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**LA THÈSE A ÉTÉ
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NODULATION AND TUMORIGENESIS BY AGROBACTERIUM
CARRYING RHIZOBIUM PLASMIDS

by

PAULINE A. DONALDSON

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

Department of Biology
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December 6, 1985

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The undersigned hereby recommend to the Faculty of Graduate Studies and Research acceptance of this thesis, submitted by Pauline A. Donaldson, B. Sc., in partial fulfillment of the requirements for the degree of Master of Science.

Chairman, Department of Biology

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December 6, 1985

ABSTRACT —

The ability of sym(biotic) plasmids of Rhizobium phaseoli, R. leguminosarum, and R. trifolii to functionally complement mutations in the Agrobacterium tumefaciens Ti plasmid virulence-genes was examined. None of the Sym plasmids introduced into A. tumefaciens restored the tumor-forming ability of the mutants. However an R. phaseoli symbiotic plasmid conferred to an A. tumefaciens Vir E- mutant the ability to form small tumors which lacked octopine synthase activity.

An R. leguminosarum and to a lesser extent an R. trifolii Sym plasmid caused attenuation of tumor-forming ability in wild-type A. tumefaciens strains. An examination of the host-specific nodulating ability of the constructed strains revealed further evidence of interference between Sym and Ti plasmids. An R. trifolii Sym plasmid carried in a Ti plasmid-free A. tumefaciens conferred a higher degree of clover root deformation and pseudonodulation than it did in the corresponding Ti plasmid containing A. tumefaciens. Relationships between Rhizobium spp. and A. tumefaciens are discussed.

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LIST OF ABBREVIATIONS

bp	base pairs
Cm	Chloramphenicol
pcr	cryptic plasmid
DNA	deoxyribonucleic acid
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	ethylenediaminetetraacetic acid
g	gram
Inc	incompatibility group
kb	kilobase pair
Km	kanamycin
L	litre
M	molar
MD	megadalton
mg	milligram
mL	millilitre
ug	microgram (10 ⁻⁶ gram)
uL	microlitre (10 ⁻⁶ litre)
Nal	nalidixic acid
nif	nitrogen fixation
Nod (Nod ⁻ or +)	nodulation (nodulation deficient or proficient)
pTi	tumor-inducing plasmid
tra ⁺ , tra ⁻	transfer proficient, transfer deficient
RNA	ribonucleic acid
rif	rifampicin
rpm	revolutions per minute
r	resistance
Sm	streptomycin
Sp	spectinomycin

s	sensitivity
Sym	symbiotic
SDS	sodium dodecyl sulphate
Tet	tetracycline
Tn	transposon
UV	ultraviolet
v.v	volume by volume
w.t.	wild type
w.v	weight by volume

INTRODUCTION

The soil is a common habitat for many bacteria and plants. Not surprisingly, complex symbiotic and pathogenic associations occur in this habitat between both these types of organisms. The molecular genetics of many important plant-bacterial associations is unclear, and this study contributes to an understanding of associations which are agronomically important.

Bacteria of the genera Rhizobium and Bradyrhizobium form an important symbiotic association with the roots of leguminous plants, an association which permits a process known as nitrogen fixation. This involves the formation of root nodules, which consist of groups of plant cells in which the bacterial symbiont resides in specialized cytosolic compartments. The bacterial symbiont converts atmospheric nitrogen (N_2) into a form of nitrogen which the plant can assimilate, thus permitting nodulated legumes to grow in nitrogen-depleted soil. The growth and consequent senescence of legumes in fields increases levels of available nitrogen in the soil. Practical research into nodulation is aimed at achieving the maximum nitrogen fixation possible for each agronomically important Rhizobium/Bradyrhizobium-legume association. Construction of Rhizobium strains to nodulate non-legumes would be extremely useful. However, plant and bacterial genes involved in symbiosis must be more fully understood before this is possible.

One of the most studied pathogenic plant-bacterial associations is crown-gall disease. Crown galls are tumors produced on many types of plants by Agrobacterium tumefaciens. Infection by this pathogen requires a wound (Lippincott and Lippincott, 1969), and so the disease is problematic where extensive wounding and cutting of plant tissues occur (Merlo 1982). Schell (1979) describes the pathogenic relationship of A. tumefaciens with plants as genetic colonization. During infection the pathogen transfers a discrete portion of its genome into the plant cell (Chilton et al., 1977). The transferred portion, the T-DNA, becomes integrated into the nuclear genome of infected cells (Thomashow et al., 1980; Zambryski et al., 1980) and is inherited by their progeny (Otten et al., 1981). T-DNA encoded genes maintain the tumorous condition of the plant cells (Braun, 1978) and the pathogen itself need not

remain (White and Braun, 1947). The T-DNA directed over-production of plant growth regulators is thought to cause the neoplastic growth of the plant cells. The T-DNA also codes for the production by tumor cells of unique amino acid derivatives, known as opines (Tempe et al 1979). Opines are ecologically significant because they can be catabolized by the virulent pathogen and thus the immediate vicinity of the tumor is a niche adapted for exploitation by the pathogen (Guyon et al. 1980).

Genetic colonization is an interesting natural phenomenon; it is also a potential tool for genetic engineering. Certain sequences of T-DNA can be replaced, *in vitro*, with other foreign DNA sequences and, if certain essential T-DNA border sequences are not interfered with, the foreign genes can be carried into plant genomes by *A. tumefaciens* (An et al. 1985). The bacterium is a natural gene vector which could be used to deliver agronomically 'useful' genes into selected plants, and thus to create 'improved' crop species. The bacterium has already been successfully used to introduce 'foreign' genes into plants (Matzke and Chilton, 1981; Herrera-Estrella et al., 1983; Schoffl and Bauman, 1985). The precise mechanism of T-DNA transfer is still unknown and so the use of T-DNA transfer as a new technology for crop improvement depends on continued research.

The two plant-bacterial associations which have been described (root nodulation and crown gall tumorigenesis) involve bacteria which are thought to be very closely related. The genera *Agrobacterium*, *Rhizobium*, and *Bradyrhizobium* involved in these associations are all currently classified in one family - the Rhizobiaceae (Jordan 1984). *Agrobacterium* and *Rhizobium* in particular share many taxonomic features and some microbiologists have suggested that the two genera be merged (eg. White, 1972). Heumann et al. (1984) have even suggested that the two genera represent different forms of one species with rearranged genomes. In the past, nodulation genes have been studied independently of tumorigenesis genes. This is not surprising since, superficially, the processes seem very dissimilar; however, they are both examples of bacteria-induced plant cell hyperplasias. Several observations suggest that *Rhizobium* and *Agrobacterium* might have some common genes contributing to both nodulation and tumorigenesis, but this possibility has not been investigated in detail.

Many of the genes contributing to the nodulating ability of *Rhizobium* (Zurkowski and

3

Lorkiewicz, 1979, Beynon et al. 1980) and to the tumor-forming ability of Agrobacterium (Van Larebeke et al. 1974) are encoded on extrachromosomal elements or plasmids, which replicate independently of the bacterial chromosome. Plasmids sometimes can be transmitted from one bacterium to another during the natural process of bacterial mating. Agrobacterium plasmids encoding many of the tumor-inducing genes are known as tumor-inducing or 'Ti' plasmids. Rhizobium plasmids encoding many of the nodulation genes are known as symbiotic or 'Sym' plasmids. When Sym plasmids are transferred to Agrobacterium, the Agrobacterium gains the ability to nodulate the legume for which the Rhizobium species from which the Sym plasmid originated is specific (eg. Hooykaas et al. 1981, 1985; Wong et al. 1983; Van Brussel et al. 1982). This supports the notion that these plasmids do indeed encode symbiotic genes. This also implies that the Agrobacterium and Rhizobium genomes either have genes which, when the Sym plasmid is present, enhance nodulation or have no genes which inhibit such nodulation. Ti plasmid genes that induce plant tumors are expressed in R. leguminosarum, R. phaseoli, and R. trifolii but not in R. meliloti (Hooykaas, 1979). The expression of Ti plasmid genes in Sym plasmid-free R. meliloti has not yet been studied. The possibility that the sym plasmid genes are responsible for inhibition of tumorigenesis has not been ruled out.

Ti plasmid tumor-inducing genes are not expressed in other bacteria such as E. coli (Holsters et al. 1978). Douglas et al. (1982, 1985) showed that certain Agrobacterium chromosomal genes are needed for tumorigenesis. The expression of Ti plasmids in several Rhizobium species and the presence of essential Agrobacterium chromosomal virulence genes suggest that these Rhizobium species may have genes with functions similar to the Agrobacterium chromosomal virulence genes.

At least two Ti plasmid regions contribute to tumorigenicity: the T-DNA already mentioned, and the virulence or vir region which is not transferred to plant cells during tumorigenesis but which is required for tumorigenesis (Garfinkel and Nester 1980; Holsters et al. 1980; Ooms et al. 1980; Iyer et al. 1982; Klee et al. 1983). Unfortunately the potential role of virulence region genes in T-DNA transfer remains unknown. The virulence region of some Ti plasmids have sequences which are structurally homologous to regions of Sym plasmids (Prakash and Schilperoort, 1982). If this structural homology represents functional

homology then there may be similar genes required for both nodulation and tumorigenesis. I have investigated this possibility by introducing symbiotic plasmids from Rhizobium into each of a series of Agrobacterium strains mutated in various virulence region loci. The tumor-forming ability of each constructed strain was compared to that of the avirulent mutants, to determine whether any of the Sym plasmids complemented the virulence region mutations. One mutation was partially complemented by an B. phaseoli Sym plasmid, although the tumors formed were not similar to tumors formed by the wild type strain. This will be discussed.

The relative contribution of chromosomal and plasmid encoded genes to nodulation and tumorigenesis is difficult to determine. When Sym plasmids are transferred to Agrobacterium, the functions associated with nodulation are usually attributed solely to the Sym plasmid. The strategy for determining Sym plasmid encoded traits or traits encoded on small nodulation gene clones of symbiotic plasmids often involves their transfer to Agrobacterium (e.g. Hirsch et al. 1985; Schofield et al. 1984). However, the chromosomes of both Agrobacterium and Rhizobium may be very similar (Hooykaas et al. 1982b) and both may thus encode genes contributing to nodulation or tumorigenesis. An interesting question is whether or not the Agrobacterium chromosomal virulence region, for example, is required for symbiotic plasmid expression in Agrobacterium.

There is some evidence that Rhizobium strains carrying both their native Sym plasmid and a Ti plasmid are impaired in their ability to nodulate and that tumors formed by these strains are small compared to those formed by A. tumefaciens (Hooykaas et al., 1979). Tumor formation in the corresponding Sym plasmid-free strains is normal. Is there interference between Ti and Sym plasmid encoded functions? No systematic survey of interactions between Ti and Sym plasmids has been done, and so I have looked at the nodulating and tumor forming ability of A. tumefaciens strains carrying various Sym plasmids with this question in mind.

LITERATURE REVIEW

2.1 Crown Gall Formation

2.1.1 General Features:

Many virulent strains of Agrobacterium tumefaciens produce crown galls or tumors on a wide range of dicotyledonous plants. Virulent strains of A. rhizogenes carry a root-inducing (Ri) plasmid and cause hairy-root formation with a mechanism similar to that used by A. tumefaciens for tumor formation (White et al. 1982). The host range of each particular bacterium varies somewhat, some have a very narrow host range (Panagopoulos and Psallidas 1973; Knauf et al. 1982). Recently Hooykaas-Van Slogteren et al. (1984) and Hernalsteens et al. (1984) demonstrated that monocotyledonous plants, once thought to be immune, can be infected by A. tumefaciens but that sometimes no visible tumor results (Hooykaas-Van Slogteren et al. 1984).

The early 'inception' phase of tumorigenesis requires the presence of viable virulent strains of A. tumefaciens (Braun 1982). A wound is one prerequisite for infection by A. tumefaciens, and may be necessary as a port of entry for the bacteria. Forty-eight to seventy-two hours after wounding in some plant tissues the first cycle of cell division, accompanied by rapid DNA synthesis, begins in the cells surrounding the wound (Lipetz 1970). Approximately forty-eight hours after wounding the cells become most susceptible to infection by A. tumefaciens (Braun 1962). At this time these cells are said to be conditioned. At this early stage of tumorigenesis temperatures greater than 28°C can inhibit tumor formation, although once tumors have been induced, these temperatures do not affect them (Braun 1947). Inter-bacterial transfer of Ti plasmids is also inhibited by these temperatures (Tempe et al. 1977) but there is no positive correlation between lack of Ti plasmid conjugative ability and avirulence (Schell et al. 1979). Wound sap may contain factors which induce bacterial virulence genes. At least one gene involved in virulence (vir C) has a promoter which is inducible with certain plant factors; however the factor/s is/are produced

by some unwounded plant tissue (Okker et al. 1984). In addition Stachel et al. (1985) have identified several *vir* regions whose transcription can be induced by certain plant factors and some which have higher levels of constitutive expression than others

A second prerequisite to tumor formation is the binding of bacteria to plant cells (Lippincott and Lippincott, 1969). Bacteria which are unable to bind are avirulent (Douglas et al. 1982, 1985). Neff et al. (1985) have demonstrated that plant cell binding sites for *Agrobacterium* are saturable. Binding on the plant cell wall is thought to involve the pectin component (Lippincott et al. 1977 and Neff et al. 1985). Binding probably involves lipopolysaccharides on the bacterial cell surface (Banerjee et al. 1981). Recently Puvanesarajah et al. (1985) reported that chromosomal mutations which render *A. tumefaciens* incapable of attaching to plant cells, also resulted in the elimination of 2-linked β -D-glucan by these mutants.

It is not clear what activities occur after the plant-bacteria binding and prior to the integration of T-DNA into the plant genome. It has been suggested that the entire plasmid enters plant cells during the early stages of tumorigenesis (Gelvin, 1984; Joos et al. 1983a). Koukolikova - Nicola et al. (1985) have suggested that T-DNA molecules are circular in the intermediate stage of the transfer of T-DNA into the plant cell. Once integrated into the plant genome, the T-DNA is transcribed (Drummond et al. 1977; Gurley et al. 1979) by the plant RNA polymerase II (Willmitzer et al. 1980). The level of expression of T-DNA genes in the bacterium is different from that of T-DNA integrated into the plant genome. Gelvin et al. (1981) observed only weak expression of octopine T-DNA genes in the bacterium, while Janssens et al. 1984 have reported that in the nopaline-type strain C58 at least seven of the T-DNA directed mRNA transcripts produced in the bacterium are similar to transcripts derived from the same T-DNA sequences in plant cells. The T-DNA transcripts in the plant are polyadenylated (Yang et al. 1980) like other eukaryotic mRNA's.

Among the T-DNA genes are those coding for crown gall specific amino acid derivatives or opines. The type of opine produced depends upon the type of Ti plasmid in the bacterial strain which has incited the tumor (Tempe et al. 1979). For example strains

with octopine-type Ti plasmids induce the formation of tumors from which octopine is produced. Encoded elsewhere on the Ti plasmid are genes specific for octopine catabolism. Strains with nopaline-type Ti plasmids induce the formation of tumors which specifically produce nopaline and these plasmids possess specific genes for nopaline utilization. Other T-DNA genes encode the synthesis of both auxins and cytokinins which are plant growth hormones (Braun 1982). The T-DNA directed over production of the growth regulators renders the plant cells hormone auxotrophic. Thus transformed plant cells are able to grow in vitro on plant growth medium without an exogenous source of plant growth regulators. Healthy, nontransformed plant cells must normally be supplied with plant growth regulators if they are to grow in vitro.

The bacteria-encoded production of plant growth regulators may play a role in the early stages of tumorigenesis. A gene for indole acetic acid (IAA) production is thought to be required for virulence (Liu et al 1982). This gene is part of the virulence region, a portion of the Ti plasmid not found in transformed tumor cells (Lemmers et al. 1980 and Thomashow et al. 1980). Another indication that IAA production may contribute to tumor initiation is that the inhibition of tumorigenesis in Agrobacterium, by an Inc W group R plasmid, pSa, is correlated with decreased levels of IAA production by Agrobacterium (Chernin et al. 1984). These observations suggest that plasmid-determined IAA production may be necessary for the initiation of tumorigenesis. In contrast to the uncertainty concerning the role of IAA production at an early stage, there is strong evidence for the role of both IAA production and cytokinin production in the maintenance of a tumor; that is, after T-DNA transfer (see section 2.1.2 a). Nopaline-type A. tumefaciens strains secrete the cytokinin trans-zeatin, which has been localized in the virulence region of the Ti plasmid pTiT37 (Akiyoshi et al. 1985). However in octopine-type A. tumefaciens strains trans-zeatin secretion does not necessarily occur (Reiger and Morris, 1982).

2.1.2 Genes Involved in Tumorigenesis:

a) The T-DNA region: Although the T-DNA region of the Ti plasmid is essential for tumor maintenance, many of the genes which it encodes are not involved in transfer of T-DNA (Lemmers et al. 1982; Joos et al. 1983b; Zambryski et al. 1983). Single mutations mapping in the T-DNA region usually lead to either an altered host range for virulence, to an altered tumor morphology (rooty, shooty or large) or to tumors unable to synthesize one or other of the opines (Ooms et al. 1981; Garfinkel et al. 1981; Ream et al. 1983). Even non-oncogenic T-DNA mutants of A. tumefaciens can still transfer T-DNA to plant cells (Hille et al. 1983).

In octopine-type tumors the size of the transferred DNA varies: some sequences are present in all octopine-type tumors. These sequences comprise the left part (the T_L -DNA) of the total T-DNA. The right part (the T_R -DNA) is not always found in octopine-type tumor cells (Thomashow et al. 1980). During infection by nopaline-type strains, the DNA which is transferred by the bacterium is less variable (Leemmers et al. 1980). The T_L -DNA of octopine-type tumors is structurally homologous to regions of the nopaline-type Ti plasmid T-DNA regions (Chilton et al. 1978; Engler et al. 1981) which are also necessary for tumor maintenance. This conserved T-DNA found in both nopaline and octopine-type tumors is known as the common or core DNA. The octopine-type common DNA (T_L -DNA) codes for eight transcriptional units (Willmitzer et al. 1982, 1983) some of which are absolutely essential for tumor maintenance. Mutations in genes one and two lead to tumors which produce shoots. This locus is referred to as ims and codes for production of indole-3-acetamide hydrolase, an enzyme involved in the biosynthesis of auxin (Inze et al. 1984; Klee et al. 1984). Mutations in gene four result in strains which form rooty tumors and this locus (known as tmr) encodes the production of DMA transferase, an enzyme involved in cytokinin biosynthesis (Akiyoshi et al. 1984; Buchmann et al. 1985). Transcript 3 codes

for octopine synthetase (Murai and Kemp 1982). The gene coding for transcript 6a has been designated ons for octopine and nopaline secretion (Messens et al. 1985). Transcript seven has been sequenced (Dhaese et al. 1983) but its function has not been reported. The octopine-type Ti plasmid T_R-DNA encodes five transcriptional units (Karcher et al. 1984) involved in the synthesis of at least agropine (Ellis et al. 1984; Salamon et al. 1984), mannopine and mannopinic acid (Komro et al. 1985).

