

***Agrobacterium* and plant genetic engineering**

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Key words: Agrobacterium tumefaciens, plant transformation, plant tumours, T-DNA, Ti plasmid, transgenic plants

Plant tumour induction

The Ti plasmid

More than eighty years ago now Smith and Townsend [141] published an article in which they presented evidence that the bacterium which is now called *Agrobacterium tumefaciens* is the causative agent of the widespread neoplastic plant disease crown gall (Fig. 1). Since then a large

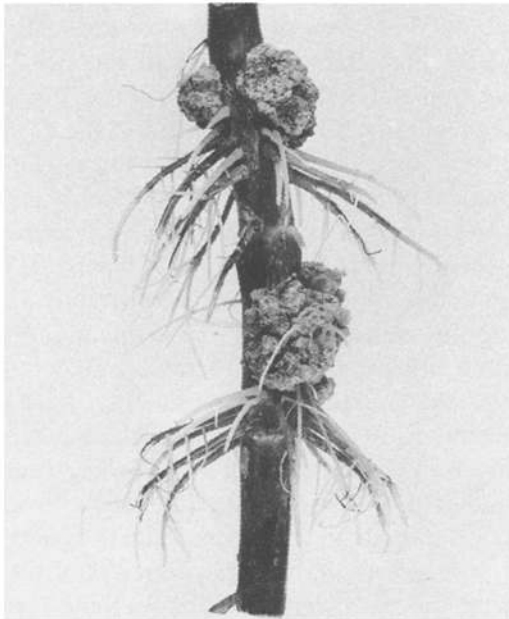


Fig. 1. Crown gall tumours induced by Agrobacterium tumefaciens.

number of scientists throughout the world have focused their research on this organism in an effort to analyse the molecular mechanism underlying the process of crown gall induction in detail. This was driven by the hope that this would lead to a better understanding of oncogenesis in general, and to the development of remedies for such diseases. After a period of diminished interest in the system *Agrobacterium* and crown gall research revived when it became apparent that oncogenic gene transfer from *Agrobacterium* to plants might form the molecular basis of crown gall induction, and thus the transfer system might be exploited for the genetic engineering of plants.

A key discovery made some fifteen years ago now was the finding that virulent strains of *A. tumefaciens* contain a large extrachromosomal element, harbouring genes involved in crown gall induction [195]. Although researchers initially were thinking of a replicative form of a tumorigenic lysogenic virus (bacteriophage) in *Agrobacterium* with similarity to the oncogenic viruses that had been discovered in animal systems by then, in fact the extrachromosomal element that was found turned out to be a plasmid of an exceptionally large size (more than 200 kb). Because of its role in plant tumour induction this plasmid was called the Ti (tumour-inducing) plasmid [171]. The introduction of the Ti plasmid into related bacterial species such as the root nodule-inducing bacterium *Rhizobium trifolii* [71] or the leaf nodule inducer *Phyllobacterium*

myrsinacearum [172] led to tumour-inducing strains, stressing the importance of the virulence determinants on the plasmid for the tumorigenicity of their bacterial hosts. However, introduction and maintenance in more distantly related bacteria such as *Escherichia coli* or *Pseudomonas aeruginosa* did not result in tumour-inducing strains [66], indicating that other factors – most likely chromosomally determined – were also important.

T-DNA structure and function

Crown gall cells are tumorous, i.e. they proliferate autonomously in the absence of the phytohormones (auxins and cytokinins) that are needed for the growth of normal plant cells [23]. Because of this property grafting of aseptic (*Agrobacterium*-free) crown gall tissue onto a normal plant results in tumour formation. In *in vitro* culture crown gall cells grow and form a callus even when the growth stimulating phytohormones are absent from the culture medium. Another feature of crown gall cells is that they produce and excrete amino acid and sugar derivatives that are not formed by normal plant cells [158]. One of the first of such compounds that was characterized was octopine, a product formed by condensation of arginine with pyruvic acid that was formerly only known from *Octopus*, hence its name. Now such plant tumour-specific compounds are generally referred to as opines (Fig. 2). The type of opines formed by crown gall cells depends on the infecting *Agrobacterium* strain [19, 123]. Thus *Agrobacterium* strains can be classified according to the typical opines present in tumours as octopine, nopaline, leucinopine and succinamopine type strains.

The fact that crown gall cells differ from normal plant cells in the two properties mentioned motivated a search for the presence of agrobacterial DNA in crown gall cells. This search led to conflicting results and was unsuccessful until restriction enzymes became available to dissect the genome into a number of discrete fragments that could be separated by gel electrophoresis. Using such isolated fragments Chilton *et al.* [32] were

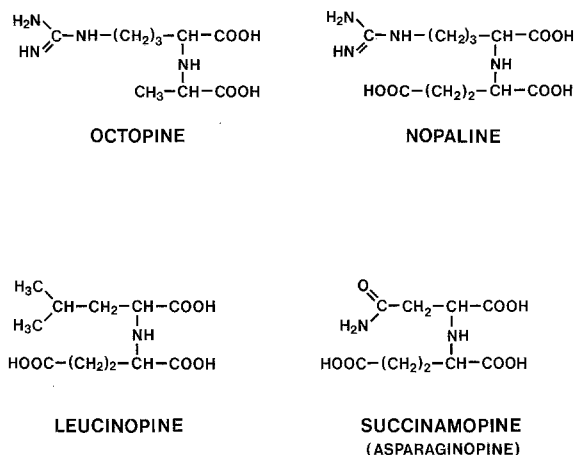


Fig. 2. Structural formulae of four characteristic opines.

the first to demonstrate the presence of about 20 copies of a segment of the octopine Ti plasmid for which a physical map had been established in the meantime [33] in an aseptic octopine crown gall line. Apparently, this piece of DNA had been introduced into plant cells by *Agrobacterium* during crown gall induction. Whether this segment of DNA had indeed oncogenic properties was however doubted soon after its discovery, when Koekman *et al.* [84] reported that in fact the deletion of this DNA stretch from the octopine Ti plasmid did not lead to a loss of oncogenicity by the host bacterium. Moreover, Ledebor [91] found that poly-adenylated transcripts homologous to another, adjacent segment of the Ti plasmid were present in plant tumour lines. The development of a new, more sensitive technique for the detection of specific, short gene sequences in large genomes by Southern [144] helped to reconcile these older, apparently conflicting data. Using the method of Southern it was found in a number of laboratories that strains with octopine Ti plasmids are exceptional in that they have two segments of Ti plasmid DNA that are independently transferred to plant cells during tumour induction [17, 43, 161]. One of these two stretches of DNA turned out to be oncogenic to plant cells and was called the left-transferred DNA (T_L-DNA). The other segment of the octopine Ti plasmid that can be transferred was found to have no oncogenic properties and was called the right-

transferred DNA (T_R -DNA). Octopine crown gall tumour lines always contain the T_L -DNA and sometimes also the T_R -DNA. In fact, the line studied initially by Chilton *et al.* [32] was exceptional in that it had a large number of copies of the T_R -DNA segment. Later on it was demonstrated that this tumour line contained the oncogenic T_L -DNA as well [161]. Tumour lines induced by nopaline, succinamopine and leucinopine strains of *Agrobacterium* contain a segment of oncogenic T-DNA that is at least partially homologous to the T_L -DNA transferred by octopine strains [17]. In all tumour lines analysed the T-DNA was invariably found to be integrated in the nuclear genome of plant cells, and to be absent from organelles [34].

By comparison of the T-DNA structure in a large number of independent tumour lines it was found that the T-DNA corresponds to a rather precisely defined segment of the Ti plasmid, and that no permutations occur during its integration into the plant genome. This latter finding suggested that the T-DNA is delivered into plant cells as a linear stretch of DNA. Sequencing of the T regions in different Ti plasmids showed that these regions are surrounded by a conserved 24 bp direct repeat [190]. Since tumour lines do not contain Ti plasmid sequences originally located outside of the (T-)region as defined by this repeat, it was logical to assume that this direct repeat functions as a recognition signal for the transfer apparatus. The copy number of the T-DNA in

transformed plant lines is usually low varying from one to a few copies, although lines with up to a dozen copies have also rarely been found. If more than one copy is present, these may be located at different loci in the plant genome, or at the same locus where they occur in a direct or inverted orientation towards each other [120, 147].

