AGROBACTERIUM MEDIATED DNA TRANSFERUSE OF *A.TUMEFACIENS* AND *A. RHIZOGENES* FOR THE GENETIC TRANSFORMATION OF TREES IN THE FAMILY *CASUARINA*CEAE

C. FRANCHE D. BOGUSZ AND E. DUHOUX
BSFT (ORSTOM/CIRAD Foret), 45 bis avenue de la Belle Gabrielle, 94736Nogent sur Marne, France.

1-*AGROBACTERIUM* TRANSFORMATION PROCESS:

1-1 Tumour induction by Agrobacterium

Agrobacterium species are Gram-negative soil bacteria within the family *Rhizobiaceae*. First described by Smith and Townsend (1907), they are ubiquitously distributed within temperate climates. A wound site is necessary for infection to occur. Because they are soil bacteria, infection in nature usually occurs at the base, or crown, of the plant; hence, the name "crown gall" disease has been attributed to the tumors caused by the most extensively studied member of this group, *Agrobacterium tumefaciens*. Depending upon the strain of *Agrobacterium tumefaciens* used to induce tumors and to the species of plant infected, tumors appears either as amorphous masses of tissue emanating from the infection wound site (these tumors are termed "unorganized") or as partially organized tumors with stem and lifelike structures (these distorted tissues are called "teratomas")(Gelvin, 1984). A.*tumefaciens* can infect numerous families of dicots; only a very few monocotyledonous plants in the families *Liliaceae* and Amaryllidaceae have been reported to be weakly susceptible to crown gall induction (DeCleene and De Ley, 1976).

Strains of *Agrobacterium* rhizogenes induce hairy root disease onto a wide variety of dicotylodonous plants (Tepfer and Casse-Delbart, 1987).Large masses of roots emanate from the wound site which the bacteria have infected. Some strains of *A. rhizogenes* can produce unorganized tumors on a limited variety of plant species.

Two additional species of *Agrobacterium* have been described: A:rubi produces tumors on a number of cane raspberries as well as few other plants; *Agrobacterium radiobacter*, strains are avirulent.

Once initiated by *A. Tumefaciens* or *A. rhizogenes*, tumorous growth can continue in the absence of bacteria and tumour tissue can grow axenically in tissue culture in media lacking exogenous supplies of auxins and cytokinins, which are normally required to promote growth of plant tissues in vitro (Braun, 1958). This observation led to postulate that some substance,

initially called the "tumor inducing principle", was transferred from the bacterium to the plant during initial stages of tumorigenesis.

A second feature of the tumor tissues is that they synthetize and excrete novel amino acid and sugar derivatives known collectively as opines (Tempe and Goldman, 1982). One of the first such compounds that was characterized was octopine, a product formed by condensation of arginine with pyruvic acid. The type of opine synthesized in the tumour(nopaline, octopine, agrocinopine, mannopine and agropine) is dependent on the strain of *Agrobacterium* that initiated tumour formation (Bomhoff *et al.*, 1976). Thus *Agrobacterium* strains can be classified according to the typical opines present in tumours. The *Agrobacterium* responsible for tumor formation selectively catabolizes the opine whose biosynthesis it has induced, using it as a source of carbon and nitrogen.

1-2 Ti plasmids of Agrobacterium tumefaciens

In 1974, large plasmids with sizes ranging from 150 to 250 kilobases were discovered in virulent strains of *Agrobacterium tumefaciens* (Zaenen *et al.*, 1974). Further experiments in which plasmids have been cured from or transferred into different *Agrobacterium* strains demonstrated that tumorigenesis was associated with these large plasmids which were called tumor-inducing plasmids or Ti plasmids (Larebeke *et al.*, 1975). With the development of Southernblot hybridizations, it appeared that a specific segment of the Ti plasmid, the "transferred DNA" or T-DNA, was integrated into the genome of the transformed plant cell and was responsible for the tumorous phenotype (Chilton *et al.*, 1977).

Unlike transposable elements. the T-DNA is stable within the plant genome and does not encode the products that mediate its transfer. The T-DNA found in the plant cell is colinear with the T-DNA present in the Ti plasmid, indicating that no major rearrangement of the sequence takes place during the transformation process. One or more copies of the T-DNA can be integrated in the plant genome and the site of integration of the T-DNA into plant DNA is apparently random.

Regions homologous to the T-DNA are found on different Ti plasmids (Reviewed in Bevan and Chilton, 1982; Kahl and Schell, 1982; Walden,1989a; Hooykaas and Schilperoort, 1992). In common nopaline strains of *Tumefaciens*, the T-DNA region consists of a single fragment of around 24 kb (Figure 1B). In some octopine strains, the T-DNA is divided into two parts, TR and TL, which can be integrated separately into the plant DNA (Figure 1A). The TL DNA is a fragment of 14 kb and is present in all transformed cell lines; it is functionally equivalent to the T-DNA found in nopaline strains. The TR DNA (7 kb) is not always present in tumor tissue; when it is, it may not be contiguous with TLfragment and its copy number can differ.

The borders of the T-DNA in the plant genome are delimited by a nearly perfect direct repeat sequence of 25 base pairs which also flanks the T-DNA in both nopaline and octopine Ti plasmids (Yadav *et al.*, 1982)The consensus sequence of the T-DNA border is

GGCAGGATATTC/GA/GGT/GTCTAAA/TT/C. The right border repeat is required for the efficient transfer of DNA to the plant cell whereas the left border repeat is not. To the right of the right T-DNA border of the octopine Ti plasmids is a 24 bp sequence called "overdrive"; this sequence is required for optimal T-DNA transfer. The other genes encoded by the T-DNA are not required for the transfer of the T-DNA to the plant cell, nor its stable maintenance in the plant genome.

Within the plant cells, the T-DNA is transcribed to produce a number of polyadenylated mRNAs (reviewed in Hooykaas and Schilperoort,1992). The level of the T-DNA transcripts is relatively low compared with other plant mRNAs and the relative abundance of each differs. The organization of the T-DNA genes and their flanking regions are similar to those found in eucaryotic genomes, except that they do not contain introns. Genetic studies have shown that the T-DNA encodes enzymes responsible for the synthesis and secretion of opines by the plant cell as well as enzymes involved in the biosynthesis of hormones which play a major role in the establishment and maintenance of the tumor phenotype. A map of a nopaline and an octopine T region is shown in Figure 2. The genes for nopaline synthase (*nos*) or octopine synthase (*ocs*) reside near the right border sequence whereas the gene for agrocinopine synthase (*acs*) is almost in the middle of the nopaline T-DNA. The secretion of nopaline or octopine is encoded by the gene 6a. The *tmr* locus encodes an enzyme involved in the synthesis of cytokinin, and mutations result in root proliferation (rooty mutant). The *tms1* and *tms2* loci are involved with the unregulated synthesis of auxins, and mutations in either of these genes result in shoot proliferation (shooty mutants). The gene *6b* is involved in the control of the size of the tumor on certain host species because mutations in this gene result in the formation of large tumors. The genes that are responsible for the tumour phenotype are considered to be the oncogenes, and are often referred to *onc* genes.

