"**Transformation**" is most simply defined as a "change". In the plant biotechnology community, transformation can be a little more precisely defined as the process of introducing exogenous DNA through the cell membrane into a target plant cell, leading to a permanent change in its genetic makeup and its derivatives.

Agrobacterium "Species" And Host Range

The genus *Agrobacterium* has been divided into a number of species. However, this division has reflected, for the most part, disease symptomology and host range. Thus, *A. radiobacter* is an "avirulent" species, *A. tumefaciens* causes crown gall disease and *A. rhizogenes* causes hairy root disease. Regardless of the current confusion in species classification, for the purposes of plant genetic engineering, the most important aspect may be the host range of different *Agrobacterium* strains. As a genus, *Agrobacterium* can transfer DNA to a remarkably broad group of organisms including numerous dicot and monocot angiosperm species and gymnosperms. In addition, *Agrobacterium* can transform fungi, including yeasts, ascomycetes and basidiomycetes. Recently, *Agrobacterium* was reported to transfer DNA to human cells…….(Debaits on genetic modified products in other lectures).

Agrobacterium tumefaciens-Mediated Transformation

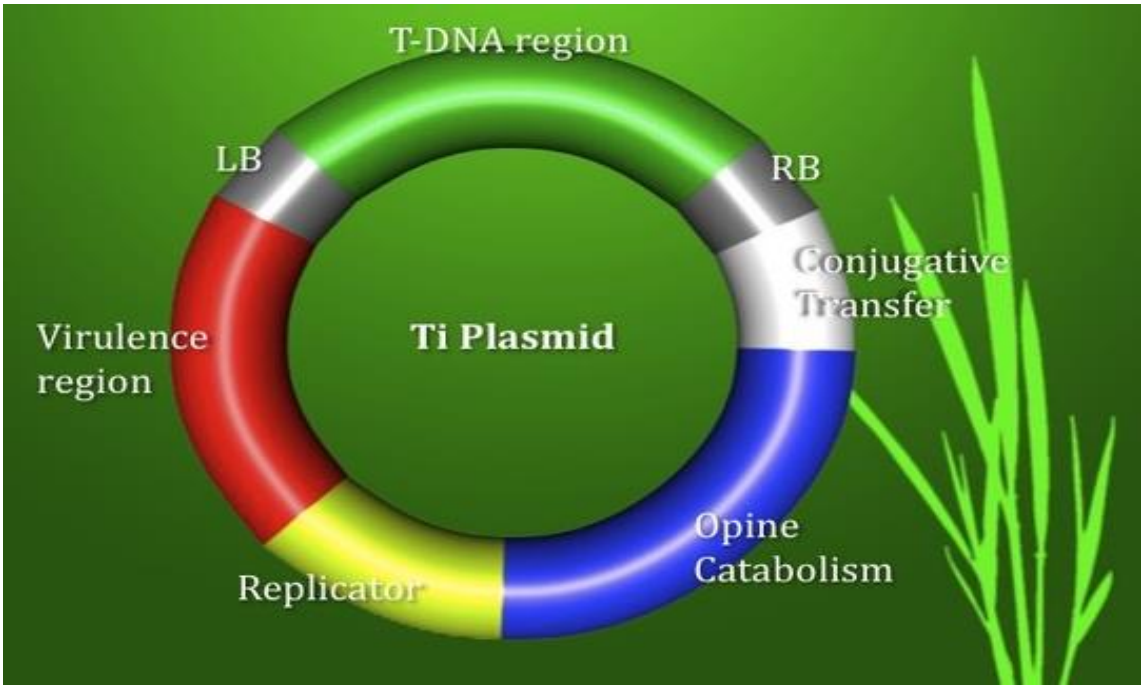
Agrobacterium tumefaciens

Agrobacterium tumefaciens is a gram-negative soil phytopathogenic bacterium which is the causative agent of crown-gall disease characterized by a tumorous phenotype, of hundreds of dicots, some monocots and gymnosperms. The disease symptoms result from the transfer, integration and expression of a specific DNA fragment (known as transferred DNA or T-DNA) from the bacterial tumor-inducing (Ti) plasmid into the plant cell genome, reprogramming them to divide and produce chemicals called opines. The bacteria use these opines as their primary food source but for the plant, they are completely useless. Once the DNA transfer is complete the tumour cells can grow and divide independently of the bacteria and so, genetically transforming the host.

