Agrobacterium-mediated genetic transformation of plants: biology and biotechnology

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Agrobacterium-mediated genetic transformation is the dominant technology used for the production of genetically modified transgenic plants. Extensive research aimed at understanding and improving the molecular machinery of *Agrobacterium* responsible for the generation and transport of the bacterial DNA into the host cell has resulted in the establishment of many recombinant *Agrobacterium* strains, plasmids and technologies currently used for the successful transformation of numerous plant species. Unlike the role of bacterial proteins, the role of host factors in the transformation process has remained obscure for nearly a century of *Agrobacterium* research, and only recently have we begun to understand how *Agrobacterium* hijacks host factors and cellular processes during the transformation process. The identification of such factors and studies of these processes hold great promise for the future of plant biotechnology and plant genetic engineering, as they might help in the development of conceptually new techniques and approaches needed today to expand the host range of *Agrobacterium* and to control the transformation process and its outcome during the production of transgenic plants.

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Introduction

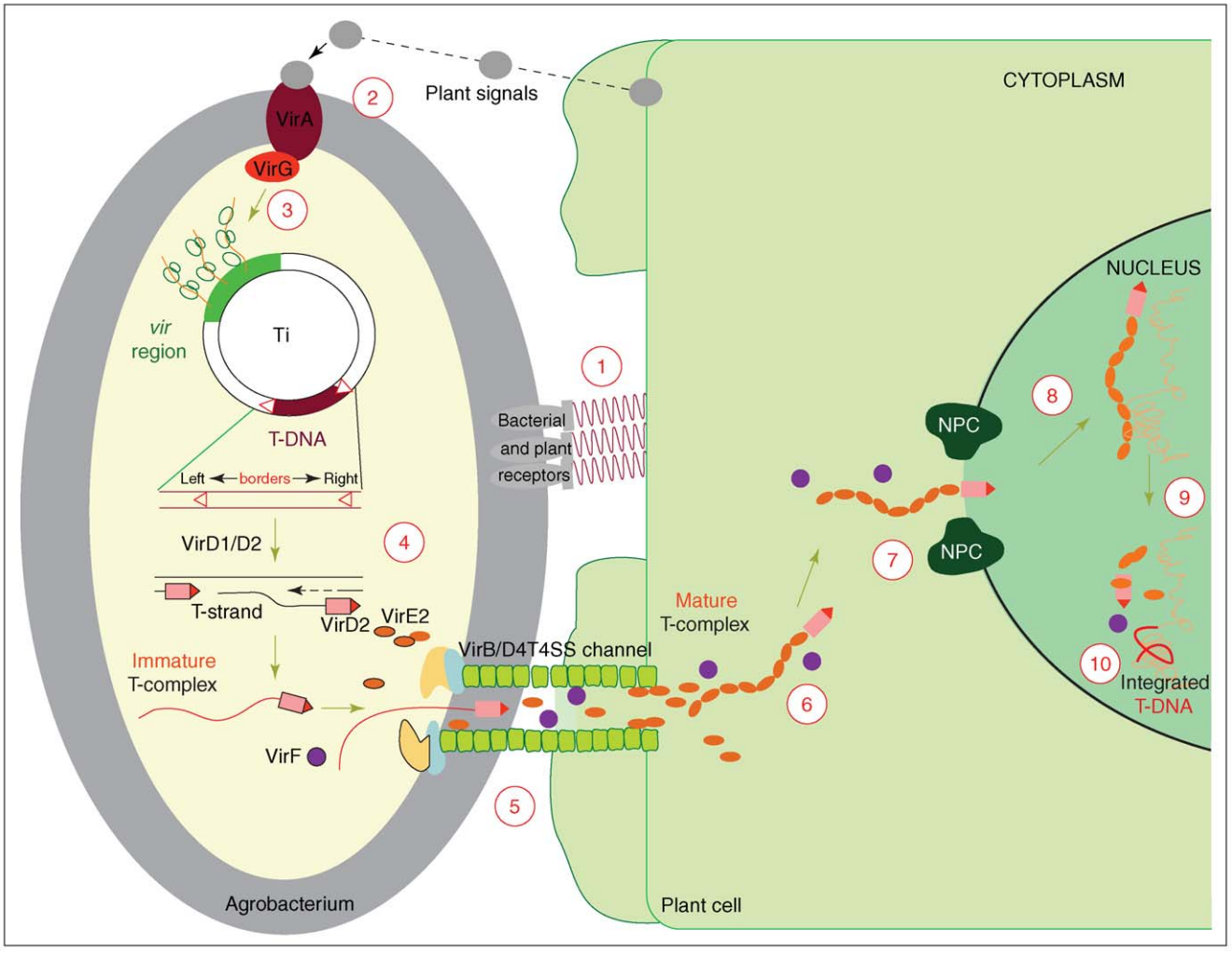
Agrobacterium genetically transforms its host by transferring a well-defined DNA segment from its tumor-inducing (Ti) plasmid to the host-cell genome [1]. In nature, the transferred DNA (T-DNA) carries a set of oncogenes [2] and opine-catabolism genes, the expression of which, in plant cells, leads to neoplastic growth of the transformed tissue and the production of opines, amino acid derivatives used almost exclusively by the bacteria as a nitrogen source. Recombinant *Agrobacterium* strains, in which the native T-DNA has been replaced with genes of interests, are the most efficient vehicles used today for the introduction of foreign genes into plants and for the production of transgenic plant species [3]. Thus, *Agrobacterium* biology and biotechnology have been the subject of numerous studies over the past few decades [4••], resulting in the establishment of many *Agrobacterium* strains, plasmids and protocols uniquely adapted for the genetic transformation of various plant species [3].