DNA-DNA hybridizations and heteroduplex mapping of different Ti plasmids have led to the identification of three additional regions of homology besides the T-DNA (Figure 1)(reviewed in Hooykaas and Schilperoort, 1992; Citovsky *et al.*, 1992):

– the ORI region encodes replication functions and the origin of replication of the Ti plasmid.

– the CON region carries the functions for conjugative transfer in *Agrobacterium*; deletion of this region does not necessarily lead to avirulence indicating that the genes are not responsible for the transfer to the plant DNA.

– the virulence region or *vir* region is highly homologous among octopine and nopaline Ti plasmids. The vir region is 30 to 40 kb and organized into seven complementation groups, *virA*, *virB*, *virC*,*virD*,*virE*,*virG* and *virH*. The *virA* and *vir G* loci encode a positive regulatory system that directs *vir* gene expression. The products of the *virD* and *virC* loci are involved in the generation and processing of the T-DNA copy, and the products of the *virB* and *virE* loci are involved in forming most of the structural components that facilitate T-DNA movement. The *virH* locus is generally not essential for virulence and *virH* mutants show attenuated pathogenicity on specific dicotyledonous plant hosts. *VirA*, *B*, *G* and *D* are absolutely essential for tumour formation, whereas *VirC*, *E* and *F* are not.

1-3 Ri plasmids of Agrobacterium rhizogenes

Virulent strains of *A. rhizogenes* contain a large plasmid, the "root inducing" or Ri plasmid, which has a *vir* region homologous to the *vir* region of the Ti plasmid and also transfers T-DNA to the plant genome (Chilton *et al.*, 1982; Tepfer and Casse-Delbart, 1987). The agropinetype Ri plasmids transfer two separate T-DNA regions, TR and TL, to the plant genome whereas the mannopine and cucumopine Ri plasmids appear to have a single T-DNA region. Although basic studies are not as developed for *A. rhizogenes* than for *A. Tumefaciens*, the mechanism of transfer of the T-DNA from the Ri plasmid to the plant cell appears to be the same as that employed by the Ti plasmid.

1-4 Molecular biology of the transformation process

Transfer of DNA from *Agrobacterium* to the plant cell involves a cascade of events requiring the active participation and interaction of both the plant cell and the bacterium. Although considerable progress as been made in the knowledge of the initial steps of the transfer within the bacterium, many other steps, particularly those involved with the passage of the DNA into the plant cell and its integration into the nuclear DNA remain unknown. The mechanism of tumor induction can be divided into a number of steps (see Figure 3) which are described below and are reviewed in the following papers: Stachel and Zambrisky, 1986; Binns and Thomashow, 1988; Hooykaas and Schilperoort,1992; Zambryski, 1992.

Chemotaxis and attachment to wounded plant cells:

The interaction of *Agrobacterium* with the plant cells occurs at a wound site. Wounded plant tissues release several phenolic derivatives into the rhizosphere, the most potent being acetosyringone, which act at low concentrations as a chemical attractant to *Agrobacterium*. Several bacterial chromosomal loci are involved in the plant-bacterial interaction and mutations in these genes result in the bacteria being unable to attach to the plant cell wall. The *chvB* gene codes for a 235kDa protein involved in the formation of a cyclic β-1,2 glucan, while there is evidence that the *chvA* gene determines a transport protein located in the bacterial inner membrane necessary for the transport of the β-1,2 glucan into the periplasm. These *chv* genes are constitutively expressed in *Agrobacterium*.

Induction of the virulence genes

Except *virA* and *virG* which are constitutively expressed at significant levels in the bacteria, the other *vir* genes are not transcribed during normal vegetative growth of

Agrobacterium; they become induced by certain plant phenolic factors such as acetosyringone, o-hydroxyacetosyringone, or lignin-precursors including coniferyl alcohol and sinapinic acid. The *virA* product specifies an inner membrane protein that recognizes and responds to the presence of plant phenolic compounds. VirA transduces this information, most likely by a mechanism involving protein phosphorylation, to the product of *virG*. *VirG* then acts as a transcriptional activator of itself and the other *vir* loci. Analysis of the sequences upstream from the VirG-induced *vir* genes has shown that there are regions of conserved sequences at which the *virG* gene product binds.

Generation of T-DNA intermediates

Following induction of *vir* gene expression, molecular reactions occur on the T-DNA element of the Ti plasmid to generate a transferable T-DNA copy. The first events are the single-stranded endonucleolytic cleavages between the third and the fourth bases of the bottom stand of the 25-bp border repeats. These nicks are then used as initiation and termination sites for the displacement of a linear ss copy of the bottom strand of the T-DNA region, designated the T-strand. The T-strand is produced at about one copy per bacterium. Two *vir*-specific products, VirD1 (16 kDa) and VirD2 (47 kDa) have been shown to be essential for T-strand synthesis. VirD1 exhibit topoisomerase activity and may relax the Ti plasmid in the vicinity of the 25 bp border sequences. VirD2 is a site specific endonuclease that acts at the 25 bp repeats. Two other polypeptides, VirC1 (23 kDa) and virC2 (26 kDa) have been shown to enhance T-DNA border nicking in *Agrobacterium*

Formation of the T-strand protein complex

Following its formation, the T-strand must traverse the bacterial cell membrane, the bacterial cell wall, the plant cell wall, and the plant cell and nuclear membranes, and consequently avoid degradation by endonucleases. The T-strand likely exists as a DNA protein complex, the T-complex, which protects it and mediate its travel. To date, at least two proteins, VirE2 and VirD2 are candidate components of the T-complex. VirE2 (60.5 kDa) is a tenacious ssDNA binding protein (SSB) that can bind any ssDNA in a nonsequence specific fashion. VirE2 renders ssDNA completely resistant to 3' and 5' exonucleases, as well as to endonucleases. This protein is the most abundant protein produced in vir-induced *Agrobacterium* cells. The VirD2 protein has been shown to be tightly linked to the 5' end of the T-strand and may contribute to the polar transfer of the T-strand.

Transfer of the T-complex to the plant cell

The first step in T-strand transfer is the passage through the bacterial membrane. Sequencing of the octopine and nopaline *virB* locu shas shown that it contains a complex operon consisting of 11 genes. Most of the proteins predicted for the *virB* operon are located

in the agrobacterial membrane and could therefore form a structure through which the T-DNA could be delivered into the plant cell. The *virB11* gene has an ATPase activity and may be involved in delivering energy required for T-DNA is transferred to the plant cell is not known. T-DNA transfer is proposed to be analogous to bacterial conjugation where the plant cell is the recipient instead of a related bacterial cell.