Crown-gall disease

Tumor-inducing (Ti) plasmid

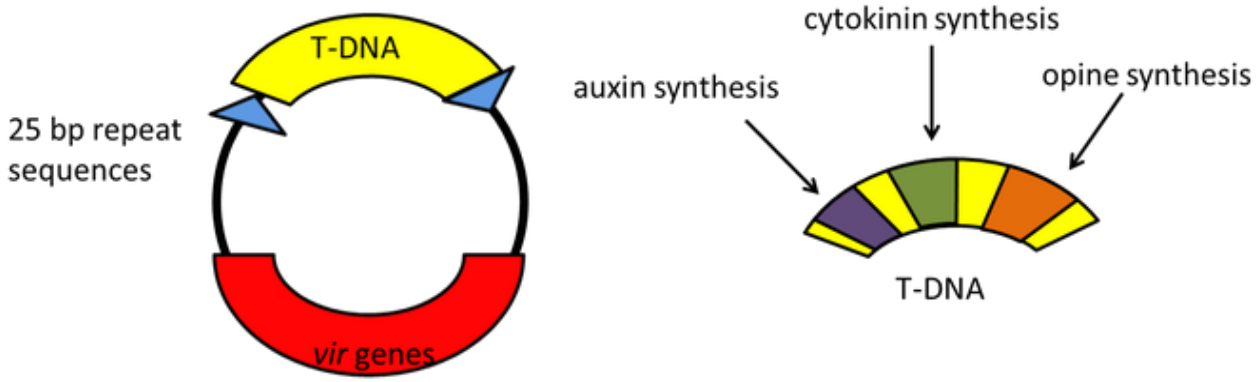
It consists mainly of 1) T-DNA containing borders sequences, oncogenic genes and opine synthesis, 2) opine catabolism, 3) origin of replication (replicator) and 4) conjugative transfer genes (oriT or Tra).



The T-region itself is not sequence-specific and is delimited by 25-bp directly repeated sequences, which are called T-DNA border sequences (left and right borders). Both borders sequences are the only parts of T-DNA needed to enable the transfer into plants. Since the T-DNA does not contain any specific sequences coding for its processing, translocation into the

host cell cytoplasm, nuclear import or integration any sequences placed between its borders will also be transferred and integrated into the host genome. The T-DNAs, when transferred to plant cells, encode enzymes for the synthesis of the plant hormones (auxin and cytokinin) as well as strain-specific low molecular weight opines. The massive accumulation of auxin and cytokinin in transformed plant cells causes uncontrolled cell proliferation responsible for tumorous phenotype. Opines are specifically synthesized and excreted by the host gall cells to be consumed by *Agrobacterium* as carbon and nitrogen sources. So by promoting plant cell division, *A. tumefaciens* increases the number of cells that are producing these opines. Based on the kind of opines produced in the tumors, Agrobacteria are classified as octopine, nopaline, succinamopine and leucinopine strains. There are more than 20 opines and each Agrobacterium strain induces and catabolizes a specific set of opines.

The Ti plasmid also contains the genes for **opine catabolism** produced by the crown gall cells, **origin of replication**, which indicate when the replication starts and the **regions for conjugative transfer** for its own integrity and stability. Many genes are involved in *A. tumefaciens*-mediated T-DNA transfer, but most of the genes required for T-DNA transfer are found on the ***vir* region** of Ti plasmid. This *vir* region comprises at least 8 operons (*virA*, *virB*, *virC*, *virD*, *virE*, and *virG*, *virF* and *virH*) encoding approximate 25 proteins. These proteins are termed virulence (vir) proteins and are required for the sensing of plant signal molecules as well as the processing, transfer, and nuclear localization of T-DNA, and the integration of T-DNA into the plant genome. Only *virA* and *virG* are constitutively transcript. The transcription of all other *vir* operons in *vir* region is coordinately induced during infection (Table below).