The molecular machinery needed for T-DNA production and transport into the host cell comprises proteins that are encoded by a set of bacterial chromosomal (*chv*) and Ti-plasmid virulence (*vir*) genes. In addition, various host proteins have been reported to participate in the *Agrobacterium*-mediated genetic transformation process [5,6••], mostly during the later stages of the process (i.e. T-DNA intracellular transport, nuclear import and integration). Because *Agrobacterium* adopts existing cellular processes (e.g. DNA and protein transport, targeted proteolysis and DNA repair) to transform its host [5,6••], understanding these general biological mechanisms of the plant cell can help expand the host range of *Agrobacterium* as a genetic engineering tool, as well as facilitating control of the transformation process and its outcome during the production of transgenic plants. In this review we focus on the key cellular factors and mechanisms used by *Agrobacterium* during the genetic transformation of its host. The application of host factors for improving the transformation efficiency of hard-to-transform plant species and the future prospects of gene targeting in plants are also discussed.

The genetic transformation process

The *vir* region, located on the *Agrobacterium* Ti plasmid, encodes most of the bacterial virulence (Vir) proteins used by the bacterium to produce its T-DNA and to deliver it into the plant cell. In wild-type *Agrobacterium* strains, the T-DNA region (defined by two 25 base pair direct repeats termed left and right T-DNA borders) is located in *cis* to the *vir* region on a single Ti plasmid. In disarmed *Agrobacterium* strains, where the native T-DNA region has been removed from the Ti plasmid, a recombinant T-DNA region usually resides on a small, autonomous binary plasmid and functions in *trans* to the *vir* region [3]. The transformation process begins with the bacterium–plant attachment (Figure 1; step 1), followed by induction of the expression of the *vir* region by specific host signals (Figure 1; steps 2 and 3). A single-stranded (ss) T-DNA molecule (T-strand) (Figure 1; step 4) is then produced by the combined action of the bacterial VirD1

Figure 1



A model for the *Agrobacterium*-mediated genetic transformation. The transformation process comprises 10 major steps and begins with recognition and attachment of the *Agrobacterium* to the host cells (1) and the sensing of specific plant signals by the *Agrobacterium* VirA/VirG two-component signal-transduction system (2). Following activation of the *vir* gene region (3), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (4) and delivered as a VirD2–DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (5). Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (6) and is actively imported into the host-cell nucleus (7). Once inside the nucleus, the T-DNA is recruited to the point of integration (8), stripped of its escorting proteins (9) and integrated into the host genome (10). A detailed model of the host cellular mechanisms and the role of plant-specific factors in the transformation process are given in Figure 2. (This illustration was reproduced, with modifications, from [28•] with permission.).