Integration of the T-DNA within the plant nucleus

The next step in transformation process is the move of the T-complex into the plant-cell nucleus. The most obvious way for T-complex nuclear uptake would be *via* its associated VirD2 and VirE2 proteins. In animal cells, most proteins that function in the nucleus contain amino acid sequences that act as signals mediating specific uptake through nuclear pores. Similar sequences have been found both inVirE2 and VirD2 suggesting an active role of these proteins in the plant cell.

The last event is the integration of the T-DNA copy into plant-cell DNA. Single T-DNA copies are frequent, but on average three copies of the T-DNA are found incorporated into the genomes of various dicotyledonous plant species; occasionally, 20-50 copies have been observed. The mechanism by which the integration of the T-DNA takes place is unknown and at present the only way to investigate this is by analyzing the T-DNA in the transformed plant cell and the plant target DNA. In general, T-DNA insertions can occur into any chromosome, and transcriptionally active regions are preferred. DNA regions undergoing transcription are presumably more accessible to invading exogenous DNA. T-DNA integration does not usually cause large rearrangements of plant DNA; however, most insertions induce small deletions, up to 79 nucleotides at the target sites. Comparison of different T-DNA insertions also show no significant sequence requirement at the plant target sites. Nevertheless, short stretches of homology (5-10 bases)between the T-DNA ends and the plant target sites may play a role in integration.

Once integrated into the plant genome, the enzymes encoded by the T-DNA start to be expressed. These enzymes synthetize auxin cytolinin which disrupt the hormonal balance of the cell and initiate disorganized growth and the synthesis and secretion of opines. Methylation of the DNA in transformed tissue can seriously effect its expression, and although the T-DNA is stably integrated, it might not always be expressed.

2.–*AGROBACTERIUM* AS A TOOL FOR PLANT TRANSFORMATION

2-1 Agrobacterium plasmid vectors

A plant vector can be defined as a system for the transfer of genetic information into a plant. Several features are necessary for any plant vector system (Gardner and Houck, 1984; Gruber and Crosby,1993): it must be capable of transferring foreign DNA into a plant cell and maintaining the transferred DNA through the cellular replication and division process, and the introduced DNA must be correctly expressed in the cell in order to affect the cell's phenotype. The natural ability of *Agrobacterium* to transfer sequences of DNA into the plant genome has been exploited in the development of a variety of plant transformation vectors (For reviews, see Bevan, 1984; Armitage *et a.*, 1988; Draper *et al.*, 1988b; Walden, 1989b).

2-2-1 Plant transformation vectors based on the Ti plasmid

The vectors based on the Te plasmid do not contain any oncogenic sequences and hence normal plant growth can be obtained following the transfer of DNA into the nucleus of the plant cell. Non-oncogenic vectors that are currently used can be divided into two types, *cis* or *trans*, depending on whether the T-DNA regions, flanked by the 25 bp direct repeat sequences, are carried on the same replicon as the *vir* genes or on a separate plasmid. The *cis* vectors are often referred to as cointegrative vectors, whist the *trans* vectors are commonly called binary vectors.

Cis vectors: there is a homologous region between the Ti plasmid and a vector plasmid containing the genes to be transferred (Figure 4A).This homologous region allows the vector to cointegrate into the Ti plasmid. Usually, the *onc* genes on the Ti plasmid are removed and replaced with a pBR322 sequence. Any plasmid which contains pBR322 sequences can be cointegrated into the disarmed Ti plasmid. The cointegration places the new sequences within the T-DNA borders, and therefore subject to transfer to the plant cell.

Trans vectors: they are based on plasmids that can replicate in both E. coli and *Agrobacterium* and which contain the T-DNA border sequences (Figure 4B). These can be designed so that the border sequences flank multiple cloning sites which allow insertion of foreign DNA, and markers that allow direct selection of transformed plant cells. The plasmids can be manipulated in *E. coli* and transferred *via* conjugation to *Agrobacterium* strains which contain a Ti plasmid which bears a *vir* region, but lacks T-DNA and 25 bp repeat sequences. Transfer of the foreign DNA on the cloning vector to the plant cell is mediated by the activity of the vir region functioning in trans.

2-2-2 Plant transformation vectors utilizing A. *rhizogènes*

Disarmed vectors derived from *A Tumefaciens* may be introduced into *A. rhizogenes* strains and will replicate stably, as long as selection is maintained. Subsequent infection of the plant by *A. rhizogenes* carrying a binary vector causes transfer of the T-DNA of the binary vector, in addition to the T-DNA of *A. rhizogenes*, into the plant genome (Simpson *et al.*, 1986). The genes to be transferred to the plant are located between the binary vector T-DNA border sequences which operate in trans after induction of the *vir* region of the resident wild-

type Ri plasmid. This is possible due to the large degree of homology, at the DNA level, between the virulence regions of *A. tumefaciens* and *A.rhizogenes*.

2-2 Transformation techniques based on Agrobacterium

There are a variety of methods that can be used to produce transgenic plants using Ti of Ri plasmid-derived vectors (Reviewed in Draper *et al.*, 1988; Weising *et al.*, 1988).

Co-cultivation of disarmed *A. tumefaciens* strains with plant protoplasts

This method involves isolating protoplasts from sterile leaf tissue and incubating them in culture medium for 2-3 days so that, although they do not divide, they begin to initiate cell wall formation. The cells are then incubated with a fresh culture of *Agrobacterium* for 1-2days. Following co-cultivation, the protoplasts are collected and cultured in the presence of antibiotics to remove any remaining bacteria. The protoplasts can be cultured to produce callus which can in turn be induced to form shoots (Wullens *et al.*, 1981). If the DNA contains a selectable marker, transformed tissue can be selected at the callus stage. The advantage of this technique is that a large number of transformants can be obtained. The major drawback is that very few economically important plant species can be regenerated from protoplasts-derived tissues.

Explant inoculation by disarmed strains of *A. tumefaciens*

Explant inoculation is possibly the most convenient way of producing transgenic material but depends on two factors: first, the wounded cells must be able to interact with *Agrobacterium* and second, the cells that have been transformed must be able to divide and regenerate into plants. A wide variety of tissue explants can be used in this sort of experiment: leaves, stems, hypocotyls, roots and tubers(Horsch *et al*, 1985). The method involves cutting sterile explants from plants and incubating them for 1-3 days with a fresh cutlture of *A.tumefaciens* on agar plates. The explants are then transferred to a media containing antibiotics to eliminate *Agrobacterium* and select transformed cells, and growth regulators to induce callus formation and shoot regeneration.

