Ti Plasmids

Secondary article

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Ti plasmids are plasmids from the genus *Agrobacterium* that encode a natural system of plant transformation. They are used extensively as vectors for plant genetic engineering.

Article Contents

Introduction

The pathogenic forms of the soil bacterium *Agrobacterium* induce tumours (commonly called crown galls) or aberrant roots (hairy roots) on a wide range of dicotyledonous plants. The tumour-inducing capacity is determined by a large plasmid, the Ti plasmid (Van Larebeke *et al*., 1974). A specific fragment of this plasmid (the T-DNA) is transferred into wounded plant cells during infection and stably integrated in the plant's DNA (Chilton *et al*., 1977). The T-DNA genes encode several functions leading to plant cell growth and the synthesis of novel low-molecular weight compounds, so-called opines that the bacterium uses for its growth. This remarkable and unique system of natural genetic engineering across kingdom boundaries has been described as genetic colonization. Recently it has been shown that *Agrobacterium* can also transfer DNA into fungi (De Groot *et al*., 1998). Most of the transformation functions are located on the Ti plasmid, although chromosomal functions, notably those involved in attaching the bacteria to plant cells, have also been described (Matthysse and McMahan, 1998).

Infection of the Plant

Agrobacterium generally infect plants through wounds situated at the transition between the roots and the stems (the crown of the plant). Wounds may be caused by rapid growth, by insects or nematodes, or by horticultural practices such as pruning or grafting. Early experiments showed that wounded plant cells are not immediately receptive to *Agrobacterium* transformation, but become competent at the time when they start to divide as part of the healing process. It has been proposed that this makes the nuclear DNA more accessible to the T-DNA.

Genetic Structure and Functions of the Ti Plasmid

Ti plasmids have sizes between 150 and 500 kb. Many different Ti plasmid types have been described. While they generally contain functions involved in tumour or hairy root induction, others are related to the spread of the Ti plasmid in the agrobacterial population. The genetic map of an octopine type Ti plasmid is shown as an example in **Figure 1**. The tumour-induction regions consist of one or more T-DNAs (the sizes of which vary between Ti plasmids) and the virulence region, or vir region (about 30 kb in size), which assures the transfer of the T-DNA to the plant cell. The opine catabolism genes encode uptake and metabolism of the opines. Some of the latter genes also permit chemotaxis towards opines. The Ti plasmid can be transferred to other agrobacteria by conjugation; this requires not only induction of the Ti plasmid-located transfer (or *tra*) genes that are induced by certain opines (the conjugative opines), but also secretion and accumulation of sufficient levels of homoserine lactone, which acts as a quorum sensor. Conjugational transfer starts at a particular Ti plasmid sequence, the origin of transfer. Replication and partitioning of the Ti plasmid is assured by its origin of replication and incompatibility functions. Ti plasmids are evolutionary chimaeras; they can rapidly change their overall structure by large-scale recombination events. Such changes can only lead to viable new structures when related functions (e.g. vir functions) are closely linked on the Ti plasmid, which is indeed what is observed.

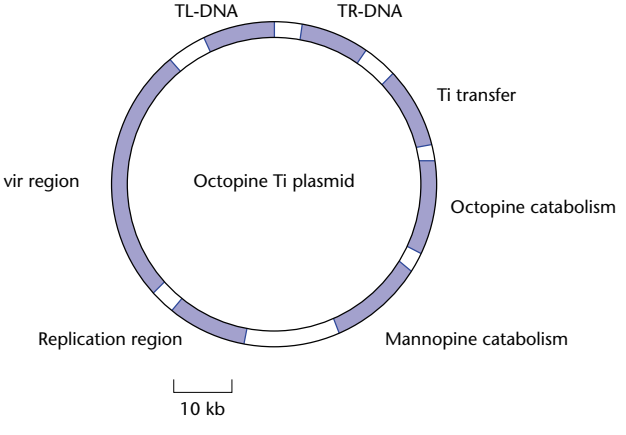

Figure 1 Genetic map of an octopine Ti plasmid.

It has been shown that bacterial insertion elements are common in *Agrobacterium* and contribute to shaping the Ti plasmids by mutation or by providing direct repeats that lead to deletion by intramolecular recombination.