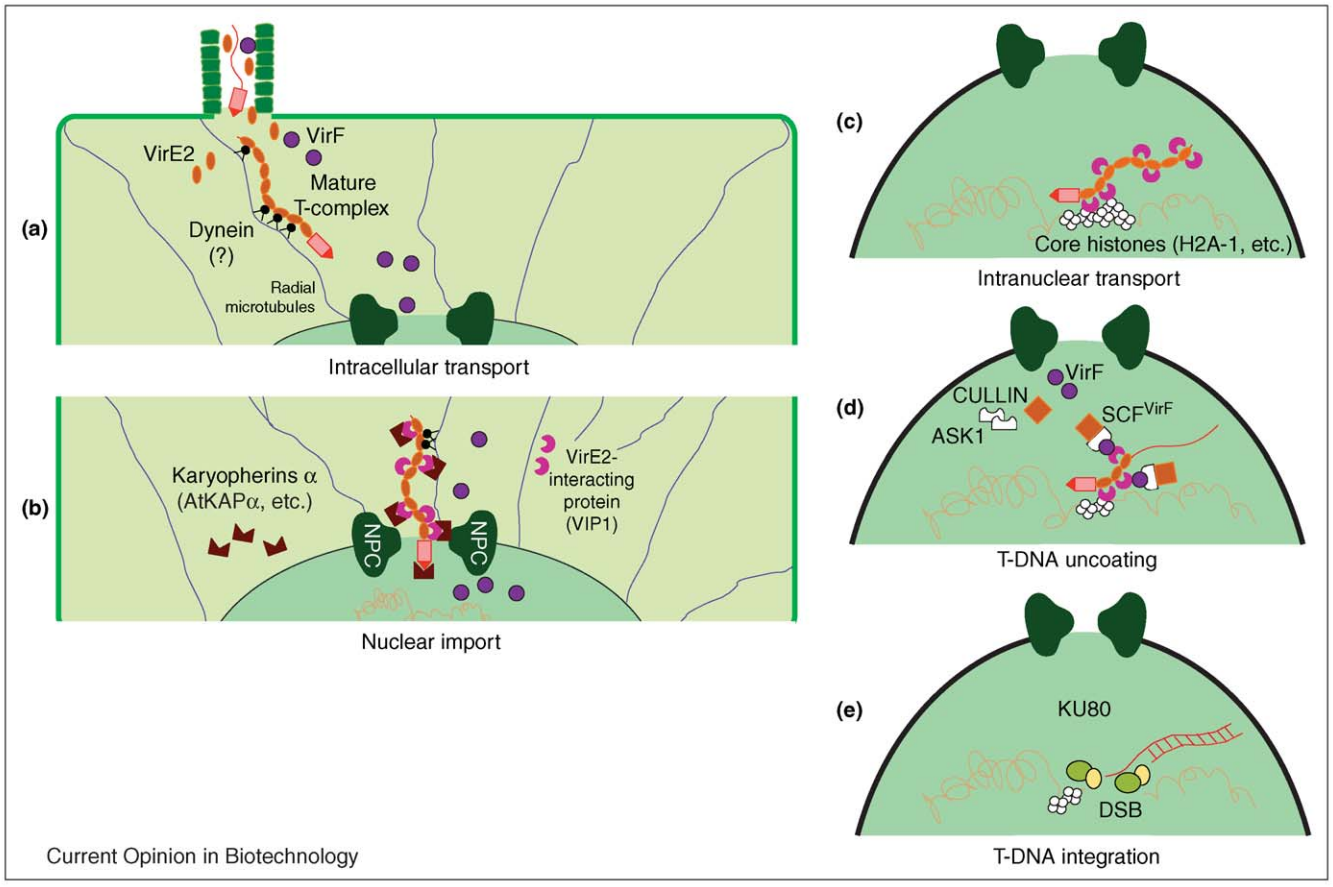
and VirD2 proteins [7]. In bacterial cells, the T-DNA exists as a ssDNA–protein complex (immature T-complex) with one VirD2 molecule covalently attached to the 5′ end of the T-strand [8]. This complex, along with several other Vir proteins [9], is exported into the host cell (Figure 1; step 5) by a VirB/D4 type IV secretion system [10], a step that requires interaction of the bacterial T-pilus with at least one host-specific protein [11]. Once inside the host-cell cytoplasm, the T-DNA is thought to exist as a mature T-complex (T-complex), in which the entire length of the T-strand molecule is coated with numerous VirE2 molecules. These molecules confer to the T-DNA the structure [12] and protection [13] needed for its travel (Figure 1; step 6) to the host-cell nucleus. It is mainly during the last steps of the transformation process — namely, transport through the cytoplasm (Figure 1; step 6), nuclear import (Figure 1; step 7), intranuclear transport (Figure 1; step 8), T-DNA uncoating (Figure 1; step 9) and integration (Figure 1; step 10) — that the *Agrobacterium* utilizes various cellular mechanisms to accomplish the genetic transformation of its host.

Agrobacterium hijacks host cellular mechanisms

The dense structure of the cytoplasm, which is composed of a mesh of microtubules, actin and intermediate fila-

ment networks, greatly restricts the Brownian diffusion of large macromolecules [14]. Thus, it is very likely that the T-complex, similar to many DNA viruses [15], is delivered to the cell nucleus with the assistance of the host intracellular transport machinery. Indeed, using biophysical particle tracking methods and fluorescently labeled VirE2–ssDNA complexes, it was recently suggested that dynein motors are required for the directed movement of the T-complex toward the nucleus [16•]. Although initially proposed on the basis of data obtained in an animal cell system, the notion that *Agrobacterium* uses the plant cytoskeleton as a track for its subcellular movement toward the nucleus is intriguing. The cellular organization of radial microtubules in plant cells, oriented with their minus-end toward the nucleus, further supports the idea that *Agrobacterium* uses the as yet unidentified dynein-like plant motor to deliver the T-complex to the nuclear pore (Figure 2a). The large size of the mature T-complex (~15.7 nm outer diameter [12]) suggests an active mechanism for its nuclear import, most likely by the nuclear-import machinery of the host cell. Indeed, both of the T-complex protein components, VirD2 and VirE2, were found to interact with host proteins for their nuclear import in host cells. VirD2 interacts with AtKAPα, a member of the *Arabidopsis* karyopherin α family, which mediates its nuclear import in permeabilized yeast cells