When tumor maintenance genes encoded in the common T-DNA are deleted, T-DNA transfer can occur but no visible tumor is formed (Hille et al. 1983). However, flanking the T-DNA in both nopaline and octopine Ti plasmids are short direct repeating sequences which seem to be necessary for T-DNA transfer. In the nopaline Ti plasmid pTiC58 deletion of a 25 bp region flanking the right border of the T-DNA results in loss of oncogenicity (Shaw et al. 1984) while removal of the 25 bp sequence flanking the left border of the T-DNA has no such effect (Joos et al. 1983b, Wang et al. 1984). When the necessary right bordering sequences of this T-DNA are moved to other sites on the Ti plasmid they are still functional (Caplan, Van Montagu and Schell, 1985). In the octopine-type plasmids both the T_L-DNA and the T_R-DNA are flanked by 23 bp direct repeats; thus to the right of the T_L-DNA or core DNA there are three separate direct repeating sequences (Simpson et al. 1982; Zambryski et al. 1982). Removal of the left end of the T-DNA including the left border repeating sequence do not affect virulence (Ream and Peralta, 1985). However when all three repeating sequences to the right of T_L-DNA are deleted virulence is abolished but DNA fragments containing the T_L- right border repeat can restore virulence to the deletion mutants when it is inserted to the right of the T-DNA in the wild type orientation (Peralta and Ream 1985). If this DNA is inserted in the opposite orientation virulence is not restored; thus this sequence functions directionally. Wang et al. (1984) reported similar directional functioning of the essential 25 bp sequence flanking the right border of the nopaline T-DNA.

b) The chromosomal virulence region: Within an 11 kb DNA region on the A. tumefaciens chromosome, a 1.5 and a 5 kb chromosomal region (known as chv A and chv B respectively) have been identified (Douglas et al. 1985). Mutations in chv A or B result in bacteria which cannot attach to isolated plant cells, and which are avirulent. Some Rhizobium strains carrying Ti plasmids are virulent (Hooykaas 1979). These Agrobacterium chromosomal genes are therefore apparently dispensable for expression of tumor-forming ability in some Rhizobium spp., although they may be substituted by analagous Rhizobium chromosomal genes. Although the ChvA- and the Chv B- mutants are defective in attachment, they still produce cellulose microfibrils. Cellulose microfibril production is a chromosomally encoded trait which assists in the anchoring and accumulation of bacteria at the wound site, but which is not absolutely essential for virulence (Matthysse, 1983). Chv B- mutants have recently been found to be defective in the production of 2-linked- β -D-glucan, and this is further evidence for the involvement of these types of molecules in the attachment of A. tumefaciens to plant cells. Several mutations leading to an altered host range have been mapped to the A. tumefaciens chromosome but the precise location of these has not been reported (Douglas et al. 1985; Garfinkel and Nester 1980). It is possible, then, that other chromosomal genes contribute to virulence on some plants.

c) The Ti plasmid virulence region: This region quite likely encodes most of the other genes involved in the early stages of tumorigenesis, those stages up to and including T-DNA transfer. This region is highly conserved among various types of Ti plasmids (Thomashow et al. 1981) and hairy-root inducing or Ri plasmids (White and Nester 1980, Risuleo et al. 1982; Huffman et al. 1984). Both nopaline-type Ti plasmids and an Ri plasmid, but not Rhizobium Sym plasmids, can direct transfer of octopine-type T-DNA from an Agrobacterium strain lacking its own virulence region (Hoekema et al. 1984b). However, the precise role of the vir region genes is still unclear, with the possible exception of the regulation of IAA production (Liu et al. 1982) and trans-zeatin production (Akiyoshi et al 1984). Mutations in the virulence region either cause complete loss of virulence, or attenuation or loss of virulence on some hosts (Ooms et al. 1980; Iyer et al. 1982; Klee et al.

1983; Hooykaas et al. 1984; Lundquist et al. 1984; Hille et al. 1984). The virulence region is able to function when it is separated from the T-DNA on another replicon (Hoekema et al. 1983) and transfer of T-DNA occurs when the T-DNA is integrated into the chromosome of a cell which also has the vir region (Hoekema et al. 1984a). These genes are obviously critical to tumor formation and so warrant the more detailed review which follows

c (i) Virulence region loci and mutant phenotypes:

The genes of the virulence region have been defined by mutagenesis and by complementation studies of vir gene mutations. Seven genetic regions in octopine Ti plasmids (vir A - E, vir F, and vir Q) (Klee et al. 1983; Iyer et al. 1982; Hooykaas et al. 1984; Hille et al. 1984) and six (complementation groups I - VI) in nopaline Ti plasmids (Lundquist et al. 1984) have been defined. Figure 1 shows maps of several virulence regions from different Ti plasmids. Regions to the right of the six complementation groups of the pTiC58 virulence region are also involved in tumor formation but have not been formally named (see for example the region mutated in pGV3187 as shown in Figure 1). For the octopine Ti plasmid pTiA6NC the vir A, B, D, and E loci seem to lie on a region which shares DNA sequence homology with the nopaline Ti plasmid pTiC58 (Klee et al. 1983). The vir C locus was reported to lie in a region of the octopine-type Ti plasmid pTiA6NC which does not show homology to the nopaline Ti plasmid (Klee et al. 1983). Subsequent reports (Hooykaas et al. 1984; Joos et al. 1983a) suggest that this vir C locus may overlap regions showing homology to the nopaline Ti plasmids. The nopaline Ti plasmid pTiC58 codes for genes analogous in function to vir A - E of octopine Ti plasmids (Hooykaas et al. 1984). On the nopaline Ti plasmid the six virulence region loci occur in regions which share DNA sequence homology with octopine Ti plasmids (Lundquist et al. 1984). Vir Q of the octopine Ti plasmid pTiB6 (Hooykaas et al. 1984) and pTiACH5 (Hille et al. 1984) occurs in a region for which there is no structural homology with the nopaline Ti plasmid pTiC58 (Hooykaas et al. 1984; Hille et al. 1984). However, mutations in this locus can be complemented by the nopaline Ti plasmid (Hooykaas et al. 1984), indicating that a functional counterpart to vir Q occurs on nopaline-type Ti plasmids. According to Hooykaas et al. (1984) and Hille et al.

Figure 1: Restriction endonuclease maps and map positions of Virulence genes for several *A. tumefaciens* Ti plasmids.

pTiC58: The map positions of vir i - vi are according to Lundquist et al. (1984).

indicates the site of a mutation causing avirulence and decreased production of IAA by the bacterium (Liu et al. 1982). *A. tumefaciens* with a mutation in the region indicated by pGV3187 is also avirulent and therefore this location represents part of the pTiC58 vir region which remains unnamed. The position of the insertion in pGV3187 is according to Holsters et al. (1980).

pTiB6: The map positions of Vir A-E and vir Q are according to Hooykaas et al (1984).

pTiA6: The map positions of Vir A-E are according to Iyer et al. (1982). Insertions 1,2, and 3 mark the site of mutation in *A. tumefaciens* A2028, A1034, and A1021 respectively (Klee et al. 1983). *A. tumefaciens* A2028 and A1021 are avirulent mutants while *A. tumefaciens* A1034 is an altered host range mutant (Klee et al. 1983) which according to Hooykaas et al. (1984) is a Vir Q mutant strain.

These indicate the approximate regions of structural homology between pTiC58 and pTiB6 DNA sequences. Engler et al. (1980) described the regions of structural homology between pTiC58 and pTiACH5, however since pTiACH5 shows extensive homology to pTiB6 the homology data for pTiC58 and pTiACH5 is often used to define the approximate regions of homology between octopine and nopaline Ti plasmids in general.

Sites of insertion of Tn3 HoHo1 in specific *A. tumefaciens* pTiA6 mutants as described by Stachel et al. (1985). These mutants are discussed in the text

(1) 10 11, 12

11	18	27	37, 39	19	23a	29	38ab	24	17	36	15	33	16	Eco R1
12		9	18	11a	27	1b			7			10		Hind III

3	14	24	11		18		1	Barn HI
2		21		4			23	Eco RI

(1984) vir Q maps immediately to the left of the insertion site of some previously defined Vir C- mutations (Klee et al. 1982; Iyer et al. 1982) and some newly defined Vir C- mutations (Hooykaas et al. 1984). According to the classification scheme of Hooykaas et al. (1984), Vir O- mutants have an altered host range phenotype and map in a region of DNA which shows no homology to pTiC58, while Vir C- mutants are completely avirulent on all plant hosts tested and have mutations in a region for which there is structural homology with pTiC58. Vir O- and Vir C- mutants are able to complement one another (Hooykaas et al. 1984). Vir E maps to the right of vir E in the octopine-type Ti plasmid pTiB6 and mutations in this locus also result in an altered host range phenotype (Hooykaas et al. 1984).

Most mutations in vir A completely abolish virulence; exceptions are two Tn3 mutations described by Iyer et al. (1982) which have an extremely attenuated virulence on some of the plant hosts tested. Most mutations in vir B and vir D completely abolish virulence; therefore these loci seem to be absolutely essential for virulence. An exception is strain A1028, isolated by Garfinkel and Nester (1980) and classified as a Vir D- mutation by Hooykaas et al. (1984), which has only a slightly attenuated virulence phenotype (Garfinkel and Nester 1980). Other mutations in vir D, however, completely abolish virulence (Klee et al. 1983; Iyer et al. 1982). Mutations in vir E, vir Q, vir E and some in vir C lead to altered host range phenotypes (Klee et al. 1983; Iyer et al. 1982; Hooykaas et al. 1984 and Hille et al. 1984). The Vir E- mutants seem to have a more restricted host range than the other altered host range mutants.

c (ii) Possible functions of vir region genes:

Most Ti plasmids virulence region genes seem to code for functions which can act in trans with respect to other vir genes (Iyer et al. 1982; Klee et al. 1983; Hooykaas et al. 1984; Hille et al. 1984; Lundquist et al. 1984). However, vir B may code in part for a cis dominant locus (Iyer et al. 1982). Conflicting reports indicate that this locus can act in trans with respect to other vir genes (Hille, van Kan, and Schilperoort, 1984). Supporting the notion of a vir B cis dominant locus is the observation that a particular Vir B- mutation in pTiB6 is not complemented in trans by an Ri plasmid, while other vir B region mutations are (Hooykaas et al. 1984). Also, Lundquist et al. (1984) have identified a cis dominant region in complementation group ii of the

nopaline-type Ti plasmid pTiC58.

A few mutations in the nopaline Ti plasmid vir region, probably in a vir E - like locus, cause avirulence and decreased levels of IAA production (Liu et al. 1982). The role of IAA production in the early stages of tumorigenesis has not been examined in detail. Of course enzymes involved in IAA synthesis are encoded on the T-DNA and do play a role in tumor maintenance (Inze et al. 1983; Klee et al. 1984). A gene for secretion of the cytokinin, trans-zeatin, has been localized on a nopaline-type Ti plasmid pTiT37, and apparently this gene occurs in the virulence region of this plasmid, although precisely where is not clear (Akiyoshi et al. 1985). This gene has a sequence similar to the T-DNA encoded tmr (rooty tumor locus) gene involved in cytokinin production. In octopine-type A. tumefaciens strains secretion of trans-zeatin could be detected if these strains contained certain nopaline-type Ti plasmids (Reiger and Morris, 1982).

Vir C- mutations of an octopine- but not a nopaline- Ti plasmid are complemented by genes carried on the hairy-root inducing plasmid (Hooykaas et al. 1984). Vir C is of particular interest since it has a promotor which is inducible by an unidentified plant factor. The factor is heat labile, probably proteinaceous, and is produced by both monocotyledonous and dicotyledonous plants even in the absence of wounding (Okker et al. 1984). So far plant factor induction has only been demonstrated for an octopine-type vir C promotor. Allenza et al. (1984) observed that co-cultivation of Agrobacterium and cultured plant cells results in induction of vir genes, and that mutations in vir A result in strains in which none of the vir genes are inducible by plant factors. Data outlined by Stachel et al. (1985) show that at five particular insertion sites of the transposon Tn3:: HoHo1 (carrying a promoter-less Lac Z gene) the gene in which insertion occurs exhibits plant factor- induced expression. These are indicated in Figure 1 and include one to the left of vir A, one to the right of vir B and one in vir D, and vir E. The possibility that other genes may exhibit plant factor-induced expression has not been ruled out.

Vir E is thought to produce either a diffusible factor or a factor acting directly on plant cells to produce an effect that can be shared by more than one strain inoculated at wound sites. When Vir E- mutants are co-inoculated on wounds with strains carrying intact vir regions (but no T-DNA), tumors are formed (Otten et al. 1984). This phenomenon, the 'helper effect', does not occur when pairs of Vir- mutants are co-inoculated or when clones of partial vir regions are

co-inoculated with Vir E- mutants (Iyer et al. 1982; Klee et al. 1983). When not only an intact vir E, but the entire vir region is present, the 'helper effect' does occur.

Vir E is responsible for another type of helper effect which differs from that observed for vir E. A five kb region of an octopine-type Ti plasmid, containing the vir E locus, enables the A. tumefaciens nopaline-type strain C58 to induce higher levels of nopaline synthesis on stem fragments of the plant Kalanchoe daigremontiana (Otten et al. 1985). This may imply that nopaline Ti plasmids do not have a functional counterpart to vir E.

The locus defined as the sixth complementation group of the nopaline Ti plasmid (pTiC58) vir region has been named hdy -for host-dependent variation (Hagiya et al. 1985). Mutations in this locus are complemented in trans by the corresponding wild-type region but the frequency with which tumors are formed varies with the plant host (Lundquist et al. 1984), suggesting that complete complementation does not occur in trans. This hdy locus encodes four transcriptional units comprising a single operon and appears to correspond to the pTiACH5 vir C gene and the pTiA6NC vir D gene (Lundquist et al. 1984).

Nopaline-type strains with mutations in possibly, a 'vir B-like' and a 'vir E-like' locus can stimulate an artificially induced proliferation (Aip) of plant cells of potatoe tuber slices incubated on a substrate containing the auxin naphthalene acetic acid (NAA) (Joos et al. 1983a). Since no nopaline or agrocinopine is produced by these cells there is no indication that T-DNA transfer has occurred and so these proliferations are said to be artificial.

d) Other possible genes involved in tumor formation: A third Ti plasmid region may contribute to tumorigenesis. This region was identified on pTiC58, and it overlaps neither the vir region nor the T-DNA (Kao et al. 1982). Mutations in this region lead to an altered host range. Joos et al. (1983a) produced pTiC58 mutations, mapping outside the known vir and T-DNA genes, which lead to strongly attenuated virulence phenotypes.

2.1.3. Naturally occurring altered host range strains :

The narrow host range of several naturally occurring Agrobacterium strains is partially due to an altered T-DNA (Thomashow et al. 1981; Knauf et al. 1983; Buchholz and Thomashow 1984 a,b; Yanofsky et al. 1985). There is also some indication of a difference between the virulence region of at least one narrow host range (NHR) Agrobacterium strain (which carries pTiAg62) and that of the well characterized wide host range (WHR) strains (Yanofsky et al. 1985). Also, several NHR-strains show weak DNA homology to a particular portion (the Hpa 1 fragment 12) of the WHR plasmid pTiB₆806 virulence region (Thomashow et al. 1981). E. W. Nester (personal communication) has made observations which suggest that in a particular NHR-A. tumefaciens strain there is no counterpart to vir C. In other cases NHR-strains seem to code for normal WHR- type virulence functions. For example pTi Ag57 from one NHR strain can transfer the T-DNA of a WHR-strain to plants which are not normally within its host range for tumor-induction (Hoekema et al. 1984b).

2.2 Nodulation

2.2.1 Nomenclature and Cross Inoculation Groups. Each Rhizobium species is capable of nodulating only a small group of plant species, known as a cross-inoculation group. According to the newly introduced nomenclature (Jordan 1984) the genus Rhizobium contains three species. Rhizobium meliloti can nodulate some but not necessarily all species of Medicago (alfalfa), Melilotus (sweet clover), and Trigonella (fenugreek). R. loti nodulates many different species, some examples are Lotus corniculatus (birdsfoot trefoil), Lupinus densiflorus (lupine), and Cicer arietinum (chick pea). R. leguminosarum is comprised of three biovars. Biovar trifolii (previously R. trifolii) nodulates many species of Trifolium (clover). Biovar phaseoli (previously R. phaseoli) nodulates temperate species of Phaseolus such as P. vulgaris (kidney bean), P. augustifolius (bean), and P. multiflorus (scarlet runner). Biovar viciae (previously known as R. leguminosarum) nodulates some, but not necessarily all species of Pisum (field pea), Lathyrus (pea), Vicia (vetch), and Lens (lentil). These three species of Rhizobium are also referred to as fast-growing Rhizobium. Slow growing Rhizobium species are now classified as Bradyrhizobium. This review of nodulation and the genes it involves deals primarily with the fast-growing Rhizobium which have been studied in more detail than the Bradyrhizobium and which are the subjects of this work. I will use the previously accepted nomenclature for the three biovars of R. leguminosarum because the new nomenclature is so recent that the previous nomenclature is still in use in the scientific journals whose publications I am citing, and in abstracts for scientific meetings as recently as August, 1985.

2.2.2. General Features of Nodule formation:

In a typical Rhizobium infection the bacteria bind to individual root hairs and stimulate a root hair curling or branching response (Yao and Vincent, 1969). The bacteria gain entry into the root hair cell and eventually into the underlying cortical cells via an infection thread which seems to be constructed of plant cell wall material (Napoli and Hubbel 1975)

Meanwhile, cells of the inner cortex begin to divide and when the infection threads carrying rows of infecting bacteria (enclosed in a plant derived membrane) encounter these cells, the bacteria are released from the thread into the plant cell cytoplasm. Here, the bacteria enlarge and differentiate, and in this form are known as bacteroids. They begin to express some genes which are not expressed in the free-living state. The important process of nitrogen fixation involves the production of the bacterially encoded nitrogenase enzyme, which catalyzes the conversion of atmospheric dinitrogen (N_2) to ammonia (NH_3); which the plant cell cytoplasm can assimilate to form other necessary nitrogen containing molecules. Many nodule specific proteins or nodulins, some of which may be encoded by the plant genome, are found in the nodule cells (Lang-Unaasch and Ausubel, 1985; Govers et al. 1985). The functions of only a few nodulins are known.

Vincent (1980) defined ten stages in the formation of nodules by Rhizobium, as shown in Table 1.

Table 1: Stages involved in nodule formation

STAGE	PHENOTYPIC CODE
Multiplication in root area, colonization	Roc
Attachment to root	Roa
Root hair curling, or branching	Hac (Hab)
Infection thread formation	Inf
Nodule initiation, development	Noi
Bacterial Release	Bar
Bacteroid differentiation	Bad
Nitrogen fixation	Nif
Complementary biochemical and physiological functions	Cof
Nodule function (stability, persistence)	Nop

Rhizobium Nod- mutants are those which do not induce nodule formation. Eff- or Fix- mutants are those which induce the formation of defective or so-called ineffective nodules in which no nitrogen is fixed. Normal, nitrogen fixing strains are usually referred to as Fix+. Nif- mutants may also form ineffective nodules because they carry mutations in the structural genes coding for the nitrogenase enzyme. Nod- or Eff- mutants might be classified further as having mutations such as Hac- or Inf- affecting specific stages of nodule formation.

Root hair binding has been postulated as the basis of the host-specificity of Rhizobium strains. One theory is that specific lectins, or carbohydrate-binding proteins on the surface of the plant cells, recognize and bind particular carbohydrate components of the bacterial cell surface (Dazzo and Hubbel, 1975 and Bohlool and Schmidt, 1974).

According to the lectin hypothesis each host cross-inoculation group has characteristic lectins, and each bacterial symbiont has characteristic cell surface carbohydrates. It has often been demonstrated that the lectins of a particular host legume do bind their own bacterial symbiont and not other heterologous Rhizobium (Bhuvaneshwari 1978; Paau et al 1981). A positive correlation between lectin binding and infectivity has been demonstrated in the clover/R. trifolii symbiotic association (Dazzo and Hubbel 1975). Bacterial extracellular (or exo) polysaccharides, postulated to be the bacterial component involved in binding, have been studied. R. phaseoli produces a particular type of extracellular acidic heteropolysaccharide which is not produced by the other Rhizobium (Jordan 1984). R. leguminosarum, R. phaseoli, and R. trifolii have acidic exopolysaccharides which differ from those of R. meliloti (Carlson 1980). Alterations in cell surface molecules are often correlated with defective nodulation. Both an R. phaseoli mutant lacking exopolysaccharides and an R. leguminosarum mutant with an altered exopolysaccharide have Nod- phenotypes (Raleigh and Signer, 1982; Sanders et al. 1979). Some reports (Seegers et al. 1985 and Wong and Shantharam 1984) describe evidence which is difficult to explain using the lectin hypothesis. Therefore the lectin hypothesis is a tentative explanation of the specificity of most Rhizobium-legume symbiotic associations. Some exopolysaccharide deficient mutants of R. meliloti do produce nodules but the nodules are defective (Leigh and Walker, 1985). It is possible therefore that bacterial exopolysaccharides may be involved in stages other than root-hair binding.

Although root-hair curling is often observed prior to infection of roots by Rhizobium, it is not an absolute prerequisite for root hair infection (Callahan and Torrey, 1981). Abnormal nodules can be formed without the involvement of infection threads in the case of some R. meliloti mutants (Finan et al. 1985a) and in the case of Agrobacterium strains carrying R. meliloti symbiotic plasmids (Truchet et al. 1984). Some naturally occurring

Bradyrhizobium typically infect root hairs without the formation of infection threads (Chandler 1978).