Inoculation of germinating seeds with disarmed *A.tumefaciens* strains

This procedure has been developed for *Arabidopsis thaliana* (Feldmann and Marks, 1987). The seeds are imbibed for 12 h and then incubated with a fresh culture of *Agrobacterium* containing a disarmed plasmid vector with a kanamycin resistance gene. Transformants are selected in the progeny of Fo plants. The advantage of this method is that it does not require tissue culture techniques, but it is still limited to *Arabidopsis* and the efficiency of transformation is low.

Transformation using wild-type *Agrobacterium tumefaciens* strains

This method is based on the co-inoculation of the plant with an *Agrobacterium* suspension containing two strains of *A. tumefaciens* (Brasileiro *et al.*, 1991): an oncogenic strain which has the property to induce tumors which spontaneously develop normal shoots, and a disarmed strain that provides the suitable marker genes in a *trans* vector. Transformed shoots exhibiting a normal morphology are selected for antibiotic resistance. This method which has been developed for the transformation of poplar has the following advantages: it does not require a regeneration procedure (the optimal hormone balance required for shoot regeneration is supplied by the oncogenic strain) and transgenic shoots can be recovered two months after plant inoculation by the *Agrobacterium* suspension.

Induction of hairy roots by *A. rhizogenes*

Hairy roots can be induced on a variety of plants simply by the inoculation of wounded stem tissue (Tepfer, 1984; Tepfer and Casse-Delbart, 1987). Generally, seedlings are grown aseptically and a freshculture of *A. rhizogenes* is applied to cut stem sections. Within 2-3 weeks, hairy roots proliferate at the site of inoculation and these can be excised and cultured further on media which promote the regeneration of whole plants. It should be noted that these plants often exhibit an aberrant phenotype which is the result of the oncogenic T-DNA insert: wrinkled leaves and reduced apical dominance. Segregation of the oncogenic T-DNA of a disarmed binary vector is usually achieved in the F1 generation and allows the recovery of fully non-oncogenic transformed plants.

2-3 Transgenic plants obtained via Agrobacterium mediated transformation

The first transgenic plant has been reported in 1983 (Fraley *et al*, 1983, Herrera-Estrella *et al*, 1983) and during the past ten years, we have witnessed a rapid progress in plant genetic engineering. Stable integration and expression of foreign genes has been obtained in many dicotyledonous and few monocotyledonous plants (Fraley *et al.*, 1986; Klee *et al.*, 1987; Weising *et al.*, 1988). A list of transgenic plants obtained after transformation with *A. tumefaciens* and *A. rhizogenes* is provided in Table 1.

It should be noted that definitive proof of stable transformation of a plant includes several tests (Potrykus, 1991). First it is important to assess whether the foreign DNA is expressed in the putative transformed tissue; this involves enzyme assays of the genetic markers which have been transferred. The organization of the foreign DNA in the plant genome should be studied by Southern blot analysis; evidence for the absence of any contaminating sequences has to be demonstrated. In cases of seed-propagated crops, analysis of the transmission of the introduced genes should also be performed in the progeny.

2-4 Limitations of Agrobacterium transformation

Although the introduction of new traits into plants *via Agrobacterium* is now a common practice, there are still important limitations to the system (Potrykus, 1990; 1991; Christou, 1993).

The first is that it seems sometimes difficult to transform the cells which are able to regenerate. It might be that these cells are in layers too deep to be reached by *Agrobacterium*, or simply these are not targets for T-DNA transfer. Another difficulty, when using disarmed strains of *A. Tumefaciens*, is that regeneration from tissue culture is a prerequisite, and somoclonal variation resulting from tissue-culture-induced mutations needs to be considered in any *Agrobacterium*-based transformation system. The long time required to regenerate transformed cells to plant is another disadvantage of the method.

The host range of *Agrobacterium* is another major limitation. Wide host range strains of *Agrobacterium* infect most dicotyledonous plants, but do not cause galls on monocots. This is a serious limitation since many important crop plants are monocots. It is suggested that the plants which are recalcitrant to *Agrobacterium* do not have the proper "wound response"; wounding results in the accumulation of phenols and leads to the death of the wound-adjacent cells. Therefore, even though *Agrobacterium* can transfer its T-DNA into cereal cells, integration of this T-DNA can not lead to transgenic clones because the receptor cells die. Problems arising from the limited host range of *Agrobacterium* have led to the development of several alternative strategies including direct gene transfer to plant cells (electroporation, high velocity microprojectiles, ...)(Reviewed in Potrykus, 1991).

3-TRANSFORMATION OF *CASUARINA* TREES USING *AGROBACTERIUM RHIZOGENES* AND *AGROBACTERIUM TUMEFACIENS*

3-1 Introduction

Gene transfer into trees provides a means of genetic analysis that can bypass sexual barriers and, to some extent, circumvent the limitations of the long breeding cycles of trees. In woody plants, considerable effort has been made to establish genetic transformation systems with many species, but the number of successful transformations has been limited to few species (Reviewed in Hanover and Keathley, 1988;Schuerman and Dandekar, 1991).

Our laboratory is currently developing genetic transformation systems for *Casuarina* trees. The *Casuarinaceae* family includes about 90 species of shrubs and trees which are primarily native to the southern hemisphere, mostly to Australia. Due to their symbiotic association with the nitrogen-fixing actinomycete Frankia, *Casuarina* trees are pioneer species, able to colonize severely disturbed sites, and are thought to contribute to the rehabilitation in these sites by stabilizing the soil and building its nitrogen content (National Research Council, 1984; Diem and Dommergues, 1990). Genetic transformation of *Casuarina* is a valuable technology to

accelerate tree improvement programs and to study the expression of plant symbiotic genes in transgenic plants.

We present the work concerning the transformation of two *Casuarina* trees, *Allocasuarina verticillata* and *Casuarina glauca*, using either *A.rhizogenes* or A *tumefaciens*.

3-2 Use of A. rhizogenes for the transformation of Allocasuarina verticillata

Three strains of *A. rhizogenes* were used in preliminary experiments of inoculation of *A. verticillata*: an agropine strain, A4 (Moore *et al*, 1979), a cucumopine strain, 2659 (Davioud *et al.*, 1988), and a mannopine strain, 8196 (Koplow *et al.*, 1984). Two month-old aseptic seedlings of *A. verticillata* were inoculated with these strains by wounding the hypocotyls with a needle dipped in the agrobacterial culture. 7 to 10 days after inoculation, about 50% of the inoculated hypocotyls developed roots showing a typical hairy root phenotype (high growth rate, extensive lateral branching, and lack of geotropism).Presence of opines was detected in the root extracts.

