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Construction of Plasmids for Studying Genetic
Events in Agrobacterium tumefaciens

by

ANDRE LOUIS HAMEL, BSc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Master of Science

Department of Biology

Carleton University

Ottawa, Ontario

October 22, 1987

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Events in Agrobacterium tumefaciens"

submitted by Andre L. Hamel, BSc.

in partial fulfilment of the requirements for
the degree of Master of Science

Thesis Supervisor

Chairman, Department of Biology

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ABSTRACT

Described in this thesis is the design and construction of several plasmids for evaluating events expected to be associated with gene transfer from Agrobacterium tumefaciens to plant cells. Two of these plasmids, pBIG and pCELGS65, comprised of fused IncP and IncQ replicons, were intended to act as miniature experimental models of the Ti plasmid. IncP and IncQ replicons were found to be stable together in A. tumefaciens. Also described is the design and construction of two plasmids that contain the sac (levansucrase) gene, and are potentially useful for studies of insertion elements in Gram-negative bacteria. One of these sac-containing plasmids, pAH1 (also a mini-Ti plasmid), was intended for studies of deletions of the T-DNA region. Experimental systems, comprised of these three mini-Ti plasmids, for studies of acetosyringone (AS) induced T-DNA and non-T-DNA circle formation in A. tumefaciens, were expected to be sensitive enough to detect circularization if it occurs at a frequency of about 10^{-3} for every cell. Studies using these systems did not detect this event. It is possible that the circularization event occurs in every cell at a frequency below 10^{-3} .

Another plasmid, designated pBRK4013, was

constructed. In addition to coding for carbenicillin resistance, pBRK4013 carries the IncP transfer functions which enable it to self-transfer between strains.

Furthermore, pBRK4013 is useful for mobilizing non-self-transmissible plasmids between bacterial strains. Small vectors that can be mobilized between bacterial strains and cannot replicate in several organisms other than Escherichia coli and its close relatives were constructed. Potentially, pBRK4013 and these small vectors can be useful for generating random and site-specific insertions in the genome of non-E. coli hosts.

Also, a suicide plasmid, designated pAH99, was constructed to isolate recA derivatives by site-directed transplacement of in vitro altered cloned DNA sequences in A. tumefaciens. Finally, further approaches that can be used for studies of Ti plasmid processing events and for making site-specific recA mutations in A. tumefaciens will be suggested.

TO MY LOVING WIFE CAROLE

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LIST OF ABBREVIATIONS

$\leq$	less than or equal to
$\geq$	greater than or equal to
μg	micro gram
μl	micro liter
μM	micro molar
$\pm$	with or without (plus or minus.)
AS	acetosyringone
<u>bla</u>	β -lactamase (gene)
<u>bp</u>	base-pair
<u>cat</u>	chloramphenicol acetyltransferase (gene)
Cb	Carbenicillin
Cb ^r	Cb resistant/resistance
Cb ^s	Cb sensitive/sensitivity
Cm	Chloramphenicol
Cm ^r	Cm resistant/resistance
Cm ^s	Cm sensitive/sensitivity
DNA	Deoxyribo Nucleic Acid
dATP	Deoxy-Adenosine-5'-Tri-Phosphate
dCTP	Deoxy-Cytosine-5'-Tri-Phosphate
dGTP	Deoxy-Guanosine-5'-Tri-Phosphate
dNTP	Deoxy-Nucleoside-5'-Tri-Phosphate
dTTP	Deoxy-Thymidine-5'-Tri-Phosphate
DR	Direct Repeat
ds	double-stranded
EDTA	Ethylene Diamine Tetra-acetic Acid
EtBr	Ethidium Bromide
EtOH	Ethanol
gm	gram
Gm	Gentamycin
Gm ^r	Gm resistant/resistance
hr(s).	hour(s)
Inc	Incompatibility group
IPTG	IsoPropyl β -Thio-D-Galactoside
IS	Insertion Sequence
kbp	kilo-base-pairs
Km	Kanamycin
Km ^r	Km resistant/resistance
Km ^s	Km sensitive/sensitivity
l	liter
LB	Left Border
M	Molar
MeOH	Methanol
mg	milli gram
min(s).	minute(s)
ml	milli liter
mM	milli Molar
MMS	Methyl Methane Sulfonate
<u>mob</u>	mobilization (gene)

Mob	inter-bacterial mobilization trait
Nal	Nalidixic Acid
NaOAc	Sodium Acetate
NH ₄ OAc	Ammonium Acetate
OD	<u>overdrive</u> sequence
<u>oriT</u>	origin of inter-bacterial transfer site
<u>oriV</u>	origin of replication site
PEG	Poly Ethylene Glycol
RB	Right Border
<u>rec</u>	recombination (gene)
<u>rep</u>	replication (gene)
Rif	Rifampicin
Rif ^r	Rif resistant/resistance
rpm	revolutions per minute
RT	room temperature (about 20°C)
<u>sac</u>	levansucrase (gene)
SDS	Sodium Dodecyl Sulfate
Sm	Streptomycin
Sm ^r	Sm resistant/resistance
Sp	Spectinomycin
Sp ^r	Sp resistant/resistance
ss	single-stranded
Suc	sucrose
Suc ^r	Suc resistant/resistance
Suc ^s	Suc sensitive/sensitivity
Tc	Tetracycline
Tc ^r	Tc resistant/resistance
T-DNA	Transferred DNA
<u>tet^r</u>	tetracycline resistance (gene)
Ti plasmid	Plant Tumor Inducing Plasmid
Tn5	Transposon 5
<u>tra</u>	transfer (gene)
Tra	inter-bacterial self-transmissibility trait
UV	Ultra Violet light
<u>vir</u>	virulence (gene)
Vir	virulence trait
w/v	weight over volume ratio
WHR	Wide Host Range
X-Gal	5-bromo-4-chloro- β -indolyl- β -D-galactoside

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INTRODUCTION

The soil bacterium Agrobacterium tumefaciens is currently attracting much attention because it can be exploited as a vehicle for transferring genes into plants. A.tumefaciens induces crown gall tumor (CGT) in a wide variety of dicotyledonous plants. The formation of CGT in plants involves the transfer of a DNA segment (T-DNA) from A.tumefaciens to plant cells and is the only known case of naturally occurring DNA transfer from bacteria to plants. The T-DNA of A.tumefaciens is carried on one of a group of large autonomously replicating extrachromosomal DNA molecules (plasmids) called the tumor-inducing (Ti) plasmid (VanLarebeke et.al.1974; Zaenen et.al.1974; Chilton et.al.1978).

T-DNA transfer from A.tumefaciens to the plant cell results in tumor formation (Garfinkel et.al.1980). T-DNA carries phytohormone genes that code for the production of plant hormones (Nester et.al.1984). After transfer of the T-DNA from A.tumefaciens to the plant cell, the T-DNA becomes integrated into the plant chromosomal DNA (Chilton et.al.1974,1977,1980; Thomashow et.al.1980a, 1980b). Expression of the T-DNA encoded phytohormone genes in the plant cells results in disorganized cell proliferation, and tumor formation (Joos et.al.1983a; Nester et.al.1984). In the hopes of better understanding the mechanism of T-DNA transfer, several researchers have extensively studied the genetics of A.tumefaciens;

yet, the precise mechanism for T-DNA transfer into plant cells is currently not known.

The virulence (vir) region of the Ti plasmid contains genes that are essential for transfer of T-DNA from A.tumefaciens to the plant cell. The vir-region is located outside the T-DNA region (Depicker et.al.1978; Garfinkel and Nester 1980; Ooms et.al.1980; Klee et.al. 1982). Several studies suggested that the vir region carries at least six genes (Iyer et.al.1982; Klee et.al. 1983; Hille et.al.1984; Hooykaas et.al.1984). Conversely, the genes found within the T-DNA are not required for its transfer into the plant cell from A.tumefaciens (Leemans et.al.1982; Ream et.al.1983).

The transfer of T-DNA from A.tumefaciens to the plant cell is induced only where there is a wound on the plant (see review by Halverson and Stacey 1986). Wounded plant cells characteristically excrete several signal molecules that are phenolic in structure (for example, acetosyringone {AS}), and can induce vir gene expression. Thus, vir gene expression appears to be regulated solely for bestowing upon Ti plasmid-bearing A.tumefaciens the capacity to recognize suitable plant wounds and initiate T-DNA transfer (Okker et.al.1984; Stachel et.al.1985a,b,1986; Bolton et.al.1986). We still do not fully understand precisely the roles in T-DNA transfer played by each of the Ti plasmid vir genes;

however, sufficient evidence supports the suggested functions for at least three of these genes, virA, virD and virG. Mutations in virA, virB, virD and virG genes completely abolish virulence (Iyer et.al. 1982; Stachel et.al.1986).

The T-DNA of all Ti plasmids is bordered by 25 base-pairs (bp) of a nearly perfect, direct repeat sequence (Zambryski et.al.1980,1982; Yadav et.al.1982; Simpson et.al.1982; Barker et.al.1983; Holsters et.al. 1983). The 25 bp border sequence does not appear in its entirety in the transformed plant genome (Yadav et.al. 1982); yet, it signals T-DNA borders and is highly conserved in all Ti plasmids analyzed to date. T-DNA transfer is dependent upon this 25 bp cis-acting border sequence, since it defines the region of the Ti plasmid that is destined for transfer.

T-DNA located on the Ti plasmid absolutely requires the rightward 25 bp sequence (or right border fragment) in order to be transferred (Ooms et.al.1982; Joos et.al. 1983b; Shaw et.al.1984; Wang et.al.1984; Caplan et.al. 1985; Hepburn and White 1985; Peralta and Ream 1985; Jen and Chilton 1986 a,b; Gardner and Knauf 1986). Efficient T-DNA transfer from the Ti plasmid, requires the right border in its native orientation and relative position (Shaw et.al 1984; Wang et.al.1984; Caplan et.al.1985; Peralta and Ream 1985; Jen and Chilton 1986a,b;

VanHaaren et.al. 1987 and Wang et.al.1987). The left border ensures the unit length transfer of T-DNA.

Incubation of A.tumefaciens, with either plant cells or AS, results in the formation of linear, single-stranded (ss) T-DNA molecules (Stachel et.al.1986b,1987; Albright et.al.1987). The linear ss-T-DNA (T-strand), results from induced vir gene expression. It has been hypothesized that the T-DNA transfers from A.tumefaciens to plants as a T-strand. This hypothesis was based upon observations that the T-strand is very similar to structures that are associated with inter-bacterial conjugation (Stachel et.al.1986b). A more recent study suggested that double-stranded (ds) T-DNA might also play a role in the transfer process (Veluthambi et.al. 1987).

Double-stranded circles comprised entirely of T-DNA were discovered by using plasmid DNA, isolated from A.tumefaciens incubated with plant cells, to transform Escherichia coli (Koukolikova-Nicola et.al.1985). T-DNA circle formation requires either AS- or plant cell-induced vir gene expression (Stachel et.al.1986a). It was suggested that ds-T-DNA circles have a role in the T-DNA transfer process as intermediates in rolling-circle replication, from which linear ss-T-DNA forms could be made (Koukolikova-Nicola et.al.1985). Also, they did not attempt to determine whether or not non-T-

DNA circles might also be produced.

Described in the present work is the design and construction of two different mini-Ti plasmid systems to test this theory. One of these systems consists of two plasmids, pBIG and pCELGS65, comprised of fused replicons. By studying pBIG and pCELGS65 in A.tumefaciens, I had expected to obtain information regarding the stoichiometry of T-DNA and non-T-DNA circle generation. Results obtained from experiments using A.tumefaciens strains carrying pBIG and pCELGS65, and potential future applications of these plasmids will be described and discussed.

The other system made use of a third, different mini-Ti plasmid, pAH1, which was used for studying deletions and insertions in the T-DNA region. Studies of E.coli and A.tumefaciens carrying pAH1, in addition to its potential future applications, will be described and discussed.

Conjugative and mobilizable plasmids of the Incompatibility (Inc)P and IncQ groups, respectively, have been important in the growth of molecular genetics in Agrobacterium, other bacterial plant pathogens, and a majority of other Gram negative bacteria. Plasmid cloning vehicles bearing either IncP or IncQ group replicons can replicate in E.coli and in A.tumefaciens, and do not influence tumorigenicity (Hille and

Schilperoort 1981). Many IncP and IncQ plasmids also carry either an IncP or IncQ origin of transfer (oriT), which allows non-self-transmissible plasmids to be mobilized between bacterial strains, by IncP transfer (Tra) functions provided in trans.

A self-transmissible plasmid, designated pBRK4013, was also constructed. Plasmid pBRK4013 contains the IncP tra and mob genes, the IncP oriT site, the pMB1 replicon, the carbenicillin resistance gene (Cb^r), and can replicate in E.coli, but not in A.tumefaciens. Potential general applications of pBRK4013 in studies of A.tumefaciens and other Gram-negative bacteria will be described.

Also described in this thesis is the design and construction of two cloning vectors, pAH69 and pAH79, which were derived from pUC13. Plasmids pAH69 and pAH79 contain the IncP oriT, the Cb^r gene (as their only dominant selectable marker), and carry the pMB1 replicon, which can replicate in E.coli, but not in A.tumefaciens. These plasmids were found to be capable of mobilization, by IncP Tra functions provided in trans, at a frequency of about one for every donor cell in matings between E.coli strains. Potential general applications of pAH69 and pAH79 will be described.

I have also constructed pAH322, a derivative of pBR322. Plasmid pAH322 contains the tetracycline

resistance gene (Tc^r) bordered by Eco RI cleavage sites. A 1.4 kbp DNA fragment, comprised entirely of the functional Tc^r gene, was generated whenever pAH322 was digested to completion with the restriction enzyme Eco RI.

Sometimes, studies of bacterial genetics require the use of strains carrying more than one recombinant plasmid. Potentially, such studies can be troublesome whenever the plasmids involved share sequence homology with one another. The use of recombination deficient A.tumefaciens, in studies of the formation of assumed intermediates of Ti plasmid-DNA processing events, could allow one to assess the role of the recombination systems. Also, recombination-deficient A.tumefaciens can be potentially useful for increasing the stability of cloned foreign genes. Accordingly, I have designed and constructed pAH99 (a derivative of pAH79), intended for making recA derivatives of A.tumefaciens by replacement mutagenesis. Reasons for using pAH99, in addition to my attempts to isolate a recA derivative of A.tumefaciens will be described and discussed.

LITERATURE REVIEW

2.1 General Features of Crown Gall Tumorigenesis

Tumor formation on plants infected by the gram-negative soil bacterium Agrobacterium tumefaciens results from the plant inheriting the T-DNA (Chilton et. al.1977; Thomashow et.al.1980; Zambryski et.al.1980). T-DNA encoded phytohormone genes, transferred from Ti plasmid-bearing A.tumefaciens into the plant cell, is eventually expressed in the plant cell after the stable integration of the T-DNA into the plant cell genome.

Tumor formation in transformed plants results from the expression of genes contained within the T-DNA. The native T-DNA genes code for functions involved in plant hormone synthesis (the causal agents of tumorigenesis) and for synthesis of novel compounds (opines) produced only by plant cells transformed by A.tumefaciens (review by Nester et.al.1984). Ti plasmid classification is based upon the types of opines that their T-DNA produces in plant tumors (Nester et.al.1984). Currently, the main types of Ti plasmids that are studied produce and catabolize the opines nopaline (as in pTiC58 and pTiT37) and octopine (pTiA6, pTiB6, pTiAch5). All Ti plasmids that have been studied to date share extensive regions of sequence homology (Drummond and Chilton 1978).

A.tumefaciens carrying the relevant Ti plasmid, is one of very few microorganisms that can utilize, as sole

carbon and energy sources, the opines produced by plant tumors. Moreover, these opines derepress Ti plasmid-encoded genes for inter-bacterial transfer of the plasmid (into avirulent Agrobacterium strains); in addition to derepressing the opine catabolism genes (see reviews by Lichtenstein 1986, 1987). Tumor formation therefore appears to be of selective advantage for this soil microbe; an intriguing strategy that Agrobacterium has evolved for inducing the plant to produce large numbers of plant cells that synthesize carbon and nitrogen sources that can only be used by that bacterial strain.