Root hairs are most susceptible to infection while they are still developing (Bauer 1981). Root hair curling and infection thread formation are thought to be consequences of an alteration in plant cell wall synthesis (Kumarashinge et al. 1977) and therefore it is reasonable to assume that the bacteria (or, alternatively the plant) is producing cell-wall modifying enzymes (Bauer 1981). There is increasing evidence that bacterial genes involved in root hair curling are conserved in many different Rhizobium strains (Djordjevic et al. 1985). Cloned root hair curling genes of R. trifolii can confer the ability to curl corn and rice root hairs (Plazinski et al. 1985). This suggests that root hair curling may not be the basis of the host specificity of Rhizobium.

One of the least understood aspects of nodulation is the division and enlargement of those plant cells which form the nodule. Mutants of R. meliloti (Finan et al. 1985a) and R. phaseoli (Vandenbosch et al. 1985) which cannot penetrate plant cells but which infect inter-cellularly, can still induce cell division. These observations suggest that cell division can be separated from the 'early stages' of nodulation. Long (1984) suggested that cell division may be necessary for an active synthesis of cell wall synthesising enzymes which may be needed during infection thread formation and growth. The observation of Calvert et al. (1984) that cortical cell division can precede infection thread formation supports this idea. The molecular basis of the cellular division is completely unknown. Rhizobium are known to synthesize IAA (Badenoch-Jones et al. 1982) and cytokinins. This may play a role in triggering the cell division.

The processes involved in maintenance of a fully effective nodule are not well understood. Often, Rhizobium mutants are isolated which are ineffective or which initiated nodules which senesce prematurely. Some of these mutants produce nodules with altered nodule specific polypeptides (Bisseling et al. 1983) or nodulin mRNA's (Fuller and Verma, 1984).

2.2.3 Bacterial genes required for nodulation:

In Rhizobium meliloti, B. leguminosarum, B. trifolii and B. phaseoli many nodulation genes are encoded on large plasmids known as symbiotic plasmids. However in R. loti most nodulation genes seem to be chromosomally encoded (Chau et al. 1985). Transfer of symbiotic plasmids among strains of B. leguminosarum, B. trifolii, B. phaseoli and B. meliloti results in the transfer of the host range for nodulation of the strain from which the plasmid originated (e.g. Van Brussel et al. 1982; Hooykaas et al. 1982a; Lamb et al. 1985; Higashi 1967). For example, when an B. trifolii sym plasmid is transferred to B. leguminosarum (Hooykaas et al. 1981) or to A. tumefaciens (Hooykaas et al. 1982a) the host range (for nodulation) of the recipient is extended to some species of Trifolium (Hooykaas et al. 1981).

a) Symbiotic plasmid genes: In the fast growing Rhizobium species the Sym plasmids often encode nodulation genes (nod) and nitrogen fixation (nif) genes which map in positions fairly close to one another on large symbiotic plasmids, although these are only recently being studied in more detail. In fact, most Sym plasmids are so large that only a small portion of the genes they encode have been characterized. The analysis of the phenotypes of Rhizobium strains with Sym plasmid mutations initially revealed that the frequency of occurrence of Nod⁻ mutants is low, suggesting that the loci involved in nodulation are small (eg. Beringer et al. 1980; Long et al. 1981; Paau et al. 1981; Rolfe et al. 1981; Forrai et al. 1983). Some nodulation genes from at least one species of each of B. meliloti, B. leguminosarum, B. trifolii and B. phaseoli have been cloned in a cosmid vector known as pLAFR1. Examples and references are given in Table 2. Cloning was accomplished in a similar manner in each case. A gene bank comprised of overlapping clones of total sym plasmid DNA was constructed in the cosmid vector, and cloned fragments capable of complementing previously defined Nod⁻ sym plasmid mutations were chosen for further study and were found to contain at least some nodulation genes.

Table 2: Rhizobium nodulation genes clones

DESIGNATION ¹	ORIGIN	SIZE ² (KB)	REFERENCE
pRmSL26	<u>R. meliloti</u> A1021	20	Long et. al. 1982
pKSK5	<u>R. meliloti</u> 41	8.7	Kondorosi et al. 1984
pRt032	<u>R. trifolii</u> ANU843	14	Schofield et al. 1984
plJ1085	<u>R. leguminosarum</u> 248	10	Downie et al. 1983
plJ1098	"	10	"
plJ1095	<u>R. leguminosarum</u> TOM	30	Hombrecher et al. 1984
plJ1111	"	30	"
plJ1097	<u>R. phaseoli</u> 8002	30	Lamb et al. 1984
plJ1098	"	30	"

¹ name of cosmid carrying clone ² size of cloned fragments only

pRmSL26 and pRt032 confer to Agrobacterium the ability to form small ineffective nodules on, respectively, alfalfa (Hirsch et al. 1985) and clover (Schofield et al. 1984). When plJ1085 and plJ1089 are transferred to a Sym-plasmidless, non-nodulating strain of R. phaseoli, the R. phaseoli gains the ability to form normal nitrogen fixing nodules on pea (Downie et al. 1983). In the case of plJ1095 and plJ1111 the ability to nodulate both a commercial pea variety and the primitive 'Afghanistan' pea is conferred (Hombrecher et al. 1984). These cosmids carry cloned nod genes from R. leguminosarum strain TOM which is capable of nodulating 'Afghanistan' peas not normally nodulated by other well characterized narrower host range R. leguminosarum strains.

There is increasing evidence that the cloned regions in the cosmids listed in Table 2 encode genes common to several Rhizobium spp. One line of evidence deals with the ability of these cloned regions to complement mutations in Nod- mutants of heterologous Rhizobium. For example the cosmid pRmSL26, carrying R. meliloti cloned nodulation genes, can complement several Nod- mutants of R. trifolii (Hirsch et al. 1985; Djordjevic et al. 1985) and R. leguminosarum (Bantolvi et al. 1981; Kondorosi et al. 1984). pRt032 carrying R. trifolii cloned nodulation genes can complement some Nod- mutants of R. meliloti.

(Djordjevic et al. 1985; Fisher et al. 1985a).

Other evidence demonstrating the presence of common nodulation genes involves the analysis of similarities between DNA sequences of different nodulation gene clones. Two reports published simultaneously in 1984 reported the sequencing of cloned nodulation genes of both *R. meliloti* (Torok et al. 1984) and *R. leguminosarum* (Rossen, Johnston and Downie 1984). The two regions showed extensive (69-72 %) structural DNA homology. The analysis of these cloned regions revealed the presence, in *R. leguminosarum* and *R. meliloti*, of three coding regions: *nod A*, *nod B* and *nod C* (Torok et al. 1984; Rossen et al. 1984). Regions of an *R. phaseoli* symbiotic plasmid showed significant homology to *R. leguminosarum* cloned nodulation genes (Lamb et al. 1985). And finally, Hombrecher et al. (1984) demonstrated that some Sym plasmid genes of *R. leguminosarum* strain 248 and cloned nodulation genes from *R. leguminosarum* strain TOM were very similar. Four genes (*nod D, A, B, C*), comprising the common nodulation genes of *R. meliloti* and *R. trifolii* have been named (Long et al. 1985b; Schofield et al. 1985) although their role in nodulation is still uncertain. Apparently the expression of *nod A, B, C* is induced by plant exudates. *Nod D* is expressed constitutively and is required for the expression of *nod A, B, C* (Fisher et al. 1985b; Kondorosi 1985).

Downie et al. (1983) propose a nodulation gene model, consisting of a core of common nodulation genes similar in different *Rhizobium* species and a host range determined by ancillary host-specificity genes. Some Nod- mutations occurring on Sym plasmids are not complemented by clones containing the common nodulation genes from heterologous *Rhizobium* strains. This suggests the presence of sym plasmid encoded genes involved in host specificity. In both *R. meliloti* strain 41 (Kondorosi et al. 1984) and *R. meliloti* strain A1021 (Long et al. 1985a), the region affected in mutations causing a Nod- phenotype not complemented by common nodulation genes from heterologous *Rhizobium*, lies between the *nif* genes and the common nodulation genes (Kondorosi et al. 1984, and Long et al. 1985a) and is referred to as the host specific nodulation region (*hsn*). A cosmid, pRmJT5, carrying the *hsn* genes of *R. meliloti* A1021, confers alfalfa nodulating ability to an *R. trifolii* strain carrying *R. trifolii* common and host specificity genes (Fisher et al. 1985a). In

strain 41 the hns genes form two (Kondorosí et al. 1984) complementation groups.

b) Rhizobium chromosomal nodulation genes: For R. meliloti, R. leguminosarum, R. trifolii and R. phaseoli there is evidence that in addition to Sym plasmid-encoded nodulation genes there are chromosomal genes contributing to effective nodulation. For example more than half of the symbiotically defective mutants isolated by Noel et al. (1984) for R. phaseoli strain CFN42 map to the chromosome. Some chromosomal mutations in R. phaseoli strain CFN42 resulted in strains which produce slowly developing nodules (Noel et al. 1984). Rhizobium plasmids other than the 'classic' symbiotic plasmids may also contribute to normal nodulation since they may code for genes involved in the production of exopolysaccharides (Finan et al. 1985b). Indirect evidence that the Rhizobium chromosome contributes to successful nodulation is that when Sym plasmids are transferred to Agrobacterium, normal Eff+ nodules are not formed (Wong et al. 1983; Van Brussel et al. 1982; Hooykaas et al. 1982a; and Leemans et al. 1984). Some R. meliloti Sym plasmids in Agrobacterium confer the ability to form only small pseudonodules or tumor-like swellings in which there are no infection threads or intra-cellular bacteria (Truchet et al. 1984). Either the Agrobacterium chromosome lacks some genes required for normal nodulation, or perhaps Agrobacterium has genes which inhibit normal nodulation. There is one report, however, of effective nodulation of beans by an Agrobacterium strain carrying an R. phaseoli symbiotic plasmid (Leemans et al. 1984).

2.3 Relatedness of Agrobacterium and Rhizobium

Several lines of evidence suggest that Agrobacterium and Rhizobium are very similar and hence their evolutions are often assumed to be closely related. It has not been possible in the past to examine the characteristics of plasmid-free Agrobacterium and Rhizobium.

An Agrobacterium strain missing both the Ti plasmid and the cryptic plasmid pATC58 was recently described (Hynes et al. 1985). However, until plasmid-free strains are available for several species of both genera it will be difficult to know, indisputably, what is a chromosomally encoded characteristic.

L.O. White (1972) compared bacteria from both genera using numerous biochemical tests, such as their ability to utilize various carbon compounds as sole carbon sources, their ability to hydrolyse casein and gelatin, the production of 3-ketolactose, etc. These tests could not always distinguish the two genera, however Rhizobium and Agrobacterium could be distinguished on the basis of pathogenicity or their ability to participate in symbiotic associations. White suggested the two genera be merged. Analysis of the ability of Agrobacterium and Rhizobium to catabolize various aromatic and hydroaromatic hydrocarbons revealed that Agrobacterium was more closely related to R. meliloti than to R. leguminosarum, R. trifolii and R. phaseoli (Parke and Ornston, 1984). Both Rhizobium and Agrobacterium produce a β - (1,2)- linked extracellular glucan (York et al. 1980). The acidic exopolysaccharides of A. tumefaciens are similar to those of R. meliloti but not to those of several other fast-growing Rhizobium (Zevenhuizen 1971). Rhizobium and Agrobacterium usually are susceptible to different types of bacteriophages (Boyd et al. 1970).

Several chromosomal genes of R. leguminosarum, R. meliloti and A. tumefaciens map in similar positions relative to one another (Kondorosi et al. 1977, 1980; Hooykaas et al. 1982b). Some of these genes also have conserved functions. Agrobacterium auxotrophic mutants become prototrophic after introduction of plasmid R-primes carrying the corresponding wild-type chromosomal genes of Rhizobium meliloti (Hooykaas et al. 1982b). The percentage of guanine (G) plus cytosine (C) in the genome of an organism is often a distinguishing characteristic of that particular organism and others 'like' it. The percentage G+C content of a) R. leguminosarum, R. trifolii and R. phaseoli, b) R. meliloti, and c) Agrobacterium strains is 59-63%, 62-63% and 57-63% respectively. (Heberlein et al. 1967). Clearly it is difficult to distinguish these species and genera on the basis of G + C content. The genome sizes of Rhizobium and Agrobacterium are $3.06-3.84 \times 10^9$ and

3.036×10^9 Daltons respectively (Chakrabarti et al. 1984). Using relative nucleic acid hybridization as a criteria of relatedness, Gibbins and Gregory (1972) concluded that the three Agrobacterium strains used were as related to the R. leguminosarum reference strain as were several Rhizobium strains. An earlier study showed that for the DNA of seven Agrobacterium strains and two R. meliloti strains the means of the degree of homology to the DNA of the reference R. leguminosarum were 50% and 59% respectively (Heberlein et al. 1967). Clones of an Agrobacterium chromosomal virulence gene hybridize to portions of an R. meliloti chromosome (Dylan et al. 1985). DNA homology between the plasmid components of these genomes has also been studied. Specifically, DNA homology is found between symbiotic plasmids of Rhizobium and various regions, including the vir region (Prakash and Schilperoort, 1982) and the T-DNA region (Hadley and Szalay 1982) of Ti plasmids. Also, Huguet et al. (1983) have shown that DNA from the Ti plasmids pTiB6 and pTiC58 will hybridize to the R. meliloti 41 symbiotic plasmid to as great an extent as DNA from another plasmid of the same R. meliloti strain. They do note, however, that the T-DNA of pTi does not share sequence similarities with pRme41. Huguet et al. (1983) conclude that in general the plasmids of R. meliloti are no more structurally similar in sequence to themselves than to other plasmids of the Rhizobiaceae.

A single table summarizing the characteristics which can be used to distinguish Rhizobium and Agrobacterium is provided in Bergey's Manual of Systematic Bacteriology Vol. 1 (Jordan, 1984). With the exception of tumor-inducing ability, these characteristics cannot be used with absolute certainty since they are either similar for both genera or they are variable for either one of the genera. However, we know that simple mating experiments can lead to the acquisition of tumor-inducing ability by Rhizobium or to the acquisition of nodulating ability by Agrobacterium. Two reports suggest the presence, in nature, of organisms which both nodulate and form tumors (Skotnicki and Rolfe, 1978 and MacGregor and Alexander, 1971). It has also been shown that Rhizobium and Agrobacterium may be different forms of one organism. When exposed to DNA-damaging agents some species of the family Rhizobiaceae may be converted by genome rearrangement to other species within the same family (Heumann et al. 1984).

Other observations reported in the literature indirectly suggest the conservation of functions between Agrobacterium and Rhizobium. These observations deal with the expression of both Ti and Sym plasmid genes in the genetic background of heterologous species. This is discussed in detail in the next section of this review

2.4 Expression of Ti and Sym plasmid genes in heterologous species of Rhizobium and Agrobacterium

The following points summarize the host range and expression of Ti and Sym plasmids within the genera Rhizobium and Agrobacterium. Experimental data from the literature supporting these statements are lengthy, and are therefore presented in detail in table-form (Tables 3-6).

2.4.1 The genetic backgrounds of R. leguminosarum, R. phaseoli and R. trifolii can all support expression of nodulation genes from each of these species and any two Sym plasmids from these species can be co-expressed in one bacterium, resulting in a strain, which can nodulate two separate cross-inoculation groups. In fact, these three species probably only differ in the type of Sym plasmid they carry. For this reason these species have recently been grouped as separate biovars of a single species (Jordan 1984). There is sometimes interference between two symbiotic plasmids carried in one bacterium. For example, strains harboring both the R. leguminosarum and the R. phaseoli Sym plasmid nodulate both peas and beans, but both are nodulated poorly (Beynon et al 1980)

2.4.2 Sym plasmids from the three strains mentioned above are not fully expressed in R. meliloti. These plasmids are probably stable: the expression of root hair curling genes (Djordjevic et al. 1985), hydrogen uptake (Hup) genes (Behki et al. 1985) and some common nod genes capable of complementing R. meliloti Nod- Sym plasmid mutations

(Fisher et al 1985a) all occur in the R. meliloti background. However these plasmids do not confer to R. meliloti the ability to nodulate the host legume for which they are specific. All of the experiments with R. meliloti have involved R. meliloti carrying their own Sym plasmids.

2.4.3 Transfer of R. meliloti sym plasmids to the other fast growing Rhizobium strains has not been widely studied. Nodulation genes carried on pRmSL26 are expressed in at least R. trifolii strains, but nodulation of alfalfa (the normal host of R. meliloti) occurs at a low frequency (Hirsch et al 1985). The clover nodulating ability of the recipient strain is seemingly unaffected, so the presence of those portions of the R. meliloti Sym plasmid cloned in pRmSL26 doesn't seem to suppress the expression of the R. trifolii Sym plasmid nodulation genes.

2.4.4 Tumor-inducing genes of Ti plasmids or root-inducing genes of Ri plasmids are not expressed in R. meliloti. Some Ti plasmid genes, such as octopine utilization genes, are expressed in R. meliloti (Hooykaas 1979). Apparently carriage of an Ri plasmid improves the nodulating ability of some R. meliloti strains (Strobel et al. 1985). Ti plasmid tumor-inducing genes are expressed in R. leguminosarum, R. trifolii, and R. phaseoli but the tumors formed are sometimes smaller than those formed by Agrobacterium strains carrying pTi (Hooykaas, 1979). It is not clear whether this reduced virulence is due to the chromosome or Sym plasmids in these Rhizobium strains. Ti plasmids in R. leguminosarum partially inhibit the ability of this strain to nodulate peas.

2.4.5 Sym plasmids from many fast-growing Rhizobium strains studied confer to A. tumefaciens the ability to form small ineffective nodules or pseudonodules. However the R. meliloti symbiotic plasmid pRme41 does not confer alfalfa nodulating ability to A. tumefaciens (Huguet et al. 1983). There is nevertheless one example of a sym plasmid which can confer effective nodulation to an A. tumefaciens (Leemans et al. 1984). The effect on virulence, of Sym plasmids in A. tumefaciens has not been examined in detail.

Figures 2 and 3 show the phenotypes of Agrobacterium and Rhizobium strains carrying

Sym and Ti plasmids respectively.

Figure 2 Nodulating and Tumor-forming ability of
A. tumefaciens carrying Sym Plasmids

- 1 Nod+ Fix- on V. sativa but Nod- on V. hirsuta and P. sativum (Van Brussel et al. 1982).
- 2 pJB5JI conferred a Nod+Fix- phenotype on V. sativa and a Nod- phenotype on V. hirsuta and P. sativum (Van Brussel et al. 1982)
pRle1001a conferred a Nod+Fix- phenotype on V. sativa and V. hirsuta; phenotype on P. sativum was not mentioned (Hooykaas et al. 1981).
- 3 Hooykaas et al. (1981)
- 4, 5 Hooykaas et al. (1985)
- 6, 7 Simon et al. (1983)
- 8 Huguet et al. (1983)
- 9 Wong et al. (1985)

* Although A. tumefaciens carrying p3622b forms ineffective nodules on P. vulgaris there is one report of an R. phaseoli Sym plasmid, p899a, which confers effective nodulation to A. tumefaciens (Leemans et al. 1984); it is unclear however if the A. tumefaciens strain in this case was pTi+ or pTi-.

