Recent Patents on *Agrobacterium*-Mediated Gene and Protein Transfer, for Research and Biotechnology

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Abstract: *Agrobacterium* has been widely used, in the last decades, for genetic transformation of a large number of plant species, and the genes and DNA sequences involved in this process have been subject of numerous patents. This review focuses on recent discoveries, which have shown new possibilities for the utilizations of this versatile microorganism. For example, the identification of an ever-increasing number of the bacterial and plant factors involved in the *Agrobacterium*-mediated DNA transfer and integration may lead to new applications in various fields of research and biotechnology. One of the main challenges in the *Agrobacterium*-mediated gene transfer technology is to achieve a better control of the integration and expression of transferred genes in the host cells and to apply it for targeted integration into the host genome or gene replacement (a technique not yet available in plants). In addition to genetic transformation of plants, under laboratory conditions, the host range of *Agrobacterium* can be extended to virtually all eukaryotic species, as demonstrated for various fungi, sea urchins, and animal cells. Not only can *Agrobacterium* transfer DNA to these very diverse hosts, but also its virulence machinery is able to inject proteins into the host cell, independently of the DNA transfer. Thus, *Agrobacterium* represents a universal gene and protein transfer machine.

Keywords: *Agrobacterium*, *Agrobacterium tumefaciens*, genetic transformation, *Agrobacterium*-mediated DNA transfer, *Agrobacterium*-mediated gene transfer technology, T-DNA integration, Gene targeting, VirB/VirD4 channel, VirB/D4 T4SS, DNA integration.

INTRODUCTION

Agrobacterium species are gram-negative phytopathogenic bacteria, which induce uncontrolled cell proliferation in a wide range of host plants. The outcome of infection may take different forms depending on the *Agrobacterium* species or strain involved; for example *A. tumefaciens* induces galls [1], whereas *A. rhizogenes* induces hairy roots [2]. The mechanism of host cell infection by *A. tumefaciens*, Fig. (**1**), relies on the transfer, integration and expression of genes from a bacterial plasmid into the host genome [3-7]. Therefore, this system represents the only known natural case in which a prokaryotic organism transfers genetic information to a eukaryotic host. This capability underlies the biotechnological uses of *Agrobacterium*, mostly employed for the genetic transformation of numerous plant species [8]. Recent discoveries have expanded the potential biotechnological uses of *Agrobacterium*; indeed, under laboratory conditions, *Agrobacterium* is able to transfer DNA and proteins to numerous non-plant species [9], including several species of yeast [10, 11] and other fungi [12, 13], as well as sea urchin embryos [14], and human cells in culture [15].

In this article, we review major host and bacterial genes implicated in the transformation process, and the patents involving these genes, with a focus on the recent potential applications of *Agrobacterium*-related technologies. Patents on DNA sequences involved in the "traditional" uses of *Agrobacterium* as an agent of plant genetic transformation, such as regulatory sequences and genes of interest to be transferred into target plants, have been reviewed elsewhere [16, 17 and references therein] and are not in the scope of the present review. The first section of this article provides a brief account of the current knowledge of the molecular mechanisms of DNA transfer from *Agrobacterium* to the host cell genome, discussing the numerous bacterial and host factors implicated in this process. The following sections focus on patents (see Table **1**) corresponding to different steps of this mechanism and on the specific genes involved.

1. GENE TRANSFER FROM *AGROBACTERIUM* TO THE HOST CELL GENOME: AN OVERVIEW

The Genes Transferred from *A. tumefaciens* to the Plant Genome

Most of the bacterial factors required for host infection are encoded by two regions of a large plasmid, named transformation-inducing (Ti) plasmid [18], the transferred DNA (T-DNA) and the virulence (*vir*) region. The T-DNA region (generally 20 to 30 Kb) is delineated by two identical sequences of 25 bp forming a direct repeat, the right and left borders [19]. The sequence between the two borders contains genes that are transferred into the host genome and are under control of promoters allowing their expression in eukaryotic host cells; these genes fall into two categories [1]. First, eight to ten genes encode proteins that modify the production of growth regulators in the transformed plant, as well as the sensitivity of plant cells to these regulators, and induce uncontrolled cell proliferation and neoplastic growths, responsible for the visible symptoms of *Agrobacterium* infection. Second, three to four genes encode enzymes required for the production of opines, condensations of an amino acid and a sugar, which can be utilized by *Agrobac-*

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Table 1. Summary of the Described Patents

Objective	Target gene	Patent number	Title	Ref.
Improving efficiency of gene transfer to plants	*vir*D2	WO07132193	Modified VirD2 and its use in improved gene transfer	[89]
	ACC deaminase	JP05312345	*Agrobacterium* microbe with improved efficiency for transducing gene to plant, and method for creating the same	[93]
	VIP1	WO04035731	Increasing host plant susceptibility to *Agrobacterium* by overexpression of the Arabidopsis VIP1 gene	[105]
	H2A	US 2007 7279336 US 2007 7276374	Enhancing plant cell transformation by adding a copy of gene involved in T-DNA integration, especially histone gene, to the plant	[106] [107]
Control of T-DNA integration	Phage integrase	US 2005 6936747	Methods for the replacement, translocation and stacking of genes in eukaryotic cells	[134]
	Cre recombinase	WO02052026	Nucleic acid integration in eukaryote	[136]
	RAD51	US 2006 7034117	RAD51 polypeptides and uses thereof	[143]
	MRE11	US 2006 7060480	MRE11 orthologue and uses thereof	[144]
Macromolecule delivery to eukaryotic cells	*vir*F	US 2004 6800791	Method for delivery of proteins to plant cells	[150]
	*vir*E2, *vir*E3, *vir*F, *vir*D5	WO01089283	Protein translocation into plant cells	[151]
	*vir*E2	WO01064923	VirE2-mediated trans-membrane delivery system	[152]
Agrobacterium T-DNA genes	*6b*	WO07138712	Use of histone chaperone activity of *Agrobacterium* 6b protein	[154]

terium as a source of carbon and nitrogen [1,5]. Different strains of *A. tumefaciens* are classified according to the type of opine produced, each strain encoding the proteins allowing the import and metabolism of its specific opine. Whereas the transferred genes may differ, the mechanism of DNA transfer is globally the same for the different *Agrobacterium* strains. Furthermore, the sequence located between the two borders of the T-DNA has no influence on the DNA transfer and can be replaced at will by genes of interest; it is this property of the T-DNA that underlies the extensive use of *Agrobacterium* for genetic engineering in research and biotechnology.