Vir genes	Contribution in transformation
Vir A	Sense acetosyringone and other phenolics secreted by wounded plant cells
Vir G	Transcriptional activator of vir box
Vir D2	Protect 5'end of T-DNA from being cleaved by exonuclease and "pilots" the mature T-strand through the translocation channel (virB/VirD4T4SS).
Vir E	Protein helps in gene transfer
Vir E2	Form virE2 protein that completely coats the T-DNA to prevent its degradation in plant cell cytoplasm.
Vir C1 C2	Forms overdrive sequence and helps in DNA transfer
Vir D	Excise T-DNA
Vir B	Form conjugational pore between plant and bacteria
Vir B11	ATPase activity-provides energy for movement of DNA
Vir H	Detoxify other adversely affecting components during the DNA transfer
Vir F	Move forward as guide to T-DNA all the way from bacteria till nucleus

Basic process of *A. tumefaciens*–mediated genetic transformation

The process of *A. tumefaciens*–mediated genetic transformation is a long journey till the insertion of T-DNA in the plant.

This process involves 1) Plant-pathogen recognition and sensing of plant signal molecules, 2) Vir genes expression by host signals and T-DNA processing, 3) Attachment of *A. tumefaciens* to plant and ssT-DNA complex export into the host, 4) Transport mature T-DNA complex through cytoplasm and nucleus and 5) T-DNA uncoating and integration.

1) Plant-pathogen recognition and sensing of plant signal molecules

A. tumefaciens is a motile organism, with peritrichous flagellae, that possesses a highly sensitive chemotaxis system which appear to be chromosomally encoded.

Wounded plants secrete acetosyringone (strong *vir* gene inducer) and other host-specific small phenolic phytochemicals exudates that act as signaling molecule to *Agrobacterium* recognition, sensing and response. These phenolics trigger VirA-VirG sensory machinery present on the bacterial membrane.

2) Vir genes expression by host signals and T-DNA processing

Sensing of signal molecules released by wounded plant cells is the first step of signal transduction leading to *vir* gene induction in *Agrobacterium*. This regulatory pathway is mediated by the VirA/VirG two-component system. VirA, an inner membrane-bound sensor protein, senses the signal molecules. The presence of extracellular acidic environment (pH 5.0 to 5.8) and phenolic compounds at a plant wound site may directly or indirectly induce autophosphorylation of VirA. The phosphorylated VirA in turn transfer its phosphate to the cytoplasmic protein VirG to be activated (phosphorylation). The activated VirG protein binds to the specific 12bp DNA sequences called *vir* box enhancer elements that acts as a transcriptional activator of *vir* genes by upstream their *vir* operons.

The activation of *vir* genes initiates a cascade of events. Following the expression of *vir* genes, some vir proteins produce the excision and transfer of the linear single stranded DNA called immature T-DNA or ssT-strand. VirD2/VirD1 protein complex is able to recognize the border sequences (left and right borders) and cleave the bottom strand of T-region at identical positions between bp 3 and 4 from the left end of each border (act as endonuclease of borders). Upon the cleavage of T-DNA border sequence, VirD2 protein remains covalently associated with the 5´-end of the ssT-strand. The excised ssT-strand is removed, and the resulting single-stranded gap in the T-region is repaired, most likely replaced by a newly synthesizing DNA strand. The association of VirD2 with the 5´-end of the ssT-strand is believed to prevent the exonucleolytic attack to the 5´-end of the ssT-strand and to distinguish the 5´-end as the leading end of the T-DNA complex (T-DNA + attached proteins) during transfer.

3) Attachment of *A. tumefaciens* to plant and ssT-complex export into the host

It is reasonable that an intimate association between pathogen and host cells is required for the transfer of both ssT-DNA and virulence proteins from *A. tumefaciens* to plant cells.