The T-DNA contains a number of genes that are expressed in the transformed plant cells. Transcript maps have been made for the octopine Ti T_L - and T_R -DNAs (Fig. 3) as well as for T-DNAs from some other types of Ti plasmids. The bacterially derived T-DNA genes are apparently surrounded by expression signals that are recognized by the transcriptional factors of the plant. Sequence analysis of the T-DNA showed in fact that the T-DNA genes have well-known 5' and 3' eukaryotic expression signals such as the TATA box for transcription initiation and the AATAAA box involved in transcription termination and poly-adenylation [10]. Besides these common sequences T-DNA genes have plant-specific regulatory sequences in which they differ from each other and which make the level of their expression controllable by tissue types or signal compounds such as phytohormones [89]. Although the T-DNA genes have their own expression signals, expression is still influenced by the neighbouring chromosomal sequences. This position effect can even lead to complete silencing of the genes. The molecular principle underlying this

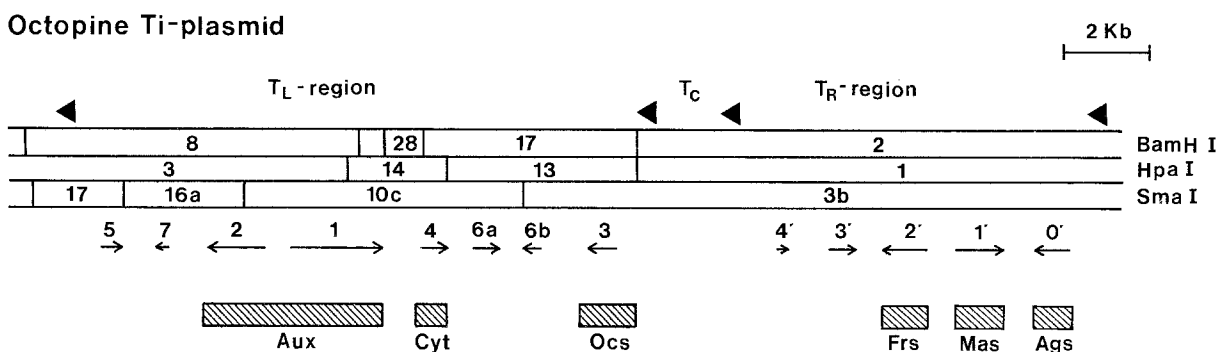


Fig. 3. Map of the transcripts encoded by the octopine Ti T-DNAs. Triangles indicate border repeats. The loci for Aux, Cyt, Ocs, Frs, Mas and Ags contain genes for IAA synthesis, isopentenyl transferase, octopine synthase, fructopine synthase, mannopine synthase and agropine synthase, respectively [53, 186].

phenomenon is unknown, but is thought to have to do with the chromatin structure at the insertion site. For some tumour lines it has been found that the expression of a few or all of the T-DNA genes is affected by DNA methylation. Treatment of such lines with the demethylating agent 5-azacytidine leads to the reappearance of T-DNA gene expression [5, 61, 176].

The T-DNA does not integrate at specific positions in the nuclear genome. Seven Ti T-DNA inserts were mapped on five different chromosomes of tomato [38], while for *Crepis capillaris* T-DNA was found to integrate into any of its three chromosomes [6]. The selection for expression of the oncogenic properties of the T-DNA will of course restrict the apparent integration sites to those regions that are transcriptionally active in the course of tumour induction and development. The DNA sequences of wild-type and T-DNA-tagged genomic loci were compared in order to find out whether integration occurs at certain preferred DNA sequences [54, 98, 100]. The results from the analysis of 17 independent insertion events showed that T-DNA integration occurs via illegitimate recombination on short stretches of DNA homology and is accompanied by short (29–73 bp) target site deletions.

T-DNA genes are responsible for the ability of crown gall cells to grow *in vitro* in the absence of phytohormones and to produce opines. Mutagenesis studies of the octopine Ti plasmid revealed that genes in the T_R region are responsible for production of the opines agropine and mannopine in transformed plant cells [85], while a gene in the T_L region is necessary for octopine formation [93]. This latter gene was cloned and expressed in *E. coli* and then found to code for the enzyme octopine synthase, which is able to convert arginine and pyruvic acid into octopine. The mutagenesis of the T_L region of the octopine Ti plasmid revealed that mutations at three loci led to changes in oncogenicity of the host bacterium. Such mutants were non-oncogenic on tomato, and formed tumours with an aberrant morphology on tobacco and kalanchoe [52, 111]. Since the supplementation of either auxin or cytokinin restored oncogenicity of such mutants on tomato,

it was concluded that the mutations had inactivated genes which cause either an auxin or a cytokinin effect in plants [111]. Therefore, two of the T-DNA genes involved were called *aux* genes and the third the *cyt* gene. The *aux* mutants induced shooty tumours on tobacco and kalanchoe, whereas the *cyt* mutants formed rooty tumours on these plant species. Because of this the *aux* genes later on were also called *tms* (tumour morphology shoot) or *shi* (shoot inhibition) genes, and the *cyt* gene the *tmr* (tumour morphology root) or *roi* (root inhibition) gene [52, 93]. These tumour phenotypes on tobacco correspond to the response of tobacco tissue to an excess of auxin or cytokinin, respectively, in *in vitro* tissue culture media [138]. Expression of the *cyt* gene in *E. coli* showed that the protein encoded by this gene was an isopentenyl-transferase capable of catalysing the formation of the cytokinin isopentenyl-AMP from isopentenyl-pyrophosphate and AMP [11]. Therefore, the *cyt* gene is often called *ipt* gene now. Similarly, the expression of each of the two *aux* genes in *E. coli* revealed that they together mediate a pathway for synthesis of the auxin indole-acetic acid (IAA). The protein encoded by the *aux-1* gene turned out to be a mono-oxygenase capable of converting tryptophan into indole acetamide (IAM), and hence the *aux-1* gene is now called *iaaM* [159]. The enzyme determined by the *aux-2* gene had hydrolase activity, and was capable of converting IAM into IAA. The *aux-2* gene is, therefore, now called *iaaH* [132]. It has to be noted here that the pathway of IAA synthesis via the intermediate IAM does not occur normally in plants, but proceeds via indole pyruvic acid as an intermediate. The (over)production of an auxin and a cytokinin via the T-DNA explains why crown gall cells proliferate even in the absence of externally applied phytohormones. Crown gall cells apparently are 'autocrine.' Production of opines, the second characteristic property of crown gall cells, is similarly explained by the finding that the T-DNAs have genes coding for opine synthases.

Besides the genes mentioned above, the octopine T_L -DNA contains some genes with a still unknown function. Inactivation of these genes

did not affect oncogenicity of the host bacterium. For one of these genes (a gene named δ^b) it was shown that it had an oncogenic effect for certain plant species even in the absence of the other T-DNA genes [70]. For another one (a gene called 5) it was recently found that it encodes an enzyme capable of forming indole-lactic acid, an inhibitor of the auxin response and thus a modulator of the effects brought about by an excess of auxin [89]. Although these genes are not of prime importance for tumour formation on most plant species, it may be that they are necessary for oncogenicity on certain specific host plants.

