. Non-oncogenic vectors that are currently in use can be divided into two types, *cis* or *trans*, depending on whether the T-DNA regions, flanked by the 25 bp direct repeat sequences, are carried on the same replicon as the *vir* genes or on a separate plasmid (Fig. 1.3). The former (*cis*-acting *vir* genes) are often referred to as co-integrative vectors; whilst the latter, with *trans*-acting *vir* genes, are commonly called binary vectors.

1.1.7.1 *Cis* vectors

These are derivatives of wild-type Ti plasmids in which the T-DNA *onc* genes have been removed and, in some cases, replaced by a specific piece of DNA which has a region of homology to a small cloning vector that can replicate only in *E. coli*. This vector strategy depends on co-integration in *A. tumefaciens* between homologous regions on the modified Ti plasmid (*vir* helper) and a small *E. coli* cloning vector (intermediate vector) which contains a selectable marker gene that will function in plant cells and unique sites for the insertion of foreign DNA.

The intermediate vector containing foreign DNA sequences is normally introduced into the *A. tumefaciens* by conjugation and, using appropriate selection, transconjugants can be obtained in which the foreign DNA has been stabilized within the T-DNA as a result of homologous recombination (Fig. 1.3A).

Table 1.1 Example co-integrative-type Ti plasmid vectors (*cis*-acting *vir* genes)

Cloning (intermediate) vector (size)	Co-integration host *vir* plasmid	Homology region	Ori	Mob/Tra helper plasmids	Bacterial selection marker	T-DNA border	Plant selection marker	Nos/ ocs	Cloning sites and comments
pMON200[1] (9.5 kb)	pTiB6S3-SE[1] (GV3111)	LIH	pBR322 (Col E1)	pR64drdll pGJ23 (JM101)	Sm/Sp	Rb (SEV) pTiT37	nos-*npt-II*	Nos	Unique sites for *Eco* RI, *Hind* III, *Xba* I, *Xho* I
pMON273[2] (10 kb)	pTiB6S3-SE (GV3111)	LIH	pBR322	JM101	Sm/Sp	Rb (SEV) pTiT37	CaMV35S-*npt*-II	Nos	Unique site for *Hind* III
pMON316[2] (11 kb)	pTiB6S3-SE (GV3111)	LIH	pBR322	JM101	Sm/Sp	Rb (SEV) pTiT37	nos-*npt-II*	Nos	Unique sites for *Bgl* II, *Cla* I, *Kpn* I and *Eco* RI between CaMV-35S promoter and Nos poly(A) site*
pGV1103[3] (6.5 kb)	pGV3850[4] (C58C1)	Ap^r gene	pBR322	JM101	Km	None	nos-*npt-II*	—	*Eco* RI Duplicate pBR322 sequences between T-DNA borders
pGV831[5] (8.9 kb)	pGV2260[5] (C58C1)	Ap^r gene	pBR322	JM101	Sm/Sp	Rb/Lb pTiB6S3	nos-*npt-II*	Ocs	*Bam* HI Duplicate pBR322 sequences are not between T-DNA borders

1 Fraley *et al.* (1985); **2** Saunders *et al.* (1987); **3** Hain *et al.* (1985); **4** Zambryski *et al.* (1983); **5** Debleare *et al.* (1985).
*pMON316 is a useful expression vector.