2.2 General Features of the Ti Plasmid

Although the precise mechanism by which A.tumefaciens transfers a part of its Ti plasmid DNA into plant cells is currently not known, the genetics of this bacterium has been extensively studied. Transfer of specific sequences of the Ti plasmid from bacterium to plant cell is absolutely dependent upon the function of another part of the Ti plasmid, the vir (virulence) region (Iyer et. al. 1982; Klee et.al. 1983; Lundquist et. al. 1984; Hagiya et.al. 1985). Although the vir genes encode functions that are necessary for T-DNA transfer into plant cells, and possibly for specific plant-

bacterium interactions, they are not themselves found in transformed plant cells (Chilton et.al.1978,1980; Thomashow et.al.1980). The vir region encoded functions can effect the bacterium to plant transfer of the T-DNA of any type of Ti plasmid (Hoekema et.al.1984a). Thus, the nopaline Ti plasmid can transfer T-DNA of the octopine Ti plasmid, and visa versa.

All Ti plasmids possess T-DNA that are flanked by 25 base-pair (bp) direct repeats. (Zambryski et.al.1980, 1982; Yadav et.al.1982; Simpson et.al.1982; Barker et.al.1983; Holsters et.al.1983). This border repeat sequence does not appear intact in the transformed plant genome (Yadav et.al.1982). The DNA sequences surrounding the 25 bp repeat sequence on octopine Ti plasmids are unrelated to the corresponding sequence of nopaline Ti plasmids (Simpson et.al.1982). Thus, the 25 bp repeat sequence signals the T-DNA borders and is of functional significance rather than being only of structural importance because it is so highly conserved in all Ti plasmids analyzed to date. Only DNA adjacent to border elements (consisting of the 25 bp sequence) in the proper orientation and position (relative to the DNA to be transferred) is capable of vir-mediated transfer from the bacterium to the plant (Holsters et.al.1980; Joos et.al.1983a,b). The 25 bp sequence defines the region of the Ti plasmid that can be destined for transfer.

Therefore, T-DNA transfer requires this cis-acting border direct repeat sequence.

Octopine type Ti plasmids, for example pTiA6, carry another sequence of 25 bp (not to be confused with the T-DNA-determining 25 bp border sequence), called the overdrive (OD) sequence. The OD sequence lies rightward of the right hand 25 bp border sequence and is required for efficient T-DNA transfer (Peralta and Ream 1985; Peralta et.al. 1986; Jen and Chilton 1986a). The OD sequence can function equally well in either orientation as long as it is adjacent to the rightward 25 bp border element. Also, there have been suggestions that the OD sequence distinguishes the right from the left border sequence, at least in octopine Ti plasmids (Peralta et. al.1986; Jen and Chilton 1986a,b). The OD sequence strongly increases the ability of the left border (LB) to act as a right border (RB) when OD is placed close to the LB (Peralta et.al.1986).

To date no sequence similar to OD has been found in nopaline Ti plasmids. Nopaline Ti plasmids function equally well in T-DNA transfer when only a synthetic 25 bp border element is present at the rightward end of the T-DNA (Wang et.al.1987). Accordingly, it can be inferred that octopine Ti plasmids require the OD-type sequences because these plasmids possess two separate T-DNAs. A sequence that indicates the difference between left and

right borders, especially where two T-DNAs are concerned, might be expected to improve the efficiency and accuracy of the T-DNA transfer process. Since nopaline Ti plasmids possess only one T-DNA, such a requirement for OD-type sequences would not be as critical because the border elements themselves should sufficiently discriminate between T-DNA and non-T-DNA regions.

We now know that T-DNA located on the Ti plasmid requires the rightward 25 bp repeat (or right border fragment) in its native orientation and relative position in order to be efficiently transferred from bacterium to plant cell (Shaw et.al.1984; Wang et.al. 1984; Caplan et.al.1985; Peralta and Ream 1985; Jen and Chilton 1986a,b; VanHaaren et.al.1987; Wang et.al.1987). Ti plasmid derivatives containing only the right border region (in its native orientation) tend to transfer varying lengths of T-DNA sequences (dominant markers are selected for in infected plant cells). Also, recent studies suggest that such Ti plasmid derivatives have the capacity to transfer no more than about 50 kilobase-pairs (kbp) of DNA in total (Caplan et.al.1985; Rubin 1986).

Ti plasmids with right border deletions tend to be incapable of transferring their T-DNA into plants (Joos et.al.1983b; Shaw et.al.1984; Wang et.al.1984; Caplan

et.al.1985; Hepburn and White 1985; Peralta and Ream 1985; Jen and Chilton 1986a,b; Gardner and Knauf 1986). On the other hand, the transfer of T-DNA into the plant cell requires that the right border on the Ti plasmid be within about 30 kbp of the gene(s) to be selected for in the plant cell. Thus, T-DNA transfer from the Ti plasmid appears to be a size dependent event. The relative position and orientation requirements of the rightward 25 bp border sequence seems to play a role in increasing the efficiency of transfer of a certain size range of DNA; which contains dominant genetic markers (T-DNA located) that are generally selected for in the transformed plant cell.

Thus, and in summary, T-DNA transfer occurs unidirectionally and initiates from the right border. The efficient transmission (transfer and integration) of a DNA segment requires flanking borders. The left border appears to provide an in cis signal for the T-DNA transfer process, thereby indicating where the tail end of the T-DNA lies, resulting in the unit length transfer of T-DNA from A. tumefaciens to the plant cell.

The vir genes are expressed in A. tumefaciens and their gene products can act in trans on the T-DNA (Hille et.al.1984). When the T-DNA and vir region are on separate replicons in the same cell (comprising a binary system), T-DNA transmission into the plant genome is not

affected (Hoekema et.al.1983, 1984b; DeFramond et.al. 1983; Bevan 1984). Binary T-DNA appears to have less stringent orientation and position requirements of the 25 bp repeats. Binary vectors carrying only a right border (in either orientation) are fully transmissible. However, with only a left border, transmission of binary T-DNA is much less efficient but is nevertheless possible (Jen and Chilton 1986a; Gardner and Knauf 1986; Grimsley et.al.1986; Jen and Chilton 1986b; Horsch and Klee 1986). Recent studies of binary T-DNA have suggested that the orientation and position requirements of the rightward border are very reminiscent of bacterial conjugation (Jen and Chilton 1986a,b; Gardner and Knauf 1986).

2.3 T-DNA Processing Events and Genes Involved

Several recent studies suggest a model for T-DNA transfer, resembling the model for the transfer of plasmids between bacterial strains; involving the unidirectional transfer of a single-stranded DNA molecule from bacterial to plant cells. Site-specific nicking occurs within the 25 bp border elements (Yanofsky et.al.1986; Wang et.al.1987; Albright et.al. 1987). The expression of vir genes is essential for the nicking process. Induction of vir gene expression

requires that Agrobacterium be exposed to certain small molecular weight metabolites, such as acetosyringone (AS), which appear to be produced only in wounded plant tissues (Stachel et.al.1985b,1986a).

Recent studies indicate that nicking occurs only on the bottom strand (as is represented conventionally) of the 25 bp border sequence, regardless where the sequence exists (Stachel et.al.1986b,1987). However, double-stranded cleavage has also been observed (Albright et. al.1987 and Veluthambi et.al.1987). The virD operon codes for a site-specific endonuclease, which cleaves a unique site within the 25 bp border sequence (Yanofsky et.al.1986; Yamamoto et.al.1987; Wang et.al.1987; Stachel et.al.1987). Increasing the copy number of the virD region on a wide-host-range plasmid also increases the quantity of observed nicking products (T-strands) (Yanofsky et. al.1986). A very recent study suggested that border nicking is confined to only the bottom strand of the 25 bp sequence, at the third or fourth nucleotide base (Wang et.al.1987). Furthermore, border cleavage in binary T-DNA constructs appears to occur at a lower frequency than when in cis to the Ti plasmid; probably the result of higher copy number of the binary plasmid (Wang et.al.1987).

Bottom strand-nicking at the border elements results in a single-strand (ss) molecule comprised of

the bottom strand DNA (T-strand) bordered by the 25 bp sequence (Stachel et.al.1986b,1987; Yanofsky et.al.1986; Albright et.al.1987). The 5' end of the T-strand is the leading end in T-DNA transfer and lies within the right border element. The transferred single-strand of conjugative bacterial plasmids also have a 5' leading end (see review by Willetts and Wilkins 1984), which is further support for the conjugative type model of T-DNA transfer between A.tumefaciens and plant cells.

Recent studies suggested that several novel T-strand molecules are produced, which are reminiscent of replacement strand synthesis intermediates (internal T-DNA structures, Stachel et.al.1986b; Stachel and Zambryski 1986b), which are known to originate at the oriT of conjugative plasmids during inter-bacterial transfer (Willetts and Wilkins 1984). Also, T-strand synthesis is limited and tightly regulated; similar to what occurs in conjugative plasmids. Apparently, every cell contains on average about one T-strand copy; detected by hybridization of DNA of Ti plasmid-bearing A.tumefaciens that had been incubated with AS (Stachel et.al.1986b). Furthermore, the relative amount of T-strand plateaus approximately eight hours after AS-induction (Stachel et.al.1986b).

2.4 The vir Genes

It has been well established that there are at least six Ti plasmid vir genes required for T-DNA transfer from bacterium to plant. These vir determinants altogether comprise a 40 kbp region of the Ti plasmid. The vir region functions with equal efficiency in the inciting of T-DNA transfer whether in cis or in trans to the T-DNA.

Tumors are induced only where there is a wound on the plant. Wounded plant cells produce a number of characteristic signal molecules that can promote the positive chemotaxis of Ti plasmid-bearing cells of Agrobacterium toward wound sites where these molecules are at higher concentrations (Ashby et.al.1987). These same signal molecules also induce vir expression. Thus, the regulation of vir gene expression can be viewed as being a desired trait for Agrobacterium carrying a Ti plasmid, which enables these cells to recognize wounds and initiate T-DNA transfer (Okker et.al.1984; Stachel et.al.1985b,1986a; Bolton et.al.1986). All vir-inducer compounds are phenolic structures (for example, acetosyringone), believed to be secondary metabolites involved specifically in the normal wound response of plants (Stachel et.al.1985b,1986a).

Precisely what roles each of the Ti plasmid vir

genes play in T-DNA transfer is not fully understood. We are now aware of apparent functions of three of these genes, virA, virD and virG.

The virA gene appears to encode a membrane-bound environment-sensing protein. Recent reports by Stachel and Zambryski (1986a), Nixon et.al.(1986) and Leroux et.al.(1987) have suggested that the virA protein has strong amino acid homology with those members of several two-component regulatory systems that respond to external environmental stimuli; for example, the nitrogen assimilation regulatory genes ntrB and ntrC (Nixon et.al.1986). Expression of virA is constitutive and not plant wound inducible. Mutations in any other vir gene does not affect virA gene expression (Stachel et.al.1985a, b,1986a; Bolton et.al.1986; Stachel and Zambryski 1986a; Stachel and Nester 1986). Also, virA mutations block the AS-inducibility of other vir genes.

The virG gene is also constitutively expressed. Also, virG expression can be significantly induced by AS plus a variety of similarly structured phenolic plant wound signal molecules (Stachel et.al.1985b,1986a; Bolton et.al.1986; Stachel and Zambryski 1986a; Stachel and Nester 1986). Most virG mutations abolish the inducible expression of the other plant influenced vir loci. Furthermore, there appears to be sufficient evidence, including the observed effects of virG

mutations, to support the role of the virG gene product as being the main positive regulator that controls its own expression and the expression of the other plant-inducible vir loci. As a result of the analysis of the DNA nucleotide sequence of the virG gene (Winans et.al. 1986; Melchers et.al.1986), it has been determined that the predicted virG protein product has extensive amino acid homology with several better-characterized positive regulatory elements.

In summary, the virA and virG genes appear to serve in jointly bringing about the positive regulation of the plant-inducible vir genes (and presumably of the T-DNA transfer process as well). Several kinds of evidence support the proposal that the protein coded by the virA gene might function in the selective transport of suitable phenolic (plant wound associated) compounds into the bacterial cell. We still do not know the mechanism by which this specific permeabilization of the signal molecules can induce the expression of the virG locus. It has been predicted that the protein coded by the virG gene has the role of functionally binding DNA sequences, namely promoter regions, thus directing transcriptional activation of other plant-inducible vir promoters (Winans et.al.1986; Melchers et.al.1986). Such a protein may undergo essential conformational change upon induction by the plant wound phenolic compounds,

allowing it to interact with specific vir promoters and to ultimately enhance their recognition by RNA polymerase. The proposed role of the virD gene will be described later on in this literature review.

It has been found that A.tumefaciens chromosomal genes are also required for virulence, since Tn5 insertions in chromosomal DNA can lead to avirulence (Garfinkel and Nester 1980). These chromosomal mutations affect the attachment of the bacterium to the target plant cell. These A.tumefaciens genes that are required for attachment to the plant cell appear to exist within an 11 kbp region, called the chromosomal virulence (chv) region (Douglas et.al.1985). So far only two chv genes have been found in Agrobacterium, chvA and chvB; and they appear to be very closely related to Rhizobium meliloti chromosomal genes that are essential for plant nodulation (Dylan et.al.1986).

Close et.al.(1985) described an A.tumefaciens isolate possessing a chromosomal mutation called ros1. The ros1 mutant is characterized by a pleiotropic phenotype which includes elevated levels of expression of the virC and virD genes, respectively increased by 300 and 12 times above background levels of expression. This mutation does not affect tumorigenesis since the ros1 mutant carrying the Ti plasmid is fully virulent. To date, the only known phenotypes affected by this

mutation, in addition to an increase in virC and virD gene expression, are its unusual colony morphology and growth temperature optimum. Close et.al.(1985) proposed that the wild type chromosomal ros gene might ordinarily code for a trans-acting negative regulator(s). In the opinion of Close et.al.(1985), the expression of the virC and virD genes might normally be negatively regulated by the product(s) of the wild type ros locus, perhaps as input regarding the general physiological state of the bacterium, and positively regulated by the virG gene product, thus allowing for a more specific response to conditions favoring infection. Perhaps Agrobacterium has a multiple regulatory system, involving the ros chromosomal locus, in addition to the virA-virG regulatory system.

Although it seems likely A.tumefaciens possesses other chromosomal genes required for virulence and attachment to plant cells, nobody has yet reported any chromosomal mutations that affect T-DNA processing (T-strand synthesis and circular-T-DNA formation).

2.5 T-DNA Circle Formation

Several models for the mechanism of T-DNA transfer from A.tumefaciens to the plant cell were reviewed. In one model T-strands were proposed to be intermediates in

T-DNA transfer. Another model, rather a variation of the single-stranded conjugation model, was suggested by Koukolikova-Nicola et.al.(1985), and was based upon the detection of double-stranded circles, comprised entirely of T-DNA sequences, generated in A.tumefaciens. The formation of T-DNA circles occurred only whenever A.tumefaciens was incubated with wounded plant cells.

As a result of interest in the T-DNA transfer process, Koukolikova-Nicola et.al.(1985) were the first investigators to report observations of double-stranded T-DNA circles, in studies of A.tumefaciens incubated with plant cells. Accordingly, Koukolikova-Nicola et.al.(1985) utilized two different Ti plasmid constructs. One of these, pGV3850, carries a T-DNA comprised chiefly of plasmid pBR322. Plasmid pBR322 is a small molecule that carries the gene (the -lactamase {bla} gene) coding for resistance toward the antibiotic carbenicillin and, has a very limited host-range replicon that can replicate in E.coli but, not in Agrobacterium. Koukolikova-Nicola et.al.(1985) isolated and purified bacterial DNA from A.tumefaciens (carrying pGV3850) that had been incubated with plant cells. They used this bacterial DNA to transform E.coli and obtained a considerable number of carbenicillin resistant transformant colonies.

Only double-stranded DNA circles can be efficiently taken up by competent bacterial cells (Maniatis et.al.

