THE USE OF THE BROAD-HOST-RANGE PLASMID, pTF-FC2, IN *Agrobacterium tumefaciens* MEDIATED PLANT TRANSFORMATION

Thabani Dube

A dissertation submitted in fulfilment of the requirements of the Degree of Doctor of Philosophy in the Faculty of Science, University of Cape Town

Cape Town, December, 2001

To my wife Thembelani

CONTENTS

CHAPTER 2 –TRANSFORMATION OF TOBACCO USING pTF-FC2

ACKNOWLEDGEMENTS

It gives me great pleasure to have this opportunity to thank the many individuals, organisations and institutions that enabled me to complete a task of this magnitude.

I am deeply indebted to my Supervisor Prof. Jennifer Thomson for all that she did for me during the period of my PhD studies. Not only did she generously offer me a place to join her research group but also her zeal, enthusiasm, energy and supervision-style enabled me to develop into a critical thinker. I shall always be thankful for the motherly support, encouragement and concern that you showed when I succumbed to illness towards the end of my studies. Thank you Jennifer for the financial assistance you so kindly rendered when funding became a major problem. You were always ready and willing to help and I never needed to make an appointment to discuss issues no matter how busy your schedule was and for this I will always be very grateful. Finally I acknowledge with thanks the meticulous proof-reading of this thesis.

I acknowledge with great thanks the support given to me by my other Supervisor Prof. Ed. Rybicki. I will always cherish his sustained interest in this work and the many critical discussions that we always had periodically. His incisiveness and enthusiasm for the field of Plant Molecular Biology especially when it came to the construction of dendograms and primer design enabled me to gain a firmer mastery of these techniques with ease.

I would also like to thank Prof. Doug Rawlings for generously donating the plasmid pDER405, which is the foundation on which this thesis is based, his interest in this work and for being readily available to give advice despite his busy schedule. I am also thankful for his critical reading of parts of this thesis.

Part of this work was done at the Friedrich Miescher Institute in Basel, Switzerland in Prof. Barbara Hohn's laboratory. I owe a very special thanks to Barbara Hohn for allowing me to join her research group and for her valuable contributions and sustained interest in this work. I am also thankful to my immediate supervisor when I was in Switzerland, Dr. Igor Kovalchuk. Through his impeccable supervision I learnt a variety of molecular techniques especially the transient GUS assays.

I would like to thank all the members of the former Microbiology Department and the current Molecular and Cellular Biology Department for creating a socially and academically stimulating environment. Special mention goes to the following from whose discussions I managed to bounce off some ideas: Dr. Kenneth Palmer, Dr. Katrina Downing, Dr. Tichaona Mangwende (a fellow friend and compatriot), and Dr. Darren Martin (the computer wizard). Special thanks also goes to Donald Solomon for his assistance in the plant growth rooms.

I feel humbled and do not have enough words to thank my dear wife Thembelani, to whom this thesis is dedicated, for her patience, encouragement and support despite the fact that she had to bear all the suffering when this thesis robbed her of all the times we could have spent together. I am glad however to say, "I am all yours now!!!!"

I am grateful to my parents, grandfather, family and extended family for their support, confidence and belief in my capabilities. Most of them will derive great pleasure by just holding this thesis in their hands.

I acknowledge with thanks the financial assistance received from the following without whose support it would have been impossible to do this work: The National University of Science and Technology, Bulawayo, Zimbabwe; DAAD; UNESCO and the Friedrich Miescher Institute.

ABBREVIATIONS

2,4-D	2,4-dichlorophenoxy acetic acid
aa	amino acid
atm	atmospheres (of pressure)
ATP	adenosine triphosphate
Amp	ampicillin
bar	bialaphos resistance gene
bom	basis of mobility
bp	base pair
BSA	bovine serum albumin
Cm	chloramphenicol
Cmr	chloramphenicol resistance
Dig	digoxygenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetra-acetic acid
GM	growth medium
gus or gusA	*Escherichia coli* β-glucoronidase (*uidA, gus or gusA gene*)
GUS	β-glucoronidase protein, gene product of the *gus* gene.

h	hour
IPTG	isopropyl-β-D-thiogalactopyranoside
Kb	kilobase
Kan	kanamycin
KDa	kilodalton
Km	kanamycin
LA	Luria-Bertani agar
LB	Luria-Bertani broth
M	molar
mRNA	messenger RNA
min	minute(s)
mob	mobilization associated protein gene
Mob	mobilization associated protein
MS	Murashige and Skoog tissue culture media (Murashige and Skoog, 1962)
MW	molecular weight
N/A	not applicable
ND	not determined
NLS	nuclear localization signal
nptII	neomycine phosphotransferase gene
OD	optical density
ORF	open reading frame
oriT	origin of transfer
p	plasmid
p	promoter
PCR	polymerase chain reaction

PPT	phosphinothricin
Rif	rifampicin
rep	replication associated protein gene
Rep	replication associated protein
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
TAE	tris-acetate EDTA buffer
TBE	tris-borate EDTA buffer
Tc	tetracycline
T-DNA	transferred DNA
TE	tris-EDTA
Ti	tumour inducing
Tn	transposon
Tris	tris(hydroxymethyl)aminoethane
µg	microgram
µg/l	micrograms per litre
µl	microlitre
µM	micromolar
vir	*Agrobacterium* virulence gene
Vir	*Agrobacterium* virulence protein
v/v	volume per volume
w/v	weight per volume

X-Glu 5-bromo-4-chloro-indolyl-β-glucoronide

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The transfer of T-DNA from *Agrobacterium tumefaciens* to eukaryotic cells is one of the most extensively studied examples of interkingdom DNA transfer. This transfer system has revolutionized plant molecular biology to the extent that the introduction of foreign genes into plant genomes is now a basic technique. Apart from the finding that a defined segment (T-DNA) of the *A. tumefaciens* Ti plasmid can be transferred to plant DNA, the discovery that the mobilization functions of the IncQ plasmid, RSF1010, can mediate its transfer from *A. tumefaciens* into plant cells as well as between Gram negative bacteria has generated considerable interest. The mobilization functions of RSF1010 substitute for the requirement of the 25-bp border T-DNA sequences of *A. tumefaciens*. In this thesis I investigated the ability of another plasmid, pTF-FC2, to transfer genes to plants.

This plasmid is a 12.2-kb, non-conjugative, broad-host-range plasmid that was originally isolated from the acidophilic biomining bacterium *Acidithiobacillus ferrooxidans* (previously called *Thiobacillus ferrooxidans*). The plasmid was found to be unique in that whilst its replicon clearly resembled that of the broad-host-range IncQ plasmids, the mobilization functions resembled those of the IncP plasmids. The unique features of plasmid pTF-FC2 combined with its similarity to RSF1010 made it an interesting plasmid for further study. Therefore the aims of this study were to investigate the possible use of pTF-FC2 as a plant vector, to elucidate the mechanism

of action for such a transfer, to develop a model system for multigene plant transformation and also to analyze and evaluate the role that the plasmid could play in multigene plant transformation.

A derivative of pTF-FC2, pDER405, was used as the backbone to construct two plasmids, pTD1 and pDER-*bar*. Plasmid pTD1 contained as the plant selectable marker, the *nptII* gene flanked by the Ti plasmid right and the left borders. Plasmid pDER-*bar* contained the bialaphos resistance gene as a selectable marker but lacked the right and the left borders. *Agrobacterium* strains C58C1(pMP90)(pTD1) and LBA4404(pAL4404)(pDER-*bar*) were generated by electroporation and were used in co-cultivation experiments using tobacco leaf discs. Large numbers of morphologically normal transgenic plants were obtained through the co-cultivation of tobacco leaf discs with LBA4404(pAL4404)(pDER-*bar*). PCR amplification and Southern blot analysis were used to confirm the stable integration of the transgenes in the T_0 and the T_1 generations. These results revealed that the *bar* gene segregated in a 3:1 ratio indicating that it had been inherited in a Mendelian fashion. No transformants could be obtained by using strain C58C1(pMP90)(pTD1). This finding demonstrated that not only were the borders unnecessary in this transfer system but that they could possibly inhibit the DNA transfer/integration process when used in conjunction with a pTF-FC2 derived binary vector.

In order to determine the extent to which the pDER-*bar* plasmid had integrated into the tobacco genome, sets of primers that covered the open reading frames of the plasmid were designed and used in PCR reactions. In eight of the randomly selected T_0 plants that were analyzed three of them showed the presence of all the open

reading frames indicating that the entire plasmid had integrated into the genomes of these plants. Apart from two lines which showed aberrant integration patterns three contained the transferred regions adjacent to each other, indicating that they were transferred as one block. These results also showed that transfer was unidirectional, from left to right, with *mobCD* leading the way into the plant cell and *mobA* coming in last.

In order to determine the mechanism of DNA transfer to plants by pTF-FC2 it was necessary to establish which protein was responsible for the nicking of the DNA strand to be transferred. VirD2 and MobA were targeted because they both have nicking activities. Initially an experiment was carried out to investigate whether VirD2 could cleave a synthetic 41-mer oligonucleotide that contained the nick region of pTF-FC2. The results showed that VirD2 could cleave the nick region of pTF-FC2 with 40% efficiency of its ability to cleave the pTiA6 right border sequence from the octopine strain of *A. tumefaciens*. In order to determine the contribution of each of the cleavage proteins, strains of *Agrobacterium* that carried the VirD2 gene and a dysfunctional MobA gene (i.e. $VirD2^{+}/MobA^{-}$) and vice-versa (ie. $VirD2^{-}/MobA^{+}$) together with the other two combinations, $VirD2^{+}/MobA^{+}$ and $VirD2^{-}/MobA^{-}$ were generated. Using GUS transient expression assays, tobacco co-cultivation experiments showed that the $MobA^{+}/VirD2^{+}$ and the $MobA^{+}/VirD2^{-}$ *Agrobacterium* strains were able to transfer their DNA into plant cells with an equal frequency. The $MobA^{-}/VirD2^{-}$ strain could not transfer plasmid DNA to plants whereas the $MobA^{-}/VirD2^{+}$ strain could only transfer plasmid DNA with a negligibly low efficiency of 0.03%. These results show that the pTF-FC2 MobA is able to mediate the transfer and

integration of DNA into plants and that the contribution made by VirD2 in this transfer is negligible.

A model system for the generation of multigene plant transformants based on the co-transformation of two compatible *A. tumefaciens* vectors was developed. The system used pBI121 with the *nptII* gene for kanamycin resistance as a selectable marker, and pPZP100, into which was cloned the *bar* gene for bialaphos resistance as a selectable marker. The strain C58C1(pMP90)(pBI121)(pPZP100-*bar*) was generated and tobacco leaf-disc co-cultivation experiments resulted in 75% double transformants in the T_0 generation. Of the 12 doubly transformed plants that were randomly selected and self-pollinated the T_1 progeny of eight of them showed that they carried the two genes in one genetic locus, thus resulting in an inheritance pattern of 3:1. These results make this system attractive for the generation of multigene plant transformants.

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Horizontal gene transfer, the mobilization and stabilization of genetic information from one organism to another, produces extremely dynamic genomes in which substantial amounts of DNA are introduced into and deleted from the chromosome (reviewed in Ochman *et al.* 2000). Horizontal gene transfer has been extensively studied in prokaryotes and eukaryotes. Examples of such transfer between different species are found with *Escherichia coli* and the yeast *Saccharomyces cerevisiae* (Heinneman and Sprague, 1989), *A. tumefaciens* and plants, and *A. tumefaciens* and yeast (Bundock *et al.* 1995; Piers *et al.* 1996). The genetic material that is transmitted between these organisms is usually in the form of plasmids. Plasmids are closed circular supercoiled DNA molecules present in some species of bacteria. Under normal growth conditions plasmids are dispensable to their host cells. However, the genes present in plasmids often confer greater versatility or adaptability on the recipient cell by providing advantageous functions that include antibiotic resistance and degradative metabolic pathways.

Plasmids are replicated, transcribed and translated in parallel with the chromosome. They have evolved an array of mechanisms that ensures their continued survival in the

host in the absence of selection (Thomas, 2000). In order to prevent the unnecessary recombination and dimerization between identical copies of a plasmid in a bacterial cell, a multimer resolution system (*mrs*) that ensures that each copy of the plasmid genome functions as a separate unit of inheritance, exists (Roberts *et al.* 1990). Another plasmid survival mechanism is the post-segregational killing (or plasmid addiction) that results in the death of plasmid-free segregants. By killing plasmid-free cells this mechanism ensures that only plasmid-containing cells predominate in a population. The inhibition of killing is either effected by RNA:RNA or protein:protein interactions and activation of the "poison" relies on different rates of processing or decay for the "poison" protein or "poison" mRNA, and the "antidote" protein or antisense RNA (Jensen and Gerdes, 1995; Gerdes *et al.* 1997).

Not all plasmids are able to coexist in the same cell. Any two plasmids known to bear this trait are described as incompatible. Plasmids, which have the same replication control functions are incompatible and thus are assigned to the same incompatibility group (Inc group). Therefore plasmids of one incompatibility group are related to each other and cannot be stably maintained in the same bacterial cell. Only different kinds of plasmids are compatible. This ensures that a cell can contain a wide variety of plasmids. Hundreds of plasmids have been sorted into incompatibility groups. Plasmids belonging to the incompatibility classes P, Q, and W are termed promiscuous because they are capable of promoting their own replication over a range of Gram-negative bacteria. Such plasmids offer the opportunity to readily transfer cloned DNA molecules into a wide range of genetic environments.

Depending upon the mode of transmission, these means of genetic exchange are called conjugation, transformation and transduction. Conjugation requires the attachment of two related species through a pilus and the presence of a self-transmissible or a mobilizable plasmid. Transformation involves the transfer of naked DNA and requires no special vehicle. Transduction is DNA transfer mediated through the action of a bacterial virus.

Conjugation can be divided into four stages:

1 Establishment of intimate physical contact between a donor and recipient cell called the mating pair formation (mpf);

2 Preparation for DNA transfer (mobilization);

3 DNA transfer;

4 Formation of a replicative functional plasmid in the recipient.

Not all types of plasmids are genetically able to carry out all of these processes. Based on these properties plasmids fall into four groups:

1 A non-transmissible plasmid lacks genes that code for mpf and DNA transfer. Such a plasmid would not only be unable to cause the formation of the pilus bridge through which genetic material from the donor to the recipient is secreted but also to process its DNA for transfer;

2 A conjugative plasmid has all of the information necessary to mediate its own transfer.

3 A mobilizable plasmid codes for the proteins that are capable of processing its DNA for transfer and this transfer relies on an mpf system from conjugative plasmids;

4. A self-transmissible plasmid is a conjugative plasmid that can mobilize other plasmids either by *trans* effects, or by cointegration;

It is very interesting to note that the mechanism of transfer of conjugative plasmids from *E. coli* to yeast is very similar to the one involved in bacterial conjugation. This transfer is not restricted to a specific bacterial transfer system since two plasmids belonging to different incompatibility groups could be mobilized into yeast cells (Heinemann and Sprague, 1989). *Agrobacterium tumefaciens*, the causative agent of crown gall tumors in plants harbors a tumor inducing (Ti) plasmid. *A. tumefaciens* has evolved a mechanism whereby only a defined segment of the Ti plasmid called the T-DNA (Transferred DNA) can be transferred to plant cells. This mechanism resembles in some aspects the bacterial conjugation system from which it probably evolved. To provide further support to this hypothesis it has also been shown that the products of the virulence region of *A. tumefaciens* normally required for T-DNA transfer can direct the conjugative transfer of an IncQ plasmid between Agrobacteria (Beijersbergen *et al.* 1992).

Although the DNA transfer process between *Agrobacterium* and plants is intriguing, the underlying processes may be neither exotic nor unique. *Agrobacterium* might best be considered an evolutionary pragmatist, borrowing existing processes to suit its ends (Zambyski, 1988). So far, data suggest that the early steps of the T-DNA transfer process use well conserved bacterial processes, from signal recognition to conjugative DNA transfer (Stachel and Zambryski, 1986). Therefore it seems plausible to think that the DNA transfer system between Agrobacteria and plants can be fully understood if an in depth study of bacterial conjugation is also undertaken.

1.1 Conjugation

Bacterial conjugation is a specialized process involving unidirectional transfer of DNA from a donor to a recipient cell by a mechanism that requires mpf. Diverse plasmids and conjugative transposons that constitute the major route for their horizontal transfer encode the process. Conjugation systems are remarkable in mediating transfer between a wide range of bacterial genera and, in some cases, from bacterial to fungal and plant cells. These properties make conjugation an important source of genetic plasticity, potentiating changes of clinical, environmental and evolutionary significance (Mazodier and Davies, 1991). Depending on the system, up to 50 transfer genes provide the basis for the conjugative machinery. Functional and genetic dissection suggest that two sets of functions direct the process of DNA transfer. The mpf apparatus mediates the initial contact between donor and recipient, whereas the DNA processing functions take care of providing the substrate to be transferred in the form of single-stranded DNA. Transfer initiates at a special origin of transfer, *oriT*, and proceeds via a rolling circle mechanism of replication.

The importance to study bacterial conjugation has many aspects, four of which are:

1 Understanding the mechanistic principles in order to contribute to the knowledge of macromolecule transport processes in bacteria in general;

2 The phylogenetic and functional relationship of the mating pair formation system;

3 Unraveling the molecular mechanisms of bacterial conjugation which provide the major pathway for the spread of resistance genes among

bacteria. This is very important as the as the increase of antibiotic resistance in pathogenic bacteria is alarming;

4 A deeper understanding of transformation systems.

Biochemical and molecular studies on enterobacteria, resulted in the discovery of conjugation as the plasmid F-encoded process of *Escherichia coli* K-12. The F plasmid is genetically complex, requiring about 40 transfer (*tra*) genes in a continuous 33-kb segment. The majority specify the mating apparatus, which includes the extracellular conjugative pilus and proteins that stabilize the aggregates of mating cells (Ippen-Ihler and Minkley, 1986; Frost *et al,* 1993). Substantial advances have come from work with other types of conjugative plasmids, in particular with RP4 and RK2 of the IncPα group (Pasengrau *et al*, 1994). Study of the naturally mobilizable plasmids, especially those of the IncQ group, has also generated some interesting results. A mobilizable plasmid typically carries a limited number of *mob* genes for its own DNA processing in conjugation, but requires a coresident conjugative plasmid to supply other essential functions. The general model of DNA processing during conjugation in Gram-negative bacteria essentially occurs in the following four steps:

1 Plasmid-specific proteins encoded by transfer or mobilization genes initiate transfer by nicking at the *oriT* site. The nicking enzyme is often called the relaxase and nicking of the DNA strand at *oriT* is carried out in a DNA protein complex called the relaxosome;

2 The nicking protein remains covalently attached to the 5' end of the nicked strand;

3 In the recipient the nicked and transferred single-strand is copied and ligated to form a supercoiled plasmid;

4 In the donor the non-transferred circular single strand is also copied to form an intact plasmid (reviewed in Waters and Guiney, 1993).

1.2 The *oriT*

The origin of transfer (*oriT*) is a nucleotide sequence that is required in *cis* for DNA transfer. It has the capacity to convert a non-transmissible plasmid into a mobilizable one (Lanka and Wilkins, 1995). *OriTs* are located in the transfer gene complex. Different plasmids within a particular incompatibility group often share extensive sequence similarities in their *oriT* regions. However significant similarities are detectable between the nick regions of different transmissible plasmids (Pansegrau and Lanka, 1991; Waters *et al.* 1991). The nick region, defined as the recognition site for relaxase, is a short stretch of up to ten nucleotides. Three groups of nick region are identifiable (Table 1.1). In each, conserved nucleotides extend for a few nucleotides upstream or around *nic*. The origins of transfer have several features in common. They include:

1 A higher AT content than in the flanking regions, probably facilitating strand separation in negatively supercoiled DNA;

2 A secondary structure conferred by direct and indirect sequence repetitions, which function as specific sites for DNA binding proteins;

3 Intrinsic bends as potential protein-binding sites to alter the *oriT* structure locally, resulting in easier access of proteins to the nick region;

4 Promoters for *tra* gene expression, which in many cases cause divergent transcription from *oriT*.

1.3 The F plasmid

Early studies on *E. coli* demonstrated that fertility, i.e. the ability to act as a donor, was a genetic trait. The fertility factor or F factor is a 94,500 bp, circular dsDNA plasmid which is generally independent of the host chromosome. The F factor controls its own replication. It has two origins of replication, *oriV,* the origin for bidirectional replication and *oriS,* the origin for unidirectional replication. The F factor also codes for proteins that regulate DNA synthesis so that its copy number is kept at a low level, and proteins that regulate the partition into the daughter cells after *E. coli* divides. Conjugation begins with the formation of a pilus bridge between a donor (F+) and recipient (F-) bacterium. DNA synthesis replaces the transferred strand in the donor and converts the transferred strand in the recipient to double stranded DNA. Pili cannot attach to other donor cells due to the presence of the proteins coded by the *traS* and *traT* genes. This phenomenon is called surface exclusion (reviewed in Wilkins and Lanka, 1993).

The Tra proteins involved in DNA metabolism during transfer are TraI, TraY, TraM, and TraD. Purified TraI (also known as DNA helicase 1) has long been known to contain DNA helicase activity (Abdel-Monem *et al.* 1983; Lahue and Matson, 1988), and it has been shown that the carboxy-terminal half of the protein is a DNA dependent ATPase (Traxler and Minkly, 1988; Reygers *et al.* 1991). Therefore, it appears that the *traI* gene encodes a bifunctional protein containing an amino domain with DNA strand scission activity and a carboxyl domain with DNA helicase activity. TraI, which is an ATP-dependant DNA helicase, apparently has two functions, nicking and unwinding and requires a single stranded region of DNA as a substrate. The *traI* locus specifies a 180- kDa, 1756-amino acid polypeptide and a related 94-

kDa, 802-amino acid polypeptide TraI* generated from an internal in-frame translational start (Bradshaw *et al.* 1988;Traxler *et al.* 1988). The role of TraI in the formation of the site and strand specific nick has been studied at the biochemical level (Matson and Morton, 1991; Reygers *et al.* 1991; Matson *et al.* 1993; Sherman and Matson, 1994; Nelson *et al.* 1995). TraI carries out a trans-esterification reaction that requires Mg^{2+}, low salt conditions, and a supercoiled DNA substrate. Once DNA has been relaxed, the 5' phosphate at the nick site remains covalently associated with TraI via the Y80 amino acid residue, whereas the 3' hydroxyl is free and available for extension synthesis by DNA polymerase I.

An analysis of *oriT* shows that the region adjacent to the nick site is complex. It contains two binding sites for the integration host factor (IHF) (Tsai *et al.* 1990), one TraY binding site (Nelson *et al,* 1993), one TraM binding site (Laurenzio *et al.* 1992), two intrinsic DNA bends (Tsai et al, 1990), and several inverted sequence repeats (Frost *et al.*1994). The biochemical role of TraY and IHF in the formation of a relaxosome at the *oriT* has been demonstrated by Nelson *et al.* (1995). These proteins were shown to stimulate the TraI-catalyzed nicking of supercoiled DNA in *vitro*, relax the topological requirement for a supercoiled DNA substrate, and relieve sodium chloride inhibition.

TraM is a 14.5-kDa cytoplasmic protein which forms tetramers in solution (Verdino *et al.* 1999). It binds to three sites in *oriT* (Di Laurenzio *et al.* 1992). The first site called *sbmC* is associated with transfer, while the other two sites *sbmA* and *sbmB* are involved in the autoregulation of *traM* transcription (Penfold *et al.* 1996). Removal of *sbmA* and *sbmB* decreases mating efficiency 100-fold, while the additional deletion of

sbmC results in a further 100-fold decrease in the efficiency of mobilization of a plasmid containing a cloned version of *oriT* (Fu *et al.* 1991). Recent studies by Fekete and Frost (2000) have confirmed earlier findings by Everett and Wilson that TraM is not required for efficient cleavage at *nic.* However a specific function for TraM has not yet been defined. It has been proposed to play a role in promoting relaxosome formation via formation of a nucleosome-like structure at *oriT* which adjusts the superhelical density and promotes cleavage and unwinding in preparation for transfer (Kupelwieser *et al.*1998). The ability to bind DNA near *nic* and interact with TraD (Disque-Kochem and Dreiseikelmann, 1997) suggests that TraM may anchor the DNA to the membrane.

TraD is an inner membrane protein whose function is to connect the relaxosome and the membrane-spanning protein complex for DNA translocation (encoded by the mpf genes), and so it has been called the "coupling protein" (Cabezon *et al.* 1997). The existence of a coupling protein is typical of all conjugation systems. The carboxyl terminus of TraD was shown to add specificity and efficiency to F-plasmid conjugative transfer such that mutating it resulted in the broadening of the range of mobilizable relaxosomes at the expense of a decrease in the efficiency of F-plasmid transfer (Sastre *et al.* 1998).

In conclusion the formation of the relaxosome at the *oriT* has been shown to be a stepwise assembly of the proteins IHF, TraY and TraI (Howard *et al.* 1995). Initially both IHF and TraY bind to their respective sites within *oriT.* This binding appears to promote TraI binding but cleavage requires the correct sequence within the TraI binding site near *nic.* TraM must be bound in *cis* to *nic* presumably to allow the

complete relaxosome to the transferosome via TraD. Each of these steps contributes to plasmid specificity (Fekete and Frost, 2000).

1.4 IncP plasmids

Studies on the broad host range IncP plasmids, of which the apparently identical RK2 and RP4 are examples, have provided insights into the initiation of DNA transfer and replication. These plasmids are of particular interest because of their ability to replicate in, and to mediate DNA transfer to, a wide variety of microorganisms, including Gram negative and Gram positive bacteria and yeast (reviewed by Guiney, 1993). Conjugative functions of 60099-bp RP4 (for the complete sequence see Pansegrau *et al.* 1994) are encoded by two distinct regions designated Tra1 and Tra2 (Barth *et al.* 1978; Lessl *et al,* 1992; Lessl *et al.* 1993). Tra1 consists of three operons, the primase, the relaxase and the leader operon (Pansegrau *et al.*1994). The transfer origin is flanked by genes involved in the formation of the relaxosome, the DNA-protein complex that initiates transfer by nicking at *oriT* (reviewed by Guiney and Lanka, 1989). The fully functional *oriT* region has been cloned as a 250-bp fragment that can be mobilized in *trans* by the RK2 transfer genes. The essential components of Tra1 include the relaxase, TraI, and two *oriT*-binding proteins, TraJ and TraK as well as TraG and TraF (Baltzer *et al.* 1994; Lanka and Wilkins, 1995; Lessl *et al.* 1993). The presence of TraM and TraL increases the transfer efficiency approximately 200-fold, but the functions are not essential (Lessl *et al.* 1993). Genes involved in the mpf and DNA transport map in the Tra2 region and include one Tra1 gene, *traF.*

Four genes encode proteins involved in *oriT* binding and relaxosome formation *in vitro*, *traH, J, I* and *K*. The TraI and TraJ proteins are sufficient *in vitro* for site specific nicking at *oriT* (Pansegrau *et al.* 1990a). Current models are based on experiments establishing TraJ binding to the nick-proximal arm of the 19-bp imperfect inverted repeat as a prerequisite of TraI binding to the DNA-protein complex (Ziegelin *et al.* 1989). Only the N-terminal portion of TraI has nicking activity in vitro, and this protein is covalently bound to the 5' end of the nicked strand (Pansengrau *et al.*, 1990b). TraK enhances relaxosome formation *in vitro* and binds specifically to multiple sites in the leading strand region of *oriT*, bending the DNA around a TraK core (Ziegelin *et al.* 1992). TraH does not bind to DNA and is contained in the relaxosome through interactions with TraI/TraJ. TraH doesn't seem to play a crucial role although it enhances nicking activity slightly in the presence of TraJ and TraI (Pansengrau *et al.* 1990a).

Negatively supercoiled *oriT* DNA is the preferred substrate for the formation of the relaxosomes in vitro. The 13.5-kDa TraJ protein binds specifically to *oriT*, recognizing a 10-bp palindrome in the right arm of the imperfect 19-bp inverted sequence repetition that is positioned upstream of *nic* (Ziegelin *et al.* 1989). The TraJ and TraI binding sites are located on the same side of the DNA helix, indicating a close contact between TraJ and TraI. Relaxosome assembly involves a cascade-like mechanism. The initial step involves the binding of TraJ to *oriT*, resulting in a nucleo-protein complex. Next, TraI (the relaxase) recognizes this complex which is unable to bind to double-stranded *oriT* DNA directly. During the relaxation reaction, TraI hydrolyses a single defined phosphodiester bond and attaches covalently to the

DNA 5' terminus via the Y22 amino acid residue (Pansengrau *et al.* 1990b). Notably the *in vitro* cleavage reaction at RP4 *oriT* is independent of host chromosome encoded proteins. Independence of host functions may contribute to the broad replication and transfer range of this plasmid (reviewed by Lanka, 1995).

TraG plays the role of coupling protein in the IncP conjugation system (Cabezon *et al.* 1997).

1.5 The IncQ plasmids

The 8684-bp plasmid, RSF1010, is a mobilizable, but not self transmissible plasmid of the IncQ group which has the remarkable capability to replicate in a broad range of bacterial hosts, including most of the Gram-negative bacteria (reviewed in Frey and Bagdasarian, 1989) and at least some of the Gram-positive species (Gromely and Davies, 1991). Mobilization of RSF1010 (essentially identical to plasmid R1162) requires a ~1.8-kb region of the plasmid (Derbyshire and Willets, 1987; Derbyshire *et al.*1987) and the presence of an appropriate conjugation system that is provided by a co-existing conjugative plasmid such as that of the IncP group. The minimal functional *oriT* is 38 bp long and is flanked upstream by *mobA*, and *mobB*, and downstream by *mobC* (Brasch and Meyer, 1987). These genes are arranged in divergently transcribed clusters. Purified Mob proteins have been used to demonstrate *in vitro* strand-specific cleavage of RSF1010 *oriT* (Scherzinger *et al.* 1992). In the presence of Mg^{2+} ions, MobA-, MobB-, and MobC-dependent cleavage was observed with linear or supercoiled DNA. Cleavage of supercoiled DNA requires larger amounts of MobC. MobA and MobC are essential components of the relaxosome, whereas MobB has a stimulatory effect. MobC protein alone cannot bind to the linear

duplex or supercoiled DNA, and it is presumably brought into position by its interaction with MobA bound to *oriT* (reviewed by Lanka and Wilkins, 1995).

MobA is the largest of the mobilization proteins and consists of a polypeptide chain of 708 amino acids of which only an amino terminal segment of 243 amino acids is required for conjugal transfer. Being an essential component of the relaxosome, MobA performs the strand specific nicking of the plasmid DNA at a unique site within *oriT*. After nicking, MobA becomes covalently attached to the 5' end of the cleaved strand via the Y24 amino acid residue and is also involved in the termination of transfer. The nicked plasmid DNA is unwound, and the linear strand transferred into the recipient cell. The strand is then ligated by the covalently attached MobA protein to form a circular molecule (Bhattachargee and Meyer, 1991; Bhattachargee and Meyer, 1993).

Experiments have shown that MobB, a protein required for efficient mobilization, increases the proportion of plasmid molecules nicked at *oriT*. The nicked molecules remain super-coiled *in vivo*, with superhelicity probably maintained by non-covalent protein-DNA binding within the relaxosome (Scherizinger *et al.*1992). An important technique in probing for single-stranded *oriT* DNA in the relaxosome has been to use potassium permanganate (Sasse-Dwight and Gralla, 1989), which selectively oxidizes unpaired pyrimidine, primarily thymine, residues. The oxidized bases can be mapped by primer extension with the Klenow fragment of DNA polymerase I because they cause pausing or termination of synthesis at, or one base pair before, the affected residue. The fact that MobB makes *oriT* DNA more sensitive to oxidation by permanganate shows that it increases the proportion of complexed plasmid molecules

rather than changing the equilibrium between nicked and covalently closed forms. However, Perwez and Meyer (1996) discovered that MobB protein increased the amount of complex formed only several fold but increased the transfer frequency approximately 1000-fold. It is therefore possible that MobB brings about a high frequency of mobilization in some way other than increasing the proportion of complexed molecules. The protein could act as a bridge to ensure proper recognition between the relaxosome and other components of the transfer machinery, encoded either by the mobilizing plasmid or by the bacterial chromosome. MobB could act indirectly by causing a conformational change in the relaxosome that could also increase stability. Such an effect of MobB on transfer would cause more rounds of transfer from each active donor cell (Perwez and Meyer, 1996).

MobC is a small protein that consists only of 94 amino acids (Scholtz *et al,* 1989). It has been shown that the frequency of conjugal mobilization of plasmid R1162 is reduced approximately 50-fold if donor cells lack MobC (Zhang and Meyer, 1997). Under these conditions, MobA can cause significant helical disruption within *oriT,* but both this strand separation and conjugal transfer become very sensitive to the amount of active DNA gyrase in the cell. Since MobC cannot separate DNA strands by itself (Zhang and Meyer, 1995) it therefore means it plays a significant role in helping to achieve MobA-induced strand separation at *oriT.* When MobC is unavailable, MobA would depend more on the helical distortion generated by supercoiling to aid in strand separation. In the relaxosome, the thymine bases located between the inverted repeat and *nic,* the MobA cleavage site, are still sensitive to cleavage when MobC is absent. However, MobC is required for detectable permanganate sensitivity of the bases at *nic* (Zhang and Meyer 1997). Thus, not only

does MobC assist in strand separation it also extends this separation to the cleavage site. This is a very important function of MobC since experimental evidence has shown that MobA requires localized melting at *nic* in order to cleave at this site. This explains why nicking and transfer require MobC even though the region of *oriT* adjacent to *nic* is still melted in its absence. The effect of MobC on both extending and enhancing strand separation suggests that it acts as a molecular wedge, inserting into the DNA and unzipping the DNA strands (Zhang and Meyer, 1997).

1.6 DNA transfer between *Agrobacterium tumefaciens* and plants

Among all bacterial conjugation systems, the one evolved by *Agrobacterium* to stably transform plant cells is especially remarkable because proteins encoded by prokaryotic genes have to achieve functions specific to eukaryotic cells. *A. tumefaciens* is a rod-shaped, peritrichous, motile, Gram-negative soil phytopathogen that inhabits the plant rhizosphere. Virulent strains are responsible for the formation of crown gall tumors, an agronomically important disease that affects a range of dicotyledonous plants and gymnosperms as well as some monocotyledonous plants. These strains of *Agrobacterium* contain a large, roughly 200-kb, tumor-inducing (Ti) plasmid (Watson *et al.* 1975), a portion of which, the T-DNA, is transferred to plant cells through fresh wound sites and becomes integrated into plant nuclear DNA during tumorigenesis (for reviews see Caplan *et al,* 1983; Nester *et al.* 1984; Zambryski *et al.* 1988; Otten *et al.*,1992; Hansen and Chilton, 1999; Gelvin, 2000; Stafford 2000). Although it has been reported that Agrobacterium can inefficiently infect plant cells in the absence of a wound site, most likely by entry through stomata (Escudero and Hohn, 1997), efficient infection normally takes place at a plant wound site or in a rapidly dividing cell suspension culture (An, 1985). Wounded plant cells

exude phenolic compounds such as acetosyringone and a wide range of amino acids and sugars. These phenolic compounds play a dual role, first as lignin precursors, necessary for wound healing, and also as chemo-attractants for *A. tumefaciens* and induce the expression of the virulence (*vir*) genes, which then mediate T-DNA transfer. This transfer requires the products of approximately 20 known *vir* genes located on the non-transferred portion of the Ti plasmid (Winans, 1992), as well as a small number of chromosomally encoded proteins (Zorregueieta and Ugalde, 1986; Cangelosi *et al.* 1989; Matthyse *et al.* 1996).

Most transferred genes can be categorized into two distinct groups. The first group leads to the production of opines. These compounds are formed by the condensation of an amino acid with a keto acid or sugar and are released by the plant for use by the inciting bacterium as nutrient sources (reviewed by Drummond, 1979; also see Oger and Farrand, 2000). Agrobacteria are often classified according to the type of opine encoded by their T-DNA, the most common strains being either nopaline or octopine specific (Hooykaas and Beijersbergen, 1994). Opine import into, and the subsequent catabolism within, the bacterial cells require specialized enzymes that are encoded by the Ti plasmid. Owing to this fact *Agrobacterium* has managed to evolve and create its own favorable niche since only a few other soil microorganisms are capable of metabolizing opines (Oger and Farrand, 2000).

The second group of transferred genes mediates the over-production of the phytohormones auxin and cytokinin which interfere with the plant's phytohormone balance and cause neoplastic growth, resulting in crown gall tumors (Akiyoshi *et al.* 1983). It has been shown that the simultaneous mutation of genes that direct the over-production of auxins and cytokinins during tumorigenesis abolishes tumor formation

but does not prevent T-DNA transfer and integration (Leemans *et al.* 1982; Ream *et al.* 1983). Therefore transfer and integration do not depend on tumor formation.

Genes coded for by the T-DNA contains no genetic information required for transfer and integration (Joos *et al.* 1983; Zambryski *et al.* 1983). Another portion of the Ti plasmid, the *vir* region, is required for tumorigenesis (Ooms *et al.* 1980; Garfinkel and Nester 1980; Holsters *et al.* 1980), but DNA from this region has never been detected in tumors (Thomashow *et al.* 1980; Lemmers *et al.* 1980). This region is therefore involved in transfer and integration of T-DNA. The Ti plasmid contains a series of about 25 linked virulence genes arranged in seven operons (Stachel and Nester, 1986). The Agrobacterium chromosome also carries a series of other virulence genes that encode proteins that determine the attraction and attachment to the plant cell, synthesis of cellulose fibrils, and additional functions that are not yet understood.

1.7 Induction of the virulence genes

In order to initiate the process of tumorigenesis a set of genes on the Ti plasmid must first be activated by three kinds of environmental signals at the wound site of a plant (reviewed by Charles *et al.* 1992; Winans, 1992; Hooykaas and Beijersbergen, 1994). These signals include specific classes of plant phenolic compounds, monosaccarides and an acidic pH (Stachel *et al.* 1985). Three proteins, VirA, VirG, and ChvE perceive the signals. The constitutively expressed VirA protein, which is anchored to the cytoplasmic membrane by two transmembrane domains, contains one N-terminal periplasmic domain and three C-terminal cytoplasmic domains (Melchers *et al.* 1989; Chang and Winans, 1992). The three cytoplasmic domains are designated the linker, kinase, and receiver domains (Fig. 1.1).

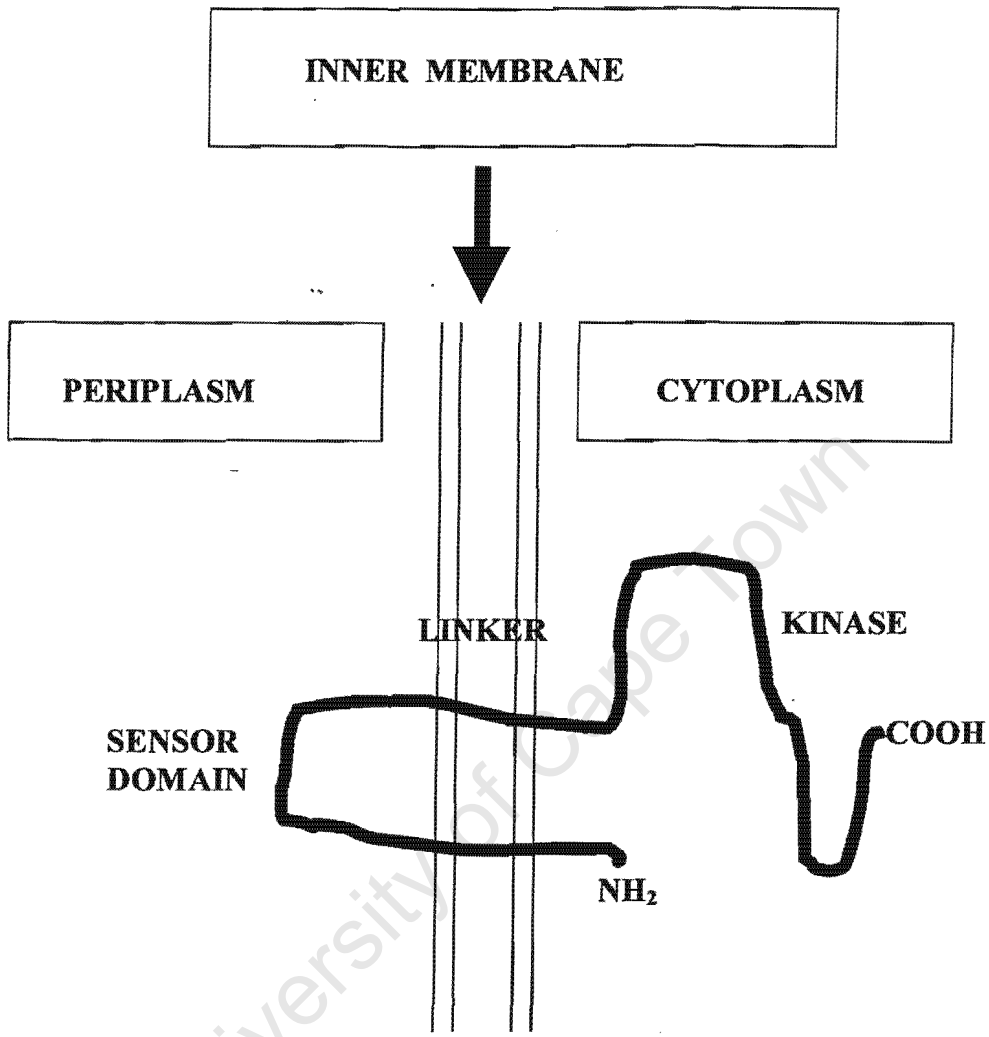


Fig. 1.1 Schematic diagram of a VirA protein. Adapted from Hansen and Chilton (1999).

The VirA protein of the octopine-type Ti plasmid is 829 amino acids and that of the nopaline-type Ti plasmid is 834 amino acids. Both proteins have basically the same domains with an identity of more than 70%. The periplasmic domain is responsible for sensing a variety of sugar-bound-monosaccharide complexes involved in *vir* gene induction. The linker domain is necessary for perceiving phenolic compounds and acidity. The function of the receiver domain is less clear (Chang and Winans, 1992). However it was further reported by Chang and Winans (1992) that the octopine VirA receiver domain may play an inhibitory role in signal transduction, because when this domain was deleted, monosaccharides alone induced *vir* gene expression in the absence of phenolic compounds. Further studies have shown that the octopine VirA receiver domain apparently restricts the recognition of phenolic inducers by VirA, since certain mutations in the domain or its removal widens the spectrum of inducers which VirA recognizes (Chang *et al.* 1996).

In response to the plant wound signals VirA is autophosphorylated at His-474, and transfers this phosphoryl group to Asp-52 within the the amino terminal receiver domain of the response regulator VirG (Jin *et al.* 1990) and then becomes the transcriptional activator for all *vir* genes including its own. It is interesting to note that the carboxy-terminal region of VirA, the receiver domain, is so called because of

its homology to the receiver domain of VirG (Chang and Winans, 1992). Thus VirA and VirG function like a two component gene regulatory system belonging to a large family of bacterial chemosensors that respond to the chemical environment (Leroux *et al.* 1987; Das 1994). Promoters of *vir* genes possess one or more 12-bp "vir box" sequences (Winans *et al.* 1987). A search for mutants that express their *vir* genes constitutively led to the discovery of *vir*GN54D by two independent groups (Pazour *et al.* 1992; Han *et al.* 1992). This mutation apparently leads to a conformation of the protein that resembles that of phosphorylated wild type *vir*G. Constitutive expression of *vir* genes by Agrobacterium eliminates the time consuming process of inducing the *vir* genes prior to inoculation onto plant tissue. The *vir*GN54D mutant might be more effective in transformation because it is in the induced state at all times. Experiments comparing the efficiency of transformation of a constitutive *virG* vector system with that of the corresponding inducible one showed that the mutant is advantageous, especially for recalcitrant plant systems (Hansen *et al.* 1994).

The chromosomally encoded ChvE is a periplasmic sugar binding protein (Cangelosi *et al.* 1990; Huang *et al.* 1990). High levels of phenolics such as acetosyringone do not induce the octopine chvE mutant strain nor is there any observable synergistic effect of monosaccharides on *vir* induction by low levels of phenolic compounds. The observation that *chv*E mutants are avirulent on a variety of plants but can cause delayed tumor formation on others may indicate that non-susceptible host plants probably lack a sufficient level of specific phenolic compounds to induce the *vir* genes. In such plants, therefore, tumor formation may require the synergistic effect of sugars on *vir* induction, which is only possible when *chv*E is expressed (Cangelosi *et al.* 1990). Apart from *vir* induction ChvE plays a major role in the uptake of specific

sugars and also in the chemotaxis to these sugars. Thus, this protein plays an important role both in virulence as well as in the physiology of Agrobacterium species in general.

The nucleotide sequence of *chv*E is similar to that of the galactose binding protein (GBP) of *Escherichia coli*, and the protein sequence is identical to the corresponding amino-terminal sequence of GBP1 (galactose binding protein) of *Agrobacterium radiobacter* (Cornish *et al.* 1988; Cornish *et al.*1989; Cangelosi *et al.* 1990; Huang *et al.*1990).

1.8 The T-DNA borders

T-DNA is flanked by short imperfect direct repeats (Yadav *et al.* 1982). Depending on the nature of the Ti plasmid used these repeats are described as being 23-bp (Peralta and Ream, 1985), 24-bp (Barker *et al.* 1983) and 25-bp (Yadav *et al.* 1982) long. However it has become the norm to represent all the borders on the basis of a 25-bp core structure. Any DNA between these borders is transferred from Agrobacterium to the plant cell (reviewed by Zambryski, 1988). Genetic analyses of the 25-bp sequences have indicated that they are polar in function (Wang *et al.* 1984; Peralta and Ream, 1985; Zambryski,). This is a consequence of the asymmetric cleavage of the T-DNA border sequence (Albright *et al.* 1987); the strand and orientation are switched if the border sequence is inverted, and a different part of the plasmid is then the initial part of the T-DNA. Furthermore, when the orientation of the right border is reversed with respect to its natural orientation on the Ti plasmid, the efficient transfer and/or integration of the T-DNA sequences is greatly attenuated. These results indicate that T-DNA transfer may occur in a rightward to leftward

fashion, determined by the orientation of the 25-bp border repeats, and suggest that transfer might be via a conjugative mechanism (Wang *et al.* 1984).