Virulence Genes Mediate the T-DNA Transfer

The virulence (*vir*) region of the Ti plasmid encodes bacterial factors required for the DNA transfer. The *vir* genes are organized in 10 to 12 operons [20, 21]. Note that, in most of the biotechnological uses of *Agrobacterium*, the *vir* and T-DNA regions are artificially separated in two plasmids (binary system) [22, 23]. The helper plasmid carries the *vir* genes, whereas the binary plasmid contains the T-DNA and can be easily manipulated and introduced into an *Agrobacterium* strain already harboring a helper plasmid. In addition to *vir* genes, several genes (named *chv*, chromosomal virulence genes) located not on the Ti plasmid, but on the bacterial chromosome are also required for *Agrobacterium* virulence [4].

The *vir* operons are activated by several environmental signals and conditions: molecules derived from the plant secondary metabolism such as the phenylpropanoid acetosyringone [24-28], mono- and oligosaccharides [29], and an acidic pH [30]. This combination of activation signals is generally found in the rhizosphere, at the proximity of wounded plant tissues which are most susceptible to the *Agrobacterium* infection. Among the *vir* genes, *vir*A and *vir*G encode the two proteins of a two-component regulatory system responsible for sensing the activation signals transcriptionally activating the other genes of the *vir* region [31]. As a result, the *vir* genes are induced and their protein products mediate the production, transfer and integration of the T-DNA in the presence of the susceptible host tissues (Fig. (**1**)). Following activation of the *vir* gene expression, the VirD1 and VirD2 proteins form an endonuclease complex that recognizes the left and right borders and excises a single-stranded segment corresponding to the non-transcribed strand of the T-DNA (T-strand), by a mechanism of strand-replacement [32]. At the end of the process, one molecule of VirD2 remains attached covalently to the 5´-end of the T-strand [33-35].

Cell-to-Cell Contact

A close contact between bacterial and plant cells is required during the transfer of the T-DNA [36, 37]. Attachment of *Agrobacterium* to the host plant cell is thought to occur in two steps, by analogy with the closely related plant-associated bacteria of the *Rhizobia* genus [37]. Initially, a physical interaction is likely mediated by plant receptors that recognize bacterial factors, Fig. (**1**). Although these plant receptors have not been definitely identified yet, some candidates have been proposed: a vitronectin analog that binds the bacterial ricadhesin [38], several cell wall