1982). The denaturation of DNA from plant cell induced-A.tumefaciens carrying pGV3850, blocks its ability to transform E.coli, whereas the renaturation of this DNA restores its transformability (Jen and Chilton 1986b). A likely conclusion that can be drawn from this evidence is that linear structures with cohesive ends are the substrates for the transformation experiments.

Koukolikova-Nicola et.al.(1985) used another Ti plasmid construct (called pTiCos7), containing a T-DNA similar with pGV3850. In addition to the bla gene, pTiCos7 has the lambda-phage cos (cohesive-ends) site. The cos site is needed in cis for in vitro packaging of DNA into phage head particles. Studies of A.tumefaciens carrying pV3850 and pTiCos7 suggested that perhaps as many as one to ten percent of induced Agrobacterium cells will carry a copy of the transformable or packageable circular T-DNA (Koukolikova-Nicola et.al. et.al.1985; Stachel et.al.1986a).

Very recent studies of AS-induced and plant cell induced A.tumefaciens carrying pGV3850 suggested that T-DNA circles cannot be detected using in vitro assay techniques (Albright et.al.1987; Wang et.al.1987); yet, ss-T-DNA forms, suggested to occur at a frequency of about one for every A.tumefaciens cell, were detected using the same in vitro assay techniques (Stachel et.al. 1986b; Albright et.al.1987; Wang et. al.1987).

Several problems are inherent with conventional transformation and bacteriophage packaging-transfection techniques. Monomeric or dimeric circles, including non-covalently closed circular DNA molecules, with cohesive ends preventing the structure from becoming linear, are the only transformable structures that can be recovered in E.coli (Maniatis et.al.1982). Furthermore, tandem linear (even circular) arrays and multimeric circles are well suited structures for the lambda packaging reaction (Maniatis et.al.1982). Therefore, experimental systems of such design do not appear to be capable of supplying unambiguous results concerning the structure of rescueable T-DNA and non-T-DNA processing events.

During the course of my studies, T-DNA circle formation has recently been observed in E.coli (Alt-Moerbe et.al.1986) and in A.tumefaciens (Machida et.al. 1986). We now know that T-DNA circle formation requires Ti plasmid vir functions plus direct repeats bordering what is to be the T-DNA (or non-T-DNA depending on the orientation of the direct repeats in the assay system used by Machida et.al.1986). Recently, Machida et.al. (1986) constructed two model mini-Ti plasmids called pTMA and pTMD, (see Figure 1 of Machida et.al.1986). Both pTMA and pTMD contain the promoter and coding regions of the kanamycin resistance (Km^R) gene. In these two model constructs, the promoter is separated from the

coding region by the plasmid pBR322, which is flanked on both ends by border region fragments (carrying the 25 bp border sequences without the overdrive sequence) from an octopine Ti plasmid. The formation of T-DNA (from pTMA) and non-T-DNA (from pTMD) circles was selected for in A.tumefaciens that had been incubated with plant cells. Complete, unprocessed constructs expressed resistance towards carbenicillin and spectinomycin. Dependent upon the induction of vir expression, the pBR322 region is excised and the promoter is fused to the coding region of the nptII gene. Therefore, processing yielded smaller molecules that expressed resistance toward kanamycin (with spectinomycin resistance unchanged); yet, they no longer expressed resistance toward carbenicillin, because the pMB1 replicon of pBR322 cannot be maintained in Agrobacterium without constant antibiotic selection (VanHaute et.al.1983).

Recent studies suggested that formation of T-DNA and non-T-DNA circles occurs in one out of about every 10^5 cells of A.tumefaciens, after incubation either with plant cells (Machida et.al.1986) or AS (Usami et.al. 1987). The frequency of T-DNA circle formation (pTMA) is the same as the frequency of non-T-DNA circle formation (pTMD). Machida et.al.(1986) speculated that this low frequency of processed circle formation may be due to the Agrobacterium chromosomal background or due to the

absence of the OD sequence in pTMA and pTMD or, could be due to the combination of these two traits. Nonetheless, we still do not know whether the inability to detect T-DNA circles by in vitro hybridization techniques may be due to the rarity of these molecules.

The virD encoded site-specific endonuclease function is required for both T-DNA circle formation (Yamamoto et.al.1987) and the generation of ss-T-DNA (T-strand) structures (Stachel et.al.1987). Recently, it was suggested that virD might code for a site-specific recombinase function in addition to its established role as a site-specific single-strand-nicking endonuclease (Yamamoto et.al. 1987).

Koukolikova-Nicola et.al.(1985) suggested a model for T-DNA transfer, which considers T-DNA circle formation. This model is reminiscent of the rolling-circle type mechanism that has been suggested to occur in the transfer of conjugative plasmid between bacterial strains (Willetts and Wilkins 1984). The model proposed by Koukolikova-Nicola et.al.(1985) suggests that double-stranded T-DNA circle formation might be required for T-DNA transfer because either, i) the circle might be more efficiently transferred to the plant or, ii) it might be easier for single strands to "peel" off such a small circular structure than from the much larger Ti plasmid. To date there is not sufficient evidence to support the

proposed double-stranded T-DNA transfer model.

The formation of T-strands, linear-ds-T-DNA and T-DNA circles has some common features. The incubation of A.tumefaciens with either AS or plant cells results in the formation of ss-T-strands (Yanofsky et.al.1986), ds-T-strands (Veluthambi et.al. 1987) and T-DNA circles (Yamamoto et.al.1987). Formation of these three types of structures either requires AS- or plant-induced expression of the virD gene. Also, all three structures appear to be due to molecular changes within the 25 bp border repeat sequence. Several recent studies suggested that T-DNA circles are formed by either recombination between LB and RB within the 25 bp sequence or, by the ds-cleavage within and rejoining between the 25 bp sequence of LB and RB (Koukolikova-Nicola et.al.1985; Alt-Moerbe et.al.1986; Machida et.al. 1986; Albright et.al.1987; Wang et.al.1987).

Thus, and in summary, all of these events, the transfer of T-DNA from A.tumefaciens to the plant cell, the formation of T-DNA circles, T-strands and ds-T-DNA structures, require the expression of the virA, virB, virD and virG genes. However, we still do not know what significance, if any, the formation of linear, ss- and ds- structures and T-DNA circles has in the T-DNA transfer process. We can still only speculate about what structural form the T-DNA might be in when it transfers

from A.tumefaciens into the plant cell genome.

2.6 Behavior of Wide-Host-Range Plasmids in Agrobacterium

Many cloning vehicles have been made that can replicate in A.tumefaciens, do not themselves influence T-DNA transfer and tumorigenicity, have a suitable size for cloning with insertional inactivation, and can also replicate in E.coli. Plasmids belonging to the IncP and IncQ groups possess these four desired traits. Thus, most cloning vehicles used in A.tumefaciens are derived from either IncP or IncQ group plasmids.

Many Agrobacterium cloning vectors that can be mobilized between bacterial strains contain either an IncP or an IncQ origin of transfer (oriT) yet, are not self-transmissible because they lack the necessary transfer (tra) genes; such mobilizable, non-self-transmissible plasmids are designated as "helped" plasmids. Plasmids carrying tra genes which can mobilize "helped" plasmids are designated as "helper" plasmids.

Conjugative plasmids of the IncP group have been especially important in the development of molecular genetics in Agrobacterium. For example, many cloning vehicles derived from the IncP plasmid RK2 have been used in complementation studies of Ti plasmid vir

mutants (Iyer et.al.1982; Klee et.al.1982,1983; Stachel et.al.1985a,1986a; Stachel and Nester 1986) and in studies of binary T-DNA vectors (Bevan 1984; Koncz and Schell 1986).

IncP and IncQ "helped" plasmids can be mobilized between bacterial strains by IncP tra genes provided in trans in the donor strain. Recent studies suggested that "helped" plasmids can be transferred at about the same frequency (10^{-3} for every recipient cell) in matings between Agrobacterium strains and in matings between E.coli and Agrobacterium strains (Hille and Schilperoort 1981; Koncz and Schell 1986).

Most self-transmissible IncP "helper" plasmids co-transfer with IncP and IncQ "helped" plasmids into the recipient strain, at a frequency of about one. Therefore, in general, selection for the transfer of the "helped" plasmid is best achieved if the "helped" plasmid carries a dominant marker different from that carried on the "helper" plasmid. Many different "helper" and "helped" IncP plasmids have been made, for example, the self-transmissible IncP "helper" plasmid pPH1JI, which carries the gentamycin resistance gene (Hirsch and Beringer 1984) and, the "helped" IncP plasmid pGS65, which carries the IncP oriT plus the kanamycin and tetracycline resistance genes (Selvaraj and Iyer 1985).

Studies requiring the use of "helper" plasmids

unable to replicate in Agrobacterium might not be well served by the use of plasmids similar to pPHrJI. For this reason, Simon et.al.(1983) developed E.coli donor (mobilization) strains that carry the transfer genes of the IncP plasmid RP4 integrated in their chromosomes; for example, strain S17-1. However, these E.coli mobilization strains must be transformed in vitro with naked "helped" plasmid DNA. Compared to mobilization of plasmid DNA between strains, in vitro transformation can be less convenient.

Figurski and Helinski (1979) constructed a self-transmissible IncP mobilization "helper" plasmid, called pRK2013, which contains the ColEI replicon, and thus cannot replicate in Agrobacterium. However, pRK2013 carries the Km^r determinant, which many useful IncP and IncQ cloning vectors also carry. Currently, there are no reports describing a Cb^r plasmid similar to pRK2013 but, not containing Km^r.

Van Haute et.al.(1983) described a method for the production of site-directed mutations in Ti plasmids. In their studies, they used pBR322 clones containing mutant Ti plasmid fragments, and took advantage of the fact that pBR322 cannot replicate in A.tumefaciens. The pBR322 clones were used as suicide vectors. In other words, generation of site-directed mutations depended upon the co-integration of the mutant clone and the

acceptor Ti plasmid after transmission of the clone from E.coli to A.tumefaciens. The full exchange of mutant and target Ti plasmid fragments resulted from a second recombination in A.tumefaciens. Also, the pBR322 portion could not be recovered after screening for the appropriate combination of selectable antibiotic resistance markers. However, transfer of the pBR322 clones from E.coli to A.tumefaciens required the use of two non-IncP group "helper" plasmids provided in trans in the donor strain. The frequency of transfer of pBR322 from E.coli to A.tumefaciens was lower than that of suicide vectors containing the IncP oriT (Simon et.al. 1983).

Binary T-DNA vectors have been made that contain IncQ replicon and IncQ mob-oriT (Waldron et.al.1985; Buchanan-Wollaston et.al.1987). Recently, Buchanan-Wollaston et.al.(1987) showed that the oriT site, in combination with the mob (mobilization) genes of the small, conjugative but not self-transmissible, IncQ plasmid, RSF1010, can substitute for the 25 bp T-DNA border sequence for its transfer into plant cells. This most recent evidence is attractive support for the conjugational-type T-DNA transfer model. We now know that the mobilization functions of a naturally occurring plasmid, with a well established capacity for inter-bacterial transfer, but with no previously known history

of gene transfer ability into plant cells, can promote the transfer of this plasmid to plants. This binary T-DNA system requires that the bacterium carries IncP tra genes, which are normally involved in the mediation of inter-bacterial plasmid transfer, in addition to the Ti plasmid vir genes.

The tra genes code for pili and structural proteins necessary for promoting inter-bacterial contact between the donor and recipient. Many tra regions also contain a gene coding for a helicase-type protein required for the unwinding of plasmid DNA prior to the transmission of a single DNA strand to the recipient cell (Willetts and Wilkins 1984). Perhaps one of the Ti plasmid vir loci might code for an enzyme of similar function or the bacterial chromosome carries this essential function.

The tra region and mob loci can function in trans to the oriT site, which is analogous to the 25 bp border sequence, itself required in cis. The mob loci produce proteins that normally promote site-specific nicking only on this plasmid DNA (RSF1010 and very closely related plasmids) at the oriT site. This initiates the unidirectional transfer of single-stranded DNA into the recipient bacterium. The oriT sequences differ between plasmids and are highly specific. Although the oriT of the non-conjugative IncQ plasmid RSF1010 is readily mobilized by the tra functions of most IncP plasmids,

the mob gene products of only IncQ plasmids can evoke this transfer (Willettts and Wilkins 1984 and Buchanan-Wollastin et.al.1987). Thus, the 25 bp sequence appears to function much like the oriT does, and perhaps the virD gene provides mob-like protein specific for this 25 bp border sequence. It can be inferred that the 25 bp border sequence is itself not essential for the transfer process, provided that Tra, Mob and Vir functions are provided in trans (Buchanan-Wollastin.et.al.1987).

3.1 Bacterial Strains Used: Growth and Maintenance

See table 3-1 for a list of the bacterial strains employed in this work.

Liquid cultures of A. tumefaciens were made by inoculation of these cells into TYC broth (final w/v of 1% Difco Bacto-Tryptone (T), 0.5% Difco Yeast Extract (Y), and 0.066% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ which was added after autoclaving and cooling the TY components to below 60°C) plus the appropriate antibiotics (see table 3-2) and incubated for ≥ 24 hours at 30°C with constant shaking.

Liquid cultures of E. coli were grown in TYS broth (same as TYC except that instead of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, NaCl was added to 0.5% w/v final prior to sterilization), plus the appropriate constituents for selection (Table 3-2), were incubated at 37°C with shaking for the necessary length of time (usually ≤ 24 hours).

Solid TYS and TYC media were prepared by the addition of 1.6% (final w/v) Difco Agar prior to sterilization, after autoclaving, cooling and addition of appropriate antibiotics, the molten agar solution was poured into petri dishes and allowed to solidify for about 1 hour at room temperature.

Isolation of single colonies on solid media (agar plates) was achieved by streaking out bacterial cultures with the aid of a sterile loop. Such streak-plated cultures, after incubation and growth were stored at 5°C

Table 3-1 Bacterial strains

	Chromosomal markers	Plasmid	Relevant plasmid markers	Source/Reference
<u>A. tumefaciens strains^a</u>				
UBAPF2	Rif ^r	Plasmid-free	-	Hynes et.al.(1985)
GV3101/pMP90	Rif ^r	pMP90(pTiC58 with its T-DNA deleted)	Gm ^r	Koncz and Schell (1986)
GV3101/pMP90RK	Rif ^r	pMP90 with pRK2 insertion	Gm ^r , Km ^r	Koncz and Schell (1986)
LBA4404	Rif ^r	pAL4404 (pTiAch5 without its T-DNA)	Sm ^r	Hoekema et.al.(1983) Klapwijk et.al.(1980)
<u>E. coli strains</u>				
HB101	Sm ^r , <u>recA</u>	Plasmid-free	-	Boyer and Roulland-Dussoix, 1969
DH5	Sm ^r , <u>recA</u>	Plasmid-free	-	Hanahan (1985)
DH5 ^b	Sm ^r , <u>recA</u>	Plasmid-free	-	Jessee (1986)
S17-1	<u>recA</u>	RP4-2-Tc::Mu-Km::Tn7 integrated into chromosome, mobilize IncP/Q plasmids	Tp ^r , Sm ^r	Simon et.al. 1983
RR1NaI ^r	NaI ^r , Sm ^r	Plasmid-free	-	Derived in this lab from RR1 of Bolivar et.al.(1977)

^a All *A. tumefaciens* strains used in this work have a C58 genetic background.

^b ϕ 80d⁺lacZ M15 genotype allows α -complementation of lacZ gene by pUC or similar vectors. This trait is useful for screening colonies on solid media containing XGal. Strains DH5 and DH5^b are more proficient in uptake of large plasmid DNA than are most other *E. coli* strains.

Table 3-2 Antibiotics

	Concentration Range Used At	
	For <i>E. coli</i>	For <i>A. tumefaciens</i>
Carbenicillin (Cb) ^a (used in preference to ampicillin)	100- 200 μ g/ml	100- 200 μ g/ml
Chloramphenicol (Cm) ^b	10- 30 μ g/ml	5- 15 μ g/ml
Gentamycin (Gm) ^a	40- 100 μ g/ml	40- 100 μ g/ml
Kanamycin (Km) ^a	40- 100 μ g/ml	50- 1000 μ g/ml
Nalidixic Acid (NaI) ^c	50- 200 μ g/ml	20- 100 μ g/ml
Spectinomycin (Sp) ^a	50- 200 μ g/ml	50- 200 μ g/ml
Rifampicin (Rif) ^d	100- 300 μ g/ml	50- 200 μ g/ml
Tetracycline (Tc) ^e	10- 15 μ g/ml	5- 10 μ g/ml

^a From 25 mg/ml aqueous stock solution (filter sterilized). Stored at 5°C in dark.