Despite the DNA sequence similarities between the right and left borders, studies of border functions have shown that T-DNA borders are differentially utilized. Analysis of the T-DNA content of different transformed plant lines has revealed that the integration of T-DNA into the plant genome takes place at (the right border) or near (the left border) these direct repeats (Slightom *et al.* 1986; Konez *et al.* 1989; Gheysen *et al.* 1991; Mayerhofer *et al.* 1991). While deletion of the left border repeat has no significant effect on pathogenicity (Joos *et al,* 1983), deletion of the right repeat totally abolishes it (Shaw *et al.* 1984; Wang *et al.* 1984; Peralta and Ream, 1985; Jen and Chilton, 1986).

The DNA sequence context around the T-DNA borders greatly influences their activity. Sequences adjoining the right border enhance, and sequences surrounding the left border reduce, the initiation of polar DNA transfer (Wang *et al.* 1987a). A *cis*-active sequence of 24 bp, called overdrive, is present next to the right border of the octopine plasmid (Peralta *et al.* 1986). Overdrive stimulates T-DNA transfer even when situated several thousand bp away from the border (Van Haaren *et al.* 1987). However, overdrive cannot mediate T-DNA transfer by itself (Peralta *et al.* 1986; Van Haaren *et al.* 1987). Several enhancer elements similar to the overdrive sequence have been found to be present in other octopine plasmids and also on the agropine plasmid (Slightom *et al.* 1987; Jouanin *et al.* 1989). The mannopine-type T-DNA right border does not contain a sequence similar to overdrive sequence but instead exhibits an8-bprelatedsequence repeated six times (Hansen *et al.* 1991). This sequence is

functionally equivalent to overdrive (Hansen *et al.* 1992). No reiterated sequence resembling overdrive has been found near nopaline Ti plasmid T-DNA borders (Wang *et al.* 1987b).

1.9 Generation of the T-DNA strand

In order for the T-DNA to be transferred to the plant cell nucleus it has to be liberated from the Ti plasmid. This process occurs soon after the induction of the *vir* genes and is facilitated by the first two genes of the *virD* operon, *virD1* and *virD2* (Alt-Moerbe *et al.* 1986; Stachel *et al.* 1986; Yanofsky *et al.* 1986; Porter *et al.* 1987; Stachel *et al.* 1987; De Vos and Zambryski, 1989; Filichkin and Gelvin 1993). VirD1 and VirD2 proteins function together as a strand transferase that carries out site and strand-specific nicks between the third and fourth bp of the bottom strand of each of the T-DNA borders (Wang *et al.* 1987b). This cleavage is thought to result in the production of a free, linear, single-stranded copy of the T-DNA element often referred to as the T-strand (Stachel *et al.* 1986). Thus, T-strand production occurs in a 5' to 3' direction, initiating at the right T-DNA border and terminating at the left border (reviewed in Citovsky *et al.* 1992).

Following cleavage, VirD2 binds to the 5' end of the T-DNA (Ward *et al.* 1988; Young and Nester, 1988; Herrera-Estrella *et al.* 1990). This protein-DNA complex is formed via the tyrosine-29 residue of VirD2 (Vogel and Das, 1992). Whilst VirD1 and VirD2 are both required for the cleavage of double stranded DNA *in vivo* (Jayaswal *et al.* 1987) and *in vitro* (Scheiffele *et al.* 1995), purified VirD2 protein alone can cleave a single-stranded T-DNA border *in vitro* (Pansegrau *et al,* 1993; Jasper *et al.* 1994). VirD2 consists of 424 amino acids of which the N-terminal 262

amino acids are responsible for border nicking and for attachment to the 5' end of the nicked DNA strand and are also sufficient for T-strand generation. However, mutations in the C-terminus abolish or attenuate tumorigenesis (Stachel *et al.* 1986; Steck *et al.* 1990). The exact function of VirD1 has not been fully defined as the first findings by Ghai and Das (1989) that it had a type 1 topoisomerase activity could not be supported by Scheiffele *et al.* (1995) who used a more purified protein.

Whilst the *virC* operon, which consists of two open reading frames, *virC1* and *virC2*, is not essential for tumorigenesis and site specific cleavage by the VirD2 strand transferase, it has been shown to enhance the efficiency of T-DNA processing through an interaction between the VirC1 protein and the overdrive sequence (Toro *et al.* 1988). De Vos and Zambryski (1989) also showed that the nopaline VirC1 protein promotes the formation of single-stranded T-DNA in a heterologous *E. coli* system only when the VirD1 and VirD2 proteins are limiting. It was later shown that VirC1 specifically bound to the overdrive, but not to the right border sequence (Toro *et al.* 1989). This interaction does not involve VirC2. However, *in vivo*, it was found that the generation of the T-strand was decreased in both *virC1* and *virC2* mutants, suggesting that the *virC2* gene also plays a role in enhancing the formation of the T-strand. Exactly how VirC2 plays its role is currently unknown although it probably associates with proteins such as VirD1 and the VirD2 strand transferase, or with the DNA substrate at or surrounding the T-DNA border region to promote T-strand production (De Vos and Zambryski, 1989). Since it was shown that the VirD2 protein interacts with both the T-DNA border and overdrive and that the VirC1 protein interacts only with the overdrive sequence, it is very likely that the interaction of the VirD2 protein with the overdrive could be via the VirC1 protein (Toro *et al.* 1988).

1.10 Transfer of the T-strand to the plant

In order to successfully infect a plant cell the *Agrobacterium* must be able to transfer an intact T-strand to the plant nucleus. This presents the bacteria with three challenges:

1 A transport system must be in place to usher the T-strand through the bacterial membrane into the plant cell;

2 The T-strand must be protected from exonucleolytic degradation, and

3 A mechanism must be in place to pilot the T-strand to the plant nucleus. Three bacterial operons have been implicated as playing major roles in dealing with the aforementioned challenges. These are, respectively, *virB*, *virE* and *virD*.

Transfer of the T-complex from *A. tumefaciens* to plant cells requires the *virB* operon and the *virD4* gene. The *virB* operon encodes eleven proteins, VirB1 to VirB11. It is now well established that pili are essential for all transfer processes and that each of the 10 genes, *vir*B2 to *virB11*, are essential for both tumorigenesis and pilus assembly (Berger and Christie, 1994; Fullner *et al.* 1996). The *virB1* gene is not absolutely required for the genetic transformation of plants, although its deletion reduces efficiency 100-fold (Berger and Christie, 1994). The VirB2 to B11 proteins are proposed to form a transmembrane channel that spans the bacterial inner and outer membranes and may transfer the T-complex into the plant cytoplasm. A number of scientists have carried out various studies to support this model. First, sequence analysis predicted membrane or periplasmic localization of most *virB*-encoded proteins (Ward *et al.* 1988; Kuldau *et al.* 1990; Shirasu *et al.* 1990). Second, PhoA

fusions and susceptibility to proteases confirmed the periplasmic location of hydrophilic VirB proteins with signal peptides (VirB1, VirB5, VirB7 and VirB9) and demonstrated periplasmic domains of membrane associated proteins (VirB3, VirB6, VirB8 and VirB10) (Fernandez *et al.* 1996). Third, cell fractionation and immunoelectron microscopy localized most VirB proteins and VirD4 to the membrane (Okamoto *et al.* 1988; Berger and Christie, 1993; Thorstenson *et al.* 1993; Fullner *et al.* 1994; Jones *et al.* 1994; Shirasu *et al.* 1994; Thorstenson and Zambryski, 1994; Finberg *et al.* 1995; Fernandez *et al.* 1996; Christie *et al.* 1997). Fourth, homologies of VirB proteins and VirD4 to components of bacterial conjugation and protein secretion systems suggest a common mechanism for traffic of DNA protein complexes and protein toxins (Okamoto *et al.* 1988; Kado, 1994; Lessl and Lanka, 1994). Furthermore VirD4 associates with the inner bacterial membrane and has a bipartite nuclear localization signal (NLS) of the type that is evolutionarily conserved in plants and animals (Sheng and Citovsky, 1996).

The accumulation of other *virB* gene products depends on the presence of other *virB* proteins suggesting that coordinate protein synthesis stabilizes individual VirB polypeptides, thus perhaps allowing them to form a multiprotein channel structure. The transport of T-complexes through the VirB channel is most likely an energy dependent process. VirB4 has a nucleotide-binding site, whereas VirB11 is both an ATPase and a protein kinase, making these two proteins the likely candidates for the provision of energy for this translocation. In addition, both proteins localize to the inner bacterial membrane (Christie, 1989). The *virE* operon encodes two polypeptides, VirE1 (7.0 kDa) and VirE2 (60.5 kDa) (Winans *et al.* 1987). VirE2 is the most abundant protein produced in *A. tumefaciens* after induction of the virulence

genes by plant phenolic compounds (Stachel, 1986). The VirE2 protein binds non-specifically, and in a highly cooperative manner, to single- stranded DNA *in vitro* (Gietl *et al.* 1987; Das, 1988; Citovisky *et al.* 1989; Sen *et al.* 1989). However it does not bind to double stranded DNA or to RNA (Das, 1988).

Due to its single-stranded DNA binding properties, VirE2 was believed to coat the T-DNA-VirD2 complex in *A. tumefaciens* and thereby form the T-complex. This hypothesis has been challenged by the following three experiments:

1 A VirE2-defective *Agrobacterium* can be complemented by co-inoculating the plant cells with a second *Agrobacterium* proficient for VirE2 production but lacking T-DNA (Otten *et al.*1984; also see Fig.1.2A);

2 VirE2 over-expressed in transgenic plants can complement an *A. tumefaciens* strain mutated in *vir*E2 to full virulence (Citovsky *et al.* 1992; also see Fig.1.2B)

3 A mutation or complete deletion of the VirE2 gene in *A. tumefaciens* does not affect the production of the T-strands (Rossi *et al.*1996).

These findings demonstrate that the essential function of VirE2 takes place in the plant cell and that it is not required for T-strand protection in *Agrobacterium* or for transfer to the plant. It should be noted therefore that these findings do not exclude the cooperative binding of *virE2* to the T-strand prior to transfer, but that the necessity for it is not documented. The VirE1 protein is required for transfer of VirE2 but not for T-strand transfer to the plant cell (Sundberg *et al.* 1996).

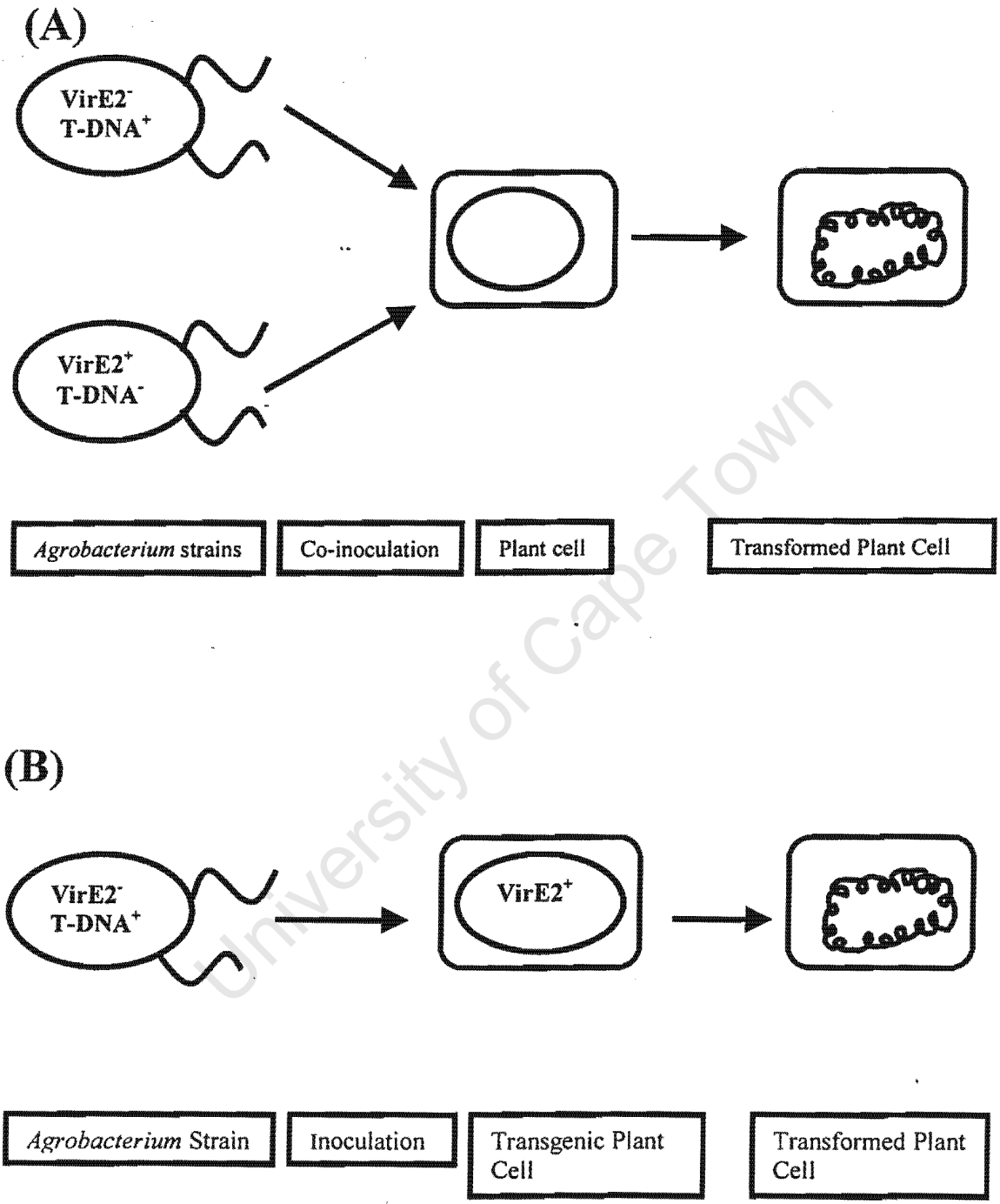


Fig. 1.2. An annotation of experiments that were carried out to demonstrate that both VirE2 and the T-DNA can be transported from Agrobacterium to the plant cell separately. Details are given in the text.

A *vir*E1 mutant Agrobacterium is avirulent. This is ascribed to its inability to deliver VirE2 protein, despite the fact that the bacterium appears to have abundant supplies of VirE2 and can transfer T-strands perfectly well (Sundberg *et al.* 1996). Although VirE2 is stable in *A. tumefaciens* without VirE1 (Sundberg *et al.* 1996), VirE1 stabilizes VirE2 in *E. coli* (Mcbride *et al.* 1988), suggesting that these proteins interact. VirE1 has been shown to bind VirE2 at sites critical for single-stranded DNA binding and self-interaction (Sundberg *et al.* 1999; Deng *et al.* 1999). Such binding by VirE1 may facilitate VirE2 export by preventing it from aggregating and binding prematurely to single stranded DNA. Thus VirE1 appears to act as a chaperone-like protein for VirE2. From the avirulence of the VirE1 mutant it seems clear that, at least in the absence of the VirE1, VirE2 does not travel to the plant aboard T-strands in sufficient quantity to enable T-DNA to transform the plant cell (reviewed by Hansen and Chilton, 2000).

VirE2 has recently been shown to associate with lipids and to be able to insert itself into artificial membranes and form channels (Dumas *et al.* 2001). These channels are voltage gated, anion selective, and single-stranded DNA-specific and can facilitate the efficient transport of single stranded DNA through membranes. Exactly how the T-DNA complex crosses the plant plasma membrane has remained a mystery. Based on the findings of Dumas and his colleagues (2001) there is a very strong possibility that VirE2 functions as a transmembrane DNA transporter in plant systems. However formation of pores large enough to allow passage of single-stranded DNA may render such plants amenable to environmental stresses. Since VirE2 transgenic plants and plants infected with Agrobacterium are viable then VirE2 channels, if they exist in plants, are probably in a closed state *in vivo*. Experiments have shown that most

membrane channels are not always open but can be closed on application of a membrane potential above a certain threshold value (Delcour *et al*, 1997; Bainbridge *et al,* 1998). This phenomenon known as voltage gating is also observed with the VirE2 channels. The voltage gating property of VirE2 *in vitro* suggests that the opening and closing of the channels may be regulated *in vivo* by a mechanism that is yet to be determined (Dumas *et al,* 2001).

The VirD2 protein contains two NLS at each end. The N-terminal sequence is of the single cluster type (monopartite), that shows some resemblance to the NLS found in the SV40 large T-antigen, whereas the C-terminal sequence belongs to the bipartite group that resembles the NLS of *Xenopus laevis*. Foreign proteins fused to either of the two NLS sequences have been shown to be targeted to the plant cell nucleus (Herrera-Estrella *et al.*1990; Howard *et al.* 1992; Tinland *et al.*1992). Thus, the VirD2 protein attached to the T-DNA may be responsible for piloting the T-DNA into the plant cell nucleus. The tyrosine 29 residue, to which the T-strand covalently attaches, is located next to the monopartite NLS. As a result of this arrangement it is unlikely that the monopartite NLS participates in the nuclear localization of the T-strand as this site becomes occluded by the connected T-strand (Shurvinton *et al.* 1992; Koukolokova-Nikola *et al.* 1993; Rossi *et al.* 1993a; Gelvin, 2000). The tightly bound VirD2 protein is known also to protect the T-strand from exonucleolytic degradation (Durrenberger *et al.* 1989).

The VirE2 protein contains two separate bipartite NLS regions that can target linked reporter proteins to plant cell nuclei (Citovsky *et al,* 1992; Citovsky *et al.* 1994). To date studies on the relative roles of the VirD2 and VirE2 NLSs in nuclear targeting

have been contradictory suggesting that more experimentation is required in this direction (reviewed by Gelvin, 2000). The following are some of the findings that have been rather contradictory: Rossi *et al.* (1993a) showed that deletion of the VirD2 bipartite NLS resulted in almost complete loss of transformation, suggesting that VirE2 NLS domain could not compensate for loss of VirD2 NLS. Later, Ziemienowicz *et al.* (1999) added more substance to Rossi's findings when his group showed that both VirD2 and VirE2 proteins were necessary for nuclear targeting of *in vitro*-synthesized T-complexes introduced into permeabilized HeLa cells, and that deletion of the VirD2 NLS could not be compensated by the presence of the VirE2 protein. However other groups (Shurvinton *et al,* 1992; Mysore *et al.* 1998) demonstrated that *Agrobacterium* strains containing a *virD2* gene with a NLS deletion retained almost full virulence and transformation capability. This result suggests that VirE2 may provide nuclear targeting capabilities in the absence of the VirD2 NLS. Further support for these findings was given by Gelvin (1998) when he showed that VirE2-producing transgenic tobacco can complement a *virE2*, *vir*D2 ΔNLS double mutant *Agrobacterium* strain. This again shows that VirE2 works in the plant to provide nuclear targeting abilities to the incoming T-strand.

1.11 T-DNA integration

Once in the nucleus, the T-DNA becomes integrated into the plant genome, although the precise mechanism of T-DNA integration is poorly understood. Integration occurs randomly and follows a mode of illegitimate recombination (for reviews see Tinland and Hohn, 1995; Tinland 1996, Gelvin, 2000). The T-DNA has been shown to integrate preferentially in transcriptionally active regions of the genome (Konez *et al.* 1989; Herman *et al.* 1990).

T-DNA has been shown to be transferred as a ss-T-DNA-VirD2 complex and probably reaches the plant cell nucleus as a complex coated by VirE2 proteins. Because of the association of VirD2 and VirE2 with the T-strand, it is likely that they play a role in the integration process. The integration of the 5' end of the T-strand into plant DNA is generally precise, with at most a few 5' nucleotides deleted during the process (Tinland *et al.* 1995; Rossi *et al.* 1996). This may be due to the protection from exonucleases that VirD2 offers to the 5' (Durrenberger *et al.* 1989).

Shurvinton *et al.* (1992) identified another region at the C terminal end of VirD2, called the omega (ω) sequence because of its location, that was very important for virulence. This region consists of highly conserved penultimate amino acid sequence DGRGG adjacent to the NLS motif. Their deletion resulted in a decrease in virulence by almost two orders of magnitude, suggesting that the ω deletion mutation affected T-DNA integration to a much greater extent than it affected T-DNA transfer and nuclear targeting. In order to confirm this hypothesis Mysore *et al.* (1998) constructed a T-DNA binary vector containing a promoterless *gusA-intron* gene just inside the

right T-DNA border. This vector also contained a luciferase-intron gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The vector was mobilized into two different strains of Agrobacterium, i.e. one that had a wild type *virD2* gene and another that had a *virD2ω* deletion/substituition. These strains were used to transform tobacco suspension cells. The results obtained indicated that only cells transformed with *Agrobacterium* that had a wild-type *virD2* gene expressed GUS activity. It was also shown that although the *A. tumefaciens* strain that contained the *virD2ω* mutation could transfer T-DNA to tobacco nuclei and transiently express a luciferase gene that contains a promoter, it could not do so with a *gusA-intron* gene that required integration into the plant genome for expression. However Bravo-Angel *et al.* (1998) obtained contradictory results when they demonstrated that a deletion of the entire five amino acids that constitute the ω region or its replacement with five glycines reduced T-DNA transfer considerably, compared with wild type, demonstrating that the ω sequence is important for the efficient transfer of T-DNAs. However, the efficiency and pattern of integration of the T-DNAs were not affected by any modifications of the ω sequence. At this stage the precise role of ω in T-DNA integration is yet to be established.

Integrated T-DNA molecules transferred from VirE2 mutant strains exhibit extensive deletions corresponding to the 3' ends of the T-strand. The 5' ends of these T-DNA's, however, remain relatively intact, probably because of the covalently bound VirD2 protein (Rossi *et al,* 1996). These results demonstrated that the efficiency of T-DNA integration was not affected by the absence of VirE2. This function is most probably taken over by plant enzymes. Evidence to support this hypothesis was collected from results that showed that *Arabidopsis* mutants hypersensitive to UV or gamma

radiations have a reduced capacity to allow integration (Sonti *et al.* 1995). Thus VirE2 may play an indirect role in T-DNA integration through its single strand binding activity and thus affording physical protection of T-strands from nucleases.

1.12 DNA transfer to plants without the use of borders

One of the most interesting findings in trans-kingdom DNA transfer between prokaryotes and eukaryotes has been the novel observation that *A. tumefaciens* is able to mediate the transfer of the broad host range plasmid RSF1010 to plant cells although this plasmid lacks the border sequences necessary for VirD2 mediated T-DNA transfer. RSF1010 belongs to the IncQ incompatibility group. This plasmid's promiscuous behaviour in bacterial conjugation, which is due to its ability to use many different transfer systems for its mobilization (Frey *et al.* 1992), probably explains why RSF1010 can be transported from bacteria to plants. Several Vir proteins encoded in the Ti-plasmid are required for this transfer (Buchanan-Wollaston *et al.* 1987). It is presumed that in this system the *oriT* and its cognate Mob proteins generate a conjugative intermediate that moves from *Agrobacterium* to plant cells by *vir*-specified transfer machinery. The transfer reaction requires the MobA protein, which in conjunction with MobB and MobC, cleaves the origin of transfer of the plasmid and then attaches covalently to the DNA to be transferred. The mobilization functions of RSF1010 have been mapped to a 2.9-kb region containing the sequences for *oriV*, the vegetative origin of replication, *oriT*, a part of the *repB* gene, and the genes which specify the *cis* acting *mob* region. Sequence analysis of this region has indicated no significant sequence homology to the T-DNA border sequence of *A. tumefaciens*. Therefore RSF1010 substitutes its mobilization functions for those of

the 25-bp T-DNA border sequence of *Agrobacterium* (Buchanan-Wollaston *et al.* 1987).

Experiments using non-polar *vir*B mutants of *Agrobacterium* have shown that the transfer of RSF1010 to plants is dependent on the eleven genes of the *virB* operon and *virD4*. VirB1 was shown to be essential only for high frequency transfer of RSF1010 and VirE2, while VirB2 to VirB11, along with VirD4, comprise a core transfer complex which is required for both pilus assembly and transfer events (Fullner, 1998). However it was also shown that the presence of a derivative of RSF1010, pJW323, in the wild-type *A. tumefaciens* A348 strain, carrying pTiA6, inhibited transfer to and/or integration of the T-DNA in plant cells as assayed by tumor formation (Ward *et al.* 1991). When the proteins VirB9, -10 and -11 were over-expressed coordinately the inhibition was reversed and tumorigenecity was restored in strain A348 carrying pJW323. Over-expressing the same proteins individually did not produce any inhibition (Ward *et al.* 1991). These results suggested that RSF1010 or its transferred intermediate blocks the transfer of the T-complex and that in its presence, VirB9, VirB10, VirB11 proteins are limiting for transfer.

In a study designed to examine the specificity of RSF1010 inhibition of T-DNA transfer the movement of other T-DNA's, in the form of binary vectors, was monitored. Extracellular complementation assays were used to characterize the effect of pJW323 on the movement of VirE2 and uncoated T-strands. The results suggest that RSF1010 or its transferred intermediate competes with VirE2 and the T-strand for limiting sites, most likely the putative VirB complexes, that are necessary for macromolecular transfer (Binns *et al.* 1995). A model consistent with these results

was proposed which suggests that the transferred DNA intermediate is the uncoated T-strand (or R-strand in the case of pJW323) and that VirE2 is transferred to plant cells independent of these intermediates. In addition the model predicts that the pJW323 intermediate sequesters some limiting factor related to transfer, perhaps part of the VirB complex. The first feature of the model is based on the following sets of results:

1 T-DNA and pJW323 can move out of *virE2* mutant cells in extracellular complementation assays (Binns *et al,* 1995).

2 *virE2* mutant strains are virulent when inoculated onto plants over-expressing VirE2 (Citovsky *et al,* 1992). These two results both support the theory that uncoated T-strands can leave the bacterium.

3 The presence of T-strands was observed in plant cells that had been infected with *vir*E mutant strains of *Agrobacterium* and it was suggested that VirE2 functioned in the plant cell to protect the T-strand from degradation (Yusibov *et al.* 1994). This result clearly shows that the VirE2 in the bacterium is not necessary for movement of the T-strand to the plant cell.

4 Intracellular overexpression of VirE2 does not increase transfer of pJW323, whereas extracellular VirE2 does (Binns *et al,* 1995). This result indicates that intracellular VirE2 is not a limiting factor in pJW323 transfer, consistent with the fact that this plasmid moves out of *Agrobacterium* cells separately from VirE2.

It is interesting to realize that MobA-mediated transformation of plant cells occurs when it only shares 18% identity at the nucleotide sequence level to VirD2. The

specificity of the interactions between the proteins accompanying the T-DNA in the process of *Agrobacterium*-plant cell transformation becomes a fundamental issue. To elucidate this issue experiments were carried out to compare the DNA transfer from MobA- or VirD2- containing bacteria to plants, as well as efficiency and precision of integration into plant DNA (Bravo-Angel *et al.* 1999). MobA and VirD2 binary plasmids, differing only by 38 nucleotides at the nick site for the respective strand transferase, were used. To make sure that the results were specific for a particular strand transferase the transfer of an *oriT*-containing plasmid was measured in a VirD2-free *Agrobacterium* strain and likewise VirD2-mediated transfer in a MobA-free *Agrobacterium* strain. The results showed that the MobA-dependent R-DNA transfer occurred with a much lower efficiency than VirD2- dependent T-DNA transfer (less than 1/1000). Further studies demonstrated that when VirE2 was over-expressed the MobA-dependent transfer was increased by up to 10-fold. This result showed that VirE2 export was limiting the transformation mediated by MobA and also confirmed the existence of the competition between MobA and VirE2 that had previously been identified by Binns *et al.* (1995). This competition suggests a high-affinity recognition of the DNA export machinery by the MobA components and may be an important feature of broad-host-range plasmids since it would allow them to parasitize other DNA transfer systems efficiently (Bravo-Angel *et al.* 1999).

Interestingly, DNAs transferred by the two proteins were integrated into the plant cell genome with similar efficiencies indicating that the R-DNA complex has integrative capacities similar to those of the T-DNA complex. In contrast, most of the integrated DNA copies transferred from a MobA-containing strain were truncated at the 5' end, a situation that is reminiscent of the properties of DNA integration mediated by a

mutant form of VirD2, VIRD2R129G, created by replacing arginine 129 with glycine (Tinland *et al.* 1995). Isolation and analysis of the most conserved 5' ends revealed patterns that resulted from the illegitimate integration of one transferred DNA within another. These complex integration patterns indicate a specific deficiency in MobA. However there is a clear difference between the integration mediated by this VIRD2R129G mutant protein and that mediated by MobA. The junctions isolated with the mutant protein never showed integration within another T-DNA molecule. The patterns of integration were clear and easy to analyze and could be interpreted as single integration events. The data on R-DNA integration patterns present a phenomenon that has never been observed before. A specific defect of MobA in ligating the R-DNA to the recipient DNA would probably not be sufficient to explain the observed pattern of integration. Rather, this indicates that certain plant cells are far more competent than others for transformation by R-DNA's. Therefore these data conform with a model according to which efficiency of integration is determined by plant enzymes and integrity is determined by bacterial proteins (Bravo-Angel *et al.* 1999).

1.13 Comparison of T-DNA transfer to bacterial conjugation

The mechanism of T-DNA transfer has strong similarities to the bacterial conjugative transfer systems of IncP (Lessl and Lanka, 1994), the F plasmid (Zambryski, 1988) and IncQ (Buchanan-Wollaston, 1987).

1. Initiation of conjugal DNA transfer

In both systems the DNA to be transferred initiates from an origin of transfer, the *oriT* for conjugational transfer and the right border for T-DNA transfer,

through the activity of a strand transferase, the nicking enzyme, that subsequently attaches covalently to the 5' end of the transferred DNA. Therefore nicking at the T-DNA border sequences by theVirD1/ VirD2 strand transferase is analogous to *oriT* nicking by the respective site-specific strand transferases, TraI/TraJ for IncP, TraI/TraY for F and MobA/MobC for IncQ). The origin of transfer sequences is very similar and the enzymes processing them contain highly conserved motifs in the N terminal part of the protein.

2. Directional transfer of donor DNA

The 5' end at the *oriT* nick provides the leading terminus for the linear transfer of plasmid DNA out of the donor cell. The T-DNA transfer process has been shown to be polar, in the direction right border to left border. The 5' end of the transferable T-DNA copy, the T-strand is at the right T-DNA border. Thus T-DNA transfer is from 5' to 3' as in bacterial conjugation.

3. Conjugative DNA synthesis

In bacterial conjugation transfer of donor single-stranded DNA is associated with synthesis of a replacement strand in the donor cell and of a complimentary strand in the recipient cell. Replacement strand synthesis in the donor cell is mediated by a DNA polymerase with the 3' OH at the *oriT* nick site as the priming site. In *Agrobacterium,* replacement strand synthesis of the lower strand of the T-DNA region presumably uses the 3' OH of the nicked right T-DNA border as a priming site.

4. Mating cell signal

The recognition of a recipient cell and the formation of a stable mating pair stimulate DNA transfer. Since the *tra* genes of the F plasmid are constitutively expressed, nicking and religation at *oriT* probably occur simultaneously in donor cells. However, following stable contact with a recipient cell, some, as yet unknown, signal is transmitted from the recipient to stimulate donor DNA transfer. Plant phenolics such as acetosyringone are essentially a mating signal to *Agrobacterium* necessary to activate expression of the T-DNA transfer process at the level of transcription.

5. Pilus formation

Elongated pili are not a formal requirement for conjugal DNA transfer, as many Gram-positive and Gram-negative bacteria transfer DNA without pili (Clewell and Gawron-Burke, 1986). F-pili primarily function to locate an appropriate recipient cell. Once a stable pair has formed the F-pili retract. Actual DNA transfer may then proceed through a transmembrane pore near the base of the pilus. The *Agrobacterium* chromosomal virulence loci, *chvA, chvB, and pscA* may provide the pilus analogous function which allows *Agrobacterium* to form an effective contact with recipient plant cells. In this case bacterial polysaccharide projections may attach to surface components of the plant cell. The presence or absence of pili in various mutants of *A. tumefaciens* was found to correlate with the capacity to effect T-DNA transfer between different *A. tumefaciens* cells. This suggests that pili are required for T-DNA transfer to plant cells (Fullner *et al.* 1996).

6. Prevention of non-productive mating

The F-specific genes *traS* and *traT* encode products that prevent sibling mating. The TraS protein is localised to the inner bacterial membrane and may block DNA transfer directly. The TraT protein is a lipoprotein located in the outer bacterial membrane that binds the pilus tip in a competitive fashion. It is presumed, but not proven, that the T-DNA transfer process is limited to plant cells. To accomplish this exclusive interaction requires that T-DNA transfer-specific proteins, either at the bacterial surface or as part of the T-DNA-protein complex, recognize plant rather than bacterial cells. The fact that the Ti plasmid has a separate conjugative operon located outside the T-DNA and the *vir* regions for the transfer of Ti plamids between bacterial cells implies that this plasmid has evolved in such a way that it possesses a specific and specialized plant mating function (De Greve *et al.*1981; Holsters *et al.* 1980).

Although similarities between these two systems have been noted there are also differences which can be summarised as follows. First, VirD2 and TraI, for example initiate the process in the same way, by nicking at their sites. They probably present their nicked DNA to the transfer system via their coupling proteins. However, in RP4 for example, the TraI-DNA complex remains attached at the mating bridge so that when replication in the donor brings the *oriT* again, the strand transferase can nick again, and rejoin the two products; the plasmid in the donor and the new plasmid in the recipient. In the T-strand transfer system, VirD2 does not remain at the mating bridge, and actually leads the way into the plant cell. Therefore it is not poised to rejoin. Finally bacterial conjugation genes are expressed in the donor and in the

recipient cell whereas in T-DNA transfer the genes encoded in the T-DNA have eukaryotic regulatory elements and are expressed only in the recipient cell. Therefore, even though the two systems may have had a common ancestor, there clearly are important differences, and one must use caution when applying information from one system to predict properties of the other (Zambryski, 1988).

1.15 Aims of this project

The main aim of this project was to explore the possibility of using the *Acidithiobacillus ferrooxidans* (previously called *Thiobacillus ferrooxidans*) plasmid pTF-FC2 as a binary vector without the T-DNA border sequences in the transformation of tobacco using *A.tumefaciens,* and also to gain an insight into the mechanism of such transformation at the molecular level. The aim was primarily based on the promiscuity of pTF-FC2 and its similarity to RSF1010 in terms of gene organization and layout. Such a possibility would result in the development of a novel plant transformation vector and would also broaden current knowledge on horizontal gene transfer between bacteria and plants. I wanted to verify whether the promiscuity of pTF-FC2 could be extended to plants in a similar fashion as had previously been shown with RSF1010 (Buchanan-Wollaston *et al.*1987). My investigations in this regard, together with a detailed account of the plasmid pTF-FC2, are presented in Chapter 2. Once this step was accomplished I sought to elucidate the mechanism of action of pTF-FC2 in plant transformation at the molecular level. The results of these investigations are presented in Chapter 3. I also investigated the possibility of generating multigene transformants by developing a simple system that utilized a strain of *Agrobacterium* carrying two genes on two separate compatible plasmids. Depending on whether this system favoured the integration of DNA to a

single locus or to multiple loci it could then be used for the generation of transgenic plants with polygenic traits such as metabolic pathways or for the generation of marker-free-plants. The implications of using the pTF-FC2-derived plasmid will also be discussed. The results of these investigations are presented in Chapter 4.

CHAPTER 2

TRANSFORMATION OF TOBACCO USING pTF-FC2

ABSTRACT

Plasmid pTF-FC2 is a 12.2-kb, non-conjugative, broad-host-range plasmid that was originally isolated from the acidophilic biomining bacterium *Acidithiobacillus ferrooxidans* (previously called *Thiobacillus ferrooxidans*). The plasmid was found to be unique in the sense that whilst its replicon clearly resembled that of the broad-host-range IncQ plasmids the mobilization functions resembled those of the IncP plasmids. Until recently the transformation of plant cells by *A. tumefaciens* represented the only known case of trans-kingdom DNA transfer. Later it was shown that the mobilization functions of the plasmid RSF1010 could mediate the transfer of plasmids from *A. tumefaciens* via a binary vector system, into plant cells, as well as transfer it between Gram-negative bacteria. Therefore the mobilization functions of RSF1010 substitute for the requirement for the 25-bp T-DNA border sequence of *Agrobacterium*. The unique features of plasmid pTF-FC2 and the realization that it was similar to but substantially different from the IncQ plasmid RSF1010 made it an interesting plasmid for further study. Therefore the aim of this study was to investigate the possible use of the plasmid pTF-FC2 as a binary vector. Using the plasmid pTF-FC2 as the backbone two plasmids were constructed namely pTD1 and pDER-*bar*. Plasmid pTD1 contained, as the plant selectable marker, the *nptII* gene flanked by the right and left borders. Plasmid pDER-*bar* contained, also as a plant selectable marker, the bialaphos resistance gene and this plasmid did not have the right and left borders. *Agrobacterium* strains C58C1(pMP90)(pTD1) and LBA4404(pAL4404)(pDER-*bar*) were generated by electroporation. Large numbers of

morphologically normal transgenic plants were obtained through the co-cultivation of tobacco leaf discs with the strain LBA4404(pAL4404)(pDER-*bar*). PCR amplification and Southern blot analysis were used to confirm the stable integration of the transgenes in the T_o generation. 320 T_1 plants generated from eight randomly selected T_o plants were tested for the presence of the *bar* gene using PCR. The results indicated a 3:1 segregation of the *bar* gene showing that the gene had been inherited in a Mendelian fashion. By designing primers which span all the open reading frames of the plasmid it was possible to determine, through PCR reactions, how much of the plasmid had integrated. These results were also able to give an indication of the direction of transfer. No plants were generated from tobacco leaf discs that were co-cultivated with the strain C58C1(pMP90)(pTD1). This result warrants further investigation as it possibly shows that not only are the borders unnecessary but also that they inhibit the DNA transfer/integration process when used in conjunction with a pTF-FC2 derived binary vector.

2.1 INTRODUCTION

Studies on the bacteria that formed the inoculum of a bioxidation plant used for the pretreatment of gold bearing arsenopyrite ores at the Fairview mine, South Africa revealed that *At. ferrooxidans* strain FC1 was the dominant bacterial strain (Rawlings *et al.* 1984). *At. ferrooxidans* is found ubiquitously in nature and has a physiology that is amongst the most remarkable of all life forms. It is an autotroph and obtains the carbon it requires for metabolism by the fixation of carbon dioxide from the atmosphere. It is in this respect similar to plants. However, unlike plants which depend upon solar energy to fix carbon dioxide, *At. ferrooxidans* obtains its energy through the oxidation of ferrous

iron to ferric iron or reduced sulphur compounds to sulphuric acid. *At. ferrooxidans* is well adapted to the large volumes of acid that it produces and grows at an optimum pH of between 1.5 and 2.5. In addition all *At. ferrooxidans* strains tested are nitrogen fixing which means that the nitrogen requirement is met by the reduction and incorporation of atmospheric nitrogen (Dean and Rawlings, 1996).

Characterization of strain FC1 showed that it contained three plasmids of which pTF-FC2 was one (Rawlings *et al.* 1983). The nucleotide sequence of the whole of pTF-FC2 has been determined (GenBank accession numbers M64981 and M35249). Four regions of the plasmid have been identified; a replicon, a mobilization region, a transposon and a plasmid addiction system (Fig. 2.1).

The pTF-FC2 plasmid is 12.2 kb in size and has been shown to replicate in a wide range of Gram-negative bacteria (Rawlings and Kusano, 1994). The minimum region required for replication has been isolated, sequenced and characterized (Dorrington and Rawlings, 1990). Although the pTF-FC2 replicon was found to resemble most closely those of the IncQ plasmids (RSF1010, R300 and R1162), the two plasmids were compatible and the replication proteins from the IncQ plasmids were unable to complement pTF-FC2 *rep* mutants. Plasmid pTF-FC2 was shown to be a non-conjugative plasmid that was capable of being mobilized at high frequency between *E. coli* strains by a co-resident IncP plasmid RP4 (Rawlings and Woods, 1985). The ability to be mobilized together with its broad-host-range replicon enables pTF-FC2 to be used in the construction of vectors that can be used in a large variety of Gram-negative bacteria (Rawlings *et al.* 1986).

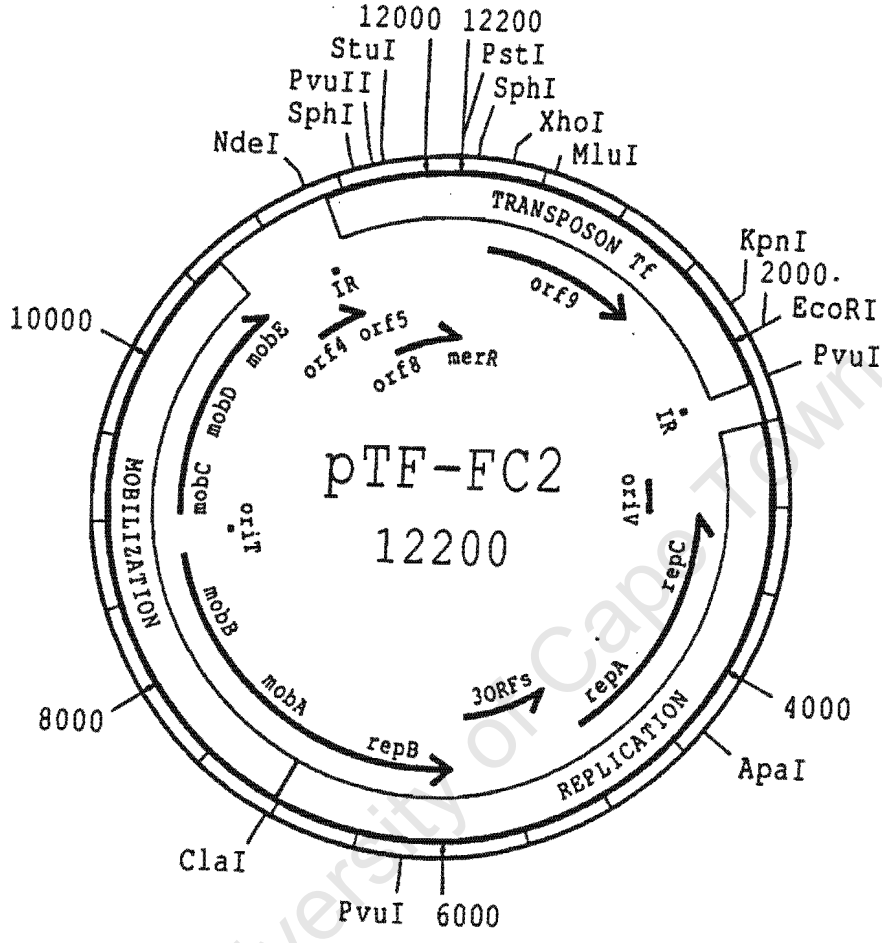


Fig. 2.1 Plasmid pTF-FC2 showing the replication, mobilization and transposon regions. The numbers indicate the distance from the *PstI* site (Smith, 1997).

The minimum region required for the mobilization of pTF-FC2 was shown to be located on a 3755-bp fragment, which consisted of an *oriT* site flanked by five genes arranged in such a way that two (*mobA* and *mobB*) were on one side and three (*mobC*, *mobD* and *mobE*) on the opposite side of the *oriT*. Of the five polypeptides, three (MobA, MobC, and MobD) were essential for mobilization while two (MobB and MobE) affected mobilization frequency (Rohrer and Rawlings,1992). In RP4, the proteins of the Tra1 region are arranged into three operons, a leader operon on one side of the *oriT*, and the relaxase and primase operons on the other (Ziegelin *et al.* 1991). There was a distinct similarity in the size, organization and amino acid sequences of four of the pTF-FC2 Mob polypeptides with the proteins of the leader and relaxase operons of the Tra1 region of RP4.

A transposon with 38-bp inverted repeat sequences that are identical to those of Tn21 is located on pTF-FC2. This transposon, Tn5467, contains three accessory genes which encode a glutaredoxin, a mercury resistance (MerR)-like regulator protein and a 43-kDa protein with similarity to multi-drug resistance transport proteins (Clennel *et al.* 1995).

Additionally pTF-FC2 also contains three small open reading frames (ORFs) that are situated between the *repB* (primase) gene and the *repA* (helicase) gene. These ORFs encode a poison-antidote plasmid stability system. The three genes were name *pasA*, *pasB* and *pasC* (plasmid addiction system), in which PasA is the antidote, PasB the toxin and PasC a protein that appears to enhance the ability of the antidote to neutralize the

toxin (Smith and Rawlings, 1997). Plasmid-encoded toxin-antidote systems counteract the loss of plasmids by killing plasmid-free segregants (Jensen and Geddes, 1995). Phenotypically this phenomenon leads to plasmid stability.

In this chapter my main aim was to investigate the possible use of pTF-FC2 as a binary vector using *Agrobacterium*-mediated leaf disc transformation of tobacco. Based on the fascinating similarity between this plasmid and RSF1010 two plasmids were constructed, one that lacked the border sequences (pDER-*bar*) and another that contained them (pTD1).

2.2 MATERIALS AND METHODS

2.2.1 Plasmid construction

All standard DNA manipulations were performed as described by Sambrook *et al.* (1989) and according to specifications of the DNA modifying enzymes (Boeringer Mannheim, Amersham or Roche). Large-scale plasmid purifications from *E. coli* were by equilibrium centrifugation in caesium chloride-ethidium bromide density gradients or by anion-exchange chromatography, using a Nucleobond™ kit (Machery-Nagel). A derivative of pTF-FC2, pDER405 (Rawlings *et al.* 1986) was used as the backbone of all the plasmids that were constructed in this study. This plasmid was constructed by cloning the 11.3-kb *Pst*1/*Nde*1fragment of pTF-FC2 into pBR325. This fragment comprises of the mobilization and the replication regions together with the vegetative origin of replication. The plasmid pDER405 is 15.5 kb and has the tetracycline (Tc) and the

chloramphenicol (Cm) resistance genes as selectable markers. A detailed map of pDER405 is shown in Appendix A.

2.2.2 Construction of pDER-*bar*

The construction of plasmid pDER-*bar* (Fig. 2.2) was done in two phases. The first phase involved the substitution of the smaller *Pvu*II fragment (containing a tetracycline resistance gene and a major part of the chloramphenicol gene) of pDER405 with a *Pst*I restricted bacterial kanamycin resistance expression cassette from pUCK4 (Vierra and Messing, 1982). Prior to cloning, the *Pst*I ends were blunted using the Klenow fragment of DNA polymerase I. This was done due to the fact that tetracycline resistance is of limited value for selection of transformed *A. tumefaciens* (Klee *et al.* 1985; Luo and Farrand, 2000). The second phase involved the replacement of the *Pst*I/*Eco*RI fragment of the resultant plasmid with the CaMV 35S promoter-*bar* expression cassette from pDPG165 (Spencer *et al.* 1990).