The T regions of nopaline, succinamopine and leucinopine Ti plasmids embrace *ipt*, *iaaH* and *iaaM* genes that are closely related to those of the octopine Ti T_L region. It is interesting to know that these same genes are also present in some other species of phytopathogenic bacteria such as *Pseudomonas syringae* pv. *savastanoi* that produce auxin and cytokinin [191]. Most *Agrobacterium* strains induce tumours on a wide range of dicotyledonous plant species. However, strains isolated from grapevine often have a limited host range for tumour induction [115]. These limited host range (LHR) strains are clearly of the octopine type, but have strongly rearranged T_L and T_R regions in the Ti plasmid [26]. Many of these LHR strains lack a functional *ipt* gene in their T_L region, and it has been demonstrated that this is one of the reasons for their limited host range. Reintroduction of the *ipt* gene from a wide host range strain resulted in an extension of the host range of the LHR strain AG57 [68]. The absence of the *ipt* gene from the T-DNA may be one of the reasons that the LHR strains are efficient tumour inducers on grapevine. A wide host range strain was found to become able to induce tumours on certain grapevine varieties only after the inactivation of its *ipt* gene [60]. Evidence is accumulating that in LHR strains the δ^b gene is a very important determinant of oncogenicity [164].

Since tumour formation had not been observed on monocotyledonous plant species [44], it was generally assumed that T-DNA transfer does not occur to such plants. However, tumour formation is the end-result of a complex process in which a

large number of discrete steps are involved including recognition of plant (target) cells by *Agrobacterium*, attachment of the bacterium to the plant cells, T-DNA transfer, T-DNA expression, T-DNA integration into the genome, and symptom expression by the transformed plant cells. It can be imagined that T-DNA transfer is not always accompanied by symptom formation. Indeed, when transformed plant cells cannot be stimulated to divide by the phytohormones that are overproduced, or when the genes for production of these phytohormones are not expressed at all, transformation would not lead to tumour formation. Since the T-DNA contains genes for opine production, opines and opine synthases can be used as alternative markers for the detection of T-DNA transfer. On the basis of these considerations in 1983 we set out to find evidence for T-DNA transfer to plant species that do not form tumours in response to infection with *Agrobacterium*, viz. the monocots *Chlorophytum capense* and *Narcissus* cv. Paperwhite [72]. These plants were infected with different types of *Agrobacterium* strains and small swellings different from typical tumours were observed some weeks after infection. In order to avoid misinterpretation due to earlier observed artefacts or to the presence of compounds unique to these plant species [37], we avoided substrate feeding and used extracts of these swellings directly in an enzyme assay that was developed earlier to detect opine synthase activity [113]. In this way we obtained evidence that not only specific opines, but also the specific opine synthases were present in tissues that had been infected with *Agrobacterium*. As expected, octopine and octopine synthase activity were only present in tissues that had been infected with octopine strains and not in those infected with nopaline strains, while nopaline was found only in tissues infected with nopaline strains. Thus, unequivocal evidence was found for the – perhaps unexpected – transfer of T-DNA to the ‘non-host’ – with regard to tumour development – monocotyledonous plant species [72]. Our findings were corroborated by subsequent similar findings in which other monocotyledonous plant species such as *Asparagus officinalis* [62],

Dioscorea bulbifera (yam) [133], *Zea mays* [55] and *Triticum aestivum* (wheat) [182] were used. Later on the even more sensitive reporter system of agroinfection was developed by Grimsley *et al.* [56] in which a plant virus is transmitted to plant cells at the infection sites concomitantly with the T-DNA. Transformed plants are then recognized by the symptoms of viral infection. Using this reporter system further clear evidence was obtained for T-DNA transfer to gramineous species such as *Zea mays* [57], *Triticum aestivum* and *Hordeum vulgare* (barley) [20]. From the data obtained so far it is obvious that the T-DNA transfer system may be exploited for the introduction of DNA into an extremely wide variety of plant species, although the efficiency with which this occurs may differ from one species to the other.

Genetic colonization

The process of crown gall induction consists of a large number of discrete, essential steps. First, wounding of the plant is necessary [22] to allow entrance of the bacteria and to make available compounds that induce its virulence system (see below). The bacteria multiply in the wound sap and attach to the walls of plant cells in the wound [95, 131]. Subsequently, the T-DNA is transferred and expressed in the plant cells even before integration [75]. After integration T-DNA expression is maintained at a particular stable level depending on the position of integration. After some time tumours develop due to cell divisions triggered by the continuous production of auxin and cytokinin via T-DNA encoded enzymes. The resulting tumours consist of a mixture of transformed (T-DNA-containing) and normal plant cells [177]. The T-DNA-containing cells produce and excrete opines that are consumed specifically by the infecting agrobacteria. Octopine strains can utilize octopine but not nopaline, while nopaline strains catabolize nopaline, but not octopine [19, 123]. The genes for opine catabolism are located on the Ti plasmid (Fig. 4). An opine may act not only as an inducer of its catabolic genes, but also as an aphrodisiac and activate the conjugative

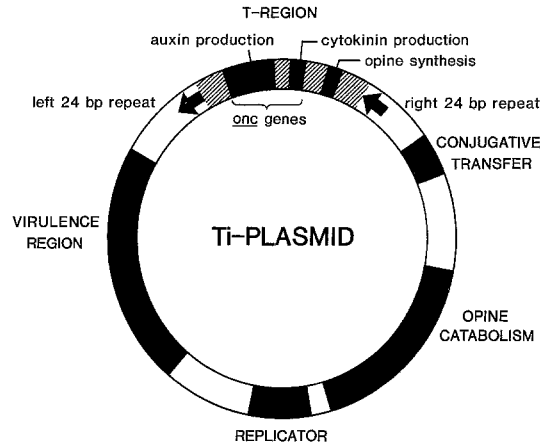


Fig. 4. Genetic map of an octopine Ti plasmid.

transfer system of the Ti plasmid [82, 158]. This is the reason that the Ti plasmid is widely spread through the bacterial population in plant tumours [81]. Since agrobacteria exploit plant cells by inducing these to produce compounds that are of specific use only to agrobacteria and do this by way of genetic engineering, the process has been called genetic colonization.

Molecular mechanism of T-DNA transfer

Virulence genes

By genetic analysis of *Agrobacterium* it was shown that besides the *onc* genes (*ipt*, *iaaM*, *iaaH*) present in the T-DNA a large number of other genes involved in tumorigenicity are present either on the Ti plasmid in a segment of 40 kb called the virulence region (*vir* genes) or on the chromosome (*chv* genes). By introducing T-DNA into plant cells *in vitro* via direct gene transfer, it was found that T-DNA by itself is sufficient for provoking the transformation of normal plant cells into tumour cells [87]. This was the first evidence that the *vir* and *chv* genes do not have an essential oncogenic function, but rather determine the apparatus necessary for *in vivo* transfer of the T-DNA from *Agrobacterium* to plant cells. Also molecular genetic experiments performed by Lee-mans *et al.* [93] and Hille *et al.* [67] showed that

none of the T-DNA genes is required for T-DNA transfer. Even when all the genes that are naturally present in the T region are inactivated or replaced with other genes the transfer of T-DNA still occurs provided that the border repeat remains intact.

The *chvA* and *chvB* genes are necessary for the attachment of *Agrobacterium* to plant cell walls [46]. It was found that the *chvB* gene codes for a 235 kDa protein involved in the formation of a cyclic β -1,2 glucan [200], 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 [29]. Mutations in another chromosomal virulence gene, which is called *pscA* or *exoC*, also lead to bacteria which do not produce β -1,2 glucan [160]. This points to a possible role of β -1,2 glucan in the attachment of agrobacteria to plant cell walls. The addition of β -1,2 glucan to *chv* mutants in plant infection experiments, however, did not result in tumour formation. More recently it was found that *chv* mutants lack a protein that was called rhicadhesin and that might be involved in attachment [139]. Addition of this protein to *chv* mutants during plant infection led to a partial restoration of the ability to induce tumours. The 40 kb *vir* region of the octopine Ti plasmid embraces 24 genes involved in virulence. These genes are present in 8 operons called *virA-virH*, which are co-regulated and thus form a regulon (see below). Most of the operons contain several genes (Fig. 5). The complete nucleotide sequence of the *vir* region of a nopaline Ti plasmid [128] and an octopine Ti plasmid [15] have been established.