^b From 10 mg/ml 100% ethanol stock solution (filter sterilized). Stored at 5°C in dark.

^c From 20 mg/ml aqueous stock solution (which was made by dissolving free acid form with NaOH prior to filter sterilization).

^d Made fresh at every use in methanol (final volume $\leq$ 0.5% methanol w/v).

^e From 10 mg/ml 50% ethanol stock solution (filter sterilized). Stored at -20°C in dark.

after wrapping in parafilm to limit the amount of evaporation. Petri plates were stored at 5°C for no longer than 60 days. Streak-plates of the A.tumefaciens strain LBA4301ros1 (Close et.al.1985) were stored at room temperature rather than at 5°C due to its inherent sensitivity to temperatures below 20°C during extended periods of growth. Long term storage of this strain at -70°C, however, did not appear to have any noticeable adverse effects toward its maintenance of viability.

Longer term storage of bacterial cultures at -70°C was achieved by doing the following: (1) growing the strain in 10 ml of liquid medium overnight, (2) harvesting the cell pellet after a 10 minute spin in a Damon/IEC Division Model HN-SII table-top centrifuge, (3) discarding the supernatant and resuspending it in 0.7 ml of sterile saline (0.9% NaCl) plus 0.4 ml 100% (w/v) glycerol, (4) transferring into 2 ml Nunc-Brand screw-cap polypropylene storage tubes, (5) incubating this cell suspension overnight at 5°C (in order to facilitate the uptake of glycerol into the cells thus ensuring high viability upon long term storage), (6) 10 minutes of quick-freezing in either a dry-ice/ethanol bath or in liquid-nitrogen prior to its storage at -70°C (good for $\geq$ 5 years with maintenance of both high viability and of relevant genetic traits).

The E.coli strain DH5 was maintained on solid agar

plates made up of M9 Minimal Salts Medium plus Thiamine (Maniatis et.al.1982).

Agrobacterium tumefaciens cultures were generally maintained and colonies obtained on medium D1M (Close et.al.1985; 5 gm/l of cellobiose, 1 gm/l of NH_4Cl , 0.3 gm/l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 gm/l of K_2HPO_4 , 1 gm/l of NaH_2PO_4 , 10 mg/l of malachite green, 15 gm/l agar) except that Bromothymol Blue <added at 100 mg/litre prior to sterilization thus turning the medium dark green-blue after autoclaving> was used instead of Malachite Green. All of the E.coli strains that were used during the course of this study are unable to utilize cellobiose as the sole carbon source, furthermore they are all auxotrophic for various amino acids, hence they are not able to grow on D1M. Characteristic yellowish, watery-mucoid colonies form when A.tumefaciens is grown on D1M, turning the surrounding medium the same color, which is due to its unique capacity for producing keto-lactose in the presence of cellobiose as the sole carbon source.

3.2 Bacterial Matings

The transfer of mobilizable and self-transmissible plasmids between different bacterial strains was done as follows: in all matings the donor to recipient ratio was 1:10. Early log phase donor cultures were used, whereas

recipients were used in late-log phase. The mixed cells suspensions were concentrated by vacuum-suction onto sterile 0.45 μ m porosity Millipore filters. Cells were kept on filters for the duration of the mating.

Matings between E.coli strains were done by placing the mating filter (containing the concentrated cell mixture) onto TYS agar plates without any added antibiotics and incubating at 37°C for ≥ 2 hours. Matings were interrupted by placing the filter in a 20 ml (2- 2.5 cm diameter) test tube containing 3 ml of sterile 0.89% NaCl, then vigorously vortexing until the cells were completely resuspended. Appropriate dilutions were serially made (in saline) and plated as 0.1 ml aliquots onto suitable selection plates and incubated at 37°C for 1- 3 days. The donor and recipient strains alone were plated undiluted as negative controls onto the same kind of selection plates.

Matings between E.coli and A.tumefaciens were carried out for ≥ 12 hours at 30°C on non-selective TYC plates. Depending upon whether the recipient strain was E.coli or A.tumefaciens, selection for transconjugants was achieved by plating out the matings on TYS, or TYC or DIM agar plates, respectively, with the appropriate antibiotics (plus counter-selective antibiotics against the donor strain) and incubated at 37°C whenever E.coli transconjugants were selected for, or incubated at 30°C

whenever A.tumefaciens was the recipient.

The mobilization of the model mini-Ti plasmid constructs, pTMA and pTMD from E.coli into A.tumefaciens was carried out essentially as described by Machida et. al.(1986) except that pGJ28 was not used. The transfer of pTMA and pTMD requires the cis-active ColEI bom (basis of mobilization, analogous with the nick or oriT) site present within the pBR322 region (Finnegan and Sheratt 1982; Van Haute et.al.1983). Transfer also requires the helper plasmids R64drd11 (Meynell and Datta 1967; Meynel and Cooke 1969; Van Haute et.al.1983) and pACYC184::ColEImob (Selvaraj unpublished 1987) in trans, providing the necessary Tra and Mob functions, respectively, which specifically recognize the ColEI bom site. Plasmid pACYC184::ColEImob lacks the ColEI bom site. Due to this fact it cannot be mobilized. Thus, it was preferentially used in place of pGJ28, which does carry the ColEI bom site. Transconjugants were selected on D1M agar plates containing 150 µg/ml Cb and 100 µg/ml Sp.

3.3 E.coli Transformation By Plasmid DNA

The transformation of E.coli bacterial cells was achieved by using the following procedure modified from Cohen et.al. (1972): (1) desired strain was grown in 10

ml of TYS broth until early log phase (about 10^7 to 10^8 cells/ml) was reached, (2) cells were pelleted by centrifugation in a table top centrifuge for 10 min., (3) the cell pellet was kept, the supernatant was carefully discarded, and (from here on until stated otherwise) all required constituents were chilled at 4°C on wet ice, (4) the cell pellet was resuspended into 10 ml of chilled sterile aqueous 100 mM MgCl_2 solution, (5) the cells were again pelleted, (6) the MgCl_2 solution supernatant was discarded and then the cell pellet was gently resuspended into 0.7 ml of sterile, chilled 75 mM CaCl_2 aqueous solution and then transferred into a prechilled, sterile 1.5 ml microcentrifuge tube, (7) a maximum state of competency was achieved by incubating overnight at 4°C , (8) DNA was then added to these cells (usually 10-100 ng of plasmid DNA in CaCl_2 solution at total volume of 100 μl was added to 100 μl of competent cells), (9) the DNA/cells mixture was incubated at 4°C for 1 hour, followed by (10) heat shock treatment by incubation at 37°C for 5 min., (11) 1 ml of 37°C preheated sterile TYS broth (without antibiotics) was added to these cells, and (12) incubated at 37°C for 1 hour in order to improve the cell viability upon, (13) selection by plating 0.1 ml aliquots onto appropriate selective solid media.

The E.coli strain DH5 α was transformed with pUC-

derived vectors and transformants were selected for on TYC agar plates containing XGal, in addition to the appropriate antibiotic(s). Solid selective media containing XGal plus IPTG were prepared as follows:

15 mg of XGal, freshly dissolved to 1.5% (final w/v) in N,N-Dimethyl Formamide, 40 mg of IPTG, freshly dissolved to 4% in water, and appropriate antibiotic(s) were together added to one liter of molten agar (between 50 and 60°C) just prior to pouring into sterile petri plates.

3.4 Acetosyringone Induction of A.tumefaciens

For A.tumefaciens Carrying pBIG or pCELGS65:

Analysis of frequency of recombination between the 25-bp repeats was accomplished by growing A.tumefaciens in TYC broth at 30°C for ≥ 24 hrs., to about 10^8 cells per ml. After centrifugation, the cell pellet was washed in MSSP liquid medium (Stachel et.al.1985b). After the MSSP wash and then recentrifugation, the cell pellet was resuspended into MSSP + AS, then incubated at 30°C with constant shaking for 3- 4 days. The MSSP medium used in this work was previously described by Stachel et.al. (1985b). The MS Mineral Salts were commercially obtained from Gibco as a powdered mixture (catalogue number 500-1117) which was used at 4.3 gm/l H₂O, and the final pH

was adjusted to between 5 and 5.5 using phosphoric acid. After autoclaving sodium phosphate was added to a final concentration of 12.5 mM then sucrose was added to 2% final (w/v).

After AS-induction, cells were plated onto TYC with or without various combinations of Km and Tc (for pBIG) (Km, Cm and Tc for pCELGS65). In another set of experiments, the cells were mated with HB101(pBRK4013) and with RR1Nal^r, then plated onto TYC containing the various combinations of antibiotics.

For *A.tumefaciens* Carrying pAH1:

Induction was performed in essentially the same way as with ~~*A.tumefaciens*~~ carrying pBIG, except that I used MSSP containing glucose in place of sucrose. Also, instead of plating the cells onto TYC with various antibiotic combinations, I had plated *A.tumefaciens* containing pAH1 onto TYC plus sucrose and onto TYC plus sucrose and Km, then replica plated the sucrose ($\pm$ Km) resistant colonies onto sucrose plus Cb.

Acetosyringone (4-acetyl-2,6-dimethoxyphenol), (AS) had been previously identified as a plant wound specific secretory compound and was demonstrated to induce the expression of the Ti plasmid vir genes (Stachel et.al. 1985b). The AS used in this work was purchased from the Aldrich Chemical Co. (catalogue number D13,440-6).

Whenever used, AS was added to MSSP liquid medium at a final concentration of $200\text{ }\mu\text{M}$ from a 510 mM stock solution in DMSO. The AS stock solution was stored frozen (in order to minimize the production of DMSO oxidation byproducts). The DMSO that was used to make the AS-stock was well purged with N_2 -gas prior to the addition of AS.

3.5 UV Light Sensitivity Testing of Bacteria

Cultures were grown to about 10^8 cell/ml in TYC broth. Then, 15 ml aliquots were harvested in a table top centrifuge and the pellets resuspended in 15 ml of saline. The cell suspension was then placed into a sterile 10 cm diameter by 1.5 cm high plastic petri dishes (with cover removed) and exposed to a UV light source (all experimentation from this point on was performed in a sterile field flow hood chamber, Canadian Cabinets Company Ltd., model BW6-99T-2). The exposure of cells to short-wave UV light employed the use of a Mineralight model UVS 11 UV lamp (Ultra Violet Products Inc., San Gabriel, California, USA). The UV light dose was determined using a Blak-Ray UV Intensity Meter model J-225 (U.V. Products Inc). The percentage survival was determined by plating 0.1 ml of serial dilutions onto TYC agar plates, and incubated at 30°C for 2- 5 days in

the dark to prevent photoreactivation. E.coli strains HB101 (recA⁻) and RR1 (recA⁺) were used as controls and, produced characteristic UV-sensitivity curves when the above methods were used (data not shown).

3.6 MMS Sensitivity Testing of Bacteria

MMS (methyl methanesulfonate, Aldrich Chemical Company Inc., Milwaukee, Wisconsin, USA, catalogue number 12,992-5) was added to molten TYC agar, prior to pouring into petri plates, at varying concentrations ranging from 0.1% to 0.5% (final w/v). The MMS was obtained from a 99% stock solution. Bacteria were patch replica plated onto TYC-MMS plates in order to ascertain whether or not they possessed a mutation causing recombination-deficiency.

3.7 Plasmids Used In This Study

See Table 3-3.

3.8 DNA Isolation and Purification Techniques

Plasmid DNA, used for restriction analysis and occasional cloning purposes, was obtained from small scale preparations using the alkaline-SDS method of

Table 3-3 Plasmids

	Relevant Characteristics	Source/Reference
pBR322	Cb ^r , Tc ^r , pMB1 replicon (limited-host-range)	Bollivar et.al.(1977)
pAH322	Cb ^r , Tc ^r , pBR322 <u>tet^r</u> gene flanked by <u>Eco RI</u> sites (Ava I site converted into <u>Eco RI</u> site)	This work
pUC13	Cb ^r , M13mp13 multiple cloning site, α -complementation, pMB1 replicon	Messing (1983)
pSF6	Sp ^r , IncW (pSa) replicon, IncP <u>oriT</u>	Selvaraj and Iyer(1984)
pAH69	Cb ^r , IncP <u>oriT</u> and lambda phage <u>cos</u> -site from pSF6 cloned into pUC13	This work
pAH79	Cb ^r , EcoRI resistant pAH69	This work
pCMR7	Cm ^r , pACYC184 bearing <u>A.tumefaciens</u> <u>recA</u>	Miles et.al.(1986)
pAH89	Cb ^r , pAH79 bearing pCMR7 Pst I <u>recA</u> fragment <u>cos</u> -site removed due to Pst I-digestion	This work
pAH99	Cb ^r , Tc ^r pAH89 bearing the pAH322 <u>tet^r</u> gene inserted within the <u>recA</u> fragment	This work
pTMA	Cb ^r , Sp ^r , pSa replicon, model mini-Ti plasmid has Km ^r gene promoter/coding region separated by pBR322. AS-induced pTi-vir mediated processing yields Km ^r T-DNA survivor	Kado et.ai.(1986)
pTMD	Same as pTMA except AS-induced pTi-vir mediated processing yields a Km ^r non-T-DNA type survivor molecule	Kado et.al.(1986)
R64d _{rd11}	Sm ^r , Tc ^r , Inc I replicon and Tra ⁺ constitutive (derepressed mutant) of R64	Meynell and Datta,1967 Meynell and Cooke,1969
pACYC184::ColEI _{mob}	Cm ^r , ColEI <u>mob</u> gene cloned into pACYC184 p15a replicon limited to <u>E.coli</u>	Selvaraj (unpublished)
pBin19	Km ^r , Inc P <u>rep,oriT</u> , M13mp9 multiple cloning <u>lacZ</u> α -complementation site. T-DNA binary vector	Bevan (1984)
pGSS33	Cb ^r /Tc ^r /Cm ^r /Sm ^r , Inc Q <u>rep, mob/oriT</u>	Sharpe (1984)
pBIG	Km ^r /Tc ^r , <u>in vitro</u> cointegrate between pBin19 and pGSS33	This work
pCEL44	Cm ^r , Inc Q <u>rep, mob/oriT</u> , T-DNA binary vector	Waldron et.al.(1985)
pGS65	Km ^r /Tc ^r , Inc P <u>rep, oriT</u>	Selvaraj and Iyer (1985)
pCELG565	Cm ^r /Km ^r /Tc ^r , <u>in vitro</u> cointegrate between pGS65 and pCEL44	This work
pRK2013	Km ^r , ColEI <u>rep</u> , Inc P <u>tra</u>	Figurski/Helinski (1979)
pBRK4013	Cb ^r , pMB1 <u>rep</u> , Inc P <u>tra</u> , <u>in vitro</u> cointegrate between pBR322 and pRK2013	This work
pUCD800	Km ^r , IncW and pMB1 <u>rep</u> , Suc ^S due to <u>sacR/B</u> gene	Gay et.al.1985
psacGSS33	Cb ^r , IncQ <u>rep</u> and <u>mob-oriT</u> , <u>sacR/B</u> from pUCD800	This work
pAH1	Km ^r , <u>sacR/B</u> -Cb ^r from psacGSS33 cloned into pBin19	This work
pGV0316	Cb ^r , pTiC58 <u>virE</u> gene cloned into pBR322	Depicker et.al.(1980)
pGV0361	Cb ^r , pTiC58 <u>virC,D (E)</u> genes in pBR322	Depicker et.al.(1980)

Birnboim and Doly (1979).

Large scale preparation of plasmid DNA was obtained by using the cesium chloride gradient centrifugation and purification technique described by Maniatis et.al. (1982). The determination of DNA concentration involved spectrophotometric analysis using an LKB Ultrospec II (model 4050, Cambridge, England).