2.2.3 Construction of pTD1

The plasmid pTD1 was constructed by cloning the *Sal*I fragment of the binary vector pART 27 (Gleave, 1992) into the *Sal*I site of pDER405. This fragment incorporates the *nptII* and the *lacZ'* genes in between the right and left borders (Fig. 2.3).

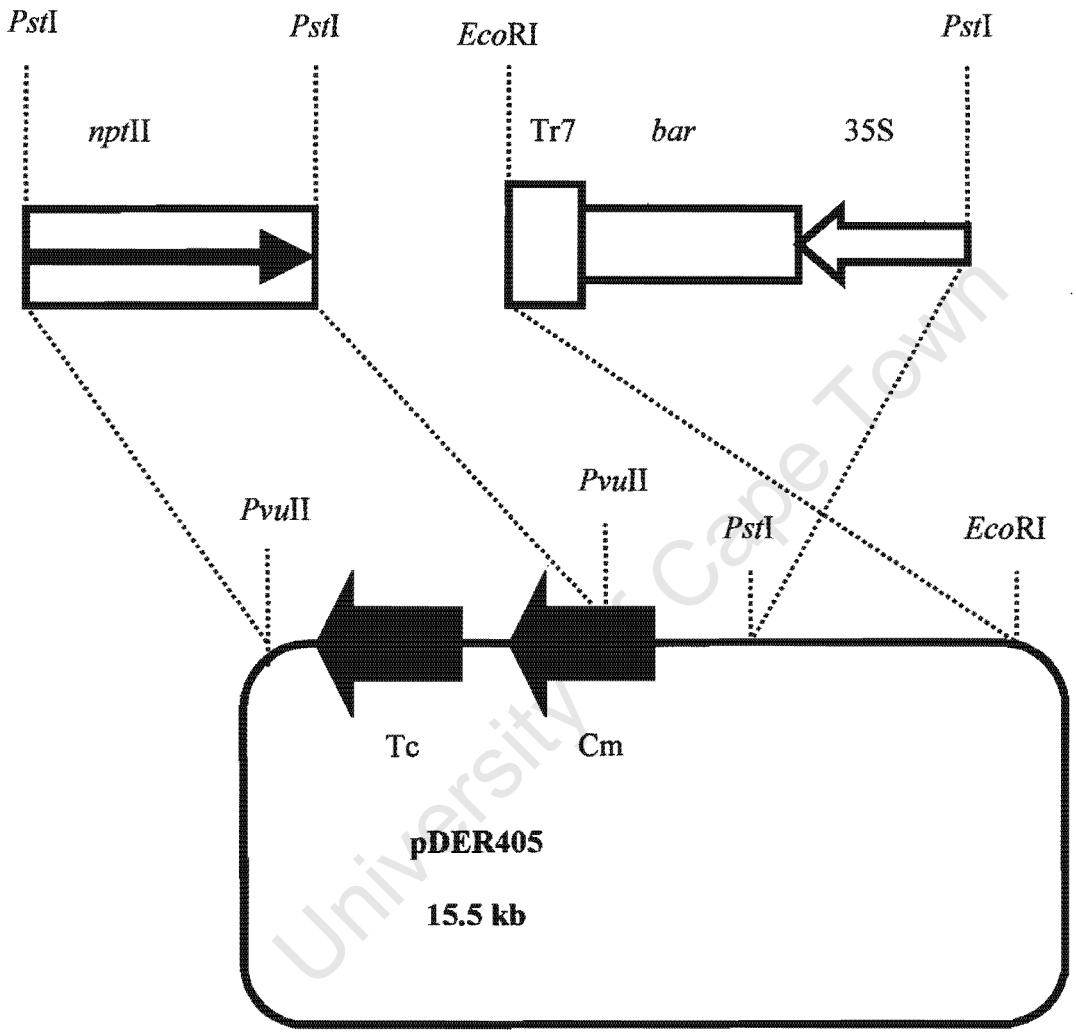


Fig. 2.2 Construction of plasmid pDER-*bar*. The construction was done in a two-stage process as detailed in Materials and Methods.

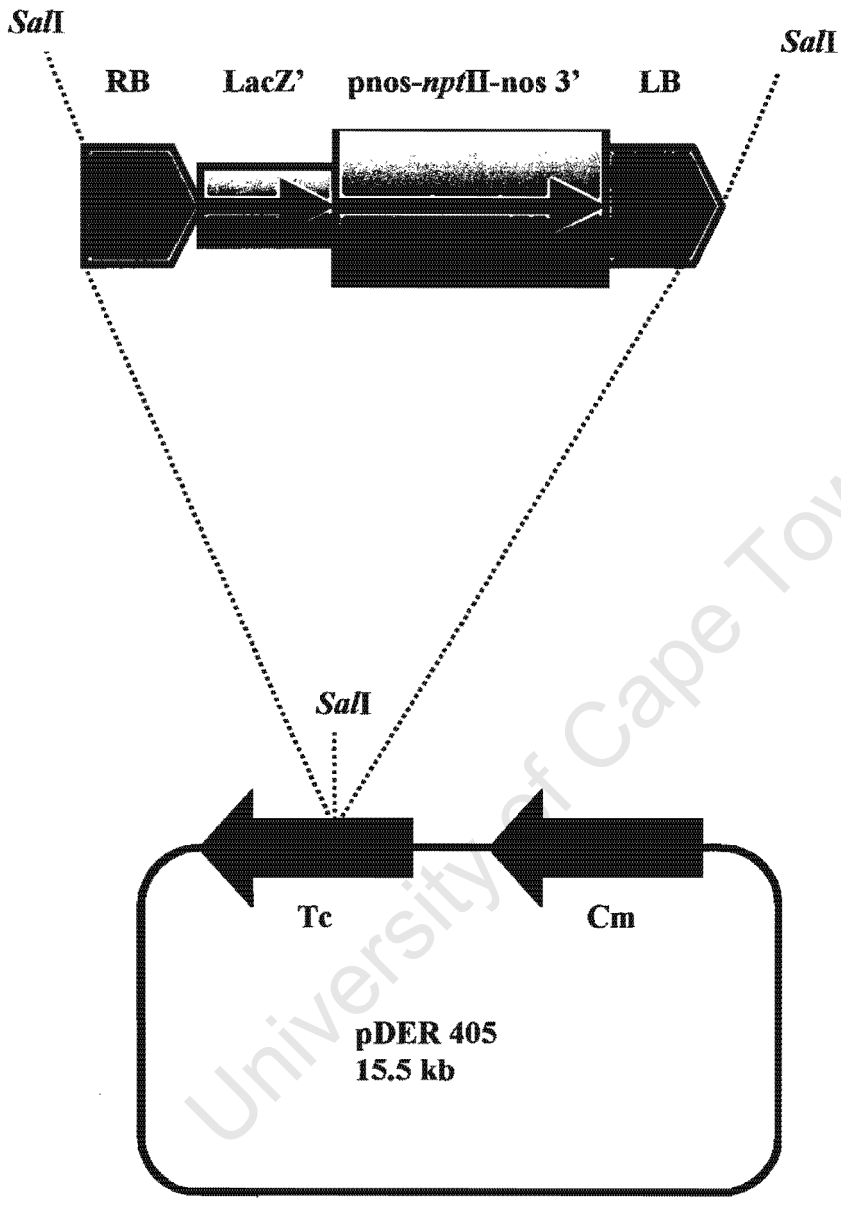


Fig. 2.3 Construction of plasmid pTD1. The right and left borders incorporating the T-DNA which carries the neomycin phosphotransferase gene (*npt*II) driven by a nopaline synthase promoter (pnos) and a nopaline synthase 3'- untranslated region (nos 3') which acts as a transcription terminator were cloned as a *Sal*I fragment from pART27 (Gleave, 1992) into the corresponding site in pDER405. Tc and Cm represent the tetracycline and chloramphenicol resistance genes respectively.

2.2.4 Transformation of *Agrobacterium* using the freeze-thaw method

Two ml of an overnight culture of either of the Rifampicin resistant (Rif^R) mutant strains of LBA4404(pAL4404) or C58C1(pMP90) were inoculated into 50 ml LB and grown at 30^0C with vigorous shaking until the cells reached an OD_{600} of between 0.5 and 1.0. The cells were harvested by centrifugation at 3000 x g for 5 minutes at 4°C. The cells were resuspended in 1 ml ice-cold 20 mM $CaCl_2$ solution. The resultant cell suspension was dispensed into pre-chilled Eppendorf tubes as 0.1 ml aliquots. One μg of plasmid DNA was added to the cells prior to freezing them in liquid nitrogen. The cells were thawed by incubating the Eppendorf tube in a 37°C water bath for 5 minutes. One ml of LB medium was added to the cells that were subsequently incubated at 30°C for 2 to 4 hours with gentle shaking in order to allow the bacteria to express the antibiotic resistance genes. The cell suspension was then centrifuged for 30 seconds in a high speed-microfuge and the pellet resuspended in 0.1 ml LB medium. The cells were plated onto LA plates supplemented with rifampicin (Rif; 100 μg/ml) and kanamycin (Km; 100 μg/ml) for LBA4404(pAL4404)(pDER-*bar*) or Rif (100μg/ml) and Chloramphenicol (Cm; 25 μg/ml) for C58C1(pMP90)(pTD1) and incubated at 30°C.

2.2.5 Transformation of Agrobacterium using electroporation

An overnight culture of either of the Rifampicin resistant (Rif^R) mutant strains of LBA4404(pAL4404) or C58C1(pMP90) *A. tumefaciens* was centrifuged in two 1.5 ml Eppendorf tubes for 30 seconds in a high-speed microfuge. The pellets were resuspended in 0.5 ml of ice-cold 300 mM sucrose in distilled water and recentrifuged. This step was repeated two more times with 0.5 ml 300 mM sucrose solution in distilled water and once

with 10% filter-sterilised glycerol. The pellets were finally resuspended in 20 µl of 10% glycerol and the contents of both tubes combined resulting in a total volume of 40 µl. One µg of plasmid DNA was then added to the cells and the tube was incubated on ice for two minutes. The cells were then transferred to a pre-chilled 0.2 cm electroporation cuvette. Electroporation was carried out using a Bio-Rad Gene Pulser set to a capacitance of 25 µF, 2.5 kV charge, and the pulse controller to 400 Ω resistance. Immediately after delivery of the high voltage pulses, 1 ml of SOC broth (see Appendix B) was added for phenotypic expression at 30°C for 4 to 6 hrs before plating 100 µl of undiluted cells onto LA plates supplemented with Rif (100µg/ml) and Km (100 µg/ml) for pDER-*bar* or Rif (100µg/ml) and Chloramphenicol (Cm; 25 µg/ml) for pTD1.

2.2.6 Isolation of genomic DNA from *A. tumefaciens*

Agrobacterium cells were grown to early log phase in 2 ml LB medium supplemented with the appropriate antibiotics and harvested by centrifugation at 4000 x g for 10 minutes. The pellet was resuspended in 300 µl of TEN buffer (10 mM Tris/HCl, 10 mM NaCl, 0.1 mM EDTA, (pH 7.8)). 100 µl of a 5% sarkosyl solution (in TEN) was added and mixed thoroughly by inverting the tube several times. 10 µl of a 50 mg/ml proteinase K solution were added and the contents mixed thoroughly prior to incubation at 37°C until the solution became viscous. This usually took between 20 and 30 minutes. In order to reduce the viscosity a 1 ml pipette was used to pipette the viscous solution up and down 5 to 10 times. This solution was extracted once with phenol (50%)-chloroform (50%) and twice with chloroform (96%)-iso-amyl-alcohol (4%). The extract was then precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5) and 2.5 volumes of

95% ethanol and this was incubated at –20°C for 2 hours. The DNA suspension was centrifuged at 12000 x g and the DNA pellet washed with 70% ethanol and allowed to dry before resuspending in 50 µl TEN. This DNA could then be used for PCR reactions or for Southern blot analysis.

2.2.7 Isolation of plant genomic DNA

Genomic DNA was extracted from fresh leaves of young tobacco plants using the Nucleon™ Phytopure Plant DNA Extraction Kit (Amersham Life Science) in accordance to the manufacturer's instructions. For large scale PCR analysis DNEASY™ Plant Extraction Kit (Qiagen) was used.

2.2.8 Southern blot analysis of *Agrobacterium* DNA

Five µg of genomic DNA was digested with *EcoRI*, separated on 0.8% agarose gel and transferred onto Hybond N^+ membrane according to the protocol of the supplier (Amersham). The PCR products of the *bar* and *nptII* genes were used as probes after ^{32}P - labeling with the random primer DNA labeling kit (Boehringer Mannheim). Southern blotting was done by standard procedures (Sambrook *et al.* 1989). Hybridization was carried out in 10% dextran sulphate (Pharmacia), 1 M NaCl, 1% SDS and 200 µg/ml denatured herring sperm DNA at 60°C. After hybridization the membrane was washed down to 0.1 x SSC at 60°C. Autoradiography was performed with Kodak X-Omat AR films according to standard procedures.

2.2.9 Southern blot analysis of plant genomic DNA

Twenty μg of DNA was digested overnight using a fourfold excess of restriction enzyme and electrophoresed in 0.8% agarose gels. The fractionated DNA fragments were transferred to a positively charged nylon membrane (Boehringer Mannheim or Amersham) according to the manufacturer's instructions. The probe used for the detection of *nptII* homologous sequences was generated by PCR in the presence of digoxygenin-labeled dUTP, with primers KanF and KanR (Table 2.1) and using 50 ng of pDER-*bar* as template DNA. The cycling conditions were 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds. The final elongation step was at 72°C for 7 minutes. This reaction generated a digoxygenin-labeled hybridization probe of 656 bp.

2.2.10 PCR analysis

PCR was routinely performed in a total volume of 50 μl using Taq polymerase in accordance to the supplier's protocol (Roche and Amersham). Between 10 and 50 ng of plasmid DNA and between 500 ng and 1 μg of plant genomic DNA were used as template. The cycle conditions were as follows: initial denaturation temperature was 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. The final extension time was at 72°C for 10 minutes. All the primers used in this study are shown in Table 2.1. The primers used the same cycling conditions as given above except that the initial denaturation step for plant genomic DNA was at 94°C for 5 minutes.

Table 2.1 Primers used in this study

Name of primer	Primer sequence 5'-3'	Size of PCR product (bp)
KanF	GCTCTGCCAGTGTTACAACCAA	
KanR	CAGATGAGATGGTCAGACT	656
MobF	CCAAGCTCCGGGCTTTTCTGCATC	
MobR	GTGAGCAGAACGGGCGTTATC	726
MobCDF	GAAATACACGACAGAGCAGC	
MobCDR	CAGCCGCGTTAATTACCACG	632
RepAF	GGCTTTAGACATTATGGCGG	
RepAR	GTCACTCATGCGCTCGGCTT	670
RepCF	CGAAAGAGGCTCAAGCTCGAT	
RepCR	CGTACTCGTTCACCGTCCAAC	720
BarF	CGTCAACCACTACATCGAG	414
BarR	GAAACCCACGTCATGCCAG	

2.2.11 Tobacco leaf disc transformation

In order to introduce genes into tobacco a modified version of the leaf disc transformation method that was described by Horsch *et al.* (1985) was used. *Agrobacterium* cells were grown to stationary phase in 5 ml LB medium supplemented with the appropriate antibiotic and centrifuged at 4900 x g for 7 minutes. The pellet was washed twice in 5 ml 0.01 M $MgSO_4$. Samples of 0.5 ml of the cell suspension were added to 9.5 ml of 0.01

M $MgSO_4$ to result in a ten-fold dilution. Leaf discs were cut out of sterile *Nicotiana tabacum* cv Petit Havana SR1 leaves (Maliga *et al.* 1973). These were floated, lower epidermis down, in a petri dish containing 10 ml of the *A. tumefaciens* suspension and incubated for 20 minutes at room temperature with gentle shaking. The leaf discs were transferred onto bacterial/plant co-cultivation medium (Appendix B) in a Petri dish for three days in the dark at 25°C, for bacterial DNA to transform the leaf discs. The leaf discs were transferred onto shooting medium containing either bialaphos (3 mg/l) or kanamycin (200 mg/l) to select for transformants and cefotaxine (1 mg/l) to kill the Agrobacterium. These plates were left at 25°C for 16 hours in the light and 8 hours in the dark. Subculturing was done every two weeks until callus tissue developed. The proliferating callus was maintained on shooting medium for plant regeneration. Two to three weeks later, regenerated plantlets were transferred to rooting medium. Plants with vigorously growing roots were transferred to soil.

2.3 RESULTS

2.3.1 Selection and plant regeneration

For plant transformation and regeneration studies, the following strains of *Agrobacterium*: LBA4404(pAL4404)(pDER-*bar*); C58C1(pMP90)(pDER-*bar*) and C58C1(pMP90)(pTD1) were used. Strain LBA4404 (pAL4404) was used as the negative control. Following tobacco leaf disc co-cultivation with the above strains, the discs were transferred to shooting medium containing bialaphos for pDER-*bar* selection and kanamycin for pTD1 selection. Within 10 to 14 days leaf discs that had been co-cultivated with *Agrobacterium* strains harbouring the pDER-*bar* plasmid developed

vigorously growing callus (Fig. 2.4). The callus was transferred to fresh shooting medium and then to rooting medium containing bialaphos. Plants were successfully regenerated and developed roots when transferred to the rooting medium. One hundred and forty (74 from LBA4404 and 66 from C58C1) independent transgenic tobacco lines were recovered. No differences between the leaf disc transformation process mediated by LBA4404 or C58C1 were observed. The 74 plants from the LBA4404 mediated transformation were allowed to grow to maturity and were self-pollinated. The negative control tobacco leaf discs and those that had been co-cultivated with *Agrobacterium* strain C58C1(pMP90)(pTD1) did not show any signs of callus formation (Fig. 2.4A and 2.4C). In fact they bleached and died when placed on medium that contained kanamycin. Leaf disc transformation with strain C58C1(pMP90)(pTD1) was repeated a further three times and still no callus could be detected.

2.3.2 Analysis of the T_0 plants

All surviving plants were shown to be transgenic by molecular analysis. Continuous selection of callus up to rooting of the plantlets on medium containing bialaphos ensured there were no escapes and confirmed the presence and activity of the *bar* gene. Initially all the 140 independently derived transgenic plants were analyzed by PCR for the presence of the *bar* gene (see Fig. 2.5 for a representative sample). The results showed that all the plants carried the gene.

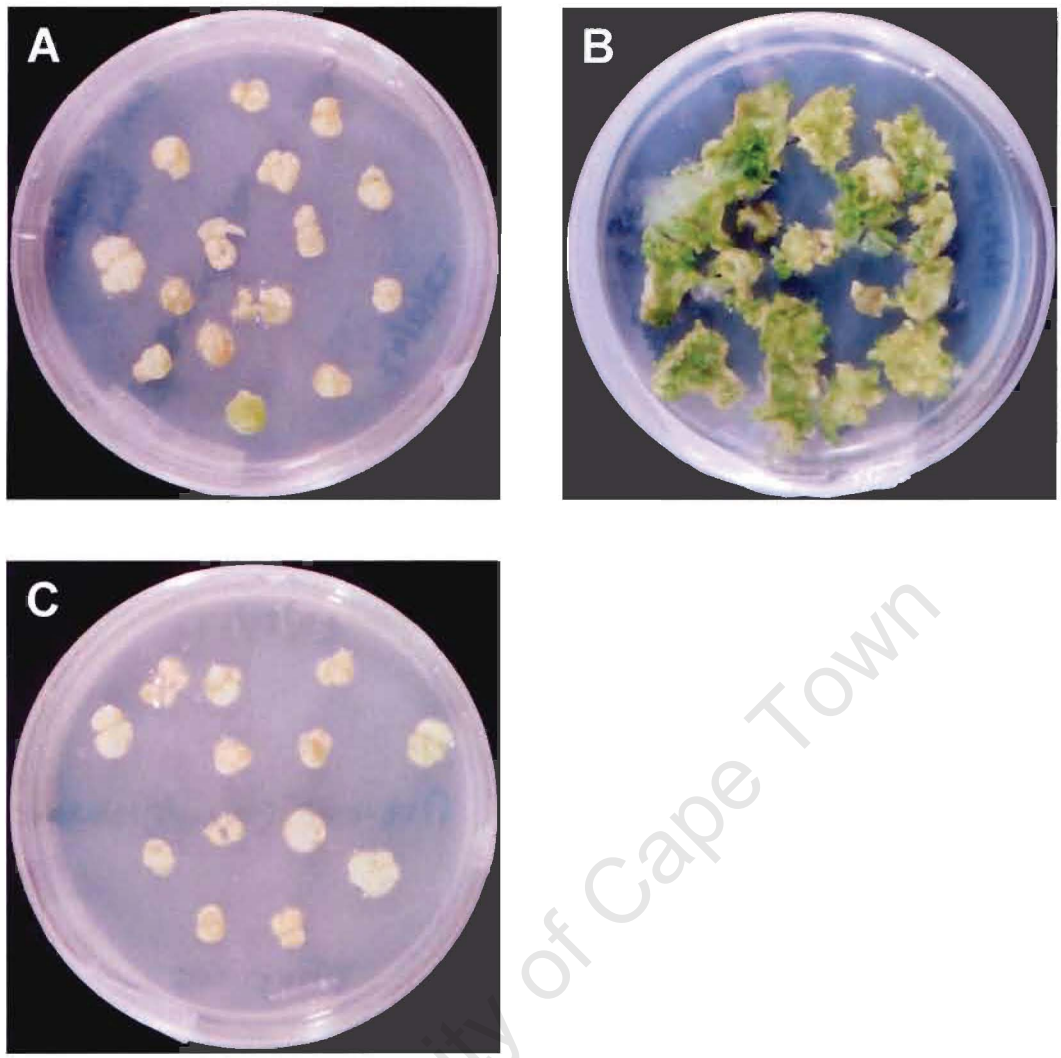


Fig. 2.4 Callus formation following tobacco leaf disc transformation by strains (A) LBA4404(pAL4404); (B) LBA4404(pAL4404)(pDER-*bar*); (C) C58C1(pMP90)(pTD1).

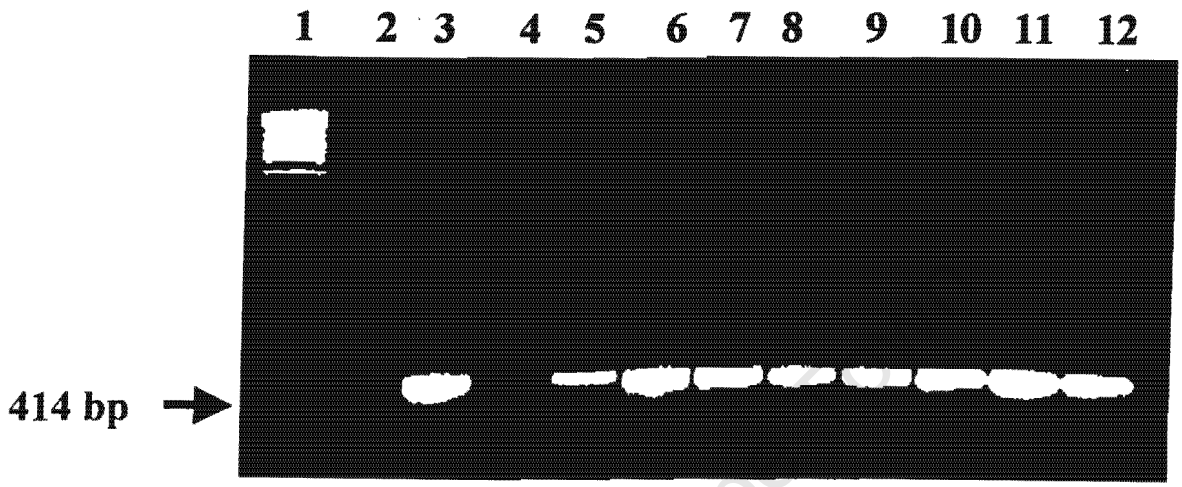


Fig. 2.5 Representative PCR analysis of T_0 plants. Lanes 1, λ/*PstI* molecular weight markers; 2, water; 3, pDER-*bar*; 4, non-transgenic plant; 5-12, transgenic plant lines 31, 35, 41, 43, 50, 52, 58 and 68 respectively. The PCR products were run on 1% agarose gel. All the transgenic plants tested positive for the *bar* gene.

To further characterize these transgenic plants in detail and to estimate the transgene copy number Southern blot analysis was done. The DIG-labeled PCR product of the *nptII* gene, which is a djacent to the *bar* gene in the pDER-*bar* plasmid was used as a probe. *Eco*R1 *and Hind*III, which both conveniently recognize a single site in the pDER-*bar* plasmid were used. However, unlike *Eco*RI, the *Hin*dIII recognition site is present within the PCR product that is used as a probe. Seven independently derived T_0 transgenic

plants were randomly selected from the plants that had been generated via LBA4404 mediated transformation. Unique and complex hybridization patterns were revealed (Fig. 2.6) indicating that these plants indeed originated from independent integration events. A rough estimate of the copy numbers were determined according to the number and intensity of hybridizing bands on Southern blots. The detection of DNA fragments with sizes different from the size (14-kb) generated by the *Eco*R1 digestion of the plasmid pDER-*bar* indicates the plasmid integration into the plant genome. The main reason for digesting genomic DNA with *Hin*dIII was to generate smaller fragments that could separate more efficiently and thus facilitating the estimation of the copy number. Typically, the copy number ranged from two to four per plant. Except for line 50, all the *EcoRI* digested lines showed hybridization signals of greater than 9 kb in length. There was no explanation as to why the *Hin*dIII digest of line 43 did not show any hybridization signal.

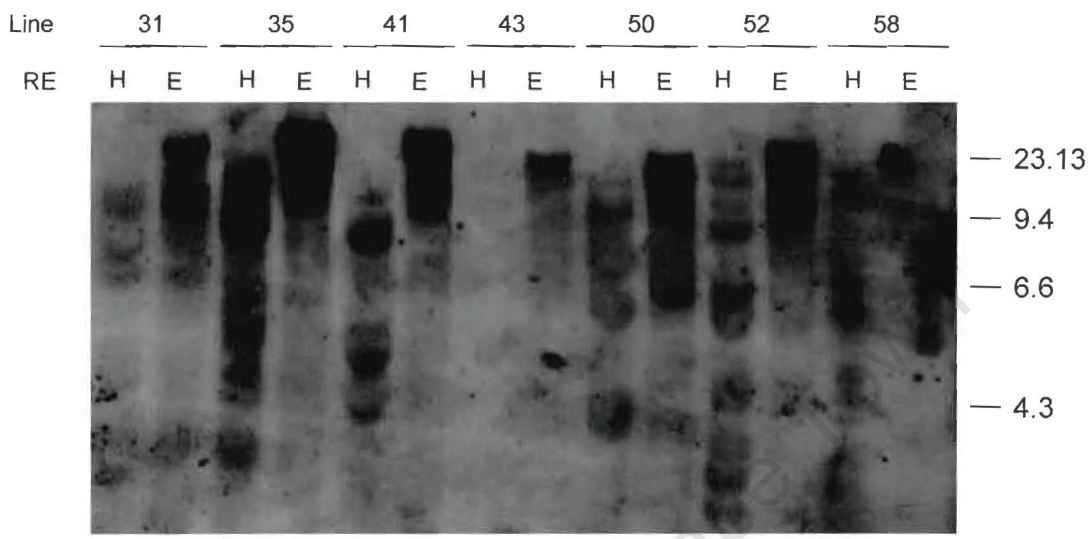


Fig. 2.5 Representative Southern blot analysis of T_0 transgenic tobacco plants. Plant genomic DNA was digested with *Hin*dIII and *Eco*RI separately, size fractionated in a 0.8% agarose gel and hybridised to a DIG-labelled *npt*II probe.

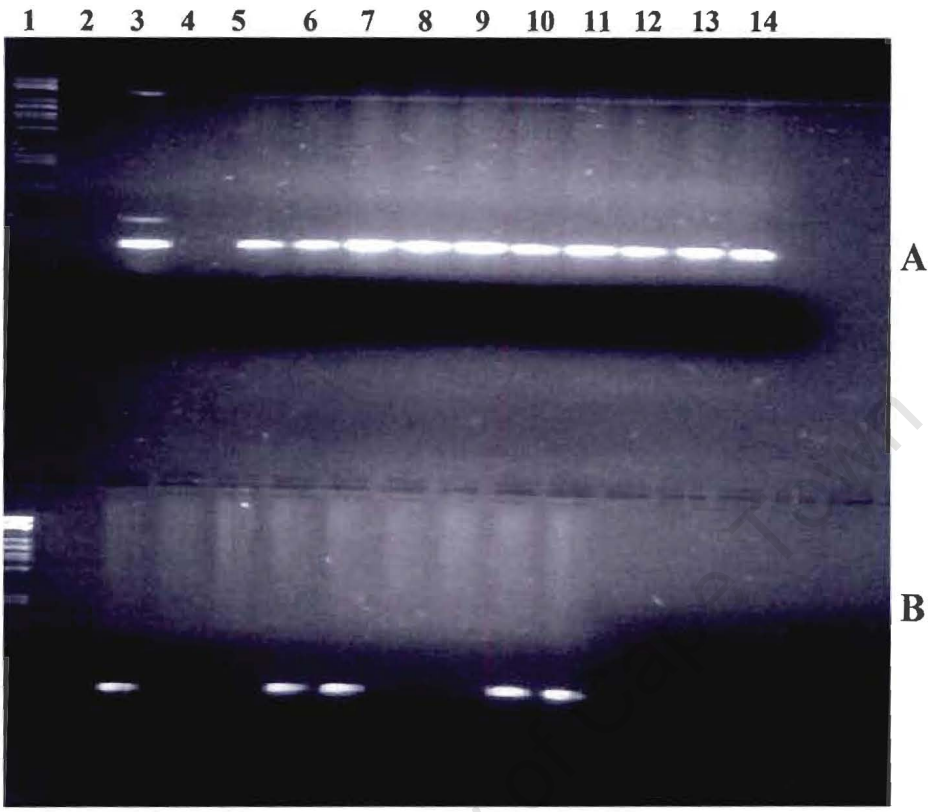


Fig. 2.7 PCR analysis of the *bar* gene of 20 T_1 progeny of line 31The PCR products were size fractionated on a 1 % agarose gel. Panel A: Lanes 1, λ/*PstI* molecular weight markers; 2, water; 3, plasmid pDER-*bar*; 4, non-transgenic SR1 plant; 5-14, T_1 progeny of the transgenic line 31 (ie. lines 31-1 to 31-10). Panel B: Lanes 1, λ/*PstI* molecular weight markers; 2-11, lines 31-11 to 31-20.

2.3.3 The Mendelian pattern of inheritance

In order to study the segregation patterns of the transgenes 40 T_1 plants from each of eight independently derived tobacco transformants (lines 31, 35, 41, 43, 50, 52, 58 and 68) were analysed using PCR. The results showed that in all the plants, except line 35, the *bar* gene segregated at a ratio of 3:1 indicating that the gene had integrated at a single genetic locus. Therefore in these plants the *bar* gene was inherited in a Mendelian fashion (Fig. 2.7 and Table 2.2).

Table 2.2 PCR analysis of the T_1 progeny of eight randomly selected transgenic lines

Plant line	*bar* positive plants/40	% of *bar* positive plants
31	31	77.5
35	35	87.5
41	32	80
43	30	75
50	29	72.5
52	28	70
58	30	75
68	31	77.5

In line 35 the *bar* gene showed a segregation ratio of 15:1 suggesting that two independent integration events had occurred. In order to show that the T_1 plants expressed the *bar* gene the T_1 seeds were germinated on media that was supplemented with PPT. The results indicated that indeed the T_1 plants expressed the *bar* gene as they were able to thrive under selection conditions as compared to the negative control plants. These results confirmed the PCR results of inheritance of the *bar* gene in a Mendelian

fashion. As expected line 35 showed a 15:1 survival ratio (Fig. 2.8 and 2.9 and Table 2.3).

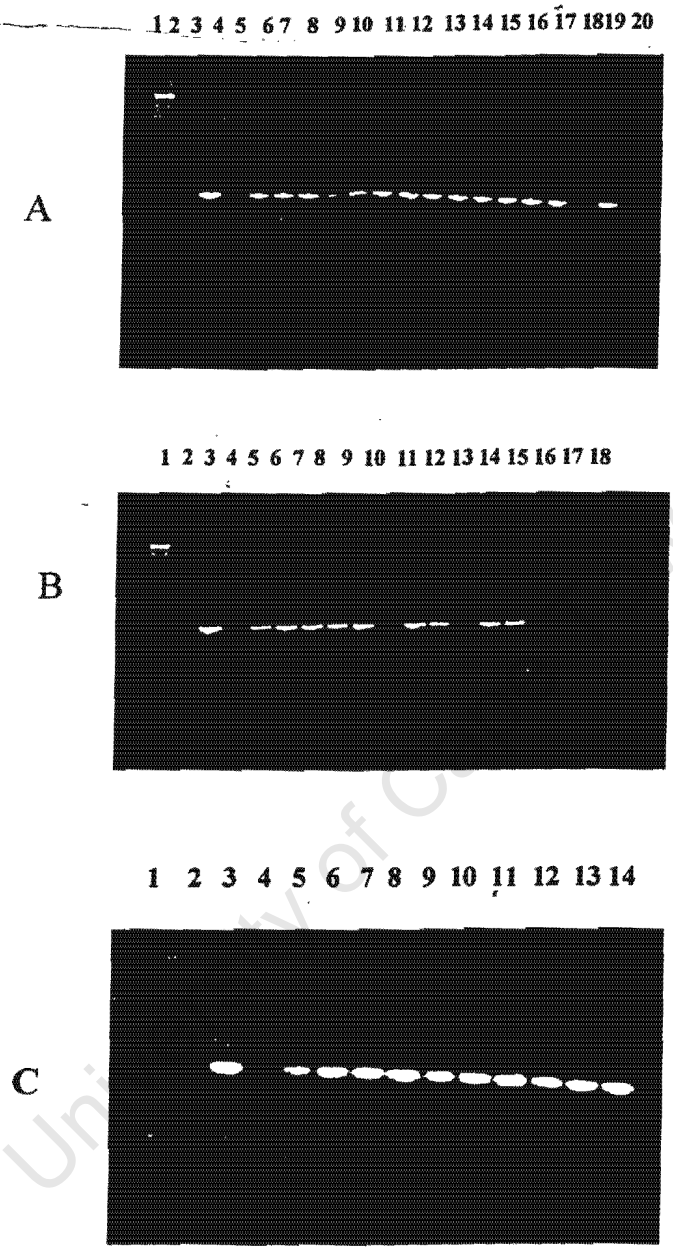


Fig 2.8 PCR analysis of theT_1 progeny of line 35. Forty plants were analysed for the presence o the *bar* gene using PCR. The PCR products were size fractionated on a % agarose gel. In al photographs lanes 1-4 contain λ/Pst 1 molecular weight markers, water, plasmid pDER-*bar* and a non transgenic SR1 plant respectively. Photograph A: Lane 5-20 T1 pogeny of line 35 (ie. Lines 35-1 to 35-16). Photograph B: lines 35-17 to 35-30. Photograph C: lines 35-31 to 35-40. Five ou of 40 plants were negative for the *bar* gene giving a 15:1 segregation ratio.

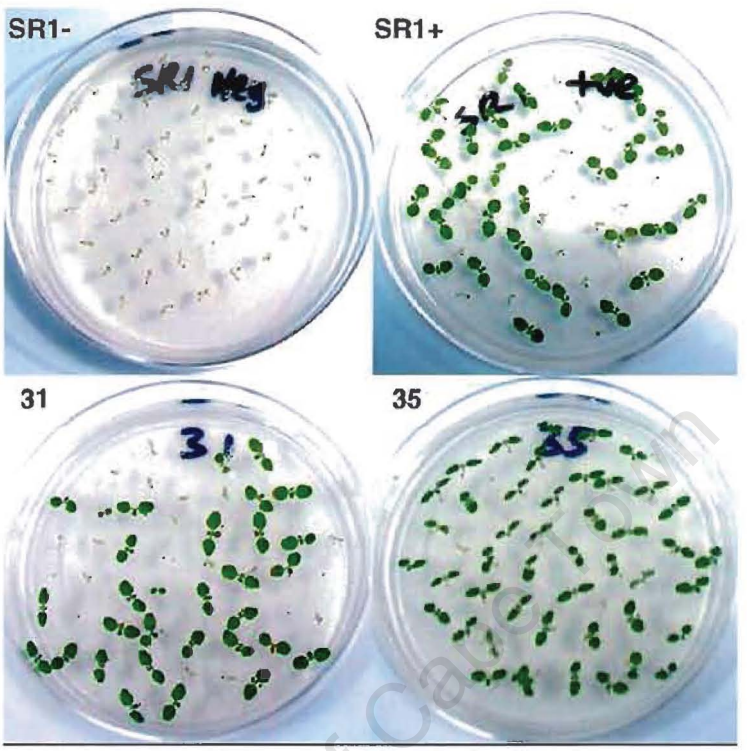


Fig 2.9 Representative PPT selection for the analysis of the segregation pattern of the *bar* gene in the progeny of T_0 plants. 50 seeds were germinated in MS media supplemented with 20mg/l PPT. Line 31 shows a 3:1 segregation pattern whereas line 35 shows a 15:1 segregation pattern. Non-transgenic tobacco seeds were used as the negative control (SR1-). Barbara Hohn kindly donated the seeds for the positive control (SR1+). These seeds were derived from the progeny of a T_0 tobacco plant that carried a single copy of the of the *bar* gene.

Table 2.3 Segregation of the *bar* gene as determined by the sensitivity and resistance to PPT of the T_1 progeny of eight randomly selected transgenic plants

Line	PPT resistant	PPT sensitive	% Resistance
31	31	14	69
35	46	3	94
41	37	7	84
43	40	10	80
50	40	7	85
52	33	14	70
58	36	11	77
68	39	8	83
SR1 (+)	33	17	66
SR1 (-)	0	50	0

2.3.4 Determination of the extent of plasmid integration into the plant genome

In order to determine what region of the plasmid had integrated into the tobacco plant genome primers that spanned the pDER-*bar* plasmid were designed (Fig. 2.10). All plants tested were positive for the *nptII*, *bar* and *mob*CD genes (Fig. 2.11 and 2.12). Three plants, lines 31, 35 and 50 tested positive for all the six fragments tested (Table 2.4).

Table 2.4 The presence (+) or absence (-) of specific regions of pDER-*bar* in transgenic plants as determined by PCR.

	MobCD	Kan	Bar	RepC	RepA	MobA
31	+	+	+	+	+	+
35	+	+	+	+	+	+
50	+	+	+	+	+	+
68	+	+	+	+	+	-
41	+	+	+	+	-	-
52	+	+	+	-	-	-
58	+	+	+	-	-	+
43	+	+	+	-	+	-

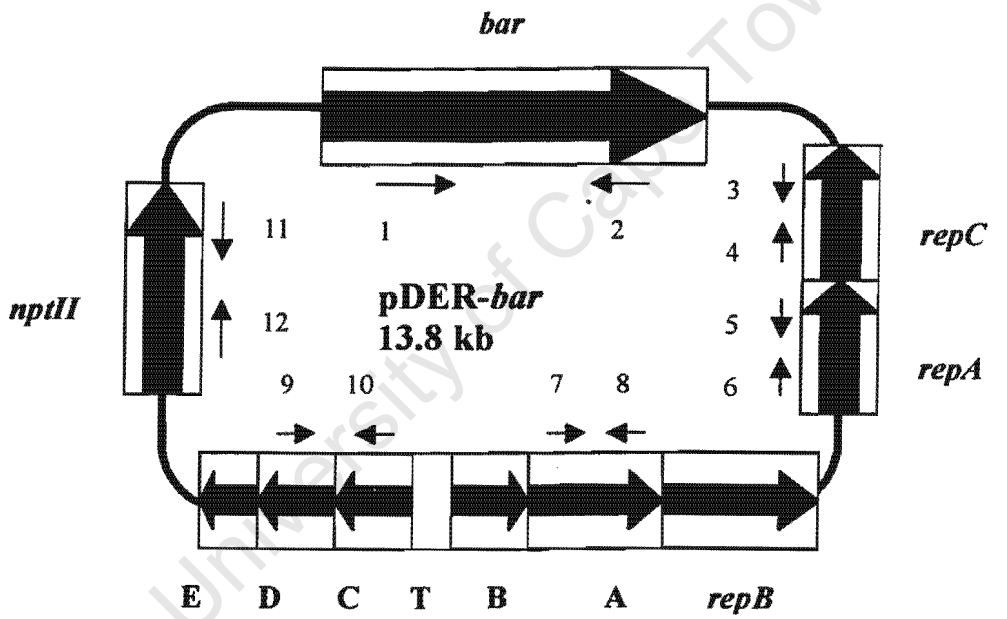


Fig. 2.10 A schematic diagram of plasmid pDER-*bar* showing the primers that were designed to amplify various regions of the plasmid in order to determine their presence in transgenic plants. The five mobilization region genes (represented by the letters A-E in the diagram above) are divergently expressed from the origin of transfer region (T). The replication region is denoted by its various components *rep*A, *rep*B and *rep*C. Primer pairs correspond to the primers that are given in Table 2.1. For example primer 1 and 2 correspond to BarF and BarR; 3 and 4 to RepCF and RepCR; 9 and 10 to MobCDF and MobCDR, etc.

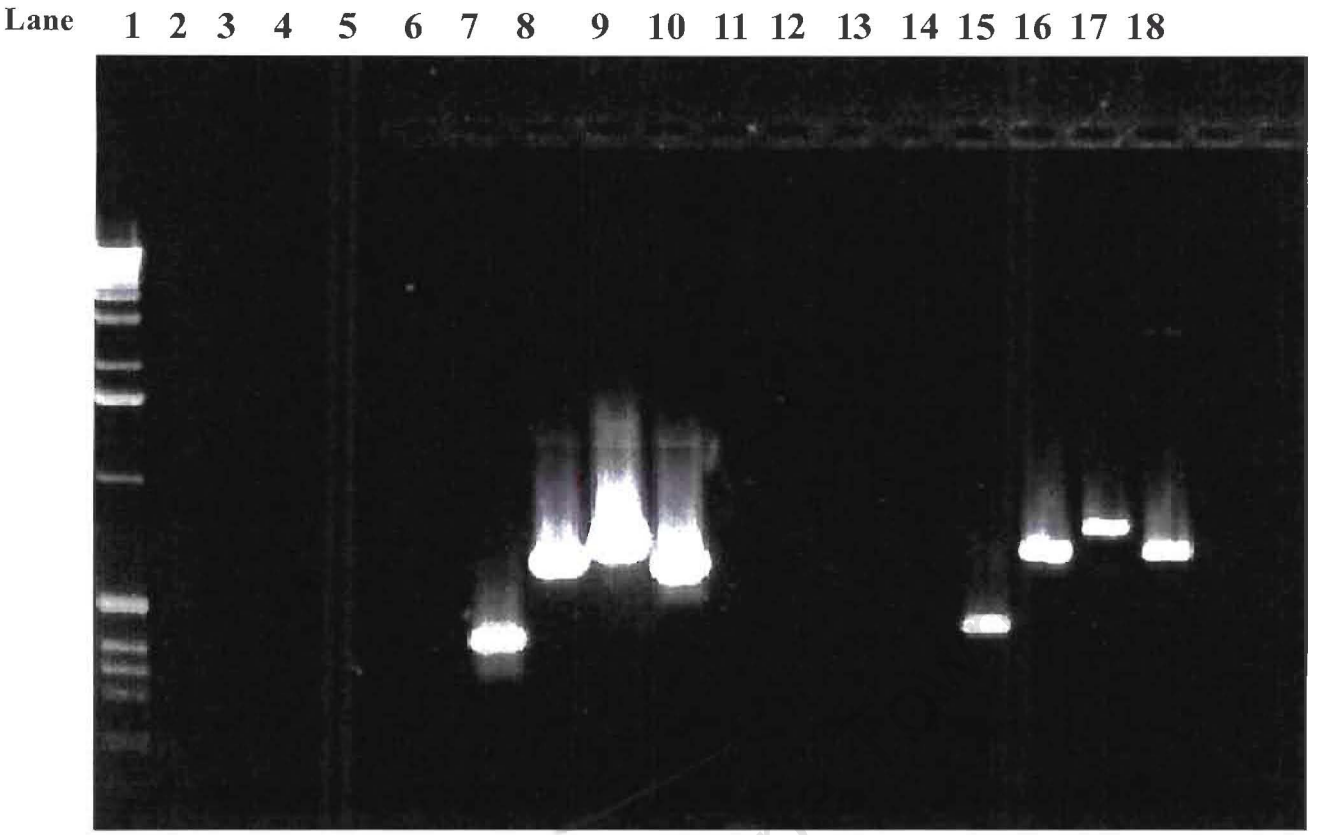


Fig. 2.11 PCR analysis to check for the presence of various fragments of pDER-*bar* that were integrated into the transgenic line 31. The primers used in this analysis were respectively BarF/BarR, KanF/KanR, MobAF/MobAR and MobCDF/MobCDR. Water and the non-transgenic (NT) plant genomic DNA were used as the negative controls whilst the pDER-*bar* plasmid DNA was used as the positive control. Lane 1 shows the molecular weight marker and lane 2 is blank.