Figure 2



The role of host factors and cellular processes in the *Agrobacterium*-mediated genetic transformation of plant cells. **(a)** Following its export into the host cell, the *Agrobacterium* T-DNA must travel through the dense structure of the cytoplasm of the host cell. Biophysical studies have indicated the possibility of microtubule- and dynein-mediated transport of mature T-complexes through the host-cell cytoplasm to the nucleus. **(b)** Host factors (karyopherin α and VIP1) and bacterial factors (VirD2, VirE2 and VirE3) cooperate during translocation of the T-complex through the nuclear-pore complex (NPC). While VirD2 is directly recognized by the host nuclear-import machinery, via direct interaction with AtKAPα, both VIP1 and VirE3 act as adaptors between VirE2 and the host karyopherins α. **(c)** The ability of VIP1 to interact with the chromosomal protein H2A-1 histone, known to function during the T-DNA integration step, and its interaction with VirE2 suggest that *Agrobacterium* uses VIP1's intranuclear mobility to deliver the T-complex to the point of integration in the host chromatin. **(d)** Recruiting the host proteasomal degradation machinery to the T-complex by interaction of the *Agrobacterium* VirF protein with VIP1 on the one hand, and with ASK1 on the other, results in proteolytic uncoating of the T-DNA before its integration into the host genome. **(e)** The role of specific host factors and the molecular mechanism of the integration process are still unclear, although the integration of double-stranded T-DNA molecules into chromosomal double-strand breaks (DSBs) by interaction with the plant factor KU80 (see Update) may represent an important pathway for T-DNA integration in plant cells.

[17]. VirE2 interacts with the plant VirE2-interacting protein 1 (VIP1) [18] and its functional homolog, the bacterial VirE3 protein [19]. Both act as molecular adaptors between VirE2 and the host-cell karyopherin α, enabling VirE2 to be 'piggy-backed' into the host-cell nucleus [19,20]. As both VirD2 and VirE2 are required for the nuclear import of ssDNA [21], the combined action of the bacterial and host proteins, including the host nuclear-import machinery, is required for translocation of the mature T-complex into the host-cell nucleus [22•] (Figure 2b).

Inside the nucleus, the T-complex needs to travel to its point of integration and be stripped of its escorting proteins before integration into the host genome. The T-complex interactions with VIP1 [18], CAK2M (plant ortholog of cyclin-dependent kinase-activating kinases) and TATA-box binding protein (TBP) [23], all members of the host transcription machinery, suggest that they may guide the T-complex to the site of integration in the host chromatin. Although the mode of action of CAK2M and TBP [23] in the transformation process is still unclear, the ability of VIP1 to interact with the H2A histone [24], a plant chromatin protein essential for T-DNA integration [25], supports the notion that *Agrobacterium* uses the affinity of VIP1, and perhaps other transcription factors, for the plant chromatin to target the T-complex to the site of integration (Figure 2c). Furthermore, biological evidence indicates that *Agrobacterium* harnesses the plant-targeted proteolysis machinery to uncoat the T-strand of its cognate proteins (Figure 2d). The molecular basis for this targeted proteolysis mechanism is the ability of VIP1 to form a ternary complex with VirE2 and VirF [26••], a bacterial F-box protein that most likely functions as a subunit of E3 ubiquitin ligase [27]. Indeed, the critical role of proteasomal degradation in the transformation process was evidenced by the ability of VirF to target VirE2 and VIP1 to degradation in yeast cells and promote destabilization of VIP1 in plant cells, and by the negative effect of a proteasomal inhibitor on T-DNA expression *in planta* [26••].

Of all the steps of the genetic transformation process, T-DNA integration is perhaps the most heavily dependent on host cellular processes [28•]. Today, it is well accepted that none of the T-complex bacterial protein components possess the DNA repair functions per se needed for T-DNA integration. Indeed, even the proposed DNA ligase activity of the VirD2 endonuclease has been recently disputed [29]. Several DNA repair and packaging proteins have been found essential for T-DNA integration in yeast [30••,31] and plant cells [25,32], and a role for chromosomal double-strand breaks (DSBs) in attracting T-DNA molecules for integration has been suggested [33–35]. Thus, although the exact molecular mechanism underlying T-DNA integration is still under debate [28•], it is safe to assume that it relies almost exclusively on the ability of the host DNA repair machinery to convert the T-strand molecule to double-stranded (ds) T-DNA integration intermediates, to recognize these molecules as broken DNA fragments, and to incorporate them into the host genome (Figure 2e; see also Update).