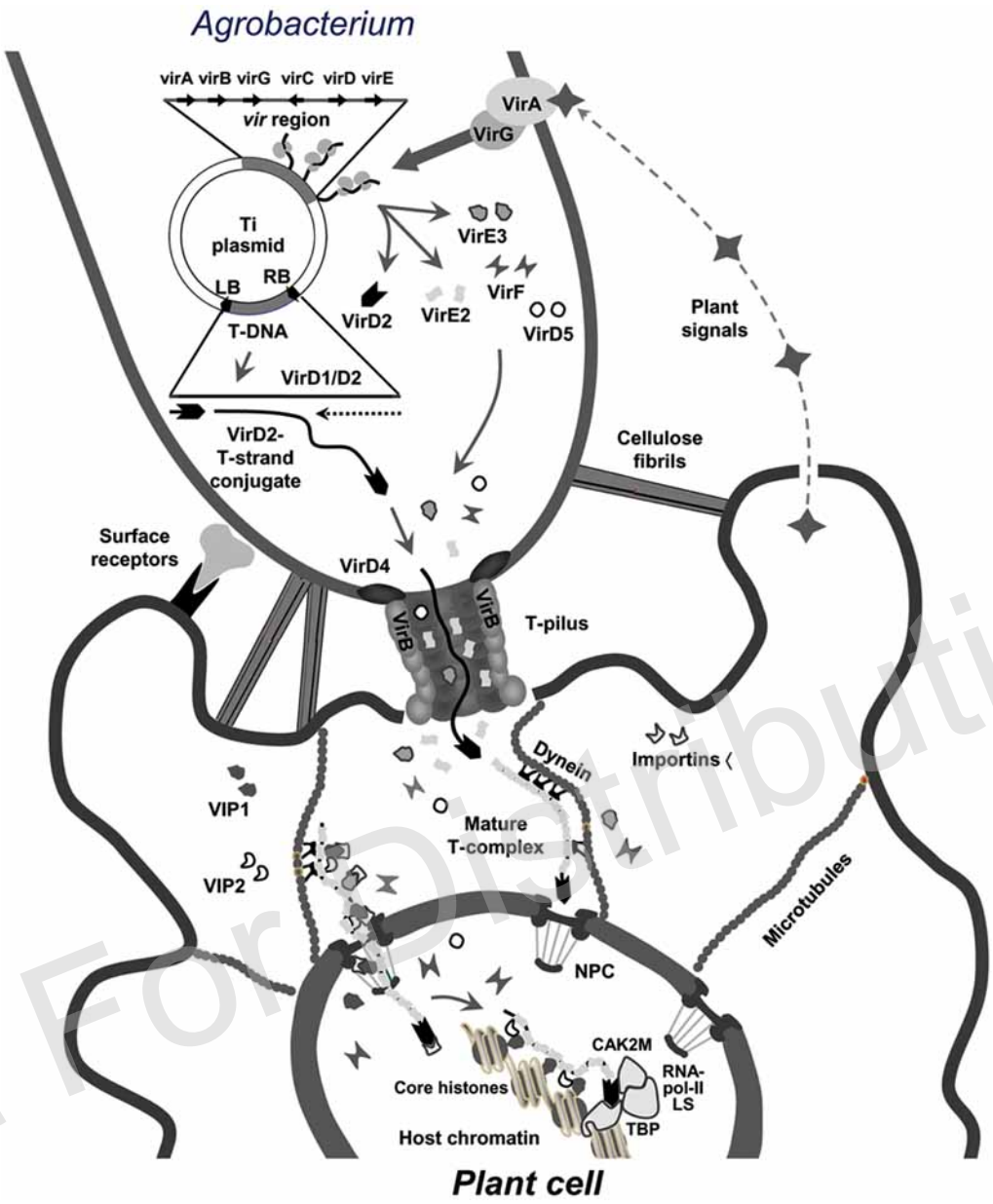

Fig. (1). A mechanism for DNA transfer from *Agrobacterium* to the host plant cell. Plant signals induce expression of *vir* genes via the *vir*A/*vir*G two-component regulation system. Attachment of *Agrobacterium* cells to host cell surface is mediated by plant and bacterial receptors and then reinforced by cellulose fibrils synthesized by *Agrobacterium*. A VirD2-T-strand complex is produced in the bacterial cell and translocated into the host cell cytoplasm, along with VirE2, VirE3, VirD5 and VirF, via the VirB/D4 secretion system. The mature T-complex, composed of VirD2-T-strand coated by VirE2 molecules, is formed in the host cytoplasm and imported into the nucleus, by a mechanism involving host factors such as importin α, VIP1 and dynein. In the nucleus, the T-complex is directed to the vicinity of the host chromatin and uncoated before T-DNA integration in the host genome.

proteins identified by screening of *Arabidopsis thaliana* insertional mutant collections for *rat* (*resistant to Agrobacterium*) mutants such as *rat1* and *rat3* [39, 40], and it was suggested that plant proteins interacting with the main component of the *Agrobacterium* pilus, VirB2, may play a role in bacterial attachment [41]. Then, this association is reinforced and stabilized by the production of cellulose fibrils by *Agrobacterium* Fig. (**1**) [42].

Macromolecular Transfer Via the VirB/VirD4 Channel

The transfer of T-DNA as well as free and T-strand-associated Vir proteins from *Agrobacterium* to the plant cell occurs via a type IV secretion system (T4SS) that shares strong homology with the bacterial conjugation system. The *Agrobacterium* T4SS is composed essentially of the proteins from the *vir*B operon (VirB1 to VirB11) and of VirD4, Fig.

(**2**), and it is usually referred to as the VirB/D4 complex [43]. The T4SS protein complex spans bacterial membrane and allows T-DNA and protein export out of bacterial cell. It also forms an external pilus composed mostly of VirB2 molecules, which might function as a channel for macromolecular transport to the host cell membrane [44], although the structure allowing the passage of T-DNA and Vir proteins through plant cell wall and membrane is not yet understood. Type IV secretion systems are widely spread in the bacterial world and serve as transporters of macromolecules; substrates that are transported via T4SS include plasmid DNA during the bacterial conjugation, and effector proteins during infection by several plant and animal pathogenic bacteria [43]. In the case of *A. tumefaciens*, the T-DNA, in the form of a VirD2/T-strand complex and several virulence proteins (namely VirE2, VirE3, VirD5 and VirF) are exported independently of each other and play a role in T-DNA transport within the host cell and integration into the host genome [45-50]. In addition to the conventional T4SS transport mechanism, another bacterial virulence protein, VirE2, is implicated in the T-strand transfer into the host cell [51]. VirE2, which is known to cooperatively bind single-stranded DNA (ssDNA) [52-55], package it into a coiled filament [56, 57] and facilitate its nuclear import [45, 46, 58-60], has been shown to pull ssDNA actively, without the need for external energy input [51]. Thus, VirE2 that has been transported into the host cell through the VirB/D4 channel may act as a "molecular winch" that cooperatively binds the incoming T-strand, folding it into a helical structure and pulling it into the host cell.

Nuclear Import of the T-DNA

After the T-strand conjugated with VirD2 enters the host cell cytoplasm, it has to be imported into the nucleus. This is achieved by cooperation between several host and bacterial factors. VirE2 molecules bind along the T-strand cooperatively [46, 53, 61], forming the mature T-complex composed of the T-strand, a single molecule of VirD2 and multiple molecules of VirE2, Fig. (**1**). In this complex, VirD2 directly interacts with karyopherin α (also known as importin α) [62] whereas VirE2 binds VIP1 (VirE2 Interacting Protein 1) [60] which in turn interacts with karyopherin α [63]. These interactions with the nuclear import machinery of the host cell promote nuclear import of VirD2 and VirE2 via a karyopherin α-dependent pathway [3, 64]. In addition, recent studies suggest that VirE2 subcellular localization also depends on structural changes upon its binding to ssDNA [51], or on serine-79 phosphorylation of VIP1 which occurs during defense response to various stimuli, including the *Agrobacterium* infection, and which was shown to enhance nuclear accumulation of VIP1 [65]. Although VirD2 and VirE2 can mediate nuclear import of short DNA molecules independently of each other, most likely they act together during the infection [66]; VirD2 likely pilots the T-complex toward the nuclear pore, while VirE2 associated with VIP1 facilitates the movement of the T-complex through the nuclear pore, Fig. (**1**). It has also been suggested [67] that the molecular motors such as dynein may interact with the T-complex and assist its movement along microtubules, toward the nucleus. Another bacterial protein exported into the plant cell through the VirB/D4 channel, VirE3 [49], was found to mimic this