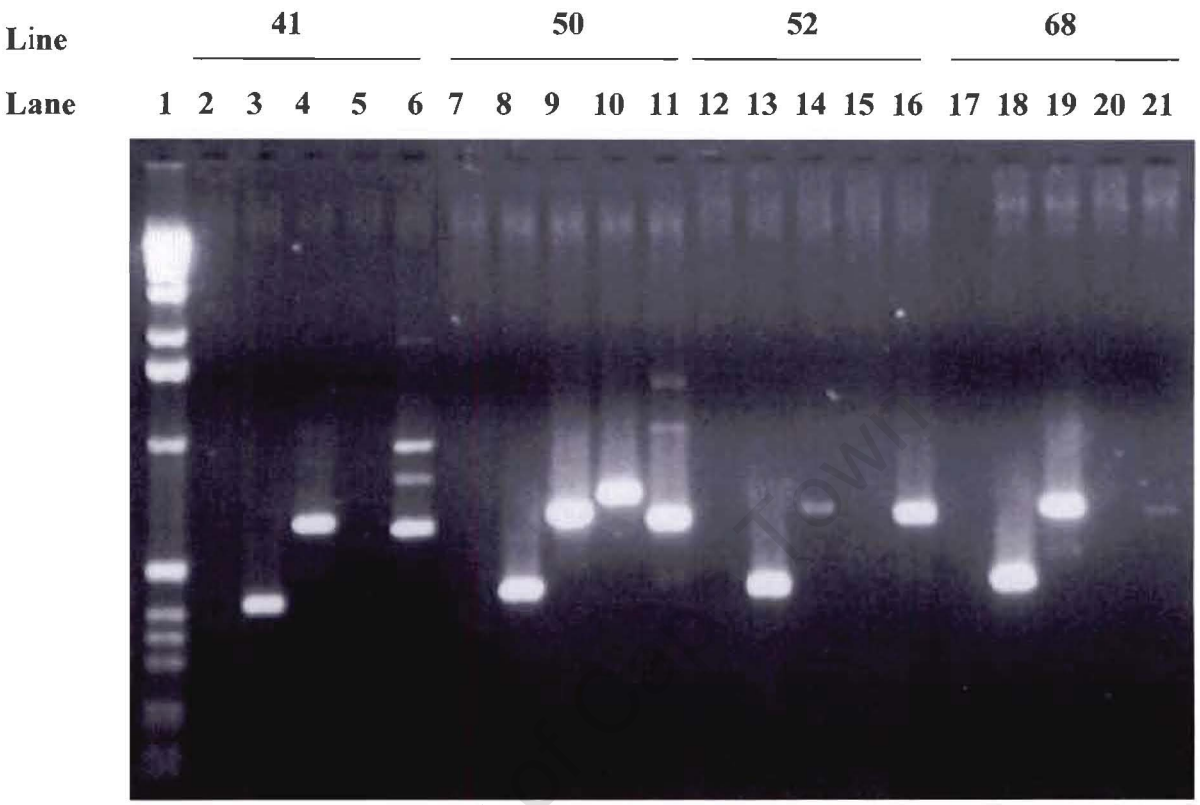


Fig. 2.12 Representative PCR analysis to determine the presence of various fragments of pDER-*bar* that were integrated in the genome of lines 41, 50, 52, and 68. The primers used were respectively BarF/BarR, KanF/KanR, MobAF/MobAR, MobCDF/MobCDR. Lanes 2, 7, 12 and 17 contain water which was used as a negative control.

2.4 DISCUSSION

This work demonstrates that the *At. ferrooxidans* plasmid pTF-FC2 can be used efficiently as a binary vector in the transformation of tobacco leaf discs using *Agrobacterium*. The transformed progeny showed no abnormalities and were as fertile as the wild type. The transformation proficiency was no different from other routinely used binary vectors notably pBI121 and pPZP100 (data not shown). In addition, the results presented here have shown that the plasmid pTF-FC2 can function equally well in both the octopine (LBA4404) and nopaline (C58C1) strains of *Agrobacterium*.

The observation that all the plants growing on bialaphos tested positive for the *bar* gene when analyzed by PCR confirms that they were indeed transgenic. The unique pattern of Southern hybridization signals confirmed that the transgenic plants analyzed were the result of independent integration events. The presence of these signals coincided with the presence of the *bar* gene and no signals were detected in the *bar*-negative progeny. The discovery that all plants that tested positive for the *bar* gene were also positive for the *nptII* gene indicates a strong linkage between the two genes. In addition, studies on the extent of plasmid integration showed that three out of the eight plants tested contained all the main regions of the plasmid pDER-*bar* indicating that the entire plasmid had integrated into the genomic DNA of these plants. The finding that the regions of the plasmid that were found in all the transgenic plants are adjacent to each other strongly indicates that they were all transferred in one block. Such a scenario gives an indication of the direction of transfer. It appears that the region *mobCD* through to *bar* is transferred first, thus leading the way into the plant cell, with *mobA* coming in last. If

this be the case, then, based on the organization of the genes on plasmid pDER-*bar* it would not be expected for *repA* to be present in the absence of *repC* or for *mobA* to be present in the absence of *repC* and *repA* as is the case with lines 43 and 58 respectively (Table 2.4). These unexpected integration patterns could be explained as internal deletions, a common phenomenon in plant transformation. It is also plausible that the position of the *bar* gene (the selectable marker) favours the observed high fidelity of integration of genes that lie upstream of it.

Not withstanding the fact that transgenic plants generated unique multiple hybridization bands on Southern blots and varied with respect to their copy numbers, segregation analysis in the T_1 generation generally showed transgene inheritance as a single Mendelian trait (3:1 segregation ratio). This shows that the linked genes had integrated into one locus, which means that they function as, and are inherited as, a single genetic unit. Judging by the amount of the plasmid that integrated, this plasmid offers great potential for producing transgenic plants with multiple genes. The generation of transgenic plants with multiple genes has been shown using the particle gun (Hadi *et al.* 1996; Chen *et al.* 1998 and Tang *et al.* 1999). Hadi and colleagues succesfully transformed soybean with 12 different plasmids and also found that the majority of soybean clones had integrated all 12 plasmids. Chen and colleagues showed that up to 13 of the 14 plasmids that they had used in their transformation experiments could be simultaneously introduced into the rice genome. Tang and colleagues successfully transformed rice using four genes that were resident in three co-transforming plasmids.

70% of the transgenic plants co-expressed all four genes. Two of these genes were of agronomic interest that conferred resistance to two separate taxa of pathogens.

The ability of the entire pDER-*bar* plasmid to integrate into the plant genome (as in lines 31, 35 and 50) makes it an attractive candidate for the delivery of multiple genes into transgenic plants. Several studies have shown that co-integration frequency of linked multiple genes is an efficient strategy (Komari *et al.* 1996; Tang *et al.* 1996). However delivery of multiple genes using a single plasmid is likely to become more difficult, both with respect to cloning and the delivery procedure itself, as the number of genes increases (Komari *et al.* 1996; Tang *et al.* 1999).

A feature of this plasmid is its lack of the usual 25-bp border sequences that delimit T-DNA. Without these sequences the transferred DNA cannot easily be defined. However the integration pattern suggests that this plasmid may operate in a similar fashion to RSF1010, the only plasmid that has been shown to transfer DNA to plants without the use of the right and left borders (Buchanan-Wollaston *et al.* 1987). In their experiment Buchanan-Wollaston and colleagues generated several RSF1010-derived binary vectors. These plasmids all contained the mobilization functions and the replication region of RSF1010. They, including pJP181, the plasmid carrying no T-DNA border sequences, were shown to transfer their DNA into plant cells. All plasmids, including those that carried the T-DNA border sequences were found to transform plants at approximately the same frequency. It was also shown that the RSF1010 *oriT* and some of its cognate Mob

proteins were responsible for the delivery of the plasmid carrying these mobilization functions to plant cells.

The results that were obtained with pJP181 are similar to the ones obtained in this study. However, in contrast with the findings of this work the RSF1010 plasmids that contained T-DNA border sequences were able to transfer DNA to plants. No transformants could be generated using plasmid pTD1 in this work. A major difference between the two plasmids is that the *mob* genes of pTF-FC2 belong to the IncP group whereas those of RSF1010 belong to the IncQ group. This would imply that each plasmid has a different mechanism of DNA transfer. In addition, pTF-FC2 seems to be a natural hybrid with a replication region belonging to the IncQ group (Rohrer and Rawlings, 1992), whereas RSF1010 belongs to the IncQ group with respect to both its mobilization and replication functions. Further studies will need to be carried out in order to establish the role that is played by the T-DNA border sequences in inhibiting DNA transfer to plants in plasmid pTD1. In order to carry out such investigations the mechanism of transfer that is in operation in the transfer of DNA to plants by the plasmid pDER-*bar* will have to be determined.

Three plants (lines 41, 43 and 52) had severe truncations at the distal end of the transferred DNA. All truncations were downstream of the *bar* gene, which had been used as the plant selectable marker. These results are consistent with the hypothesis that the *mob*C and *mob*D genes are transferred first. Based on this direction of transfer it is not surprising that the regions of the pDER-*bar* plasmid that are downstream of the *bar* gene are deleted and/or truncated. Grevelding *et al.* (1993) obtained similar results.

Using binary and cointegrate vectors that had selectable markers located proximal to the right border they showed that 63% of their leaf disc-derived transgenic *Arabidopsis* plants had lost at least 3 kb of the left border regions compared to 15% that had lost at least 1.1 kb of the right border region. T-DNA transfer is thought to occur in a polar fashion beginning at the right border (Zambryski *et al.* 1992).

It has been shown that the integration of complete transferred DNA is dependant on the activity of VirE2 protein (Rossi *et al.* 1996). However it is not clear whether the truncations observed in this work were a result of the lack of, or the inadequacy, of VirE2. The fact that the entire pDER-*bar* plasmid integrated in three of the plant lines (31, 35 and 50) suggests that the VirE2 protein was sufficient in these plants. In addition Rossi and colleagues did not observe a plant that showed the integration of a complete T-DNA unit when they used a VirE2 mutant strain of *Agrobacterium*. Therefore in these plants it could be said that the deletions that were observed were due to the unavailability of VirE2. Based on these findings it is plausible to suggest that the role played by VirE2 in the deletions and/or truncations observed in this work is negligible. Truncations beyond the *bar* gene could not have been detected because this would have prevented the selection of an intact *bar* gene.

The transgenic line 35 showed a 15:1 segregation ratio indicating that two independent integration events had taken place. This is one of the three plants that showed the integration of the whole plasmid. It is possible that in this plant the two integration events may not be the result of the integration of whole plasmids but rather that of two plasmid fragments that complement each other in areas that have been deleted or

truncated. The PCR analysis that was used in this study could not detect such an eventuality. Many scientists have reported multiple and aberrant T-DNA insertions in tobacco (Spielmann and Simpson, 1986), tomato (McCormick *et al.* 1986; Jorgensen *et al.* 1987; Sukhapinda *et al.* 1987), petunia (Delores and Ganer, 1988; Jones *et al.* 1988) and *Arabidopsis* (Grevelding, 1987).

In conclusion, the results presented in this chapter show that a pTF-FC2-derived plasmid can be used as a binary vector. The proficiencies of transformation obtained in this study are comparable to the best that can be achieved currently using commercially available binary vectors such as pBI121. The fact that the entire plasmid can be integrated into the plant genome raises the possibility that this plasmid may be used as a pTF-FC2-based "super" co-integrate vector capable of introducing multiple genes into one locus in the plant genome. It is interesting to note that this vector does not require the right and left border sequences in order to perform its functions. Even more interesting is the fact that no transformants could be generated by plasmid pTD1 that contained the border sequences. This may mean that the border sequences are not only unnecessary but that they also inhibit or hinder the DNA transfer process to plants. Plasmid pTF-FC2 has been shown to be highly promiscuous (Rawlings *et al.* 1986). It is interesting to note that this promiscuity can be extended to plants as well. Derivatives of the pTF-FC2 plasmid will certainly offer a unique range of plant vectors. In order to use them in the best possible way there is a need to understand the mechanism that the plasmid uses in integrating into the plant genome. This is the subject of the investigations in Chapter 3.

CHAPTER 3

MECHANISM OF DNA TRANSFER TO TOBACCO MEDIATED BY pTF-FC2

ABSTRACT

The similarity between *Agrobacterium*-mediated DNA transfer in plants and conjugative DNA transfer between bacteria is well documented. Further evidence was provided by the fact that *A. tumefaciens* is able to transfer to plants plasmid RSF1010, despite the fact that this plasmid lacks the border sequences upon which the VirD2 protein acts. Transfer of RSF1010 into plant cells was shown to be dependent on the *mob* and *oriT* regions and on several of the *vir* genes of the Ti plasmid, suggesting that in Agrobacterium cells, MobA and *oriT* of RSF1010 are the functional equivalent of VirD2 and the border sequences of the Ti plasmid. In the previous chapter I showed that *Agrobacterium* is able to transfer to tobacco cells the plasmid pTF-FC2, which like RSF1010, lacks the border sequences upon which the VirD2 protein acts. The aim of this study was to elucidate the mechanism of action. In it I show that although VirD2 is able to cleave, *in vitro*, the *oriT* region of pTF-FC2 with an efficiency of 40%, it can barely do so *in vivo*. Two plasmids were constructed, pDER-*bar*-GUS, which has intact and functional *mob* genes, and pDER-*bar*-GUS-ΔMobA, with a specific deletion in *mobA* gene which renders it non-functional. These plamids were electroporated into *Agrobacterium* strains GV3101 (pPM6000), which has an intact and functional *virD2* gene and GV3101 (pPM6000K), which has a mutated and non-functional *virD2* gene, resulting in strains GV3101 (pPM6000)(pDER-*bar*-GUS), GV3101 (pPM6000)(pDER-*bar*-GUS-ΔMobA), GV3101

(pMP6000K)(pDER-*bar*-GUS) and GV3101 (pPM6000K)(pDER-*bar*-GUS-ΔMobA). Co-cultivation experiments showed that the MobA+/VirD2+ and the MobA+/VirD2- strains were able to transfer their plasmid DNA to plants with an equal frequency. This was measured using the transient expression GUS assay. Both strains produced calli with the same efficiency when they were co-cultivated with young tobacco seedlings. Stable integration analysis showed that there was no difference in the ability to transfer and to integrate DNA between these strains. The MobA-/VirD2- strain could not transfer plasmid DNA to plants or produce resistant callus as expected. The MobA-/VirD2+ strain showed a transfer efficiency of 0.03% which is a negligible fraction, compared with the other two strains. These results show that the pTF-FC2 MobA is able to mediate the transfer and the integration of DNA into plants.

3.1 INTRODUCTION

A. tumefaciens transforms plant cells by transporting DNA, mobilized from a tumor inducing (Ti) plasmid located in the virulent bacterium, into the plant cell nucleus. Expression of this transferred DNA (T-DNA) leads to the formation of crown gall tumors in many dicotyledonous plants. The Ti plasmid also contains the virulence region, which provides several gene products that mediate transformation. The initiator of DNA processing, VirD2, in conjunction with VirD1, cleaves the bottom strand at the 25-bp border repeats and remains covalently attached to the 5' end of the released strand. This DNA-protein complex is then transferred to the plant cell accompanied by the single stranded DNA binding protein, VirE2. VirE2 has been shown to protect the T-DNA from nucleolytic attack and also to cause the formation of pores or channels in plant

membranes through which the T-DNA may pass. These processes have been extensively reviewed in Chapter 1.

It has long been known that processing and transfer of the T-DNA region to plant cells shares structural and functional characteristics with events occurring during plasmid conjugation (Stachel and Zambryski, 1986). For example, the highly conserved T-DNA border sequences are the functional equivalents of the *oriT*, acting as recognition and cleavage sites for the site-specific nicking complexes (Yanofsky *et al.* 1986; Wang *et al.* 1987; Pansegrau *et al.* 1990). In addition the cleaving molecule remains covalently attached to the 5' end (Stachel *et al.* 1987; Bhattacharjee and Meyer, 1991). Furthermore, both transfer processes involve single stranded DNA transfer intermediates (Cohen *et al.* 1986; Albright *et al.* 1987) and both require cell to cell contact (Ream, 1989). Derivatives of the mobilizable IncQ plasmid RSF1010 that carry cognate *mob* genes can be mobilized to plant cells by the Ti plasmid-encoded Vir system (Buchanan-Wollaston *et al.* 1987).

To add to this growing list of similarities I have shown, in the previous chapter, that a derivative of the plasmid pTF-FC2 is able to transfer DNA to plants without the use of the right and left borders. These results require the elucidation of the mechanism of action at the molecular level. My strategy will initially assume that the mechanism of DNA transfer to plants is similar if not identical to that used by plasmid RSF1010. The difficulty here could be the nature of differences that exist between these two plasmids. Whilst RSF1010 is an IncQ plasmid, pTF-FC2 is a hybrid with an IncQ-like replication

region and an IncP-like mobilization region (Rawlings and Tietze, 2001). This means that these plasmids share similarities in areas that do not affect plant transformation *ie.* the replication region and differ quite extensively in the area that does *ie.* the mobilization region. However both plasmids have a similar gene organization. In RSF1010 three proteins are required for its conjugal mobilization: MobA, which cleaves and ligates the transferred strand (Bhattacharjee and Meyer, 1991) and two accessory proteins MobB and MobC. MobC assists in localized separation of the DNA strands at *oriT* (Zhang and Meyer, 1997); MobB stabilizes the complex of Mob proteins at *oriT* and also has an additional function in the transfer (Perwez and Meyer, 1999). In addition, RSF1010 encodes three replication proteins (Scherzinger *et al.* 1984), a helicase and an iteron binding protein, the products of the *repA* and *repC* genes, respectively, and a primase. The primase is encoded in the *repB* and is translated both as the C-terminal domain of MobA and separately (Scholz *et al.* 1989). Both forms of the primase are active and sufficient for plasmid replication both *in vitro* (Scherzinger *et al.* 1991) and *in vivo*. However it has also been shown that the MobA-linked primase is the only replication protein of RSF1010 that is required for conjugal mobilization (Henderson and Meyer, 1999).

The gene arrangement in pTF-FC2 is essentially identical to the one that I have just described for RSF1010 (Dorrington *et al.* 1991; Rohrer and Rawlings, 1992). Although the mobilization regions are different at the sequence level the similarities at the functional level are remarkable. The only notable difference is that the three critical proteins for mobilization in pTF-FC2 are MobA, MobC and MobD. MobB and MobE

were found to affect the frequency of mobilization (Rohrer and Rawlings, 1992). It is also interesting to note that the region where pTF-FC2 changes from IncP-like to IncQ-like lies within the large continuous MobA-RepB reading frame (Rohrer and Rawlings, 1992).

These similarities in function suggested that it was still worthwhile to continue with the assumption that had been made. The first step was to determine which protein was involved in the cleavage of the DNA that was transferred to plants. MobA or VirD2 were picked as the likely candidates. The second step was to design experiments that would identify the actual protein involved. Due to the large number of plants that had to be analyzed a GUS transient assay method (Rossi *et al.* 1993) would be used because it was quick and efficient. This method would make it possible to determine the efficiency of transfer mediated by each of the two test cleavage proteins. In order to be able to do GUS assays the *uidA* gene had to be incorporated into the pDER-*bar* construct. A *mobA* deletion was generated from the resultant construct in order to be able to determine its role in the cleavage of DNA. It was also necessary to show *in vitro* whether or not VirD2 could cleave the *oriT* region of pTF-FC2.

3.2 MATERIALS AND METHODS

3.2.1 Molecular methods

Unless otherwise stated all DNA manipulations were performed according to Sambrook *et al.* (1989).

3.2.2 Sequencing reactions

Sequencing was done using the ALFexpress DNA automated sequencer (Amersham Pharmacia Biotech AB, Upsalla, Sweden. The thermo sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP was used according to the manufacturer's instructions (Amersham Pharmacia Biotech).

3.2.3 *In vitro* cleavage by VirD2

The VirD2 protein that was used in this experiment was a kind donation from Barbara Hohn. It had previously been over-expressed in *E. coli* and purified by FPLC. The oligodeoxyribonucleotides GGTATATATCCTG^CCAGTCTTGATGCCGCGCGCAT, derived from the right border of the octopine-type plasmid pTiA6, and AACGGTCATCCTG^TATTGCTCAACCGCTCTACTATCATATC, derived from the *oriT* of the plasmid pTF-FC2, were used to test the *in vitro* activity of VirD2. 5'-end-labelling was performed using (γ^{-32}P) ATP (Amersham, Little Chalfont, UK) and T4 polynucleotide kinase (Boeringer Mannheim, Mannheim, Germany). *In vitro* cleavage was performed as described earlier (Pansengrau *et al.* 1993). However varying concentrations of VirD2 were used, ranging from 5 to 20 μg. Oligonucleotides were incubated at 37°C with VirD2 for 3 hours in a 20 μl reaction buffer (20 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 50 mM NaCl). The reaction products were separated on a 20% acrylamide gel containing 8 M urea.

3.2.4 Quantification of radioactive signal

Autoradiography was quantified using a PhosphoImager device and imageQuant software version 3.3 (Molecular dynamics, Sunnyvale, CA).

3.2.5 Construction of pDER-*bar*-GUS

The construction of plasmid pDER-*bar*-GUS was done by the introduction of the *EcoRI* fragment of pGUS23 (Appendix A) that contained the GUS gene (*uidA*) driven by the 35S cauliflower mosaic virus promoter (35S) and which also contained a nopaline synthase (Nos 3') transcription terminator into the *EcoR1* site of plasmid pDER-*bar* (Fig. 3.1).

3.2.6 Construction of pDER-ΔMobA-*bar* -GUS

In order to create an internal deletion within the *mobA* gene two divergent primers, MobΔAF and MobΔAR were used to amplify the rest of the plasmid pDER-*bar*-GUS. This was achieved by making use of the Expand Long Template PCR kit according to the manufacturer's instructions (Roche, Mannheim, Germany). Ten ng of plasmid DNA were used as template. The cycle conditions were as follows: initial denaturation at 94°C for 3 minutes, followed by 20 cycles at 94°C for 20 seconds, 64°C for 30 seconds and 68°C for 15 minutes with an additional 20 seconds added after each cycle. The final cycle at 68°C had an elongation time of 7 minutes. The PCR product that was generated was then cloned into the pGEM®-T Easy vector in accordance to the manufacturer's method (Promega, USA). The resultant plasmid, pGEMΔMobA (Fig. 3.2), was then sequenced to check for the precision of the deletion. After sequencing, the fragment

containing the pGEM®-T Easy vector was excised and discarded by using the *Not*I restriction enzyme. The remaining fragment consisting of the pDER-*bar*-GUS plasmid that contained the MobA deletion was purified from the agarose gel using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). This fragment was self-ligated, resulting in plasmid pDER-*bar*-GUS-ΔMobA (Fig. 3.2), in which 1084 bp of the 1228-bp-long open reading frame of *mobA* were deleted.

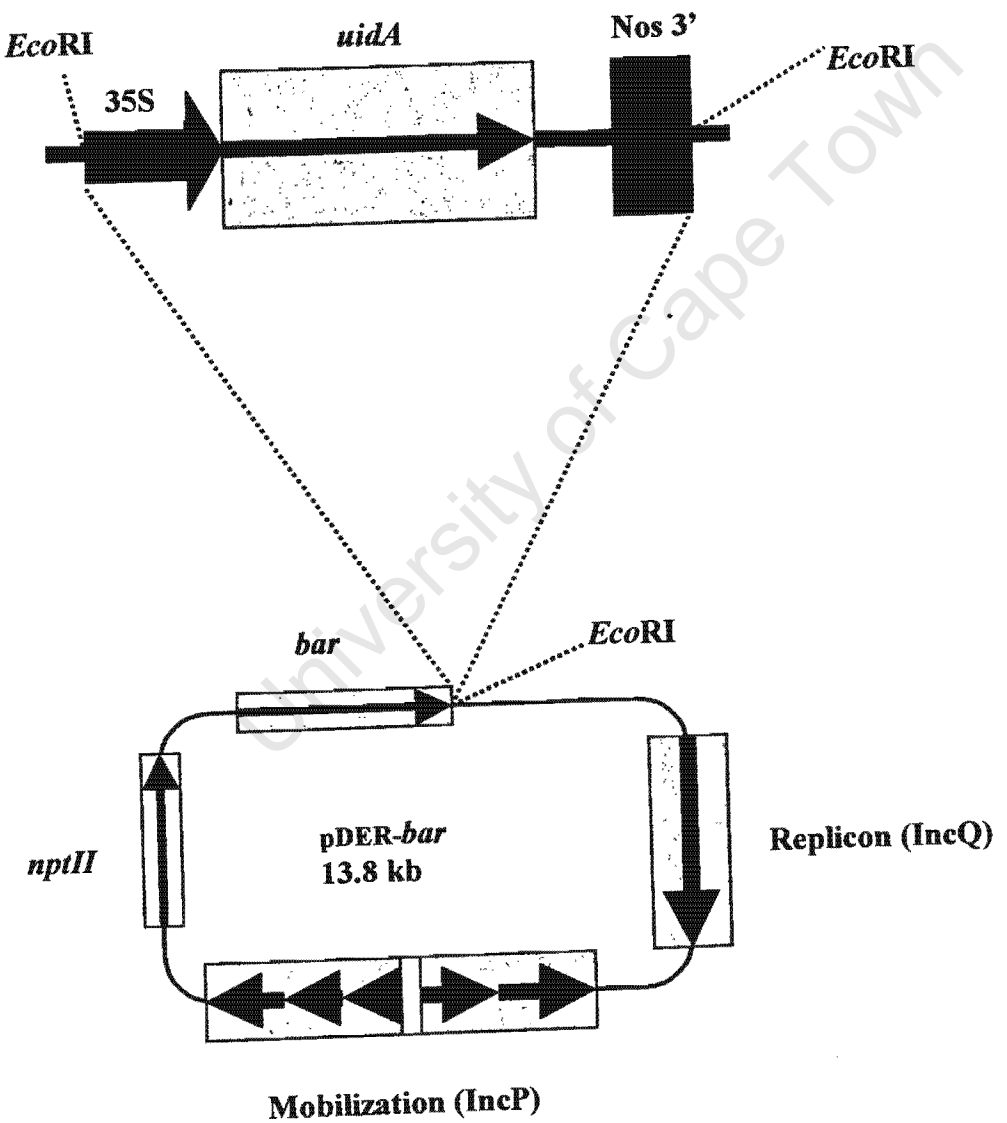


Fig. 3.1 Construction of the plasmid pDER-*bar*-GUS. Construction of the plasmid pDER-*bar* is described in Chapter 2. The GUS expression cassette was obtained as an *Eco*RI fragment from plasmid pGUS23. See text for details.

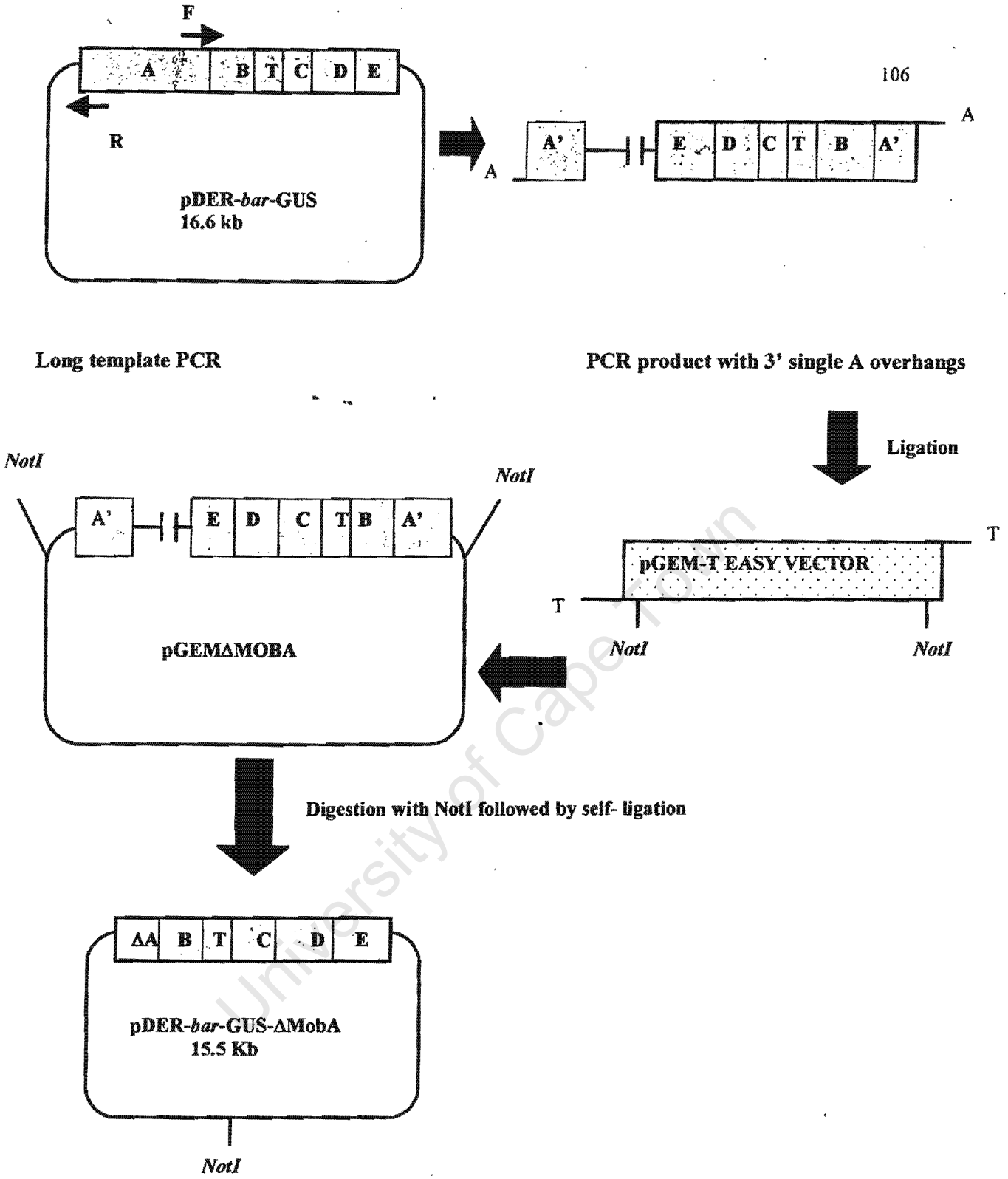


Fig. 3.2 Strategy used to prepare the plasmid pDER-*bar*-GUS-ΔMobA. The deletion was verified by sequencing and restriction enzyme analysis. The shaded areas represent the mobilization region. Different genes of this region are marked A to E. T represents the origin of transfer. A' represents the remnants of *mob*A after PCR. ΔA represents the truncated *mob*A gene. The primers used are shown by the arrows marked F(ΔMobAF) and R(ΔMobAR). The drawings are not to scale. More details about the construction are available in the Materials and Methods section.

3.2.7 *A. tumefaciens* strains used

In order to study the roles played by *mobA* and *vir*D2 an *A. tumefaciens* strain devoid of *virD2*, GV3101 (pPM6000K), and GV3101 (pPM6000), which had a functional and intact *virD2*, were used. Strain GV3101 (pPM6000), a cured C58 nopaline strain containing the pTiAch5 derivative pPM6000, which is deleted in the T-DNA (Bonnard *et al.* 1989) was modified in the *vir*D2 gene of the Ti plasmid, yielding strain GV3101 (pPM6000K). The latter strain lacks 70% of the coding sequence of the VirD2 protein, which is essential for T-DNA transfer. This strain was shown to be transfer defective for T-DNA transfer (Rossi *et al.* 1993). A summary of the strains used is given in Table 3.1.

Table 3.1 Agrobacterium strains used in this study

STRAINS	RELEVANT PHENOTYPE[a]	
	VirD2	MobA
GV3101(pPM6000)(pDER-*bar*-GUS)	+	+
GV3101(pPM6000K)(pDER-*bar*-GUS)	-	+
GV3101(pPM6000)(pDER-*bar*-GUS-ΔMobA)	+	-
GV3101(pPM6000K)(pDER-*bar*-GUS-ΔMobA)	-	-

[a] Additional information about these strains and plasmids is provided under materials and methods.

3.2.8 Transformation of *A. tumefaciens*

The introduction of the test plasmids pDER-*bar*-GUS and pDER-*bar*-GUS-ΔMobA into each of strains GV3101 (pPM6000) and GV3101 (pPM6000K) was done by electroporation using the method described in Chapter 2.

3.2.9 Co-cultivation with tobacco cotyledons

The co-cultivation experiment was performed essentially as described by Rossi *et al.* 1993. About 400 *Nicotiana tabacum* SR1 seedlings (two weeks old) were incubated for three days with the following *A. tumefaciens* strains: GV3101 (pPM6000)(pDER-*bar*-GUS), GV3101 (pPM6000)(pDER-*bar*-GUS-ΔMobA), GV3101 (pPM6000K)(pDER-*bar*-GUS) and GV3101 (pPM6000K)(pDER-*bar*-GUS-ΔMobA). The non-transformed strain GV3101 (pPM6000K) was used as a negative control. A 5 ml overnight culture of *A. tumefaciens* grown in YEB medium containing the antibiotics rifampicin (100 mg/ml) and kanamycin (100 mg/ml) was washed twice with an equal amount of 10 mM $MgSO_4$ and resuspended in 10 ml GM medium to a final optical density of 0.6 at 600 nm. Different dilutions, 1:10 to 1:1000, of all the strains with the test plasmids were made. About 200 seedlings were added to the bacterial suspension. The mixture was exposed to a reduced pressure (0.15 atm) in a sterile vacuum chamber for 5 minutes. The seedlings were later placed on GM plates (1% agar) and further co-cultivated for three days in a growth chamber at 25 °C with 16 hours light/day. The plantlets were washed in sterile 10 mM $MgSO_4$ and blotted dry in a sterile vacuum chamber for 5 minutes. Transient expression of *uidA* was determined by measurement of β-glucoronidase activity via a histochemical assay using 5-bromo-4-chloro-3-indolyl-glucoronide (X-glu) as described

(Rossi *et al.* 1993). After co-cultivation, plants were added to 5 ml of GUS staining solution. A soft vacuum of about 0.15 atm was applied for 5 minutes in order to standardize the distribution of the substrate into the cells of different tissues in the plantlets. The reaction was allowed to proceed for 24 hours at 37°C. The plantlets were washed in water and bleached with ethanol. The blue sectors present on the plantlets were counted under microscope. The remaining 200 seedlings were analyzed for T-DNA integration. The seedlings were washed with 10 mM $MgSO_4$ and placed on MS medium containing 0.1 mg/ml naphtylacetic acid, 1 mg/ml benzylamino purine, 100 mg/ml kanamycin, 500 mg/ml vancomycin and 500 mg/ml claforan. The seedlings were transferred to fresh plates every week. Calli were counted after 6 to 8 weeks.

3.3 RESULTS

The *A. tumefaciens* strain used to transform tobacco in Chapter 2 synthesized two types of proteins which have been shown to be involved in the excision of the DNA that is to be transferred to plants: VirD2 and MobA. It was not clear at this stage whether the protein that was responsible for the recognition and nicking of the *oriT* region of pTF-FC2 plasmid and its subsequent transfer to plants was VirD2 or MobA or both. In order to determine the contribution of each of these two proteins I had to show that they can recognize and cleave the *oriT* sequence and also that they can separately or in combination transfer plasmid DNA into plant cells.

3.3.1 Construction of pDER-*bar*-GUS-ΔMobA

The best way to study the role and function played by any known gene is to generate a mutation in it. In order to study the role played by the MobA protein plasmid pDER-*bar*-GUS-ΔMobA that contained an internal deletion of MobA had to be constructed. Through the use of Expand Long template PCR an internal deletion of 1084 bp (Figs. 3.2 and 3.3) was created. Considering that the pTF-FC2 *mobA* gene is 1228 bp the internal deletion was extensive and rendered the gene non-functional. Rawlings and Woods (1985) identified the mobilization region of pTF-FC2 and cloned it into a non-mobilizable Bluescript SK vector. The resultant plasmid, pTF100, was mobilizable at high frequency by plasmid RP4 or *E. coli* S17-1. Rohrer and Rawlings (1992) tested ordered sequential deletions from both ends of pTFmob100 for their ability to be mobilized by the chromosomally located IncP plasmid of *E. coli* S17-1. The results showed that deleting 319 bp from the 5' end of the *mobA* gene reduced the mobilization frequency by 9000-fold. With the removal of a further 391 bp mobilization was no longer detectable. Thus, removing a total of 710 bp renders *mobA* completely inactive. According to this reasoning an internal deletion of 1084 bp should result in inactivation of the *mobA* gene product.

The presence of the specific *mobA* deletion was confirmed by sequencing (results not shown) and restriction enzyme analysis (Fig. 3.3).

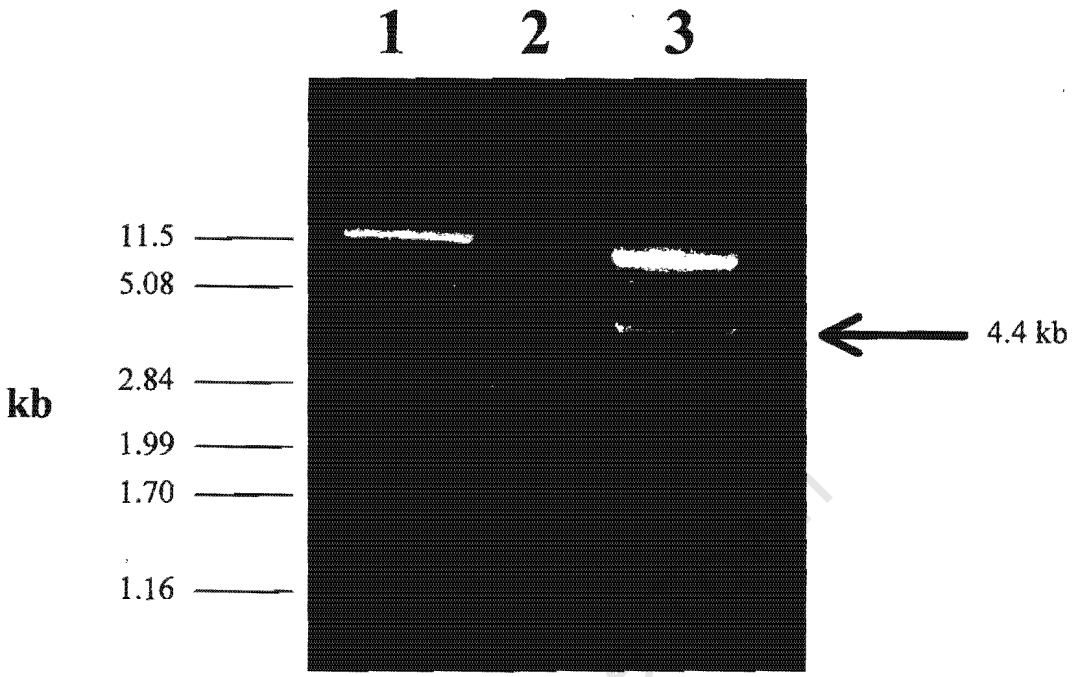


Fig. 3.3 Restriction analysis to confirm the presence of the MobA deletion in pDER-*bar*-GUS-ΔMobA. Lanes 1, λ/*Pst*I molecular weight markers; 2, *Cla*I restriction digest of pDER-*bar*-GUS, yielding a 5.484-kb fragment that contains the entire mobilization region; 3, *Cla*I restriction digest of pDER-*bar*-GUS-ΔMobA, producing a band of 4.4 kb which shows a deletion of 1084 bp in the *mob* region.

3.3.2 Sequence alignment

The use of DNA and protein sequences for the generation of phylogenetic trees (or dendograms) is an established technique. Since the aim of this study was to determine the mechanism of action of DNA transfer to plants by pTF-FC2 it was necessary to establish the evolutionary relationship of the cleavage proteins from various bacteria. Of particular interest was the phylogeny of those cleavage proteins that are known to mediate the transfer of DNA to plants, notably VirD2 and RSF1010 MobA. These would be compared with pTF-FC2 MobA.

A wide range of cleavage proteins (relaxases) were analyzed using DNAMAN (version 4.0) and it was clear that while all the proteins shared the same root, VirD2 had evolved separately from the rest (Fig. 3.4). The clustering of pTF-FC2 MobA with R751 TraI and RP4 TraI shows that they are similar at the amino acid level. The RSF1010 MobA is in a cluster of its own although the degree of relatedness is higher between it and pTF-FC2 than it is with VirD2.

Sequence alignment showed a 19% identity between VirD2 and pTF-FC2 MobA (Fig. 3.5). The nuclear localization signal and motifs I, II and III were either not, or were poorly, conserved in pTF-FC2 MobA. It was however of interest to note that the ω sequence was well conserved in the two proteins.

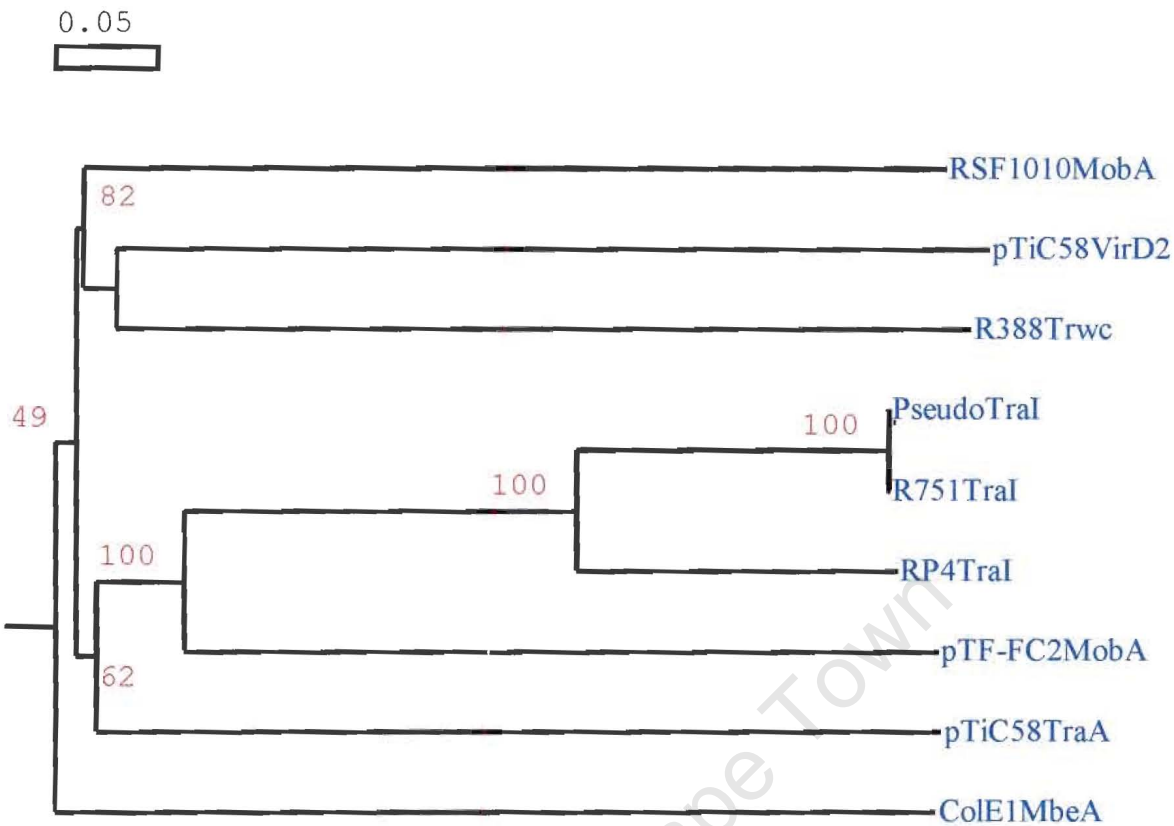


Fig. 3.4 A rooted neighbour joining tree illustrating the amino acid relationships of the relaxase family of proteins. This tree was generated from protein sequence data and the output sequence file was subjected to phylogenetic analysis using DNAMAN 4.0 with ColE1MbeA as an outgroup. All vertical lines are arbitrary, while horizontal lines are proportional to sequence divergence.

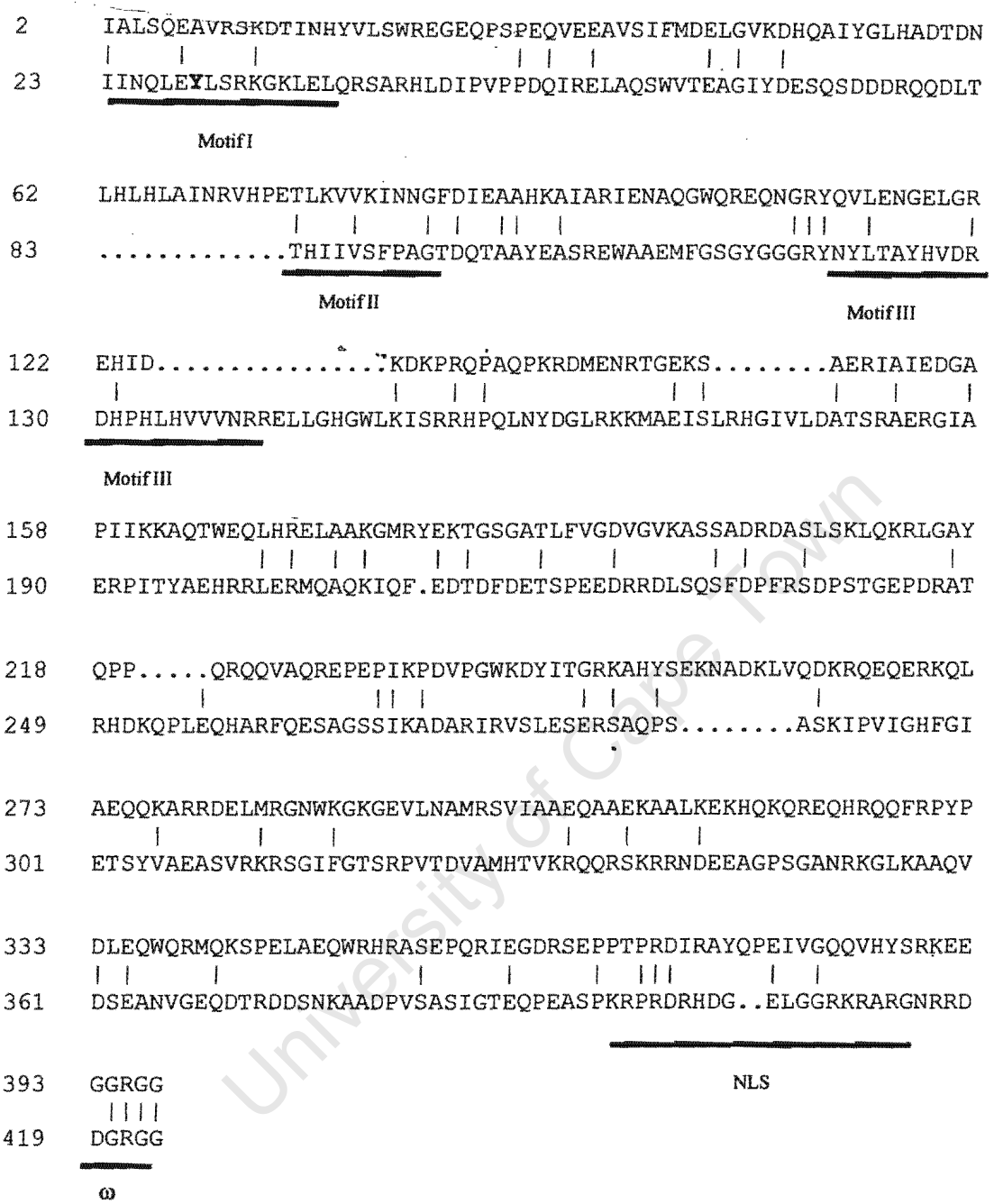


Fig. 3.5 Comparison of the amino acid sequences of the MobA (top line) and VirD2 (bottom line) proteins encoded by plasmids pTiA6 and pTF-FC2, respectively. This alignment reveals a 19% identity between the two proteins. Motifs I, II and III correspond to the conserved domains characterizing the members of the relaxase family to which VirD2 belongs. Y29 (boldface) of motif I of VirD2 was shown to be involved in the phosphotyrosine bond establishing the liason with the T-strand (Pansegrau *et al.* 1994). NLS represents the nuclear localization signal, while ω represents the omega sequence.