Purification of DNA fragments after electrophoresis in agarose gels made use of the NaI-glassmilk system of the GenecleanTM Kit (BIO 101 Inc., P.O.Box 2284, La Jolla, California, 92038-2284).

3.9 DNA Modifying Enzymes Used In This Study

i/ Restriction endonucleases were obtained from Amersham (Canada Ltd., Oakville, Ontario), Bethesda Research Labs Ltd. (Gaithersburg, MD, USA), Boehringer Mannheim (Canada, Dorval, Quebec) and Pharmacia (Canada, Dorval, Quebec).

ii/ TA Buffer was used for T₄-DNA Polymerase treatment of DNA (see below) and for DNA digestion with all restriction endonucleases except for Sal I (where upon TA Buffer was modified for use with this enzyme by the addition of NaCl to 200 mM).

iii/ Eco RI Methylase was obtained from and used as directed by the manufacturer (New England Biolabs

(Beverly, Maryland, USA).

iv/ The protruding 5'-end-filling and 3' → 5' exonuclease functions of T₄-DNA Polymerase (purchased from Amersham) were used in this work. Reactions were held at 37°C for 30 min. in 1 x TA buffer (O'Farrell et. al. {1980}: 33 mM Tris Acetate, 66mM Potassium Acetate, 10mM Magnesium Acetate, 100 µg/ml Gelatine and 0.5 mM DTT) in addition to 0.2 mM of each required dNTP. About 2- 3 units of enzyme per µg of DNA was used.

v/ DNA ligation was carried out as prescribed by manufacturer using T₄-DNA Ligase. The ligation buffer used was supplied by BRL as a 5x stock solution containing 25% glycerol. Reactions were carried out at 15°C overnight. When transforming ligation reactions into competent bacterial cells, the reaction had to be diluted in 75 mM CaCl₂ such that the volume of ligation reaction is 1/20 of the volume of competent cells. This diluted the various components of the ligation reaction which may decrease transformation efficiency if not diluted. 5'- phosphorylated ds-Eco RI linker DNA was purchased from Pharmacia.

vi/ Alkaline phosphatase treatment of plasmid DNA was carried out under the conditions specified by the manufacturer (Boehringer Mannheim). The buffer used was as follows: 1x buffer; 50 mM Tris-pH 9, 1 mM MgCl₂, 0.1 mM ZnCl₂, 1 mM Spermidine.

3.10 Electrophoretic Analysis of DNA

DNA samples were analyzed by using agarose gel electrophoresis as described in Maniatis et.al.1982. The electrophoresis buffers used in this work were TBE (89 mM Tris base, 89 mM boric acid, 1 mM Na₂EDTA, pH 8) and TAE (40 mM Tris base, 12 mM acetic acid, 1 mM Na₂EDTA, pH 8). TBE buffer was generally used for high voltage/current runs and was reused several times due to its high buffering capacity. TAE buffer was generally used only for preparative work such as DNA fragment separation prior to Geneclean Kit purification.

The horizontal mini-gel apparatus used in this work was purchased from International Biotechnologies Inc. (IBI, New Haven, Connecticut, USA), model QSH (catalogue number 50000). The larger horizontal gel apparatus was purchased from Biorad Canada Ltd of Mississauga, Ontario and the regulated power supply used in this work was obtained from Savant Instruments Inc., model CVR350.

Reaction stop and loading buffer (an aqueous solution of 7 M urea, 70% sucrose, 50 mM Na₂EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol) was added at 1/10 volume to DNA samples prior to loading into 0.7% agarose gels.

Mini (5 x 8 cm, 0.6 cm thick) gels were run at about 60 volts for 2- 3 hrs depending on molecular

weight of DNA samples. Larger (15 x 15 cm, 1 cm thick) gels were generally run at 60 volts for 3- 5 hrs depending on molecular weight of samples.

After electrophoretic migration, gels were stained in 0.5 $\mu\text{g/ml}$ ethidium bromide aqueous solution (as described in Maniatis et.al.1982).

3.11 Radiolabelling of DNA Probes by Nick Translation

Plasmid DNA probes were radiolabelled with the Amersham Nick Translation Kit (catalogue number N-5000). The nick translation reactions were carried out as defined by the manufacturer whenever α - ^{32}P -dCTP (3000 Ci/mmmole specific activity from Amersham) was used. Where α - ^{32}P -dATP (3000 Ci/mmmole) was used instead of α - ^{32}P -dCTP, the enzyme solution provided in the nick translation kit was used, but not the nucleotide solution. In place of the nucleotide solution, the reaction were carried out with final concentrations of the following: 0.2 mM each of dTTP, dGTP and dCTP; 10 μM dATP; 1x Polymerase A buffer (50 mM Tris pH 7.5, 10 mM MgCl_2); DNA, water and ^{32}P -dATP to bring volume up to required amount.

Nick translation reactions were stopped by the addition of 1/3 volume of 10 M NH_4OAc plus 2.5 volumes of ethanol. After 30 min. of incubation at RT, the DNA

was pelleted by 30 min. of centrifugation at high speed in a microcentrifuge, then washed twice in 70% ethanol/30% H₂O and once in 100% ethanol. Afterward, the DNA was dissolved in H₂O. Generally, the probe exhibited specific radioactivity of about 10^8 cpm/ μ g.

Prior to hybridization, the ³²P-probe was boiled for 5 min., along with appropriate volume of sheared salmon sperm DNA.

3.12 Colony Transfer onto Filter Membranes/Filter Hybridization

Colonies were transferred from agar plates onto membrane filter circles and then hybridized to radiolabelled DNA probes essentially as is described in Maniatis et.al.(1982), except that Hybond-N nylon membrane circles (Amersham catalogue number RPN 82N for 10 cm diameter circles, using the procedure given by the manufacturer) were used instead of nitrocellulose filter circles.

RESULTS and DISCUSSION

Studies of A.tumefaciens carrying pGV3850 had suggested that the generation of T-DNA circles (rescued by conventional transformation of CaCl_2 -induced competent E.coli) might occur at a frequency of about one for every 100 A.tumefaciens cells (Koukolikova-Nicola et.al.1985; Stachel et.al.1986b). To test this suggestion, I have designed and constructed two different systems for studies of genetic events that occur in A.tumefaciens. One of these systems consisted of two mini-Ti plasmids and the second system made use of a third yet, different mini-Ti plasmid. I will describe how these mini-Ti plasmids were employed, and discuss their potential advantages and drawbacks.

4.1 Fused Wide-Host-Range Replicons

One system that I have designed and constructed, consisted of two different mini-Ti plasmids, pBIG and pCELGS65 (Figures 1 and 2, respectively). These two constructs were made in vitro (see respective Figures 1 and 2 for details of construction). Plasmids pBIG and pCELGS65 are cointegrates of two compatible wide-host-range (WHR) replicons. Each replicon is flanked by direct repeats of the 25 bp border sequence. In other words, each mini-Ti plasmid consists of T-DNA and non-T-DNA halves, with a different WHR-replicon in each half.

Figure 1 pBIG is a co-integrate plasmid, constructed in vitro by ligating Eco RI-Sst I digested pBin19 and pGSS33, and using this ligation mixture to transform DH5 α . pBIG was isolated from transformants resistant to Km and Tc, and its structure was determined by Bam HI-Eco RI, Sal I-Sst I, Hind III-Eco RI and Hind III-Sst I restriction analysis (data not shown). pBIG contains all pBin19 sequences, and has all pGSS33 sequences except for the Cb, Cm and Sm resistance genes. pBIG can replicate both in E.coli and A.tumefaciens, and presumably has functional IncP rep, IncP oriT, IncQ rep, IncQ mob and IncQ oriT sites. Left and right borders are indicated by LB and RB respectively. Open arrows represent the polarity of the 25 bp sequence. Genetic regions of interest are indicated: Km^r, Tc^r (kanamycin and tetracycline resistance genes respectively); and the Inc group of each rep, mob, and oriT sites are given. The T-DNA borders are from the nopaline Ti plasmid pTiT37, which does not contain the overdrive site (see the Literature Review section of this thesis). Sequences derived from pBin19 (Bevan 1984) are represented by the thick line, and sequences derived from pGSS33 (Sharpe 1984) are represented by the thinner line. Restriction enzyme cleavage sites are as follows: B (Bam HI), C (Cla I), E (EcoRI), H III (Hind III), S (Sal I) and Ss (Sst I). pBIG codes for Km resistance (Km^r) and Tc resistance (Tc^r).

Figure 2 pCELGS65 is a cointegrate plasmid, made in vitro by ligating Bgl II digested pGS65 (Selvaraj and Iyer 1985) and pCEL44 (Waldron et.al.1985), and using this mixture to transform DH5. pCELGS65 was isolated from transformants resistant toward Km, Tc and Cm, and its structure was determined by Bam HI-Eco RI, Eco RI-Hind III, Sal I-Eco RI and Eco RI-Pst I restriction analysis (data not shown). Genetic regions and restriction enzyme cleavage sites are the same with Figure 1, in addition to Bg (Bgl II), P (Pst I), Ac (Acc I), Av I (Ava I), Av II (Ava II), X (Xho I). cat indicates the chloramphenicol resistance (Cm^r) gene. In pCELGS65, the T-DNA and non-T-DNA regions are in the reverse location of that in pBIG; the T-DNA in pCELGS65 contains the IncP replicon and IncP oriT functions in addition to Km^r and Tc^r, whereas the non-T-DNA portion carries the IncQ replicon and mob-oriT functions, plus Cm^r. pGS65-derived sequences are represented by the thick solid line, and sequences derived from pCEL44 are represented by the thinner solid line. pCELGS65 contains all pGS65 and pCEL44 sequences, can replicate both in E.coli and A.tumefaciens, and is assumed to have functional IncP rep, IncP oriT, IncQ rep, IncQ mob and IncQ oriT sites. pCELGS65 codes for Km, Tc and Cm resistance.

Plasmids pBIG and pCELGS65 were used with the intention of detecting the T-DNA and non-T-DNA apart from one another. As a part of the studies of pBIG and pCELGS65, it was presumed that T-DNA and non-T-DNA circles would be stable together in the same cell. Whenever this system was used, no selection was applied at the point that resolution was assumed to occur. Consequently, it was expected that studies of the stoichiometry of T-DNA and non-T-DNA circle formation might be possible. A.tumefaciens strains carrying pBIG and pCELGS65 were incubated with AS at 30°C for 96 hrs., and spread-plated onto TYC containing no antibiotics. After incubation for three days at 30°C, several hundred colonies were picked at random and replica-plated onto several different antibiotic-containing TYC plates. Thus, and in summary, this study was expected to 1) find some colonies carrying either T-DNA or non-T-DNA circles and 2) determine the stoichiometry of formation of these structures presumed to result from resolution of pBIG and pCELGS65 into separate replicons in A.tumefaciens (Figure 3).

It was assumed that if the Ti plasmid can resolve into two smaller DNA circles, in A.tumefaciens incubated with AS, then mini-Ti plasmids pBIG and pCELGS65 can act as experimental models the Ti plasmid. In order for the system to work as intended, I would have had to detect

Figure 3 . Reason for using mini-Ti plasmids pBIG and pCELGS65. Region A represents the T-DNA. Region A contains Tc^R and the IncQ rep & mob-oriT functions in the case of pBIG. pCELGS65, on the other hand, has Km^R , Tc^R plus the IncP rep & oriT functions in region A. Region B represents the non-T-DNA. In the case of pBIG, region B contains Km^R plus the IncP rep & oriT, whereas the non-T-DNA portion of pCELGS65 contains Cm^R plus the IncQ rep & mob-oriT. The open arrows indicate the 25 bp border sequence, RB and LB (see Figures 1 and 2). Steps 1- 7 represent: 1/ LBA4404 carrying pBIG and LBA4404 carrying pCELGS65 were grown until about 10^8 cells/ml, in liquid TYC containing relevant antibiotics needed to select for both regions A and B; 2/ these cells were transferred into MSSP $\pm$ AS; 3/ incubated for 96 hrs. at $30^\circ C$; 4/ suitable serial dilutions were spread plated onto relevant antibiotic-containing TYC plates and onto non-antibiotic-containing TYC plates; 5/ the percentage viable cell density was determined as the ratio of the titers obtained from every plate containing antibiotics over the one lacking antibiotics; 6/ colonies from all different antibiotic and non-antibiotic-containing plates were replica plated onto every relevant type of antibiotic-containing TYC plates; 7/ the percentage of colonies that grew under these conditions was determined individually for each type of plate. Replica plating of colonies picked at random was intended to confirm any of

the four possible scenarios that are represented in this figure: i/ colonies capable of growth in the presence of antibiotics selecting for region A but not in presence of selection for B must have had region B separated from the original mini-Ti plasmid; ii/ colonies capable of growth in presence of selection for B, but not A, must have had region A separate from the original mini-Ti plasmid; iii/ colonies capable of growth in presence of selection for both A and B might have had either, iii a/ no change in the original dimeric mini-Ti plasmid or, iii b/ division of the mini-Ti plasmid into two separate monomeric plasmids, designated as circles A and B.

some colonies carrying only one of the two sets of antibiotic resistance markers originating from pBIG and pCELGS65.

A.tumefaciens strain LBA4404 carries the T-DNA-less Ti plasmid pAL4404 and can transfer binary T-DNA into plant cells (Hoekema et.al.1983). Plasmids pBIG and pCELGS65 were separately mobilized from S17-1 into LBA4404. LBA4404 carrying pBIG and LBA4404 carrying pCELGS65, at a titre of about 10^8 /ml, were separately incubated in MSSP $\pm$ AS, for 96 hrs at 30°C. Afterward, appropriate serial dilutions were spread onto TYC plates containing no antibiotics. A total of about one thousand randomly picked colonies, obtained from duplicately run investigations of each strain, were replica plated onto relevant antibiotic-containing TYC plates. In other words, about one thousand colonies each obtained from LBA4404 derivatives carrying pBIG, or pCELGS65 and incubated with and without AS were replica plated onto TYC plates with and without the following combinations of antibiotics: for pBIG, Km alone, Tc alone and Km,Tc; for pCELGS65, Cm alone, Km,Tc and Cm,Km,Tc.

Each replica plated colony was found to grow on every relevant antibiotic plate.

Each mini-Ti plasmid half carries either an IncP or IncQ origin of transfer (oriT). The oriT is required in cis for the plasmid to be mobilized between strains. The

oriT is mobilized by transfer functions provided in trans on a separate "helper" plasmid. The oriT of different Incompatibility (Inc) group plasmids tend to be best mobilized by the transfer functions (see review by Willetts and Wilkins 1984) of only a specific Inc group of plasmids. Both IncP and IncQ oriTs are specifically mobilized by the IncP tra (Willetts and Wilkins 1984).

Additionally, the transfer functions of an IncP plasmid were intended to be used to mobilize the mini-Ti plasmids and their presumed processed circles from A.tumefaciens to E.coli in triparental matings. Thus, the second reason for using pBIG and pCELGS65 was that tri-parental matings would hopefully bring about the separation of the mini-Ti plasmid and presumed processed circles from one another. For this purpose, the Cb^r "helper" plasmid pBRK4013 (see Figure 4 for details of its construction and structure) was designed and constructed. Plasmid pBRK4013 was found to transfer on average at a frequency of about one for every donor cell in 4 hr. matings between E.coli strains.