Agrobacterium as a tool for plant genetic engineering

During the past two decades, we have witnessed a significant increase in the number of reports on the successful *Agrobacterium*-mediated genetic transformation of various plant species, variants and cultivars [36]. Moreover, numerous publications have demonstrated the expansion of *Agrobacterium*'s host range to non-plant species, ranging from prokaryotes to yeast and many other fungi through to human cells [37••]. Interestingly, most of the progress achieved to date in establishing protocols for the transformation of new host species has relied on a relatively small number of binary vectors and genetically modified Ti-helper plasmids, and on an even smaller number of disarmed *Agrobacterium* strains and isolates. Thus, progress in the genetic transformation of different plant species has been mostly achieved by matching the inoculated plant tissue to the suitable *Agrobacterium* strain, by genetic modification of *Agrobacterium*, and by developments in tissue culture and transgene selection techniques [36]. Nevertheless, we realize that we may have now reached the limit in our ability to expand the host range of *Agrobacterium* through manipulation of the bacterium, and that further progress in improving the transformation efficiency of hard-to-transform plant species and widening the host range to recalcitrant species will be achieved by genetic manipulation of the host genome [38]. Recent studies of *Agrobacterium*–host interactions that focus on revealing the functions of host proteins in the transformation process [5,38] hold great potential for the future of the biotechnology of plant genetic engineering.

Genetic manipulation of the host to improve transformation efficiency

The search for specific host factors involved in the integration process has yielded a wide range of proteins and genes proposed to function at different steps of the transformation process [39•]. As mentioned above, these include proteins involved in the initial bacterium–host contact [11,40], nuclear import of the T-complex [18] and its intranuclear transport [24], uncoating [26••], and integration [25,30••,31,32]. Although the exact molecular function of many of these host proteins is still unknown, overexpression of three of them in transgenic plants has been shown to render the plants more susceptible to *Agrobacterium* infection [20,25]. Firstly, the *Arabidopsis rat5* mutant, knocked out in the histone H2A coding gene, was blocked at the T-DNA integration step of *Agrobacterium*-mediated genetic transformation, and its overexpression in wild-type *Arabidopsis* plants signifi-

cantly increased their susceptibility to *Agrobacterium* infection [25]. Likewise, overexpression of VIP1 (a plant protein essential for T-DNA nuclear import [18]) in tobacco plants significantly increased their susceptibility to *Agrobacterium*-mediated genetic transformation [20]. Finally, overexpression of VirB2-interacting protein (BTI), a plant protein reported to interact with the *Agrobacterium* T-pilus protein VirB2, increased the susceptibility of *Arabidopsis* plants to *Agrobacterium* infection [11]. Thus, overexpression of key host proteins that function not only in the nuclear import, chromatin targeting, uncoating, and integration steps of the transformation process (i.e. steps that occur within the host cell and in which the *Agrobacterium* relies heavily on the host cellular mechanisms), but also during the initial *Agrobacterium*–host contact, is useful for increasing the transformation efficiency of model plants.

Naturally, the application of host factors to improve the transformation efficiency of hard-to-transform plant species can be somewhat tricky, as these plants would be recalcitrant to genetic manipulation using *Agrobacterium* in the first place. One way to overcome this technological barrier could lie in the transient expression of specific host factors during the inoculation step using *Agrobacterium*-independent means for their delivery (e.g. microbombardment). A more intriguing possibility is the use of *Agrobacterium* for the expression and delivery of host proteins into the host cell during the transformation process itself. The ability of *Agrobacterium* cells to transport several Vir proteins, independently of the T-DNA, to the host cell [9] and the identification of the relatively short export signal needed for this transport [41] suggest a possible technology in which host factors could be fused to the export signal, expressed in *Agrobacterium* cells, and delivered to the host by *Agrobacterium* concomitantly with the delivery of the transforming T-DNA. Indeed, the export to *Arabidopsis* cells of a chimeric Cre recombinase fused to the VirF protein export signal [41] indicates the feasibility of using such technology for the export of various proteins of interest to host cells.

Gene targeting and homologous recombination

The very low rate of homologous recombination (HR) between T-DNA and the plant DNA is a major drawback in developing the much needed and highly desired technology for gene targeting in plant cells [42]. In fact, only a few examples have been reported to date of targeted integration by HR in higher plants (e.g. [43]). Experimental evidence suggests that the lack of HR between T-DNA and plant DNA may be a direct result of its mechanism of integration. DSBs in the host genome have been reported to increase the T-DNA integration rate [44], and T-DNA molecules can even be directed into specifically induced genomic DSB sites [33–35]. The fact that, in plant cells, DSBs are mainly repaired by non-homologous end-joining (NHEJ) and not by HR [45] may provide the molecular explanation for the inefficiency of *Agrobacterium*-mediated gene targeting in plants: if the integration requires the presence of DSBs in the host genome, and if the integration occurs via NHEJ, T-DNA molecules cannot utilize an HR pathway for their integration. Indeed, in yeast cells, where both HR- and NHEJ-mediated integration of foreign DNA can occur, integration of the *Agrobacterium* T-DNA can be directed to either pathway by eliminating specific host DNA repair proteins: in the absence of KU70, a key protein in the NHEJ pathway, T-DNA integrates only via the HR pathway [31], whereas in the absence of Rad52, a key factor in the HR pathway, T-DNA integration occurs via NHEJ [30••]. Deletion of both proteins, by mutations of their corresponding genes, completely inhibits T-DNA integration [30••]. In plants, HR is stimulated in the absence of Rad50 [46], further supporting the notion that genetic manipulation of the host cell can facilitate our ability to control the integration process and to achieve HR in plant cells. This, in turn, will allow site-specific integration of a transgene in a pre-determined location in the host genome, representing a major breakthrough in the use of *Agrobacterium* for gene replacement for plant breeding and research purposes (see also Update).