3.3.3 *In vitro* cleavage by VirD2

Purified VirD2 was used to investigate its ability to cleave a synthetic 41-mer oligonucleotide that contained the nick region of the pTF-FC2 or the pTiA6 right border sequences from the octopine strain of *A. tumefaciens*. I also used various concentrations (5mg, 10mg and 20mg) to show the effect of increasing the protein concentration on the efficiency of cutting. Cleavage products obtained following incubation of VirD2 with the 41-mer pTF-FC2 *oriT* oligonucleotide and the 35-mer pTiA6 right border oligonucleotide were separated in a denaturing polyacrylamide gel. In both cases a product with the electrophoretic mobility of a 13-mer was observed (Fig 3.6). These results showed that VirD2 is able to recognize the *oriT* region and is able to cleave the synthetic oligonucleotide. The radioactive signal was quantified by use of a phosphoImager device that showed that VirD2 could cleave the oligonucleotide with an efficiency of 40% as compared to the efficiency of cleaving the right border. The occurrence of a 13-mer in the cleavage of the pTF-FC2 *oriT* gives the exact cleavage site of the *oriT* nick region. The identification of this site had hitherto not yet been proven experimentally.

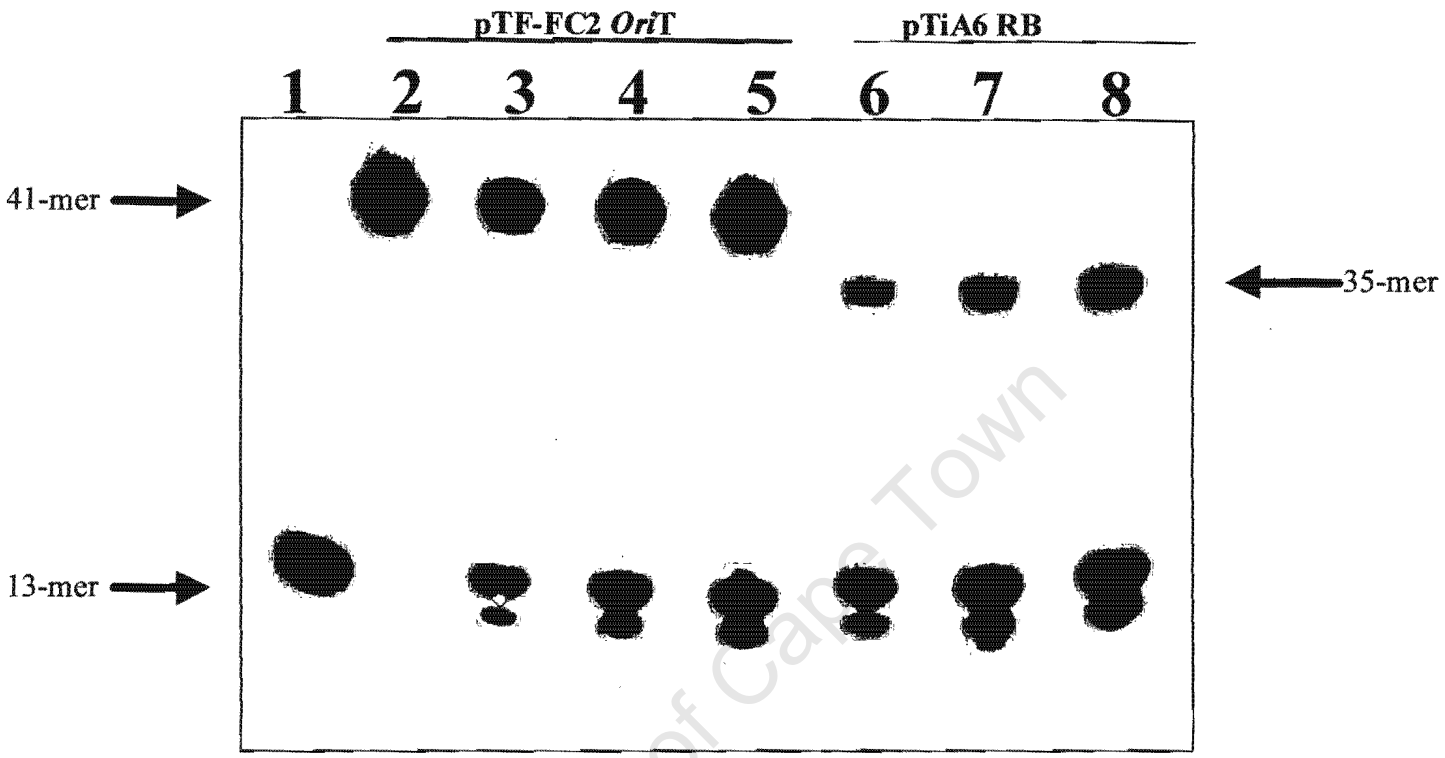


Fig. 3.6 Site specific cleavage of oligonucleotides by purified VirD2 using as substrates the pTiA6 right border (RB): GGTATATATCCTG^CCAGTCTTGATGCCGCGCGCAT and pTF-FC2 *oriT* : AACGGTCATCCTG^TATTGCTCAACCGCTCTACTATCATATC. Positions of the cleavage sites are marked ^. Lanes 1, labeled 13-mer used as a control; 2, labeled 41-mer that was not incubated with VirD2; 3-5, cleavage products (13-mers) of the 41-mer pTF-FC2 *oriT* generated by increasing concentrations (5, 10 and 20 mg) of VirD2; 6-8, cleavage products (13-mers) of the 35-mer pTiA6 RB after receiving the same treatment as pTF-FC2 *oriT*.

3.3.4 Transient expression GUS assays

In this study I set out to test the efficiency of DNA transfer to young tobacco seedlings by the Agrobacterium strains given in Table 3.1. In this assay the activity of β-glucoronidase transiently expressed by the *uidA* gene carried on vectors pDER-*bar*-GUS and pDER-*bar*-GUS-ΔMobA represents a quantitative measure of transferred DNA molecules which arrive in the plant cell nucleus without necessarily being integrated into the genome (transfer efficiency). Thus transfer efficiency can be described as the number of blue spots appearing on a seedling after histochemical staining. Transient expression experiments (Fig. 3.7) showed nearly equal numbers of blue spots on cotyledons transfected with GV3101 (pPM6000K)(pDER-*bar*-GUS) and GV3101 (pPM6000)(pDER-*bar*-GUS) (Table 3.2A and Table 3.2B). This suggests that MobA is able to transfer T-DNA as efficiently as the combination of VirD2 and MobA. Transfection of tobacco cotyledons with GV3101 (pPM6000K)(pDER-*bar*-GUS-ΔMobA) showed no DNA transfer and transfection with an undiluted GV3101 (pPM6000)(pDER-*bar*-GUS-ΔMobA) strain showed very little transfer, about 0.03% of the transfer efficiency of the 1:100 diluted strain of either GV3101 (pPM6000)(pDER-*bar*-GUS) or GV3101(pPM6000K)(pDER-*bar*-GUS). This result shows that although VirD2 is able to cleave the *oriT* region *in vitro* with a 40% efficiency, it is barely able to do so *in vivo*. However there is a possibility that VirD2 is able to cleave the *oriT* region efficiently but is unable to transfer this DNA to plant cells.

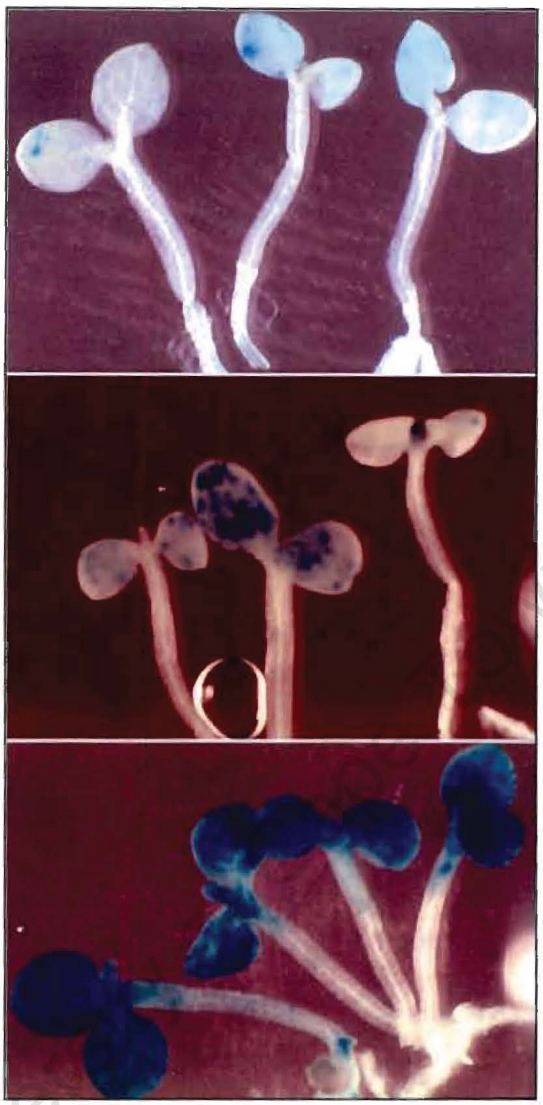


Fig. 3.7 Representative transient expression of *uidA* gene in tobacco seedlings that were co-cultivated with various dilutions of strain GV 3101(pPM6000K)(pDER-*bar*-GUS). Photograph A represents the undiluted strain; B, 1:10 dilution; C,1:100 dilution). Strain GV 3101(pPM6000K) was used for the dilutions as it contained a non-functional VirD2 protein.

3.3.5 Tobacco cotyledon co-cultivation

The efficiency of T-DNA integration into the plant chromosomal DNA was estimated after selection for PPT resistant calli (Table 3.2A 3.2B). The relative transformation frequency of a strain corresponds to the number of PPT resistant calli/seedling compared with this number for a wild type bacterium (Tinland *et al.* 1995). Therefore the integration efficiency is defined as the ratio of transformation efficiency to transfer efficiency. This value represents the fraction of T-DNA molecules that integrate from a pool that entered the nucleus. Experimentally integration efficiency is defined as the number of PPT resistant calli divided by the number of blue spots (Tinland *et al.* 1995).

The experiment that I had designed to test for stable integration of the plasmid DNA showed that both strains GV3101 (pPM6000)(pDER-*bar*-GUS) and GV3101 (pPM6000K)(pDER-*bar*-GUS) transformed tobacco seedlings with a similar frequency, 15.4% and 15.6% respectively. Considering that these two strains showed the same transfer efficiency it is not surprising that they have the same integration efficiency as well (Table 3.2B).

Table 3.2A Raw data obtained during the transient GUS expression experiments and the subsequent formation of callus.

Phenotype of strains used		Transient GUS expression (Spots/seedlings)		Calli formation (Calli/seedlings)
VirD2	MobA	UNDILUTED	DILUTED 1:100	
-	+	Blue	1550/298	192/1234
+	+	Blue	1561/332	145/942
-	-	0/365	N/A	0
+	-	2/350	0	0

Table 3.2B Efficiency of transfer[a], transformation and integration mediated by MobA and/or VirD2[d]

Phenotype of strains used		Transfer efficiency: Spots/seedling	Transformation efficiency: Calli/seedling	Integration efficiency: Transformation/transfer
VirD2	**MobA**			
-	+	5.2	0.156	0.031
+	+	4.7	0.154	0.033
-	-	N/A[b]	0	——[c]
+	-	0	0	

[a] This table only shows results that were obtained after 1:100 dilution
[b] Not applicable
[c] Not determined
[d] The results shown in this table are a result of the average of three independent experiments

3.4 DISCUSSION

VirD2 is one of the key *A. tumefaciens* proteins involved in T-DNA processing and transfer. In addition to its strand transferase domain, necessary for nicking the T-DNA borders, VirD2 contains a bipartite C-terminal nuclear localization sequence (NLS), necessary for piloting the T-strand to the plant nucleus, and a conserved region called ω that is important for virulence (reviewed in Chapter 1). The conjugative plasmid RSF1010 can be transferred by *A. tumefaciens* either to plant cells or to other Agrobacteria. Three proteins required to process plasmid DNA for conjugal transfer assemble at the *oriT* to form a complex called the relaxosome. The largest and best known of these proteins, MobA, locally disrupts the helical structure of the *oriT* DNA in the relaxosome and then cleaves one of the strands (reviewed in Chapter 1). As there was no resemblance between the mobilization region of RSF1010 and that of pTF-FC2 (Fig. 3.4), it was necessary to gain an insight into the mechanism that was operational in the pTF-FC2 transfer system. This was done by establishing which protein was involved in the processing of the DNA. The VirD2 and MobA cleavage proteins were targeted. However amino acid sequence alignment showed that the two proteins had a 19% identity (Fig. 3.5).

The results presented here have shown that VirD2 is able to cleave pTF-FC2 *oriT in vitro* with an efficiency of 40% (Fig.3.6). The cleavage site of the pTF-FC2 *oriT* has also been identified. It falls immediately after the perfectly conserved hexamer, ATCCTG (Table1.1). This is consistent with other cleavage sites (Lanka and Wilkins, 1995).

In the previous Chapter strain LBA4404(pAL4404)(pDER-*bar*), which contains intact and functional VirD2 and MobA cleavage proteins was shown to be able to mediate the transfer of plasmid DNA to plants. To verify the contribution of each of these proteins in this transfer I measured the MobA-mediated transfer in a VirD2-free *Agrobacterium* strain [GV 3101(pPM6000K)(pDER-*bar*-GUS)] and the VirD2-mediated transfer in a MobA-free *Agrobacterium* strain [GV 3101(pPM6000)(pDER-*bar*-GUS-ΔMobA)]. The results obtained from these experiments were compared with those obtained from strain GV 3101 (pPM6000)(pDER-*bar*-GUS) which contained intact and functional VirD2 and MobA cleavage proteins.

The fact that the strains GV3101 (pPM6000)(pDER-*bar*-GUS) and GV3101 (pPM6000K)(pDER-*bar*-GUS) transformed the plants with the same frequency indicates that VirD2 does not interfere with MobA. Thus it can be said that despite the fact that the two proteins co-exist in the same strain they act independent of each other. This finding is consistent and comparable with that made by Shadenkov *et al.* (1996) and Bravo-Angel *et al.* (1999) who both found that DNA transfer by MobA of RSF1010 is neither dependent on nor inhibited by the presence of VirD2. It is also interesting to note this similarity in functionality between RSF1010 and pTF-FC2 when yet they have no similarity in their mobilization regions at the DNA sequence level (Rohrer and Rawlings, 1992).

Although VirD2 was able to cleave the pTF-FC2 *oriT in vitro* with an efficiency of 40% it may be barely able to do so *in vivo*. Arbitrarily setting the transfer efficiency by the

strains GV3101(pPM6000)(pDER-*bar*-GUS) and GV3101(pPM6000K)(pDER-*bar*-GUS) to 100% and also taking into account the dilution factor VirD2 could only achieve a 0.03% transfer efficiency in an environment devoid of MobA. In this case the contribution of VirD2 *in vivo* can be considered to be negligible since the two strains GV 3101 (pPM6000)(pDER-*bar*-GUS) and GV 3101(pPM6000K)(pDER-*bar*-GUS) showed a similar transfer efficiency. Another explanation could be that VirD2 is able to cleave the pTF-FC2 *oriT* but, as has already been shown, is unable to transfer DNA to the plant cells. It is a well established fact that in order for VirD2 to pilot the T-strand to the plant nucleus it has to remain tightly bound to the 5' end of the T-strand (Howard *et al.* 1992; Shurvington *et al.* 1992; Tinland *et al.* 1992; Rossi *et al.* 1993b). However to my knowledge such binding between VirD2 and an *oriT*-derived T-strand has not been documented. This means that even if VirD2 could cleave the pTF-FC2 *oriT* it may fail to pilot the T-strand to the plant cell or the mobilization accessory proteins MobB, C and D may not be able to associate with VirD2 as they do with MobA.

In the previous Chapter it was shown that strain C58C1(pMP90)(pTD1) which contained intact VirD2 and MobA proteins and also contained the right and left border sequences could not mediate the transfer of DNA to plant cells. This finding, taken together with the results of the experiments that are presented in this Chapter, can shed more light on the mechanism of action that mediates the transfer of DNA into plants by pTF-FC2. The co-existence of the VirD2 and MobA proteins does not cause any interference as others (Shadenkov *et al.* 1996 and Bravo-Angel *et al.* 1999) and I have already shown. In addition each of these proteins had its on specific and appropriate cleavage site.

Therefore there was no competition at the level of the cleavage site. However based on the construction of pTD1 and the location of the cleavage sites, transformed plants would only result from VirD2-mediated cleavage. This is so because if both VirD2 and MobA cleave at their respective sites MobA will transfer to the plant a fragment that does not carry the selectable marker gene (Fig. 3.8). However even so no transformants were obtained, which means VirD2 was unable to pilot the T-strand carrying the *nptII* gene into plant cells. These observations imply that there is some inhibition mechanism that prevents VirD2 from carrying out its normal functions.

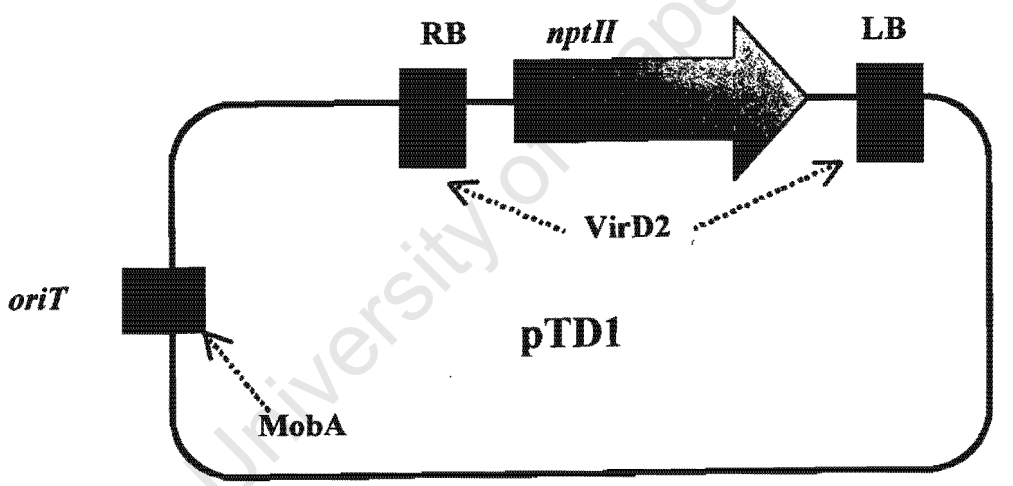


Fig. 3.8 Schematic diagram of plasmid pTD1 showing the possible cleavage sites for MobA and VirD2. The area between the right border and the origin of transfer (*oriT*) does not contain a selectable marker gene. Therefore during plant transformation if both MobA and VirD2 cleave their respective sites then MobA will transfer a DNA fragment that does not carry a selectable marker gene.

Several studies, which relate to the inhibition of the transfer of the T-strand have been undertaken by several scientists. RSF1010 was shown to inhibit the transfer and or integration of pTiA6 in a manner that is reversed by the over expression of *virB9*, *virB10*, and *virB11* (Ward *et al.* 1991). In this study the authors suggested that RSF1010 or its transferred intermediate blocks the transfer of the T-complex and that in its presence VirB9, VirB10 and VirB11 become limiting for transfer. Further studies showed that the R-strand (ie. the transferred DNA from RSF1010), the T-strand and VirE2 utilize a common transport site and that the transferred intermediate of RSF1010 has an advantage over VirE2 and VirD2-T strand in accessing this site (Binns *et al.* 1995). The most obvious candidate site of competition is the hypothetical transfer complex encoded by *virB* and *virD4* (Okamoto *et al.* 1991). It was further shown that the formation of an intermediate of RSF1010 capable of conjugal transfer is necessary for the inhibition of tumorigenesis and VirE2 movement (Stahl *et al.* 1998). The major finding in these experiments was that RSF1010 or its transferred intermediate preferentially interacts with the *virB* encoded transfer system and thus sequestering it in a manner that leaves this intermediate in a state that is more likely to take advantage of extracellular VirE2 than the T-DNA intermediate made in the same bacterium. This competition between RSF1010 products and VirE2 suggests a high affinity recognition of elements of the DNA export machinery by the MobA components and may be an important feature of broad-host-range plasmids since it would allow them to utilize other DNA systems efficiently (Bravo-Angel *et al.* 1999). Perhaps more importantly, the RSF1010 transferred intermediate is probably present in *Agrobacterium* species even in the absence

of *vir* induction, as it is in *E.coli*, in which the relaxed MobA-capped intermediate of RSF1010 is found constitutively (Nordheim *et al.* 1980). The fact that VirB proteins are potentially limiting and that they are only produced after *vir* induction means that they can easily be sequestered by the RSF1010 transfer intermediate.

Furthermore the presence in *Agrobacterium* of the IncW plasmid pSa has been shown to suppress tumorigenesis (Loper and Kado, 1979; Farrand *et al.* 1981). Further studies have localized the oncogene suppression gene to a 3.1 kb region of pSa (Close and Kado 1991). This region contains the *osa* (oncogenic suppression activity) gene that is the fourth gene of a four-gene operon. The presence of *osa* alone in *A. tumefaciens* is sufficient for suppressing ocogenicity gene (Close and Kado, 1992; Chen and Kado, 1994). It was also shown that the presence of OSA protein in *A. tumefaciens* inhibits tumorigenesis not by blocking T-DNA transfer, but rather by inhibiting VirE2 protein export from the bacterium to the plant cell (Lee *et al.* 1999). The finding that the *osa* gene by itself, rather than the formation of a conjugal intermediate with pSa, blocks transformation suggests that the mechanism of oncogenic suppression by *osa* may differ from that of the IncQ plasmid RSF1010.

With regards to the lack of transformants when using C58C1(pMP90)(pTD1) I propose the following scenario: The pTF-FC2 intermediate (ie. the protein-nucleic acid strand to be transferred) inhibits the VirD2-T strand transfer in a similar fashion as described for RSF1010. However although the pTF-FC2 intermediate, by virtue of its perceived inhibitory capacity, has a competitive advantage over the VirD2-T strand, no

transformants are generated because the fragment that it transfers to the plant cell does not carry a selectable marker (Fig. 3.8). The selectable marker gene, *nptII* is upstream of the *oriT* and is delimited by the right and the left borders. The observation that no transformants results is an indication that the VirD2 protein cleaves the right border efficiently. If it were not so, some MobA-mediated transformants would be obtained as a result of the non-cleaved border sequences. Thus failure by VirD2 to cleave the borders efficiently would enable MobA to pilot to the plant cell a strand that contains the selectable marker.

The fact that Buchanan-Wollaston *et al.* (1987) showed that binary vectors derived from RSF1010 were able to mediate the transfer of DNA to plants whether or not they contained borders suggests a different mechanism of transfer between this plasmid and pTF-FC2. However, this could also mean that pTFC2 and RSF1010 function the same way with the Vir transfer system, but that the juxtaposition of *oriT* and borders on pTD1 is different from that on the RSF1010 derivatives.

Bravo-Angel *et al.* (1999) conducted experiments that confirmed that the export of VirE2 proteins can be inhibited by the presence of RSF1010-derived plasmids in Agrobacterium, consequently leading to a decrease in transfer efficiency. This question was not addressed in this work although the possibility that the truncations that were observed in Chapter 2 may have been due, in part, to the low concentration of VirE2 that was delivered to the plant cells cannot be excluded. Rossi *et al.* (1996) Showed that in

the absence of VirE2, a large majority of the T-DNA molecules are integrated in truncated versions, with variable extents of left parts of the T-DNA missing.

Without further experimentation the results presented in this Chapter do not allow extensive speculation about the mechanism of transfer. However it is clear that the MobA protein of pTF-FC2 and the *oriT* mediate the transfer of DNA into plant cells. It is also clear that the products of pTF-FC2 inhibit the export of VirD2-T strand into plant cells. The results obtained so far point to a model that suggests that the mechanism in operation in the inhibition of VirD2-T strand transfer by pTF-FC2 is very similar to that of RSF1010 and different from that of pSa.

It has been reported that RSF1010 MobA-mediated transformation occurs with a much lower efficiency compared to VirD2-dependent T-DNA transfer (Bravo-Angel *et al.* 1999). However the pTF-FC2 MobA-mediated transformation occurred with a high proficiency that was similar to VirD2-dependent T-DNA by a strain that carried the commercially available vector pBI121(results not shown). Another deficiency observed in the use of RSF1010 MobA were the severe truncations at the 5' end of the transferred DNAs that were integrated into the plant genome (Bravo-Angel *et al.* 1999). Typically most of these deletions were over 500 bp long. The results presented here show that primer MobCDF located 121 bp from the MobA nicking site consistently produced the right sized PCR product when used in combination with primer MobCDR in all the plants tested. This shows that the 5' deletions in these plants, if any, were less than 121 bp. It is therefore possible that pTF-FC2 MobA is able to integrate the 5' end of the transferred

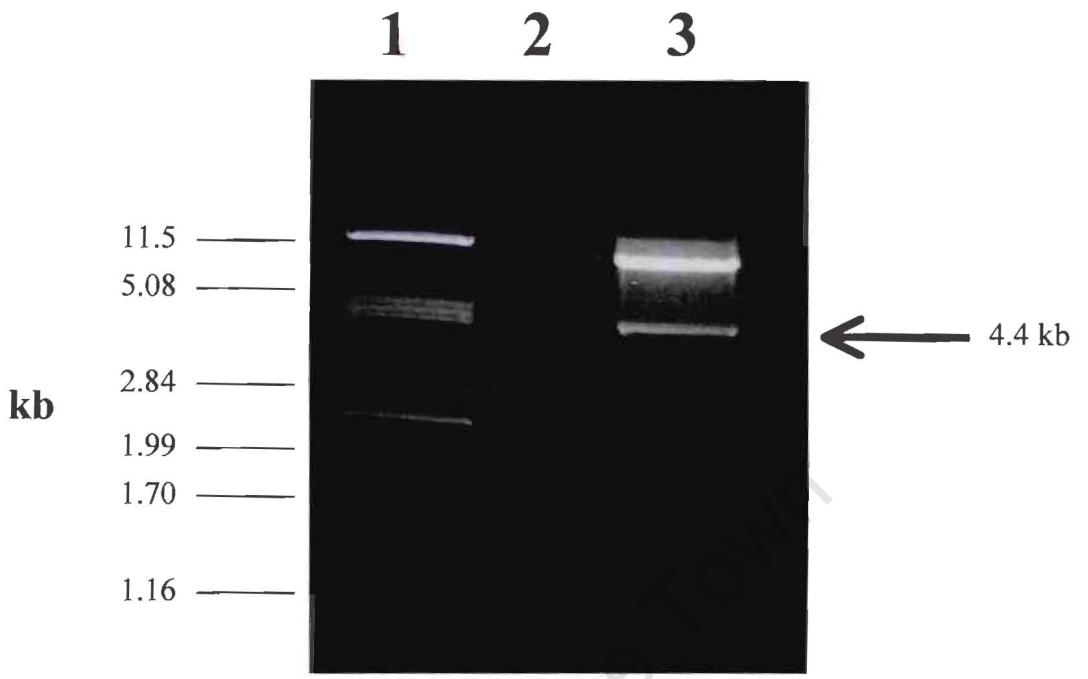


Fig. 3.3 Restriction analysis to confirm the presence of the MobA deletion in pDER-*bar*-GUS-ΔMobA. Lanes 1, λ/*Pst*I molecular weight markers; 2, *Cla*I restriction digest of pDER-*bar*-GUS, yielding a 5.484-kb fragment that contains the entire mobilization region; 3, *Cla*I restriction digest of pDER-*bar*-GUS-ΔMobA, producing a band of 4.4 kb which shows a deletion of 1084 bp in the *mob* region.

DNA more precisely into the plant genome. Of the fifteen plants that Bravo-Angel *et al.* (1999) analyzed only one was found to be correctly ligated to the plant genome without any 5' deletion. Based on the available results it is likely that pTF-FC2 MobA can achieve a higher frequency of correctly ligated DNA compared to the one by RSF1010 which Bravo-Angel and colleagues observed.

My results suggest that pTF-FC2 MobA may be more efficient than RSF1010 MobA at maintaining the integrity of the 5' end of the transferred DNA during the process of integration. It is interesting to note that of the important domains of VirD2 the ω domain is the only one that is well conserved between VirD2 and pTF-FC2 MobA (Fig. 3.5). In the absence of a clearly defined role of ω (reviewed by Gelvin, 2000) I propose that it is this domain that gives pTF-FC2 its proficiency both in DNA transfer and in integration.

CHAPTER 4

CO-TRANSFORMATION OF TWO COMPATIBLE *AGROBACTERIUM tumefaciens* BINARY VECTORS AND THEIR USE IN PLANT TRANSFORMATION

ABSTRACT

The introduction of foreign genes into the plant genome is now a basic technique. However the realization that most agronomic traits are polygenic in nature has directed researchers to focus more on multiple transformation systems. Most methods that have so far been used to generate multigenes use the co-bombardment method. In order to achieve multigene plant transformation, in this study, a strain of *Agrobacterium* carrying two compatible binary vectors was used to transform tobacco cells. The bialaphos resistance gene (*bar*) and the kanamycin resistance gene (*nptII*) were the two genes used. *Agrobacterium* strains were transformed by electroporation and/or freeze thaw methods resulting in C58C1(pMP90)(pBI121)(pPZP100-*bar*). A large number of morphologically normal transgenic plants were obtained by co-cultivation of tobacco leaf discs with the above strains of *Agrobacterium*. Using PCR analyses the T_o generation showed that the frequency of double transformation, defined as the frequency with which cells transformed with a first T-DNA contained a second unselected T-DNA, was 75%. This represented 12 of the 16 plants that were analysed. The 12 doubly transformed T_o plants were self-pollinated. The results showed that in eight of the plants the two genes had integrated into one genetic locus, with an inheritance conforming to a 3:1 Mendelian fashion. The progeny of one plant showed a 15:1 segregation pattern indicating that the two genes had integrated in two unlinked sites in the genome. The progeny of the other

three plants failed to thrive on medium supplemented with bialaphos suggesting that this gene had either been silenced, truncated, deleted or mutated.

4.1 INTRODUCTION

Agrobacterium has largely been used in leaf disc transformation for the transfer of single trait genes (Zhu *et al.* 2000; Zupan *et al.* 2000). Of particular importance has been the production of hepatitis B surface antigen for oral immunization (Richter *et al.* 2000), the production of transgenic plants that express cationic peptide chimeras that exhibit broad-spectrum resistance to phytopathogens (Osusky *et al.* 2000) and the production of biopharmaceuticals (Giddings *et al.* 2000). The most exciting factor is that one transgenic plant-derived biopharmaceutical, hirudin, is now being produced commercially in Canada for the first time. It would therefore seem that transgenic plants hold great potential for the safe production of relatively inexpensive pharmaceutical proteins and peptide products.

However since most agronomic characteristics are polygenic in nature, plant genetic engineering will require the manipulation of complex regulatory or metabolic pathways involving multiple genes or gene complexes (Chen *et al.* 1998). For instance in order to express the polyhydroxybutyrate polymer or Guy's 13 antibody, for example, single genes were first introduced into individual transgenic plants, then these plants were back-crossed to reconstitute the entire pathway or the complete protein (Navrath *et al.* 1994; Ma *et al.* 1995). It took Ye and his colleagues (2000) seven years to introduce three genes for a short biosynthetic pathway that resulted in β-carotene expression in rice (De

Cosa *et al.* 2001). Therefore it is quite clear that redirecting complex biosynthetic pathways and modifying polygenic agronomic traits requires the integration of multiple transgenes into the plant genome, while ensuring their stable inheritance and expression in succeeding generations (Chen *et al.* 1998). This poses the single biggest technical limitation in plant DNA transformation today.

Strategies to produce multiple transgenes in plants have been developed. Most of these utilize co-bombardment as a means of introducing genes into plants (Hadi *et al.* 1996; Chen *et al.* 1998; De Cosa *et al.* 2001). Co-bombardment is viewed as a simple process in which genes carried on separate plasmids are mixed prior to transfer. Hadi and colleagues demonstrated that it was possible to genetically transform soybean by co-bombardment of embryogenic suspension tissues using a "cocktail" of 12 different gene constructs. However, in this case, no information was provided on the integration, coexpression, or inheritance of the transgenes in regenerated plants.

Through detailed molecular and genetic studies Chen and colleagues showed that it was possible to transform embryogenic tissues with a mixture of 14 different pUC-based plasmids. Eighty-five percent of these transgenics contained more than two, and 17% more than nine of the target genes. Integration of multiple transgenes occurred at either one or two genetic loci with inheritance conforming to a 3:1 Mendelian ratio. In this case integration and behaviour of multiple transgenes inserted by co-bombardment was similar to that for a single transgene, and exhibited the patterns of stable and predictable inheritance necessary for incorporation into plant breeding programmes. Although all

transgenes, except for one, were morphologically normally only 63% produced viable seed. In addition, whilst it is clear that the integration of many genes at one or few loci could not be attributed to chance alone, the mechanisms governing this phenomenon remain unknown. Therefore, this method may not be universally suitable for all crops.

De Cosa and colleagues (2001) demonstrated another strategy for engineering multiple genes in plants. In this case multiple genes were introduced into the plastid genome unlike in all of the aforementioned examples where genes were transferred into the nuclear genome. This strategy offers a some advantages notably the fact that integration of foreign genes into the plastid genome enhances gene containment because in many crop plants plastids are inherited from the maternal parent preventing the pollen-mediated spread of the transgenes (Maliga *et al.* 1993; Daniell *et al.* 1998; Scott *et al.* 1999). Although not completely proven, in principle this removes the possibility of antibiotic resistance gene transfer to weeds or microorganisms in the environment or gut (Lamtham and Day, 2000).

The presence in transgenic crop plants of marker genes conferring antibiotic or herbicide resistance has raised a great deal of concern (reviewed by Daniell, 1999 and Puchta, 2000). Although no scientific basis has been determined for these concerns, removal of marker genes would likely hasten the public acceptance of transgenic crops (Zuo *et al.* 2001).

In their study De Cosa and colleagues used the *Bacillus thuringiensis* (*Bt*) *cry*2Aa2 operon as a model system to demonstrate operon expression and crystal formation via the

chloroplast genome. The *cry*2Aa2 is the distal gene of a three-gene operon. The open reading frame immediately upstream of *cry*2Aa2 codes for a putative chaperonin that facilitates the folding of Cry2Aa2 (and other proteins) to form proteolytically stable cuboidal crystals (Crickmore *et al.* 1992; 1994; Ge *et al.* 1998). Since it has been shown that Cry protein levels decrease in plant tissues late in the growing season or under physiological stress it was part of the objectives of this study to produce a more stable protein that would be expressed at high levels in the chloroplast throughout the growing season and in so doing also help in the elimination of the development of *Bt* resistance. The results obtained showed that the foreign protein accumulated at 45.3% of the total soluble protein in mature leaves and remained stable even in old bleached leaves (46.1%), thereby increasing the efficacy and safety of transgenic plants throughout the growing season.

However, a major disadvantage with chloroplast genetic engineering in higher plants may be the utilization of antibiotic resistance genes as the selectable marker to confer streptomycin/spectinomycin resistance. Initially, selection for chloroplast transformation utilized a cloned mutant 16 S rRNA gene that did not bind the selection antibiotic and this conferred spectinomycin resistance (Svab *et al.* 1990). Subsequently the *aadA* gene product that inactivates the antibiotic by transferring the adenyl moiety of ATP to spectionomycin/streptomycin was used (Svab and Maliga, 1993). These antibiotics are commonly used to control bacterial infection in humans and animals (Danielle *et al.* 2001). However, given the high number of chloroplast genome copies in the cell, transformation unavoidably yields thousands of copies of antibiotic resistance genes in

the chloroplast genome, thus increasing the probability of gene transfer from plants to bacteria living in the gastrointestinal tract or soil.

In the light of all the aforementioned problems the simplicity and specificity of T-DNA transfer may provide a solution for the engineering of multigenes in plants. In addition *Agrobacterium* offers defined transgene integration, potentially low copy number and preferential integration into transcriptionally active regions of the chromosome (Konez *et al.* 1989). Studies have shown that two unlinked T-DNAs can transform the same tobacco plant cell and segregate in the F_1 generation (De Frammond *et al.* 1986). Further studies have also shown that it is possible to obtain plant cells transformed by two distinct T-DNA elements (An *et al.* 1985; Depicker *et al.* 1985; Jones *et al.* 1987; McKnight *et al.* 1987). Jorgensen and colleagues (1987) carried out a detailed study of DNA sequences transferred to the plant genome via *A. tumefaciens*. They discovered that seven out of eleven transgenic tomato plants carried T-DNA at a single genetic locus. Their major discovery was that two distinct T-DNA elements could be physically joined in the plant genome. However Spielmann and Simpson (1987) carried out studies on transgenic tobacco plants that were produced by inoculation of leaf discs with *A. tumefaciens* harboring a disarmed vector containing soybean leghemoglobin Lbc3 and glycinin G2 genes. The transferred DNA was found at multiple genetic loci in five of the six cases examined. In one plant, kanamycin-resistance traits were at four independent chromosomal positions, although two were genetically linked at about three centimorgans. These results were different from those by other researchers who showed that distinct T-DNAs were always integrated at the same locus. The difference was

attributed to the fact that Spielmann and Simpson (1987) used *Agrobacterium* strain LBA4404 which is an octopine strain whereas the other researchers used C58, a nopaline strain. The frequency of multiple transformation was shown to be relatively high (Petit *et al.* 1986). Petit and colleagues speculated that some of the transformants with multiple T-DNA inserts must result from multiple transformations of a single plant cell by different individual bacteria. This was due to the fact that in their experiments, which used opines as markers, about 40% of individual roots that developed on carrot discs inoculated with a mixture of manopine and cucumopine type strains of *Agrobacterium* contained both opines.

Previously work by Depicker and colleagues (1985) had provided interesting findings. In their experiments they wished to determine whether a plant cell is transformed by one or many Agrobacteria during cocultivation, since there is a 100-fold excess bacteria versus plant cells. Also, they wanted to know if the transformation frequency in cocultivation experiments is limited either by the number of plant cells capable of being transformed and/or by the frequency with which effective bacterial contacts are established with each plant cell. They approached these questions by measuring the co-transformation frequencies of two different selectable T-DNAs when present either in the same bacterium or in two different bacteria. The first T-DNA that they used was the wild type T-DNA of the plasmid C58; the hormone balance of the plant cell is changed by expression of the T-DNA genes allowing selection of hormone-independent growth. In addition, this T-DNA codes for nopaline synthase, an additional marker to detect plant cell transformation. The second T-DNA construction was a mini-T-DNA construction

containing a selectable marker gene, consisting of fusions of plant promoters to the neomycin phosphotransferase coding sequence of Tn5. Their interpretation of the transformation frequencies was based on the following rationale. If the frequency of doubly transformed plant cells would be equal to the product of each single transformation, then this would indicate that co-transformation is the result of independent transformation events, and that every plant cell is equally competent, and can equally well stabilize the introduced T-DNA copies presumably through integration. They defined the competence of a plant cell as the ability to interact with *Agrobacterium* and to stabilize accepted T-DNAs during the course of co-cultivation. They also reasoned that if the number of co-transformed cells would be higher than the number of cells with only one type of T-DNA, this would imply that the plant cell competence to acquire, integrate, and express a T-DNA is more limiting than the *Agrobacterium* competence to transform a cell.

The results of their experiments showed that the co-transformation frequency of two distinguishable T-DNAs from different bacteria by a mixed co-cultivation infection is nearly identical to the frequency predicted for independent events. This observation seemed to imply that each plant cell has enough attachment sites for several bacteria. Based on the fact that there is no limitation for other bacteria to superinfect a transformed plant cell their results suggested that most of the plant cells are transformed by only one or a few bacteria. They also found that the co-transformation frequency of two different T-DNAs contained within one bacterium is much higher than expected for independent transformation events; in different experiments it ranged between 60% and 70% relative

to single transformed plant cells. Thus they had shown that a single bacterium can transfer and integrate two separate T-DNAs during one infection event. This result indicated that plant cells are more likely to acquire several T-DNAs from one bacterium than one each from several bacteria.

De Block and Debrouwer (1991) and Denis *et al.* (1995) both cotransformed hypocotyl explants of *Brassica* using the same transformation method. Denis *et al.* (1995) found that six out of nine cotransformed plants contained the two different T-DNAs genetically linked. De Block and Debrouwer (1991) performed a genetic and molecular analysis on eighteen cotransformed plants and found that fourteen of these plants contained linkages of the two different T-DNAs. Thus the observation that two different cotransferred T-DNAs are freqently linked to each other has been made so far for one transformation method and one species.

De Neve *et al.* (1997) extended these observations by verifying whether linkage of cotransferred T-DNAs was a general phenomenon occurring also with other transformation methods and plant species. In order to achieve this they transferred two different T-DNAs into two model plant species, *Nicotiana tabacum* and *Arabidopsis thaliana*, using three different transformation methods: Arabidopsis leaf disc and root transformation and Nicotiana protoplast transformation. Their results showed that the T-DNAs derived from different bacteria were frequently integrated at the same locus, independent of the plant species or transformation method used. Southern analysis

revealed that 12 out of 27 Arabidopsis transformants contained the cotransferred T-DNAs linked to each other.

Based on the work by the aforementioned researchers the aim of this study was to develop a simple system using a tobacco leaf disc transformation system based on Agrobacterium that would generate multigene transformants (Fig. 4.1). This would be achieved by generating a strain of Agrobacterium that harbours two compatible plasmids, each carrying a separate T-DNA. The rationale of this experiment was based on the fact that plant cells are more likely to acquire several T-DNAs from one bacterium than one each from several bacteria (Depicker *et al.* 1985). Additionally the frequency of transformation of two T-DNAs contained within one Agrobacterium is much higher than expected for independent transformation events (Depicker *et al.* 1985; Komari *et al.* 1996).

4.2 MATERIALS AND METHODS

4.2.1 Molecular methods

All routine DNA manipulation techniques were performed as outlined in Sambrook *et al.* (1989).

4.2.2 DNA isolation

Agrobacterium DNA isolation and plant DNA extraction was done as described in Chapter 2. DNA concentrations were determined using a Beckman DU®-64 spectrophotometer and samples were stored at -20°C.

4.2.3 Construction of pPZP100-*bar*

The plasmid pPZP100-*bar* was constructed by inserting the *Hin*dIII/*Eco*RI fragment of plasmid pDGP165 (Spencer *et al.* 1990), which contains the CaMV 35S promoter-*bar* expression cassette, into the respective site of plasmid pPZP100 (Hajdukiewicz *et al.* 1994). The plasmid pPZP100-*bar* incorporates the *bar* expression cassette within the right and left borders (Fig. 4.1).

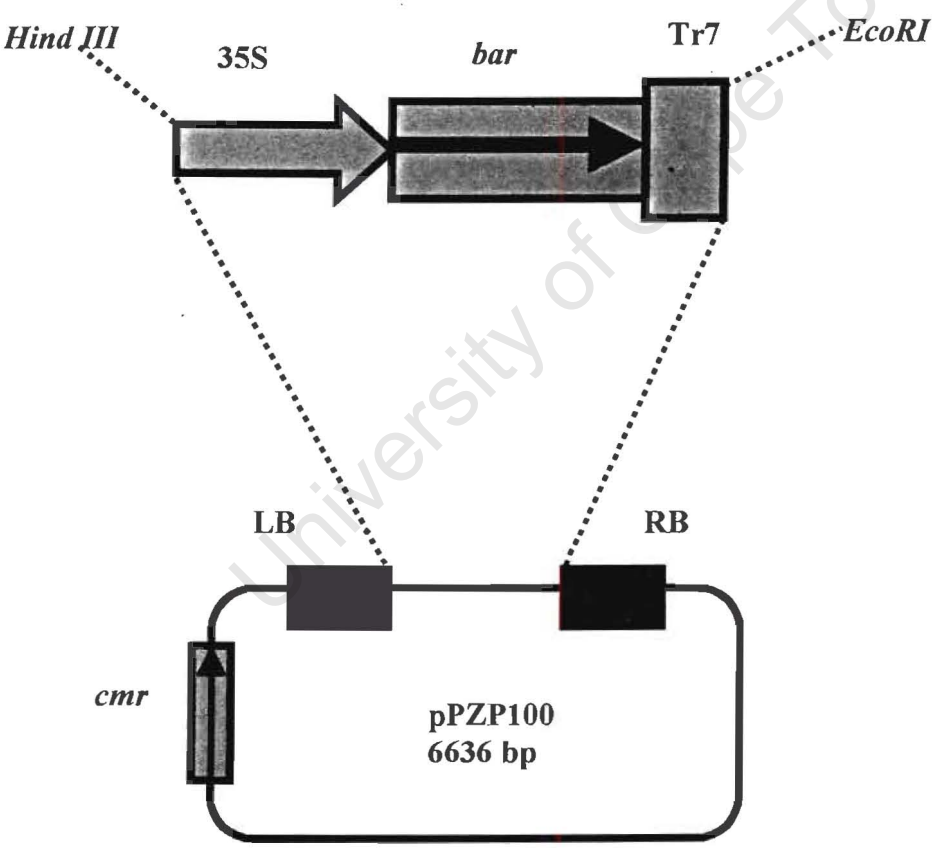


Fig. 4.1 Construction of plasmid pPZP100-*bar*. The *bar* expression cassette was derived from plasmid pDPG165 as a *Hin*dIII/*Eco*RI fragment and then directionally cloned into the corresponding site of plasmid pPZP100 flanked by the right (RB) and left (LB) borders. The *bar* gene is driven by the Cauliflower Mosaic 35S promoter (35S) and has a Tr7 transcription terminator. The plasmid pPZP100 has a chloramphenicol resistance (*cmr*) gene.