Regulation of the virulence genes

With the exception of the *virA* and *virG* genes, the *vir* operons are not transcribed during normal vegetative growth [150]. Therefore, one of the early steps in plant tumour induction concerns the coordinate activation of the virulence system, when the bacteria are present near (wounded) plant tissues and sense plant cell exudate factors [150]. While the known *chv* genes are constitutively expressed, the *vir* genes are silent until they become induced by certain plant factors. Stachel *et al.* [148] identified these plant factors from tobacco as being the phenolic compounds acetosyringone and α -hydroxyacetosyringone (Fig. 6). These compounds are released from plant tissue, especially after wounding, which has been long known to be a prerequisite for plant tumorigenesis via *Agrobacterium*. Further work by several groups including our own showed that besides (α -hydroxy)acetosyringone several other phenolic compounds can act as *vir* inducers including well-known lignin precursors such as coniferyl alcohol and sinapinic acid [103, 143, 145]. Recent evidence suggests that also some flavonoids known as *nod* inducers in *Rhizobium* may act as *vir* inducers [197].

For different plant species or plant tissues different substituted phenols may be responsible for induction. For instance, although (α -hydroxy)-acetosyringone was found to be the most prominent inducer in solanaceous plants such as tobacco, tomato and potato, other species rather tended to have derivatives of benzoic acid or cinnamic acid as inducers [143, 146]. For wheat suspension cells for instance the *vir* inducer re-

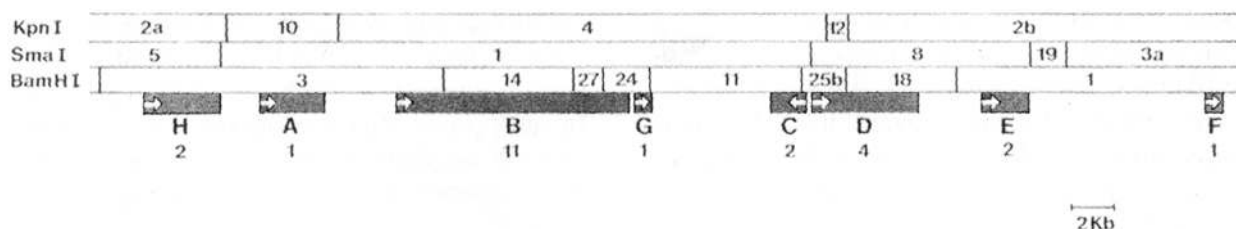


Fig. 5. Loci present in the virulence region of the octopine Ti plasmid. Letters refer to names of the *vir* operons, numbers to the number of genes in these operons. The direction of transcription is indicated.

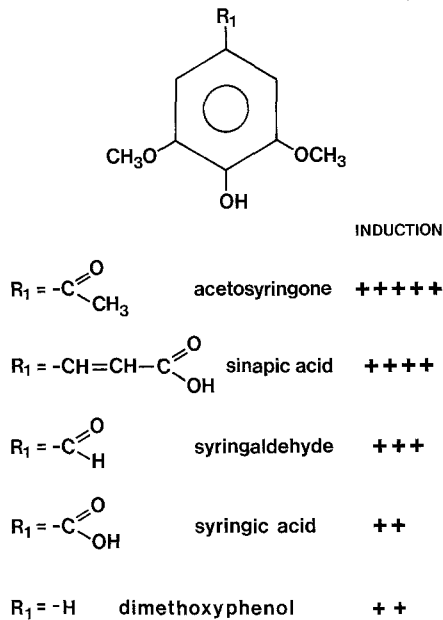


Fig. 6. Induction of the virulence genes by acetosyringone and some other phenolic compounds.

leased was found to be ethyl ferulate [107]. Plants may also excrete compounds that are inhibitory to *vir* induction. Certain maize varieties have an inhibitor in their root exudate that turned out to be 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) [130]. In a few cases the amounts of *vir*-inducing compounds were found not to be optimal for tumour induction to occur. In such cases the addition of acetosyringone during infection stimulated tumour induction. This was the case for leaf disc infection of *Arabidopsis thaliana* [135] and tuber disc infection of the monocot *Dioscorea bulbifera* [133].

The induction of the *vir* system can be monitored most easily for indicator strains carrying a *vir* gene promoter linked to a reporter gene such as the *lacZ* gene (encodes the enzyme β -galactosidase) of *E. coli*. The presence of β -galactosidase is easily measured with substrates such as 0-nitrophenyl- β -d-galactopyranoside (ONPG) or 5-bromo-4-chloro-3-indolyl- β -d-galactopyranoside (X-gal) that release coloured compounds after β -galactosidase action. Using cultures of such indicator strains it was found that particular conditions have to be met – even in the pres-

ence of an inducer – in order to obtain optimal *vir* induction [4, 104, 150, 167]: (1) the pH of the medium must be between 5 and 6, (2) the temperature must be between 20 and 30 °C, (3) the presence of yeast extract in the medium must be avoided, (4) a high sugar content must be present in the medium. Recently, it was found that some specificity exists in the sugars that are required for optimal *vir* induction [28, 136, 142]. In 'conditioned' plant medium the presence of a high level of inositol enhances *vir* expression [142]. Also some non-catabolizable sugars such as 2-deoxy-*d*-glucose and 6-deoxy-*d*-glucose have such a stimulatory effect [7, 136]. This sugar effect is seen most clearly when phenolic inducers are limiting. Although the *vir* systems of different Ti plasmids are similar and are inducible in similar induction media, there are small, but significant differences in their prerequisites for optimal induction [167]. For instance octopine and leucunopine Ti strains require a lower pH for optimal induction than nopaline and succinamopine Ti strains. This probably reflects small differences in the regulatory proteins that control the *vir* system. The maximal level to which the *vir* system can be induced also varies for different strains. While the *vir* system in LHR strains is only inducible by acetosyringone to a low level, it is possible to induce the supervirulent leucinopine Ti strain Bo542 to a much higher level than octopine or nopaline Ti strains under the same conditions. There are some indications that the level to which the *vir* system can be induced correlates with virulence on certain host plants [127, 187].

Two proteins encoded by the virulence region, VirA and VirG, mediate the activation of the other *vir* genes in the presence of phenolic inducers [150]. Sequence analysis revealed that the *virA* [94, 105] and *virG* [106, 188] genes resemble genes of other two-component regulatory systems such as *envZ-ompR*, *ntrB-ntrC*, *dctB-dctD* [108]. In such two-component systems the VirA-like proteins are thought to be sensors for a specific signal (phenolic compounds in the case of VirA), while the VirG-like proteins are thought to be DNA-binding activator proteins. Recent data obtained with the NtrB-NtrC [80], EnvZ-OmpR

[2] and CheA-CheY [21] systems have shown that the sensor protein can become phosphorylated and then can act as a specific protein phosphorylase for the accompanying activator protein. *In vitro* binding of the OmpR protein to the *omp* promoters, on which this protein acts, turned out to be efficient if the protein preparation was from cells that had been grown in a high-osmolarity medium, but inefficient if the preparation was from cells grown in a low-osmolarity medium [50]. Osmolarity is what is being sensed by the EnvZ protein which acts on OmpR. For the *vir* system we and others have shown that the VirA protein is present in the bacterial inner membrane [94, 105] and therefore is theoretically in the proper position to sense phenolic compounds directly. The topology of the VirA protein was analysed in more detail by making fusions with the *E. coli* protein PhoA (for alkaline phosphatase) devoid of its signal sequence [104, 189]. Since the alkaline phosphatase protein only becomes active after transport across the bacterial inner membrane, it can be used as a tool to probe the topology of predicted transported proteins [96]. Since only PhoA-VirA fusions in the predicted periplasmic domain of VirA resulted in alkaline phosphatase activity, these experiments support the model in which the VirA protein has a cytoplasmic N-terminus, a first hydrophobic transmembrane α -helix (TM1), a periplasmic domain, a second hydrophobic transmembrane α -helix (TM2) and a large C-terminal cytoplasmic domain (Fig. 7). With this topology VirA resembles many other sensor proteins (e.g. EnvZ, Tar, Tsr). In order to find out where the receptor function for acetosyringone was located in VirA, we made hybrids between VirA and the *E. coli* Tar protein (sensor for aspartate and maltose) and assayed the function of such hybrid proteins in a *virA* mutant background [104]. Our results showed unexpectedly that the periplasmic domain does not contain the receptor for acetosyringone, but point to the possibility that an acetosyringone receptor domain is located in the TM2 region or in a neighbouring cytoplasmic portion of VirA [104]. Acetosyringone is a lipophilic compound that easily would accumulate in the bacterial inner