The roots transformed by the strain 2659 were then excised and grown on nutritive hormone-free medium. Shoot regeneration occurred spontaneously on 90% of the roots and transgenic rooted plants were obtained within five months following plant inoculation (Phelep *et al.*, 1991). The transformation was demonstrated by Southern blot analysis. Transgenic *Allocasuarina* plants have retained the ability to benodulated by Frankia and they still fix nitrogen. Nevertheless, they exhibit an alteration of their phenotype which is characterized by an extensive lateral branching and a lack of geotropism due to the expression of the *vol* genes of *A. rhizogenes*.

3-3 Use of Agrobacterium tumefaciens

Transfer of foreign genes in *Allocasuarina verticillata* and in *Casuarina glauca* using the disarmed strain C58C1(BIN19-GUSINT)(Vancanneyt *et al.*, 1990) was studied. The plasmid vector carries the *nptII* gene conferring resistance to kanamycin and a derivative of the β-glucuronidase reporter gene expressed only upon transfer to the plant cells and not in *Agrobacterium*. Explants excised from one to two month old seedlings of *A. verticillata* and *C. glauca* were co-cultivated with a fresh culture of *A. tumefaciens*, and transferred onto callus induction medium containg antibiotics. Calli emerging from the wound edges of the explants were observed three weeks after transformation. 80 to 90% of the kanamycin resistant calli expressed the reporter geneat different levels. PCR analysis demonstrated the presence of the β-glucuronidase gene.

Several parameters influencing the transformation efficiency were studied with *C. glauca*: use of acetosyringone duing the co-cultivation, time of co-cultivation, pretreatment of the

explants with high velocity microprojectiles, age of the explants, and pH of co-cultivation Optimal transformation was achieved with explants which were excised from one month old seedlings and co-cultivated for three days with *A tumefaciens* in presence of 25 µM of acetosyringone, at pH 5.6.

Transgenic plants of *A. verticillata* have been obtained, whereas a range of hormone conditions are currently investigated to obtain regeneration of shoots from transgenic calli of *C. glauca*

4. CONCLUSION

Over the past years, remarkable progress has been made in delopingan understanding of the *Agrobacterium* infection and transformation process. This knowledge has already provided several clues for improving gene transfer by *Agrobacterium*, specially to dicotylodonous plants. As more details of the biology of T-DNA transfer to plant cells are revealed, new opportunities for manipulating the host-range may be realized. In the meantime, host/vector systems which optimize the expression of essential factors required for T-DNA transfer and integration to plant chromosomes may contribute further to the optimization and the facility of this natural system.

REFERENCES

Armitage, P., Walden, R. and Draper, J (1988) Vectors for the transformation of plant cells using *Agrobacterium*. In: Plant genetic transformation and gene expression, a laboratory manual, Armitage, P.,Walden, R. and Draper, J (eds), pp. 3-67, Blackwell Scientific Publications.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. Nucl. Acids Res., 12, 8711-8721.

Bevan, M.W. and Chilton, M.D. (1982) T-DNA of the *Agrobacterium* Ti and Ri plasmids. Ann. Rev. Genet., 16, 357-384.

Binns, A.N. and Thomashow, M.F. (1988) Cell biology of *Agrobacterium* infection and transformation of plants. Ann. Rev. Microbiol., 42, 575-606.

Bomhoff, G., Klapwijk, P.M., Kester, H.C.M., Schilperoort, R.A.,Hernalsteens, J. P., and Schell, J. (1976) Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of *A.tumefaciens*. Mol. Gen. Genet., 145, 177-181.

Brasileiro, A.C.M., Leple, J.C., Muzzin, J.,Ounnoughi, D, Michel, M.F.,Jouanin, L. (1991) An alternative approach for gene transfer in tree using wild-type *Agrobacterium* strains. Plant Mol. Biol., 17, 441-452.

Braun, A.C. (1958) A physiological basis for autonomous growth of crowngall tumor cells. Proc. Natl. Acad. Sci. USA, 44, 344-349.

Chilton, M.D., Drummond, M.H., Merlo, D.J., Sciaky, D., Montoya, A.L.,Gordon, M.P. and Nester, E.W. (1977) Stable incorporation of plasmidDNA into higher plant cells: the molecular basis of crown galltumori genesis. Cell, 11, 263-271.

Chilton, M.D., Tepfer, D.A., Petit, D.C., Casse-Delbart, F.and Tempe, J.(1982) *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant cells. Nature, 295, 432-434.

Christou, P. (1993). Philosophy and practice of variety-independent gene transfer into recalcitrant crops. In Vitro Cell. Dev. Biol., 29P,119-124.

Citovsky, V., Mc Lean, B.G., Greene, E., Howard, E. and Kuldau, G. (1992) *Agrobacterium*-plant cell interaction: induction of *vir* genes and T-DNA transfer. CRC Rev., pp. 169-199.

Davioud, E., Petit, A., Tate, M.E., Ryder, M.H., Tempe, J. (1988)Cucumopine-a new T-DNA encoded opine in hairy root and crown gall. Phytochemistry, 27, 2429-2433.

De Cleene, M. and De Ley, J. (1976) The host range of crown gall. Bot.Rev., 42, 389-466.

Diem, H.G., Dommergues, Y.R. (1990) Current and potential uses and management of *Casuarina*ceae in the tropecs and subtropics. In "The Biology of *Frankia* and Actinorhizal Plants", Schwintzer, C.R., Tjepkema, J.D. (eds), New York, Academic Press, pp 317-342.

Draper, J., Scott, R. and Hamil, J. (1988) Transformation of dicotyledonous plant cells using the Ti plasmid of *Agrobacterium* tumefaciens and the Ri plasmid of *A. rhizogenes*. In: Plant genetic transformation and gene expression, a laboratory manual, Armitage, P.,Walden, R. and Draper, J (eds), pp. 69-160, Blackwell Scientific Publications.

Feldmann, K.A. and Marks, M.D. (1987) *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non-tissue culture approach. Mol. Gen. Genet., 208, 1-9.

Fraley, R.T. Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P.,Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R.,Goldberg, S.B., Hoffman, N.L. and Woo, S.C. (1983) Expression of bacterial genes in plant cells. Proc. Nat. Acad. Sci. USA, 80,4803-4807.

Fraley, R.T., Rogers, S.G. and Horsch, R.B. (1986) Genetic transformation in higher plants. CRC Critical Rev. Plant Sci., 4, 1-46.

Gardner, R. and Houck, C. M. (1984) Development of plant vectors. In Plant-Microbe interactions, Molecular and genetic perspectives, voll, Kosuge, T. and Nester, E. (eds), pp. 146-167, Mcmillan PublishingCompany.

Gelvin, S.B. (1984) Plant tumorigenesis. In Plant-Microbe interactions, Molecular and genetic perspectives, voll, Kosuge, T. and Nester, E.(eds),pp. 343-337, Mcmillan Publishing Company.