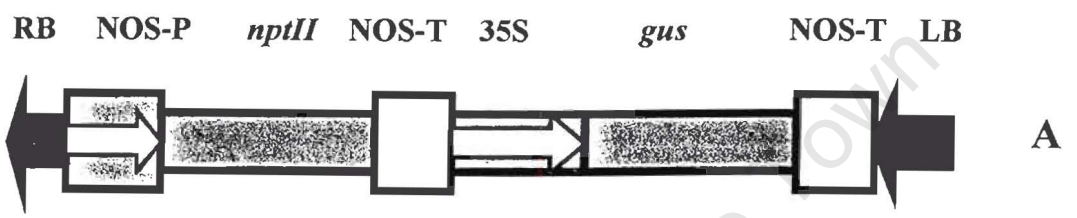


Fig. 4.2 The 800 bp T-DNA fragment of plasmid pBI121. The fragment carries an *nptII* gene, which confers resistance to kanamycin, driven by a nopaline synthase promoter (NOS-P) and transcription is terminated by a nopaline synthase terminator (NOS-T). It also has a *gus* gene driven by a 35S promoter and has a nopaline synthase transcription terminator (NOS-T). Both genes are flanked by the RB and LB.

4.2.4 Transformation of Agrobacterium

Transformation of Agrobacterium was done by either electroporation or freeze-thaw methods as described in Chapter 2. In the strain that contained two separate binary vectors the plasmids were introduced sequentially.

4.2.5 The strategy used to generate tobacco multigene transformants in this study

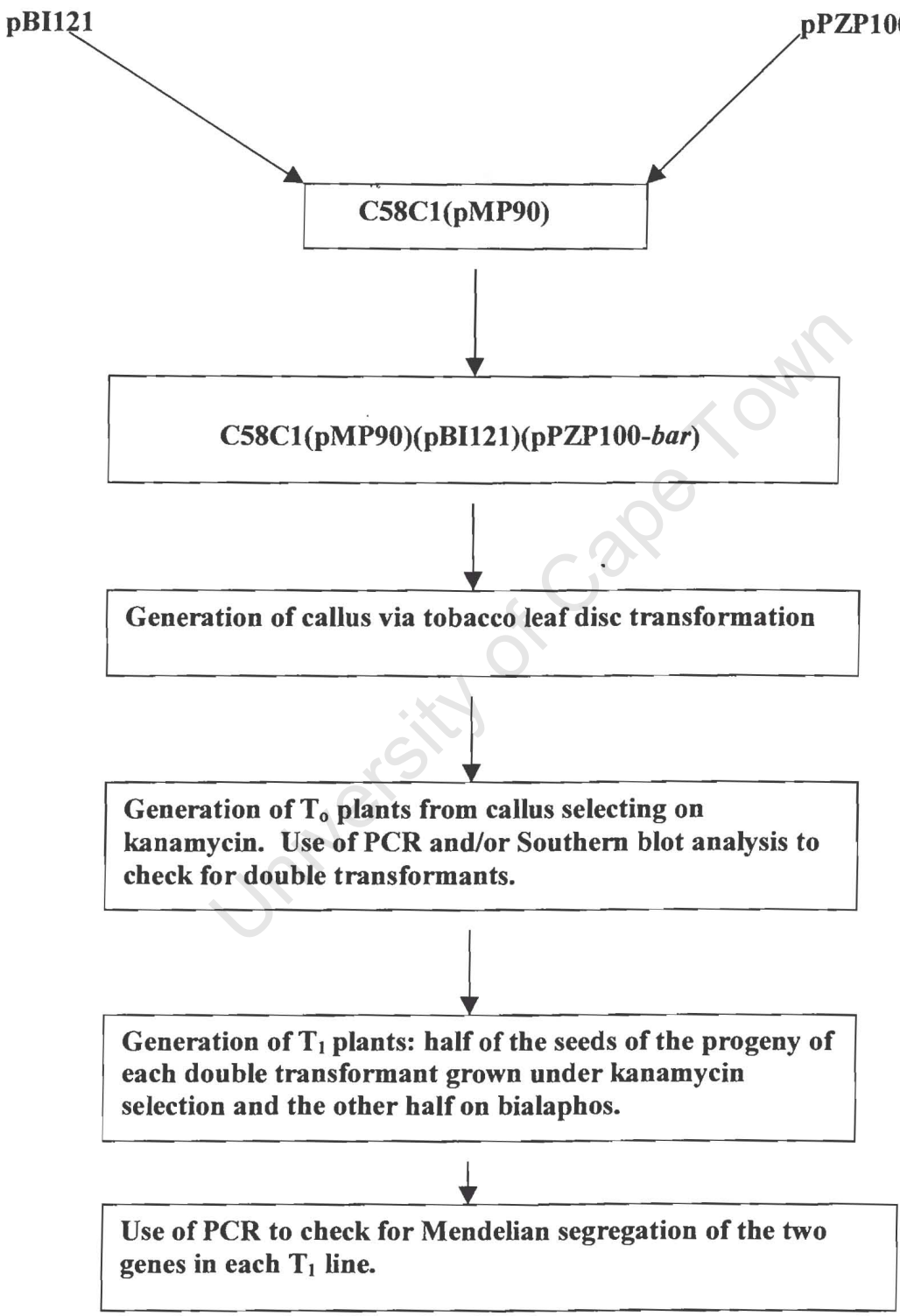


Fig. 4.3 A flow diagram of the strategy used for the generation of tobacco multigene transformants. See text for more details.

4.2.6 Southern blot analysis of *Agrobacterium* and PCR analysis

Southern blot analysis and PCR analysis of *Agrobacterium* was done as described under Materials and methods in Chapter 2.

4.2.7 Tobacco leaf disc transformation

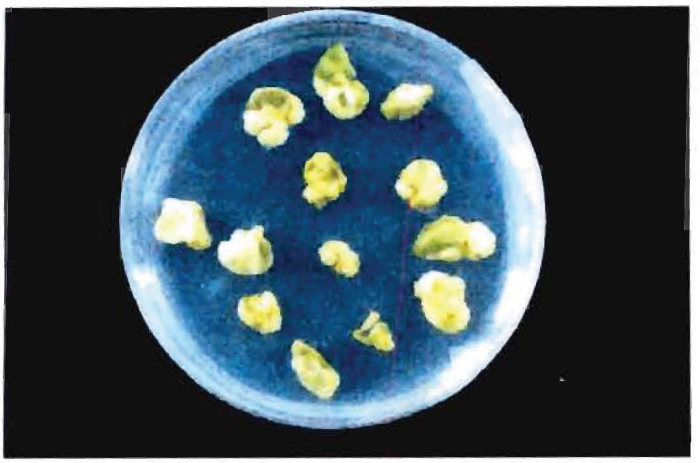
Tobacco leaf disc transformation was performed as described under Materials and Methods in Chapter 2.

4.3 RESULTS

4.3.1 Tobacco leaf disc co-cultivation using *A. tumefaciens* strain C58C1(pMP90)(pBI121)(pPZP100-*bar*)

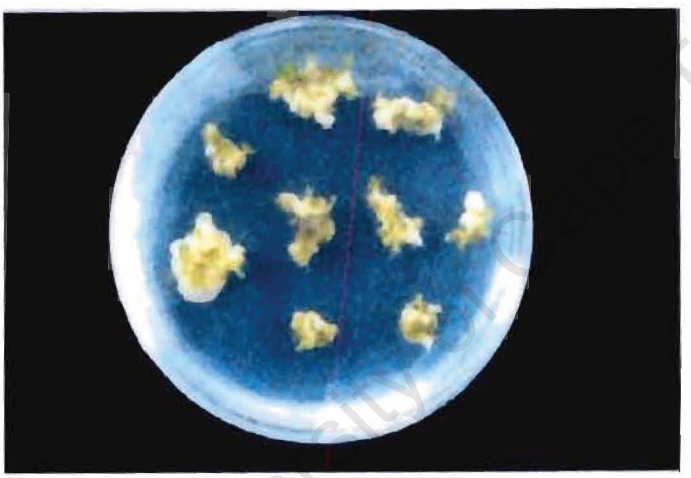
Following co-cultivation with the above strain of *A. tumefaciens*, the tobacco leaf discs were cultured on callus induction medium containing kanamycin (100 mg/l) to inhibit growth of non-transformed tobacco cells, and supplemented with claforan (500 mg/l) to inhibit fungal growth. These conditions resulted in the appearance of vigorously growing callus tissue around the edges of the leaf discs. No callus developed in the leaf discs that were transformed with strain C58C1(pMP90) (Fig. 4.4). The 28 leaf discs that had developed callus tissue were cut into smaller pieces and transferred to regeneration medium, and then to rooting medium containing kanamycin (100 mg/l). Continuous selection of callus up to rooting of the plantlets on medium containing kanamycin ensured there were no escapes (Christou *et al.* 1991; Sudhakar *et al.* 1998; Vain *et al.* 1998; Tang *et al.* 1999). In an attempt to ensure independently derived plants 28 plants were recovered, one each from the 28 leaf discs. These plants constituted the T_o generation (Fig. 4.5).

A



C58C1(pMP90)

B



C58C1(pMP90)(pPZP100-*bar*)

Fig. 4.4 Representative photographs of tobacco leaf disc transformation using *Agrobacterium* strains C58C1(pPMP90) and C58C1(pMP90)(pBI121)(pPZP100-*bar*). The photographs were taken three weeks after callus initiation. More details are available in the text.

Fig. 4.5 Flourishing T_o Tobacco plants growing on soil. These plants were generated from callus that had been a result of leaf disc transformation which was co-cultivated with the strain C58C1(pMP90)(pBI121)(pPZP100-*bar*) and selected on kanamycin. See text for more details.

4.3.2 Characterization of T_0 plants

Of the 28 T_0 plants, 16 were randomly selected for further studies and molecular analysis was carried out using PCR (Table 4.1 and Fig. 4.6A). The aim of this analysis was to determine which of the 16 plants were double transformants. The results showed that 12 of the 16 plants were double transformants (Fig.4.6B). These plants contained the unselected *bar* gene.

4.3.3 Characterization of T_1 plants

In order to generate T_1 plants the flowers of the 12 T_0 plants that were double transformants were self-pollinated by covering them with paper bags to ensure that no cross pollination took place. Seeds were harvested and planted in ½ MS medium in a petri dish. Half of the seeds from each plant were grown on medium supplemented with kanamycin (100 mg/l) and the other half on medium supplemented with bialaphos (3 mg/l). The plantlets that were resistant to kanamycin or bialaphos thrived whereas those that were sensitive bleached and died (results not shown). Of the 12 T_1 plants that emanated from double transformants, eight of them (lines 200, 206, 212, 213, 215, 221, 226 and 228) showed segregation patterns of 3:1 (Table 4.1; Fig. 4.7C). One plant, line 207, showed a segregation ratio of 15:1 (Table 4.1; Fig 4.7D). However three plant lines (201, 219 and 223) did not grow on bialaphos despite the fact that they were progeny of double transformants (Table 4.1; Fig. 4.7A).

Table 4.1. Molecular and genetic analysis of the T_0 and T_1 tobacco transformants.

Plant Line	PCR analysis of T_0 plants Bial[a]	Kan[b]	Growth on Bialaphos selection of T_1 plants Total no. tested	R[c]	S[d]	Segregation ratio
200	+	+	116	84	32	3:1
201	+	+	84	0	84	
203	-	+	NT[e]			
206	+	+	92	67	25	3:1
207	-	+	112	107	5	15:1
212	+	+	131	95	36	3:1
213	+	+	114	86	28	3:1
215	+	+	104	73	31	3:1
218	-	+	NT			
219	+	+	107	0	107	
221	+	+	96	71	25	3:1
223	+	+	119	0	119	
224	-	+	NT			
226	+	+	91	67	24	3:1
227	+	+	NT			
228	+	+	87	75	22	3:1

[a] Bialaphos; [b] Kanamycin; [c] Resistant; [d] Sensitive; [e] Not tested

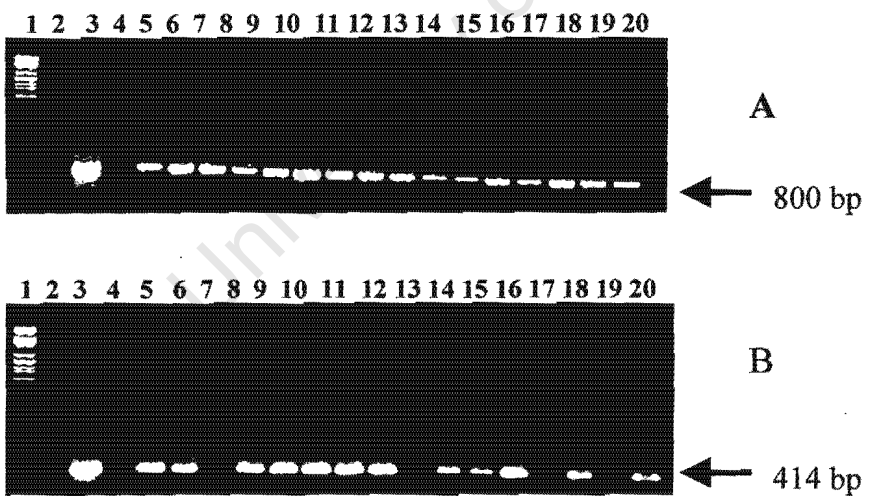


Fig. 4.6 PCR analysis of the T_0 plants generated from callus. Panel A shows the 16 plants that were selected on kanamycin (lanes 5-20). These plants were positive for the 800 bp *nptII* gene. The same plants were analysed for the presence of the 414 bp *bar* gene (Panel B) in order to determine the double transformants. In both panels lanes 1,2 and 4 contain λ/*Pst 1*, water and the non transgenic tobacco plant. Lane 3 contains plasmid positive control, pBI121 in panel A and pPZP100-*bar* in panel B.

Despite repeated attempts Southern blot analysis was not successful in this study. Line 212 was randomly selected for further analysis using PCR. This was done in order to affirm that the expression was a result of the presence of the DNA. The results showed that all the 14 plants that were selected on kanamycin tested positive for the *bar* gene whilst the 10 plants that were selected on bialaphos also tested positive for the *bar* gene (Fig. 4.8). The 14 plants that were selected on kanamycin were all positive for the *nptII* gene and the 10 plants that were selected on bialaphos were all positive for the *nptII* gene (Fig 4.9).

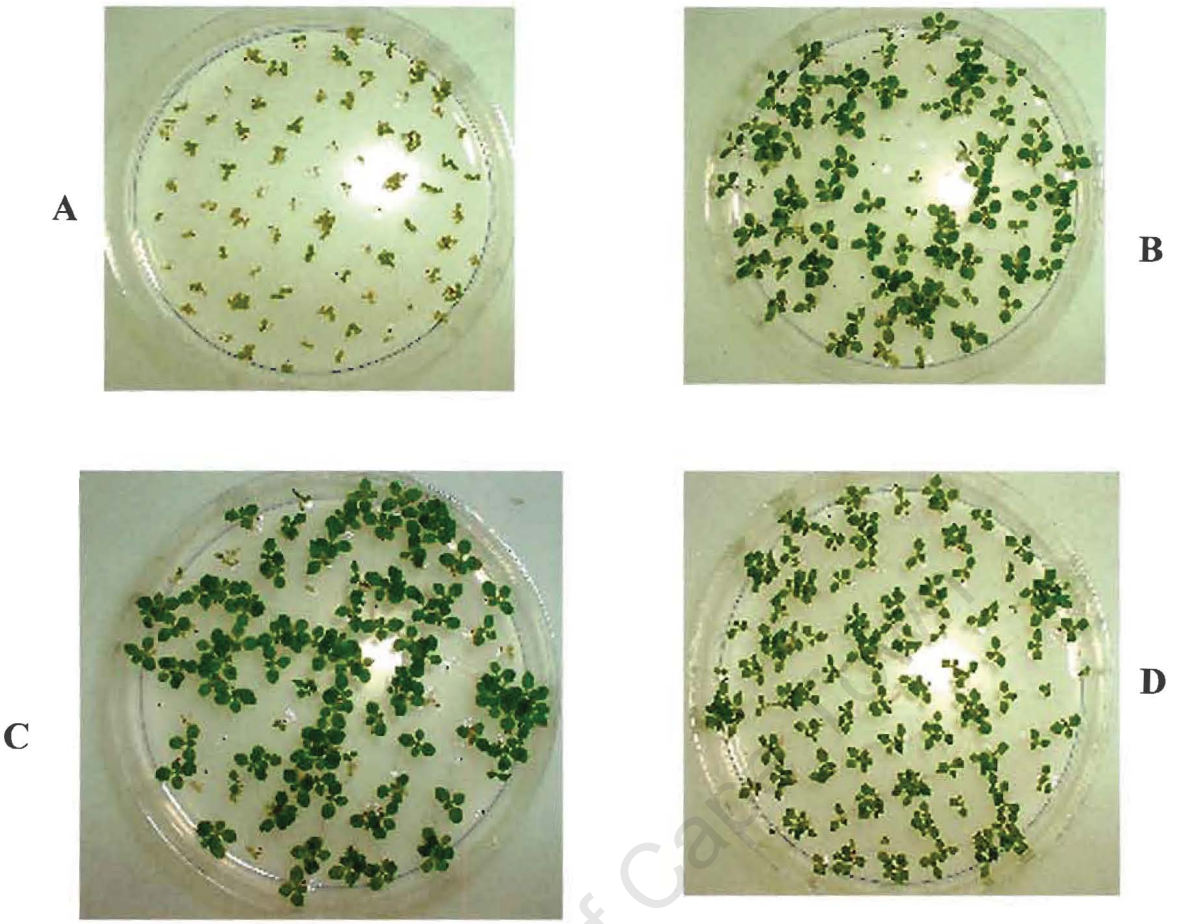


Fig. 4.7 Growth of T_1 plants on selection medium. Panel A shows the progeny of line 201 which failed to thrive on medium supplemented with bialaphos although it could grow on medium supplemented with kanamycin (Panel B). Panel C shows the progeny of line 228 being a representative of the 3:1 Mendelian segregation ratio. Panel D shows the progeny of line 207, the only plant to give a 15:1 Mendelian segregation ratio.

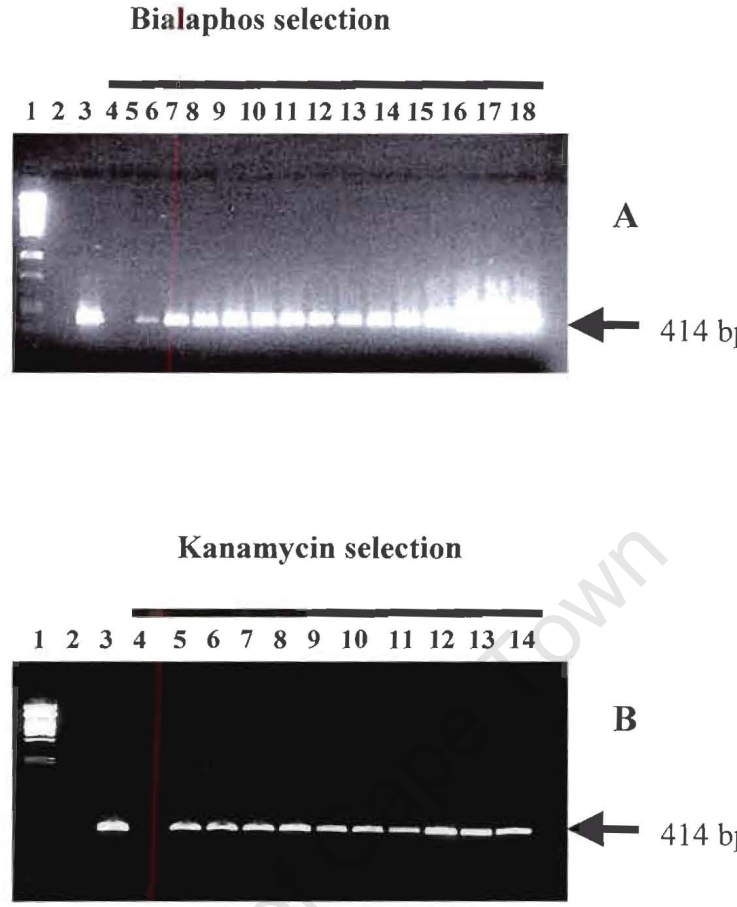


Fig. 4.8 Analysis of the progeny of 212. The 14 plants (Panel A) that were selected on bialaphos (lanes 5-18) are all positive for the 414-bp *bar* gene as expected. The 10 plants (Panel B) that were selected on kanamycin (lanes 5-14) are also positive for the *bar* gene. Lanes 1-4 of both panels are identical and they contain; 1, λ/*Pst 1* molecular weight markers; 2, water; 3, plasmid pPZP100-*bar*; 4, a non transgenic tobacco plant.

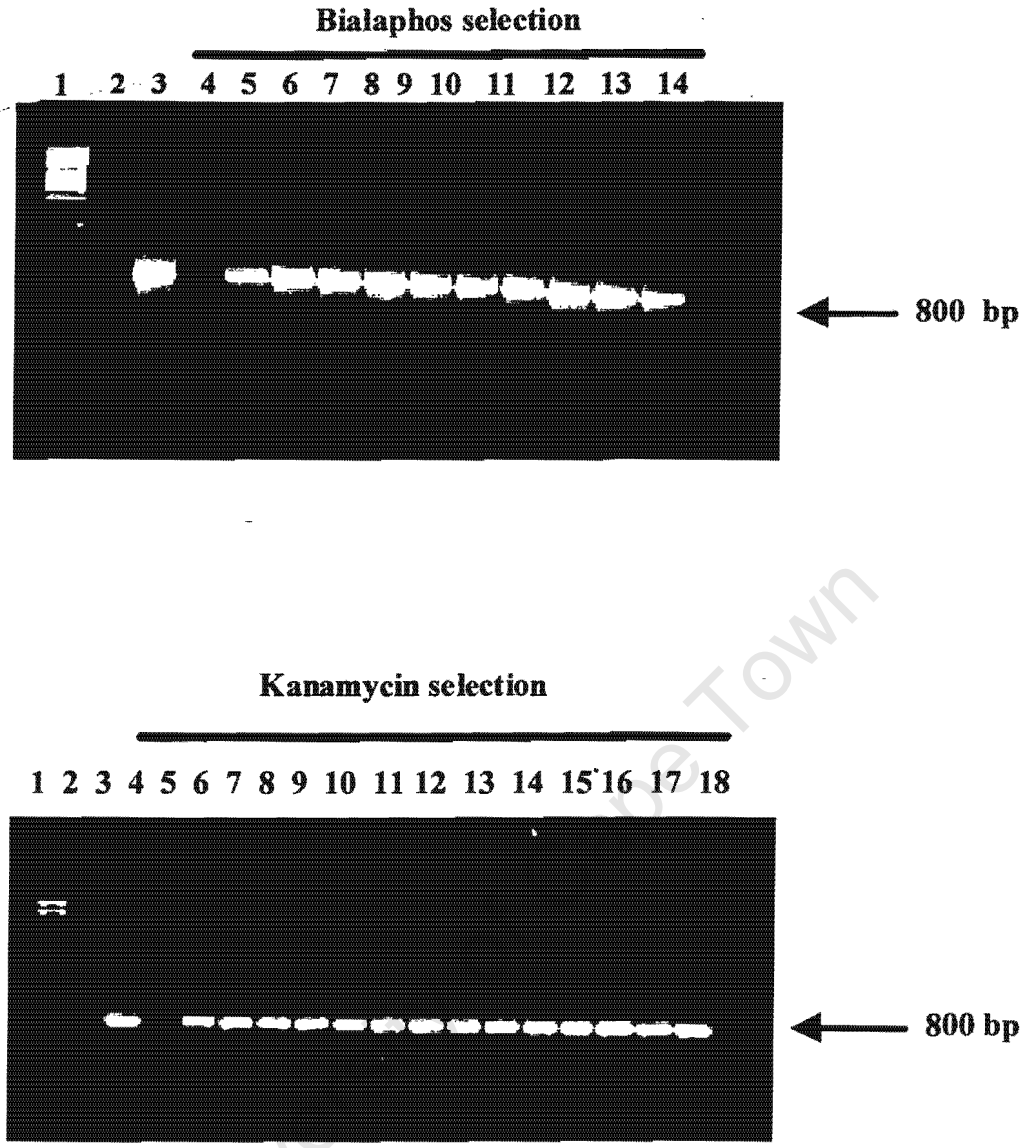


Fig. 4.9 Analysis of the progeny of line 212. The 10 plants (Panel A) that were selected on bialaphos (lanes 5-14) are all positive for the 800-bp *nptII* gene. The 14 plants (Panel B) that were selected on kanamycin (lanes 5-18) are also positive for the *nptII* gene as expected. Lanes 1-4 of Panels A and B are identical and they contain; 1, λ/*Pst I* molecular weight markers; 2, Water; 3, pBI121; 4, a non transgenic tobacco plant. A detailed explanation is given in the text.

4.4 DISCUSSION

The ability of introducing blocks of foreign genes in a single operon would avoid complications inherent in putting one gene at a time into random locations in the nuclear genome. Cloning several genes into a single T-DNA does not avoid the compounded variable expression problem encountered in nuclear transgenic plants (Bogorad, 2000). The size of such multiple-gene plasmids may determine the amount of transgene rearrangement, as foreign DNA is known to undergo rearrangement before or during integration (Stam *et al.* 1997; Kohli *et al.* 1998; 1999). Although plants have been transformed with very large constructs of up to 150 kb (Hamilton *et al.* 1996), the optimal size range needed to maintain a high ratio of intact to rearranged/truncated transgenic sequences is not known (Tang *et al.* 1999).

The aim of this study was to investigate the possibility of introducing two genes carried on two separate and compatible *A. tumefaciens* binary vectors to a single locus using the single strain method. The results obtained in this study show that 12 out of 16 (i.e. 75%) plants were double transformants, defined as the frequency with which cells transformed with a first T-DNA contain a second unselected T-DNA. The frequency of double transformation obtained in this work is higher than that observed by Jones *et al.* (1987) and Jorgensen *et al.* (1987) who observed a frequency of just over 50% and De Neve *et al.* (1997) who found that 12 out of 27 transgenic plants (44%) were double transformants. De Block and Debrouwer (1991) evaluated the frequency of co-

transformation and linkage of a large number of *Brassica napus* transformants and obtained a co-transformation frequency of 37%. This result is also consistent with the findings of Depicker *et al.* (1985) who showed that the frequency of double transformation was much higher than is expected of individual transformation events if the T-DNAs were contained in one bacterium.

However when the seeds generated from the 12 double transformants were grown on selection medium only eight plants gave the expected Mendelian segregation of the two genes (Fig 4.6 and Table 4.1). In other words 75% of the T_1 plants contained the two genes in one locus. Considering that these plants were growing on selection media, it is plausible to think that the two genes were being expressed. This is particularly important as Jones *et al.* (1987) showed that the particular position of insertion of the T-DNA can influence the expression of the introduced gene. It is therefore conceivable to suggest that the two genes were preferentially integrated in transcriptionally active regions of the chromosome. This phenomenon has also been observed by Konez *et al.* (1989).

The progeny of line 207 gave a segregation ratio of 15:1 indicating that the two genes had integrated in two independent loci (Table 4.6). Thus, taken together these results confirm the findings of De Buck *et al.* (1999) that after *Agrobacterium*-mediated transformation, multiple T-DNAs frequently integrate at the same locus in the plant genome. They went on to show that this integration often results in the formation of inverted and direct repeats. However these inverted repeats cannot be amplified or analysed by PCR. The orientation of the T-DNAs could not be determined in this experiment due to the

unavailability of Southern blot analysis results. However it has been shown that co-transformed T-DNAs integrate as inverted copies, oriented in an inverted or tandem configuration in more than 70% of the transformants (De Neve *et al.* 1997). This observation is of particular importance in view of the correlation between transgene repeats and transgene silencing. It has been reported that genes or transgenes that are present in multiple copies as clustered units seem to have an enhanced susceptibility for both transcriptional and post-transcriptional silencing (Meyer and Saedler, 1996; Stam *et al.* 1997; De Neve *et al.* 1999). Thus frequent co-transformations, as a result of applying optimal transformation conditions, may be an important reason for silencing and variation of transgene expression in transgenic plants (De Buck *et al.* 1998).

In this experiment the progeny of three lines (lines 201, 219 and 223) could not grow on media supplemented with bialaphos (represented by Fig. 4.7A) despite the fact that these plants were generated from parents that contained the *bar* gene as demonstrated by PCR. These three plants were however able to grow on media supplemented with kanamycin (represented by Fig. 4.7B). These results indicated that the lack of *bar* expression was due either to the phenomenon of transgene silencing setting in early during development (Matzke and Matzke, 1995), or to the absence of an intact copy of the gene cassette due to rearranged transgenic sequences. De Buck *et al.* (1999) analysed nine direct or inverted T-DNA border junctions at the sequence level and showed that precise end-to-end fusions were found between two right border ends, whereas imprecise fusions and filler DNA were present in T-DNA linkages containing a left border. Their conclusion was that end-to-end ligation of double stranded T-DNAs occurs especially between right

T-DNA ends and that illegitimate recombination on the basis of microhomology, deletions, repair activities, and insertion of filler DNA is involved in the formation of left border T-DNA junctions. Based on these findings it is possible that the lack of *bar* expression observed in the three lines was a result of the formation of left border T-DNA junctions. Considering that Tingay *et al.* (1997) demonstrated that in different transgenic barley plants with up to at least ten copies of the *gus* gene there was no correlation between copy number and the levels of transgene expression observed, it is unlikely that the possible silencing observed in this experiment could have been due to the variation in copy number. However it is still interesting to note that the progeny of three out of twelve transgenic plants could not express the *bar* gene.

The fact that most of the transgenic plants contained the two genes in one locus is a very common one. However the reason for this phenomenon is subject to speculation. This, and other experiments used a derivative of a nopaline-type strain C58. In this work, linked loci were favoured despite the fact that the two genes were carried on two separate plasmids in a single strain. Remarkably, all the experiments that showed the integration of foreign DNA at multiple loci utilized the octopine LBA4404 strain of *Agrobacterium* (Spielman and Simpson, 1986; Komari *et al.* 1997). The method of transformation used and the type of plant or explant used do not affect the type of T-DNA integration (De Neve *et al.* 1997). Based on these observations it is plausible that the use of a derivative of an octopine strain may favour unlinked co-transformation.

In this experiment only one plant out of twelve double transformants (line 207) showed that the two genes had integrated at two independent loci. This low incidence of multiple independent integration sites makes the single strain method, with two genes on separate plasmids, a suitable option for introducing multigene families. Apart from the high frequency of transformation that the single strain method is known for, the method described in this experiment will be much easier to perform with respect to the construction of the plasmids used. In other experiments researchers have resorted to using a mixture method that has been shown to produce a low frequency of double transformants. Construction of co-integrate vectors that contain two T-DNAs for use in the single strain method will certainly be more difficult (Komari *et al.* 1997). The only setback with the method that has been described here is that the vectors need to be compatible. However this is a lesser problem than having to construct a special co-integrate vector. It is also possible that several compatible plasmids each carrying a separate gene can co-exist in a single *Agrobacterium* bacterium cell and still be able to transfer these genes into a single locus.

CHAPTER 5

CONCLUSIONS AND GENERAL DISCUSSION

The overall aim of this research was to investigate the possibility of using pTF-FC2 as a binary vector and also to develop a simple system for the generation of multigene transformants. In this Chapter I present an evaluation of the major findings of this research and, where appropriate, the implications that this might have in the field of plant biotechnology.

The introduction of genes into plants is now a basic technique. Most genes are introduced into plants via plasmid vector systems. T-DNA vectors must have the right and left borders. These vectors must either replicate in *Agrobacterium* on their own (binary vectors) (De Frammond *et al.* 1983; Hoekema *et al.* 1983) or be designed with a partner plasmid that does so (cointegrate vector; Zambryski *et al.* 1983; Fraley *et al.* 1985). Binary vectors rely on two plasmids, one with the T-DNA and another with the *vir* genes. The *vir* gene plasmid, often called a helper, is normally a Ti plasmid from which the T-DNA has been deleted. Helper plasmids in common use include the octopine type pAL4404 (Hoekema *et al.* 1983), the nopaline type pMP90 (Konez and Schell, 1986) and the succinomopine type EHA101 (Hood *et al.* 1986). Most binary vectors are derived from the wide host range plasmid RK2, e.g pBIN19 (Bevan, 1984), or alternatively from pVS1 e.g the pPZP series of vectors (Hajdukiewcz *et al.* 1994).

Plasmid pTF-FC2, like RSF1010 or their derivatives, offers a very attractive possibility for the development of novel binary vectors. These are likely to be adapted for the

transfer of bigger fragments of DNA than the plasmids that are currently in use. The *oriT* is the only region of the vector that is recognized and nicked by the MobA protein. Therefore the release of the DNA strand to be transferred to the plant cell will result from two successive nicks of the *oriT* site, in which the second nick occurs only after the initially nicked *oriT* site has been restored by lagging strand synthesis. In other words *oriT* plays the role of the right and left borders and the size of the transferred DNA corresponds to that of the entire plasmid.

The fact that the entire plasmid may integrate within the plant genome may be a cause for concern but this is not any different from the T-DNA binary vectors in which it was shown that up to 75% of the plants studied contained the vector backbone sequences (Kononov *et al.* 1997). In this case the authors confirmed a fact that scientists generally do not discuss and, that is if VirD2 serves as a 'pilot protein' to guide the T-strand from the bacterium to the plant cell, it should also be able to serve as a 'pilot protein' to initiate transfer of the non-T-DNA portion of a Ti plasmid (or the vector backbone sequences of a binary vector) to the plant. The association of VirD2 and the 5' end of the non-T-DNA portion of the Ti plasmid has already been shown (Durrenberger *et al.* 1989). Since then other scientists have also shown the transfer into plants of non-T-DNA sequences (Martineau *et al.* 1994; Ramanathan and Veluthambi, 1995). The fact that the *oriT* and the *mob* genes can be separated and still remain functional opens up the possibility of generating two smaller plasmids, one in which the genes of interest and *oriT* are resident and another one that supplies the *mob* functions in *trans*. Such an arrangement will make it possible to clone even bigger fragments of DNA than those that are currently be cloned

using the ordinary vectors. The low transformation efficiency of RSF1010 and its lack of precision in ligating the 5' end of the transferred DNA makes it less attractive as a binary vector (Bravo-Angel *et al.* 1999). However, the results that were obtained in this research show a specific advantage of pTF-FC2 in this regard. Ensuring that the genes of interest are all cloned upstream of the selectable marker and downstream of the *oriT nic* site will avoid any 3' deletion in the genes of interest. This has become a standard rule even when using T-DNA binary vectors. In this case the right border provides the *nic* site. The only problem in using pTF-FC2 derivatives as binary vectors could arise from the fact that they tend to inhibit T-DNA transfer by other binary vectors. This means that a pTF-FC2 derived binary vector cannot be used in the system of co-transformation described in Chapter 4 where a strain of *Agrobacterium* has to carry two binary vectors.

If integration of DNA took place at a single locus, then it would be plausible to think that such an integration was due to the use of a nopaline type strain C58. Even more surprising is the fact that in all cases where researchers performed co-transformation experiments using the octopine strain LBA4404 the integration pattern favoured multiple loci. If that be the case then the system described in Chapter 4 may have a dual purpose. The first one would be that it could be used as a way of generating multigene transformants. This is very important as most traits are polygenic in character. Secondly, by using the octopine strain LBA4404 the system may be used for the generation of unlinked loci. Such a scenario makes it possible to generate marker-free plants. The process of plant transformation usually utilizes a gene that confers antibiotic or herbicide (marker genes) resistance, along with the gene of interest to impart a desired trait.

Regenerating transformed cells in antibiotic-or herbicide-containing growth media permits selection of only those cells that have incorporated the foreign genes. Once transgenic plants have been selected, these marker genes serve no useful purpose but they continue to produce their gene products. It is for this reason, among others, that transgenic crop plants have generated a number of environmental and consumer concerns (Zechendorf, 1994). Of particular concern is the presence of clinically important antibiotic resistance gene products that could inactivate oral doses of the antibiotic (reviewed by Pucta, 2000; Daniell, 1999). Another concern is that the antibiotic resistance genes could be transferred to pathogenic microbes in the gastrointestinal tract or soil rendering them resistant to treatment with such antibiotics (Daniell *et al.* 2001). Risk assessment reports have argued that there are no scientific, health or safety reasons to restrict the use of the neomycin phosphotransferase (*nptII*) selectable marker gene (Nap *et al.* 1992; Fuchs *et al.* 1993). This may not be true of other selectable markers and as such necessitates a lengthy and expensive risk assessment process of each marker gene and its product. This could cause considerable delays in the release of a transgenic crop. Furthermore, regardless of the assurances provided in risk assessment reports, it is consumer acceptance of a product that governs its market performance (Gleave *et al.*1999). Therefore it would be sensible to alleviate perceived risks by eliminating the antibiotic resistance genes.

Komari *et al.* (1996) used what they called super binary vectors by virtue of the fact that they carried two separate T-DNAs on one plasmid in order to generate marker-free plants through *A. tumefaciens*-mediated co-transformation of higher plants. They used the

octopine LBA4404 strain and found that they obtained a high frequency of integration of the T-DNAs at unlinked loci using the single strain method. The frequency of co-transformation with the two T-DNAs was greater than 47%. However, the system described in Chapter 4 has a good potential for generating marker-free plants as the construction of a super binary vector with two T-DNAs may be more difficult than simply cloning the two T-DNAs on two separate plasmids. Furthermore the frequency of double transformation is likely to be higher than the 47% they obtained.

The results described in this work show that the mobilization functions of plasmid pTF-FC2 can transfer the plasmid carrying these functions into plant cells. This is the second naturally occurring plasmid, after RSF1010, that is known to perform this function. Both these plasmids are small wide-host-range plasmids that can be transferred amongst Gram-negative bacteria. Therefore this system, as Buchanan-Wollaston *et al.* (1987) proposed, has the potential to allow transfer of DNA from different bacteria into plant cells (Fig. 5.1). Based on these results the possibility of gene flow between Gram-negative bacteria and plants is higher than previously thought. This is of considerable evolutionary significance as it means that any gene normally found in Gram-negative bacterium could be transferred by a plasmid such as pTF-FC2 into plants. It is also very likely that the *mob* and *oriT* regions of other as yet unidentified plasmids could function in the same way as those of RSF1010 and pTF-FC2.

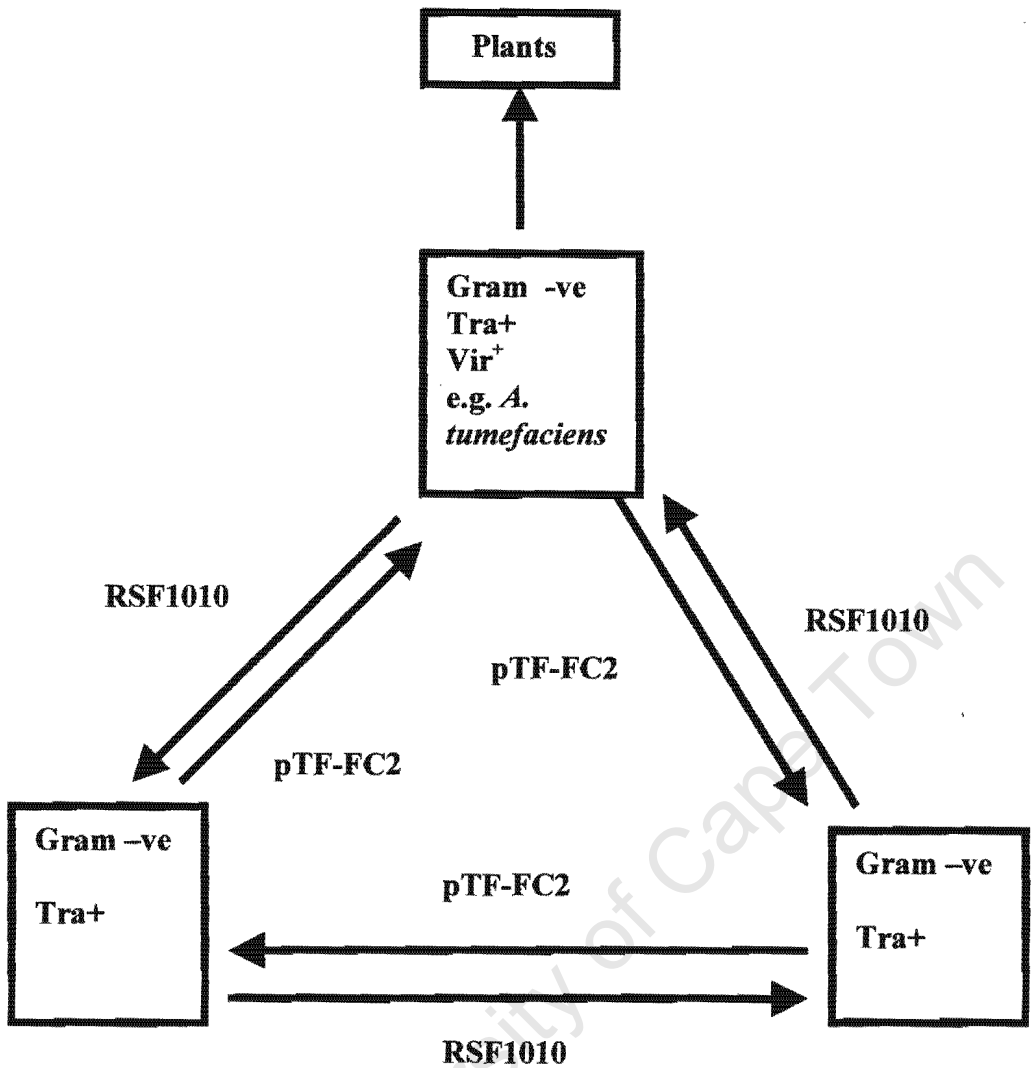


Fig. 5.1 Diagram to represent the potential spread of RSF1010 and pTF-FC2 amongst plant and bacterial populations. The plasmids can be mobilized into Vir^+ bacteria like *A. tumefaciens* and thence into plant cells, or they can be transferred amongst other Gram-negative bacteria. At any stage they could incorporate bacterial DNA which could then be transferred to the next recipient. Adapted from Buchanan-Wollaston *et al.* (1987).

Another an attractive feature of the pTF-FC2-derived binary vectors will be their use in the expansion of knowledge of the similarities between the transfer of T-DNA and conjugal plasmids. RSF1010 has already provided the much needed evidence that *vir* genes are functionally interchangeable with bacterial conjugal transfer genes and that this indicates a very strong similarity between the two (Winans, 1992). Plasmid pTF-FC2 brings with it additional features which will need to be studied further in order to understand and gain more insights into DNA transformation systems. So far indications are that conjugal transfer systems are more promiscuous than previously thought.

The results presented here make it possible to determine the direction of transfer of T-DNA although this was not part of the aims of this study. The plasmid genes were transferred in a polar fashion with the *mobC* end of *oriT* leading the way into the plant cell (Fig. 5.2). These results are consistent with those obtained by Kim and Meyer, (1989) who showed that the transfer of the plasmid R1162 (essentially identical to RSF1010) was unidirectional. This phenomenon has also been shown for T-DNA transfer into plants (Wang *et al.* 1984).

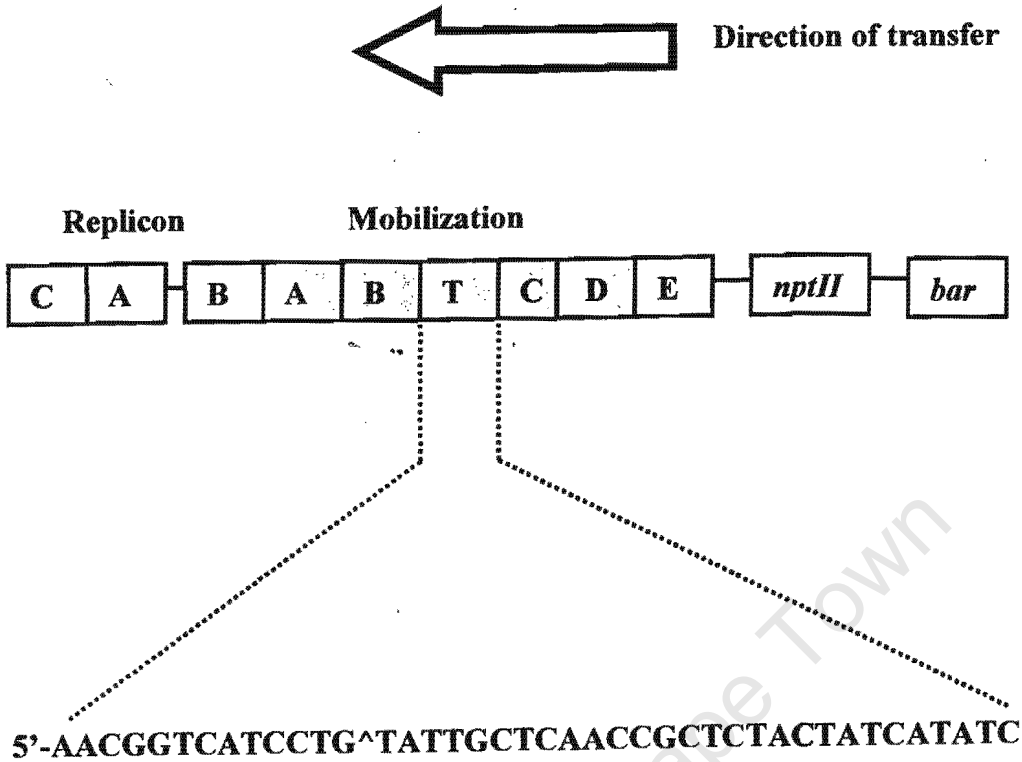


Fig 5.2 Simplified map of pDER-*bar* showing the location of *ori*T (**T**) within the region required for mobilization (shaded). The direction of transfer (indicated with an arrow) and the *nic* site (^) have been determined from this work.