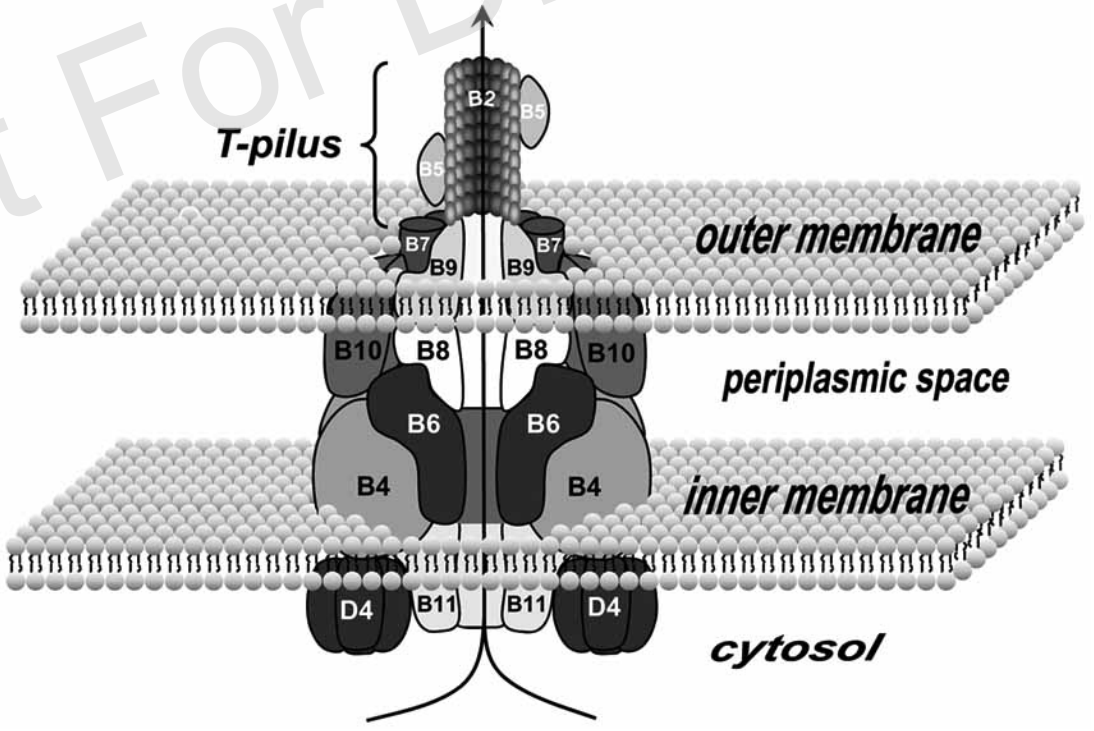

Fig. (2). A schematic structure of the VirB/D4 channel for macromolecular transport between *Agrobacterium* and the host cell. The *Agrobacterium* T4SS is composed of the 11 proteins encoded by the *vir*B operon, and of VirD4. These proteins form a complex spanning *Agrobacterium*'s inner membrane, periplasmic space and outer membrane, and an external pilus composed mainly of VirB2 molecules. This complex mediates the export of macromolecules out of bacterial cell and into the plant cell, likely via the VirB2 pilus.

activity of VIP1 and assist nuclear import of VirE2. Finally, in certain strains of *A. rhizogenes*, the absence of VirE2 may be compensated by another bacterial protein, GALLS, albeit most likely via a different mode of action [68, 69].

T-DNA Uncoating and Integration

Inside the nucleus, the T-complex has to reach its potential site of integration in the host chromatin, be uncoated of its associated protein components, and the T-DNA must then be integrated into the host genome, Fig. (**3**). Recent evidence suggests that VIP1 interacts with the plant core histones and potentially targets the entire T-complex to the host chromatin [70, 71]. Furthermore, VIP1 binds *in vitro* to purified plant nucleosomes and mediates association of nucleosomes with VirE2 as well as with VirE2-ssDNA complexes (BL and VC, unpublished data). Subsequently, the uncoating of the T-complex presumably is achieved by proteasomal degradation. Indeed, one of the bacterial proteins exported into the host cell, VirF, contains an F-box domain. F-box proteins are known to target their substrates to degradation via the SCF pathway [72], and VirF was shown to mediate degradation of VIP1 and VirE2 also by the SCF pathway [73].

Following uncoating, the T-DNA is integrated into the host DNA by a mechanism as yet not completely elucidated. Several models have been proposed for this event [74]. The SSGR (single strand gap repair) model is based on micro-homologies between the T-DNA sequence and the sequence of the host genome where the integration occurs; in this model, the T-DNA is integrated as a single strand, and the synthesis of the second strand follows the integration event [75]. However, some patterns of T-DNA integration are not compatible with the SSGR model [76, 77]; thus a different model was proposed in which the second strand synthesis occurs before integration [78, 79]. Moreover, non-homologous end joining (NHEJ) machinery likely plays an important role in T-DNA integration into double-stranded breaks (DSBs) in the host DNA, where the T-DNA most likely integrates in a double-stranded form [74]. Note that the co-existence of both integration pathways in the same host is also possible.

Unlike the host proteins involved in other stages of the infection process, the host factors that play a role in integration are poorly characterized. These host functions may include VIP2 (VirE2 Interacting Protein 2), which is required for the T-DNA integration and which functions by a mechanism that may involve expression of the core histones [80]. Consistent with this idea, core histones, besides their role in the T-complex chromatin targeting [70, 71], have been implicated in the T-DNA integration process [81], Fig. (**1**).