N.D. no data available

Transconjugants phenotype

Figure 3: Nodulating and Tumor-forming ability of
Rhizobium spp. carrying Ti plasmids

- 1 B. leguminosarum LPR1112, and LPR1802 (Hooykaas 1979)
- 2 "poor " nodulation of pea (Hooykaas 1979)
- 3 small tumors are formed on tomato and petunia (Hooykaas 1979)
- 4 B. trifolii LPR5011 (Hooykaas 1979; Hooykaas et al. 1977)
- 5 Nod- on I. parviflorum and Nod+ Fix+ on I. pratense (Hooykaas et al. 1977)
- 6 B. phaseoli LPR9001 (a Fix- strain)
- 7 small tumors are formed on tomato (Hooykaas 1979)
- 8 B. meliloti LPR2120 (Hooykaas 1979)

N.D. = no data available

Transconjugants phenotype

Table 3: Nodulating ability of *B. leguminosarum*, *B. phaseoli*, and *B. trifolii* Carrying Symbiotic Plasmids from Heterologous Sources

DONATED PLASMID	RECIPIENT STRAIN	USUAL PLANT HOST OF DONOR	NODULATION BY TRANSCONJUGANT ON USUAL HOST OF RECIPIENT	COMMENTS	REFERENCE
pRL1JI	<i>B. trifolii</i> 6661, 6710	Nod + Fix +/-	Nod + Fix +	Low level of N ₂ fixation	Johnston et al. 1978
pJ35JI	<i>B. trifolii</i> T37	Nod + Fix +	Nod + Fix +	plasmids of donor and recipient may be incompatible	Christensen and Schubert, 1983
pRia1001a	<i>B. trifolii</i> LPR5-	Nod + Fix +	Nod + Fix +		Hooijkaas et al. 1982
pJ35JI	<i>B. trifolii</i> LPR509pSym-	Nod + Fix +	Nod - (Hac +)		Hooijkaas et al. 1982
	<i>B. trifolii</i> LPR5039	Nod + Fix +	Nod - (Hac +)		Van Brussel et al. 1982
	<i>B. trifolii</i> ANU855pSym-	Nod + Fix +	Nod - (Hac +)	Nod + Fix - on <i>V. firsutia</i>	Van Brussel et al. 1982
	<i>B. trifolii</i> ANU851Nod-	Nod + Fix +	Nod + Fix +	nodulation of clover delayed	Djordjevic et al. 1985
pJ35JI	<i>B. phaseoli</i> 1233	Nod + Fix +	Nod + Fix +	poor nodulation of both plant hosts, plasmid incompatibility	Beynon et al. 1980
pRL1JI	<i>B. phaseoli</i> 8401pSym-	Nod + Fix +	n m		Downe et al. 1983
pJ1083		Nod + Fix +	n m		Downe et al. 1983
pRL5JI		Nod + Fix nm	n m		Hembrécher et al. 1984
pRL1JI	<i>B. phaseoli</i> 1233	Nod + Fix +	Nod + Fix +	some isolates Fix - on bean	Johnston et al. 1978
pJ35JI	<i>B. leguminosarum</i> 1105	Nod + Fix +	Nod + Fix +		Hooijkaas et al. 1981
	<i>B. leguminosarum</i> 1120 Fix-	Nod + Fix +	Nod + Fix -		Hooijkaas et al. 1981
	<i>B. leguminosarum</i> 1802Nod-	Nod + Fix +	Nod -		
pRP2JI	<i>B. leguminosarum</i> B157pSym-	Nod + Fix +	Nod -	pSymfot <i>B. trifolii</i> is incompatible with p3622b	Lamb et al. (1982)
p3622b	<i>B. trifolii</i> LPR5pSym+	Nod + Fix -	Nod -		Hooijkaas et al. 1985

p3622b carrying 10 kb of *B. leguminosarum* Sym plasmid pRL1JI
n m. Not mentioned in published report
NOTE: pRia1001a is a *B. trifolii* Sym plasmid and pRph2JI and p3622b are
B. phaseoli Sym plasmids; the others are *B. leguminosarum*
Sym plasmids

Table 4: Nodulating ability of *B. melioidi* carrying heterologous sym plasmids and of other *Rhizobium* carrying Cloned nodulation genes of *B. melioidi*

DONATED PLASMID	RECIPIENT STRAIN	USUAL PLANT HOST OF DONOR	NODULATION BY TRANSCONJUGANT ON USUAL HOST OF RECIPIENT	COMMENTS	REFERENCE
PIJ1008	<i>B. melioidi</i> MW37	Nod -	Nod + Fix +	*Hup + in nodules on alfalfa	Benkó et al. 1985
-	GS1002	Nod -	Nod + Fix +		
-	GS1022	Nod -	Nod + Fix +		
pJBSJ1	<i>B. melioidi</i> GY1823Nod	n.m.	Nod + Fix +		Bartolaj et al. 1981
-	<i>B. melioidi</i> 102F51 Nod	n.m.	Nod + Fix +		
-	<i>B. melioidi</i> ZB157 Nod	n.m.	Nod + Fix +	(note: restoration of ability to nodulate alfalfa)	
pJBSJ1	<i>R. melioidi</i> ZB157 Nod	Nod -	Nod + Fix +	(nodulation delayed 7-9 days)	Djordjevic et al. 1985
pBRJAN	<i>B. melioidi</i> 1126Nod	Nod - (Hac +)	Nod +		Djordjevic et al. 1985
pRi038	-	Nod - (Hac +)	Nod -	early senescence of alfalfa nodules	
pRi032	-	Nod - (Hac +)	Nod + Fix -		
pRimSL26	<i>B. trifolii</i>	Nod + Fix -	Nod + Fix +	low frequency of alfalfa nodulation	Hirsch et al. 1985
-	<i>B. trifolii</i> ANU851Nod	Nod - (Hac -)	Nod + Fix +	delayed nodulation of clover	Djordjevic et al. 1985
-	<i>B. trifolii</i> pSym	Nod + Fix -	Nod -	low frequency of alfalfa nodulation	Hirsch et al. 1985
-	<i>B. trifolii</i> ANU845pSym	Nod -	Nod - (Hac -)	conflicts with data above	Djordjevic et al. 1985

Note pBRJAN, pRi038, pRi032 derived from an *B. trifolii* sym plasmid
 pRimSL26 was described previously (from *B. melioidi*)
 pJ1008 and pJBSJ1 are *B. leguminosarum* symbiotic plasmids
 n.m. Not mentioned in the published report

*the Hup + (hydrogen uptake) phenotype encoded by pJ1008 is expressed

Table 5: Nodulating Ability and Virulence of *Agrobacterium* carrying Symb plasmids

DONATED PLASMID	RECIPIENT A. tumefaciens	NODULATION BY TRANSCONJUGANT*	TUMOR INDUCING ABILITY OF TRANSCONJUGANT	COMMENTS	REFERENCE
pRme41 pRme41 pRme 2011	CS8 (pTi+) GV3101 (pTi-) 290 (pTi-) 275 (pTi+)	Nod- Nod + Fix- Nod + Fix- Nod + Fix-	n.m. n.m. n.m. n.m.	no bacteria in plant cells	Huguet et al. 1983 Wong et al. 1985 Simon et al. 1983
pRmsL26 pRmsL26 pRmsL30 pRms30 pRms51 pRms51	A348 A136 (pTi-) A348 A136 (pTi-) A348 A136 (pTi-)	Nod + Fix- (Hac-) Nod + Fix- (Hac-) Nod + Fix- (Hac-) Nod + Fix- (Hac-) Nod + Fix- (Hac-) Nod + Fix- (Hac-)	n.m. n.m. n.m. n.m. n.m. n.m.	15.4% of plants Nod+ 12.1% of plants Nod+ 23.8% of plants Nod+ 14.8% of plants Nod+ 13.6% of plants Nod+ <3% of plants Nod+	Hirsch et al. 1985
<i>B. leuconingosa</i> pB5J	LBA201 (pTi+)	Nod + Fix-	Vir +	Nod + Fix- on <i>V. sativa</i> but Nod- on <i>V. hirsuta</i> and <i>P. sativum</i>	Van Brussel et al. 1982
pB5J pRie1001a	LBA4301 (pTi-) ____ (pTi-)	Nod + Fix- Nod + Fix-	n.m. n.m.		Hooykaas et al. 1982
<i>B. lotum</i> pU5a pR1038	LBA288 (pTi-) ____ (pTi-)	Nod + Fix- (Hac +) Nod + Fix-	n.m. n.m.	nodulation delayed in 12 days nodules begin to senesce	Hooykaas et al. 1981 Schroeld et al. 1984
<i>B. phaseoli</i> p42d p899a p3622b	____ (pTi?) ____ (pTi?) LBA202 (pTi-) LBA1010 (pTi+)	Nod + Fix- Nod + Fix + Nod + Fix- Nod + Fix-	n.m. n.m. n.m. vir+		Leemans et al. 1984 Hooykaas et al. 1985

* nodulation only of the usual host plant of the *Rhizobium* from which the plasmid originated
 ** cosmids carrying cloned nodulation genes from *B. meliloti* A1021

Table 6: Modulating Ability and Virulence of *Rhizobium* carrying Ti and Ri plasmids

DONATED PLASMID	RECIPIENT STRAIN	NODULATION BY TRANSCONJUGANT*	TUMOR INDUCING ABILITY	COMMENTS	REFERENCE
PTB6::RP4	<i>B. melioli</i> LPR2120	Nod + Fix +	Vir -	small tumors, and poor nodulation of pea plants	Hooijkaas 1979
PTB6::RP4	<i>B. leguminosarum</i> LPR1112, and LPR1802	Nod + Fix -	Vir +		
PTB6::RP4	<i>B. phaseoli</i> LPR9001(Fix-)	Nod + Fix -	Vir +		
PTB6::RP4	<i>B. trifolii</i> LPR5001	Nod + Fix +	Vir +		
PTB6S3::RP4	<i>B. trifolii</i> 5	Nod + Fix +	Vir +	Nod- on <i>I. garvillanum</i> , Nod+Fix+ on <i>I. pratense</i>	Hooijkaas et al. 1977
PRiR105-2::Ti5	<i>B. melioli</i> 102F77	Nod + Fix +	Vir -	improved alfalfa nodulation	Sirobel et al. 1985

* nodulation only of the usual plant host of the *Rhizobium* from which the plasmid originated

MATERIALS AND METHODS

3. 1 Growth and Maintenance of Bacterial Strains

a) Agrobacterium and Rhizobium were grown at 30°C in sterile liquid TYC medium containing 'Difco' tryptone (0.5%, w:v), 'Difco' yeast extract (0.3%, w:v) and 'Baker' anhydrous calcium chloride (0.066 %, w:v) (Beringer 1974). For the growth of strains carrying plasmids with antibiotic resistance genes, antibiotics were added to the TYC medium. The concentration of antibiotics used for growth and selection of strains is given in Table 7. Aliquots of filter-sterilized antibiotic stock solutions were added to the autoclaved medium. A stock solution of the CaCl₂ was autoclaved separately and then added to the medium. For long-term storage of strains, a liquid culture, combined in a ratio of 1:1 (v:v) with a sterile 80% (v:v) solution of glycerol, was stored in sterile plastic vials at -20°C or at -90°C. For the growth of new cultures of each strain, approximately 0.01 mL of the frozen culture was added to 5 mL of liquid medium and grown at 30°C in a shaker bath for 2-3 days until the desired cell density was reached.

b) E. coli strains were grown at 37°C in a tryptone-yeast extract-NaCl liquid medium described by Selvaraj (1979), which was supplemented with the appropriate antibiotic when necessary for plasmid maintenance. They were stored in glycerol as described above for Agrobacterium and Rhizobium strains.

Table 7 : Concentration of Antibiotics In Medium

Antibiotic	Concentration (ug/mL) used for growth of:	
	<u>Rhizobium</u> or <u>Agrobacterium</u>	<u>E. coli</u>
kanamycin	100	30
carbenecillin	100	n.u.
chloramphenicol	30	30
spectinomycin	100 or 250*	n.u.
rifampicin	150	100
tetracycline	10	10
streptomycin	100	n.u.
nalidixic acid	50	n.u.

* 250 ug/mL of spectinomycin was used only for the growth of Rhizobium or Agrobacterium strains carrying pRL1Jl::Tn1831.

NOTE: in liquid medium the concentration of antibiotic is one-half that listed
Antibiotics are from Sigma, except tetracycline which was from Bristol Labs
n.u. = not used

3.2 Bacterial Strains Used:

Rhizobium strains are listed in Table 8. Table 9 lists the characteristics of Agrobacterium strains used. E. coli strains and plasmids are described in Tables 10 and 11. A. tumefaciens strains constructed during this study are listed in Table 12. pRmSL26 is a cosmid vector carrying cloned nodulation genes from the Sym plasmid of R. meliloti A1021 (Long et al. 1982), and is carried in E. coli strain HB101.

3.3 Bacterial Matings

To determine the characteristics of A. tumefaciens strains carrying Sym plasmids from Rhizobium it was first necessary to transfer each Sym plasmid into a series of A. tumefaciens strains. Plasmids may replicate independently of bacterial chromosomes and can sometimes be transferred from one bacterial cell (the donor) to another bacterial cell (the recipient) by inter-bacterial mating or conjugation to produce a so-called 'transconjugant' bacterium. Self-transmissible plasmids encode 'transfer' proteins including those which form an inter-bacterial bridge or pilus between the donor and recipient cells. Non self-transmissible plasmids do not encode transfer proteins but if they have an origin of transfer (oriT) and 'mobilization' genes they can be transferred during conjugation by using the transfer system of a self-transmissible plasmid carried in the same cell. In the following paragraphs the conditions used to promote conjugation between A. tumefaciens and Rhizobium spp. and the procedure used to isolate transconjugants are described.

Aliquots of early log-phase liquid cultures of donor and recipient bacteria were usually combined in a ratio of 1:10 (v:v). The mixture was pipetted onto a sterile millipore filter, (porosity 0.45 μ m; Millipore Corp.). Aliquots of the donor and recipient strain were placed individually on separate filters. The medium was suctioned through the filters while the bacteria were retained on the surface. The filters were then placed, with the bacterial layer facing up, on agar solidified TYC medium. The plates were inverted and incubated at 30°C for 18-24 hours. Following incubation the filters were transferred to sterile tubes containing 5 mL of sterile NaCl (0.85% w/v).

Table 8: Rhizobium strains Used

Strain	Parental Strain	Sym Plasmid Present	Reference/Source
<u>R. phaseoli</u> 3622	natural isolate	p3622b	Rothamstead, Harpenden, U.K
<u>R. phaseoli</u> PD101(Spr)	<u>R. phaseoli</u> 3622	p3622b	this study
<u>R. leguminosarum</u> B151 (rif ^r)	<u>R. leguminosarum</u> 128C53	no pSym	Brewin et al 1982
<u>R. leguminosarum</u> B151 (sp ^r)	"	"	Behki et al 1985
<u>R. leguminosarum</u> PD102	<u>R. leguminosarum</u> B151 (rif ^r)	pPD102 ¹	this study
<u>R. leguminosarum</u> B316	<u>R. phaseoli</u> 8401 ^a	pRL5J1 ²	N. J. Brewin
<u>R. trifolii</u> RBL5523	<u>R. trifolii</u> RBL5515	pRL1J1::Tn1831 ³	C J J Wijffelman
<u>R. trifolii</u> LPR5035	<u>R. trifolii</u> LPR5	ptr5a	Hooykaas et al 1981
<u>R. meliloti</u> JJ1(rif ^r)	<u>R. meliloti</u> IZ450	pSym <u>R. meliloti</u>	Selvaraj & Iyer 1983
<u>R. meliloti</u> JJ1C10 ⁴ (KN1001)	<u>R. meliloti</u> JJ1	"	Adachi et al 1983

¹ pPD102 is p3622b::Tn5.A1; construction of pPD102 is described in the text

² an R. leguminosarum Sym plasmid originating in R. leguminosarum TOM, the plasmid used here is a Tn5 insertion derivative of the original plasmid referred to as pRL5J1

³ an R. leguminosarum Sym plasmid pRL1J1::Tn5 originating in R. leguminosarum 248

⁴ heat cured of a 135 MD cryptic plasmid (pTA2) present in R. meliloti JJ1; this strain has also been referred to as KN1001.

^a this is an R. phaseoli strain which is cured of its own Sym plasmid

Table 9 Agrobacterium strains Used

Strain	Parental Strain	Plasmids Present	Phenotype	Reference or Source
Sm240	A136	pATC58, pTiA6NC, pPH1J1 ¹	VirA-	E.W. Nester
Sm243	"	"	VirB-	"
Sm37	"	"	VirB-	"
Sm365 ²	"	"	VirC-	"
Sm311	"	"	VirD-	"
Sm355	"	"	VirD-	"
Sm341	"	"	VirE-	"
Sm36	"	"	VirE-	"
A136	NT1	pATC58	pTi-	Watson et al. 1975
A348	A136	pATC58, pTiA6NC	Vir+	Sciaky et al. 1977
A1020	NT1	pATC58, pTiB ₆ 806	chvB-	Douglas et al. 1985
A1011	NT1	pATC58, pTiB ₆ 806	chvB-	"

pATC58 = a cryptic plasmid carried in all of the A. tumefaciens strains used.

Note: Tn3::HoHo1 inserted into the Ti plasmid of the A. tumefaciens Vir A-E mutants confers resistance to Cb

¹ plasmid introduced into these strains during the original strain construction

² an altered host range VirC- mutant, not similar to the avirulent Vir C- mutants described by Hooykaas et al. (1984)

Table 10. *E. coli* strains Used

Strain	Associated characteristics	Reference
HB101	Pro- Leu- Thy- thi- SmR	Bolivar et al 1977
GS500	as above but also rif ^r	Selvaraj & Iyer 1983
Wa803	Met-Thi-	Wood, 1966

Table 11 *E. coli* plasmids:

Plasmid	Markers	Replicon	Transfer System	Reference
pGS39	Km, Cm	P15a	tra-	Selvaraj, 1983
pGS9	Km, Cm	p15a	N tra+	Selvaraj & Iyer 1983
pRK2013	Km	colE1	N tra+	Figurski & Helinski 1979
pRK2	Ap, Sm, Tc, Km	P	P tra+	"

Table 12 *A. tumefaciens* strains derived in this study:

Designation	Parental Strain	Plasmid Introduced
PD40	A348	pPD102
PD41	A136	"
PD43	Sm240	"
PD44	Sm243	"
PD45	Sm311	"
PD46	Sm341	"
PD47	Sm355	"
PD48	Sm361	"
PD49	Sm365	"
PD50	A348	pRL5JI
PD51	A136	"
PD54	Sm240	"
PD55	Sm311	"
PD56	Sm341	"
PD57	Sm355	"
PD58	Sm361	"
PD60	A348	ptr5a
PD61	A136	"
PD63	Sm240	"
PD64	Sm243	"
PD65	Sm311	"
PD66	Sm341	"
PD67	Sm355	"
PD68	Sm361	"
PD69	Sm365	"
PD91	A136	pCrS ¹
PD92	"	pSym-JJ1C10 ²
PD93	"	pCrS
PD94	"	pSym-JJ1C10
PD30	A348	pRL1JI::Tn1831
PD31	A1011	"
PD32	A1020	"

¹ small cryptic plasmid originating following mating of *A. tumefaciens* A136 with *R. meliloti* JJ1C10::Tn5A1 pooled insertion mutants

² Sym plasmid from *R. meliloti* JJ1C10

The bacteria were resuspended in the saline by vortexing. Aliquots of the suspensions of the mating mixture, the donor, and the recipient were each plated on separate plates containing agar-solidified TYC medium containing a combination of two antibiotics on which the transconjugant bacteria, but neither the donor nor the recipient, should grow. The selective medium used depended upon the transconjugant being isolated. Serial dilutions of the mating mixture suspension were also plated on medium on which only the donor could grow. The plates were incubated at 30°C for up to one week or until colonies appeared. The total number of viable donor cells used in the mating were determined by counting colonies growing on plates which allow for the selective growth of donors. The frequency of occurrence of transconjugants is expressed as the number of transconjugants per unit volume of 'mating mixture' divided by the number of donor cells per unit volume of 'mating mixture'. When a transconjugant colony was to be studied further it was purified twice, by streaking on selective medium to isolate single colonies. Single colonies from the second single colony purification were propagated in liquid medium and stored as described in section 3.1.

When the donor carried a non self-transmissible plasmid, 1.0 mL of a liquid culture of *E. coli* HB101 carrying the self-transmissible plasmid pRK2013 was incorporated into the mating mixture to allow mobilization of the non self-transmissible plasmid. When this third strain was used it was also plated on selective medium as I have described for the donor and recipient strain.

3. 4 Construction of *A. tumefaciens* strains carrying Sym plasmids:

a) *A. tumefaciens* strains carrying *B. trifolii* ptr5a and *B. leguminosarum* pRL5JI:

These strains were constructed in order to determine whether ptr5a from *B. trifolii* LPR5035 or pRL5JI from *B. leguminosarum* B316 could complement *vir* region mutations in *A. tumefaciens*, and to determine whether host-specific nodulation is conferred in *A. tumefaciens*. ptr5a and pRL5JI are Sym plasmids carrying the transposable element Tn5 which confers kanamycin resistance to the bacterial cell in which they reside. The chromosome of the *A. tumefaciens* recipients encodes resistance to nalidixic acid (Nal). Ti

plasmids in the mutant *A. tumefaciens* strains listed in Table 9, but not the Ti plasmid in the wild-type *A. tumefaciens* A348, carry the transposon Tn3::HoHo1 which confers to them resistance to carbenecillin (Cb). The *Rhizobium* donors are Nal^S and Cb^S while the *A. tumefaciens* recipients are Km^S. *R. trifolii* LPR5035 and *R. leguminosarum* B316 were each mated with each of the *A. tumefaciens* Vir- mutants and also with *A. tumefaciens* A348 and *A. tumefaciens* A136. Transconjugant *A. tumefaciens* carrying Sym plasmids were selected on medium containing nalidixic acid and kanamycin. For the *R. leguminosarum* x *A. tumefaciens* matings sometimes transconjugant colonies were identified on the basis of their morphology which differed from that of colonies of *R. leguminosarum*, a few of which arose on the selective medium. To determine if both pTi::Tn3 HoHo1 and pSym::Tn5 associated antibiotic resistance markers were present, the transconjugants selected on medium with Nal + Km were screened for resistance to Cb. The sizes of plasmids in the transconjugants were examined by agarose-gel electrophoresis as described in section 3.6. The tumor-forming and nodulating ability of *A. tumefaciens* carrying the Sym plasmids were determined as will be described in sections 3.7 and 3.9.

b) *A. tumefaciens* carrying *R. phaseoli* 3622b:

R. phaseoli 3622 does not contain a Sym plasmid with an antibiotic resistance marker, as do *R. trifolii* LPR5035 and *R. leguminosarum* B316. Therefore, in order to construct and analyze strains of *A. tumefaciens* carrying the *R. phaseoli* Sym plasmid it was necessary to label, or 'tag' the Sym plasmid with an antibiotic resistance marker. A derivative of the transposable element Tn5 was used for this purpose. The strategy used to accomplish this is outlined in Figure 4 and is described below.