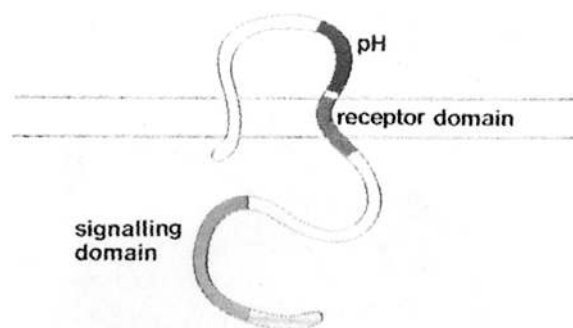


Fig. 7. Topology of the VirA protein. Positions of the receptor domain and signalling (kinase) domain are indicated; pH refers to an area influencing pH sensitivity of induction.

membrane or pass through it. Certain mammalian receptors such as the adrenergic receptors are known to have their receptor domain (for phenolic compounds such as dopamine) formed by a number of transmembrane helices [51, 154]. It is rather striking that serine and cysteine residues that form part of the binding pocket of the adrenergic receptors are also present and conserved in the TM2 domains of VirA proteins from various Ti plasmids. Another interesting feature of the VirA TM2 domain is its potential to form a leucine zipper structure. It may therefore be that dimerization of VirA plays a role in the signal transduction via VirA.

Part of the cytoplasmic domain of VirA is homologous to other sensor proteins. It was found that this domain can act as an autokinase phosphorylating itself on a conserved histidine residue [74, 78]. The phosphorylated VirA protein has the capacity to transfer its phosphate to a conserved aspartate residue in the VirG protein *in vitro* [77]. It is likely that phosphorylation modulates the DNA-binding VirG protein in a way that it stimulates *vir* gene transcription (Fig. 8). The VirG protein binds to specific areas of *vir* promoters probably as a dimer or multimer [118, 155]. The structure of the N-terminal part of the VirG protein has been predicted on the basis of the crystal structure of the homologous CheY protein of *E. coli* which revealed that the VirG protein has an acidic pocket similar to that of CheY where phosphorylation occurs [125]. The

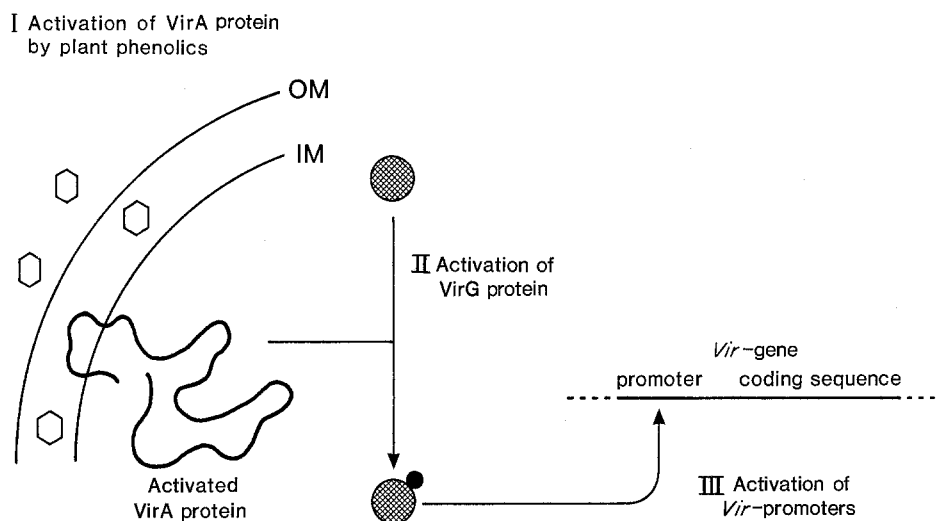


Fig. 8. Activation of the *vir* genes via VirA and VirG.

C-terminal part of VirG has been shown to have DNA-binding activity [118].

It is remarkable that the C-terminal 110 amino acids of VirA have similarity to the N-terminal (phosphorylation) domain of VirG. The function of this domain is unknown, but one could speculate about a regulatory (modulating) role in the process of *vir* regulation. Deletion of part of this region strongly reduced the activity of the VirA protein, but the deletion of the complete 110 amino acid domain led to a VirA mutant protein that was in *in vitro* assays almost as active as the wild-type VirA protein [166, 187].

T-DNA processing and transfer

After induction of the *vir* system single-stranded (ss) molecules (called T-strands) that represent the bottom strand of the T-region can be detected in *Agrobacterium* [149]. However, with lower frequency also double-stranded (ds) T-molecules have been detected by physical or genetic analysis [47, 151, 163], and therefore it is still a matter of debate in what form the T region segment is transferred to plant cells. The formation of both ss- and ds-T molecules is dependent on the activity of two proteins called VirD1 and VirD2 that are encoded by the *virD* operon of the *vir* region

[194]. These proteins together determine an endonuclease activity capable of nicking (introducing ss breaks) the border repeats at a precise site, which is of course in agreement with their suspected role as recognition signals for the transfer system. Since nicking occurs at a precise site which is conserved in all border repeats sequenced so far, it was a surprise to find that mutagenesis of this nick site in the right-border repeat of the T-region (conversion of 3'G-T5' into 3'A-T5') did not lead to avirulence of the host bacterium [169].

It is likely that the nick sites act as starting points for DNA synthesis in the 5' → 3' direction. T-strands will then be released by displacement (Fig. 9). Alternatively, such nicks may be used by recombination systems to dissociate the T-region in a ds form from the Ti plasmid at a low frequency [163]. That the nick sites in the border repeats define the DNA segment that is transferred to plant cells became evident from the fact that from transformed plant cells 3 bp at most from the right-border repeat and 21 bp from the left-border repeat can be recovered [9]. It had been observed earlier that the deletion of the right-border repeat almost completely abolished T-DNA transfer [112, 134], whereas the deletion or mutation of the left repeat only led to a slightly lower frequency of transfer [67]. This observed

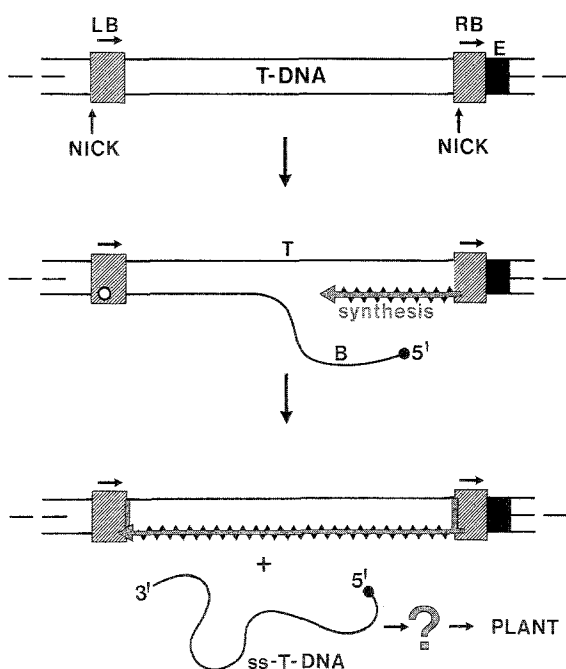


Fig. 9. Model for formation of T-strands in *Agrobacterium tumefaciens*. Black dot at the 5' end of the T-strand indicates the covalently bound VirD2 protein.

polarity of the system is of course in agreement with the importance of T-strands as intermediates since these are probably formed after 5' → 3' displacement synthesis from right border to left border repeat. The absence of a right border repeat would be lethal to such a system since T-strand synthesis would not start at all, while it can be imagined that termination of the process might occur – albeit with lower efficiency – even when a left border repeat is absent. For the formation of ds T-molecules via recombination left and right-border repeat would be equally important. Therefore, recombination is apparently not involved in formation of T-DNA intermediates. However, one can still speculate that ds intermediates are formed in alternative ways.