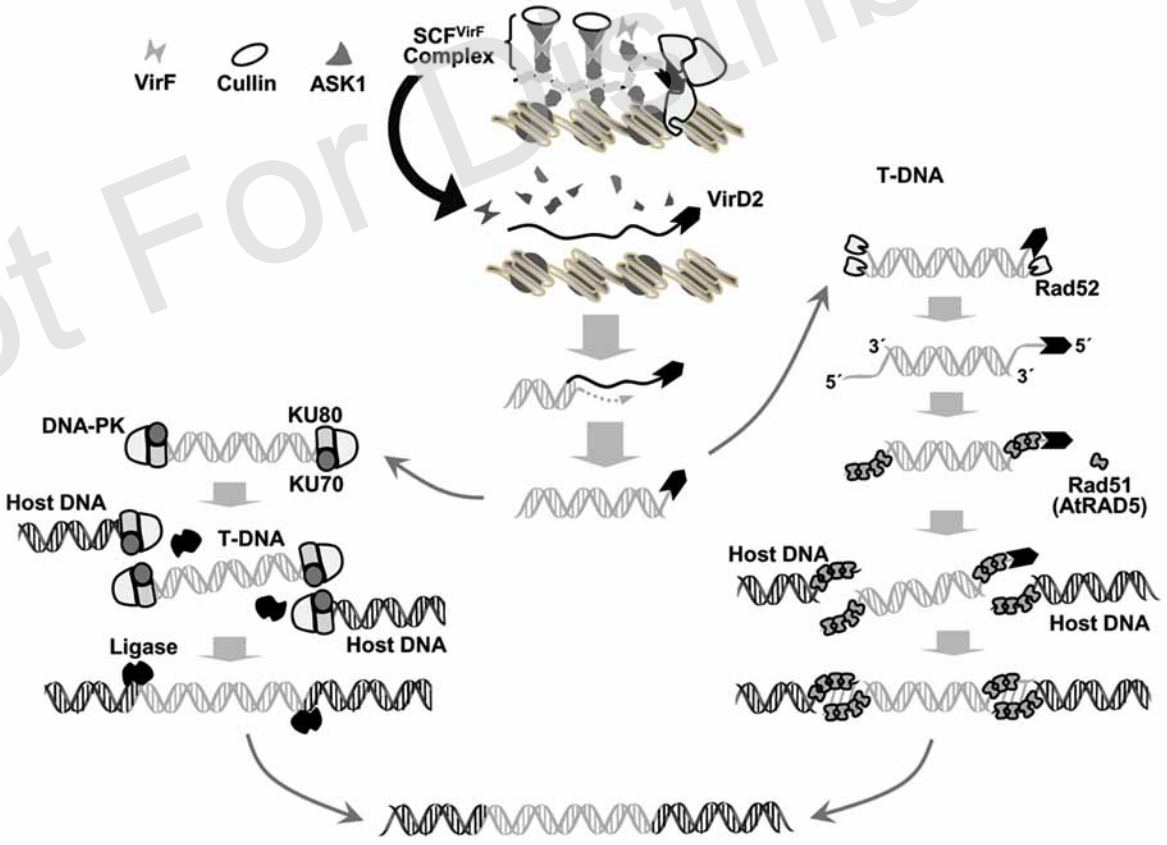

Fig. (3). A model for T-DNA integration into the host cell genome. T-DNA uncoating presumably is mediated by the SCF^{virF} complex, removing VIP1 and VirE2 from the T-strand. Then, T-DNA second strand synthesis occurs most likely before integration. Depending on the host, the T-DNA integration may occur by non-homologous recombination, involving such host factors as Ku80, Ku70 and an as yet unidentified ligase, or by homologous recombination with the help of Rad51 and Rad52.

2. IMPROVING THE EFFICIENCY OF PLANT GENETIC TRANSFORMATION BY *AGROBACTERIUM*

Whereas a large number of plant species can be transformed by *Agrobacterium*, efficient and reliable transformation protocols are available only for a relatively few of them. Thus, numerous plant species, among them many agronomically-important crops, are still considered "recalcitrant" to *Agrobacterium*-mediated genetic transfor-mation, meaning that current protocols achieve only very low efficiency of their genetic transformation [8]. For improvement of these protocols, efforts have been made to modify the handling of *Agrobacterium* cultures (such as virulence-induction treatments) [82], the use of supervirulent strains that either occur naturally [83] or have been engineered to carry extra copies of some *vir* genes [84, 85], or to render plant cells/tissues supercompetent for transformation, for example, by a pre-cultivation of explants with various growth regulators or other biologically active compounds [e.g. 86, 87]. The continuing discovery of bacterial and host factors involved in different stages of the *Agrobacterium*-mediated genetic transformation has led to the idea that over-expressing or modifying these factors will further enhance the transformation efficiency [88].

Manipulation of *Agrobacterium* Virulence Factors

Patent WO07132193 (modified VirD2 and its use in improved gene transfer) [89]. Increasing understanding of the interactions of the Vir proteins with plant factors provided valuable insights into the manner in which the corresponding vir genes can be modified to improve genetic transformation efficiency. This is illustrated by the patent WO07132193 [89], which employs a modified form of VirD2, a pilot protein covalently attached to the T-strand, see above and Fig. (**1**). VirD2 contains a sequence similar to known target sequences for caspase-mediated proteolysis [90]. Though caspases have not been conclusively identified in plants, several studies have shown the presence of caspase-like activities in plant tissue extracts [91]. In particular, VirD2 was degraded in planta by what has been suggested to represent a defense reaction pathway against the *Agrobacterium* infection [90]. Based on this idea, an *Agrobacterium* strain expressing a modified VirD2 with a point mutation abolishing its sensitivity to caspase-like degradation was developed [92]. This *Agrobacterium* mutant displayed an enhanced virulence, as assessed by transient expression of a GFP reporter, in six different plant species [92]. The authors of these studies filed a patent advancing this modified *Agrobacterium* strain as a tool for improvement of the efficiency of plant genetic transformation.

Inhibition of Plant Defense Response

Patent JP05312345A2 (*Agrobacterium* microbe with improved efficiency for transducing gene to plant, and method for creating the same) [93]. Generally, Agrobacterium does not induce extensive defense reactions-such as hyper-sensitive response resulting in local necrosis of plant tissues as observed with some bacterial or viral infections [94, 95]-in its host plants. However, several studies indicate that some plant defense genes [96-98] as well as an RNA silencing response [99] are induced during *Agrobacterium* infection. In one of these studies, defense-related genes were initially induced, but then they became repressed, suggesting that *Agrobacterium* may encode or induce a repressor of plant defense [96]. Moreover, inhibition of plant defense responses by manipulating the levels of salicylic acid, an important signal molecule in plant defense, improved the efficiency of plant genetic transformation by *Agrobacterium* [100]. Ethylene is another important signal molecule involved in plant reaction to several types of stress and challenge with pathogens [101]. Because ethylene emission by the host plant has been suggested to inhibit *Agrobacterium* virulence [102], Eomo *et al*. [93] proposed to enhance *Agrobacterium* virulence by inhibiting ethylene production. The patented system is based on the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase that catalyzes the cleavage of the direct precursor of ethylene, ACC, into α-ketobutyrate and ammonia [103]. This enzyme is present in several species of plant growth-promoting bacteria, and it is able to reduce the level of ethylene in the plant, by sequestering and degrading ACC [103]. A vector expressing ACC deaminase was introduced into Agrobacterium and shown to enhance *Agrobacterium* virulence in the model plant Arabidopsis and in melon [104]. Collectively, these studies suggest that plant defenses may represent one of the reasons for recalcitrance of some plant species to Agrobacterium, and that the strategy aimed at inhibiting defense responses may be useful for improving genetic transformation of these plant species.