R. phaseoli 3622 was reported to contain a 250 MD Sym plasmid p3622b and a 100 MD cryptic plasmid p3622a (Prakash and Schilperoort 1982). A spontaneous spectinomycin resistant derivative of *R. phaseoli* 3622 was isolated by plating approximately 10⁹ *R. phaseoli* 3622 cells on TYC medium with 100ug/mL spectinomycin (Sp). A Sp^r derivative, designated *R. phaseoli* PD101, was purified. Analysis of the plasmid content and nodulating ability of this strain verified that PD101 was indistinguishable from the original *R. phaseoli* 3622 except that it was Sp^r. The plasmid pGS39 was used to deliver Tn5-A1 into the genome of PD101. This

Figure 4: Strategy Used to Transfer p3622b to A. tumefaciens

1. Tn5.A1 mutagenesis of R. phaseoli PD101 using the suicide vehicle pGS39 to create insertions of Tn5.A1 into either the chromosome, pSym (p3622b) or pCr1 (p3622a) of R. phaseoli PD101
2. Transfer of Km^r to the R. leguminosarum intermediate strain to produce R. leguminosarum B151rif carrying either pSym or pCr1
3. Identification of an R. leguminosarum B151 isolate which carries the Tn5.A1 labelled R. phaseoli Sym plasmid. This isolate is positively identified by its ability to nodulate P. vulgaris (bean). This isolate is then mated with A. tumefaciens

■ represents bacterial chromosome

▼ represents the transposon Tn5.A1

pCrRL cryptic plasmid (also known as pRL13JI) of R. leguminosarum B151

R. phaseoli PD101
(= R. phaseoli 3622sp^r)

E. coli GS500 (pgs39)

E. coli HB101 (PRK2013)
tra⁺

R. leguminosarum B151 rif^r

Sp^r Km^r
Cm^s

E. coli HB101 (PRK2013)
tra⁺

Rif^r Km^r
Sp^s

3

Nod⁺
Nod⁻

plasmid is known to be unstable in *B. meliloti* strains and is thus referred to as a suicide vehicle (Selvaraj and Iyer 1984). Tn5-A1 is a derivative of Tn5 carrying, in addition to a gene for kanamycin resistance, mobilization (mob) genes. *E. coli* GS500 (pGS39) was mated with *B. phaseoli* PD101 and transconjugants were selected on medium containing Sp and Km. Since pGS39 is not self-transmissible it was necessary to include a strain carrying a self-transmissible plasmid in the mating mixture; therefore *E. coli* HB101 carrying pRK2013 was included. Since pGS39 is unstable in *Rhizobium* strains it was expected that the acquisition of Km resistance by *B. phaseoli* PD101 would occur only by the transposition of Tn5-A1 from the suicide vehicle pGS39 into the genome of *B. phaseoli* PD101. Chloramphenicol (Cm) resistance genes in pGS39 are not encoded on a transposable element and so to verify that pGS39 was absent in the Sp^r , Km^r transconjugants, resistance to Cm was tested.

The individual isolates are expected to be derivatives of *B. phaseoli* PD101 in which Tn5-A1 is inserted in either the chromosome, in p3622b or in p3622a. More than seventy of these transconjugants (*B. phaseoli* PD101::Tn5.A1) were purified and then groups of individually grown liquid cultures of purified transconjugants were pooled, concentrated and mated with a recipient strain, *B. leguminosarum* B151 rif. This *B. leguminosarum* strain is cured of its own Sym plasmid, and its chromosome encodes resistance to the antibiotic rifampicin (rif). Following mating bacteria were selected on medium containing rif and Km. Neither the donor pool, nor the recipient *B. leguminosarum* could grow on this medium. The transconjugants were screened to ensure that they were Sp^S and therefore not spontaneous rif^r derivatives of the donor bacterium. It was expected that *B. leguminosarum* B151rif could acquire resistance to Km by receiving either p3622a::Tn5.A1 or p3622b::Tn5.A1. These two classes of transconjugants were distinguished by their bean nodulating ability, pigment production and plasmid content as determined by agarose-gel electrophoresis (see section 3.6). Black pigment production in *B. phaseoli* strains is usually Sym plasmid encoded (Jordan 1984). Bacteria were grown for 10 days on ~~TYC~~ agar medium to which 10mg/L $CuSO_4 \cdot 5H_2O$ and 30 mg/L L-tyrosine (from Sigma) - filter sterilized was added to induce pigment production. *B. leguminosarum* B151rif carrying the Tn5.A1 labelled Sym plasmid was designated PD102. This strain was used as a donor in matings

with A. tumefaciens. A. tumefaciens strains carrying p3622b::Tn5.A1 were selected on medium containing nalidixic acid and kanamycin. Following verification of the plasmid content of presumptive transconjugants, the strains were tested for their nodulating and tumor-forming ability.

c) A. tumefaciens strains carrying a Sym plasmid from B. meliloti JJ1C10

The Sym plasmid from B. meliloti JJ1C10 was transferred to A. tumefaciens A136 by mating pools of B. meliloti cells carrying insertions of Tn5.A1. The procedure used was as described for the transfer of the labelled Sym plasmid from B. phaseoli PD101 except that the pool used was prepared previously and provided for this study by G. Selvaraj. Transconjugants were selected on medium containing nalidixic acid and kanamycin. Also transconjugants of A. tumefaciens A136 carrying the Sym plasmid were identified in part by their ability to nodulate alfalfa. Several $\text{Nal}^r \text{Km}^r$ transconjugants receiving plasmids differing in size from the Sym plasmid of B. meliloti JJ1C10 were also tested for their ability to nodulate alfalfa.

d) A. tumefaciens carrying pRmSL26

The cosmid pRmSL26 carrying cloned nodulation genes of the B. meliloti A1021 Sym plasmid was transferred to several Vir- A. tumefaciens mutants, and to A. tumefaciens A348 during the mating of HB101 (pRmSL26) with each of the A. tumefaciens strains. Since pRmSL26 is not self-transmissible, E. coli HB101 (pRK2013) was also included in the mating mixture. pRmSL26 carries genes for resistance to the antibiotic tetracycline and therefore transconjugant A. tumefaciens strains carrying pRmSL26 were selected on medium containing nalidixic acid and tetracycline. Presumptive transconjugants were screened for their plasmid content and their respective tumor-forming ability and alfalfa-nodulating ability.

3.5 Determination of the Stability of the Sym and Ti plasmids in the Constructed strains:

The nodulating and tumor-forming ability of the constructed strains was tested under conditions which are not necessarily advantageous for the maintenance of the Sym plasmid

and the Ti plasmid. Prior to testing, the strains are grown in medium which ensures that both of these plasmids are maintained, however at wound sites or on the roots of plants antibiotics are not present to provide selective pressure for the stable maintenance of both plasmids. Therefore it was necessary to determine if both the Ti and Sym plasmids in the constructed strains were stably maintained in the absence of antibiotics. This was determined in several ways. First bacteria isolated from nodules formed by the constructed strains were tested to determine whether or not both plasmids were still present. The isolation of bacteria from nodules is described in section 3.8. The stability of both pTi and pSym-linked antibiotic resistance phenotypes following growth in non-selective medium was also tested. After 2 days growth viable counts of the early-log phase culture were determined on two different medium, TYC without antibiotics, and TYC + 100ug/mL Cb + 100 ug/mL Km. I assumed that loss of either of the plasmids would result in a significant reduction of viability on the selective medium in comparison with viability on the non-selective medium. This procedure was used with only one representative strain, i.e. A. tumefaciens pTi::Tn3HoHo1/pSym, for each type of Sym plasmid which was introduced. Since A. tumefaciens A348 derivatives do not have a Cb^r phenotype linked to pTi they could not be examined in this way, however in these strains the presence of the w.t. Ti plasmid was confirmed by tumor-forming ability on at least some hosts (see RESULTS) demonstrating the presence of a Ti plasmid-linked phenotype in these constructed strains.

3.6 Analysis of the Plasmid Content of Bacterial Strains:

The plasmid content of strains was determined by agarose-gel electrophoresis of total cell lysates by a modification of a procedure described by Eckhardt (1978). Rosenberg et al. (1982) described a modification of the Eckhardt (1978) procedure which improves the resolution of very large plasmids such as those found in Rhizobium and Agrobacterium strains. I have used the 'analytical' procedure which Rosenberg et al. (1982) described, with minor modifications. 0.1-0.5 mL of bacterial suspension was spun for 3 minutes in a Beckman Eppendorf microfuge. The cell pellet was resuspended in 1.0 mL of 0.1% sarkosyl (w:v) in Tris-Borate buffer (89mM Tris-Base, 89mM boric acid, 2.5mM disodium EDTA at pH 8.2) and the cells were again pelleted as above. This was followed by a wash

with 1.0 mL of Tris-Borate buffer. Next the pelleted cells were resuspended in 40ul of 'Lysis Mixture' [20% (w:v) ficoll (Ficoll 400, Pharmacia), 7,500 units/mL lysozyme (Grade 1 Sigma) 0.3units/mL ribonuclease III-B (Sigma), and 0.01% bromophenol blue in Tris-borate buffer] and then vortexed for 3 seconds and immediately loaded into the wells (8 x 12 x 3 mm) of an 0.7% (w:v) agarose [Ultra-Pure DNA Grade, BioRad] (in Tris-borate buffer) vertical gel using Eppendorf pipette tips with approximately 0.5 cm of the end of the tip removed. Within 5 minutes of well loading 40ul of a solution of 0.2% (w:v) SDS (Sigma), 10% (w:v) ficoll in Tris-Borate buffer were added and mixed gently with the cell layer using a toothpick. 100ul of a solution of 0.2% SDS, 5% ficoll in Tris-Borate buffer were carefully loaded above this layer. The wells were sealed with the softened 0.7% (w:v) agarose. Vertical gel-electrophoresis was carried out for 1.5 hours at 20 volts and then for 18-24 hours at 60 volts in a running buffer of Tris-Borate. Gels were stained in an 0.4ug/mL solution of ethidium bromide and then photographed under long-wave UV illumination. Plasmid migration distances were compared to those of a 350 MD pSym and a 135 MD cryptic plasmid present in B. meliloti JJ1 (Adachi et al. 1983) or to pATC58 (270 MD), pTi (130 MD) and pPH1J1 (36 MD) carried in A. tumefaciens strains listed in Table 9 and kindly provided by E. W. Nester.

3.7 Determination of the Nodulating Ability of Bacterial Strains:

a) Growth of Plants:

Sterilized seeds of Phaseolus vulgaris L. cv. Kentucky Wonder, Trifolium repens L. Medicago sativa L. cv. Saranac and Pisum sativum L. cv. Novella were prepared according to the following protocol. The seeds were:

(i) Rinsed in 70% (v:v) ethanol for approximately 1 minute

(ii) Stirred in a 15 % (v:v) Javex solution for 30 minutes

(iii) Rinsed three times for two minutes each in sterile distilled water. The

seeds germinated on TYC agar plates at 30°C. After two days incubation, any seeds in contact with bacterial or fungal growth were discarded. Germinated seeds free of contamination were transferred, using sterile forceps, to containers for further growth. Bean

seedlings were grown in sterile 500 mL flasks containing 250ml of Jensens' nitrogen-free plant nutrient solution which was solidified with 1.5 % (w:v) [Difco] agar. Pea plants were grown in sterile 1 litre plastic bottles containing sterile vermiculite moistened with sterile Jensens nitrogen-free nutrient solution. Alfalfa and clover seedlings were grown in large 150mm x 25mm sterile glass tubes, on slopes of Jensens nitrogen-free nutrient solution which was solidified with 1.7 % (w:v) [Difco] Agar. Jensen's nitrogen-free medium contained, in g/L 0.272, KH_2PO_4 ; 0.349, K_2SO_4 ; 0.249, MgSO_4 ; 0.004 H_3BO_4 ; 1.034, CaSO_4 ; 0.015, Fe-EDTA; and in mg/L 0.990 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$; 0.575, ZnSO_4 ; 0.125, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.050, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. Chemicals were from Baker, Sigma and Fisher Scientific. The pH was 6.5-7.0 and the medium was agitated before it was dispensed.

b) Inoculation of Plants with Bacterial Strains:

After the seedlings were transferred to the appropriate containers they were grown for 5-7 days before being inoculated with bacteria. The bacteria were grown in liquid medium selective for either the presence of the sym plasmid or the Ti plasmid, or for both, depending on the strain being grown. They were pelleted by centrifuging for 10 minutes at 3,000 rpm in a Sorvall Benchtop centrifuge. The pelleted cells were resuspended in sterile distilled water and immediately pipetted over the surface of the roots. For clover and alfalfa seedlings approximately 10^8 bacterial cells suspended in 0.1 mL of sterile distilled water were inoculated. For bean seedlings approximately 10^9 bacterial cells suspended in 5 mL of sterile distilled water were inoculated. Pea plants were inoculated with approximately 10^{10} bacterial cells suspended in 25 mL of sterile distilled water. The plants were grown in 'controlled- environmental' rooms with a light photoperiod of 18 hours at a light intensity which was 8600 lux at the top of the plants. The temperature was between 23 and 25°C.

c) Analysis of Nodulation in Infected Plants:

The inoculated clover, alfalfa, and bean plants were observed periodically during their growth. Pea roots were observed only after plants were uprooted approximately 50 days after inoculation. Initial experiments revealed that pea plants grown on agar in flasks could not be nodulated and therefore vermiculite was used.

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All inoculated plants were examined for the presence of visible root deformations which were not characteristic of uninoculated plants or plants inoculated with control non-nodulating strains. The number and type of root deformations observed was recorded. These included marked root curling or waviness, thick short roots (tsr), root swellings, or structures resembling nodules, which I refer to as pseudonodules. Sometimes the time of appearance (ie. # of days post-inoculation) of these structures was noted.

3.8 Isolation of Bacteria from Nodules:

To determine that nodules were formed by the original inoculum, and to determine whether the inoculated strain had undergone changes during passage through the nodule the bacteria from the nodules were isolated, purified, and later, characterized. The nodules were surface-sterilised before bacteria were isolated from them. The nodules were excised intact, from the roots and placed in sterile distilled water for 2 minutes, 95% (v:v) ethanol for 30 seconds, sterile distilled water for 2 minutes. Next they were placed in a dilute solution of hydrogen peroxide (1 part 33% (v:v) hydrogen peroxide (Baker) to 2 parts sterile distilled water) for one minute and rinsed in sterile distilled water for 3 minutes. The 'surface-sterilized' nodules were transferred to a sterile plastic petri-dish and crushed with a sterile scalpel. The resulting pulp was suspended in 0.5 mL of a sterile solution of NaCl (0.85 % w:v). 100ul of the suspension was plated on TYC agar medium with no antibiotics and incubated at 30°C. Single colonies were purified and tested for their ability to grow in the presence of antibiotics to which the original inoculum bacteria was resistant. The plasmid profiles of the nodule isolates were compared to those of the original inoculated strains. The tumor-forming ability of some of the nodule isolates was tested.

3.9 Determination of the Tumor-forming ability of Bacterial Strains:

a) Growth of Plant Hosts:

Kalanchoe daigremontiana, Lycopersicum esculentum (tomato) cv. Wonder Boy, Nicotiana tabacum (tobacco), and Daucus carota (carrot) were the plant hosts used to test

the tumor-forming ability of the constructed strains. For the first three species whole greenhouse-grown plants were inoculated. K. daigremontiana leaves, and tomato and tobacco stems were inoculated. For Daucus carota sterile root slices were used. Carrot roots were first cleaned in soapy water, peeled, and placed in a 5% (v.v) Javex solution for 30 minutes. After three rinses in large volumes of sterile distilled water they were sliced with a sterile knife and the slices were placed, proximal side down, on sterile moistened absorbent paper discs in sterile petri-dishes.

b) Infection of Wounds with Agrobacterium and Rhizobium

Whole plants of K. daigremontiana, tomatoe and tobacco were wounded using sterile tooth-picks. Approximately 10^7 bacterial cells suspended in 25ul of sterile distilled water were applied to the wounds or, alternatively to the surface of the sterilized carrot root slices. For up to one week following wounding the leaves and stems of infected greenhouse-grown plants were left undisturbed during watering so that cross-contamination of wounds was avoided. After this time the wounds are expected to be no longer susceptible to infection. Wounded, uninoculated plants were included in all tests of virulence to verify that cross-contamination did not occur. With the exception of K. daigremontiana each plant was inoculated with only one strain of bacteria. For K. daigremontiana several strains were sometimes inoculated on a single leaf. Control wounds containing no bacteria were included on each leaf to assure that no cross-contamination occurred. In no case did uninoculated wounds develop into tumors.

The basal side of the carrot root slices were inoculated. The orientation of the slice was important since during initial attempts to examine tumor formation on the root discs I observed callusing on the proximal side of about 90% of the slices, even when they had not been inoculated. Ryder et al. (1985) have recently reported a similar observation. This 'natural' or 'spontaneous' callusing was not observed on the basal side of the root slice. Therefore it was assumed that callusing of the inoculated basal side of the root slices was a result of the inoculated bacteria.

c) Scoring of Tumor-inducing Ability:

A subjective judgement was made of the tumor size-the relative size in comparison with

tumors formed by the wild-type Agrobacterium strains on the same type of plant and within the same length of time. Tumors scored as WT were indistinguishable from those formed by the wild-type A. tumefaciens strain A348. Tumors scored as medium (M) were considerably smaller than those formed by A. tumefaciens A348. Small (S) tumors were smaller than medium sized tumors. A very weak positive response of wounded plants to some strains was sometimes observed. This is scored as weak (WK). Such a response was never observed in uninoculated wounds and therefore was considered to be a consequence of the inoculated strain

3.10 Assay of Octopine Synthase Activity in Tumor extracts:

The presence of the octopine synthesizing enzyme, octopine synthase, is a positive indicator of plant cell transformation by A. tumefaciens carrying octopine-type Ti plasmids. Tumors formed by the constructed strains were analysed for the presence of octopine synthase activity. The procedure used was similar to that described by Otten and Schilperoort (1978). Extracts of tumor tissue are incubated with substrates (arginine and pyruvate) necessary for the biosynthesis of octopine. Following incubation cell extracts are spotted on paper and electrophoresed. Staining with phenanthrenequinone allows visualization under ultraviolet light of octopine, arginine and other guanidine compounds. The modified procedure I have used is described by Holbrook, Haffner and Miki (in press) and involves the use of ^{14}C -labelled arginine. Radioactive compounds formed from the arginine are visualized, following paper chromatography, using a fluorographic procedure.

Freshly excised tumor tissue was weighed and then placed in an Eppendorf tube. Twice the volume (eg. 10ul for 5mg) of extraction buffer [0.1M Tris pH8.0, 0.1% ascorbic acid, 0.1% cysteine, and 0.5M sucrose] was added to each. Samples were kept on ice. A small amount of sand was added to the sample and the tissue was crushed with a glass rod. The sample was spun for three minutes at speed 5 in a Beckman microfuge TM/2. Two uL of sample were withdrawn and placed in clean microfuge tubes; this was mixed carefully with two uL of reaction buffer [30mg NADH, 190ul of 150mM pyruvate, 70ul 1M Na_3PO_4 , 0.1mM ^{14}C -arginine [New England Nuclear] in 300ul] and incubated for 1.5 hours at room

temperature. The tubes were then placed at -90°C for several minutes to arrest enzymatic activity. Three microlitres of each sample were spotted on MN 214 chromatography paper. Horizontal gel-electrophoresis was carried out for 1 hour at 400 volts with a running buffer of formic acid/acetic acid/water (5: 15: 80 parts respectively). Chromatograms were impregnated with 40 mL of an 8% (w/v) solution of 2,5 diphenyloxazole in ether and then air-dried in a fume hood. The dried chromatograms were placed for 48 hours with Kodak X-omat film in a Kodak developing cassette with intensifying screen; this was incubated at -90°C . The fluorogram was developed in Kodak Developer for 2.5 minutes, rinsed in water for 2.5 minutes, and then fixed in Kodak Fixer for 3 minutes and rinsed for 10 minutes in running tap water.