Gruber, M. Y. and Crosby, W. L. (1993) Vectors for plant transformation. In: Methods in plant molecular biology and biotechnology, Glick, B.R. and Thompson, J.E. (eds), CRC Press.

Hanover, J. W., Keathley, D. E. (1988) The genetic manipulation of woody plants, vol 44. In: Basic Life Sciences, Plenum, New York, 519p.

Herrera-Estrella, L., Depicker, A., Van Montagu, M. and Schell, J. (1983) Expression of chimeric genes into plants using a Ti-plasmid derived vector. Nature, 303, 209-213.

Hooykaas, P.J.J. and Schilperoort, R.A. (1992) *Agrobacterium* and plant genetic engineering. Plant Mol. Biol., 19, 15-38.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.Y. (1985) A simple and general method for transferring genes into plants. Science, 227, 1229-1231.

Kahl, G. and Schell, J. (1982) Molecular biology of plant tumors. New-York, Academic Press.

Klee, H., Horsch, R. and Rogers, S. (1987) *Agrobacterium*-mediated plant transformation and its further applications to plant biology. Annu. Rev. Plant Physiol., 38, 467-486.

Koplow, J., Byrne, M.C., Jen, G., Tempe, J., Chilton, M.D. (1984)Physical map of the *Agrobacterium rhizogenes* strain 8196 virulence plasmid. Plasmid, 11, 130-140.

Larebeke, N.,Genetello, C.,Schell, J., Schilperoort, R.A. Hermans, A.K.,Hunalsteens, J.P. and Van Montagu, M. (1975) Acquisition of tumor-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. Nature, 255, 742-743.

Moore, L.W., Warren, G., Strobel, F. (1979) Involvement of a plasmid in the hairy root disease of plants caused by "A*grobacterium* rhizogenes. Plasmid, 2, 617-626.

National Research Council (1984) *Casuarinas*: Nitrogen Fixing Trees for Adverse Sites. National Academy Press, Washington DC.

Phelep, M., Petit, A., Martin, L., Duhoux, E., Tempe, J. (1991)Transformation and regeneration of anitrogen-fixing tree, Allo*casuarina*verticillata Lam. Bio/Technol., 9, 461-466.

Potrykus, I (1990) Gene transfer to cereals: an assessment. Bio/Technology, 535-542.

Potrykus, I. (1991) Gene transfer to plants: an assessment of published approaches and results. Ann. Rev. Plant Physiol. Plant Mol. Biol., 42,205-225.

Schuerman, P.L. and Dandekar, A.M. (1991) In: Subcellular Biochemistry, Plant Genetic Engineering, Vol 17, Biswas B.B. and Harris J.R. (eds),Plenum Press, pp 81-105.

Simpson, R.B., Spielmann, A., Margossian, L. and Mc Knight, T.D. (1986)A disarmed binary vector from *Agrobacterium tumefaciens* functions in *Agrobacterium rhizogenes*. Frequent cotransformation of 2 distinct T-DNA.Plant Mol. Biol., 6, 403-415.

Smith, E.F. and Townsend, C.D. (1907) A plant tumor of bacterial origin. Science, 25, 671-673.

Stachel, S.E. and Zambrisky, P.C. (1986) *Agrobacterium tumefaciens* and the susceptible cells: a novel adaptation of extra cellular recognition and DNA conjugation. Cell, 46, 325-333.

Tempe, J. and Goldmann, A. (1982) Occurence and biosynthesis of opines. In Molecular biology of plant tumors, Kahl, G. and Schell, J.(eds), pp.427-449. Academic Press, New York.

Tepfer, D. (1984) Transformation of several species of higher plants by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. Cell, 37, 959- 967.

Tepfer, D. and Casse-Delbart, F. (1987) *Agrobacterium rhizogenes* as a vector for transforming higher plants. Microbiol. Sci., 4, 24-28.

Vancanneyt, G., Schmidt, R., O'Conner-Sanchez, A., Willmitzer, L.,Rocha-Sosa, M. (1990) Construction of an intron containing marker gene-splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium* mediated plant transformation. Mol. Gen. Genet., 220, 245-250.

Walden, R. (1989a) The biology of tumor formation by *Agrobacterium*. In: Genetic transformation in plants, Walden, R. (ed), pp. 25-48, Prentice Hall.

Walden, R. (1989b) Vectors based on the Ti and Ri plasmids of *Agrobacterium*. In Genetic transformation in plants, Walden, R.(ed), pp.25-48, Prentice Hall.

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

Wullems, G.J., Molendijk, L., Ooms, G., Schilperoort, R.A. (1981)Differential expression of crown gall tumor markers in transformants obtained after *in vitro Agrobacterium tumefaciens*-induced transformation of cell wall regenerating protoplasts derived from *Nicotiana tabacum*. Proc. Natl. Acad. Sci. USA, 78, 4344-4348.

Yadav, N.S., Vanderleyden, J., Bennett, D.R., Barnes, W.M. and Chilton,M.D. (1982) Short direct repeats flank the T-DNA on a nopaline Tiplasmid. Proc. Natl. Acad. Sci. USA, 79, 6322-6326.

Zaenen, I., Van Larebeke, N., Teuchy, H., Van Montagu, M., Schell, J. (1974) Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. J. Mol. Biol., 86, 109-127.

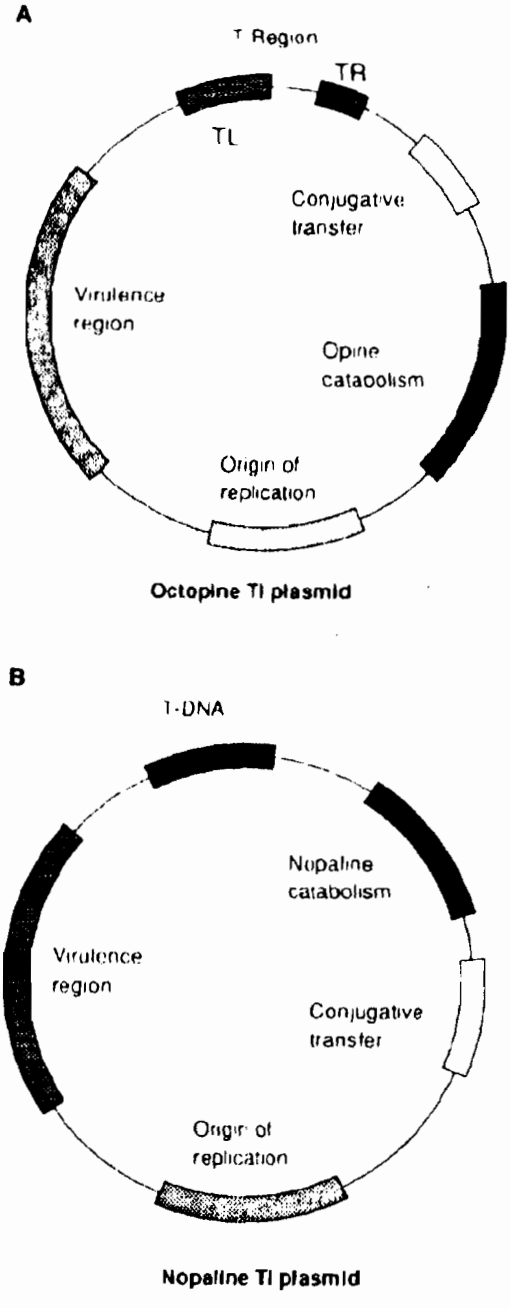
Zambryski, P.C.(1992) Chronicles from the *Agrobacterium*-plant Cell DNA transfer story. Annu. Rev. Plant Physiol. Plant Mol. Biol., 447,465-490.