Manipulation of Host Factors

Patent WO04035731 (Increasing host plant susceptibility to *Agrobacterium* by overexpression of the Arabidopsis VIP1 gene) [105]. This patent proposes that elevated expression of the plant VIP1 protein in the host organism may increase the transformation efficiency. VIP1, initially discovered as an interactor of the exported bacterial protein VirE2 [60], is thought to participate in several critical steps in T-DNA transfer and integration, from nuclear import of the T-complex to its chromatin targeting and to proteasomal uncoating, see above and Fig. (**1**). The enhancing effect of VIP1 over-expression on Agrobacterium-mediated gene transfer was demonstrated in model plants, tobacco and Arabidopsis [63]. The patent WO04035731 [105] extends this system to other plants, particularly crop species that are difficult to transform. Specifically, the authors of the patent propose either to express VIP1 transiently by co-inoculation with an *Agrobacterium* strain carrying the VIP1 expression cassette in its T-DNA and the strain containing the gene of interest, or to produce transgenic plants stably expressing VIP1 that can be used as a starting material for a second transformation with a gene of interest.

Patents US 2007 7279336 and US 2007 7276374 (Enhancing plant cell transformation by adding a copy of gene involved in T-DNA integration, especially histone gene to the plant and expressing additional copies of the gene) [106, 107]. These patents employ a similar approach aiming to express a plant factor essential for Agrobacterium-mediated transformation in order to enhance the efficiency of gene transfer. The applications of this patent focus on the Arabidopsis gene encoding the core histone H2A. Several recent studies have demonstrated the importance of core

histones in the final steps of *Agrobacterium* infection, i.e., chromatin targeting of the T-complex and T-DNA integration [81, 108]. An Arabidopsis mutant with the disrupted H2A gene displayed an *Agrobacterium*-resistant rat phenotype [109], while overexpression of several variants of AtH2A conferred increased susceptibility to *Agrobacterium* [110]. Subsequently, H2A as well as other core histones were shown to be important for *Agrobacterium*-mediated genetic transformation of other plant species, such as *Nicotiana benthamiana* [111]. In support of these findings, VIP1 was found to interact with core histones, including H2A, *in vitro* and *in vivo* [70, 71]. Therefore, the authors of this patent propose to express, transiently or stably, the histone H2A in order to increase the efficiency of the *Agrobacterium*-mediated gene transfer.

3. TARGETING OF T-DNA INTEGRATION

One of the main challenges in plant genetic transformation is to specify the site of integration of the T-DNA in the host genome. In plants, the main pathway of integration of foreign DNA (regardless of the technique used to introduce the DNA) is non-homologous or illegitimate recombination, Fig. (**3**), whereas homologous recombination may occur only at a very low rate [112-114]. Indeed, numerous analyses of T-DNA integration sites in the plant genome have shown that integration is generally random [115, 116]. Some of these studies suggested preferential T-DNA integration in transcriptionally-active chromatin regions as well as into 5' and/or 3' untranslated sequences in plants [117-120] and in fungi [121]. However, the apparent bias for integration into euchromatin may derive simply from requirement for expression of selection markers to recover the transformed plants [122]. Indeed, several recent studies analyzing T-DNA integration sites in transgenic plants generated under non-selective conditions demonstrated that integration occurs to the same degree in euchromatic and heterochromatic regions [122-124]; however, the results of these studies do not explain the apparent preference for 5´ and 3' untranslated gene regions as integration sites [115, 118].

Unlike plants, yeast and other fungi can integrate the *Agrobacterium* T-DNA by homologous recombination, Fig. (**3**), if T-DNA contains the appropriate sequence homology to the host genome [10]. Thus, the bacterial T-DNA transfer machinery most likely does not dictate the pathway of T-DNA integration, which depends mainly on the host factors. Consistent with this idea, expression of a yeast gene involved in homologous recombination enhanced *Agrobacterium*-mediated gene targeting in *Arabidopsis* [125], see below.

Advances in this direction of the *Agrobacterium* research are highly desirable for two main reasons. First, variability of transgene expression is a major concern in *Agrobacterium*-mediated genetic transformation of plants; targeted integration of the T-DNA into a specific site in the genome, which can be chosen for its constitutive and consistent expression, would circumvent these difficulties. Second, the ability to target the T-DNA integration will allow specific gene disruption or gene replacement, greatly facilitating research in plant functional genomics as well as production of desired mutants for agricultural applications. Below, are presented the patents for gene targeting in plants that use either integrase/recombinase of phage origin or modification of the host machinery for DNA repair and recombination. Another approach to targeted DNA integration is the use of zinc finger DNA binding domains, which represents the focus of several patents [126]. Specifically, zinc finger domains can be engineered to recognize virtually any DNA sequence in the host genome [127]. These "custom-tailored" DNA binding protein domains are then fused to an endonuclease [127, 128], resulting in a chimeric endonuclease that will create double strand breaks at the specific sites within the host genome, which serve as preferential sites for T-DNA integration [129].