Transfer of T-DNA

T-DNAs are transferred through the action of the Ti plasmid-located virulence (*vir*) genes (**Figure 2**). These are arranged in at least eight complementation groups.

All T-DNAs are delimited by direct imperfect repeats of 24 nucleotides, called border sequences. These are recognized and cleaved on the lower DNA strand by the VirD1/VirD2 endonuclease. Elongation of the DNA from the free 3′-hydroxyl ends liberates a single-stranded DNA molecule (the T strand) that is exported to the plant nucleus. Various Vir proteins are required for efficient transfer of the T strand. The VirD2 protein attaches itself covalently at its 5′-end. The VirE2 protein binds in a nonspecific way to single-stranded DNA and protects the T strand in the plant cell against endonucleases, thus forming the T complex. Both the VirD2 and VirE2 protein contain a nuclear localization signal (NLS) that directs the T complex to the plant nucleus. The virulence of *virE* and *virF* mutants can be restored by expressing VirE2 or VirF in the target plant, showing that both are required in the plant cell. The role of the VirF protein is still unknown.

The T strand is integrated randomly into the nuclear DNA. Integration leads to various changes in the target DNA. Some T-DNAs are integrated as inverted repeat structures.

Transfer of the T complex involves at least 10 of the 11 *virB* genes (*virB2* to *virB11*) and *virD4*. These proteins form a multimeric protein complex (called core transfer complex) and pili with a diameter of 10 nm (Fullner *et al*., 1996). The *virB* operon encodes several proteins, which are related to proteins of bacterial conjugation systems like those of the broad host range plasmid RP4. The T-DNA borders are similar to bacterial origin of transfer (oriT) sequences. These similarities make it very likely that the T-DNA transfer system is derived from a bacterial conjugation system.

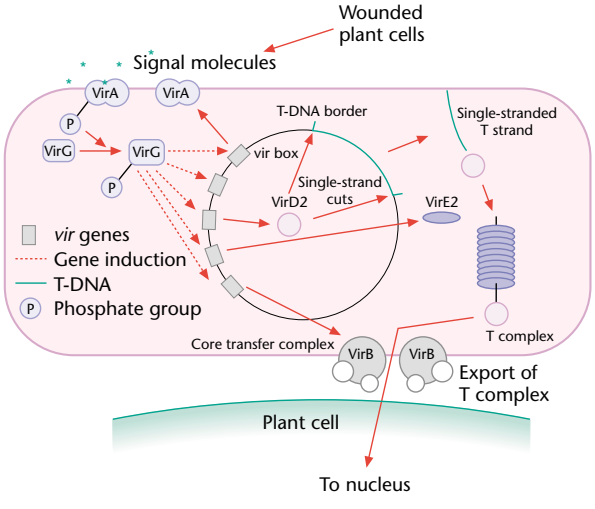

Figure 2 Transfer of the T-DNA to the plant cell and its control by the *vir* genes.

The expression of the virulence genes is tightly controlled and relies on a two-component regulatory system involving VirA and VirG. The dimeric transmembrane protein VirA detects an acidic pH and signal molecules released by wounded plant cells. These include phenolic compounds such as acetosyringone, derived from plant cell wall degradation processes and monosaccharides. Detection of monosaccharides by VirA also requires the chromosomally encoded ChvE protein. VirA activation involves phosphorylation of a histidine residue of the VirA protein, which transfers the phosphor group to an aspartic acid residue of the cytoplasmic VirG protein; the latter acts as a transcriptional activator for *vir* genes. The promoter sequences recognized by VirG have been defined and are called 'vir boxes'.

T-DNAs can vary in size from 3.5 kb to 25 kb. It has been shown that artificial T-DNAs of more than 50 kb can be transferred intact (Hernalsteens *et al*., 1980). The number of T-DNAs on a Ti plasmid can vary from one to three. Multiple T-DNAs are transferred independently; their relative rate of transfer is probably determined by the structure of their border sequences. T-DNAs may contain internal sequences with sufficient similarity to border sequences to be recognized by the VirD2 endonuclease; these pseudoborders may lead to truncated T-DNAs.