Marker-gene excision or replacement

The ability to delete or replace a marker gene after it has been used for the selection of transgenic plants represents another important feature for plant molecular breeding. In site-specific recombination systems (e.g. Cre/LoxP and FLP/FRT [47]), transgenic parental lines with an established recombination site serve as a source for marker-gene excision before their end use in agricultural applications. In this approach, *Agrobacterium* is often used for the production of the transgenic parental lines and for the delivery of the new target gene, but no advantage is taken of the mechanism of T-DNA integration. The observation that DSBs and dsT-DNA intermediates may play an important role in the integration process [33–35] suggest an alternative strategy in which the host DNA repair machinery could actively participate in the gene excision and replacement. Specifically, transgenic plants expressing the transgene of interest are produced using binary vectors in which the marker gene is flanked with sequences recognized by a rare-cutting restriction endonuclease. Then, these plants are retransformed with a new T-DNA that contains a gene coding for the rare-cutting restriction endonuclease which is itself flanked by the recognition sequences of the same enzyme. Transient expression of this enzyme from the invading T-DNA will remove the marker gene from the genome and prevent stable integration of the restriction enzyme gene itself, resulting in a plant line transgenic only for the specific gene of interest. In a variation of this strategy, the restriction-endonuclease-containing T-DNA can carry

yet another transgene of interest, which will be preferentially integrated into the DSBs created following excision of the marker gene, effectively replacing the marker gene and producing a plant line carrying two transgenes of interest. The lines with the excised marker genes can be easily identified by their loss of marker activity (e.g. antibiotic resistance).

Conclusions and future prospects

Over a century has passed since Erwin Smith began his studies on the plant pathogen *Agrobacterium* [4••], not knowing that this unique bacterium would bring us into the new era of plant molecular breeding. The golden years of *Agrobacterium* research led us to understand many of the bacterium's biological processes and mechanisms, and laid the foundation for establishing *Agrobacterium* as the major tool for plant genetic engineering. Indeed, with an ever-expanding host range that includes many commercially important crops, flowers, and tree species, *Agrobacterium* is guaranteed a place of honor in nearly every plant molecular biology laboratory and biotechnology company for a long time to come. Furthermore, its recent application to the genetic transformation of non-plant species, from yeast to cultivated mushrooms, and even human cells [37••], places *Agrobacterium* at the forefront of future biotechnological applications [48]. Naturally, this new use of *Agrobacterium* will require the design and construction of binary plasmids specifically tailored for each host species, and the identification of *Agrobacterium* strains and isolates more suited to the task of transforming non-plant species.

In recent years, *Agrobacterium* research has enjoyed a revival, marked by vast progress in the identification of the host factors and cellular pathways involved in the transformation process. Although this research has only just uncovered the tip of the iceberg of information that host cells may provide about the transformation process, it holds great promise for improving the transformation efficiency of hard-to-transform plant species [49]. For example, super-virulent *Agrobacterium* strains can be generated that augment their infectivity by producing and exporting into the host cell proteins derived from plant factors that maximize transformation and that might be lacking in plants recalcitrant to transformation.

In addition, new approaches and techniques for controlling and affecting DNA integration can be designed based on the *Agrobacterium*-mediated genetic transformation. The foundation for such new and intriguing '*Agrobacterium*'-like technologies was recently laid by showing that gene transfer to plant species can be achieved with diverse species of bacteria outside of the genus *Agrobacterium* [50••]. Driven by the complexity of the patents and intellectual property issues that limit the use of *Agrobacterium* in both public and private sectors [51], Broothaerts *et al.* [50••] have rationalized the search for non-*Agrobacterium* species capable of transforming plant species. By providing *Sinorhizobium meliloti*, *Rhizobium* sp. NGR234 and *Mesorhizobium loti* with a disarmed Ti and binary plasmids, these plant-associated symbiotic bacteria were shown capable of transferring T-DNA fragments to various plant species [50••]. Although it is not likely that these 'revolutionary' bacterial species present a threat to *Agrobacterium*'s throne as the 'tzar of genetic engineering', they may certainly represent the birth of a new era in which the hegemony over plant genetic transformation will be divided among a more egalitarian compilation of bacterial species.