The Use of Integrase/Recombinase from Phage Origin

In phage-derived integration systems, an integrase or recombinase mediates integration of a specific DNA donor sequence, attP, into a specific destination sequence, attB [130]. In the case of bacteriophage λ integrase for example, its site-specific activity [131] is used in the commercial Gateway cloning system (Invitrogen). Employing such system for gene targeting in plants requires two rounds of plant transformation: first with the destination sequence, and then with the T-DNA in which the sequence of interest is contained between the two donor sequences. Furthermore, the second transformation must be performed in presence of the integrase/recombinase required for the site-specific integration; this condition can be achieved, for example, by transient expression of integrase/recombinase following coinfection with the corresponding *Agrobacterium* strain. It is expected that T-DNA will be preferentially integrated into the destination site, and plants with a single integration events will be obtained by subsequent segregation of progeny out of the initial transformants. These methods have been successfully used for site-specific integration into the human genome [132, 133]. Thus, whereas the two patents described below are primarily designed for use in *Agrobacterium*-mediated genetic transformation of plants, they can also be applicable to other organisms.

Patent US 2005 6936747 (Methods for the replacement, translocation and stacking of gene in eukaryotic cells) [134]. Based on the φC31 phage integrase from *Streptomyces* [130], this approach first produces transgenic host organisms that contain the destination attB sequence. These transgenic lines are then transformed with a construct containing the gene of interest inserted between two donor attP sequences, in presence of transiently coexpressed integrase. The expected result is a higher level of targeted integration of the gene of interest into the destination site attB. In the case of the φC31 phage, integration and excision are performed by two different enzymes, integrase and excisase, respectively; thus, the integration event is irreversible in the absence of excisase. The patent description includes also the possibility of using other phage integrase systems that allow irreversible integration. Whereas there are no published data supporting the feasibility of this approach for the Agrobacterium-mediated plant genetic transformation, a similar strategy was used in human cells [132], and the activity of the φC31 integrase was demonstrated in plant plastid transformation [135].

Patent WO02052026 (Nucleic acid integration in eukaryote) [136]. This patent utilizes the Cre/lox recombination system from the P31 phage [130]. Technically and conceptually, this approach is similar to that of the φC31-based technology, with the Cre recombinase exchanging the DNA segment contained between two lox sites in the host plant genome with another sequence on the T-DNA also flanked by two lox sites [137, 138]. In this system, however, integration is potentially reversible because the Cre recombinase can perform both integration and excision; this difficulty can be circumvented by the use of heterospecific lox sites that are incompatible with each other and, thus, cannot be excised [139]. The Cre/lox system was successfully tested in the model plant *Arabidopsis*, allowing efficient selection of *Agrobacterium*-mediated gene targeting events and recovery of the transgenic plants [139].

Modification of Host DNA Repair and Recombination Pathways

Another approach to manipulate integration is to introduce or modify the DNA repair and recombination machinery, enhancing the homologous recombination and/or inhibiting the non-homologous recombination pathways to favor homologous integration. Experiments with yeast mutants have shown that their integration pathway can be manipulated by modifying factors involved in DNA repair, Fig. (**3**). Indeed, some of these factors, such as Ku70, Rad50 or Mre11, were required for non-homologous recombination [140], while others, such as Rad51 and Rad52, were required for homologous recombination [141]. The validity of this strategy was also demonstrated in plants using transgenic *Arabidopsis* expressing the yeast *RAD54* gene [125]. *RAD54* is a member of the *SWI2/SNF2* superfamily of chromatin remodeling genes known to promote recombination between homologous DNA segments in yeast [142]; moreover, *RAD54* disruption led to lower rates of targeted gene integration. In *RAD54* expressing plants, the gene targeting efficiency of *Agrobacterium* via homologous recombination was enhanced by one to two orders of magnitude [125].

Patent US 2006 7034117 (RAD51 polypeptides and uses thereof) [143] and patent US 2006 7060480 (MRE11 orthologue and use thereof) [144]. These patents involve the sequences of two maize genes, Rad51 and Mre11, important for DNA repair and recombination. In yeast, RAD51 is involved in homologous DNA pairing and strand exchange reaction [145] and, during yeast transformation by *Agrobacterium*, RAD51 was required for T-DNA integration via the homologous recombination pathway [141]. Interestingly, an Arabidopsis mutant in the RAD5 gene, which is closely related to the yeast RAD51, displayed a reduced susceptibility to *Agrobacterium* [146]. The Mre11 protein functions in complex with Rad50 and Xrs2, and it has an exonuclease activity that plays a role in both homologous and non-homologous recombination [147]. In Agrobacterium-infected yeast cells, Mre11 was necessary for T-DNA integration via the non-homologous recombination pathway [140]. It is expected that these genes, either stably expressed in transgenic plants or expressed transiently during *Agrobacterium* coinoculation, will allow to control the pathway and the efficiency of T-DNA integration.

4. MACROMOLECULE DELIVERY INTO EUKARYOTIC CELLS

Several bacterial virulence proteins are exported from *Agrobacterium* to host cells during the infection process, see above and Fig. (**1**). The first indications that this protein transfer can occur independently of the T-DNA relied on observations that VirE2, although being a ssDNA binding protein that should be able to bind T-strands already within the bacterial cell, does not necessarily need to be expressed in the same bacterial cell that carries the T-DNA. Specifically, the virulence of avirulent *Agrobacterium* mutants with the disrupted *vir*E2 gene but carrying a functional T-DNA can be complemented by coinoculation with *Agrobacterium* lacking its T-DNA but expressing the functional VirE2 [148]. The complementation of virulence likely occurred within the plant cell following independent export of T-DNA and VirE2. This observation was consistent with the known function of VirE2 in the host, rather than bacterial, cell [45, 46] and with the known ability of T4SS channels to transport DNA and protein substrates [149]. Later, the transport of VirE2 as well as VirD5, VirE3 and VirF from *Agrobacterium* to plant and yeast cells was demonstrated directly [47, 48, 50]. These discoveries naturally suggested the use of *Agrobacterium* as a system to deliver proteins of interest into various eukaryotic hosts irrespective of the T-DNA transport.