The expression of the T-DNA genes depends largely on the site of T-DNA insertion (position effect) and can vary enormously from cell to cell (Peach and Velten, 1991). In natural crown gall induction, tumours result from the combined growth of several independently transformed cell lines. Expression has been found to diminish during the early phases of transformation; the initial high level of expression has been called transient expression and is supposed to result from nonintegrated T-DNA copies. After integration, expression can further diminish because of methylation or cosuppression.

Mechanism of Tumour and Hairy Root Formation

Tumour and hairy root formation result from the expression of the tumour genes (also called oncogenes) located on the T-DNA(s). A biochemical function has been found for only a few of these oncogenes.

The isopentenyl transferase (*ipt*) gene encodes the Ipt protein, which catalyses the synthesis of isopentenyladenine from dimethylallylpyrophosphate and adenine. Isopentenyladenine and its derivatives are well-known plant hormones of the cytokinin type. This type of hormone generally induces shoots.

The indoleacetic acid (*iaa*) genes consist of the tryptophan monooxygenase (*iaaM*) and indoleacetamide hydrolase (*iaaH*) genes. The first one leads to synthesis of indoleacetamide from tryptophan, that is converted into indoleacetic acid (IAA) by the product of the *iaaH* gene. IAA is an auxin that induces roots on certain plants. Together, and in the right proportions, cytokinins and auxins lead to undifferentiated growth. Cells with *ipt* and *iaa* genes can stimulate the growth of nearby cells by secretion of the two hormone types and tumours can therefore contain considerable amounts of untransformed cells.

Both *iaa* and *ipt* genes occur as bacterial genes in *Agrobacterium*, *Pseudomonas* and *Erwinia*, where they induce plant growth without genetic transformation. Most probably, these genes represent the ancestral forms of the T-DNA *ipt* and *iaa* oncogenes.

Hairy roots are induced by *Agrobacterium rhizogenes* through the combined action of the *rolA*, *B*, *C* and *D* genes (*rol* for 'root locus'). The *rol* genes stimulate growth and differentiation of transformed cells but not of neighbouring cells; they are 'cell-autonomous'. When one of these genes is mutated, the morphology of the hairy roots changes. Unfortunately, we do not yet know how these genes act; such knowledge could be of great interest for understanding the normal regulation of root growth and may lead to various practical applications.

No bacterial variants of the *rol* genes have yet been found. However, they have been detected in certain nontransformed plant species, such as *Nicotiana tabacum* or *Nicotiana glauca*, where they are expressed (Ichikawa *et al*., 1990). It has been proposed that these cellular *rol* genes (*c-rol*) result from an ancient transformation event by *A. rhizogenes*; the resulting hairy roots would have regenerated spontaneously into fertile plants. Alternatively, the *rol* genes are of plant origin and were somehow acquired by the bacterium. However, the occurrence of *rol* genes in some plant species but not in others argues against the latter possibility. Further research is required to establish the possible role of *rol* genes in plant evolution.

T-DNAs of both *A. rhizogenes* and *A. tumefaciens* have been found to contain other, minor oncogenes. Some induce growth by their own on certain plant species (gene *6b*, *orf13*, *lso*, gene *3′*), while others modify the growth response induced by the rest of the T-DNA (gene *e*, gene *5*). Many of these genes are remotely related to each other and to genes *rolB* and *rolC* (Otten and Schmidt, 1998). Several have been introduced into plants under the control of their own promoter or under the control of a strong, constitutive promoter; various phenotypes have been described, but no biochemical function has yet been attributed to these genes.

Opines

The *raison d'être* of the Ti plasmid is undoubtedly its capacity to induce opine synthesis in plant cells (**Figure 3**). Tumour or root induction would merely permit further amplification of opine synthesis. The importance of opine synthesis is indicated by the following findings: in natural tumours, opines are produced in very large amounts; these compounds may constitute as much as 7% of the tumour's dry weight. Second, a large part of the Ti plasmid is occupied by the opine degradation genes that encode regulatory proteins, permeases and oxydases; in some cases several enzymes are involved in a stepwise degradation process. Third, opines play a crucial role in the control of Ti plasmid conjugation. Finally, agrobacteria are specifically attracted by the opines they degrade.