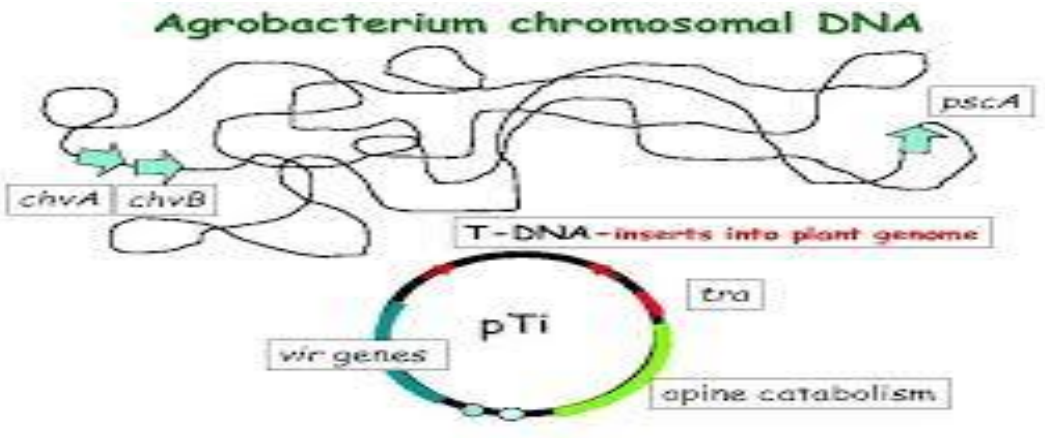
Bacterial genes involved:

The binding of *A. tumefaciens* to host plant cells seems to require the participation of specific receptors that may exist on the bacterial and plant cell surface. A large number of bacterial chromosomal genes are involved in the bacterial attachment to host cells. These genes are identified to mainly locate on two regions of the bacterial chromosome.

The binding of bacteria to host cells is thought to be a two-step process:

- The binding in the first step is loose and reversible because the bound bacteria are easy to being washed from the binding sites by shear forces, such as water washing or vortexing of tissue culture cells. Genes involved in this step are identified to locate on the *att* gene region (more than 20 kb in size) of the bacterial chromosome. This group includes genes for ATPase as well as a number of biosynthetic genes, which include the transacetylase required for the formation of an acetylated capsular polysaccharide, which is required for the bacterial attachment to some plants.

- The second step in the bacterial attachment to the host results in tight binding of the bacteria to the plant cell surface because the bound bacteria can no longer be removed from the plant cell surface by shear forces. This step requires the synthesis of cellulose fibrils by the bacteria, which recruits larger numbers of bacteria to the wound sites. The genes required for the synthesis of bacterial cellulose fibrils (*cel* genes) are identified to locate on the bacterial chromosome near, but not contiguous with the *att* gene region. Some other chromosomal virulence genes *chvA*, *chvB*, and *pscA* are believed to be involved indirectly in bacterial attachment to host. These genes are involved in the synthesis, processing, and export of a cyclic β-1,2-glucan, which has been implicated in the bacterial binding to plant cells.



Plant factors involved:

In addition to bacterial factors, some plant factors are essential for the attachment of *A. tumefaciens* to plant cells. Two plant cell wall proteins: a vitronectin-like protein and a rhicadhesin-binding protein have been proposed to mediate the bacterial attachment to plant cells. Vitronectin is an animal receptor that is specifically utilized by different pathogenic bacteria. A plant vitronectin-like protein is reported to occur in several *A. tumefaciens* host plant.

Following the production of ssT-DNA and attachment to the host cells, *Agrobacterium* transports ssT-DNA and virulence proteins into the host. The transportation must cross the bacterial cell membrane and wall, as well as host cell membrane and wall.

Transfer apparatus

The type IV secretion systems of Gram-negative bacteria are evolutionarily related to bacterial conjugation systems. Gram-negatives use type IV secretion systems for a variety of biological functions including the exchange of genetic material with other bacteria and the translocation of oncogenic DNA and effector proteins into eukaryotic host cells. The secretion apparatus itself typically comprises a macromolecular complex that spans the bacterial inner and outer membranes and can also span the membrane of eukaryotic host cells. This assembly is typically composed of up to 12 proteins.