Studies of the mobilization of pCELGS65 and pBIG by pBRK4013, in triparental matings, were done in duplicate and held at 30°C for 2 days. These tri-parental matings involved an E.coli donor (DH5 carrying pBRK4013), an A.tumefaciens donor an E.coli recipient, RR1Nal^r. Tri-

Figure 4 pBRK4013 was made in vitro by ligating Eco RI-Sal I double-digested pBR322 and pRK2013, and using this ligation mixture to transform DH5. pBRK4013 was isolated from Cb^r transformants that were also sensitive to Tc and Km. Genetic regions that are indicated in this figure are as follows: Cb^r (carbenicillin resistance), kil and kor regions are loci that affect viability of the host bacterium, rep (pMB1 replicon of pBR322), pri (primase locus), oriT (nick-origin of transfer site), Tra regions (trans functional genes required for transfer) and the trf regions (trans-acting replication functions of the wide-host-range IncP replicon, which in this plasmid are incomplete and non-functional, see review in PhD Thesis of John Nash 1986). The solid line represents pRK2013-derived DNA, while pBR322-derived DNA is given by a jagged line. pBRK4013 can replicate in E.coli but not in A.tumefaciens, can transfer itself between E.coli strains at about 100% for every donor cell, can transfer itself from E.coli to A.tumefaciens at about 1% per donor cell, and can mobilize "helped" plasmids (which carry either an IncP or IncQ oriT, yet are not self-transmissible), on average, at frequencies of between about 10% and 100% for every donor cell in matings between E.coli. Restriction enzyme cleavage sites are given: ApI (Apa I), AvI (Ava I), B (Bal I), E (Eco RI), EV (Eco RV), HII (Hind II), HIII (Hind III), K (Kpn I), P (Pst I), Pv (Pvu II), S (Sal I), Sm (Sma I), Sp (Sph I), St (Sst II).

parental matings using A.tumefaciens carrying pBIG and A.tumefaciens carrying pCELGS65 were carried out separately. In such tri-parental matings, plasmids pBIG and pCELGS65 were able to transfer, on average, at a frequency of about one for every 10^3 A.tumefaciens donor cells.

Studies of LBA4404 carrying pBIG and LBA4404 carrying pCELGS65 were performed separately and in duplicate. These strains were incubated in MSSP $\pm$ AS at 30°C for 96 hrs and afterward subjected to separate tri-parental matings 30°C for 24 hrs. Both pBIG and pCELGS65 were found to transfer on average at a frequency of about 10^{-4} for every A.tumefaciens donor cell in such (24 hr.) tri-parental matings. Thus, incubation of these A.tumefaciens strains, with and without AS, did not appear to have any obvious affect on the transfer frequency of pBIG and pCELGS65, in tri-parental matings.

I have replica plated, about one thousand Km^R -RR1Nal^R colonies, obtained from tri-parental matings involving pBIG-bearing-LBA4404 that were incubated with AS; and about 10^3 Tc^R-RR1Nal^R colonies, obtained from tri-parental matings involving pBIG-bearing-LBA4404 that were incubated with AS; and about 10^3 Km^R -RR1Nal^R colonies, obtained from matings involving pBIG-bearing-LBA4404 that were incubated without AS; and about 10^3 Tc^R-RR1Nal^R colonies, obtained from matings involving

pBIG-bearing-LBA4404 that were incubated without AS; onto three types of antibiotic-containing TYS plates (all containing Nal: Km alone, Tc alone and Km,Tc). It was found that every replica plated colony could grow on all three types of antibiotic-containing TYC plates.

Similarly, I have replica plated about one thousand Cm^{r} -RR1Nal $^{\text{r}}$ colonies, obtained from tri-parental matings involving pCELGS65-bearing-LBA4404 that were incubated with AS; and about 10^3 Km^{r} , Tc $^{\text{r}}$ -RR1Nal $^{\text{r}}$ colonies, obtained from tri-parental matings involving pCELGS65-bearing-LBA4404 that were incubated with AS; and about 10^3 Cm^{r} -RR1Nal $^{\text{r}}$ colonies, obtained from matings involving pCELGS65-bearing-LBA4404 that were incubated without AS; and about 10^3 Km^{r} , Tc $^{\text{r}}$ -RR1Nal $^{\text{r}}$ colonies, obtained from matings involving pCELGS65-bearing-LBA4404 that were incubated without AS; onto three types of antibiotic-containing TYS plates (all containing Nal: Cm alone, Km,Tc and Cm,Km,Tc). It was observed that each replica plated colony was able to grow on every relevant type of antibiotic-containing TYC plate.

Spontaneous Nal $^{\text{r}}$ donors were observed to occur at a frequency of about one in every 10^9 cells. Km^{r} , Tc $^{\text{r}}$ and Cm^{r} recipient cells were detected at a frequency of about one for every 10^{10} cells.

Additionally, pBin19 and pGSS33 were found to be very stable together in the same cell. An LBA4404

strain, carrying both pBin19 and pGSS33 together, that was grown for 100 generations in TYC containing no antibiotics, was found to grow on TYC plates containing Km,Tc, at a density of about 50% of the viable titre. Both pBIG-bearing-LBA4404 and pBIG-bearing-UBAPF2, that were grown for 100 generations in TYC containing no antibiotics, were found to grow on TYC plates containing Km,Tc at a density of about 70% of the viable titre. Also, both pCELGS65-bearing-LBA4404 and UBAPF2 carrying pCELGS65, that were grown for about 100 generations in TYC containing no antibiotics, were found to grow on TYC plates containing Cm,Km,Tc at a density of about 70% of the viable titre.

The inability to detect T-DNA circles after tri-parental mating might have been due in part to the low transfer efficiency of my mini-Ti plasmids. The mobilization of IncP and IncQ oriTs by the IncP tra was found to occur at a frequency of about one for every donor cell, in matings between E.coli strains. Accordingly, the IncP and IncQ oriTs were mobilized non-specifically. Thus it is very likely that they were co-mobilized. Also, studies of the conjugative transfer of plasmids are complicated by the fact that physiological states of the recipient and donor strains must be taken into account (Willetts and Wilkins 1984). The transfer of IncP plasmids from E.coli to A.tumefaciens occurs at

a lower frequency than that between E.coli strains. In addition, the transfer of IncP plasmids from A.tumefaciens to E.coli tends to occur at an even lower frequency (Koncz et.al.1986). If the processing event occurred at a frequency of about 10^{-3} for every cell, then it is possible that an inefficient and non-specific mobilization system, in combination with the stability of separate IncP and IncQ replicons together in the same cell, accounted for the inability to detect the T-DNA and non-T-DNA circles, even by colony screening.

Jen and Chilton (1986b) had clearly indicated that the transformability of the pGV3850-derived DNA that had been used by Koukolikova-Nicola et.al.(1985), was destroyed by physical denaturation and restored by renaturation. Accordingly, it can be inferred that these T-DNA "circles" were linear structures containing cohesive ends. Perhaps under certain conditions mechanical breakage, at places where ss-nicks are generated (by the virD product), might account for the production of ds-T-DNA structures which can be used for transforming E.coli.

Recently, Stachel et.al.(1986b) and Albright et.al. (1987) had shown by hybridization that linear ss-T-DNA structures, but not ds-T-DNA circles, could be detected. Stachel et.al.(1986b) had suggested that linear ss-T-DNA structures (T-strands) are formed in every cell, at a

copy number of about one. Accordingly, it can be inferred that ds-T-DNA circles cannot be detected by in vitro hybridization because these molecules are generated at a low frequency (much lower than about 10^{-2} for every cell).

T-DNA and non-T-DNA circles had been detected by selecting for their formation in A.tumefaciens (Machida et.al.1986). Recent studies had suggested that these circles are generated at a frequency of between about 10^{-5} and 10^{-7} for every cell (Machida et.al.1986; Yamamoto et.al.1987; Usami et.al.1987). Accordingly, it can be inferred that the inability to detect T-DNA and non-T-DNA circles in the studies of pBIG and pCELGS65 is related to the rarity of the circularization event in A.tumefaciens.

Assuming that circularization is a rare event, then I would have had to replica-plate and screen about 10^6 colonies before detecting a processing event, in my studies of pBIG and pCELGS65. On the other hand, if the circularization event were to occur at a frequency of about one for every 10^3 cells, then it might have been detected. I have done studies of pTMA and pTMD, by separately examining their processing in derivatives of GV3101 carrying the T-DNA-less Ti plasmid pMP90. Circle formation was detected in the studies of these two strains, where incubation was held at 30°C in MS&P + AS

for 96 hours with constant shaking. The same conditions were used in my studies of LBA4404 carrying pBIG and pCELGS65.

In my studies of pTMA and pTMD, colonies resistant to both Km and Sp yet sensitive to Cb were found to occur at a frequency of about one for every 10^7 cells. Machida et.al.(1986) had incubated pTMA-, and pTMD-carrying A.tumefaciens with plant protoplasts, rather than with AS, and in NT medium instead of in MSSP; and suggested that processing occurred at a frequency of about one for every 10^5 cells. Very recently, Usami et. al.(1987) demonstrated that when A.tumefaciens carrying pTMA, was incubated in MSSP containing $200\mu\text{M}$ AS, the generation of $\text{Km}^r, \text{Cb}^s, \text{Sp}^r$ colonies carrying processed T-DNA circles occurred at a frequency of about one for every 10^5 A.tumefaciens cells. Therefore, I am confident that I have incubated A.tumefaciens carrying pBIG and pCELGS65 under conditions suitable for inducing the vir-mediated processing of my mini-Ti plasmids. Although every plausible alternative approach was surely not examined, this first system was nevertheless too impractical.

In general, failure to detect an event may be due to (1) use of an inefficient detection system, or (2) the event itself is rare. In practice, it could be due to a combination of these two factors. My assessment of

the results that had been obtained in these two studies was that the formation of T-DNA and non-T-DNA circles could have been detected only if the event occurred in every cell at a frequency greater than 10^{-3} .

Potentially, pBIG and pCELGS65 can be useful for studies of cointegrate plasmids. Furthermore, pBIG and pCELGS65 might also be useful for studies of linear, single-stranded and double-stranded T-DNA and non-T-DNA molecule formation in A.tumefaciens. The Ti plasmid is much too large for studies of non-T-DNA linear, ss- and ds-molecule formation. Accordingly, it can be inferred that the mini-Ti plasmids could be analyzed in their entirety because they are much smaller than the natural Ti plasmid.

Further experimental approaches could be taken in for studies of the stoichiometry of circle formation in A.tumefaciens. I suggest that one could begin by making plasmids similar to pBIG and pCELGS65, but containing an IncW oriT in place of the IncQ oriT-mob functions. Potentially, such constructs can be used the same way that pBIG and pCELGS65 were used, except that one could discriminate between T-DNA and non-T-DNA circles. Their mobilization by specific "helper" plasmids might be more selective. The IncP "helper" should be expected to mobilize the IncP oriT much more readily than the IncW oriT; likewise, the IncW "helper" would be expected to

selectively mobilize the IncW oriT at a much higher frequency than the IncP oriT. In this laboratory, Radhika Basheer has found that the IncW oriT can be mobilized by the IncW tra at a frequency of about one for every donor cell and by the IncP tra at a frequency of about 10^{-4} for every donor cell. She has also shown that the IncP oriT was mobilized by the IncW tra at a frequency of about one for every 10^4 donor cells (R.Basheer, personal communication 1987).

The construct, pBRK4013, is a plasmid of about 40.4 kbp derived from pRK2013 and pBR322; pBRK4013 carries the Cb^r gene and the pMB1 replicon on a DNA segment derived from pBR322. It was observed that both pBRK4013 and pRK2013 were capable of self-transfer between E.coli strains, on average, at a frequency of about one for every donor cell. Both pBRK4013 and pRK2013 were able to mobilize pSF6 (Selvaraj and Iyer (1984); a non-self-transmissible IncW replicon carrying Sp^r and the IncP oriT) on average at a frequency of about one for every donor cell, in matings between E.coli strains. Also, pSF6 was found to be mobilized by both pBRK4013 and pRK2013, from E.coli to Agrobacterium, on average at a frequency of about one for every 10^3 donor cells. Both pBRK4013 and pRK2013 were found to co-transfer with pSF6 at a frequency of one, in matings between E.coli strains.

4.2 A Mini-Ti Plasmid for Studying Deletions and Insertions in the T-DNA

The second system that was used in the present investigation consisted of the mini-Ti plasmid pAH1. Plasmid pAH1 was designed and constructed pAH1 for studies of deletions and insertions in the T-DNA. My aim was redirected to the development of an assay and detection system from one involving colony screening toward one based upon colony selection. Recently, Gay et.al.(1985) had constructed a WHR vector system for the positive selection and isolation of insertion sequence (IS) elements in Gram-negative bacteria. This vector, pUCD800, contains structural (sacB) and regulatory (sacR) genes which were cloned from Bacillus subtilis. The sacB gene codes for levansucrase, an enzyme secreted in culture medium by B.subtilis after induction with sucrose. Levansucrase catalyzes the transfructorylation from sucrose to various acceptors on the bacterial cell, resulting in two main physiological reactions: (i) levan synthesis and (ii) the hydrolysis of sucrose. The production of levansucrase is lethal to Gram-negative bacteria grown in the presence of sucrose.

The mini-Ti plasmid (pAH1) has a cassette of genes consisting of the sacR/B region adjacent to the gene coding for carbenicillin resistance (Cb^r). This sacR/B:: Cb^r cassette is flanked by direct repeats of the 25 bp

border sequence and is situated on a WHR replicon (see Figures 5 and 6 for details of its construction and structure).

The reason for using pAH1 was that insertions in the T-DNA region of pAH1 should inactivate the sacR/B gene and result in $\text{Suc}^r/\text{Km}^r/\text{Cb}^r$ cells. Deletions of the T-DNA region had been expected to result in $\text{Suc}^r/\text{Km}^r/\text{Cb}^s$ cells carrying a smaller, solely non-T-DNA plasmid. Thus, and in summary, the processing of pAH1 in A.tumefaciens, presumably by AS-induced vir activity, was hoped to result in $\text{Suc}^r/\text{Km}^r/\text{Cb}^s$ cells carrying a plasmid both smaller than pAH1 and containing solely, a non-T-DNA portion of pAH1; I had expected this to occur because the T-DNA part of pAH1 cannot replicate in bacteria (in plasmid pAH1, the ~~T-DNA~~ lacks a replicon (Figure 7)).

Theoretically, at least some Suc^r (survivor) colonies should have displayed Cb^s due to loss of the T-DNA portion of pAH1. I had expected Suc^r survivors to contain an almost complete copy of pBin19 (with a hybrid junction similar to that described by Machida et.al.1986 and Alt-Moerbe et.al.1986).

Incubation of GV3101 carrying pMP90 and pAH1, with and without AS, had not generated any $\text{Suc}^r/\text{Km}^r/\text{Cb}^s$ cells. The titers of Km^r , Cb^r and Km^r/Cb^r cells were, on average about $10^9/\text{ml}$. The titers of Suc^r/Km^r , Suc^r/Cb^r

Figure 5 Map of plasmid psacGSS33. Plasmid pUCD800 (an IS-element-trapping vector, Gay et. al.1985) was cleaved with Pst I and then made blunt-ended with T₄-DNA Polymerase. This blunt-ended, linearized DNA was cleaved with Bam HI to yield the 2.6 kbp DNA fragment containing the sacR/B (levansucrase) gene, represented by the thick line. Plasmid pGSS33 was cleaved with Eco RI, blunt-ended with T₄-DNA Polymerase and then Bam HI/Hind III double digested. Afterward, this blunt, linear pGSS33 DNA was alkaline phosphatase treated (to prevent self-ligatability). Subsequently, this dephosphorylated pGSS33 DNA was ligated to the 2.6 kbp sacR/B DNA fragment and this ligation reaction was used to transform DH5. psacGSS33 was isolated from Cb^r-Km^s-Suc^s transformants, and its structure was determined by Sal I, Sal I-Eco RI, Sal I-Pst I, Bam HI-Pst I, Eco RI-Pst I, Eco RI-Sst I and Hind III-Pst I restriction analysis (data not shown). psacGSS33 can replicate both in E.coli and A.tumefaciens, has functional IncQ mob-oriT sites, encodes sensitivity to sucrose and resistance to Cb. Restriction enzyme cleavage sites are given: B (Bam HI), E (Eco RI), H (Hind III), P (Pst I), S (Sal I), Ss (Sst I).