The Use of the VirB/D4 T4SS for Protein Delivery

The two following patents rely on the same strategy, taking advantage of the VirB/D4 macromolecular translocation system, Fig. (**2**). The principle is to introduce into *Agrobacterium* a construct expressing a fusion between the protein of interest and one of the *Agrobacterium* exported Vir proteins or a partial sequence of this protein corresponding to its export signal. The fusion protein, expressed in the bacterial cell, will be exported into the eukaryotic host cell, allowing direct introduction of a protein of interest into the target cell, without the need for genetic transformation. These methods may be useful, for example, for introduction of a biologically active protein that may modify the host cell metabolism, when DNA delivery is not possible or not desirable.

Patents US 2004 6800791 (Method for delivery of proteins to plant cells) [150]. In this patent, the system involves the fusion of the gene of interest to virF, and its expression from the virF promoter. The use of a vir gene promoter is interesting, because it allows inducible expression of the fusion protein in coordination with expression of the rest of the *Agrobacterium* virulence machinery, mimicking the natural expression pattern of all other Vir proteins exported from Agrobacterium, see above. This likely ensures optimal timing and expression levels of the fusion protein, presumably facilitating its translocation. However, the choice of the full length virF gene might represents a caveat of this method because VirF functions as an F-box protein component of the SCF complex [73], and it might promote degradation of its fusion partner within the target cell, effectively defeating the purpose of this technology. More generally, the use of a full length Vir protein "carrier" protein may be problematic because Vir proteins possess their own biological activities within the host cell which may

potentially interfere with the target cell physiology and/or function of the fused protein of interest. These pitfalls can be avoided by using partial sequence of a Vir protein containing the export signal, but not other active domains as described below.

Patent WO01064923 (Protein translocation into plant cells) [151]. The system proposed in this patent utilizes a fusion between the gene of interest and the three amino-acid-long sequence, Arg-Pro-Arg, believed at the time of filing of this patent to represent the Vir protein export signal. The advantage of this system is that only a very short sequence is added to the protein of interest, which is unlikely to affect significantly its function or the physiology of the target cell. Unfortunately, later studies revealed that this 3-amino acid sequence is necessary, but not sufficient for translocation; instead, the signal is now believed to be a longer, ca. 40-amino acid sequence rich in arginine and positively charged residues [50]. Still, this signal is substantially shorter than any of the exported Vir proteins, and its use for mediating export remains advantageous over full-length Vir protein fusions.

The Use of VirE2 as Membrane Channel for Transport of Macromolecules

Patent WO01064923 (VirE2-mediated trans-membrane delivery system) [152]. VirE2 is a truly multifunctional protein and, in addition to its roles in packaging the T-DNA, pulling it through the VirB/D4 channel, facilitation of nuclear transport, chromatin targeting and uncoating, VirE2 was shown to form transmembrane channels able to traffic single-stranded oligonucleotides across artificial membranes [153]. Although this function of VirE2 has not yet been confirmed *in vivo*, the patent WO01064923 [152] proposes to take advantage of the channel-forming property of VirE2 to mediate transport of DNA or other negatively charged macromolecules through membranes of various target cells. Additionally, VirE2 may be modified to accommodate to other types of substrates.

5. UTILIZATION OF THE NATURAL *AGROBACTERIUM* T-DNA GENES

Whereas patents reviewed so far rely on different uses and modifications of the *Agrobacterium* DNA transfer machinery, it is also possible to take advantage of the genes naturally present on the T-DNA, termed oncogenes as they induce formation of tumorous growths, that promote host cell proliferation and opine production.

Patent WO07138712 (Use of histone chaperone activity of *Agrobacterium* 6b protein) [154]. This recently filed patent proposes to use the *Agrobacterium*-encoded oncogenic protein 6b for altering the target cell physiology and chromatin structure. Unlike most other T-DNA-encoded oncogenes, the biological function and activity of oncogene 6b have been investigated in detail. Expression of 6b modified plant morphology [155, 156] by altering the balance of growth regulators [157, 158]. A recent work shed light on the molecular mode of action of 6b [159], showing that it interacts with histone H3 and acts as a protein chaperone. These activities of 6b protein modified the structure of the plant chromatin and induced changes in gene expression, notably the genes involved in auxin synthesis. The authors of the patent suggest several possible applications for the 6b protein. For example it can be used to modify expression of its natural targets in transgenic plants, thereby inducing physiological and morphological alterations in the target plant. Or, the 6b protein can be fused with a protein interacting with a specific DNA sequence, thus targeting the 6b-induced modifications of chromatin structure and gene expression to a specific site in the host genome.

CURRENT & FUTURE DEVELOPMENTS

For decades, *Agrobacterium* has served as a major tool for development of plant biotechnology, by providing an efficient, simple and cost-effective system for plant genetic transformation [5]. This unique property of *Agrobacterium* most likely stems from its ability to “insinuate” into and take advantage of such complex cellular pathways as nuclear import, chromatin targeting, proteasomal degradation, and DNA repair. Thus, an exceptional research effort has been made to decipher the major aspects of the interactions between *Agrobacterium* and its hosts [4, 160]. In many ways, *Agrobacterium* has become a model organism, for example for the bacterial T4SS [43], and a probe to investigate the host cell biology. The in-depth knowledge of mechanisms underlying the T-DNA transfer, and in particular the *Agrobacterium* ability to transport macromolecules not only to plant cells, but also to other eukaryotic organisms, allows us to envision many new possibilities for the use of *Agrobacterium* in basic and applicative research.

For example, future advances in *Agrobacterium* research will make possible more efficient genetic transformation of more plant species, among them agronomically-important crops. T-DNA integration, as well as transgene expression, will be better controlled, and may be methods for targeted gene integration will become available. Furthermore, *Agrobacterium* genetic transformation of non-plant organisms will likely gain more importance, as it has already been the case for fungi [12, 13]. Finally, the direct transfer of proteins and multiprotein complexes, employing *Agrobacterium* machinery, but avoiding genetic transformation, may emerge as a promising tool for research and biotechnology.

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