Table 1: Transgenic plants obtained via *Agrobacterium*-mediated transformation

DICOTS	METHOD
Solanaceae	
Lycoperscon esculentum	*A. tumefaciens & rhizogenes*
Lycopersicon peruvianu	*A.rhizogenes*
Nicotiana cleverlandii	*A. tumefaciens*
Nicotiana edwarsonii	*A. rhizogenes*
Nicotiana glauca	*A. tumefaciens*
Nicotiana tibacum	*A. tumefaciens*
Petunia hybrida	*A. tumefaciens*
Solanum melongena	*A. tumefaciens*
Solanum nigrum	*A. rhizogens*
Solanum tuberosum	*A. tumefaciens*
Leguminosae	
Glycine canescens	*A. rhizogenes*
Glycine max	*A. tumefaciens*
Lotus comiculatus	*A. rhizogenes*
Medicago sativa	*A. tumefaciens*
Medicago varia	*A. tumefaciens*
Pisum sativaum	*A. tumefaciens*
Stylosanthes spp	*A. tumefaciens*
Trifolium repens	*A. tumefaciens*
Cruciferae	
Arabidopsis thaliana	*A. tumefaciens*
Brassica juncea	*A. tumefaciens*
Brassica napus	*A. tumefaciens & A. rhizogenes*
Brassica oleracea	*A. rhizogenes*
Linum usitatissimum	*A. tumefaciens*
Other dicos	
Apium graveolens	*A. tumefaciens*
Armracia olapathifolia	*A. rhizogene*
Atropa belladona	*A. tumefaciens*
Beta vulgaris	*A. tumefaciens*
Catharanthus	*A. rhizogens*
Convolvulus	*A. rhizogens*
Cucumis melo	*A. tumefaciens*
Cucumus sativas	*A. rhizogenes & A. tumefaciens*
Daucus carota	*A. rhizogenes*
Fragaria×ananassa	*A. tumefaciens*
Gossypium hirsutum	*A. tumefaciens*
Helianthus annuus	*A. tumefaciens*
Kaoanchoe laciniata	*A. tumefaciens*
Lactuca sativa	*A. tumefaciens*

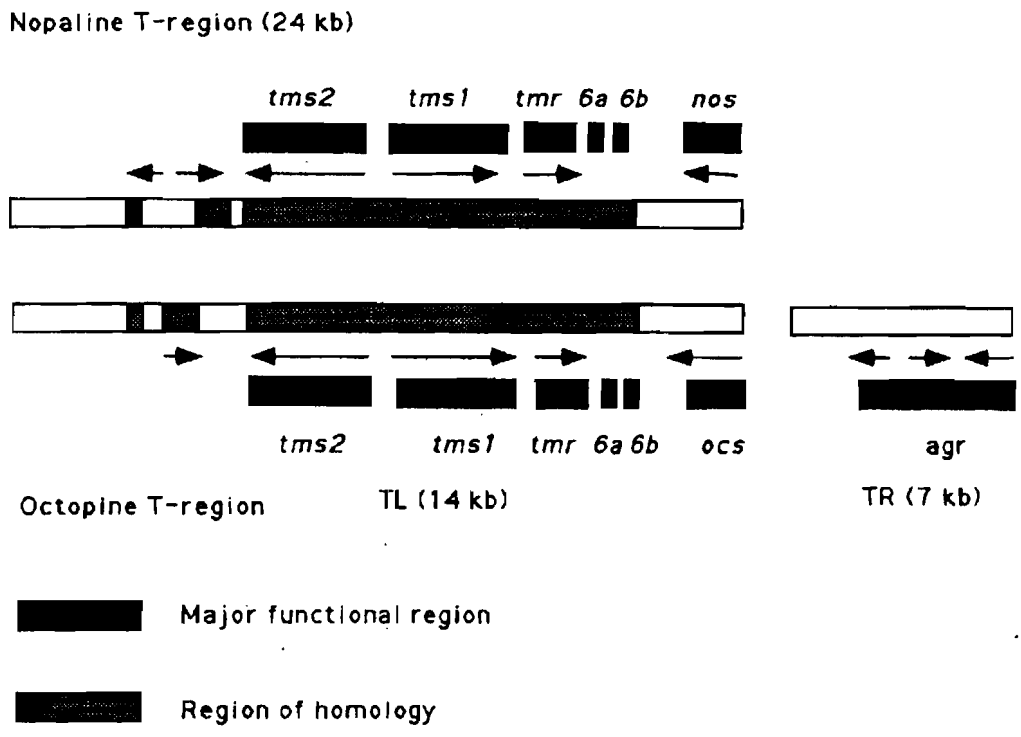
Ribes nigrum	*A. tumefaciens*
MONOCOTS	
Asparagus officinalis	*A. tumefaciens*
TREES	
Allocasuarina veerticillata	*A. rhizogenes*
Agadirachta indica	*A. tumefaciens*
Juglans regia	*A. tumefaciens*
Malus pumila	*A. tumefaciens*
Popuous spp	*A. tumefaciens*
Vitis rupestris	*A. tumefaciens*
Liquidambar styraciflua	*A. tumefaciens*
Robinia pseudoacacia	*A. rhizogenes*