Figure 6 pAH1 is a mini-Ti plasmid derived from psacGSS33. pAH1 was constructed in vitro by ligating Bam HI-Sst I-digested psacGSS33 (purified 4.8 kbp sacR/B-Cb^r fragment) and pBin19, and using this ligation mixture to transform DH5α. pAH1 was isolated from Cb^r transformants and its structure was determined by Sal I, Eco RI, Bam HI-Eco RI, Hind III, Hind III-Eco RI restriction analysis (data not shown). In pAH1, the T-DNA portion contains the Cb^r and sacR/B genes and lacks a replicon, while the non-T-DNA portion carries the IncP rep & oriT functions and the Km^r gene. pAH1 can replicate both in E.coli and A.tumefaciens, has a functional IncP oriT, codes for sucrose sensitivity, Cb^r and Km^r. Processing of pAH1 in AS-induced A.tumefaciens (carrying a Ti plasmid) was expected to result in Cb^s-Suc^r-Km^r cells. (see Figure 7). Other genetic regions are the same with Figures 1 and 2. Restriction endonuclease cleavage sites are given: B (Bam HI), E (Eco RI), H (Hind III), P (Pst I), S (Sal I), Ss (Sst I).

Figure 7 Reason for using the mini-Ti plasmid pAH1. Open arrows represent the 25 bp border sequence and are arranged to indicate their relative position and orientation (direction), as direct repeats. LB and RB represent the leftward and rightward border repeats, respectively. Genetic symbols are the same with Figure 6. Step 1; GV3101 carrying both pMP90 & pAH1 was grown in liquid TYC containing Gm, Km and Cb until about 10^8 cells/ml. Step 2; cells were transferred into MSSP (containing glucose instead of sucrose) $\pm$ AS and then incubated for 96 hrs at 30°C. Step 3; the appropriate serial dilutions were spread plated onto TYC containing 5% Suc, onto TYC containing Suc and Km and onto TYC alone. After incubation at 30°C for between 2 and 10 days, the frequency of formation of Suc^r colonies was determined as the ratio of the titers of Suc^r cells (observed on TYC containing Suc $\pm$ Km) over the titer obtained from plain TYC. The Suc^r colonies were replica plated onto TYC plus Suc, TYC plus Suc/Km and onto TYC plus Suc/Km/Cb, and then these plates were incubated at 30°C for up to 10 days. Step 4; the percentage of colonies that grew after replica plating on all three types of TYC plates was determined (numbers given in parenthesis). Replica plating of colonies and the restriction analysis of their plasmid DNA was intended to confirm which one of the following schematically represented scenarios might be the appropriate: i/ no

detectable change in size or restriction endonuclease digest pattern of pAH1, except that pAH1 no longer expresses sensitivity toward sucrose; ii/ deletion of T-DNA region; and iii/ loss of pAH1 altogether.

and $\text{Suc}^{\text{r}}/\text{Km}^{\text{r}}/\text{Cb}^{\text{r}}$ cells were, on average about $10^4/\text{ml}$ and the titer of Suc^{r} cells was, on average about $10^6/\text{ml}$. The viable cell density was, on average about $10^9/\text{ml}$. These titers were obtained from two separate experiments (each ran in duplicate).

Curiously, there were high background levels of sucrose resistant cells (on average about one for every 10^3 cells). A total of about 800 $\text{Km}^{\text{r}}/\text{Suc}^{\text{r}}$ and about 800 Suc^{r} colonies had been replica plated onto TYC containing Cb. All of these replica plated colonies were found to be Cb^{r} . It is possible that indigenous IS elements in the A.tumefaciens chromosome caused the insertional activation of the sacB gene.

Studies of E.coli carrying pAH1 and portions of the Ti plasmid vir region (cloned in pBR322) has generated results that were very similar with the studies of pAH1 in A.tumefaciens. E.coli strains RR1 (recA⁺) and HB101 (recA⁻) are isogenic strains. In this study, HB101 and RR1 derivatives carrying pGV0316 and pAH1, and HB101 and RR1 carrying pAH1 and pGV0361, were used. The plasmids pGV0316 and pGV0361 are recombinant pBR322 derivatives that carry the cloned pTic58 virE gene, and virC, virD genes respectively. These E.coli strains were incubated in TYS containing neither AS nor antibiotics, rather than in MSSP ± AS. However, I was not able to check for Cb^{r} colonies because both pGV-plasmids carry Cb^{r} .

Unfortunately, Gay et.al.(1986) did not report the relative frequency (per cell) of inactivation of the sacB gene. It is possible that potential alternative solutions to this high Suc^r problem were overlooked in my studies of pAH1. Gay et.al.(1985) had shown by positive selection using pUCD800, that certain IS elements exist in some A.tumefaciens strains and not in others. However, these findings could not be readily correlated with my own observations concerning the inactivation of sacB in those same strains that Gay et.al.(1985) had studied. Apparently, Gay et.al.(1985) had entrapped only one IS element. Studies of Agrobacterium and its very close relative, Rhizobium, have suggested that they probably have more than one IS element in their genomes (Ramakrishnan et.al.1986; S.Denes of this laboratory, personal communication 1986).

Recently, Alt-Moerbe et.al.(1986) had studied the formation of T-DNA circles in E.coli carrying cloned vir genes. The system that Alt-Moerbe et.al.(1986) had used was very similar to one described in this thesis. They had constructed a mini-Ti plasmid containing a gene (galK) in the non-T-DNA region. When their plasmid was introduced into a certain E.coli strain, the host was prevented from growing unless the galK gene was removed in vivo by vir-mediated processing. Therefore, surviving cells contained T-DNA circles.

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The construct, pAH1, was similar in principal with the plasmid used by Alt-Moerbe et.al.(1986), except that the surviving DNA molecule had been anticipated to be the non-T-DNA circle. In both systems, host cells were subjected to negative selective pressure (by respective sac and galK genes) toward molecules which under normal conditions are beneficial substrates for cell growth. Accordingly, it is possible that if pAH1 was processed into two circles, then (1) the T-DNA region, or (2) the enzyme levansucrase, or (3) a combination of (1) and (2), would have remained long enough in the cell to prevent growth on sucrose-containing TYC.

In the studies of pAH1 carried by A.tumefaciens, Suc^r/Cb^s/Km^r colonies might have formed if the AS-incubated cells were allowed to grow for several generations in the absence of sucrose prior to selection for Suc^r colonies. On the other hand, the system devised by Machida et.al.(1986) selected for the formation of T-DNA circles (or non-T-DNA circles depending on whether pTMA or pTMD was studied) based upon events bringing together the promoter and coding regions of the Km^r gene. The plasmid pBR322 does not contain any functions that are detrimental to the cell, under the conditions that are used in these experiments. Also, pBR322 cannot replicate in A.tumefaciens, but even if it was able to, it is expected that the formation of Km^r colonies would

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not be affected. However, I probably would have designed pTMA and pTMD in such a way that the Cb^r marker was attached to only one replicon, for example the WHR, IncW one. Accordingly, it may be inferred that the results obtained from my studies of pAH1 in E.coli and A.tumefaciens were not unexpected.

Further approaches for studying pAH1 in E.coli carrying cloned vir genes could involve the construction of a modified pAH1; carrying an antibiotic resistance marker other than Cb^r in the T-DNA (for example Tc^r), potentially allowing one to screen for loss of that T-DNA marker and so indicate the presence of a processed plasmid.

The constructs psacGSS33 and pAH1 will have other future applications. Potentially, they can be useful for the positive selection and entrapment of IS elements in Gram-negative bacteria. Whereas plasmid pUCD800 lacks any mob-oriT sequences, pAH1 carries the IncP oriT and psacGSS33 contains the IncQ oriT and mob functions. It is expected that both psacGSS33 and pAH1 should be able to replicate in most Gram-negative bacteria without the need for constant selection. Furthermore, pAH1 and psacGSS33 can be efficiently mobilized, by IncP Tra functions provided in trans, from E.coli to Agrobacterium; this should increase their usefulness, because potentially they can be mobilized from E.coli

into other Gram-negative bacteria, especially if the non-E.coli cell cannot be efficiently transformed with plasmid DNA.

4.3 A Plasmid Constructed for Making recA Derivatives of Agrobacterium by Replacement Mutagenesis

Many studies of bacterial genetics involve using more than one recombinant plasmid that shares sequence homology with another replicon together in the same bacterium. Recombination deficient bacteria are used in such studies in order to eliminate the possibility of recombination occurring between the homologous DNA. Evidence suggesting that Agrobacterium possesses a recA locus was described by Better and Helinski (1983), who also made a recA strain of Rhizobium meliloti. The proof of a recA locus in Rhizobium, and the apparent homology of the E.coli recA gene with part of the A.tumefaciens chromosome stimulated the recent cloning and sequencing of the A.tumefaciens recA gene (Miles et.al.1986).

The recA gene of E.coli has been established as being essential for homologous recombination (Dressler and Potter 1985). Better and Helinski (1983) made it clear that the recA gene of Rhizobium meliloti is also required for homologous recombination. The recA strain of R.meliloti, described by Better and Helinski (1983) was produced by site-specific exchange insertion of

transposon Tn5 with the bacterial chromosome. Double homologous recombination between the chromosomal recA and a cloned, mutagenized homologue (with a site specific Tn5 insertion) resulted in the exchange of these recA genes. This site-specific Tn5 insertion inactivated the chromosomal recA gene.

The production of site-directed mutations in Ti plasmids has been well documented. One such method involves an approach similar with the one described by Better and Helinski (1983), except that incompatibility between the resident recombinant and incoming plasmids is not employed. Instead, the inability of the recombinant plasmid to replicate in A.tumefaciens is employed. In general, the most commonly used replicons in such plasmids are p15a and pMB1 type, which can replicate in E.coli without constant selection, but not Agrobacterium (Simon et.al.1983, VanHaute et.al.1983). Releasing selection for these replicons decreases their capacity to be maintained in Agrobacterium. Thus, when recombinant plasmids are involved, the likelihood for double-homologous recombination between sequences of interest is increased after selection for the plasmid is released. Accordingly, it can be inferred that such an approach potentially can be useful for the generation of site-specific insertion mutations in the chromosomes of bacteria other than E.coli and its close relatives.

Miles et.al.(1986) kindly sent me the recombinant plasmid pCMR7, which carries about 4.8 kbp of cloned A.tumefaciens chromosomal DNA. Within this cloned DNA lies the intact recA gene. It was found that the sample of pCMR7 that had been sent to me could indeed complement the recA mutation in E.coli strain DH5. Thus, pCMR7 was used for transforming DH5. It was observed that DH5 carrying pCMR7 could grow on TYS agar containing MMS, but not DH5 without pCMR7. Afterward, I felt justified to go ahead with my designing and making of pAH99 (Figure 10). I have sub-cloned a major portion of the cloned recA gene of pCMR7 into the vector pAH79 (which carries the IncP oriT, Figure 8), thus creating pAH89 (Figure 10). Plasmid pAH99 was made by subcloning the tet^r gene from pAH322 (Figure 9) into the unique EcoRI site of pAH89, within the recA fragment (Figure 10).

S17-1 carrying pAH99 was mated with UBAPF2, at 30°C for 48 hours, and this mating mixture was spread plated onto D1M agar containing 10 µg/ml-tetracycline (Tc). About one hundred Tc^r UBAPF2 colonies were obtained after incubation at 30°C for 5 days (from my own experience, A.tumefaciens generally grows slower on D1M than on TYC). It was observed that S17-1 carrying pAH99 and UBAPF2 could not grow on D1M containing 10 µg/ml Tc, even when 10⁹ cells were applied onto a plate and incubated for twelve days at 30°C. Spontaneous Tc^r

Figure 8 Plasmid pAH69 was constructed in vitro by ligating Eco RI-Pst I-double digested pSF6 and pUC13, and using this ligation mixture to transform DH5 α . pAH69 was isolated from Cb-resistant transformants, and its structure was determined by Eco RI, Pst I, and Eco RI-Pst I restriction analysis (data not shown). pAH69 contains all pUC13 sequences and a 1.5 kbp fragment of pSF6 containing the IncP oriT and the lambda-bacteriophage cos (cohesive ends site). pAH69 can be mobilized from E.coli strain S17-1 into RR1Nal^r at a frequency of about 100% per donor cell, and into A.tumefaciens strain UBAPF2 at about 10^{-2} per donor cell. pAH69 is no longer capable of α -complementing DH5 α . I also made pAH79, which is an Eco RI-resistant derivative of pAH69. pAH79 was made in vitro by ligating T₄-DNA polymerase-blunt-ended, Eco RI-digested pAH69, and using this self-ligation mixture to transform DH5. pAH79 was isolated from Cb-resistant transformants, whose plasmid DNA was restriction analyzed, screened for the presence of a 4.1 kbp, Eco RI resistant plasmid (data not shown). Restriction enzyme cleavage sites are indicated as follows: E (Eco RI), P (Pst I), X (Xba I).

Figure 9 pAH322 was made in vitro by the following steps: 1/ Ava I-digested pBR322 (linearized near one end of the tetracycline resistance (tet^r) gene) was, 2/ treated with Eco RI Methylase, which prevented Eco RI from cleaving at the unique recognition site, which is on the other side of the tet^r gene (opposite to the side that the Ava I site is on), 3/ this Eco RI Methylase treated, Ava I-cleaved linear ds-DNA was made blunt-ended by treatment with T₄-DNA Polymerase. The blunt ends were then, 4/ dephosphorylated by treatment with alkaline phosphatase. This dephosphorylated vector DNA was unable to self ligate in presence of DNA-Ligase. Afterwards, 5/ this DNA was ligated to phosphorylated Eco RI linker DNA, followed by, 6/ Eco RI-digestion, 7/ self ligation in presence of DNA Ligase and, 8/ used to transform DH5. pAH322 was isolated from Cb^r, Tc^r transformants, and its structure was determined by Eco RI; Eco RI-Pst I, Pst I-Sal I and Eco RI-Bam HI restriction analysis (data not shown). pAH322 contains all pBR322 sequences, which presumably includes a functional pMB1 bom site (basis of mobilization, analogous to oriT; Finnegan and Sherratt 1982). The complete digestion of pAH322 with Eco RI yields two DNA fragments, one of 2.9 kbp (containing the pMB1 replicon, the Cb-resistance gene and the bom site) and the other 1.4 kbp in size (comprised entirely of the functional Tc-resistance gene).

Figure 10 The suicide plasmid pAH99, which was intended for in vitro site-specific-recA-mutagenesis of the A.tumefaciens chromosome. pAH99 was made in vitro by the following steps: 1/ the 0.8 kbp DNA fragment containing most, but not all, of the recA gene (and was therefore non-functional) was isolated from plasmid pCMR7 (Miles et.al.1986) by Pst I digestion and NaI-glassmilk (GeneClean Kit) purification; 2/ Pst I-digested pAH79 was, 3/ dephosphorylated using alkaline phosphatase; 4/ these two DNAs were ligated together; 5/ this ligation mixture was used to transform DH5; 6/ pAH89 was isolated from Cb-resistant transformants after screening colonies using colony filter lift and hybridization to ³²P-labelled purified 0.8 kbp Pst I recA fragment DNA probe. The structure of pAH89 was determined by Eco RI, Pst I and Eco RI-Pst I restriction analysis (data not shown). pAH89 contains all pAH79 sequences and the 0.8 kbp Pst I recA fragment of pCMR7. pAH99 was made in vitro by ligating Eco RI-digested pAH79 and 1.4 kbp tet^r fragment (from pAH322), and using this ligation mixture to transform DH5. pAH99 was isolated from Tc, Cb-resistant transformants, and its structure was determined by Eco RI, Pst I and Eco RI-Pst I restriction analysis. (data not shown). pAH99 contains all pAH89 sequences and the 1.4 kbp tet^r fragment. All four plasmids, pAH69, pAH79, pAH89 and pAH99 were capable of being mobilized from S17-1 to E.coli

recipient at a frequency of about one for every donor cell. pAH89 codes for resistance to only Cb, pAH99 codes for Cb and Tc resistance. Restriction enzyme sites in this figure as the same as in Figure 8.

UBAPF2 were detected on TYC plates containing 2.5 and 5 $\mu\text{g/ml}$ Tc, at a frequency of about one for every 10^8 cells. On the other hand, spontaneous Tc^r RR1Nal^r were detected on TYS plates containing 5 $\mu\text{g/ml}$ Tc, at a frequency of about one for every 10^{10} cells. Spontaneous Tc^r UBAPF2 mutants were never observed on TYC plates containing 10 and 15 $\mu\text{g/ml}$ Tc, even when more than 10^9 cells were applied on a plate. Furthermore, S17-1 was never able to grow on D1M with and without Tc and Cb, even when about 10^9 cells were applied on a plate.