Most of the opines are imine conjugates of amino acids with sugars or keto acids (arginine and pyruvate yield octopine, arginine and α-ketoglutaric acid yield nopaline). Agrocinopine is a combination of L-arabinose and sucrose (Ryder *et al*., 1984). Some opines like octopine and nopaline are synthesized in a single step, while others like agropine require several enzymes. Mannopine and agrocinopine synthesis genes are found in different T-DNA contexts, illustrating the chimaeric nature of the Ti plasmids.

The genes encoding synthesis of agrocinopine and mannopine are related to those encoding their catabolism, indicating that the two systems arose by duplication. It has

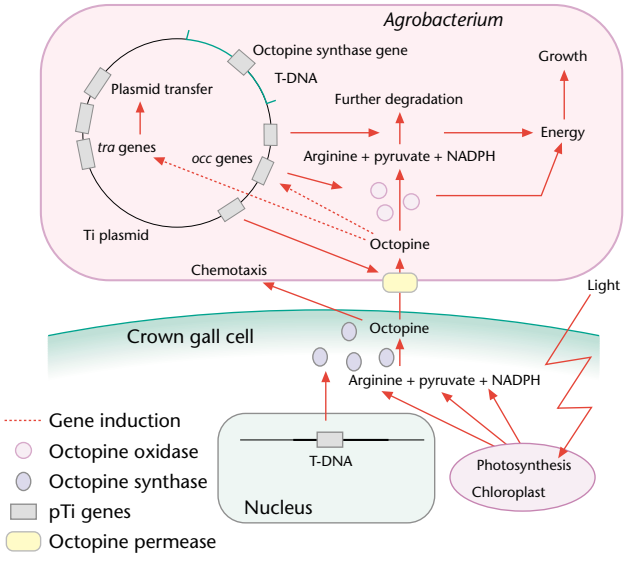

Figure 3 Induction of opine synthesis in the crown gall cell and use of opines by the bacterium.

also been shown that some opine synthesis genes like vitopine and octopine synthase genes are related.

Oxidation of opines liberates the original compounds and also a certain amount of chemical energy that is used by the bacterium for its growth. It has been shown that transgenic opine-producing plants (carrying an opine synthesis gene) favour the growth of opine utilizers in their rhizosphere; these synthetic symbiotic systems may be used to create artificial couples of plants and beneficial bacteria, e.g nitrogen-fixing Rhizobia or growth-promoting *Pseudomonas* species (Guyon *et al*., 1993).

Ecology of Ti Plasmids

Most *Agrobacterium* research has been directed towards elucidation of the basic mechanisms of T-DNA transfer and other Ti functions, using a few model plasmids. Very little is known about the distribution and ecological properties of these plasmids. In the case of *A. vitis* it has been possible to conduct such a study: this particular species is only found on grapevine (*Vitis vinifera*) and many isolates are available from vineyards all over the world. It was shown that Ti plasmids and chromosomal types of *A. vitis* are strongly linked, suggesting only limited Ti plasmid transfer (Otten *et al*., 1996). These studies also revealed that grapevine strains carry genes for the degradation of tartrate, an abundant compound in grapevine. The tartrate degradation genes are clustered on an 11-kb DNA fragment found on three different plasmids, one of them a Ti plasmid. Thus, besides opines, agrobacteria can also exploit natural plant compounds. Competition studies between a wild-type strain and a tartrate-utilization mutant showed that tartrate degradation confers a selective advantage both in the healthy grapevine plant and in the tumours (Salomone *et al*., 1998). Growth in healthy plant material could constitute a first step in colonization before tumour induction.

Interestingly, the great majority of natural soil isolates of *Agrobacterium* are avirulent, and virulent strains have mostly been isolated from crown galls. It appears therefore that Ti plasmids are lost when there is no need for them. Close to tumours, the conjugative opines would rapidly induce spreading of the Ti plasmid through the agrobacterial population. In this way, the burden of Ti plasmid replication would be diminished.