Figure 1 Genetic map of an octopine (A) and a nopaline (B) Ti Plasmid



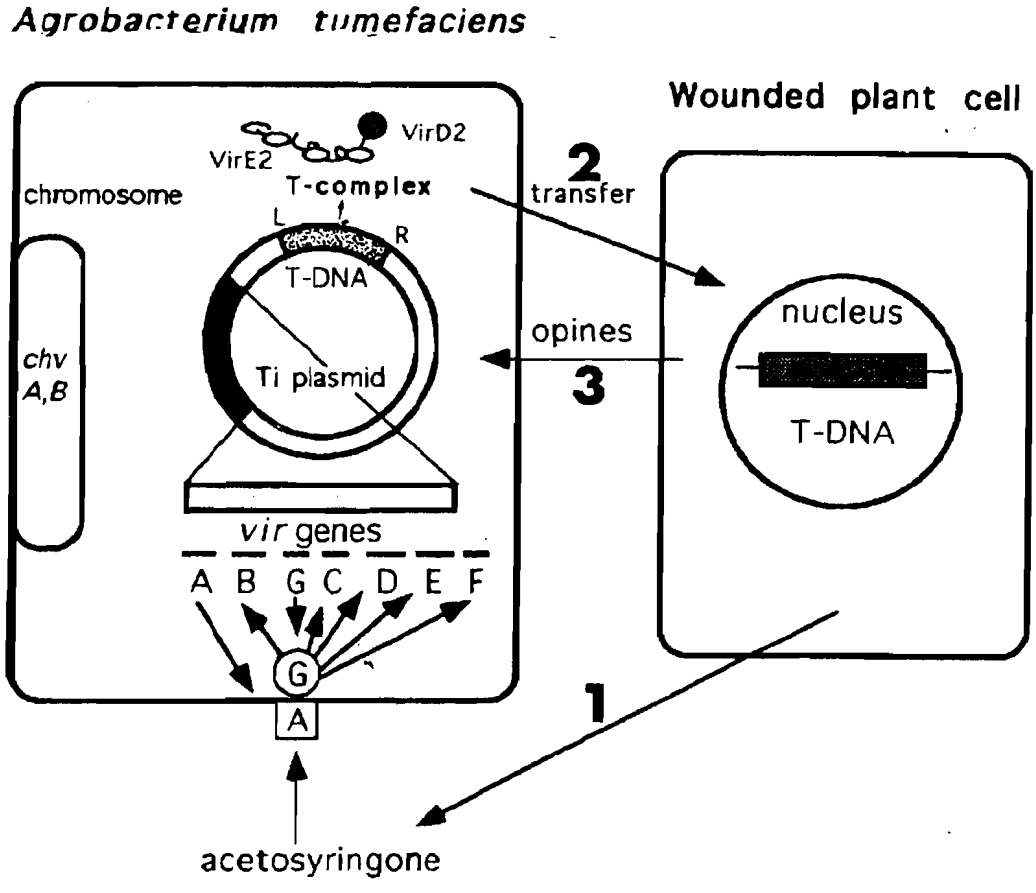
These maps show the relative positions of the major functional regions in Tiplasmids of *Agrobacterium tumefaciens*.
TL and TR are the regions containing the T (Transferred)-DNA.

Figue 2 Maps of a nopaline and octopine T-region



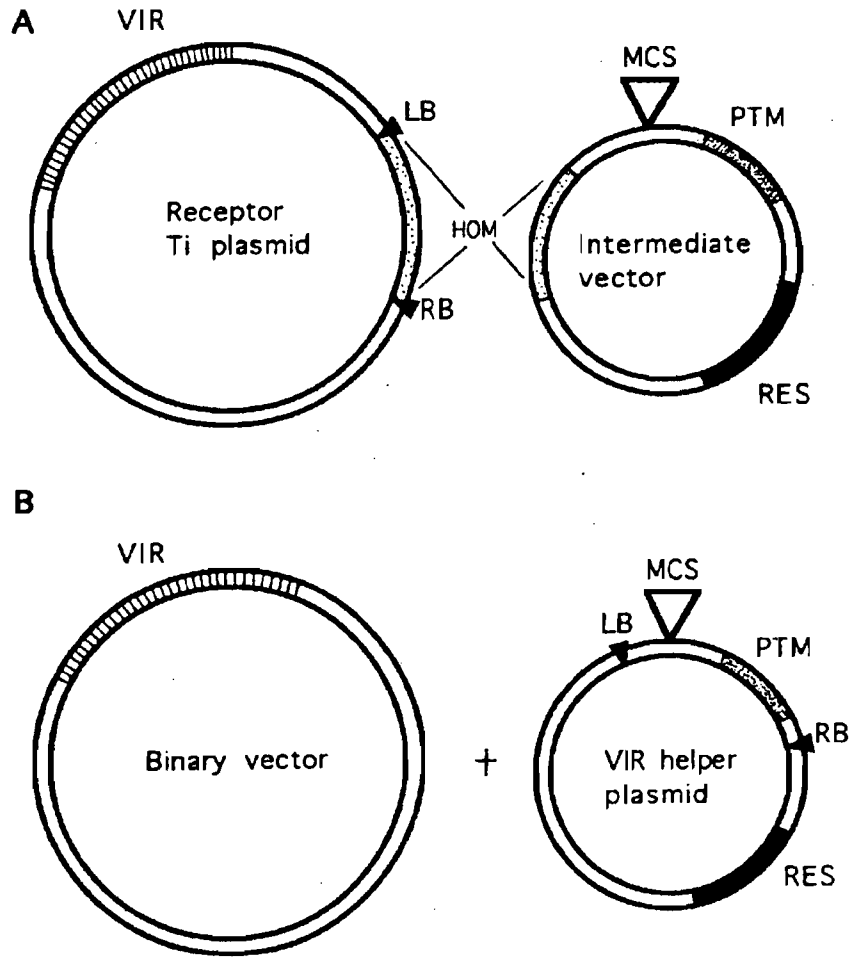
Products of transcription which have been identified are :
- *nos*: nopaline synthase
- *ocs*: octopine synthase
- *tms1*: tryptophan mono-oxygenase
- *tms2*: indole-3-acetamide hydrolase
- *tmr*: DMA transferase
- *agr*: transcripts required for agropine synthesis

Figure 3 Schematic diagram of hte transformation of a plant call by *Abrobacterium tumefaciens*



chvA,B are chromosomal genes involved in the plant-bacterial interaction. The products of *virA* and *virG* genes recognize acetosyringone, a signal molecule derived from the wounded plant cell and trigger the activation of the other *vir* loci. Vir products are involved in the formation and the transfer of the T-strand, which is protected as a DNA-protein complex called the "T-complex". The T-DNA is then integrated in the plant nucleus and the genes encoded by the T-DNA starts to be expressed.

Figure 4 Schematic diagram of co-integrative (A) and binary (B) vectors



VIR: Virulence region; RB,LB: right and left border; HOM: homologous regions within which recombination may occur for co-integration; MCS: multiple cloning site; PTM: plant transformation marker.

Franche Claudine, Bogusz Didier, Duhoux Emile (1994)

Agrobacterium mediated DNA transferuse of *A. Tumefaciens* and *A. Rhizogenes* for the genetic transformation of trees in the family *Casuarinaceae*

In : Jiaguo X. (ed.), Shimin L. (ed.) Genetic engineering and crop improvement. Wuhan : Huazhon Agricultural University, 69-90

Genetic Engineering and Crop Improvement, Wuhan (CHN), 1994/04/11-15

ISBN 91-85798-398