Fifty Tc^r UBAPF2 colonies were picked at random and tested for sensitivity to Cb and MMS. The 50 Tc^r UBAPF2 clones and wild type UBAPF2 were found to be capable of growing on TYC containing MMS (0.01%, 0.05%, 0.1% and 0.3% final v/v). The wild type UBAPF2 and 50 Tc^r clones were not able to grow on TYC containing 100 $\mu\text{g/ml}$ Cb. Furthermore, I have also tested two of the Tc^r clones for sensitivity to UV; both Tc^r clones and wild type UBAPF2 displayed identical percent survivor curves when treated with UV (Figure 11).

It is possible that this mating experiment resulted in the selection of Tc^r derivatives of UBAPF2. The Tc^r colonies, that were obtained from selection on D1M containing 10 $\mu\text{g/ml}$ Tc, very much appeared to be A. tumefaciens, judging from the colony morphology (texture, size, shape and odor), the characteristic

Figure 11 UV sensitivity curves for recA^+ UBAPF2 (open circle) and putative recA^- clones of UBAPF2 (open triangle and X). Cells from 15 ml log-phase liquid culture grown in TYC (about 10^8 cells/ml) were washed once in sterile saline then resuspended in 15 ml saline. The culture suspensions were then placed in sterile, uncovered petri dishes and irradiated with UV light (see Methods and Materials section).

yellow-orange hue that surrounded these colonies, and when streaked onto TYC plates, were all typical of A.tumefaciens-type growth. Spontaneous Cb^r UBAPF2 mutants arose on TYC and D1M plates containing 150 $\mu\text{g}/\text{ml}$ Cb , at a frequency of about 10^{-5} . Accordingly, it is conceivable that Tc^r mutations arose at a higher frequency in UBAPF2 than in E.coli.

Better and Helinski (1983) made a recA R.meliloti strain by site-specific mutagenesis using a mobilizable recombinant plasmid that contained 32 kbp of R.meliloti chromosomal DNA. Within this large cloned region, the recA gene was mutagenized in E.coli by a $Tn5$ insertion. After the clone had been mobilized into R.meliloti, homologous recombination between the plasmid-borne and genomic recA sequences gave rise to marker exchange (homogenotization), resulting in a recA Rhizobium derivative.

Plasmid pAH99 possesses 0.8 kbp of A.tumefaciens chromosomal DNA. It is possible that a recA derivative of UBAPF2 was not isolated because the cloned region was not large enough. Theoretically, a larger fragment of chromosomal DNA in pAH99 should be more likely to recombine with the bacterial genome. Studies by Simon et.al.(1983) and Jagadish et.al.(1985) had described the production of site-specific mutant derivatives of R.meliloti by using plasmids that are very similar to

pAH99, but contained cloned DNA about 20 to 30 kbp in size. Simon et.al.(1983) and Jagadish et.al.(1985) had obtained site-specific chromosomal insertion derivatives of R.meliloti by using suicide plasmids that cannot replicate in non-E.coli target cells. The suicide plasmids that they had used were efficiently mobilized from S17-1 into R.meliloti. I have also intended to mobilize pAH99 from S17-1 into A.tumefaciens. Thus, constructs similar to pAH99, but containing a much larger fragment of cloned Agrobacterium chromosomal DNA, potentially can be useful for obtaining recA derivatives of Agrobacterium.

Another approach for obtaining recA derivatives of A.tumefaciens is that a co-integrate between pAH99 and, an IncP plasmid which can replicate in A.tumefaciens, might be used. Thus, pAH99 could be maintained in Agrobacterium, yet the introduction of an incompatible plasmid into the pAH99-bearing strain would be expected to lead to the isolation of the desired derivative (in manner similar with Bétter and Helinski (1983)).

Yet another approach could simply involve the transformation of Agrobacterium with pAH99 DNA. If pAH99 did not appear to be capable of inciting a recA mutation after conjugative transfer from E.coli into Agrobacterium (which involves single stranded transfer), then it is possible that transformation of Agrobacterium

with naked plasmid pAH99 DNA can accomplish this objective.

Potentially, the procedure for obtaining site-specific mutants can be easy and rapid. However, one should use plasmids containing the largest sized piece of cloned DNA of interest in order to increase the probability of homologous recombination.

I have found that GV3101 carrying the self-transmissible T-DNA-less Ti plasmid, pMP90RK (Koncz and Schell 1986) can grow on TYC containing 1 mg/ml Km. I have also observed that pMP90 cannot self-transfer from GV3101 into another bacterium. This observation was not unexpected because pMP90 has the Gm^r gene inserted within the regulatory region of the nopaline catabolism operon (noc); the expression of the inter-bacterial transfer functions of the nopaline Ti plasmid is controlled by noc. Thus, studies of pTMA and pTMD carried by LBA4301 ($\pm$ ros1) were not done.

During my studies of the Agrobacterium recA gene, I have designed and constructed five potentially useful vectors. Plasmid pAH322 contains the Tc^r gene bordered by an Eco RI cleavage site on each end; and is useful for removing the intact Tc^r gene for subcloning purposes (Figure 9). The highly mobilizable plasmids, pAH69 and pAH79, carry Cb^r , the IncP oriT, the bacteriophage lambda cos site, the M13mp9 multiple cloning site and a

pMB1 replicon (Figure 8). Therefore, my constructs pAH69 and pAH79, potentially can be useful as vectors for random and site-specific insertion mutagenesis in non-E.coli genomes.

6

Further Discussion and Conclusions

My attempts to detect Ti plasmid processing into T-DNA and non-T-DNA circles were not successful. The approaches that involved screening of individual colonies were not sufficiently sensitive or efficient. Fused IncP and IncQ replicons were found to be stable without selection in A.tumefaciens. In addition, these two replicons were stable on separate molecules together in A.tumefaciens, and this trait had been previously observed in other Gram-negative species; for example E.coli, Pseudomonas sp., Methylophilus methylotrophus, Salmonella sp. and Klebsiella pneumoniae (J.Nash, personal communication 1987).

Nonetheless, it is possible that fused replicons may be useful in studies of A.tumefaciens and other Gram-negative bacteria; for example, studies of incompatibility between IncP and IncQ group plasmids. Furthermore, because pBIG and pCELGS65 are much smaller than the Ti plasmid, it is possible that they can be useful for studies of the formation of linear, ss- and ds-, T-DNA and non-T-DNA structures in A.tumefaciens.

Circularization of the T-DNA and non-T-DNA in A.tumefaciens had been suggested to occur in every cell at a frequency of about 10^{-5} (Machida et.al.1986). Alternately, it had been proposed that circle formation is not quite so rare, possibly occurring in every cell at a frequency of about 10^{-2} (Koukolikova-Nicola et.al.

1985). This apparent contradiction may be attributable to the differences between the systems that had been used (in the way that circles were rescued). It is possible that my studies of circle formation will emphasize the need for exercising caution in comparing the results of experiments where circles were rescued by different methods. Specifically, these methods are (1) using plant cell-induced A.tumefaciens plasmid DNA for transforming E.coli (Koukolikova-Nicola et.al.1985) and (2) selecting for the circularization event in E.coli (Alt-Moerbe et.al.1986) and A.tumefaciens (Machida et.al.1986). In spite of this shortcoming, studies of the formation of T-DNA and non-T-DNA circles are potentially valuable because this event occurs under conditions that are associated with the transfer of T-DNA from A.tumefaciens to the plant cell. It is likely that selection for circularization (for example, Machida et.al.1986) is more efficient than screening for this event (for example, my studies of pBIG).

Construct pAH1 was apparently not suitable for the selection of A.tumefaciens carrying non-T-DNA circles. Possible reasons for this are (1) activity of IS elements of the bacterial genome, (2) persistence of the sacR/B T-DNA fragment in the cell after processing, or a combination of (1) and (2). Nonetheless, pAH1 and psacGSS33 are likely to be useful in studies of

incompatibility and replication of plasmids, and insertion elements in gram-negative bacteria.

The binary T-DNA vectors pBin19 (Bevan 1984), from which pBIG and pAH1 were derived, and pCEL44 (Waldron et.al.1985), from which pCELGS65 was derived, are capable of transfer from A.tumefaciens carrying a vir-bearing plasmid to plant cells. By design, the in vitro construction of pAH1, pBIG and pCELGS65 did not disrupt the T-DNA border repeats. Accordingly, it was expected that the mini-Ti plasmids pBIG, pCELGS65 and pAH1 sufficiently resembled the Ti plasmid, considering their possession of T-DNA and non-T-DNA halves. Furthermore, I have shown that the medium (MSSP containing AS), in which LBA4404 derivatives carrying pBIG and pCELGS65 were incubated, was suitable for inducing GV3101 carrying pMP90 to process pTMA and pTMD into T-DNA and non-T-DNA circles, respectively. Very recently, Usami et.al.(1987) confirmed that MSSP containing AS is a suitable medium for inducing the same event in A.tumefaciens carrying pTMA and a complete Ti plasmid.

Plasmid pMP90 is a T-DNA-less derivative of the nopaline Ti plasmid, pTiC58 (Koncz and Schell 1986). The T-DNA-less Ti plasmid, pAL4404 (carried by LBA4404), is a derivative of the octopine Ti plasmid pTiAch5 (Hoekema et.al.1983). Machida et.al.(1986) had observed different processing frequencies of pTMA among various strains of

A.tumefaciens. They had suggested that the differences might be due to the chromosomal background rather than the type of Ti plasmid. A question that can be asked is whether or not the differences of processing frequency, obtained in my studies of the circularization event, were due to differences in the processing ability between these T-DNA-less Ti plasmids or the chromosomal background of the strains that were used. Further experiments could be carried out to investigate this question; for example, studies of the processing of pTMA in LBA4404, and pBIG in GV3101 carrying pMP90.

Potentially, pBRK4013 ~~can~~ be useful as a "helper" for mobilizing "helped" plasmids (for example, pSF6, pGS65 and pBin19) containing non-Cb^r markers. Also, it is expected that pBRK4013 can be useful as a "suicide" vehicle for the delivery of transposons, for example Tn5, into the chromosomes and resident plasmids of bacteria other than E.coli and its close relatives. The transposon Tn5 encodes resistance to kanamycin, is a well-studied representative of a class of moveable genetic elements and has been widely used for making insertion and deletion mutations, for example in the Ti plasmid (Garfinkel and Nester 1980; Iyer et.al.1982; Klee et.al.1982,1983; Lundquist et.al.1984) and in the chromosome of A.tumefaciens (Douglas et.al.1982,1985).

I have designed and constructed the cloning vectors

pAH69, pAH79 and pAH322. Also constructed was pBRK4013, which can be useful for the mobilization of non-self-transmissible "helped" plasmids containing either the IncP oriT or IncQ mob-oriT. It is expected that these constructs will be useful for studies of genetic events in the majority of Gram-negative bacteria, particularly those other than E.coli and its close relatives.

During the course of my studies, Buchanan-Wollaston et.al.(1987) very recently reported that the oriT of an IncQ plasmid (which also requires the IncQ mob gene functions provided in trans) can replace the 25 bp border sequence with equal efficiency for T-DNA transfer from A.tumefaciens into the plant cell. Studies of the processing of mini Ti plasmid constructs carrying the IncQ oriT in place of the 25 bp sequence at the T-DNA borders are expected to be possible. One could ask the question of whether or not such constructs would be processed into two smaller ds-circles, and would require directly repeated oriT borders. Potentially, processing of such a construct can generate linear ss-molecules. Further experiments could involve A.tumefaciens carrying the required mob (and/or virD), along with various IncP tra genes and/or vir genes. Thus, and in summary, it is suggested that further studies of the role of directly repeated oriT sequence, acting as T-DNA borders, might increase our understanding about T-DNA circularization

and Ti plasmid processing in general.

Recent reports indicate that Ti plasmid vir gene expression in E.coli (Alt-Moerbe et.al.1986) and in A.tumefaciens (Koukolikova-Nicola et.al.1985; Machida et.al.1986) can cause the formation of double-stranded circles comprised entirely of T-DNA sequences. Sequence analysis of these double-stranded T-DNA circles shows the presence of a single 25 bp-type of border sequence (the hybrid junction sequence); a hybrid between the left and right border sequences. Machida et.al.(1986) had suggested that the generation of T-DNA circles in A.tumefaciens occurs by the intramolecular homologous recombination between the right and left 25 bp border repeat sequences.

Thus, and in summary, under conditions that appear to simulate gene transfer into plants, processed forms of the Ti plasmid DNA can be detected in A.tumefaciens. The notion that a linear, double-stranded or single-stranded molecule is the true intermediate in T-DNA transfer leaves open the origin or role, if any, of the processed double-stranded circular DNA molecules. Yet, no definitive evidence currently exists in support of the notion that it is in either of these forms that Ti plasmid DNA is transferred into the plant cell.

To date no one has determined the role played by the homologous recombination system of A.tumefaciens in

the formation of processed circular T-DNA recombinants. All of the previously described studies on T-DNA circle formation used recombination proficient host bacteria. Although the junction sequence of the processed T-DNA circle appears to be associated with circle formation, we still can only speculate about the mechanism producing the hybrid border junction. Based upon the evidence that has been published to date, it can be postulated that the A.tumefaciens recombination system(s) might contribute to the recombination observed between the left and right, directly repeated 25 bp border sequence of the T-DNA. Apparently, the ability of dimeric plasmid DNA circles (possessing direct repeat sequences) to undergo intramolecular recombination and result in monomeric circle formation is a rather general phenomenon not limited solely to Ti plasmid processing (see review by Gellert and Nash 1987).

Earlier, Klapwijk et.al.(1979), had isolated a recombination-deficient strain of A.tumefaciens, called LBA4301. Miles et.al.(1986) had suggested that LBA4301 is not a recA mutant because its mutant phenotype cannot be complemented by the cloned A.tumefaciens recA gene. However, we still do not know the precise cause for the recombination-deficiency of LBA4301. Currently there is no recA mutant strain of Agrobacterium. The isolation of such a mutant would be useful not only for studies of

the role of recA in T-DNA processing, but also because of its relevance to the stability of cloned foreign genes maintained in Agrobacterium.

In this thesis I have described the construction of a small suicide plasmid carrying a site-specific insertion of the tetracycline resistance gene within most, but not all of the A.tumefaciens recA gene. Also, several further approaches that could be used for making a recA Agrobacterium derivative have been suggested. It is possible that in the near future, recA derivatives of A.tumefaciens will be isolated. Potentially, studies of processing of the Ti plasmid and of the mini-Ti plasmids pTMA and pTMD, in recA strains of A.tumefaciens, can generate more insights into the mechanism of T-DNA and non-T-DNA circularization. Accordingly, it is probable that the role of T-DNA and non-T-DNA circle formation, if any, in the T-DNA transfer process, will be determined from studies of well characterized recombination-deficient A.tumefaciens. For example, studies of LBA4301 and recA R.meliloti (a Km^r Tn5 insertion mutant) carrying constructs similar in design with pTMA, might be useful until a recA Agrobacterium derivative is isolated. One might expect to obtain from such studies the following scenario of suggested experimental outcomes:

1/ If T-DNA circularization is ever found to be

dependent upon bacterial recombination system(s), whereas T-DNA transfer from bacterium to plant is not, then one might conclude that T-DNA circle formation does not play any major role in the T-DNA transfer process.

2/ If T-DNA circularization and T-DNA transfer are ever found to be independent upon bacterial recombination system(s), then circle formation might be the cause of genetic determinant(s) other than the bacterial recombination system(s). We would still not know whether or not T-DNA circularization plays any role in the transfer process.

3/ If the T-DNA transfer process is ever found to be dependent upon bacterial recombination system(s), whereas T-DNA circle formation is not, then a very interesting puzzle would arise. The role of T-DNA circularization would still not be determined. One would have to investigate into the role played by bacterial recombination system(s) and also determine which gene(s) cause T-DNA circularization.

4/ If T-DNA circularization and T-DNA transfer together are ever found to be dependent upon the bacterial recombination system(s), then one might conclude that T-DNA circularization plays a role in the T-DNA transfer process. Further investigation would still be necessary in order to determine whether or not the T-DNA circularization and transfer processes can be separated.

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