A. tumefaciens uses a type IV secretion system (T4SS) called VirB/VirD4 T4SS channel to transfer ssT-DNA and effector proteins (needed for activation and success) to its host cells. In *A. tumefaciens*, it is assembled from 11 proteins (VirB1 to VirB11 encoded by the *virB* operon) and VirD4. VirD4 protein might recruit ssT-complex (ssT-DNA-VirD2) to pass through the T4SS transporter after its maturation. The ssT-complex will be completely coated with VirE2 proteins to form a transported form of T-DNA (mature). VirE2 is a single-stranded DNA-binding protein that can bind single-stranded DNA without sequence specificity. When bound to single-stranded DNA, VirE2 can alter the ssDNA from a random-coil conformation to a telephone cord-like coiled structure and increases the relative rigidity. It coats the T-strand along its length to protect the T-strand from the nucleolytic degradation because single-stranded T-DNA is believed to be susceptible to nucleases. Mature VirD2-T-

DNA-VirE2 strand complex (leaded/pilot by its VirD2) will be transported into the host cells through the VirB/VirD4 T4SS translocation channel. The Vir C1 C2 forms an overdrive sequence to enhance the transfer the mature VirD2-T-strand complex to plant. Vir F proteins move forward as guide to mature VirD2-T-strand complex all the way through the channel till nucleus.

4) Transport mature T-DNA complex through cytoplasm and nucleus

Following the entry of the mature T-DNA strand complex in plant cell cytoplasm, it interacts with plant proteins, likely forming "*super-T-complex*", which is responsible for subcellular travelling of T-strand from cytoplasm through nuclear membrane into nucleus, and to the chromatin, thus facilitating T-DNA integration into host genome.

In Host cytoplasm

The dense structure of the cytoplasm, which greatly restricts the free diffusion of macromolecules, and the size of the "super-T-complex", which far exceeds the 60 kDa size exclusion limit of the nuclear pore, indicate that active transport processes are required for the nuclear import of T-complex. Once in the plant cell, VirD2 may function in additional steps of the transformation process. VirD2 contains nuclear localization signal (NLS) sequences that may help direct it and the attached T-DNA to the plant nucleus. VirD2 may not be sufficient to direct T-complex to the nucleus; so importin-α proteins and two VirE2-interacting proteins (VIP1 and VIP2) interact with NLS-containing proteins and guide the super-T-complex to the nucleoporin complex (NPC) of the nucleus.

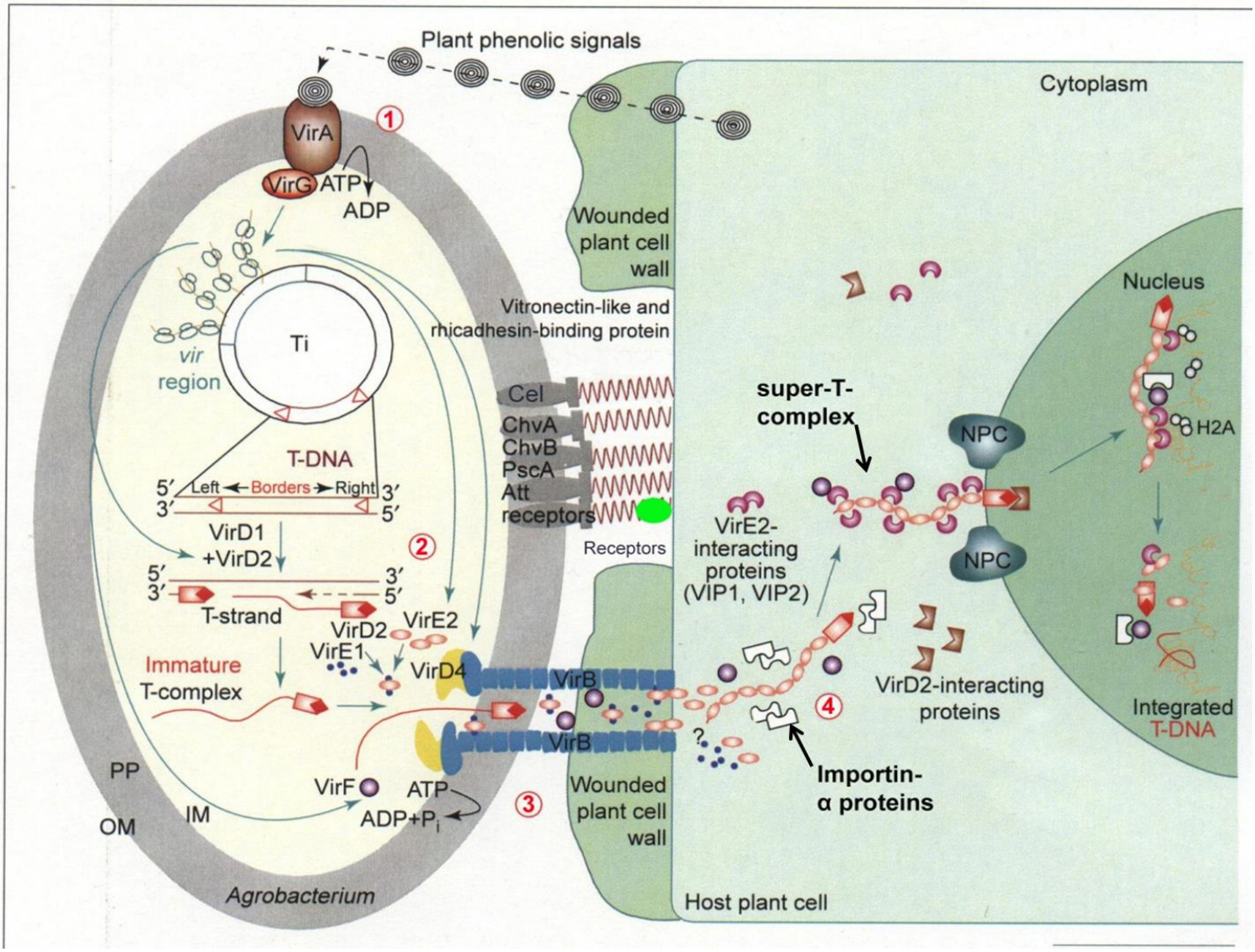
Integration of T-DNA into plant genome (In nucleus)