Octopine synthase activity (identified by the presence of octopine 'spots' on the fluorogram) in the tumor tissue being tested was compared to that found in tumors formed by A. tumefaciens A348 and also to levels in transformed and non-transformed Brassica oleraceae tissue previously characterized by Holbrook, Haffner and Miki (in press).

RESULTS AND DISCUSSION

4.1 Construction of A. tumefaciens carrying Sym plasmids from R. leguminosarum, R. phaseoli and R. trifolii

4.1.1 Transfer of a non self-transmissible R. phaseoli Sym plasmid to A. tumefaciens

The strategy outlined in Section 3.4 to transfer the symbiotic plasmid of R. phaseoli 3622 into A. tumefaciens was successful. First a spontaneous spectinomycin resistant (Sp^r) mutant of R. phaseoli 3622 was isolated. This isolate, R. phaseoli PD101, had a plasmid profile similar, if not identical, to that of R. phaseoli 3622 and was able to nodulate beans effectively. This demonstrated that the acquisition of Sp^r has not impaired the nodulating ability of this strain.

a) Tn5.A1 Mutagenesis and transfer of pSym::Tn5.A1 to R. leguminosarum

In order to label p3622b with an antibiotic resistance marker, R. phaseoli PD101 was mutagenized using an E. coli strain carrying the suicide plasmid vehicle pGS39. This suicidal plasmid was previously shown to be capable of delivering Tn5.A1 to the genome of R. meliloti (Selvaraj 1983). In the present work it is shown to be useful for the Tn5.A1 labelling of the sym plasmid of R. phaseoli. A triparental mating between R. phaseoli PD101 E. coli HB101(pGS39) and E. coli HB101 (pRK2013) produced approximately 6×10^{-6} (average of two separate trials) Sp^r Km^r transconjugants per donor. Of one-hundred derivatives tested three were chloramphenicol resistant suggesting that these three isolates retained pGS39. The original R. phaseoli PD101 recipient was Cm sensitive (Cm^S). The presence of pGS39 in the three Cm^r isolates was not verified by further tests, but these isolates were omitted from further steps since in these Tn5.A1 may not be incorporated into the genome of R. phaseoli PD101.

b) Transfer of p3622b::Tn5.A1 to R. leguminosarum

A pool of Tn5.A1 insertion derivatives of R. phaseoli PD101 consisting of twenty-three individually purified insertion derivatives, was mated with R. leguminosarum B151 rif^r as described (section 3.3) and five transconjugants designated R. leguminosarum PD102,

PD103, PD104, PD105, and PD106 were obtained following selection on medium supplemented with rifampicin and kanamycin. The individual components of the mating- the pooled isolates, the cured *R. leguminosarum* B151 rif, and the *E. coli* strain carrying pRK2013- did not grow on the selective medium.

To determine which, if any, of the presumptive transconjugants could be used to donate a Tn5.A1-labelled *R. phaseoli* Sym plasmid to *A. tumefaciens*, *R. leguminosarum* PD102, PD103, PD104, PD105, and PD106 were tested further. Several characteristics of these transconjugants are listed in Table 13.

Table 13: Properties of Presumptive Transconjugants PD102-106

STRAIN	RIF ^r	SP ^r	PIGMENT ¹	NODULATION ²	PLASMID CONTENT (approx. size in MD)
PD102	+	-	+	+	250, 230
PD103	+	-	-	-	230
PD104	+	+	+	+	250, 100
PD105	+	-	-	-	230
PD106	+	-	-	-	230, 100

¹ production of black pigment after growth on TYC medium supplemented with cupric sulphate and tyrosine

² ability of presumptive transconjugants to nodulate *P. vulgaris* L.

* An approximation based on a comparison of migration distances with that of plasmids of *R. phaseoli* 3622 -containing a 250 MD and a 100 MD plasmid (Prakash and Schilperoort, 1982) and *R. leguminosarum* B151 carrying a single plasmid of approx. 230 MD (Brewin et al. 1982).

The characteristics of *R. leguminosarum* PD102 suggested that it indeed carried a Tn5.A1-labelled *R. phaseoli* Sym plasmid. *R. leguminosarum* PD103 and PD105 are probably spontaneous Km^r derivatives of *R. leguminosarum* B151rif since they have plasmid profiles similar to *R. leguminosarum* B151 rif^r and can neither nodulate bean nor produce a black pigment. *R. leguminosarum* PD104 is probably a spontaneous rif^r derivative of *R. phaseoli* PD101 since it maintains resistance to spectinomycin which was not selected for following mating and since its plasmid profile is like that of *R. phaseoli* PD101. When *R. leguminosarum* PD104 was mated with *A. tumefaciens* A136 conjugal transfer of Km resistance was not observed (frequency was $\ll 4.1 \times 10^{-10}$ per donor); therefore in this strain Tn5.A1 is probably present on the chromosome. PD106 probably carries the cryptic plasmid

p3622a since this strain could transfer Km resistance to A. tumefaciens A136 during a triparental mating which also included E. coli HB101(pRK2013) and transfer of Km (occurring at a frequency of 3.5×10^{-5} per donor) was correlated with the receipt of a plasmid similar in size to R. phaseoli p3622a. Carriage of this plasmid did not confer pigment production or bean nodulating ability to A. tumefaciens A136 suggesting that this smaller plasmid from R. phaseoli PD101 does not carry nodulation determinants. This is expected since Prakash et al (1981) showed that p3622b, but not p3622a hybridized to a nif- gene probe from K. pneumoniae. Finally, the characteristics of R. leguminosarum PD102 were as follows. It was sensitive to spectinomycin, produced a black pigment after growth in medium supplemented with tyrosine, and could nodulate beans effectively. The parental R. leguminosarum B151^{nif} did not nodulate beans under the conditions used to assay nodulation, and did not produce black pigment.; therefore it was concluded that R. leguminosarum PD102 did contain the R. phaseoli Sym plasmid labelled with Tn5.A1 and that the insertion of Tn5.A1 did not interfere with the ability of p3622b::Tn5.A1 to confer bean nodulating ability. This Sym plasmid derivative, p3622b::Tn5.A1, was named pPD102.

c) Transfer of pPD102 from R. leguminosarum PD102 to A. tumefaciens

R. leguminosarum PD102 carrying pPD102 was used as a donor in matings with A. tumefaciens strains. Kanamycin resistance was transferred from this strain to the A. tumefaciens recipients, using E. coli HB101(pRK2013) for mobilization. The frequencies of transfer of Km resistance (given in Table 14) varied somewhat according to the recipient A. tumefaciens strain.

To determine if pPD102 could be transferred without the help of a self-transmissible plasmid, R. leguminosarum PD102 was mated with A. tumefaciens A136 but the E. coli strain HB101(pRK2013) was not used. Under these conditions pPD102 was not transferred. The frequency of transfer of Km resistance per donor cell was $< 5 \times 10^{-9}$ suggesting that pPD102 is not a self-transmissible plasmid. Hooykaas et al. (1985) recently demonstrated the mobilization of p3622b using the P-incompatibility group plasmid pRL180 and also found that conjugal transfer of p3622b occurred only in the presence of a mobilising plasmid.

Table 14 also indicates that the Vir B- mutant Sm 37 was a poor recipient for the labelled R. phaseoli Sym plasmid. It was also a poor recipient for the E. coli plasmids pRK2

and pGS9. During mating of *E. coli* DF102 (pRK2) and *E. coli* HB101 (pGS9) with *A. tumefaciens* Sm 37, it was found that *A. tumefaciens* Sm 37 received these plasmids at a frequency which was approximately 100 fold lower than the frequency of transfer to the other *A. tumefaciens* Vir B- mutant Sm 243. Frequencies of transfer of pRK2 (which has a P transfer system) to *A. tumefaciens* Sm37 and Sm 243 were $< 2 \times 10^{-10}$ and 4×10^{-8} respectively. *E. coli* carrying pGS9, which encodes an N transfer system, transferred Km^r to *A. tumefaciens* Sm37 and *A. tumefaciens* Sm243 at frequencies of 4×10^{-9} and 4×10^{-7} respectively. The reason for the poor recipient ability of *A. tumefaciens* Sm37 is unclear. It would be of interest to determine if the poor recipient ability is due to the Vir- mutation or to some other change in this strain, since if it is due to the mutation in *vir B* then an investigation of the molecular basis of the poor recipient ability may reveal an aspect of the function of this gene.

Table 14: Frequencies of transfer of Km resistance from PD102 to the *A. tumefaciens* Recipients

<i>A. tumefaciens</i> Recipient	Frequency of Transfer ¹
A348	4×10^{-6}
A136	2×10^{-7}
Sm 240 (Vir A-)	4×10^{-7}
Sm 37 (Vir B-)	$< 2 \times 10^{-9}$
Sm 243 (Vir B-)	4×10^{-7}
Sm 365 (Vir C-)	1×10^{-7}
Sm 311 (Vir D-)	9×10^{-8}
Sm 355 (Vir D-)	7×10^{-8}
Sm 341 (Vir E-)	6×10^{-8}
Sm 361 (Vir E-)	4×10^{-7}

¹ approximate frequency of transfer of Km resistance per donor *R. leguminosarum* PD102

* no transfer to this strain was detected (frequency $< 7 \times 10^{-11}$ in another trial)

Although no growth of the donor *R. leguminosarum* PD102 was observed on the selective plates several $Nal^r Km^r$ presumptive transconjugants derived from plating of the mating mixture were found to be rif^r suggesting that they could be spontaneous Nal^r derivatives of the donor *R. leguminosarum* PD102. Only rif^s isolates were chosen for further study. Once transconjugants from each mating were purified they were tested further. The Vir- *A.*

tumefaciens recipients of pPD102 were still Cb^r suggesting that pTiA6NC::Tn3HoHo1 is still present. When the plasmid content was examined each isolate was found to contain plasmids corresponding in size to pATC58 (the cryptic A. tumefaciens plasmid), pTi and pPH1J1. An additional plasmid migrating very close to pATC58 was also seen. This may correspond to pPD102 since it migrates to approximately the same extent as does pPD102 from B. leguminosarum PD102. However since pATC58 and pPD102 are so similar in size, the two could not always be resolved during electrophoresis. In spite of this difficulty the presence of pPD102 was confirmed by the acquisition of pigment production and bean pseudonodulating ability by these strains. A. tumefaciens A348, assumed to be carrying pPD102, could form tumors and could also pseudonodulate bean suggesting again that both pTi and pSym are present.

To determine whether both the Ti and the Sym plasmid were stably maintained in these strains A. tumefaciens Sm240 carrying pPD102 was tested as described in section 3.5. The viable counts on TYC and on TYC plus Cb and Km were 5.9×10^9 and 4.7×10^9 respectively; of 50 single colonies growing on TYC all were able to grow on medium carrying TYC plus Cb and Km when they were streaked onto these plates. This suggests that during the brief absence of selective antibiotics there is no substantial reduction in the number of bacteria in the population which carry both antibiotic resistance markers. All of the A. tumefaciens strains carrying pPD102 were able to produce a black pigment normally characteristic of B. phaseoli strains and were able to form small ineffective nodules on P. vulgaris cv. Kentucky Wonder. I conclude that genes on pPD102 which are responsible for these phenomenon are expressed in all of the A. tumefaciens strains whether or not the Ti plasmid is present. Bacteria which were isolated from the nodules induced by the wild-type A. tumefaciens A348 strain carrying pPD102 were still oncogenic, indicating that the Ti plasmid was maintained in these strains during passage through nodules. Bacteria isolated from nodules formed by mutant Agrobacterium, carrying pPD102, still carried the Ti-plasmid linked Cb^r phenotype, again suggesting that both the Ti and the Sym plasmid were stably maintained during passage through nodules. Agarose-gel electrophoresis of nodule isolates showed that they contained plasmids similar in size, if not identical, to those in the original strains which were inoculated.

4.1.2 Transfer of a self-transmissible *R. trifolii* Sym plasmid to *A. tumefaciens*

It was not necessary to alter the *R. trifolii* sym plasmid ptr5a prior to transfer since it is self-transmissible and carries the transposon Tn5 (Hooykaas et al. 1981) which confers resistance to kanamycin. The *R. trifolii* Sym plasmid ptr5a was successfully transferred to the *A. tumefaciens* recipients during mating, at frequencies which varied from 5×10^{-5} to 1×10^{-8} per donor cell. Transfer to the *A. tumefaciens* Sm37 (Vir B-) strain was not attempted. The introduction of ptr5a into the *A. tumefaciens* strains did not appear to lead to any loss of the indigenous plasmids of these strains. Plasmids corresponding in size to pATC58, pTi and pPHIJ1 were present and an extra plasmid was acquired which corresponded in size to ptr5a from *R. trifolii* LPR5035. Several observations suggested that both the Ti and the Sym plasmid were stably maintained in these strains. Transconjugant bacteria from matings with *A. tumefaciens* (pTi::Tn3 HoHo1), which were selected on medium containing nalidixic acid and kanamycin, were still Cb^r. Also, transconjugants from matings with *A. tumefaciens* A348 were capable of inducing tumors and could also induce root deformations and pseudonodules on clover. The nodulating and tumor-forming abilities of these strains will be described in more detail in Sections 4.2 and 4.3. When the viability of one *A. tumefaciens* pTi::Tn3HoHo1/ptr5a strain was tested no marked difference in viable counts was observed on TYC medium compared with the counts on TYC containing Km and Cb, suggesting that both pTi and pSym were stably maintained. In all cases *A. tumefaciens* had acquired the ability to induce root deformations or pseudonodules on clover roots. *A. tumefaciens* strains not carrying ptr5a were unable to deform clover root hairs. Bacteria isolated from the nodules formed on plants inoculated with *A. tumefaciens* pTiA6NC::Tn3HoHo1/ptr5a were found to contain both the Ti and the Sym plasmid associated antibiotic resistance markers and plasmids with sizes similar to those in the original inoculated strains.

4.1.3 Transfer of the Self-transmissible *R. leguminosarum* Sym plasmid pRL5JI

The *R. leguminosarum* Sym plasmid pRL5JI (carried in *R. leguminosarum* B316) is a self-transmissible plasmid carrying the transposon Tn5. After the mating of *A. tumefaciens*

with R. leguminosarum B316, transconjugants were selected on medium containing nalidixic acid and kanamycin. There was some background growth of the donor R. leguminosarum B316 on the selective plates, however it was possible to distinguish transconjugants from the donor background growth. Several colonies growing on the selective plates inoculated with the mating mixture appeared to be less mucoid in morphology, in comparison with colonies arising from the donor R. leguminosarum B316 plated on selective medium. Such transconjugants were purified and often found to carry, as expected, 4 plasmids corresponding approximately in size to the cryptic plasmid of A. tumefaciens, pATC58, pTi, pRL5J1 and pPHI1 (see example shown in PLATE 1, Lane 3). Therefore it was assumed that these isolates are A. tumefaciens which carry pRL5J1 and indeed these were found to have Ti plasmid-associated markers (ie. Cb resistance in the case of A. tumefaciens pTi::Tn3HoHo1 recipients) and to be capable of pea root deformation. When bacteria were reisolated from these nodules both pTi and pSym (except in bacteria isolated from nodules formed by A. tumefaciens A136/pRL5J1) were apparently present (an assumption based on plasmid profile) suggesting that during passage through nodules both plasmids were stably maintained. Several initial isolates were found to contain five plasmids (see example shown in PLATE 1, Lane 2). Such transconjugants were isolated from matings of R. leguminosarum with A. tumefaciens strains A348, Sm361 and Sm311 but these particular transconjugants were not used in further experiments; other transconjugants with plasmid profiles indicating that only one plasmid (corresponding in size to pRL5J1) had been received from R. leguminosarum B316 were used. It is possible that similar transconjugants arose from matings with other of the A. tumefaciens strains, but following identification of an isolate carrying a plasmid corresponding in size to pRL5J1 and conferring pea nodulation was identified for each mating further colonies were not screened. Several attempts to transfer pRL5J1 to A. tumefaciens Sm240 VirA- and to Sm365 VirC- were unsuccessful. Following mating, colonies forming on selective plates were usually found to be Cb^S and were morphologically similar to colonies of the donor strain. Four separate matings of R. leguminosarum B316 with A. tumefaciens Sm365 and six with Sm240 yielded no true transconjugants.

Plate 1: Agarose-gel electrophoresis of cell lysates of (LANE 1) A. tumefaciens Sm311 carrying pATC58 (A), pTi (B), and pPHIJ1 (C); (LANE 2 and LANE 3) B. leguminosarum B316 X A. tumefaciens 311 Nal^r Cb^r Km^r transconjugants; (LANE 4) B. leguminosarum B316 carrying two cryptic plasmids (marked but not labelled) and pRL5J1 (D). Fragments of chromosomal DNA occurring in all of the cell lysates are also visible (E).

1

2

3

4

A

B

C

D

E

4.2 Tumor-forming ability of A. tumefaciens recipients of pPD102, ptr5a or pRL5J1:

4.2.1 Effect of the *R. phaseoli* Sym plasmid pPD102 on tumor-forming ability in *A. tumefaciens*

The virulence phenotypes of the A. tumefaciens recipients of pPD102 are given in Table 15. The first column in this table shows the tumor-forming ability of each of the A. tumefaciens strains. This initial phenotype was compared to that of the A. tumefaciens strains carrying pPD102 or other Sym plasmids.

The virulence of the majority of the strains was not altered by the presence of pPD102. The avirulent A. tumefaciens mutants and the Ti-plasmidless A. tumefaciens strain remained avirulent; the wild type A. tumefaciens A348 control strain carrying pPD102 remained virulent, and the host range of the altered host range strains A. tumefaciens Sm 365 Vir C- and Sm 361 Vir E- were unaltered. The observation that A. tumefaciens A348, Sm 365, Sm 341 and Sm 361 remain virulent on plants on which they are normally virulent is further evidence that the Ti plasmid is still present and suggests that carriage of the *R. phaseoli* Sym plasmid does not impair tumorigenicity in these strains. The continued avirulence of the other mutant strains carrying pPD102 indicates that pPD102 did not complement the function of the mutated *vir* genes. However A. tumefaciens Sm 341 (Vir E-) carrying pPD102 formed tiny tumors when inoculated on wounded leaves of *K. daigremontiana*. Neither A. tumefaciens Sm 341 nor *R. leguminosarum* PD102 alone caused tumor formation on *K. daigremontiana*. The presence of pPD102 in A. tumefaciens Sm 341 did not alter its tumor-forming ability on the other plant hosts, i.e. it remained avirulent on tobacco and carrot and formed small tumors on tomato as did A. tumefaciens Sm 341.