A question that remains is why the DNA segment to the left of the left border repeat is not transferred with high efficiency to plant cells. Of course transfer of this area would not lead to tumour formation, but the same is true for the T_R region of the octopine Ti plasmid which is de-

tected regularly in tumour lines. The reason for this turned out to be the fact that left border areas are much less efficient in acting as starting sites for DNA synthesis than right borders [122, 168]. This difference is not due to the subtle differences in the nucleotide sequences of the left and right 24 bp border repeats, but rather is caused by the presence of an enhancer next to the right sequence [121]. This T-DNA transfer enhancer, also called 'overdrive' by Peralta and Ream [121], strongly enhances T-strand formation in *Agrobacterium* [170]. It is called an enhancer because it functions in both orientations and at different positions and distances from the border repeat sequence [170]. The deletion of the enhancer sequence leads to a diminished virulence of the host bacterium [122, 168]. Toro *et al.* [165] have found that the VirC1 protein specifically binds to the overdrive sequence. Mutations in the *virC* operon result in an attenuation of virulence. In order to find out how plant cells would deal with ssDNA, we have introduced ssDNA into tobacco protoplasts via electroporation [126]. Results indicate that ssDNA is quickly converted into dsDNA in plant cells. In spite of this, ssDNA turned out to be a more effective vehicle for stable plant cell transformation (somewhat higher transformation frequencies) than dsDNA [126]. If *Agrobacterium* indeed introduced ssDNA into plant cells, this result may at least partially explain why *Agrobacterium* is so efficient in transforming plant cells. One should note that it is likely, however, that *Agrobacterium* does not introduce naked DNA molecules into plant cells. Recent evidence indicates that T-strands retain the VirD2 protein covalently attached to the 5' terminus [63, 183]. The presence of VirD2 makes the 5' end of the T-strand less vulnerable to an attack by exonucleases [47]. Besides the VirD2 protein may act as a pilot to direct the T strand to the nucleus of the transformed plant cell, since it contains nuclear targeting sequences [64]. The 69 kDa VirE2 protein encoded by the second open reading frame (orf) of the *virE* operon is a ssDNA-binding protein, which is able to coat the T-strands by cooperative binding leading to long, thin nucleo-protein filaments [39]. However, the

presence of such nucleoprotein complexes (T-complexes) in transformed plant cells has not been demonstrated yet.

The introduction of nicks at border repeats by the VirD system followed by ss T-strand formation is reminiscent of what occurs in the initial steps of bacterial conjugation. In this latter process a specific nick is made at a sequence called origin of transfer (*oriT*) via Mob- or Tra-proteins, which is followed by the formation of single-stranded molecules via (rolling circle) displacement synthesis [185]. In bacteria ssDNA is transferred from donor to recipient via a conjugative pore (encoded by Tra proteins) that is formed after the bacteria have been brought into close contact via the sex pilus (encoded by other Tra proteins) [185]. Recently, Pansegrau and Lanka [116] observed that there was not only homology between the border repeat sequences of Ti plasmids and the *oriT* of *incP* plasmids, but also between the nicking enzymes VirD2 of Ti and TraI in *incP* plasmids. In fact, the *virD* operon of Ti plasmids, which contains four genes called *virD1*, *virD2*, *virD3* and *virD4*, seems analogous to the mobilization operon of the *incP* plasmids containing the genes *traJ*, *traI*, *traH* and *traG*, although only *traG* and *virD4* share strong DNA homology [199]. It is interesting to note that the *traK* gene of *incP* plasmids, which does not bind to the nick site of *oriT* but to a neighbouring enhancing sequence, shares a high proline content with the product of the *virC1* gene, which binds to the T-DNA transfer enhancer.

The recent data described above make clear that the initial steps of T-DNA transfer and bacterial conjugative transfer are similar. They are in line with the initially surprising finding of Bucha-

nan-Wollaston *et al.* [25] that *incQ* plasmids are transferred to plant cells from agrobacteria harbouring the Ti virulence genes provided that the *oriT* sequence and the *mob* genes of the *incQ* plasmids are functional. These results point to a strong relationship between T-DNA transfer from *Agrobacterium* to plant cells and conjugative DNA transfer between bacteria. Since in the latter case ssDNA is transferred from donor to recipient, this might be taken as an extra argument in favour of ssDNA being the material that is introduced by *Agrobacterium* into plant cells.

Above the roles played by VirD, VirC and VirE proteins in T-complex formation are described in detail, as well as the way *vir* expression is regulated via VirA and VirG. The remaining Vir-proteins are not involved in regulation of expression or T-strand formation; only those encoded by the *virB* operon are essential for virulence. Sequencing of the octopine [162, 184a] (and nopaline [88, 128]) Ti *virB* locus showed that it contains a complex operon consisting of 11 genes (Fig. 10). Most of the proteins predicted for the *virB* operon are located in the membrane, and we and others have therefore suggested that these proteins together may form a structure (conjugal pore or pilus) through which the T-DNA is delivered into the plant cell [162, 184]. The *virB11* gene has an ATP binding site [162], and more recently the protein was found indeed to have ATPase activity [35]. It may therefore be involved in delivering energy required for T-DNA transfer. Remarkably, the *virB11* gene has clear DNA homology with the *comG* gene of *Bacillus subtilis* that is involved in ssDNA uptake by competent cells of this bacterium [3]. The VirB10 protein was found to form aggregates sticking from the inner

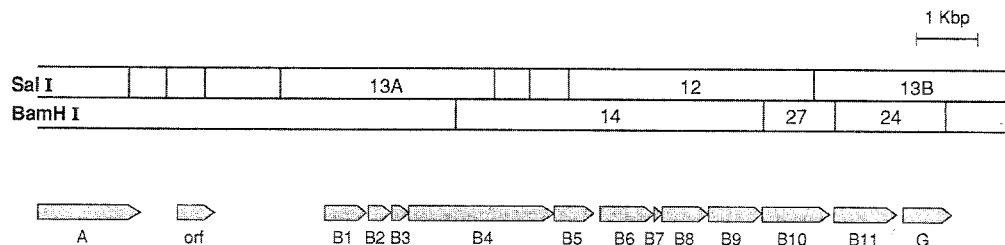


Fig. 10. Structure of the *virB* operon as determined by nucleotide sequence analysis [88, 128, 162, 184].

membrane into the periplasm [184]. Via the *phoA* system (described above for determining the VirA topology in detail) further evidence for export or membrane location was obtained for the *virB1*, *virB2*, *virB5*, *virB6*, *virB7* and *virB10* gene products [14]. In this way it was also demonstrated that the small open reading frame corresponding to the *virB7* gene indeed encoded a protein that is exported over the inner membrane and may have an outer membrane location [14].

Recently, it was found that the conjugative transfer system of *incP* plasmids can be used to introduce DNA into yeast cells [59, 137]. Apparently the *incP* type conjugal pore can be formed even between such widely diverse organisms as yeasts and bacteria. Since it might be that the *virB* operon determines a transfer apparatus similar to that of conjugative plasmids, we tried to find some experimental evidence for this. The approach we took was to investigate whether indeed the *vir* system could replace the *tra* system of conjugative plasmids in the mobilization of the non-conjugative wide host range *incQ* plasmids between bacteria. Hereby, we speculated that the transfer apparatus determined by the *virB* system would not be specific to bridge bacterial cells with plant cells but would also be able to bring together bacterial donors and recipients. Of course we used an octopine Ti plasmid from which the (octopine-inducible) conjugative transfer genes had been deleted in these experiments [13]. In full agreement with our hypothesis we found that the *vir* system was able to mobilize *incQ* plasmids into recipient *A. tumefaciens* and *E. coli* cells. As expected the system only was operative after induction with acetosyringone. Mutagenesis experiments showed that the mutation of *virA*, *virG*, *virB* or *virD4* led to a complete loss of *incQ* transfer ability [13]. This corroborates with the proposed role of the VirB and VirD4 proteins in determining a transfer apparatus similar to that of conjugative plasmids.