The integration of the incoming ssT-strand of the T-complex into plant genome is the final step of the *Agrobacterium*-mediated genetic transformation. Whether or not the host can be successfully transformed is highly dependent on whether the T-DNA could be integrated into the suitable sites of the host genome. There are microsimilarities involved in the integration of both the right and left borders of the T-DNA insertions. These microsimilarities occur only in a stretch of 3 to 5 bp and can be between any T-DNA and genomic sequence. This mini-match of 3 to 5 bp basically allows T-DNA to integrate at any locus in the genome (randomly). It was also showed that T-DNA integration is favored in plant DNA regions with an A-T-rich content.

Integration mechanism

Once the T-DNA complex enters the nucleus, core histone (H2Aproteins) removes all the uncoated bacterial chaperons. The T-DNA (with virD2 and virE2) can be self-integrate in the random part of the plant DNA. virD2 and virE2 acts as 2 endonucleases which cleave the internal covalent bonds of the polynucleotide chain. This cut single strand will be removed from plant DNA. Next, the T-DNA will be integrated from the 5'-end (where virD2 was attached) to the 3'-end (where the VirE2 was attached) and both virD2 and virE2 will be removed. The upper complementary strand of the plant DNA (original) was cut out by exonucleases. The plant polymerase will start to replicate the complementary strand of the T-DNA to fill the gap. Once complete replication is ended, the plant cell will express the new phytohormones (auxins and cytokinins) and opines synthesis. The phytohormones will uncontrolled cell divisions of the plant cell causing tumor. Opines will be synthesized by the reprogrammed plant cell and used as nutrients for the bacteria (C and N-sources).

Note: By cutting out the Ti-inducing sequence in genetic engineering, every gene of interest can be inserted in and will be expressed.


Plant phenolic signals
Cytoplasm
VirA
VirG
ATP
ADP
1
Wounded plant cell wall
Vitronectin-like and rhicadhesin-binding protein
Ti
vir region
Cel
ChvA
ChvB
PscA
Att receptors
Receptors
super-T-complex
NPC
NPC
Nucleus
H2A
T-DNA
5′ Left Borders Right 3′
3′ 5′
VirD1 +VirD2
2
5′ 3′
3′ 5′
T-strand
VirD2
VirE2
VirE1
VirD4
VirB
VirB
Immature T-complex
VirE2-interacting proteins (VIP1, VIP2)
VirD2-interacting proteins
4
?
Integrated T-DNA
Importin-α proteins
PP
OM
IM
VirF
ATP
ADP+Pi
3
Wounded plant cell wall
Agrobacterium
Host plant cell

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