The tiny tumors induced by A. tumefaciens Sm 341/pPD102 on *K. daigremontiana* leaves are shown in Plate 2. These always appeared approximately 1.5 months after inoculation (never sooner) and were formed on thirty per-cent of the *K. daigremontiana* leaf wounds inoculated. These structures, however, contained negligible octopine synthase activity in

Table 15: Tumor-forming ability of *A. tumefaciens* carrying *Rhizobium* Sym plasmids

<i>A. tumefaciens</i> Recipient	Virulence of <i>A. tumefaciens</i> recipient of				Plant Host
	no.pSym	pPD102	ptr5a	pRL5Jl	
A348 w.t.	52/52 WT	20/20WT	10/10M	9/10M	<i>K. daigremontiana</i>
	10/10WT	10/10WT	10/10M	10/10S	<i>L. esculentum</i>
	10/10WT	10/10WT	4/10WT	0/10	<i>N. tabacum</i>
	36/38	10/10	8/10	7/10	<i>D. carota</i>
A136 pTi-	0/40	0/20	0/10	0/10	<i>K. daigremontiana</i>
	0/10	0/10	0/10	0/10	<i>L. esculentum</i>
	0/10	0/10	0/10	0/10	<i>N. tabacum</i>
	0/10	0/10	0/10	0/10	<i>D. carota</i>
240 (VirA-)	0/22	0/22	0/10	N.S.A.	<i>K. daigremontiana</i>
	2/10S	0/10	0/10	N.S.A.	<i>L. esculentum</i>
	0/10	0/10	0/10	N.S.A.	<i>N. tabacum</i>
	0/10	0/10	0/10	N.S.A.	<i>D. carota</i>
243 (VirB-)	0/32	0/12	0/10	0/10	<i>K. daigremontiana</i>
	0/10	0/10	0/10	0/10	<i>L. esculentum</i>
	0/10	0/10	0/10	0/10	<i>N. tabacum</i>
	0/10	0/10	0/10	0/10	<i>D. carota</i>
365 (VirC-)	22/32M	9/12M	10/10S	N.S.A.	<i>K. daigremontiana</i>
	10/10M	10/10M	4M1S/10	N.S.A.	<i>L. esculentum</i>
	0/10	0/10	0/10	N.S.A.	<i>N. tabacum</i>
	8/10	9/10	16/18	N.S.A.	<i>D. carota</i>
311 (VirD-)	0/34	0/14	0/10	0/10	<i>K. daigremontiana</i>
	0/10	0/10	0/10	0/10	<i>L. esculentum</i>
	0/10	0/10	0/10	0/10	<i>N. tabacum</i>
	0/10	0/10	0/10	0/10	<i>D. carota</i>
355 (VirD-)	0/44	0/22	0/10	0/10	<i>K. daigremontiana</i>
	0/10	0/10	0/10	0/10	<i>L. esculentum</i>
	0/10	0/10	0/10	0/10	<i>N. tabacum</i>
	0/10	0/10	0/10	0/10	<i>D. carota</i>
341 (VirE-)	0/42	11/36Wk	0/10	0/10	<i>K. daigremontiana</i>
	10/10S	10/10S	8/10S	5/10S	<i>L. esculentum</i>
	0/10	0/10	0/10	0/10	<i>N. tabacum</i>
	0/10	0/10	0/10	0/10	<i>D. carota</i>
361 (VirE-)	0/34	0/14	0/10	0/10	<i>K. daigremontiana</i>
	10/10S	10/10S	8/10S	4/10S	<i>L. esculentum</i>
	0/10	0/10	0/10	0/10	<i>N. tabacum</i>
	0/10	0/10	0/10	0/10	<i>D. carota</i>

pPD102= *B. phaseoli* pSym, ptr5a= *B. trifolii* pSym, pRL5Jl= *B. leguminosarum* pSym

N.S.A.= no strain available

wild-type (WT), medium (M), small (S) tumors. WK = a weak response (shown in Plate 2)

Plate 2 K. daigremontiana leaf tumors and assay of octopine synthase
in tumor tissue

Upper Tumors formed on K. daigremontiana leaves infected with A. tumefaciens
A348/pPD102 (A) and A. tumefaciens Sm 341/pPD102 (B). The tumors
were photographed 70 days after infection. (Magnification 1.5 x)

Lower Fluorogram of octopine synthase assay performed as described in section
3.10. (Magnification 1.5) This procedure was according to Holbrook, Haffner
and Miki (in press). Presence of an octopine spot (OCT) was considered to be
indicative of opine synthase activity. Arginine (ARG) 'spots' are also visible

- 1 Brassica oleraceae L. A6NC tumor callus (positive control)
- 2 Brassica oleraceae L. non-transformed tissue (negative control)*
- 3 K. daigremontiana L., A. tumefaciens A348 tumor tissue (positive control)
- 4 K. daigremontiana L., A. tumefaciens A348/pRL5JI tumor tissue
- 5 K. daigremontiana L., A. tumefaciens A348/pPD102 tumor tissue
- 6 K. daigremontiana L., A. tumefaciens Sm341/pPD102 tumor tissue.

* described in Holbrook, Haffner and Miki (in press)

A

B

1

2

3

4

5

6

ARG

OCT

comparison with levels found in tumor tissue induced by the *A. tumefaciens* A348/pPD102 (see Plate 2). Several other plant types not used in the original tumorigenesis assay were tested in an attempt to identify other plant hosts outside the host-range of *A. tumefaciens* Sm341 which might also display this positive response to *A. tumefaciens* Sm341/pPD102. However inoculations of stems of intact *Nicotiana rustica*, petunia and *Brassica juncea* plants revealed that the *A. tumefaciens* Sm341 mutant was tumorigenic, albeit attenuated, eliminating the possibility of using these plants to test for restoration of virulence by pPD102.

It was of interest to determine if the small tumors or calli of the type formed by *A. tumefaciens* 341/pPD102 would be formed if pPD102 was provided to *A. tumefaciens* Sm 341 in a different way. Following the co-inoculation of *A. tumefaciens* Sm341 and *B. leguminosarum* PD102 on leaf wounds of *K. daigremontiana*, small tumors arose as they did following inoculation with the single strain of *A. tumefaciens* Sm341/pPD102. The frequency of tumor formation was quite low as it was for infection with *A. tumefaciens* 341/pPD102. After 65 days five of the twenty wounds inoculated simultaneously with the two strains, developed tiny tumors. Inoculation with *B. leguminosarum* PD102 alone failed to induce tumor formation at all of the 35 inoculation sites tested.

4.2.2 Effect of the *B. trifolii* Sym plasmid ptr5a on tumor-forming ability:

The tumor-forming ability of *A. tumefaciens* strains carrying ptr5a is also given in Table 15. The presence of ptr5a did not enhance the tumor-forming ability of any of the *A. tumefaciens* strains. Therefore there was no evidence for functional complementation, by ptr5a, of any of the mutated *vir* loci. Rather, the presence of ptr5a in *A. tumefaciens* seemed to have a minor inhibitory effect on tumorigenicity. The virulence on *L. esculentum* and *K. daigremontiana*, of *A. tumefaciens* A348/ptr5a and Sm 365/ptr5a was slightly reduced in comparison with that of the corresponding sym plasmid-free *A. tumefaciens* strains.

4.2.3 Effect of B. leguminosarum Sym plasmid pRL5JI on tumor-forming ability

The tumor-forming ability of A. tumefaciens strains carrying pRL5JI is listed in Table 15. The presence of pRL5JI did not enhance the tumor-forming ability of any of the avirulent mutants or altered host range mutants. Apparently no functional complementation of mutated vir genes occurred under the conditions used here. However in A. tumefaciens A348 the presence of pRL5JI resulted in an attenuation of virulence. No tumors were formed on N. tabacum. The size of tumors formed on K. daigremontiana and L. esculentum was decreased. Also an attenuation of the tumor-forming ability of A. tumefaciens Sm 341 (Vir E-) and Sm 361 (Vir E-) carrying pRL5JI was observed on tomato plants.

4.3 Nodulating ability conferred by pPD102, ptr5a and pRL5JI in A. tumefaciens

4.3.1 Nodulating ability of A. tumefaciens carrying pPD102

The A. tumefaciens strains carrying pPD102 produced small white nodules on beans. Although the number of nodules varied somewhat from plant to plant it was never as high as on plants inoculated with the control Rhizobium strains which induced the formation of effective nodules. For example P. vulgaris L. inoculated with B. leguminosarum PD102 or B. phaseoli 3622 produced many large rounded pink-brown nodules. Table 16 lists the number of bean plants inoculated with A. tumefaciens strains carrying pPD102 and the number of plants which developed small rounded white nodules.

That all strains are capable of inducing pseudonodules indicates that pPD102 can confer pseudonodulating ability in A. tumefaciens regardless of the presence of the Ti plasmid. In each case the A. tumefaciens strain carrying pPD102 stimulated the production of only a few (1-4) pseudonodules.

Table 16: Bean Nodulating ability of A. tumefaciens carrying pPD102

<u>A. tumefaciens</u> Recipient	#Plants Pseudonodulated	<u>A. tumefaciens</u> Recipient	#Plants Pseudonodulated
A348	6/16	311 (VirD-)	3/9
A136	6/12	355 (VirD-)	3/9
240 (VirA-)	4/9	341 (VirE-)	4/9
243 (VirB-)	4/9	361 (VirE-)	2/9
365 (VirC-)	2/9		

[†] carrying pPD102

Note: P. vulgaris L. were inoculated and roots examined 40 days later. R. leguminosarum PD102 effectively nodulated 7/10 plants and R. phaseoli 3622 effectively nodulated 10/10 plants while each A. tumefaciens strain not carrying pPD102 was unable to nodulate P. vulgaris (a total of 20 plants tested per strain)

4.3.2 Clover Nodulating Ability conferred by ptr5a in A. tumefaciens

The clover nodulating ability of A. tumefaciens recipients of ptr5a confirmed that ptr5a was in fact present and able to express some genes in A. tumefaciens. In no case was effective nodulation conferred by ptr5a in A. tumefaciens. Only root swellings, root thickenings or small, rounded, black (or occasionally white) pseudonodules were observed. In contrast the nodules induced by R. trifolii LPR5035 were elongated and pink, and growing out at right angles from the roots. Plants with such nodules remained green and continued to grow until they were considerably larger than those plants inoculated with A. tumefaciens strains carrying the plasmid ptr5a. Plants inoculated with A. tumefaciens /ptr5a began to senesce about 3 weeks after inoculation as did control uninoculated plants.

The nodulating ability conferred by ptr5a in A. tumefaciens seemed to vary according to the presence of the Ti plasmid. In the Ti-plasmidless strain A. tumefaciens A136, ptr5a conferred a higher frequency of clover root deformation and pseudonodulation than in A. tumefaciens A348, suggesting that some portion of the Ti plasmid may inhibit the formation of these structures. Table 17 (below) gives the nodulating and root deformation frequencies for A. tumefaciens A136/ptr5a and A348/ptr5a. Fifty days after inoculation approximately 60% of plants inoculated with A. tumefaciens A136/ptr5a showed visible root deformities while 31% of plants inoculated with A348/ptr5a showed deformities. The majority of the clover root

deformations induced by A. tumefaciens A348/ptr5a were small swellings or root thickenings (shown in Plate 3) and these began to appear about 10 days later than those induced by A. tumefaciens A136/ptr5a. A graph showing the percentage of clover plants showing root deformations vs. the number of days after inoculation for infection with A. tumefaciens A136/ptr5a or A348/ptr5a is given in FIGURE 5. Another difference in these two strains was their ability to induce the formation of distinct pseudonodules (shown in PLATE 3) rather than root swellings or thickenings. Pseudonodules were formed on 31% of the plants inoculated with A. tumefaciens A136/ptr5a and on 8% of plants inoculated with A. tumefaciens A348/ptr5a. Also, plants which were inoculated with A. tumefaciens A348/ptr5a had fewer pseudonodules, when they had any, than plants inoculated with A. tumefaciens A136/ptr5a

Table 17 : Clover Nodulating ability of A. tumefaciens recipients of ptr5a

Strain	# plants inoculated	# with root deformations (and %)	# with pseudonodules (and %)	Total# of pseudonodules (av. per plant)
A348/ptr5a	35	11(31%)	3(8%)	3 (1)
A136/ptr5a	35	21(60%)	11(31%)	79 (7)
240/ptr5a	10	6 (60%)	6	48 (8)
243/ptr5a	10	3 (30%)	1	1 (1)
365/ptr5a	30	8 (27%)	4	10 (3)
311/ptr5a	10	2 (20%)	0	
355/ptr5a	10	3 (30%)	1	2 (2)
341/ptr5a	10	2 (20%)	0	
361/ptr5a	10	5 (50%)	5	25 (5)

[†] including those plants with only pseudonodules.

Observations made 50 days after inoculation. A. tumefaciens strains not carrying ptr5a were unable to induce root deformations or pseudonodules (total 20 plants tested per strain).

Figure 5: Graph of percentage of clover root deformations vs. # of days post-Inoculation

The data shown is for clover plants infected with A. tumefaciens A348 carrying the R. trifolii Sym plasmid ptr5a [A348/ptr5a] and A. tumefaciens A136 carrying ptr5a [A136/ptr5a]. Data is from a total of 35 infected plants for each strain.

A. tumefaciens-induced Root Deformations of Clover

Plate 3: Clover roots with pseudonodules and swellings induced by A. tumefaciens carrying an B. trifolii Sym plasmid. Small, black pseudonodules (indicated at A) are found on plants inoculated with A. tumefaciens A136/ptr5a, while swellings (indicated at B) are more common in plants infected with A. tumefaciens A348/ptr5a. (MAGNIFICATION x 3)

The root deforming and pseudonodulating ability of the *A. tumefaciens* Vir- strains carrying ptr5a is also given in Table 17 above. The pseudonodulating ability of *A. tumefaciens* 240/ptr5a is certainly greater than that of *A. tumefaciens* A348/ptr5a suggesting that inactivation of *vir A* may suppress the inhibitory effect of the Ti plasmid and therefore *vir A* might be directly or indirectly responsible for the inhibitory effect of the Ti plasmid on pseudonodulation. However, further testing will be required to prove this.

4.3.3 Nodulating Ability conferred by pRL5J1

All of the *A. tumefaciens* transconjugants carrying pRL5J1 formed small white nodules on some of the *P. sativum* L. plants inoculated (see Table 18) demonstrating that pRL5J1 genes involved in pea nodulation are expressed in *A. tumefaciens* whether or not the Ti plasmid is present. More often these strains induced root deformations such as root thickenings and shortness, root waving or kinking and root swellings. Such deformations were not observed on uninoculated plants or on those inoculated with *A. tumefaciens* not carrying pRL5J1.

Table 18: Pea Nodulating ability of *A. tumefaciens* recipients of pRL5J1

Strain carrying pRL5J1	Parental Strain ²	#Plants Pseudonodulated	# Plants with Root Deformations ¹
PD50	A348	1/12	3/12
PD51	A136	2/12	4/12
PD54	243	3/10	4/10
PD55	311	2/10	5/10
PD57	355	3/10	6/10
PD56	341	2/12	3/12
PD58	361	1/12	3/12
B316 ²	10/10	10/10	10/10

¹ all plants with any root deformities including those with pseudonodules or nodules.

² *Rhizobium* strain carrying pRL5J1 (original donor of pRL5J1 to *A. tumefaciens*)

Note: For each *Agrobacterium* not carrying pRL5J1 the nodulation frequency was 0/10

4.4 Mobilization of an *R. meliloti* Sym plasmid to *A. tumefaciens* A136

The self-transmissible plasmid pRK2013 was used to mobilize an *R. meliloti* Sym plasmid from a pool of *R. meliloti* JJ1C10- Tn5.A1 insertion derivatives to *A. tumefaciens* A136. A mating between *A. tumefaciens* A136 and a pool of Tn5.A1 Km^r insertion mutants of *R. meliloti* JJ1C10 resulted in the acquisition of Km resistance by *A. tumefaciens* A136. An analysis of the plasmid profiles of nine transconjugants revealed that not all transconjugants had received a plasmid of similar size. One class of transconjugant (represented by five transconjugants) had acquired a plasmid of a size similar to the Sym plasmid in *R. meliloti* JJ1C10. A second class of transconjugant (represented by three transconjugants) contained a plasmid, which I refer to as pCr (cryptic plasmid), which is smaller than any of the plasmids which have been characterized in either *R. meliloti* JJ1C10 or in *A. tumefaciens* A136 and thus its origin is unclear. All of the transconjugants contain in addition, a plasmid corresponding in size to the cryptic plasmid, pATC58. The nodulating ability of the transconjugants was tested. Only the first class of transconjugant formed ineffective nodules or clearly visible swellings on alfalfa roots. The frequency of inoculated plants which have these structures is given in Table 19. The other class, carrying pCr, elicited no response on alfalfa roots while *R. meliloti* JJ1C10 produced normal effective nodules on alfalfa.

Table 19 : Nodulating ability of *A. tumefaciens* A136 x *R. meliloti* JJ1C10 Transconjugants

Transconjugant	Class	Plasmids Present ¹	#Alfalfa Nodulated (of 11 inoculated)
PD91	2	pATC58, pCr ²	0
PD92	1	pATC58, pSym <i>R. meliloti</i>	5*
PD93	2	pATC58, pCr	0
PD94	1	pATC58, pSym <i>R. meliloti</i>	10*
Control inoculum			
<i>A. tumefaciens</i> A136		pATC58	0
<i>R. meliloti</i> JJ1C10		pSym <i>R. meliloti</i>	11

* ineffective nodules or swellings

¹ estimation based on approximate size of plasmids and on pseudonodulating ability

² plasmid of approximately 130 MD

2 OF/DE 2

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Attempts to transfer pSym B. meliloti JJ1C10m from A. tumefaciens PD92 to other A. tumefaciens strains or to an intermediate strain (B. leguminosarum B151Spr) were unsuccessful due to problems encountered selecting against the donor cells using Cb + Km or Sp + Km respectively. Therefore attempts to construct A. tumefaciens carrying the entire Sym plasmid of B. meliloti JJ1C10, in order to examine the effect on tumorigenicity, were unsuccessful to date.

4.5 Characteristics of A. tumefaciens carrying pRmSL26

The cosmid pRmSL26 carrying B. meliloti A1021 cloned nodulation genes and a gene conferring resistance to tetracycline, was mobilized from E. coli HB101 into A. tumefaciens using pRK2013 carried in E. coli. After the triparental mating, A. tumefaciens carrying pRmSL26 were selected on medium containing nalidixic acid and tetracycline. Although the frequency of transfer was very low (10^{-8} - 10^{-9} per donor) some Tet^r A. tumefaciens strains were identified and following agarose-gel electrophoresis were found to have acquired a plasmid corresponding in size to pRmSL26. These were tested for their ability to nodulate alfalfa. Twenty alfalfa plants were inoculated with each strain. One month after inoculation none of the strains carrying pRmSL26 were found to have induced root deformations. During this time B. meliloti strain JJ1C10 formed numerous nodules on 19/20 of the plants inoculated. However between 46 and 66 days after inoculation some root deformations were observed in plants inoculated with A. tumefaciens carrying pRmSL26 as indicated in Table 20.

Table 20. Alfalfa nodulating ability of A. tumefaciens carrying pRmSL26

Strain inoculated	# of alfalfa with root deformations
<u>E. coli</u> HB101 (pRmSL26)	6/20 plants have thick-short roots (tsr)
<u>A. tumefaciens</u> Sm240/pRmSL26	2/20 tsr
" Sm243/pRmSL26	4/20 tsr, 1/20 with 1 pseudonodule
" Sm 365/pRmSL26	4/20 with root swellings
" Sm 355/pRmSL26	2/20 tsr
" Sm 361/pRmSL26	2/20 with 1 pseudonodule
<u>A. tumefaciens</u> Sm A348/pRmSL26	2/20 with 1 pseudonodule
<u>B. meliloti</u> JJ1C10	19/20 nodulated effectively
<u>A. tumefaciens</u> A348	1/20 with root swellings
<u>A. tumefaciens</u> A136	0/20
<u>A. tumefaciens</u> 240,243,365,355 and 361	0/20 for each strain

Although carriage of pRmSL26 conferred the ability to produce root deformations, these occurred only after prolonged incubation as I have mentioned and appeared long after a noticeable senescence had occurred in these plants growing in nitrogen free medium. When swellings or pseudonodules were observed these were very tiny in comparison with the deformations of alfalfa roots observed with *A. tumefaciens* A136 carrying the entire Sym plasmid from *R. meliloti* JJ1C10. Unfortunately it is difficult to test the stability of pRmSL26 in the *A. tumefaciens* strains because it is expected that in the absence of tetracycline in the culture medium this cosmid will be easily lost (Long et al. 1982). Hirsch et al. (1984) originally described the ability of pRmSL26 to confer nodulating ability to an *A. tumefaciens* strain carrying a Ti plasmid; but they did not describe the tumor forming ability of this strain. I tested the tumor-forming ability of several *A. tumefaciens* recipients of pRmSL26 on carrot root slices. *A. tumefaciens* Sm 240/pRmSL26, Sm 243/pRmSL26, Sm 355/pRmSL26 and 361/pRmSL26 were avirulent on 10/10 carrot root slices inoculated, suggesting that in these strains, pRmSL26 did not restore the ability to transform carrot root tissue. Within 28 days of inoculation of carrot root slices with *A. tumefaciens* Sm 365/pRmSL26 a total of 7/10 positive responses were observed, compared with 8/10 positive responses to Sm 365, 0/10 to *E. coli* HB101/pRmSL26 and 10/10 to *A. tumefaciens* A348. *A. tumefaciens* A348 carrying pRmSL26 was still capable of tumor-formation on carrot slices; other plant hosts were not tested. This suggests that in *A. tumefaciens* A348 or in Sm 365 the presence of pRmSL26 does not eliminate the ability to induce tumors. Thus at least the portion of pRme1021 cloned in pRmSL26 does not eliminate tumor-forming ability. It is possible then that nodulation genes in *R. meliloti* may not be responsible for the lack of expression of tumor-inducing genes in *R. meliloti* strains reported by Hooykaas (1979). Further experiments will be necessary to determine if whole Sym plasmids of *R. meliloti* can interfere with tumorigenesis.