The natural distribution of *Agrobacterium* strains has also been investigated in the case of crown gall of fruit trees. It was shown that, in most cases, nopaline strains were responsible for infection. A natural antagonist of these strains, the avirulent *Agrobacterium* isolate K84 (Clare *et al*., 1990) was found to produce an antibiotic, agrocin K84. This compound is an analogue of agrocinopine and at the same time a fraudulent nucleotide. Since many nopaline strains induce the synthesis of agrocinopine in tumours and take it up via an agrocinopine permease, they will also take up agrocin, which is then incorporated into the DNA, leading to replication arrest. The genes for agrocin K84 synthesis have been localized on a plasmid; a second, conjugative plasmid carries nopaline and agrocinopine degradation genes, allowing the antagonist strain to benefit from the opines in the tumours induced by its victim. The K84 synthesis plasmid contains agrocin immunity genes and can be mobilized by the nopaline-degradation plasmid. This would preclude the use of strain K84 as a natural pesticide since virulent strains may acquire the immunity genes. A nontransmitting variant (K1026) has been constructed and is now commercialized for use on a large scale. Whether the use of this biocontrol strain will lead to efficient elimination of *Agrobacterium* infection, to the appearance of agrocin-resistant nopaline strains or to the replacement of nopaline strains by other strain types remains to be studied.

Biotechnological Applications of Ti Plasmids

When it became clear that the Ti plasmid enables transfer of DNA to the plant cell, it was rapidly realized that the system could be put to use for the genetic engineering of plants (Hernalsteens *et al*., 1980). Various Ti-derived vector systems were developed. The most commonly used ones (Hoekema *et al*., 1983) consist of a so-called disarmed Ti plasmid that contains virulence genes but lacks T-DNAs and a second, smaller plasmid with an artificial T-DNA (**Figure 4**). The virulence functions act in *trans* on the T-DNA of the smaller plasmid; this system is called a binary system and the T-DNA carrying vector is called a binary vector. Selection of transformed plant cells is generally based on the antibiotic resistance gene neomycine phosphotransferase (*nptII* gene); a visible marker gene like the

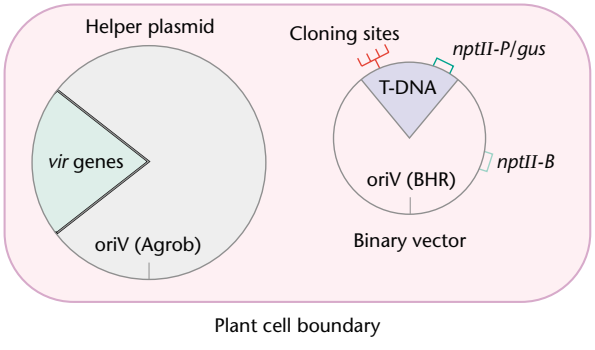

Figure 4 *Agrobacterium* strain for genetic engineering. The strain contains a T-DNA-less helper plasmid and a binary vector with an artificial T-DNA. *nptII-P* and *nptII-B* designate genes which are expressed in the plant (P) or in the bacterium (B); oriV (BHR), broad host range origin of replication.

β-glucuronidase gene (*uidA* or *gus* gene) can also be added. As starting material one often uses leaf fragments, incubated with an *Agrobacterium* suspension, followed by selection on kanamycin and regeneration of transformed shoots. Other transformation systems use embryo cultures, protoplasts or even whole plants that can be vacuum infiltrated with agrobacteria; in the latter case, transformants can be selected among the progeny of the infected plants (Bechtold *et al*., 1993). Highly efficient variants of *vir* genes have been used for the construction of supervirulent strains (Hood *et al*., 1986). Ongoing studies on the mechanism of transfer may one day allow the construction of artificial infectious T strands that would eliminate the need for agrobacterial infection.

The T-DNA has also been successfully employed as a mutagen. Since the site of the insertion contains a T-DNA copy, the mutated region can be easily cloned using the T-DNA as a probe.

Apart from their vector properties, Ti plasmids are also interesting sources of growth-modifying genes. Placed under appropriate promoter control, they may be of interest to modify the growth of crop plants (see, for example, Schmülling *et al*., 1989).

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