In conclusion this work has shown that the *mob* and *oriT* mobilization functions of pTF-FC2 are able to promote its transfer to tobacco (Chapter 2). Furthermore the plasmid is likely to be more proficient in DNA transfer and integration than RSF1010 (Chapter 3). The results presented in Chapter 4 give a simple and efficient system for the generation of multigene plant transformants. Depending on the strain of *Agrobacterium* used this can be adapted to linked loci or multiple loci.

REFERENCES

Abdel-Monem M., Taucher-Scholz G. and Klinkert M-Q. (1983) Identification of *Escherichia coli* helicase I as the *traI* gene product of the F sex factor. Proceedings of the National Academy of Sciences USA **80**: 4659-4663.

Akiyoshi D.E., Morris R.O., Hinz R., Mischke B.S., Kosuge T., Garfield D.J., Gordon M.P. and Nester E.W. (1983) Cytokinin-auxin balance in crown gall tumours is regulated by specific loci in the T-DNA. Proceedings of the National Academy of Sciences USA **80**: 407-411.

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. Journal of Bacteriology **16**: 1046-1055.

An G. (1985) High efficiency transformation of cultured tobacco cells. Plant Physiology **79**: 568-570.

Alt-Moerbe J., Rak B. and Schroeder (1986) A 3.6 kb from the *vir* region of Ti plasmids contains genes responsible for border sequence-directed production of T-region circles in *E . coli*. EMBO Journal **5**: 1129-1135.

Ankenbauer R.G., Best E.A., Palanca C.A., and Nester E.W. (1991) Mutants of the *Agrobacterium tumefaciens virA* Gene Exhibiting Acetosyringone-Independent Expression of the *vir* Regulon. Molecular Plant-Microbe Interactions **4**(4): 400-406.

Anthony K.G., Klimke W. A., Manchak J., and Frost L. S. (1999) Comparisons of Proteins Involved in Pilus Synthesis and Mating Pair Stabilization from the Related Plasmids F and R100-1: Insights into the Mechanism of Conjugation. Journal of Bacteriology **181**(17): 5149-5159.

Ashby A.M., Watson M.D, Loake G.J. and Shaw C.H. (1988) Ti plasmid-specified chemotaxis of *Agrobacterium tumefaciens* C58C1 toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. Journal of Bacteriology **170**: 4181-4187.

Bainbridge G., Gokce I. and Lakey J.H. (1998) Voltage gating is a fundamental feature of porin and toxin beta-barrel membrane channels. FEBS Letters **43**(3): 305-308.

Bakkeren G., Koukolikova-Nicola Z., Grimsely N. and Hohn B. (1989) Recovery of *Agrobacterium tumefaciens* T-DNA from whole plants early after transfer. Cell **57**: 847-857.

Baltzer D., Pansegrau W. and Lanka E. (1994) Essential motifs of relaxase (TraI) and TraG proteins involved in conjugative transfer of plasmid RP4.

Banta L.M., Joerger R.D., Howitz V.R., Campbell A.M., and Binns A.N., (1994) Glu-255 Outside the Predicted ChvE Binding Site in VirA is Crucial for sugar Enhancement of Acetosyringone Perception by Agrobacterium tumefaciens. Journal of Bacteriology **176:** 3242-3249.

Barker R.F., Idler K.B., Thompson D.V., and Kemp J.D., (1983) Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTil5955. Plant Molecular Biology **2**: 335-350.

Barth P.T., Grinter N.J. and Bradley D.E. (1978) Conjugal transfer system of plasmid RP4: analysis by transposon 7 insertion. Journal of Bacteriology **133**: 43-52.

Becker D., Kemper E., Schell J. and Masterson R. (1992) New plant binary vectors with selectable markers located proximal to the left T-DNA border. Plant Molecular Biology **20**: 1195-1197.

Bedbrook J.R., Lehrach H., and Ausubel F.M. (1979) Directive segregation is the basis of ColE1 plasmid incompatibility. Nature **281**: 447

Beijersbergen A., Dulk-Ras A.D. Schilperoort R.A. and Hooykaas P.J.J. (1992) Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. Science **256**: 1324-1327.

Berger B. R., and Christie P.J. (1993) The *Agrobacterium tumefaciens virB4* Gene Product Is an Essential Virulence Protein Requiring an Intact Nucleoside Triphospate-Binding Domain. Journal of Bacteriology **175**(6): 1723-1734.

Berger B. R., and Christie P.J. (1994) Genetic complementation analysis of the Agrobacterium tumefaciens *virB* operon : *virB2* through *virB11* are essential virulence . Journal of Bacteriology **176**: 3646-3660.

Bevan M. (1984) Binary Agrobacterium vectors for plant transformation. Nucleic Acids Research **12**: 8711-8721.

Bhattacharjee M., Rao X-M., and Meyer R.J. (1992) Role of the Origin of Transfer in Termination of Strand Transfer during Bacterial Conjugation. Journal of Bacteriology **174**(20): 6659-6665.

Bhattacharjee M.K. and Meyer R.J. (1991) A segment of a plasmid gene required for conjugal transfer encodes a site specific, single-strand DNA endonuclease and ligase. Nucleic Acids Research **19**: 1129-1137.

Bhattacharjee M.K. and Meyer R.J. (1993) Specific binding of MobA, a plasmid encoded protein involved in the initiation and termination of conjugal DNA transfer, to single stranded *ori*T DNA. Nucleic Acids Research **21**: 4563-4568.

Binns A.N. and Thomashow M.F. (1988) Cell biology of Agrobacterium infection and transformation of plants. Annual Review of Microbiology **42**: 575-606.

Binns A.N., Beaupre C.E. and Dale E.M. (1995) Inhibition of VirB-mediated transfer of diverse substrates from *Agrobacterium tumefaciens* by the IncQ plasmid RSF1010. Journal of Bacteriology **177**: 4890-4899.

Bogorad L. (2000) Engineering chloroplasts: an alternative site for foreign genes, proteins, reactions, and products. Trends in Biotechnology **18**: 257-263.

Bonnard G., Tinland B., Paulus F., Szegedi E. and Otten L. (1989) Nucleotide sequence, evolutionary origin and biological role of a rearranged cytokinin gene isolated from a wide-host-range biotype III Agrobacterium strain. Molecular and General Genetics **216**: 428-438.

Bradshaw H.D. Jr., Traxler B.A., Minkley E.G. Jr., Nester E.W. and Gordon M.P. (1990) Nucleotide sequence of the *traI* (Helicase I) gene from the sex factor F. Journal of Bacteriology **172**: 4127-4131.

Brasch M. A., and Meyer R.J. (1987) A 38 Base-pair Segment of DNA is required in *cis* for Conjugative Mobilization of Broad Host-range Plasmid R1162. J. Mol. Biol. **198**:361-369.

Bravo-Angel A.M., Hohn B. and Tinland B. (1998) The omega sequence of VirD2 is important but not essential for efficient transfer of T-DNA by *Agrobacterium tumefaciens*. Molecular and Plant Microbe-Interactions **11**: 57-63.

Bravo-Angel A.M.., Gloeckler V., Hohn B. and Tinland B. (1999) Bacterial conjugation MobA mediates integration of complex DNA structures into plant cells. Journal of Bacteriology **181** (18): 5758-5765.

Broek A.V., and Vanderleyden J. (1995) The Role of Bacterial Motility, Chemataxis, and Attachment in Bacteria- Plant Interactions. Molecular and Plant-Microbe Interactions **8**(6): 800-810.

Buchanan-Wollaston V., Passiatore J.E. and Cannon F. (1987) The *mob* and *ori*T of a bacterial plasmid promote its transfer to plants **328**: 172-175.

Bundock. P., den Dulk-Ras., Beijersbergen A. and Hooykaas P.J.J. (1995) Trans-kingdom T-DNA transferfrom Agrobacterium to *Saccharomyces cerevisiae*. EMBO Journal **14**: 3206-3214.

Cabezon E., Ignacio Sastre J., and de la Cruz F. (1997) Genetic evidence of a coupling role for the TraG protein family in bacterial conjugation. Molecular and General Genetics **254**: 400-406.

Cangelosi G.A., Huang L., Martinetti G., Leigh J.A., Lee C.C., and Theines C. (1989) Role of *Agrobacterium tumefaciens chvA* protein in export of β-1,2-glucan. Journal of Bacteriology **171**: 1609-1615.

Cangelosi G.A., Ankenbauer R.G., and Nester E.W. (1990) Sugars induce the Agrobacterium Virulence genes through a periplasmic binding protein and a transmemrane signal protein. Proceedings of the National Academy of Sciences USA **87**: 6708-6712.

Caplan A., Herrera-Estrella L., Inze D., Van Haute E., Van Montagu M., Schell J. and Zambryski P. (1983) Introduction of genetic material into plant cells. Science **222**: 815-821.

Chang C-H. and Winans S.C. (1992) Functional Roles Assigned to the Periplasmic, Linker, and Receiver Domains of the *Agrobacterium tumefaciens* VirA Protein. Journal of Bacteriology **174**(21): 7033-7039.

Chang C-H. and Winans S.C. (1996) Resection and Mutagenesis of the Acid pH-Inducible P2 promoter of the *Agrobacterium tumefaciens virG* Gene. Journal of Bacteriology **178**(15): 4717-4720.

Chang C-H., Zhu J., and Winnans S.C. (1996) Pleitropic phenotypes caused by genetic ablation of the receiver Module of the *Agrobacterium tumefaciens* VirA Protein. Journal of Bacteriology **178**(15): 4710-4716.

Chattoraj D. K., (2000) Control of plasmid DNA replication by iterons: no longer paradoxical. Molecular Microbiology **37**(3): 467-476.

Chen C-Y. and Kado C.I. (1994) Inhibition of *Agrobacterium tumefaciens* oncogenecity by the *osa* gene of pSa. Journal of Bacteriology **176**: 5697-5703.

Chen L., Marmey P., Taylor N.J., Brizard J-P., Espinoza C., D'Cruz C., Huet H., Zhang S., De Kochko A., Beachy R.N. and Fauquet C.M. (1998) Expression and inheritance of multiple transgenes in rice plants. Nature Biotechnology **16:** 1060-1064.

Christie P.J., Ward J.E. Jr.,Gordon M.P., and Nester E.W. (1989) A gene required for transfer of T-DNA to plants encodes an ATPase with autophosphorylating activity. Proceedings of the National Academy of Sciences USA **86**: 9677-9681.

Christie P.J. (1997) *Agrobacterium tumefaciens* T-complex transport apparatus; a paradigm for anew family of multifunctional transporters in eubacteria. Journal of Bacteriology **179**: 3085-3094.

Christou P., Ford T.L. and Kofron M. (1991) Production of (*Oryxa sativa L.*) plants from from Agronomically important *Indica* and *Japonica* varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/technology **9**: 957-962.

Chyi Y.S., Jorgensen R.A. Goldstein D., Tanksely S.D. and Loaiza-Figueroa F. (1986) Locations and stability of Agrobacterium-mediated T-DNA insertions in the *Lycopersicon* genome. Molecular and General Genetics **204**: 64-69.

Citovsky V., Zupan J., Warnick D. and Zambryski P. (1992) Nuclear localization of Agrobacterium VirE2 protein in plant cells. Science **256**: 1802-1805.

Citovsky V., Warnick D. and Zambryski P. (1994) Nuclear import of Agrobacterium VirD2 and VirE2 proteins in Maize and tobacco. Proceedings of the National Academy of Sciences USA **91**: 3210-3214.

Clennel A-M. Johnston B. and Rawlings D.E. (1995) Structure and function of Tn5467, a Tn21-like transposon located on the *Thiobacillus ferooxidans* broad-host-range plasmid pTF-FC2. Applied environmental microbiology **61**: 4223-4229.

Close S.M.and Kado C.I. (1991) The *osa* gene of pSa encodes a 21.1 kilodalton protein that suppresses *Agrobacterium tumefaciens* oncogenicity. Journal of Bacteriology **173**: 5449-5456.

Close S.M.and Kado C.I. (1992) A gene near the plasmid pSa origin of replication encodes a nuclease. Journal of Molecular Microbiology **6**: 521-527.

Cohen A., Fisher W.D., Curtis III R. and Adler H.I. (1986) DNA isolate from *Escherichia coli* mini cells mated with F^+ cells. Proceedings of the National Academy of Sciences USA **61**: 61-68.

Conley D.L., and Cohen S. N.,(1995) Isolation and Characterization of Plasmid Mutations That Enable Partitioning of pSC101 Replicons Lacking the Partition (*par*) Locus. Journal of Bacteriology **177**(4): 1086-1089.

Cornish A., Greenwood J.A. and Jones C.W. (1988) Binding-protein-dependent sugar transport by *Agrobacterium radiobacter* grown in glucose limited continuous culture. Journal of General Microbiology **134**: 3099-3110.

Cornish A., Greenwood J.A. and Jones C.W. (1989) Binding-protein-dependent sugar transport by *Agrobacterium radiobacter* and *A. tumefaciens* grown in glucose limited continuous culture. Journal of General Microbiology **135**: 3001-3013.

Crickmore L. and Ellar D. (1992) Involvement of a possible chaperonin in the efficient expression of a cloned cryIIA δ-endotoxin gene in *Bacillus thuringiensis*. Molecular Microbiology **6**: 1533-1537.

Crickmore N., Wheeler V. and Ellar D. (1994) Use of an operon fusion to induce expression and crystallization of *Bacillus thuringiensis* δ-endotoxin encoded by a cryptic gene. Molecular and General Genetics **242**: 365-368.

Dale E.M., Binns A.N., and Ward J.E. Jr., (1993) Construction and Characterization of Tn*5virB*, a Transposon That Generates Nonpolar Mutations, and Its Use To Define *virB8* as an Essential Virulence Gene in *Agrobacterium tumefaciens*. Journal of Bacteriology **175**(3): 887-891.

Daniell H. (1999) GM crops: public perception and scientific solutions. Trends in Plant science. **4**: 467-469.

Daniell H. (1999a) New tools for chloroplast genetic engineering. Nature Biotechnology **17**: 855-856.

Daniell H., Datta R., Gray S., Varma S. and Lee S. (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nature Biotechnology **16**: 345-348.

Daniell H., Datta R., Varma S., Gray S., and Lee S-B., (!998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nature Biotechnology **16**:345

Daniell H., Muthukumar B. and Lee S.B. (2001) Marker-free transgenic plants: engineering the chloroplast genome without the use of antibiotic selection. Current Genetics **39**: 109-116.

Das A. (1988) *Agrobacterium tumefaciens vir*E operon encodes a single stranded DNA binding protein. Proceedings of the National Academy of Sciences USA **85**: 2909-2913.

Das A. (1994) Regulation of *Agrobacterium tumefaciens* virulence gene expression.In Molecular Mechanisms of Bacteria Virulence. Kado C.I. aaand Crosa J.H. Kluwer Academic Publishers. Netherlands, 477-489.

Das A and Xie Y-H., (1995) Replication of the broad-host range plasmid RK2: isolation and characterization of a spontaneous deletion mutant that can replicate in *Agrobacterium tumefaciens* but not in *Escherichia coli.* Mol Gen Genet **246**: 309-315.

De Block M. and Debrouwer D. (1991) Two T-DNAs co-transformed *into Brassica napus* by a double Agrobacterium infection are mainly integrated at the same locus. Theory of Applied Genetics **82**: 257-263.

De Buck S., Jacobs A., Van Montagu M. and Depicker A. (1998) *Agrobacterium tumefaciens* transformation and co-transformation frequencies of *Arabidopsis thaliana* root explants and tobacco protoplasts. Molecular and Plant Microbe Interactions **11**: 449-457.

De Buck S., Jacobs A., Van Montagu M. and Depicker A. (1999) The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. The Plant Journal **20** (3): 295-304.

De Cosa B., Moar W., Lee S-B., Miller M. and Daniell H. (2001) Over-expression of the *Bt cry*2Aa2 in chloroplasts leads to formation of insecticidal crystals. Nature Biotechnology **19**: 71-74.

De Framond A.J., Back E.W., Chilton W.S., Kays L. and Chilton M-D. (1986) Two unlinked T-DNAs can transform the same tobacco plant cell and segregate in the F1 generation. Molecular and General Genetics **202**: 125-131.

De Greve H., Decraemer H., Seurinck J., Van Montagu M. and J. Schell (1981) The functional organization of the octopine *A. tumefaciens* plasmid pTiB6S3. Plasmid **6**: 235-248.

Delcour A.H. (1997) Function and modulation of bacterial porins: insights from electrophysiology. FEMS Microbiological Letters **151**(2): 115-123.

De Neve M., De Buck S., Jacobs A., Van Montagu M. and Depicker A. (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from ligation of separate T-DNAs. The Plant Journal **11**: 15-29.

De Neve M., De Buck S., De Wilde C., Van Houdt H.I., Van Montagu M. and Depicker A. (1999) Gene silencing results in instability of antibody production in transgenic plants. Molecular and General Genetics **260** (6): 582-592.

De Vos G. and Zambryski P. (1989) Expression of Agrobacterium nopaline-specific VirD1, VirD2, and VirC1 proteins and their requirement for T-strand production in *E. coli.* Molecular Plant-Microbe Interactions **2**: 43-52.

Dehio C., and Meyer M. (1997) Maintenance of Broad-Host-Range Incompatibility Group P and Group Q Plasmids and Transposition of Tn5 in *Bartonella henselae* following Conjugal Plasmid Transfer from *Escherichia coli.* Journal of Bacteriology **179**(2): 538-540.

Del Solar G. and Espinosa M. (2000) Plasmid copy number control: an ever-growing story. Molecular Microbiology **37**(3): 492-500.

Denis M., Renard M. and Krebbers E. (1995) Isolation of homozygous transgenic Brassica napus lines carrying a seed specific chimaeric 2S albumin gene and determination of linkage relationships. Molecular breeding **1**: 143-153.

Deng W., Chen L., Peng W-T., Liang X., Sekiguchi S., Gordon M.P. and Nester E.W. (1999) VirE1 is a specific molecular chaperone for the exported single-stranded-DNA binding-protein VirE2 in Agrobacterium. Molecular Microbiology **31**: 1795-1808.

Depicker A., Herman L., Jacobs A., Schell J., and Van Montagu M., (1985) Frequencies of simultaneous transformation with different T-DNAs and their relevance to the Agrobacterium/plant cell interaction. Molecular and General Genetics **201**: 477-484.

Derbyshire K.M. and Willets N. (1987) Mobilization of the non-conjugative plasmid RSF1010: a genetic analysis of its origin of transfer. Molecular and General Genetics **206**: 154-160.

Derbyshire K.M., Hatfull G., and Willets N. (1987) Mobilization of the non-conjugative plasmid RSF1010: A genetic and DNA sequence analysis of the mobilization region. Molecular and General Genetics **206**:161-168.

Deroles S.C. and Gardner R.C. (1988) Analysis of the T-DNA structure in a large number of transgenic petunias generated by Agrobacterium-mediated transformation. Plant Molecular Biology **11**: 365-377.

Di Laurenzio L., Frost L.S. and Paranchych W. (1992) The TraM protein of the conjugative plasmid F binds to the origin of transfer of the F and the ColE1 plasmids. Molecular Microbiology **6**: 2951-2959.

Disque-Kochem C. and Dreiseikelmann B. (1997) The cytoplasmic DNA-binding protein TraM binds to the inner membrane protein TraD in vitro. Journal of Bacteriology **179**: 6133-6137.

Dorrington R. and Rawlings D. (1990) Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity beween pTF-FC2 and the IncQ plasmids. Journal of Bacteriology **172**: 5697-5705.

Dorrington R., Bardien S. and Rawlings D. (1991) The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*. Gene **108**: 7-14.

Doty S.L., Chang M., and Nester E.W. (1993) The Chromosomal Virulence Gene, *chvE*, of *Agrobacterium tumefaciens* is regulated by a LysR family member. Journal of Bacteriology **175**(24): 7880-7886.

Doty S.L., Yu M.C., Lundin J.I., Heath J.D., and Nester E.W. (1996) Mutational analysis of the input domain of the VirA protein of *Agrobacterium tumefaciens*. Journal of Bacteriology **178**(4): 961-970.

Drummond M. (1979) Crown gall disease. Nature **281**: 343-346.

Durrenberger F., Crameri A., Hohn B. and Koukolikova-Nicola Z. (1989) Covalently bound VirD2 of *Agrobacterium tumefaciens* protects the the T-DNA from exonucleolytic degradation. Proceedings of the National Academy of Sciences USA **86**: 9154-9158.

Ebinuma H., Sugita K., Matsunaga E., and Yamakado M. (1997) Selection of marker-free isopentenyl transferase gene. Proceedings of the National Academy of Sciences USA **94**: 2117-2121.

Escudero J. and Hohn B. (1997) Transfer and integration of T-DNA without cell injury in the host plant. Plant cell **9**: 2135-2142.

Farrand S., Kado C.I. and Ireland C.R. (1981) Suppression of tumorigenecity by the IncW R plasmid pSa in *Agrobacterium tumefaciens*. Molecular and General Genetics **181**: 44-51.

Fekete R. A., and Frost L. S. (2000) Mobilization of Chimeric *oriT* Plasmids by F and R100-1: Role of Relaxosome Formation in defining Plasmid Specificity. Journal of Bacteriology **182**(14): 4022-4027.

Fernandez D., Dang T.A.T., Spudich G.M., Zhou X-R., Berger B.R and Christie P.J. (1996) The *Agrobacterium tumefaciens vir*B7 gene product, a proposed component of the T-complex transport apparatus, is a membrane associated lipoprotein exposed at the periplasmic surface. Journal of Bacteriology **178**: 3156-3157.

Fernandez D., Dang T.A.T., Spudich G.M., Zhou X-R. and Christie P.J. (1996) The *Agrobacterium tumefaciens vir*B7 lipoprotein is required for stabilization of *vir*B proteins during assembly of the T-complex apparatus. Journal of Bacteriology **178**: 3168-3176.

Filichkin S.A. and Gelvin S.B. (1993) Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* VirD1, VirD2 endonuclease . Molecular Microbiology **8**: 915-926.

Finberg K.E., Muth T.R., Young S.P., Maken J.B., Heiritter S.M., Binns A.N. and Banta L. (1995) Interactions of virB9, -10 and –11 with the membrane fraction of *Agrobacterium tumefaciens*: solubility studies provide evidence for tight associations. Journal of Bacteriology **177**: 4881-4889.

Fraley R.T., Rodgers S.G., Horsch R.B., Eichholtz D.A., Flick J.S., Fink C.L., Hoffman N.L., and Sanders P.R. (1985) The Sev System: a new disarmed Ti plasmid vector system for plant transformation. Bio/Technology **3**: 629-635.

Frey J. and Bagdasarian M. (1989) The molecular biology of IncQ plasmids, in C.M. Thomas (ed.) Promiscuous plasmids of Gram-negative bacteria. Academic Press London. 79-94.

Frey J., Bagdasarian M.M. and Bagdasarian M. (1992) Replication and copy number control of the broad-host-range plasmid RSF1010. Gene **113**: 101-106.

Frost L.S. (1993) Bacterial conjugation, ed. D.B. Clewell. New York: Plenum. 189-212.

Frost L.S., Ippen-Ihler and Skurray R.A. (1994) Analysis of the sequence and gene products of the F sex factor. Microbiological Reviews **58**(2): 162-210.

Fu Y.H., Tsai M., Luo Y.N. and Deonier R.C. (1991) Deletion analysis of the F plasmid *oriT* locus. Journal of Bacteriology **173**: 1012-1020.

Fuchs R.L., Heeren R.A., Gustafson R.E., Rogan G.J. Bartnicki D.E., Leimgruber R.M., Finn R.F., Hershman A. and Berberich S.A. (1993) Safety assessment of the neomycin phoshotransferase II (*nptII)* protein. Bio/Technology **11**: 1537-1547.

Fullner K.J., Stephens M. and Nester E.W. (1994) An essential virulence protein of *Agrobacterium tumefaciens*, *vir*B4, requires an intact mononucleotide binding domain to function in transfer of DNA. Molecular and General Genetics **245**: 705-715.

Fullner K.J., Lara K.C. and Nester E.W. (1996) Pilus assembly by T-DNA transfer genes. Science **273**: 1107-1109.

Fullner K.J. (1998) Role of Agrobacterium *vir*B genes in the transfer of T-complexes and RSF1010. Journal of Bacteriology **180**: 430-434.

Furuya N. and Komano T. (2000) Initiation and termination of DNA transfer during conjugation of IncI1 Plasmid R64: roles of two sets of inverted repeat sequences within *oriT* in termination of R64 Transfer. Journal of Bacteriology **182**(11): 3191-3196.

Gabant P., Newman P., Taylor D., and Couturier M. (1993) Isolation and location on the R27 map of two replicons and incompatibility determinant specific for IncHI1 plasmids. Journal of Bacteriology **175**(23): 7697-7701.

Garfinkel D.J. and Nester E.W. (1980) *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. Journal of Bacteriology **144**: 732-743.

Ge B. ***et al.*** (1998) Differential effects of helper proteins encoded by the *cry*2A and *cry*II2A operons on the formation of cry2A inclusions in *Bacillus thuringiensis*. FEMS Microbiology Letters **165**: 35-41.

Gelvin S. B. (1998) Agrobacterium VirE2 Proteins Can Form a Complex with T Strands in the Cytoplasm. Journal of Bacteriology **180**(16): 4300-4302.

Gelvin S.B. (2000) Agrobacterium and plant genes involved in T-DNA transfer and integration. Annual Review of Plant Physiology and Plant Molecular Biology **51**: 223-256.

Gerdes K., Gultiayev A.P., Franch T., Perdersen K. and Mikkelson N.D. (1997) Antisense RNA-regulated programmed cell death. Annual Review of Genetics **31**: 1-34.

Gerdes K., Moller-Jansen J. and Jensen R.B. (2000) Plasmid and chromosome partitioning surprises from phylogeny. Molecular Microbiology **37**(3): 455-466.

Ghai J. and Das A. (1989) The *virD* operon of *Agrobacterium tumefaciens* Ti plasmid: a DNA relaxing enzyme. Proceedings of the National Academy of Sciences USA **86**: 3109-3113.

Gheysen G., Villarrroel R. and Van Montagu M. (1991) Illegitimate recombination in plants: A model for T-DNA integration. Genes and Development **5**: 287-297.

Giddings G., Allison G., Brooks D. and Carter A. (2000) Transgenic plants as factories for biopharmaceuticals. Nature Biotechnology **18**: 1151-1155.

Gietl C., Koukolikova-Nicola Z and Hohn B. (1987) Mobilization of T-DNA from Agrobacterium to plant cells involves a protein that binds single stranded DNA. Proceedings of the National Academy of Sciences USA **84**: 9006-9010.

Gleave A.P. (1992) A versatile binary vector system with a T-DNA organizational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Molecular Biology **20**: 1203-1207.

Gleave A.P., Mitra D.S., Mudge S.R. and MorrisB.A.M. (1999) Selectable marker-free transgenic plants without sexual crossing:transient expression of *cre* recombinase and use of a conditional lethal dominant gene. Plant Molecular Biology **40**: 223-235.

Gromely E.P. and Davies J. (1991) Transfer of plasmid RSF1010 by conjugation from *Escherichia coli* to *Streptomyces lividans* and *Mycobacterium smegmatis*. Journal of Bacteriology **173**: 6705-6708.

Grevelding C., Fantes V., Kemper E., Schell J. and Masterson R. (1993) Single-copy insertions in Arabidopsis are the predominant form of integration in root-derived

transgenics whereas multiple transformations are found in leaf discs. Plant Molecular Biology **23**: 847-860.

Guiney D.G. and Lanka E. (1989) Conjugative transfer of IncP plasmids, in C. Thomas (ed.) Promiscuous plasmids of Gram-negative bacteria. Academic Press, London. 27-56.

Guiney D.G. (1993) Bacterial conjugation, ed. D.B. Clewell. New York: Plenum. 75-100.

Hadi M.Z., McMullen M.D. and Finer J.J. (1996) Transformation of 12 different plasmids into soybean via particle bombardment. Plant cell Reporter **15**: 500-505.

Hajdukiewicz P., Svab Z. and Maliga P. (1994) The small, versatile pPZP of Agrobacterium binary vectors for plant transformation. Plant Molecular Biology **25**: 989-984.

Hamilton C. M., Lee H., Li P-L., Cook D. M., Piper K.R., von Bodman S. B., Lanka E., Ream W., and Farrand S. K. (2000) TraG from RP4 and VirD4 from Ti Plasmids confer relaxosome specificity to the conjugal transfer system of pTiC58. Journal of Bacteriology **182**(6): 1541-1548.

Hamilton C.M. Frary A., Lewis C. and Tanksley S.D. (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. Proceedings of the National Academy of Sciences USA **93**: 9975-9979.

Han D.C., Chen C.Y., Chen Y.F. and Winans S.C. (1992) Altered-function mutations of the transcriptional regulatory gene virG of *Agrobacterium tumefaciens*. Journal of Bacteriology **174**: 7040-7043..

Hansen G., Larribe M., Vaubert D., Tempe J., Biermann B., Montaya A.L., Chilton M-D. and Brevet J. (1991) *Agrobacterium rhizogenes* pRi8196 T-DNA: mapping and DNA sequence of functions involved in mannopine synthesis and hairy root differentiation. Proceedings of the National Academy of Sciences USA **88**: 7763-7767.

Hansen G., Tempe J. and Brevet J. (1992) A T-DNA transfer enhancer sequence in the vicinity of the right border of *Agrobacterium rhizogenes* pRi8196. Plant Molecular Biology **20**: 113-122.

Hansen G. and Chilton M.D. (1999) Lessons in gene transfer to plants by a gifted microbe. Current Topics in Microbiology and Immunology **240**: 21-58.

Heath J.D., Boulton M.I., Raineri D.M., Doty S.L., Mushegian A.R., Charles T.C., Davies J.W., and Nester E.W. (1997) Discrete regions of the sensor protein VirA determine the strain-specific ability of Agrobacterium to agroinfect maize. MPMI **10**(2): 221-227.

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

Henderson D. and Meyer R. (1999) The MobA-linked primase is the only replication protein R1162 required for conjugal mobilization. Journal of Bacteriology **181**(9): 2973-2978.

Herman L., Jacobs., Van Montagu M. and Depicker A. (1990) Plant chromosome/marker gene fusion assay for study of normal and truncated T-DNA integration events. Molecular and General Genetics **224**: 248-256.

Herrera-Estrella A., Chen Z., Van Montagu M. and Wang K. (1988) VirD proteins of Agrobacterium proteins are required for the formation of a covalent DNA-protein complex at the 5' terminus of T-strand molecules. EMBO Journal 7:4055-4062.

Herrera-Estrella A., Van Montagu M. and Wang K. (1990) A bacterial peptide acting as a plant nuclear targeting signal:the amino terminal of Agrobacterium VirD2 protein directs a β-galactosidase fusion protein into tobacco nuclei. Proceedings of the National Academy of Sciences USA **87**:9534-9537.

Hoekema A., Hirsch P.R., Hooykaas P.J.J. and Schilperoort R.A. (1983) A binary plant vector strategy based on separation of *vir* and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature **303**: 179-180.

Holsters M., Silva B., Van Vliet F., Genetello C., De Block M., Dhaese P., Depicker A., Inze D., Engler G., Villarroel R., Van Montagu M. and Schell J. (1980) The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. Plasmid **3**: 212-230.

Hood E.E., Helmer G.L., Fraley R.T. and Chilton M.D. (1986) The hypervirulence of Agrobacterium tumefaciens A281 is encoded in a region of pTiBo542 outside of T-DNA. Journal of Bacteriology **168**: 1291-1301.

Horsch R.B., Fry J.E., Hoffman N.L., Eichholtz D., Rogers S.D. and Fraley R.T. (1985) A simple and general method of transferring genes into plants. Science **227**: 1229-1231.

Howard E.A., Winsor B.A., De Vos G. and Zambryski P. (1989) Activation of the T-DNA process in Agrobacterium results in the generation of a T-strand-protein complex : tight association of VirD2 with 5' ends of T-strands. Proceedings of the National Academy of Sciences USA **86**: 4017-4021.

Howard E.A., Zupan J.R., Citovsky C. and Zambryski P. (1992) The VirD2 protein of *Agrobacterium tumefaciens* contains a C-terminal bipartite nuclear localization signal: implications for nuclear uptake in DNA in plant cells. Cell **68**: 109-118.

Howard M.T., Nelson W.C. and Matson W.S. (1995) Stepwise assembly of a relaxosome at the F plasmid origin of transfer. Journal of Biological Chemistry **270**(47): 28381-28386.

Hoykaas P.J.J. (1989) Transformation of plant cells via Agrobacterium. Plant Molecular Biology **13**: 327-336.

Hoykaas P.J.J. and Beijersbergen A.G.M. (1994) The virulence system of *Agrobacterium tumefaciens*. Annual Review of Phytopathology 32: 157-179.

Huang M.-L., Cangelosi G.A., Halperin W. and Nester E.W. (1990) A chromosomal Agrobacterium tumefaciens gene required for effective plant signal transduction. Journal of Bacteriology **172**: 1814-1822.

Ippen-Ihler E.A. and Minkley E.G.J. (1986) The conjugation system of F, the fertility factor of *Escherichia coli*. Annual Review of Genetics **20**: 593-624.

Jagura-Burdzy G., Khanim F., Smith C.A., and Thomas C.M. (1992) Crosstalk between plasmid vegetative replication and conjugative transfer: repression of the *trfA* operon by *trbA* of broad host range plasmid RK2. Nucleic Acids Research **20**(15): 3939-3944.

Jasper F., Konez C., Schell J. and Steinbiss H-H. 1994) Agrobacterium T-strand production *in vitro*: Sequence-specific cleavage and 5' protection of single stranded DNA templates by purified VirD2 protein. Proceedings of the National Academy of Sciences USA **91**: 694-698.

Jayaswal R.K., Veluthambi K., Gelvin S.B. and Slightom J.L. (1987) Double stranded T-DNA cleavage and the generation of single stranded T-DNA molecules in *E. coli* by a virD-encoded border-specific endonuclease from *A. tumefaciens*. Journal of Bacteriology **169**: 5035-5045.

Jefferson R.A., (1989) The GUS reporter gene system. Reprinted from Nature **342**(6251): 837-838.

Jen G.C., and Chilton M-D. (1986) Activity of T-DNA Borders in Plant Cell Transformation by Mini-T Plasmids. Journal of Bacteriology **166**(2): 491-499.

Jensen R.B. and Gerdes K. (1995) Programmed cell death in bacteria: proteic plasmid stabilization systems. Molecular Microbiology **17**: 211-220.

Ji J.M., Martinez A., Dabrowski M., Veluthambi K., Gelvin S.B. and Ream W. (1988) The *overdrive* enhancer sequence stimulates production of T-strands from the Agrobacterium tumor inducing plasmid. Molecular and Cell Biology **101**: 19-31.

Jin S., Prusti R.K., Roitsch T., Ankenbauer R.G. and Nester E.W. (1990) Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role of the biological activity of VirG. Journal of Bacteriology. **172**: 4945-4950.

Jin S., Prusti R.K., Roitsch T., Ankenbauer R.G., Gordon M.P. and Nester E.W. (1990) The VirA protein of *Agrobacterium tumefaciens* is autophosphorylated and is essential for *vir* gene induction. Journal of Bacteriology.**172**: 525-530.

Jin S., Roitsch T., Christie P.J. and Nester E.W. (1990) The regulatory VirG protein specifically binds to a *cis*-acting regulatory sequence involved in transcriptional activation of *Agrobacterium tumefaciens* virulence genes. Journal of Bacteriology **172**: 531-537.

Jones J.D.G., Gilbert D.E., Grady K.L. and Jorgensen R.A. (1987) T-DNA structure and gene expression in petunia plants transformed by *Agrobacterium tumefaciens* C58 derivatives. Molecular and General Genetics **207**: 478-485.

Jones J.D.G., Svab Z., Harper E.C., Hurwitz C.D. and Maliga P. (1987a) A dominant nuclear streptomycin resistant marker for plant transformation. Molecular and General Genetics **210**: 86-91.

Jones A.L., Shirasu K. and Kado C.I. (1994) The product of the *virB4* gene promotes the accumulation of VirB3 protein. Journal of Bacteriology **176**: 5255-5261.

Jorgensen R., Snyder C. and Jones J.D.G. (1987) T-DNA is organized predominantly in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Molecular and General Genetics **207**: 471-477.

Joos H., Timmermann B., Van Montagu M. and Schell J. (1983) Genetic analysis of transfer and stabilization of Agrobacterium DNA in plant cells. EMBO Journal **2**: 2152-2160.

Jouanin L., Bouchez D., Drong R.F., Tepfer D. and Slightom J.L. (1989) Analysis of TR-TDNA/plant junctions in the plant genome of a *Convolvulus arvensis* transformed with *Agrobacterium rhizogenes* strain A4. Plant Molecular Biology **12**:75-85.

Kado C.I. (1994) Promiscous DNA transfer system of *Agrobacterium tumefaciens*: Role of the *virB* operon in sex pilus assembly and synthesis. Molecular Microbiology **12**: 17-22.

Khan M.S., and Maliga P. (1999) Fluorescent antibiotic resistance marker for tracking plastid transfomation in higher plants. Nature Biotechnology **17**: 910

Khan S. A. (2000) Plasmid rolling-circle replication: recent developments. Molecular Microbiology **37**(3): 477-484.

Kim H-Y., Banerjee S. K., and Iyer V. N. (1994) The IncN plasmid replicon: two pathways of DNA polymerase I-independent replication. Journal of Bacteriology **176**(24): 7735-7739.

Kim K. and Meyer R.J. (1989) Unidirectional transfer of broad-host-range plasmid R1162 during conjugative mobilization. Evidence for genetically distinct events at *ori*T. Journal of Molecular Biology **208**: 501-505.

Klee H., Horsh R. and Rogers S. (1987) Agrobacterium-mediated plant transformation and its further applications to plant biology. Annual Review of Plant Physiology. **38**: 467-486.

Kohli A., Griffiths S., Palacios N., Twyman R.M., Vain P., Laurie D.A. and Christou P. (1999) Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology-mediated recombination. The Plant Journal **17**: 591-601.

Kohli A., Leech M., Vain P., Laurie D.A. and Christou P. (1998) Transgene organization in rice engineered through direct DNA transfer supports a two phase integration mechanism mediated by the establishment of integration hot spots. Proceedings of the National Academy of Sciences USA **95**: 7203-7208.

Komari T., Hiei Y., Saito Y., Murai N. and Kumashiro T. (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated *by Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant Journal **10**: 165-174.

Konez C. and Schell J. (1986) The promoter of TL-DNA gene 5 controls the tissue specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Molecular and General Genetics **204**: 383-396.

Konez C. ***et al.*** (1989) High-frequency T-DNA-mediated gene tagging in plants. Proceedings of the National Academy of Sciences USA **86**: 8467-8471.

Kononov M.E., Bassuner B. and Gelvin S.B. (1997) Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence of multiple complex patterns of integration. The Plant Journal **11**(5): 945-957.

Krause S., Pansegrau W., Lurz R., De La Cruz F., and Lanka E. (2000) Enzymology of type IV macromolecule secretion systems: the conjugative transfer regions of plasmids RP4 and R388 and the *cag* pathogenicity island of *Helicobacter pylori* encode structurally and functionally related nucleoside triphosphate hydrolases. Journal of Bacteriology **182**(10): 2761-2770.

Kuldau G.A., De Vos G., Owen J., MacCaffrey G. and Zambryski P. (1990) The *vir*B operon of *Agrobacterium tumefaciens* pTiC58 encodes 11 open reading frames. Molecular and General Genetics. **221**: 256-266.

Kupelwieser G., Schwab M., Hogenauer G., Koraimann G. and Zechener E.L. (1998) Transfer protein TraM stimulates TraI-cleavage of the transfer origin of the plasmid R1 *in vivo*. Journal of Molecular Biology **275**: 81-94.

Lahue E.E. and Matson S.W. (1988) Escherichia coli DNA Helicase I catalyzes a unidirectional and highly processive unwinding reaction. Journal of Biological Chemistry **263**: 3208-3215.

Lamtham S. and Day A. (2000) Removal of antibiotic resistance genes from transgenic tobacco plastids. Nature Biotechnology **18**: 1172-1176.

Lanka E. and Wilkins B.M. (1995) DNA processing reactions in bacterial conjugation . Annual Review of Biochemistry **64**: 141-169.

Lanka E.` and Lessl M., (1994) Common mechanisms in bacterial conjugation and Ti-mediated T-DNA transfer to plant cells. Cell **77**:321-324.

Lee L-Y., Gelvin S.B. and Kado C.I. (1999) pSa causes oncogenic suppression of Agrobacterium by inhibiting VirE2 protein export. Journal of Bacteriology **181**: 186-196.

Leemans J., Deblaere R., Willmitzer L., De Greve H., Hernalsteens J.P., Van Montagu M. and Scell J. (1982) Genetic identification of functions of TL-DNA transcripts in octopine galls. EMBO Journal **1**: 147-152.

Lemmers M., Beuckeleer M., Holsters M., Zambryski P., Depicker A., Hernalsteens J.P.,Van Montagu M. and Schell J. (1980) Internal organization, boundaries and integration of Ti plasmid DNA in nopaline crown gall tumours. Journal of Molecular Biology **144**: 353-376.

Leroux B., Yanofsky M.F., Winans S.C., Ward J.E., Ziegler S.F. and Nester E.W. (1987) Characterization of the *virE* locus of *Agrobacterium tumefaciens*: a transcriptional regulator and host range determinant. EMBO Journal **6**: 849-856.

Lessl M., Balzler D., Pansengrau W. and Lanka E. (1992) Sequence similarities between the RP4 Tra2 and the Ti *virB* region strongly support the conjugation model for T-DNA transfer. Journal of Biological Chemistry **267**: 20471-20480.

Lessl M., Balzler D., Pansengrau W. and Lanka E. (1993) The mating pair formation system of plasmid RP4 defined by RSF1010 mobilization. Journal of Bacteriology **175**: 6415-6425.

Li P-L., and Ferrand S. K. (2000) The replicator of the nopaline-type Ti plasmid pTiC58 is a member of the *repABC* family and is influenced by the TraR-dependent quorum-sensing regulatory system. Journal of Bacteriology **182**(1): 179-188.

Liu Z., Guiliani N., Appia-Ayme C., Borne F., Ratouchniak J., and Bonnefoy V. (2000) Construction and Characterization of a *recA* Mutant of *Thiobacillus ferrooxidans* by marker exchange mutagenesis. Journal of Bacteriology **182**(8): 2269-2276.

Loper J.E. and Kado C.I. (1979) Host range conferred by the virulence-specifying plasmid of *Agrobacterium tumefaciens.* Journal of Bacteriology **139**: 591-596.

Luo Z.O., Clemente T.E. and Farrand S.K. (2001) Construction of a derivative of Agrobacterium tumefaciens C58 that does not mutate to tetracycline resistance. Molecular and Plant Microbe Interactions **14**(1): 98-103.

Ma J. K-C., Hiatt A., Hein M., Vine N.D., Wang F., Stabila P., Van Dolleweerd C., Mostov K. and Lehner T. (1995) Generation and assembly of secretory antibodies in plants. Science **268**: 716-719.

Machida Y., Usami S., Yamamoto A. and Takebe I. (1986) Plant-inducible recombination between the 25-base-pair border sequence of T-DNA in *Agrobacterium tumefaciens*. Molecular and General Genetics **204**: 374-382.

Maliga P. (1993) Towards plastid transformation in plants. Trends in Biotechnology **11**: 101-107.

Martineau B., Volker T.A. and Sanders R.A. (1994) On defining T-DNA. Plant Cell **6**: 1032-1033.

Matson S.W. and Morton B.S. (1991) *Escherichia coli* DNA helicase I catalyzes a site- and strand-specific nicking reaction at the F plasmid *oriT*. Journal of Biological Chemistry 266: 16232-16237.

Matson S.W., Nelson W.C. and Morton B.S. (1993) Characterization of the *oriT* nicking reaction catalyzed by *Escherichia coli* DNA Helicase I. Journal of Bacteriology **175**: 2599-2606.

Matzke M.A. and Matzke A.J.M. (1995) How and why do plants inactivate homologous (trans) genes? Plant Physiology **107**: 679-689.

Matthysse A.G., Yarnall H.A. and Young N. (1996) Requirement for genes with homology to ABC transport systems for attachment and virulence of *Agrobacterium tumefaciens*. Journal of Bacteriology **178**: 5302-5308.

Mayerhofer R., Konez-kalman Z., Nawrath C.,Bakkeren G., Crameri A., Angelis K., Redei G.P., Schell J., Hohn B.and Konez C. (1991) T-DNA integration: a mode of illegitimate recombination in plants. EMBO journal. **10**: 697-704.

Mazodier P. and Davies J. (1991) Gene transfer between distantly related bacteria. Annual Review of Genetics **25**: 7-71

McBride K.E. and Knauf V.C. (1988) Genetic analysis of the *virE* operon of the Agrobacterium Ti plasmid pTiA6. Journal of Bacteriology. **170**: 1430-1437.

McNight T.D., Lillis M.T. and Simpson R.B. (1987) Segregation of genes transferred to one plant cell from two separate Agrobacterium strains. Plant Molecular Biology **8**: 439-445.

Melchers L.S., Regensburg-Tuink A.J.A., Schilperoort R.A. and Hooykaas P.J.J. (1989) Specificity of signal molecules in the activation of Agrobacterium virulence gene expression. Molecular Microbiology **3**: 969-977.

Meyer R. (2000) Identification of the *mob* genes of plasmid pSC101 and characterization of a hybrid pSC101-R1162 system for conjugal mobilization. Journal of Bacteriology **182**(17): 4875-4881.

Miller C.A., and Cohen S. N. (1993) The partition *(par)* locus of pSC101 is an enhancer of plasmid incompatibility. Molecular Microbiology **9**(4): 695-702.

Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plantarium **15**: 473-497.

Mysore K.S., Bassuner B., Deng X-b., Darbinian N.S. and Motchoulski A. (1998) Role of the *Agrobacterium tumefaciens* protein VirD2 in T-DNA transfer and integration. Molecular and Plant-Microbe Interactions **11**: 668-683.

Nap J.P., Bijvoet J. and Stiekema W.J. (1992) Biosafety of kanamycin resistant plants: an overview. Transgenic Research **1**: 239-249.

Navrath C., Poirier Y. and Somerville C. (1994) Targeting of the polyhydroxybutyrate biosynthetic pathway to the chloroplasts of *Arabidopsis thaliana* results in high levels of polymer accummulation. Proceedidings of the National Academy of Sciences USA **91**: 12760-12764.

Nelson W.C., Morton B.S., Lahue E.E. and Matson S.W. (1993) Characterization of the *Escerichia coli* F factor *traY* gene product and its binding sites. Journal of Bacteriology **175**: 2221-2228.