4.6 Expression of Pseudonodulating ability in wild-type and Chv B- A. tumefaciens strains

Matings between B. trifolii LPR5523 (carrying an B. leguminosarum pSym pRL1JI::Tn1831) and each of A. tumefaciens A1020 (Chv B-), A1011 (Chv B-) and A. tumefaciens A348 (w.t) were carried out in order to transfer B. leguminosarum nodulation determinants to A. tumefaciens. Transconjugants were selected in medium containing Km and Sp when A. tumefaciens Chv B- strains were the recipients and on Nal and Sp when A. tumefaciens A348 was the recipient. The transconjugants isolated were tested to ensure they were sensitive to 150 ug/mL Rif since the donor strain is Rif^r. Rif sensitive transconjugants were chosen for further examination. The nodulating ability of the transconjugants was tested on P. sativum L. (cv. Novella) and is described in Table 21.

Table 21: Pea Nodulating ability of A. tumefaciens Chv B- mutants and of A. tumefaciens A348 carrying pRL1JI::Tn1831

Strain inoculated	# of Plants ¹ with pseudonodules	# of Plants with ² other root deformations
A348	0	0
A348/pRL1JI	5	2
A1011	0	0
A1011/pRL1JI	1	0
A1020	0	0
A1020/pRL1JI	1	2

1 # of P. sativum L. (cv. Novella) inoculated per strain is 24

2 Plants with root swellings or marked root curling (not including those which are pseudonodulated)

In each A. tumefaciens transconjugant the ability to induce root deformations was acquired. In the A. tumefaciens A1020 transconjugant the two plants with minor root deformations had several root thickenings and root kinkings. However, even in the w.t. A. tumefaciens A348 recipient the pseudonodulating frequencies were low and therefore it is difficult to assess the effect of the Chv B- mutation in A. tumefaciens A1011 and A1020 transconjugants on the ability of pRL1JI::Tn1831 to confer pseudonodulating. Clearly though, some degree of pseudonodulation did occur even in the Chv B- strains carrying pRL1JI::Tn1831 suggesting that chv B is not absolutely essential for expression of pseudonodulating ability in A. tumefaciens. It should be noted however, that pseudonodule isolates from pseudonodules formed by the Chv

B- transconjugants were not tested to determine whether in these the Chv B- mutation was unaltered. However, since this work was initiated other work by Dylan et al. (1985) seems to have dealt more directly with the role of 'Chv B-like' genes involved in nodulation in the genetic background of Rhizobium spp., and this will be discussed in the following section.

FURTHER DISCUSSION AND CONCLUSIONS

This investigation of the relationships among the genes involved in nodulation and tumorigenesis proceeded along three lines: i) I investigated the extent to which Rhizobium Sym plasmids complement the function of the A. tumefaciens Ti plasmid virulence genes; ii) I examined whether the presence of Sym or Ti plasmids interferes with, respectively, tumorigenesis and nodulation in A. tumefaciens; iii) I investigated whether a Sym plasmid can confer pseudonodulating ability to A. tumefaciens chromosomal Chv B- mutants.

Since Agrobacterium and Rhizobium are closely related and both produce hyperplasias of plant tissues the possibility was considered that functionally analogous or homologous genes may be carried by both these types of organisms. Several workers have compared the DNA sequences of the Agrobacterium and Rhizobium genetic elements (ie. Sym and Ti plasmids) which play a major role in the induction of these hyperplasias. Huguet et al. (1983) found that there are similar DNA sequences encoded on some Ti plasmids and an R. meliloti Sym plasmid. Prakash and Schilperoort (1982) showed that certain Sym plasmids have DNA sequences similar to portions of pTiC58 and pTiACH5 DNA including the virulence (vir) region which is critical to tumor formation. An important question arising from this observation is do these Sym plasmids sequences code for functions similar to those of the A. tumefaciens vir region, and if so, what, if any, is their role in nodulation? If these Sym plasmid sequences code for functions similar to the vir region and if they do play a role in symbiosis then an eventual determination of that role might suggest a role for the vir genes in tumorigenesis. To determine if Sym plasmids do have genes similar in function to the Ti plasmid vir region I have introduced various Sym plasmids into avirulent or altered host range Vir- A. tumefaciens strains and then examined the tumor-forming ability of the 'hybrid' strains. Since pSym and pTi are probably maintained on separate replicons, functional complementation of the mutated virulence region by Sym plasmid genes requires that the complementing gene act in trans. Many vir genes, in fact are known to be capable of acting in trans with respect to each other (Iyer et al. 1982; Klee et al. 1983; Hille et al. 1984; Lundquist et al. 1984) and with respect to the T-DNA (Hoekema et al. 1983).

I found that the presence of pPD102, ptr5a or pRL5J1 did not restore the tumor-forming ability of the avirulent or of the altered host-range A. tumefaciens mutants which were tested. Therefore one could conclude that these Sym plasmids do not have genes with functions similar to these Ti plasmid vir genes and that structural homology between Sym plasmid sequences and Ti plasmid vir genes reflects the presence of sequences which are sufficiently different as to encode non-analogous or non-homologous functions. It would still be of interest to establish if these sequences play a role in nodulation.

Other factors may contribute to the lack of complementation by these Sym plasmids. First, hypothetical 'vir-gene like' regions in Rhizobium Sym plasmids may be subject to regulation such that they are not expressed under the conditions I have used to test tumorigenesis. There is some evidence that certain Rhizobium Sym plasmid genes involved in nodulation require legume host root-factors for induction (Fisher et al. 1985). On the other hand certain B. trifolii nodulation genes can cause root-hair curling in corn and rice plants, presumably in the absence of legume root factors. In either case the mode of regulation of all Sym plasmid encoded genes has not been established and also it cannot be assumed at this time that any Sym plasmid regions structurally similar to vir genes are also involved in nodulation or are induced by root-factors from the normal host legume.

A second reason for the lack of restoration of tumor-forming ability could be the loss of either pTi or pSym at the wound sites. If pSym is lost then of course complementation by pSym is not possible. It is however unlikely that pSym or pTi are lost at wound sites. The following observations suggest that both pTi and pSym stably co-exist in the constructed strains. Sym plasmid-containing derivatives of A. tumefaciens A348 are still capable of forming tumors on one or more plant hosts. Bacteria isolated from pseudonodules formed by such derivatives were also tumorigenic on one or more hosts even after passage through nodules. From pseudonodules formed by Sym plasmid-containing derivatives of mutant A. tumefaciens retain both Cb and Km resistance. Finally following growth on non-selective medium Cb and Km resistance is retained by these strains. Additional evidence for the stability of B. trifolii, B. leguminosarum, and B. phaseoli Sym plasmids with pTi::RP4 cb-integrates was also reported by Hooijkaas (1979).

A possible third factor contributing to the lack of restoration of tumor-forming ability may

be an overriding inhibitory effect of Sym plasmids on tumor-forming ability which masks any underlying complementary action. I have shown for example that the presence of pRL5Jl and to a lesser extent, ptr5a impairs the tumor-forming ability of the wild-type A. tumefaciens strain A348.

Until the possibilities mentioned above, are investigated further, I can only conclude that under the conditions used here the carriage of any of the Rhizobium Sym plasmids used does not restore tumor-forming ability, and therefore that there is no evidence to suggest genes functionally analogous to Vir are expressed by Sym plasmids in A. tumefaciens. In spite of the apparent lack of complementation two observations do suggest that the presence of Sym plasmids does affect the tumor-forming ability of A. tumefaciens in a different way.

First the B. phaseoli Sym plasmid pPD102 affected a change when introduced into A. tumefaciens Sm341 (Vir E-). Instead of being completely non-tumorigenic on leaf wounds of K. daigremontiana, the Vir E- mutant carrying pPD102 induced the formation of tiny, slow-growing proliferations at about 30% of the wound sites. The proliferations contained negligible octopine synthase activity in comparison with tumors induced by A. tumefaciens A348 or A. tumefaciens A348-carrying pPD102. Neither A. tumefaciens Sm341 nor B. leguminosarum PD102 alone, induced these proliferations but co-inoculation of A. tumefaciens Sm 341 and B. leguminosarum PD102 did result in the production of these proliferations on a low frequency of inoculation sites. This suggests that the effect of pPD102 is mediated via a diffusible factor, or one acting directly on plant cells. This effect was not seen on the other host plants on which A. tumefaciens Sm 341 is avirulent, ie. on tobacco or carrot. Also none of the other Vir- mutants (VirA-, Vir B-, Vir C-, or Vir D-) carrying pPD102 were altered in their tumor-forming ability. These non-octopine producing proliferations occur only with A. tumefaciens Sm341/pPD102, and their presence is to some extent host-dependent.

Non-opine producing proliferations (referred to as artificially-induced proliferations or Aip's) were observed by Joos et al. (1983a) when the nopaline-type A. tumefaciens strain C58 mutated in a 'vir B-like' gene or in a 'vir E-like' gene was inoculated on potato tuber slices incubated on medium containing the auxin NAA. These proliferations which,

according to Joos et al. (1983a) occurred in the absence of the stable integration of T-DNA but only when pTi was present, suggest that besides T-DNA tumor-maintenance genes the Ti-plasmid encodes one or more other functions that influence the growth properties of the infected cells. These artificially induced proliferations (Aip's) may reflect an early step in the formation of tumors but would of course be masked by true transformations if infection was with a wild-type *A. tumefaciens* strain. The interesting point here is, why does the presence of NAA induce these proliferations? As I mentioned in Section 2 the role of growth regulators in tumor-induction prior to T-DNA integration is unclear. Possibly the cellular proliferations (which have negligible octopine synthase activity) which I observed on *K. daigremontiana* leaves are Aip's produced as a consequence of the action of an auxin present because of pPD102. Certainly *Rhizobium* strains do produce plant growth regulatory substances, including auxins (Badenoch-Jones et al. 1982). Although it is not known to be so, if this production is coded for by Sym plasmid genes, it may be capable of mimicking the NAA-induced effect observed by Joos et al. (1983a) and thus may be responsible for the induction of the tiny tumors on *K. daigremontiana* by *A. tumefaciens* Sm341/pPD102.

I have mentioned that the proliferations of *K. daigremontiana* leaf tissue occurred not only upon inoculation of *A. tumefaciens* Sm341 carrying pPD102 but also upon co-inoculation on *A. tumefaciens* Sm341 (VirE-) and of *R. leguminosarum* carrying pPD102. This inter-cellular effect is probably not comparable to the inter-cellular complementation of Vir E- mutants by wild-type *vir* regions described by Otten et al. (1984) since in that case full virulence was restored and full complementation required the presence of an entire intact *vir* region. For these reasons I propose that the proliferation I observed probably do not correspond to true complementation but rather may be similar to those Aip's described by Joos et al. (1983).

In summary then I have found no firm evidence for complementation of mutated *vir* genes by either pSym ptr5a, pPD102, or pRL5J1. As a final speculative note on the possible role of the *vir* genes in tumorigenesis I should draw attention to several independent observations about crown-gall formation which hitherto have been largely neglected by

researchers. First, Liu et al (1982) reported that a mutation in the pTiC58 vir region caused a decrease in the bacterial production of IAA, and that such mutants could not form tumors. It was not clear whether these mutants were capable of T-DNA transfer, but since the intact T-DNA should produce disease symptoms if transferred to the plant cells, that such transfer did occur is unlikely. In light of the intense, ongoing research of the role of vir genes in T-DNA integration, further investigation of the observation of Liu et al. (1982) is certainly due; particularly regarding other types of Ti plasmids. One question, for example, might be: is the apparent involvement of the pTiC58 vir region in the regulation of IAA levels dependent upon the involvement of T-DNA encoded hormone producing genes? The deletion from a Ti plasmid of those T-DNA genes involved in cytokinin and auxin production in transformed plant cells, results in bacteria from which T-DNA transfer, but not visible tumor formation occurs. This implies that these genes are not required for T-DNA transfer. Furthermore, the presence of pSa in A. tumefaciens strains results in decreased levels of auxin production by the bacteria and also causes avirulence, although it is not clear whether T-DNA transfer can still occur. If T-DNA transfer does not occur this suggests that non-T-DNA encoded auxin production might be required for T-DNA transfer, a notion confirmed by the fact that, when IAA is supplied, tumor formation does occur. The role of the various vir or T-DNA genes in auxin production by the bacteria (not the transformed plant cells) would seem to merit considerably more attention than has been afforded to date.

My examination of both the tumor-forming and nodulating ability of the A. tumefaciens strains carrying Sym plasmids suggested that interference occurs between Ti and Sym plasmids. The reasons for this interference are unclear. In section 2 (Figures 2 and 3) I outlined the information currently available regarding the expression of i) Sym plasmid encoded nodulation genes in the presence of Ti plasmids and ii) of Ti plasmid tumor-inducing genes in the presence of Sym plasmids. The information is also relevant to an understanding of the relative contribution of chromosomal genes and Sym or Ti plasmid genes to both tumorigenesis and nodulation. For example, Hooykaas (1979) observed an attenuated tumor-forming ability (on petunia and tomato) and poor pea nodulation by an R. leguminosarum strain carrying a Ti plasmid, however, an R. trifolii strain carrying a Ti

plasmid nodulated clover normally, and formed "normal" sized tumors. This *B. trifolii* strain was also found to nodulate pea although as Hooykaas (1979) mentions this could have been due to a mutation in the *B. trifolii* strain and not to the presence of the Ti plasmid. It has been assumed in the past that the *Rhizobium* chromosome does not provide an altogether suitable environment for expression of Ti plasmid tumor-inducing genes, since as Hooykaas (1979) has shown, tumor-formation by *B. leguminosarum* /pTi is attenuated. I suggest, however, that this interpretation be re-considered. In this work I found that the introduction of an *B. leguminosarum* Sym plasmid pRL5JI into *A. tumefaciens* A348 resulted in attenuation of tumor-forming ability. Although pRL5JI is not the same as the *B. leguminosarum* Sym plasmids present in the strain used by Hooykaas (1979) the reduction of virulence he observed could be due to the presence of the *B. leguminosarum* sym plasmid and not necessarily to the heterologous chromosomal background of the *B. leguminosarum* strain which carried the Ti plasmid. Therefore the *Rhizobium* chromosome may be as suitable as that of *A. tumefaciens* for supporting the expression of Ti plasmid tumor-inducing ability. As Figure 3 indicates there is little known about the expression of Ti plasmid genes in sym plasmid-free *Rhizobium*. In such strains tumor-forming ability conferred by pTi might be the same as that conferred in *A. tumefaciens*.

Additional evidence suggesting that *Rhizobium* could support normal tumorigenesis comes from an observation of Dylan et al. (1985) that genes homologous in structure to the *A. tumefaciens* chromosomal virulence gene *chv B* occur in the chromosomes of several *Rhizobium* spp., including *B. meliloti*. Since Ti plasmid tumor-forming ability is apparently normal in *B. trifolii* and not so in *B. leguminosarum* (Hooykaas 1979), and since the recent re-classification of *Rhizobium* spp. (Jordan 1984) implies that *B. trifolii* and *B. leguminosarum* are essentially the same except for the presence of particular Sym plasmids, one could suppose that the presence of the *B. leguminosarum* Sym plasmid is responsible for attenuated virulence of *B. leguminosarum* strains carrying Ti plasmids.

I found pRL5JI to have an inhibitory effect on tumorigenesis in *A. tumefaciens*. Other *B. leguminosarum* Sym plasmids may have a similar effect. The inhibition of tumorigenesis may prove to be a general characteristic of *B. leguminosarum* Sym plasmids. The basis of the inhibitory effect of pRL5JI is not clear. It may exert an effect similar to that of pSa which

causes a reduction in the bacterially produced IAA levels when it is carried in A. tumefaciens. Alternatively it may alter the cell surface molecules in A. tumefaciens resulting in a decreased ability to bind to plant cells effectively. In fact the Sym plasmid may be capable of inducing any number of physiological responses in the plant cells which might render them less susceptible to infection by A. tumefaciens. These possibilities can be investigated further, and tobacco may be useful for the study of inhibition of A. tumefaciens/pTiA6NC tumorigenesis by pRL5J1.

In B. meliloti LPR2120 (containing its own Sym plasmid) the presence of a Ti plasmid does not result in the acquisition of tumor-forming ability (Hooykaas 1979). Therefore either the B. meliloti chromosome lacks factors necessary for tumor-forming ability, or either the chromosome or plasmids in B. meliloti directly inhibit tumor formation. Although B. meliloti Sym plasmids have been transferred to A. tumefaciens strains carrying Ti plasmids (Simon et al. 1983; Huguet et al. 1983) there is no data available as to the tumor-forming ability of these strains; so an inhibitory effect of B. meliloti Sym plasmids themselves cannot be ruled out. In this study I did not transfer an B. meliloti Sym plasmid to a Ti plasmid-containing A. tumefaciens strain; however, when pRmSL26 carrying cloned nodulation genes of pRme1021 was transferred to A. tumefaciens A348 or Sm 365, tumor-forming ability on carrot slices was retained. This suggests that at least this portion of an B. meliloti Sym plasmid does not eliminate tumor-forming ability.

I have shown that the presence of each of the Rhizobium Sym plasmids in A. tumefaciens conferred ineffective nodulation of the appropriate legume host. This observation only substantiates earlier observations (outlined in Figure 2) by other researchers. For the expression of an B. trifolii Sym plasmid in a Ti plasmid containing A. tumefaciens strain there was no data available, but I found that even when pTi was present clover pseudonodulation was conferred by an B. trifolii Sym plasmid. The different frequencies of pseudonodulation and root deformation by A. tumefaciens A348/ptr5a and A136/ptr5a, which I observed, suggests that the Ti plasmid inhibits pseudonodulating ability. In a practical sense this implies that Ti plasmid-free A. tumefaciens strains should be used for experiments in which cloned regions of Sym plasmids are transferred to A. tumefaciens to establish either a) the role of these genes in nodulation or b) the role of the Rhizobium

chromosome in nodulation, since obviously the Ti plasmid affects the degree of nodulation conferred at least by *ptr5a*. Since, as I have suggested in the my discussion of the results in section 4.3, the *vir* genes may be responsible for the Ti plasmid-associated inhibition of pseudonodulation, the basis of this interference is worth investigating. Supraoptimal levels of auxins are known to interfere with nodulation and since loss of Ti plasmids is correlated with decreased *A. tumefaciens* auxin production (Liu et al. 1982), it may be that high auxin levels contribute to the reduced clover pseudonodulating ability of *A. tumefaciens* A348/*ptr5a* compared with A136/*ptr5a*.

A third area I examined briefly in this work was the role of the *A. tumefaciens* chromosome in the expression of Sym plasmid encoded nodulation genes in *A. tumefaciens*. Two observations suggested that a gene similar to the *A. tumefaciens* chromosomal virulence genes might be present in *Rhizobium* spp. First, Douglas et al. (1982, 1985) showed that the *A. tumefaciens* chromosomal genes *chv A* and *chv B* are essential for tumorigenesis in *A. tumefaciens*. Second when Ti plasmids are transferred to *Rhizobium* spp. tumor-formation occurs, suggesting that some *Rhizobium* genes have substituted for the 'essential' *chv A* and *chv B*. Assuming there are such genes in *Rhizobium* they may also play a role in nodulation. My observations were as follows: *A. tumefaciens* Chv B- mutants carrying genes from *B. trifolii* pRL1JL::Tn1831 were capable of a certain degree of nodulation of *P. sativum* L., suggesting that the proper functioning of *chv B* is not required for at least some expression of nodulation genes. From the results of this work, however, it is difficult to assess just what effect these Chv B- mutations did have since pseudonodulation even in the wild-type *A. tumefaciens* A348 (ie. a derivative of A348 assumed to be carrying pRL1JL::Tn1831) also occurred at a very low frequency. I can only conclude, then, that some pseudonodulation can occur even in *A. tumefaciens* Chv B- mutants.

Since this work was completed, experiments by Dylan et al. (1985) seem to have dealt more directly with the role of *chv B*- like genes in nodulation. They report that mutations in an *B. meliloti* chromosomal gene structurally homologous to *A. tumefaciens chv B* leads to defective nodulation by the *B. meliloti* strain, showing conclusively that this gene is involved

in Rhizobium nodulation. The observations I have made are certainly not incompatible with this recent report. Mutations of the A. tumefaciens chv B gene may simply reduce the already defective nodulation conferred by the Sym plasmid in A. tumefaciens.

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