Update

Recent work has shown that the plant factor KU80 is involved in the T-DNA integration process, most likely by bridging between double-stranded T-DNAs and DSBs [52•]. In addition, Shaked *et al.* [53•] reported that overexpression of the yeast Rad54 protein led to high-frequency gene targeting in transgenic plants. These two reports further support the notion that integration of T-DNA molecules is promoted by host cellular factors and open a new direction for plant gene targeting by genetic manipulation of the host genome.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Gelvin SB: **The introduction and expression of transgenes in plants**. *Curr Opin Biotechnol* 1998, **9**:227-232.

2. Gaudin V, Vrain T, Jouanin L: **Bacterial genes modifying hormonal balances in plants**. *Plant Physiol Biochem* 1994, **32**:11-29.

3. Draper J, Scott R, Armitage P, Walden R: *Plant Genetic Transformation and Gene Expression, A Laboratory Manual*. London: Blackwell Scientific Publications Ltd; 1988.

4. •• Nester E, Gordon MP, Kerr A: *Agrobacterium tumefaciens: from Plant Pathology to Biotechnology*: American Phytopathological Society; 2005.
This book contains a unique collection of scientific papers, published over a period of more than a century, tracing the history of *Agrobacterium* research — from works on *Agrobacterium* pathogenicity in the early years of the last century to the sequencing of the complete *Agrobacterium* genome in recent years. Commentaries by a number of the original authors of these seminal papers provide new insights into the fundamental research described in these publications.

5. Tzfira T, Citovsky V: **Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium***. *Trends Cell Biol* 2002, **12**:121-129.

6. •• Gelvin SB: ***Agrobacterium*-mediated plant transformation: the biology behind the 'gene-jockeying' tool**. *Microbiol Mol Biol Rev* 2003, **67**:16-37.

This comprehensive review summarizes the basic biology of *Agrobacterium* while highlighting its uses as a tool for plant genetic engineering. The review also describes our current knowledge on the roles of the basic biological processes, in both the bacterium and the host cell, and their function in the transformation process. The possible implications of this knowledge for extending the use of *Agrobacterium* to the genetic transformation of recalcitrant species are emphasised.

7. Filichkin SA, Gelvin SB: **Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* VirD1, D2 endonuclease**. *Mol Microbiol* 1993, **8**:915-926.

8. Ward E, Barnes W: **VirD2 protein of *Agrobacterium tumefaciens* very tightly linked to the 5′ end of T-strand DNA**. *Science* 1988, **242**:927-930.

9. Vergunst AC, Schrammeijer B, den Dulk-Ras A, de Vlaam CMT, Regensburg-Tuink TJ, Hooykaas PJJ: **VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells**. *Science* 2000, **290**:979-982.

10. Christie PJ: **Type IV secretion: the Agrobacterium VirB/D4 and related conjugation systems**. *Biochim Biophys Acta* 2004, **1694**:219-234.

11. Hwang HH, Gelvin SB: **Plant proteins that interact with VirB2, the *Agrobacterium tumefaciens* pilin protein, mediate plant transformation**. *Plant Cell* 2004, **16**:3148-3167.

12. Abu-Arish A, Frenkiel-Krispin D, Fricke T, Tzfira T, Citovsky V, Grayer Wolf S, Elbaum M: **Three-dimensional reconstruction of Agrobacterium VirE2 protein with single-stranded DNA**. *J Biol Chem* 2004, **279**:25359-25363.

13. Citovsky V, Wong ML, Zambryski PC: **Cooperative interaction of *Agrobacterium* VirE2 protein with single stranded DNA: implications for the T-DNA transfer process**. *Proc Natl Acad Sci USA* 1989, **86**:1193-1197.

14. Luby-Phelps K: **Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area**. *Int Rev Cytol* 2000, **192**:189-221.

15. Dohner K, Sodeik B: **The role of the cytoskeleton during viral infection**. *Curr Top Microbiol Immunol* 2005, **285**:67-108.

16. • Salman H, Abu-Arish A, Oliel S, Loyter A, Klafter J, Granel R, Elbaum M: **Nuclear localization signal peptides induce molecular delivery along microtubules**. *Biophys J* 2005, **89**:2134-2145.

This biophysical study provides a direct look at the possible molecular mechanism of T-complex intracellular movement. Using an automated tracking method coupled with statistical analysis, the authors calculate the directed movement of artificial T-complexes along microtubules of sea urchin axoneme segments.

17. Ballas N, Citovsky V: **Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein**. *Proc Natl Acad Sci USA* 1997, **94**:10723-10728.

18. Tzfira T, Vaidya M, Citovsky V: **VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity**. *EMBO J* 2001, **20**:3596-3607.

19. Lacroix B, Vaidya M, Tzfira T, Citovsky V: **The VirE3 protein of Agrobacterium mimics a host cell function required for plant genetic transformation**. *EMBO J* 2005, **24**:428-437.

20. Tzfira T, Vaidya M, Citovsky V: **Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis VIP1* gene**. *Proc Natl Acad Sci USA* 2002, **99**:10435-10440.