The octopine and nopaline Ti plasmids have a few accessory *vir*-genes that are specific for these plasmids and affect the host range for tumour formation. In the octopine Ti plasmid these are *virF* and *virH*, in the nopaline Ti plasmid a gene

called *tzs*. The *tzs* gene codes for an enzyme that is similar to that determined by the T-DNA gene *ipt* and is involved in cytokinin production that is excreted from the cells as *trans*-zeatin [12]. The presence of this gene might result in enhanced tumorigenicity on certain host plants [198]. The *virH* operon consists of two genes that code for proteins that show some similarity to cytochrome P450 enzymes [79]. These proteins may therefore have a role in the detoxification of certain plant compounds that might otherwise adversely affect the growth of *Agrobacterium*. Enhanced tumorigenicity was observed for bacteria having the *virH* genes as compared to those lacking these on certain hosts [79]. The *virF* operon encodes one 23 kDa protein which shows no obvious homology to any of the proteins for which sequences are available in data banks [102]. Presence of the *virF* gene in octopine Ti strains makes these vastly superior to nopaline strains in transferring DNA to *Nicotiana glauca* and some other plant species. Using reporter genes we recently found that *virF* plays a role in T-DNA delivery rather than symptom formation [124]. A striking feature of *virF* is that it like *virE* shows 'trans-complementation', i.e. bacteria lacking *virF* can be complemented for tumour formation by coinfection with bacteria lacking a T region but having *virF*. Cell exudates or cell extracts of *virF*⁺ cells do not give trans-complementation [124]. Therefore, it may not be a product made via *virF* that is needed for complementation but rather the VirF protein itself. Indeed, trans-complementation only works if the complementing bacterium carries a complete *vir* system. Localization experiments showed that the *virF* gene product has at least partially a membrane location, but evidence for secretion was not found [124]. All these data point to the possibility that the VirF protein is delivered into plant cells via the *vir* system and functions there. In order to test this we made transgenic *N. glauca* plants in which the *virF* gene is expressed from the CaMV 35S promoter. Such engineered *N. glauca* plants are now equally good hosts for *virF*⁺ as for *virF* strains, showing that indeed the VirF protein can exert its function when present in plant cells [124]. Together our results indicate that proteins are

delivered into plant cells via the *vir*-system even in situations when there is no T-DNA transfer. In view of the similarities between T-DNA transfer and conjugative plasmid transfer the same may be true for the latter process.

Applications

Vector systems

Although besides the T-DNA no other parts of the Ti plasmid become integrated into the genome of plant cells [17], it has long been debated whether the entire Ti plasmid or just the T-DNA segment was introduced into plant cells via *Agrobacterium*. Experiments in which the T-region was separated from the rest of the Ti plasmid [45, 69]. Genetic experiments showed that these two parts were maintained on independent replicons indeed, and did not form a cointegrate again [69]. This firmly established that no physical linkage between the T-region and the rest of the Ti plasmid was necessary for T-DNA transfer to occur. As described above the transfer system is determined by the *vir* and *chv* genes, while the 24 bp

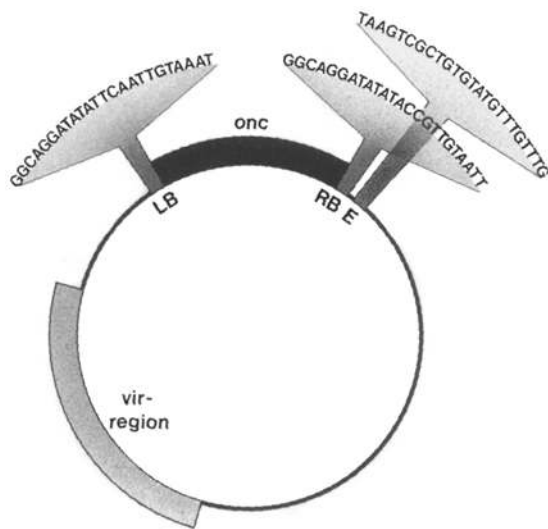


Fig. 11. Regions of the Ti plasmid important for tumorigenicity: *vir* region, border repeats (RB, LB) and enhancer (E) are involved in T-DNA transfer, and the T-DNA with *onc* genes brings about symptoms on plants.

direct repeat which flanks the T-region is essential as recognition signal for the transfer apparatus (Fig. 11). On the basis of these results vector systems for the transformation of plants have been developed (Fig. 12). These can be distinguished into two types: (1) *cis* systems in which new genes are introduced via homologous recombination into an artificial T-DNA already present on the Ti plasmid [196], (2) binary systems in which new genes are cloned into plasmids containing an artificial T-DNA, which are subsequently introduced into an *Agrobacterium* strain harbouring a Ti plasmid with an intact *vir* region, but lacking the T region [16, 45, 69].

Transgenic plant cells carrying a wild-type (oncogenic) Ti T-DNA are tumorous and cannot be regenerated into plants. However, plant cells transformed with disarmed, i.e. non-oncogenic T-DNA behave in the same way as untransformed plant cells of the same species in tissue culture and during regeneration.

After use of *Agrobacterium* for the delivery of disarmed T-DNA, mature transformed plants are being obtained for an ever increasing list of plant species including crops such as tobacco [73], potato [153], rapeseed [31] and asparagus [27]. Such transgenic plants were indistinguishable from untransformed plants, although sometimes aberrations were observed due to somaclonal variation occurring during tissue culture. In order to be able to detect or select transformed plant cells new markers have been developed. Selection markers are based on the sensitivity of plant cells to antibiotics and herbicides. It was found that

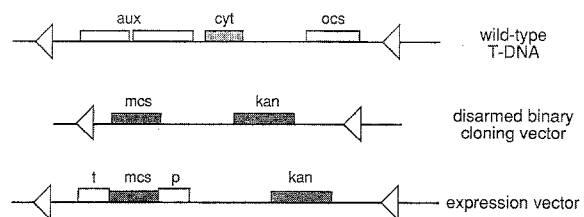


Fig. 12. Construction of plant vectors in which the *onc* genes in the T region are replaced by genes that do not disturb plant development. mcs, multiple cloning site; kan, kanamycin resistance gene for plants; p, promoter; t, terminator.

expression of (bacterial) genes coding for enzymes capable of detoxifying such compounds in plant cells can make these resistant. To this end chimaeric genes were constructed in which the (bacterial) sequence coding for the detoxifying enzyme was surrounded by plant expression signals that were obtained from the cauliflower mosaic virus (19S, 35S promoter), T-DNA genes (e.g. for octopine or nopaline synthase) or endogenous plant genes. Vectors are now available which allow selection for instance for kanamycin resistance [18] via the neomycin phosphotransferase (NPTII) gene from the bacterial transposon Tn5, hygromycin resistance [173] via the hygromycin phosphotransferase (HPT) gene from *E. coli*, methotrexate resistance [48] via the dihydrofolate reductase (DHFR) gene from mouse, or bialaphos (a herbicide) resistance [42] via the *bar* gene from *Streptomyces hygroscopicus*. In certain instances herbicides may be preferable because these can be sprayed and are well taken up by plants. Screening for transformation can be done by using the genes for opine synthase activities. Relatively new plant reporter genes include those coding for the enzymes luciferase [114], which gives light emission, β -galactosidase [157] and β -glucuronidase [76]. Because of the presence of endogenous β -galactosidase activity in many plant tissues, the use of β -glucuronidase is usually preferred. Reporter enzyme activity can be measured quantitatively using umbelliferoyl derivatives, which release umbelliferone after enzymatic activity that can be measured fluorometrically. Histological staining for the reporter enzymes can be done using 5-bromo-4-chloro-3-indolyl derivatives, which release a compound after enzymatic activity that is quickly converted into indigo (blue) with oxygen. In order to avoid expression of β -glucuronidase by *Agrobacterium*, gene constructs were made in which the gene lacked a bacterial ribosome-binding site [75] or contained an intron in its coding sequence [178]. Such constructs are being used for an early detection of transformation via *Agrobacterium* [75].