Nelson W.C., Howard M.T., Sherman J.A., and Steven S.W. (1995) The *traY* Gene product and intergration host factor stimulate *Escherichia coli* DNA Helicase I-catalyzed

nicking at the F Plasmid *oriT*. The Journal of Biological Chemistry **270**(47): 28374-28380.

Nester E.W., Gordon M.P., Amasino R.M. and Yanofsky M.F. (1984) Crown gall: a molecular and physiological analysis. Annual Review of Plant Physiology **35**: 387-413.

Nordheim A., Hashimoto-Gotoh T. and Timmis K.N. (1980) Location of two relaxation nick sites in R6K and single sites in pSC101 and RSF1010 close to origins of vegetative replication: implication for conjugal transfer of deoxyribonucleic acid. Journal of Bacteriology **144**: 923-932.

Ochman H., Lawrence J.G., and Groisman E.A. (2000) Lateral gene transfer and the nature of bacterial innovation. Nature **405**: 299-304.

Oger P. and Farrand S.K. (2002) Two opines control conjugal transfer of an *Agrobacterium* plasmid by regulating expression of separate copies of the quorum-sensing activator gene *traR*. Journal of Bacteriology **184**(4): 1121-1131.

Okamoto S., Toyoda-Yamamoto A., Ito K., Takebe I. and Machida Y. (1988) Localization and orientation of the VirD4 protein of *Agrobacterium tumefaciens* in the cell membrane. Molecular and General Genetics **228**: 24-32.

Ooms G., Klapwijk P.M., Poulis J.A. and Schilperoort R.A. (1980) Characterization of Tn904 insertions in octopine Ti plasmid mutants of *Agrobacterium tumefaciens*. Journal of Bacteriology **144**: 82-91.

Osusky M., Zhou G., Osuska L., Hancock R.E., Kay W.W. and Misra S. (2000) Transgenic plants expressing cationic peptide chimeras exhibit broad-spectrum resistance to phytopathogens. Nature Biotechnology **18**: 1162-1166.

Otten L., DeGreve H., Leemans J., Hain R.and Hoooykaas P. (1984) Restoration of virulence of *vir* region mutants of *Agrobacterium tumefaciens* strain B6S3 by coinfection of normal and mutant Agrobacterium strains. Molecular and General Genetics **195**: 159-163.

Otten L., Canaday J., Gerard J-C., Fournier P., Crouzet P., and Paulus F. (1992) **5**(4): 279-287.

Pansegrau W., Balzer D., Kruft V., Lurz R., and Lanka E. (1990a) *In vitro* assembly of relaxosomes at the transfer origin of plasmid RP4. Proceedings of the National Academy of Sciences USA **87**: 6555-6559.

Pansegrau W., Ziegelin G. and Lanka E. (1990b) Covalent association of the *traI* gene product of plasmid RP4 with the 5'-terminal nucleotide at the relaxation site. Journal of Biological Chemistry **18**: 10637-10644.

Pansegrau W. and Lanka E. (1991) Common sequence motifs in DNA relaxases and nick regions from a variety of DNA transfer systems. Nucleic Acids Research **19**: 3455.

Pansegrau W., Schoumacher F., Hohn B. and Lanka E. (1993) Site specific cleavage and joining of single stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: Analogy to bacterial conjugation. Proceedings of the National Academy of Sciences. USA **90**: 11538-11542.

Pansengrau W., Lanka E., Barth P.T., Figurski D.H. and Guiney D.G. (1994) Complete nucleotide sequence of Birmingham InPα plasmids: Compilation and comparative analysis. Journal of Molecular Biology **239**: 623-663.

Pazour G.P., Ta V.N. and Das A. (1992) Constitutive mutations of *Agrobacterium tumefaciens* transcriptional activator VirG. Journal of Bacteriology **174**: 4169-4174.

Penfold S.S., Simon J. and Frost L.S. (1996) Regulation of the expression of the *traM* gene of the F sex factor of *Escherichia coli*. Molecular Microbiology **20**: 549-558.

Peralta E.G. and Ream L.W. (1985) T-DNA border sequence required for crown gall tumorigenesis. Proceedings of the National Academy of Sciences USA. **82**: 5112-5116.

Peralta E.G., Hellmiss R. and Ream L.W. (1986) Overdrive, a T-DNA transmission enhancer on the *A. tumefaciens* tumor inducing plasmid. EMBO journal. **5**: 1137-1142.

Perwez T. and Meyer R. (1996) MobB protein stimulates nicking at the R1162 origin of transfer by increasing the proportion of complexed plasmid DNA. Journal of Bacteriology **178**: 5762-5767.

Perwez T. and Meyer R. (1999) Stabilization of the relaxosome and stimulation of transfer are genetically functions of the R1162 protein MobB. Journal of Bacteriology **181**: 2124-2134.

Petit A., Berkaloff A. and Tempe J. (1986) Multiple transformation of plant cells by Agrobacterium may be responsible for the complex organization of T-DNA in crown gall and hairy root. Molecular and General Genetics **202**: 388-393.

Piers K.L., Heath J.D., Liang X., Stephens K.M. and Nester E.W. (1996) *Agrobacterium tumefaciens*-mediated transformation of yeast. Proceedings of the National Academy of Sciences USA **93**: 1613-1618.

Prembroke J. T., and Murphy D.B. (2000) Isolation and analysis of a circular form of the IncJ conjugative transposon-like elements, R392 and R997: implications for IncJ incompatibility. FEMS Microbiology Letters **187**: 133-138.

Puchta H. (2000) Removing selectable marker genes: taking the shortcut. Trends in Plant Science **5**: 273-274.

Ramanathan V. and Veluthambi K. (1995) Transfer of non-T-DNA portions of the Agrobacterium tumefaciens Ti plasmid pTiA6 from the left terminus of TL-DNA. Plant Molecular Biology **28:** 1149-1154.

Ramirez-Romero M. A., Soberon N., Perez-Oseguera A., Tellez-Sosa J., and Cevallos M. A. (2000) Structural elements required for replication and incompatibility of the *Rhizobium etli* symbiotic plasmid. Journal Of Bacteriology **182**(11): 3117-3124.

Rawlings D.E. and KusanoT. (1994) Molecular genetics of *Thiobacillus ferooxidans.* Microbiology Review **58**: 39-58.

Rawlings D.E. and Woods D.R. (1985) Mobilization of *Thiobacillus ferooxidans* plasmids among *Escherichia coli* strains. Applied Environment and Microbiology **49**: 1323-1324.

Rawlings D.E., Pretorius I. and Woods D.R. (1984) Expression of a *Thiobacillus ferooxidans* origin of replication in *E. coli.* Journal of Bacteriology **158**: 737-738.

Rawlings D.E., Pretorius I. and Woods D.R. (1986) Expression of *Thiobacillus ferooxidans* plasmid functions and the development of genetic systems for the Thiobacilli.Biotechnology and Bioengineering Symposium **29**: 291-297.

Rawlings D.E. and Tietze E. (2001) Comparative Biology of IncQ and IncQ-like Plasmids. Microbiology and molecular Biology Reviews **65**(4)

Ream L.W., Gordon M.P. and Nester E.W. (1983) Multiple mutations in the T region of *Agrobacterium tumefaciens* tumour inducing plasmid. Proceedings of the National Academy of Sciences USA **80**: 1660-1664.

Reygers U., Wessel R., Muller H. and Hoffmann-Berling H. (1991) Endonuclease activity of *Escherichia coli* DNA helicaseI directed against the transfer origin of the F-factor. EMBO Journal **10**: 2689-2694.

Richter L.J., Thanavala Y., Arntzen C.J. and Mason H.S. (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nature Biotechnology **18**: 1167-1171.

Risseeuw E., Franke-van Dijk M.E.I., and Hooykaas P.J.J., (1997) Gene targeting and instability of *Agrobacterium* T-DNA loci in the plant genome. The Plant Journal **11**(4): 717-728.

Roberts R.C., Burioni R. and Helinsiki D.R. (1990) Genetic characterization of the stabilizing functions of a region of the broad host range plasmid RK2. Journal of Bacteriology **172**: 6204-6216.

Rohrer J. and Rawlings D. (1992) Sequence analysis and characterization of the mobilization region of a broad-host plasmid, pTF-FC2, isolated from *Thiobacillus ferooxidans*. Journal of Bacteriology **174**: 6230-6237.

Rosche T. M., Siddique A., Larsen M. H., and Figurski D. H. (2000) Incompatibility Protein IncC and Global Regulator KorB Interact in Active Partition of Promiscuous Plasmid RK2. Journal of Bacteriology **182**(21): 6014-6026.

Rossi L., Hohn B. and Tinland B. (1993a) The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plants. Molecular and General Genetics **239**: 345-353.

Rossi L., Escudero J., Hohn B. and Tinland B. (1993b) Efficient and sensitive assay for T-DNA-dependent transient gene expression. Plant Molecular Biology Reporter **11**: 220-229.

Rossi L., Hohn B. and Tinland B. (1996) Integration of complete transferred T-DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*. Proceedings of the National Academy of Sciences USA **93**: 126-130.

Rubin R.A. (1986) Genetic studies on the role of octopine T-DNA border regions in crown gall tumor formation. Molecular and General Genetics **202**: 312-320.

Sambrook J., Fritsch E.F. and Maniatis T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Samuels A. L., Lanka E., and Davies J. E. (2000) Conjugative Junctions in RP4-Mediated Mating of *Escherichia coli*. Journal of Bacteriology **182**(10): 2709-2715.

Sasse-Dwight S. and Gralla J.D. (1989) $KMnO_4$ as a probe for *lac* promoter for DNA melting and mechanism *in vivo*. Journal of Biological Chemistry **264**: 8074-8081.

Sastre J.I., Cabezon E. and de la Cruz F. (1998) The carboxyl terminus of protein TraD and specificity and efficiency to F-plasmid conjugative transfer. Journal of Bacteriology **180**: 6039-6040.

Scheiffele P., Pansengrau W. and Lanka E. (1995) Initiation of *Agrobacterium tumefaciens* T- DNA processing : Purified protein VirD1 VirD2 catalyze site-and strand specific cleavage of super helical T-border DNA *in vitro*. Journal of Biological Chemistry **270**: 1269-1276.

Scherzinger E., Bagdasarian M.M., Scholz P., Lurz R., Ruckert B. and Bagdasarian M. (1984) Replication of the broad-host-range plasmid RSF1010: requirement for three plasmid-encoded proteins. Proceedings of the National Academy of Sciences USA **81**: 654-658.

Scherzinger E., Lurz R., Otto S. and Dobrinski B. (1992) *In vitro* cleavage of double and single-stranded DNA by plasmid RSF1010-encoded proteins. Nucleic Acids Research **20**: 41-48.

Schocher R. J., Schillito R. D., Saul M.W., Paszkowski J.,and Potrykus I. (1986) Co-Transformation of unlinked foreign genes into plants by direct gene transfer.Bio/Technology **4**:1093

Scholtz P., Haring V., Wittmann-Liebold B., Ashman K., Bagdasarian M., and Scherzinger E. (1989) Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene **75**: 271-288.

Scott S.E. and Wilkinson M.J. (1999) Low probability of chloroplast movement from oil seed rape (*Brassica napus*) into wild *Brassica rapa*. Nature Biotechnology **17**: 390-392.

Sen P., Pazour G.J., Anderson D and Das A. (1989) Cooperative binding of *Agrobacterium tumefaciens* VirE protein to single-stranded DNA. Journal of Bacteriology. **171**: 2573-2580.

Shaw C.H., Watson M.D., Carter G.H. and Shaw C.H. (1984) The right hand copy of the nopaline Ti plasmid 25 bp repeat is required for tumour formation. Nucleic Acids Research **12**: 6031-6041.

Sheng J. and Citovsky V. (1996) Agrobacterium-plant cell transport: Have virulence proteins, will travel. Plant Cell **8**: 1699-1710.

Sherman J.A. and Matson S.W. (1994) *Escherichia coli* DNA Helicase I catalyzes a sequence-specific cleavage /ligation at the F plasmid Origin of Transfer. Journal of Biological Chemistry **269**: 26220-26226.

Shirasu K., Morel P. and Kado C.I. (1990) Characterization of the *virB* operon of *Agrobacterium tumefaciens* Ti plasmid; nucleotide sequence and protein analysis. Molecular Microbiology **4**: 1153-1163.

Shirasu K., Koukolikova-Nicola Z., Hohn B. and Kado C.I. (1994) An inner membrane associated virulence protein essential for T-DNA transfer from *Agrobacterium tumefaciens* to plants exhibits ATPase activity and similarities to conjugative transfer genes. Molecular Microbiology **11**: 581-588.

Shurvinton C.E., Hodges L., and Ream W. (1992) A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. Proceedings of the National Academy of Sciences USA **89**: 11837-11841.

Slightom J.L., Durand-Tardif M., Jouanin L. and Tepfer D. (1986) Nucleotide sequence analysis TL-DNA *Agrobacterium rhizogenes* type plasmid: Identification of open reading frames. Journal of Biological Chemistry **261**: 108-121.

Smith A.S.G. (1997) An investigation into the partition functions of the broad-host-range plasmid pTF-FC2. PhD thesis, University of Cape Town.

Sonti R.V., Chiruazzi M., Wong D., Davies C.S. and Harlow G.R. (1995) Arabidopsis mutants deficient in T-DNA integration. Proceedings of the National Academy of Sciences USA **92**: 11786-11790.

Spencer T.M., O'Brien J.V., Start W.G., Adams T. R., Gordon- Kamm W.J., and Lemaux P.G. (1992) Segregation of transgenes in maize. Plant Molecular Biology **18**:201-210.

Spielman A., and Simpson R.B. (1986) T-DNA structure in transgenic tobacco plants with multiple independent integration sites. Molecular and General Genetics **205:** 34-41.

Stachel S.E., Messens E., Van Montagu M. and Zambryski P. (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature **318**: 624-629.

Stachel S.E. and Nester E.W. (1986) The genetic and transcriptional organization of the vir region of the A6 Ti plasmid of Agrobacterium tumefaciens. EMBO Journal **5** : 1445-1454.

Stachel S.E. and Zambryiski P.C. (1986) *Agrobacterium tumefaciens* and the susceptible plant cell: A novel adaptation of extracellular recognition and DNA conjugation. Cell **47**: 155-157.

Stachel S.E. and Zambryiski P.C. (1986a) VirA and VirG control the plant-induced activation of the T-DNA transfer processof *A. tumefaciens*. Cell **46**: 325-353.

Stachel S.E., Timmerman B. and Zambryski P. (1987) Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 region: requirements for the 5' *vir*D gene products. EMBO Journal **6**: 857-863.

Stachel S.E., Timmerman B., and Zambryski P. (1986) Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature **322**: 706-712.

Stafford H. A., (2000) Crown Gall Disease and *Agrobacterium Tumefaciens*: A Study of the History, Present Knowledge, Missing Information, and Impact on Molecular Genetics.The Botanical Review **66**(1)

Stahl L.E., Jacobs A. and Binns A.N. (1998) The conjugal intermediate of RSF1010 inhibits *Agrobacterium tumefaciens* virulence and VirB-dependent export of VirE2. Journal of Bacteriology **180**: 3933-3939.

Stam M., De Bruin R., Kenter S., Van der Hoorn R.A.L., Van Blockland R., Mol J.N.M. and Kooter J.M. (1997) Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. The Plant Journal **12**: 63-82.

Steck T.R., Lin T-S.and Kado C.I. (1990) VirD2 gene product from the nopaline plasmid pTiC58 has at least two activities required for virulence. Nucleic Acids Research **18**: 6953-6958.

Sudhakar D., Fu X., Stoger E., Williams S., Spence J., Brown D.P., Bharathi M., Gatehouse J.A. and Christou P. (1998) Expression and immunolocalization of the snowdrop insecticidal protein GNA, in transgenic rice plants. Transgenic Research **7**: 371-378.

Sundberg C., Meek., Carrol., Das A. and Ream W. (1996) VirE1 protein mediates export of single-stranded DNA binding protein VirE2 from *Agrobacterium tumefaciens* into plant cells. Journal of Bacteriology 178: 1207-1212.

Sundberg C.D., and Ream W. (1999) The *Agrobacterium Tumefaciens* Chaperone-Like Protein, VirE1, Interacts with VirE2 at Domains Required for Single-Stranded DNA Binding and Cooperative Interaction. Journal of Bacteriology **181**(21): 6850-6855.

Svab Z. and Maliga P. (1993) High frequency plastid transformation in tobacco by selection of a chimeric *aadA* gene. Proceedings of the National Academy of Sciences USA **90**: 913-917.

Svab Z., Hajdukiewicz P. and Maliga P. (1990) Stable transformation of plastids in higher plants. Proceedings of the National Academy of Sciences USA **87**: 8526-8530.

Tang K., Tinjuangjun P., Xu Y., Sun X., Gatehouse J.A., Ronald P.C., Qi H., Lu X., Christou P. and Kohli A. (1999) Particle bombardment-mediated co-transformation of elite Chinese rice cultivars with genes conferring resistance to bacterial blight and sap-sucking insect pests. Planta **208**: 552-563.

Thomas C. M. (2000) Paradigms of plasmid organization. Molecular Microbiology **37**(3): 485-491.

Thomashow M.F., Nutter R., Montoya A.L., Gordon M.P. and Nester E.W. (1980) Integration and organization of Ti plasmid sequences in crown gall tumours. Cell **19**: 729-739.

Thompson C.J., Movva N.R., Tizard R., Davies J.E., Lauwereys M., and Botterman J., (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus.* The EMBO Journal **6**(9): 2519-2523.

Thorstenson Y.R., Kuldau G.A and Zambryski P.C., (1993) Subcellar Localisation of Seven VirB Proteins of *Agrobacterium tumefaciens*: Implications for the Formation of a T-DNA Transport Structure. Journal of Bacteriology **175**(16): 5233-5241.

Thortenson Y.R. and Zambryiski P.C. (1994) The essential virulence protein VirB8 localizes to the inner membrane of *Agrobacterium tumefaciens*. Journal of Bacteriology : 1711-1717.

Tingay S., McElroy D., Kalla R., Fieg S., Wang M., Thornton S. and Brettel R. (1997) Agrobacterium mediated barley transformation. The Plant Journal **11** (6): 1369-1376.

Tinland B., Koukolikova-Nicola Z., Hall M.N. and Hohn B. (1992) The T-DNA-linked VirD2 protein contains two distinct nuclear localization signals. Proceedings of the National Academy of Sciences USA **89**: 7442-7446.

Tinland B., Schoumacher F., Gloeckler V., Bravo-Angel A.M. and Hohn B. (1995) *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. EMBO Journal **14**: 3585-3595.

Tinland B. and Hohn B. (1995) Recombination between prokayotic and eukaryotic DNA: Integration of *Agrobacterium tumefaciens* T-DNA into the plant genome. In Genetic Engineering , ed. J.K Setlow. New York: Plenum Press.

Tinland B. (1996) The integration of DNA into plant genomes. Trends in Plant Science **1**: 178-184.

Toro N., Data A., Yanofsky M. and Nester E.W. (1988) Role of the overdrive sequence in T-DNA border cleavage in Agrobacterium. Proceeedings of the National Academy of Sciences USA **85**: 8558-8562.

Toro N., Data A., Carmi O.A., Young C., Prusti R.K. and Nester E.W. (1989) The *Agrobacterium tumefaciens virC1* gene binds to overdrive, a T-DNA transfer enhancer. Journal of Bacteriology **171**: 6845-6849.

Traxler B.A. and Minkley E.G. Jr. (1988) Evidence that DNA helicaseI and site-specific nicking are both functions of the F TraI protein. Journal of Molecular Biology **204**: 205-209.

Tsai M-M., Fu Y.H.F. and Deonier R.C. (1990) Intrinsic bends and integration host factor binding at F plasmid *oriT.* Journal of Bacteriology **172**: 4603-4609.

Van Haaren M.J.J., Seede N.J.A., Schilperoort R.A. and Hooykaas P.J.J. (1987) Overdrive is a T-region transfer enhancer which stimulates T-strand production in *A. tumefaciens*. Nucleic Acids Research **15**: 8983-8997.

Verdino P., Keller W., Stromaier H., Bischof K., Lindner H. and Koraimann G. (1999) The essential transfer protein TraM binds to DNA as a tetramer. Journal of Molecular Biology **274**: 37421-37428.

Vieira J. and Messing J. (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with universal primers. Gene **19**: 259-268.

Wang K., Herrera-Estrella A. and Van Montagu M. (1990) Overexpression of *vir*D1 and *vir*D2 genes in *Agrobacterium tumefaciens* enhances T-complex formation and plant transformation. Journal of Bacteriology. **172**: 4432-4440.

Wang K., Herrera-Estrella L., Van Montagu L. and Zambryski P. (1984) Right 25-bp terminus sequences of the nopaline T-DNA is essential for and determines and direction of transfer from Agrobacterium to the plant genome. Cell **38**: 390-399.

Wang K., Genetello C., Van Montagu M. and Zambryski P. (1987a) Sequence context of the T-DNA border repeat element determines its relative activity during T-DNA transfer to plant cells. Molecular and General Genetics **210**: 338-346.

Wang K., Stachel E., Timmerman B., Van Montagu M. and Zambryski P. (1987b) Site specific nick in the T-DNA border sequence as a result of Agrobacterium *vir* gene expression. Science.**235**: 587-591.

Ward E.R. and Barnes W.M. (1988) VirD2 protein of *Agrobacterium tumefaciens* very tightly linked to the 5' end of T-strand DNA. Science. **242**: 927-930.

Ward J.E., Akiyoski D.E., Regier D., Datta A., Gordon M.P. and Nester E.W. (1988) Characterization of the *vir*B operon from an *Agrobacterium tumefaciens* Ti plasmid. Journal of Biological Chemistry. **263**: 5804-5814.

Ward J.E., Dale M., Christie P.J., Nester W.E. and Binns A.N. (1990) Complementation analysis of *Agrobacterium tumefaciens* Ti plasmid *virB* genes by use of a *vir* promoter expression vector: *virB9*, *virB10*, and *virB11* are essential virulence genes. Journal of Bacteriology. **172**: 5187-5199.

Ward J.E., Dale M.E. and Binns A.N. (1991) Activity of the Agrobacterium T-DNA transfer machinery is affected by the *virB* gene products. Proceedings of the National Academy of Sciences USA **88**: 9350-9354.

Waters V.L., Hirata K.H., Pansegrau W., Lanka E. and Guiney D.G. (1991) Sequence identity in the nick regions of IncP plasmids transfer origins and T-DNA

borders of Agrobacterium Ti plasmids. Proceedings of the National Academy of Sciences USA **88**: 1456-1460.

Waters V.L. and Guiney D.G. (1993) Processes at the nick region link conjugation, T-DNA transfer and rolling circle replication. Molecular Microbiology **9**: 1123-1130.

Watson B., Currier T.C., Gordon M.P. Chilton M.D. and Nester E.W. (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. Journal of Bacteriology **123**: 255-264.

Wilkins B. and Lanka E. (1993) Bacterial conjugation. Ed W. Clewell. New York Plenum. 369-393.

Willmitzer L., Dhaese P., Schreier P.H., Schmalenbach W., Van Montagu M., and Schell J., (1983) Size, location and polarity of T-DNA-encoded transcripts in nopaline crown gall tumors; common transcripts in octopine and nopaline tumors. Cell **32**:1045-1056.

Winans S.C. (1992) Two-way chemical signaling in Agrobacterium-plant interactions. Microbiological Reviews **56**(1): 12-31.

Winans S.C., Allenza P., Stachel S.E., McBride K.E. and Nester E.W. (1987) Characterization of the *virE* operon of the Agrobacterium Ti plasmid pTiA6. Nucleic Acids Research. **15**: 825-837.

Winans S.C., Kerstetter R.A. and Nester E.W. (1988) Transcriptional regulation of the *virA* and *virG* genes of *Agrobacterium tumefaciens.* Journal of Bacteriology.**170**: 4047-4054.

Yadav N.S., Vanderleyden J., Bennett D.R., Barnes W.M., and Chilton M-D. (1982) Short direct repeats flank the T-DNA on a nopaline Ti Plasmid. Proceedings Of the National Academy of Sciences USA **79**:6322-6326.

Yamada Y., Yamada M., and Nakazawa A., (1995) A ColE1-encoded gene directs entry exclusion of the plasmid. Journal of Bacteriology **177**(21): 6064-6068.

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

Ye X., Al-Babilli S., Kloti A., Zhang J., Lucca P., Beyer P. and Potrykus I. (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science **287**: 303-305.

Yoder J.I., and Goldsbrough A.P., (1994) Transformation systems for generating marker-free transgenic plants. Bio/Technology **12**: 263

Young C. and Nester E.W. (1988) Association of the VirD2 protein with the 5' end of T-strands in *Agrobacterium tumefaciens*. Journal of Bacteriology. **170**: 3367-3374.

Youngren B., Radnedge L., Hu P., Garcia E., and Austin S., (2000) A plasmid partition of the P1-P7*par* family from the pMT1 virulence plasmid of *Yersinia pestis*. Journal of Bacteriology **182**(14): 3924-3928.

Zambryski P. (1988) Basic processes underlying Agrobacterium-mediated DNA transfer to plant cells. Annual Review of Genetics. **22**: 1-30.

Zambryski P., Joos H., Genetello 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 Journal **2**: 2143-2150.

Zambryski P.C. (1992) Chronicles from the Agrobacterium-plant cell DNA transfer story. Annual Review of Plant physiology and Plant Molecular Biology **43**: 465-490.

Zechendorf B. (1994) What the public thinks about biotechnology. Bio/Technology **12**: 870-875.

Zhang S. and Meyer R. (1997) The relaxosome protein MobC promotes conjugal plasmid mobilization by extending DNA strand separation to the nick site at the origin of transfer. Molecular microbiology **25**: 509-516.

Zhang S., and Meyer R.J., (1995) Localised denaturation of *oriT* DNA within relaxosomes of the broad-host-range plasmid R1162. Molecular Microbiology **17**(4): 727-735.

Zhou X-R., and Christie P.J., (1999) Mutagenesis of the *Agrobacterium* VirE2 Single-stranded DNA-binding protein identifies regions required for self-association and interaction with VirE1 and a permissive site for hybrid protein construction. Journal of Bacteriology **181**(14): 4342-4352.

Zhu J., Oger P.M., Schrammeijer B., Hooykaas P. J. J., Farrand S. K., and Winnans S. C. (2000) The bases of crown gall tumorigenesis. Journal of Bacteriology **182**(14): 3885-3895.

Ziegelin G., Pansegrau W., Strack B., Kroger M., Kruft V. and Lanka E. (1989) Nucleotide sequence and organization of genes flanking the transfer origin of promiscous plasmid RP4. DNA Sequencing Mapping **1**: 303-327.

Ziegelin G., Pansegrau W., Strack B., Balzer D., Kroger M., Kruft V. and Lanka E. (1991) Nucleotide sequence and organization of genes flanking the transfer origin of promiscuous plasmid RP4.DNA sequence Journal. DNA Sequencing mapping **1**: 303-327.

Ziemienowicz A., Gorlich D., Lanka E., Hohn B. and Rossi L. (1999) Import of DNA into mammalian nuclei by proteins originating from plant pathogenic bacterium. Proceedings of the National Academy of Sciences USA **96**: 3729-3733.

Zorreguieta A. Ugalde R.A. (1986) Formation in Rhizobium and Agrobacterium spp. of a 235-kilodalton protein intermediate in β-d-(1,2) glucan synthesis. Journal of Bacteriology **167**: 947-951.

Zuo J., Niu Q-W., Moller S.G. and Chua N-H (2001) Chemical regulated, site-specific DNA excision in transgenic plants. Nature Biotechnology **19**: 157-161.

APPENDIX A

Plasmids used in this study

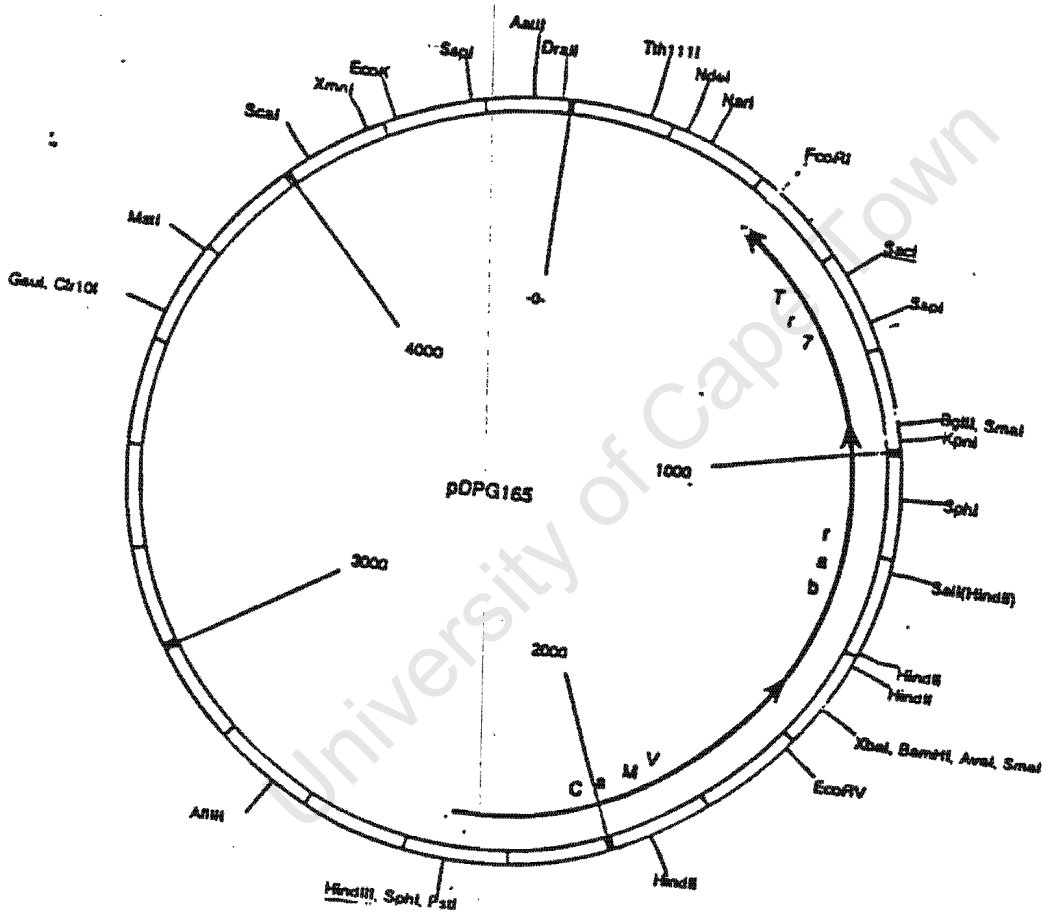


Fig. A.1 Physical map of plasmid pDPG165 that was used as a source for the *bar* gene expression cassette (Spencer *et al.* 1990).

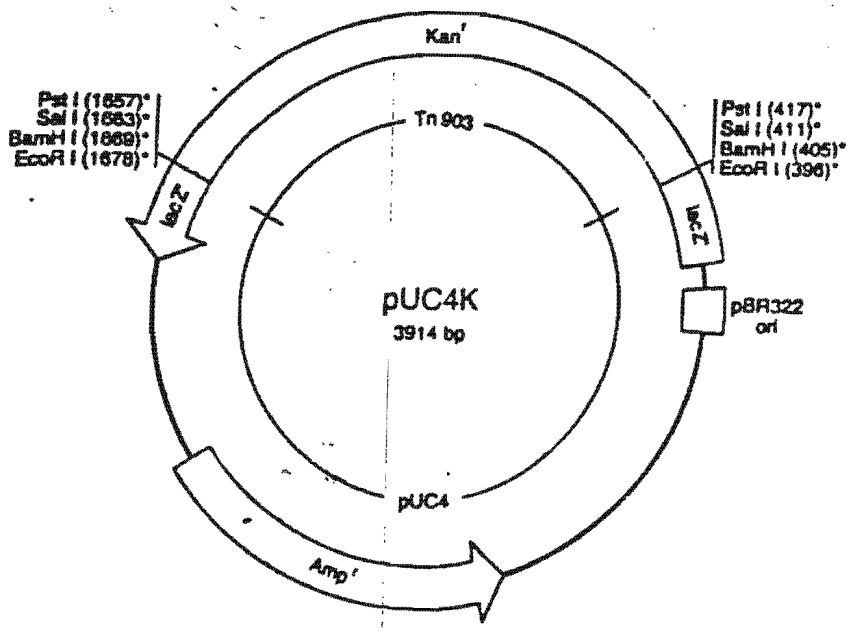


Fig. A.2 Physical map of pUC4K carrying a drug resistance marker from the transposon Tn903 which encodes an aminoglycoside 3' phosphotransferase gene, conferring resistance to kanamycin and neomycin (Viera and Messing, 1992).

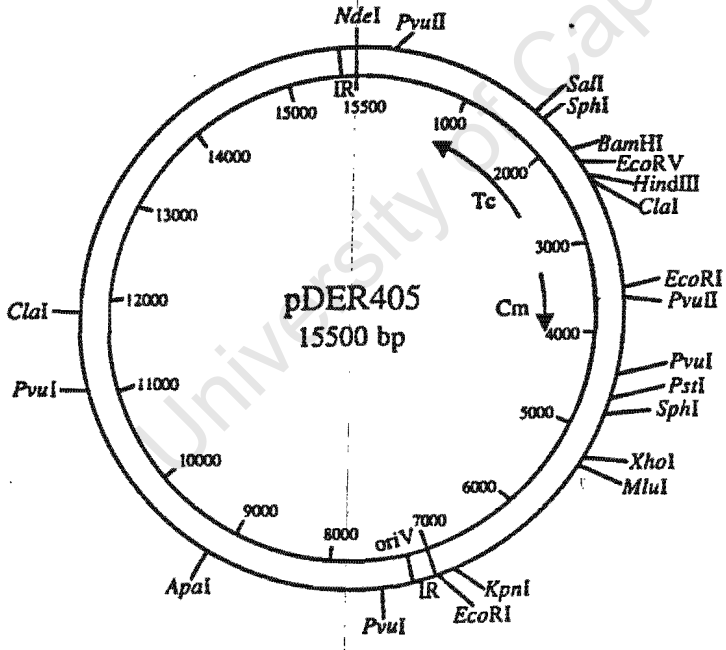


Fig. A.3 Physical map of the broad-host-range plasmid pDER405 constructed by cloning a 4.2-kb *PstI/NdeI* fragment, carrying the mobilization region from the *Acidithiobacillus ferrooxidans* plasmid pTF-FC2, into pBR325 (Rawlings *et al.* 1986).

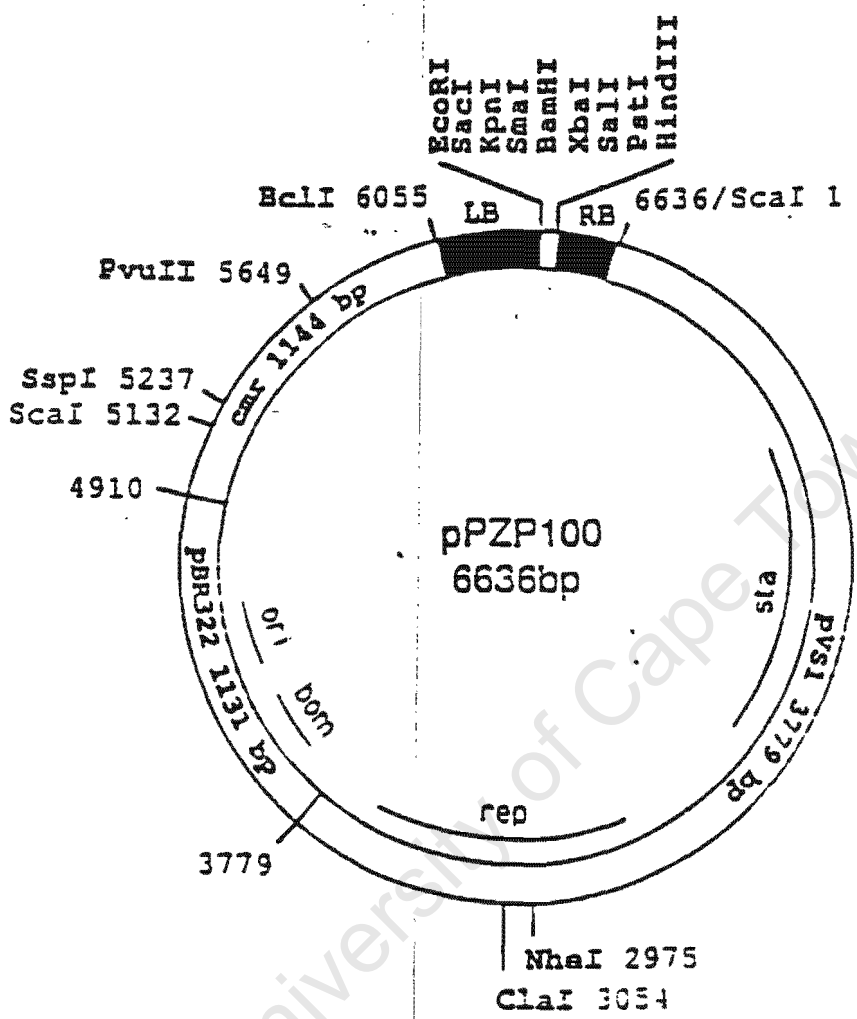


Fig. A.4 Physical map of the pPZP100 Agrobacterium binary vector (Hajdukiewicz *et al.* 1994).

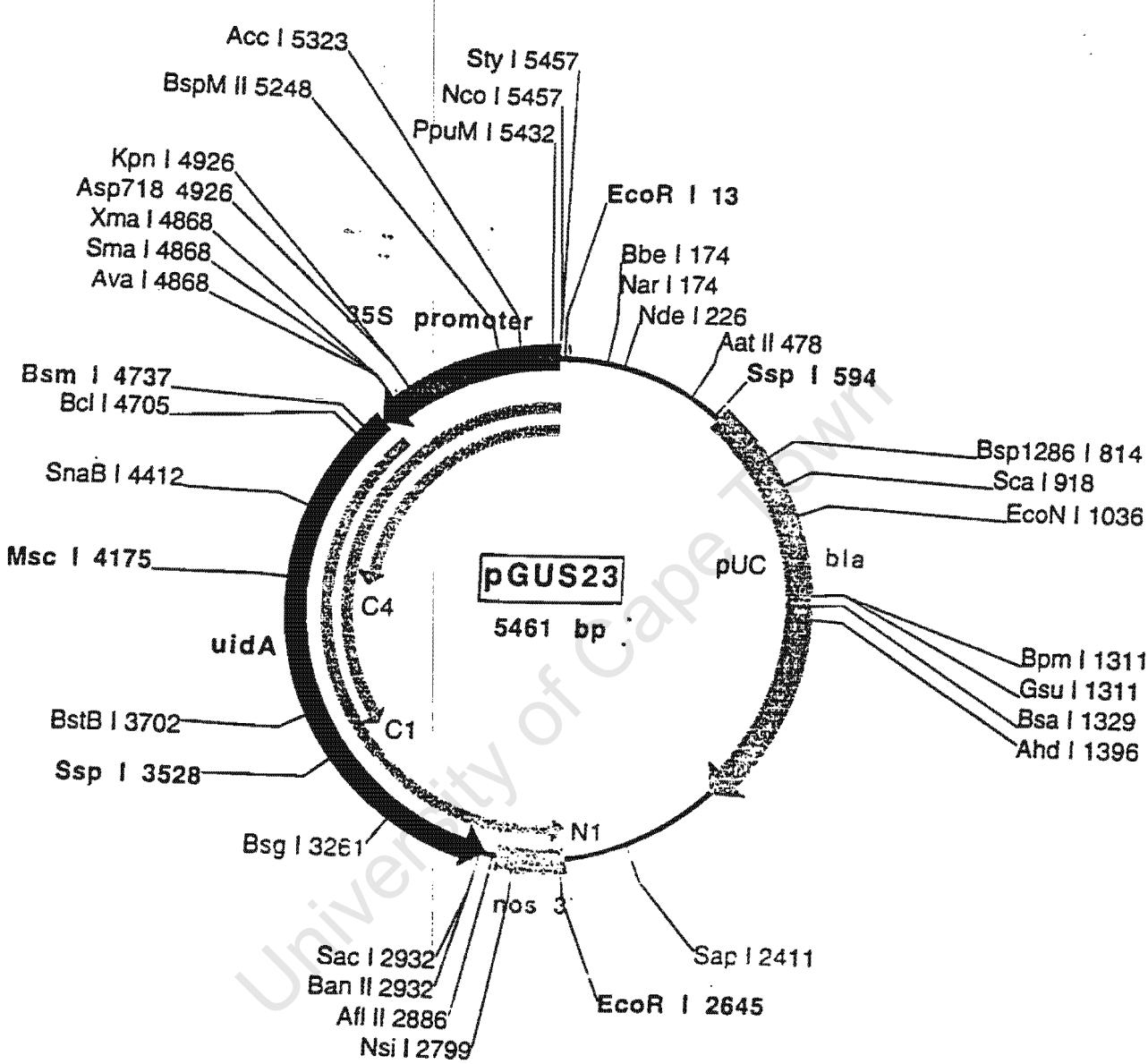


Fig. A.5 Physical map of pGUS23 which was used as a source of the *gus* gene expression cassette.

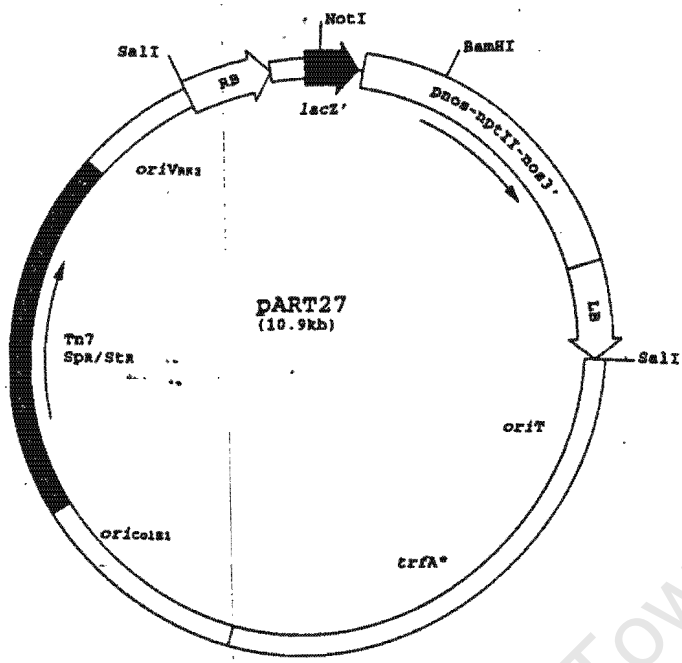


Fig. A.6 Physical map of the plasmid pART27. (Gleave, 1992)

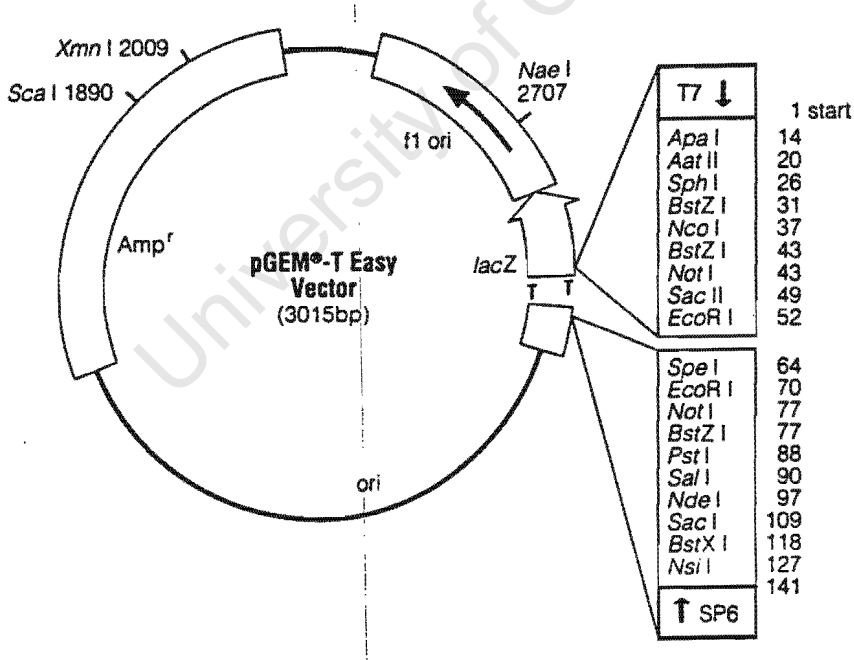


Fig. A.7 Physical map of pGEM®-T Easy vector showing sequence reference points (Promega, USA)

APPENDIX B

Plant tissue culture media used in this study

TOBACCO INFECTION WITH *AGROBACTERIUM tumefaciens*

	1l Co-cultivation medium	1l Shooting medium	1l Rooting medium	1l Basic or Growth medium (GM)
MS stock	25ml	25ml	25ml	25ml
4x micronutrient stocks- KI, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$	1ml of each	1ml of each	1ml of each	1ml of each
MS vitamins stock	10ml	10ml	10ml	10ml
NAA	0.1mg	0.1mg	-	-
BAP	1mg	1mg	-	-
Cefotaxine	-	250mg	-	-
Kanamycin or Bialaphos or PPT	- - -	100mg 3mg 20mg	100mg 3mg 20mg	- - -
MES	-	-	0.5g	-
Sucrose	30g	30g	30g	30g
Agar	8g	8g	8g	8g
pH	5.8	5.8	5.7	5.75

MS VITAMINS STOCK

	Mg/500ml
Glycine	50
Nicotinic acid	125
Thiamine-HCl	250
Pyridoxine-HCl	250
Myo-inositol	2.5

Bacterium culture media used in this study

Luria broth (LB)

10g Tryptone
5g Yeast extract
10g Sodium chloride

Make up to a litre with sterile water and autoclave.

Luria agar (LA)

10g Tryptone
5g Yeast extract
10g Sodium chloride
15g Agar

Make up to a litre with sterile water and autoclave.

SOC broth

20g Tryptone
5g Yeast extract
0.5g Sodium chloride

Dissolve in 950ml distilled water. Add 10ml of 250mM KCl. Adjust pH to7.0 with 10N sodium hydroxide and make up to 975ml. Autoclave, cool, and add 20ml sterile 1M glucose and 5ml sterile 2M $MgCl_2$.