The *Agrobacterium* vector system is being used extensively now for the transfer of various traits to (crop) plants as well as for the study of gene

function in plants. Applications include the transfer of genes affecting such widely diverse traits as: resistance to viruses [1], herbicide tolerance [42], altered flower colour [175], altered shelf life of tomato [140], male sterility [97], cold tolerance [65], altered source-sink relationships [180], altered starch composition [179], starch derivatization to cyclodextrin [109], and resistance to pathogenic bacteria [8]. Although none of the transgenic crops produced is ready for marketing, field tests have been performed for quite a few of such modified crops and it is likely therefore that we shall begin to see these on the market in years to come.

Although the introduction of new traits into plants via the *Agrobacterium* system is now a common practice, there are still shortcomings in the system. The first is that it seems sometimes difficult to transform those cells in a tissue that are able to regenerate. It might be that these are in layers too deep to be reached by *Agrobacterium*, or simply are not targets for T-DNA transfer. Recently, an alternative system was developed for plant transformation with which it is – in contrast with previous alternatives such as Ca^{2+} /PEG coprecipitation, electroporation and microinjection – possible to introduce DNA sequences directly into cells rather than into protoplasts (cell walls removed) that have to be used with most of these alternative methods. In this novel procedure a particle gun is used with which small tungsten or gold microprojectiles that are coated with DNA are shot into plant tissues [83]. When a microprojectile reaches the nucleus, the DNA segment that it brought along is able to integrate into the genome and express its genes [192]. Such transformed plant cells can be regenerated into fertile, mature plants even for difficult species such as soybean [101] and rice [36, 129]. Although this has not been studied in detail, delivery of DNA via the particle gun may have the same disadvantages as other naked DNA transformation methods, i.e. scrambling of DNA copies and integration of multiple DNA copies that may be prone to recombination, rearrangement or silencing. The *Agrobacterium* system does not have such disadvantages, which is probably due to the structure

with which the T-complex is delivered into plant cells and the activity of Vir proteins in the plant cells. It may therefore be a good idea to use the particle gun to deliver DNA-protein complexes into plant cells that are similar to the T-complex known from *Agrobacterium*.

Perspectives

Although genes that are introduced into plant cells are usually expressed there may be large variations in the levels at which the genes are expressed. Such 'position' effects can affect even genes closely linked on one T-DNA in a different manner. The reason for this is unknown, but it may have to do with the chromatin structure at the integration site. Also general regulatory systems including those that act via the methylation of DNA may be involved in this [99]. The copy number of T-DNA often does not correlate with the expression level [119]. It has been observed that the introduction of extra T-DNA copies even can lead to gene inactivation, a phenomenon for which there is as yet no clear explanation and that has been called co-suppression [99]. For mammalian cells it has been found that transformation with genes that are surrounded by matrix attachment regions (MARs), sequences that form the contact points for chromatin proteins, leads to expression that is independent of the integration position [152]. For such genes there was found to be a direct correlation between copy number and expression level [152]. Thus the addition of MARs, which have been isolated from plants as well in the meantime [58], to genes that are to be delivered into plants, may help to avoid variations in the level of expression after transformation.

An alternative way to avoid position effects would be to target the genes to predetermined sites in the genome where expression is guaranteed. This may be accomplished by using either site-directed or homologous recombination systems. To this end the bacterial Cre-*loxP* [41] system was introduced into plant cells. Systems for gene targeting via homologous recombination would have the additional advantage that they

could also be used for the replacement or modification of genes endogenous to the plant genome. Unfortunately, in contrast to lower eukaryotes such as yeasts, fungi and protists, where integration occurs preferentially via homologous recombination, plants like mammalian cells integrate new segments of DNA only efficiently via illegitimate recombination. However, recent results show that homologous recombination can occur with a low frequency between an incoming DNA segment and a homologous copy endogenous to the plant genome. Whether the DNA was introduced via direct DNA transfer [117] or via the *Agrobacterium* vector system [92, 110] did not make much difference for the frequency with which recombination was observed, i.e. in 1 out of 10^4 to 10^5 transformants. From our results in this area we obtained unequivocal evidence that gene targeting, i.e. the modification of a locus in the plant genome, can occur in plant cells after the introduction of a homologous repair construct via *A. tumefaciens* [110]. In mammalian cells initially similar low frequencies for gene targeting were obtained. However, extensive further research led to the identification of variables that affect the frequency of gene targeting [30] and now gene targeting is used as a standard tool for the modification of the mammalian genome and the analysis of gene function. Therefore, one may hope that a similar development will be possible for plants if the process of homologous recombination is studied more carefully in these organisms.

The *Agrobacterium* vector system has also been used to tag and therefore identify plant genes influencing plant morphology (plant height, flower morphology, trichome formation). This approach has been especially successful for *Arabidopsis thaliana* for which Feldmann developed a simple seed transformation protocol with which large numbers of independent T-DNA-tagged mutants were obtained [49]. Using a T-DNA-tagged homeotic mutant, the *Arabidopsis* gene *agamous* was identified, which was found to encode a transcriptional regulator necessary for flower development [193].

Also special purpose T-DNA vectors have been developed for the identification of particular plant

genes. These include vectors that have a promoterless resistance or indicator gene located close to the border repeat [156]. Activation of expression can occur after integration into a transcriptionally active area. Unexpectedly, it was found that such gene activation occurs with an extremely high frequency, i.e. 30–50% of the plant cells transformed expressed the promoterless reporter or resistance gene [86]. This was similar for plants with a small genome (*Arabidopsis thaliana*: 10^8 bp) and those with a large genome (*Nicotiana tabacum*: 5×10^9 bp), which suggests that T-DNA integrates preferentially in potentially transcriptionally active areas. By the analysis of the gene expression pattern of the reporter construct in the tagged transgenic plants genes may be identified that have a tissue- or organ-specific expression pattern. Besides these promoter/enhancer trap constructs more recently also other novel types of T-DNA vectors were constructed, such as promoter/enhancer-out constructs which have a strong promoter/enhancer near one of the border repeats [174, 181]. It is hoped that with these genes can be identified involved in the regulation of growth and development. Tumour formation in mammalian systems is often due to the unregulated (over) expression of genes involved in the control of growth, and it can therefore be imagined that activation of similar genes in plants by an outward directed promoter/enhancer in the T-DNA may lead to tumour formation or an otherwise aberrant development. Another novel type of T-DNA vector contains a promoterless toxic gene such as that for diphtheria toxin, which is toxic to plant cells [40], near the border repeat [174]. It can be imagined that integration into a tissue- or developmentally-specific gene will lead to ablation of the tissue or a halt in development at a specific stage. With this latter type of construct also cell ablation experiments can be done by fusion of the toxin gene to well characterized promoters in the same way as is done in mice [24]. Another novel type of *Agrobacterium*/T-DNA vector system was recently described by Ludwig *et al.* [90]. This concerned the construction of a special *Agrobacterium* strain that expressed the *E. coli lamB* gene and since it expressed the LamB

protein became sensitive for *E. coli* bacteriophage λ . Thus cosmids can easily be introduced into this strain. A special cosmid vector was constructed containing between T-DNA borders plant selectable markers and a *cos* site. A cosmid bank containing genomic fragments from *Arabidopsis thaliana* was established in this *Agrobacterium* strain, which will no doubt become important in complementation experiments in the near future.

Looking back on developments in the field of plant molecular biology in the last decade we like to conclude with saying that the development of plant vectors on the basis of what was known about the *Agrobacterium* T-DNA transfer system in the early stages of this decade was one of the important factors that made a vast increase in knowledge in the field of plant molecular biology possible during the past ten years. We sincerely believe that further detailed knowledge of the molecular mechanism of T-DNA transfer will contribute to the further development of the field of plant molecular biology by making the genetic modification of plants more precise and sophisticated.

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