21. Ziemienowicz A, Merkle T, Schoumacher F, Hohn B, Rossi L: **Import of *Agrobacterium* T-DNA into plant nuclei: two distinct functions of VirD2 and VirE2 proteins**. *Plant Cell* 2001, **13**:369-384.

22. • Tzfira T, Lacroix B, Citovsky V: **Nuclear import of Agrobacterium T-DNA**. In *Nuclear Import and Export in Plants and Animals.* Edited by Tzfira T, Citovsky V. Landes Bioscience/Kluwer, Academic/ Plenum Publishers; 2005:83-99.

T-DNA nuclear import is a central step in the transformation process. This chapter summarizes our current knowledge on the functions of bacterial and host proteins and draws a model for the nuclear import and intranuclear transport of the *Agrobacterium* T-complex.

23. Bako L, Umeda M, Tiburcio AF, Schell J, Koncz C: **The VirD2 pilot protein of *Agrobacterium*-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants**. *Proc Natl Acad Sci USA* 2003, **100**:10108-10113.

24. Li J, Krichevsky A, Vaidya M, Tzfira T, Citovsky V: **Uncoupling of the functions of the *Arabidopsis* VIP1 protein in transient and stable plant genetic transformation by Agrobacterium**. *Proc Natl Acad Sci USA* 2005, **102**:5733-5738.

25. Mysore KS, Nam J, Gelvin SB: **An *Arabidopsis* histone H2A mutant is deficient in *Agrobacterium* T-DNA integration**. *Proc Natl Acad Sci USA* 2000, **97**:948-953.

26. •• Tzfira T, Vaidya M, Citovsky V: **Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium***. *Nature* 2004, **431**:87-92.

In this report, the ability of *Agrobacterium* to utilize the targeted proteolysis mechanism of the host cell is discussed. The function of the bacterial F-box protein, VirF, in proteasomal degradation of VirE2 and VIP1 (the T-complex host and bacterial chaperones) is described.

27. Schrammeijer B, Risseeuw E, Pansegrau W, Regensburg-Tuïnk TJG, Crosby WL, Hooykaas PJJ: **Interaction of the virulence protein VirF of *Agrobacterium tumefaciens* with plant homologs of the yeast Skp1 protein**. *Curr Biol* 2001, **11**:258-262.

28. • Tzfira T, Li J, Lacroix B, Citovsky V: ***Agrobacterium* T-DNA integration: molecules and models**. *Trends Genet* 2004, **20**:375-383.

This review describes recent studies revealing the importance of host proteins involved in DNA repair and maintenance for T-DNA integration. The models for microhomology-based T-strand integration and for the DSB-mediated integration of dsT-DNA molecules are presented.

29. Ziemienowicz A, Tinland B, Bryant J, Gloeckler V, Hohn B: **Plant enzymes but not *Agrobacterium* VirD2 mediate T-DNA ligation *in vitro***. *Mol Cell Biol* 2000, **20**:6317-6322.

30. •• van Attikum H, Hooykaas PJJ: **Genetic requirements for the targeted integration of *Agrobacterium* T-DNA in *Saccharomyces cerevisiae***. *Nucleic Acids Res* 2003, **31**:826-832.

An excellent paper showing how the function of host and cellular mechanisms can be efficiently studied using non-host species. Employing yeast mutants knocked down in various enzymes of the DNA repair machinery, the authors pinpoint two specific regulators of the integration process, yRad52 and yKu70. The authors conclude that yKu70 is required for T-DNA integration via non-homologous recombination, whereas yRad52 is essential for T-DNA integration via the homologous recombination pathway.

31. van Attikum H, Bundock P, Hooykaas PJJ: **Non-homologous end-joining proteins are required for *Agrobacterium* T-DNA integration**. *EMBO J* 2001, **20**:6550-6558.

32. Friesner J, Britt AB: **Ku80- and DNA ligase IV-deficient plants are sensitive to ionizing radiation and defective in T-DNA integration**. *Plant J* 2003, **34**:427-440.

33. Salomon S, Puchta H: **Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells**. *EMBO J* 1998, **17**:6086-6095.

34. Chilton M-DM, Que Q: **Targeted integration of T-DNA into the tobacco genome at double-strand breaks: new insights on the mechanism of T-DNA integration**. *Plant Physiol* 2003, **133**:956-965.

35. Tzfira T, Frankmen L, Vaidya M, Citovsky V: **Site-specific integration of *Agrobacterium* T-DNA via double-stranded intermediates**. *Plant Physiol* 2003, **133**:1011-1023.

36. Herrera-Estrella L, Simpson J, Martinez-Trujillo M: **Transgenic plants: an historical perspective**. *Methods Mol Biol* 2005, **286**:3-32.

37. •• Lacroix B, Tzfira T, Vainstein A, Citovsky V: **A case of promiscuity: Agrobacterium's endless hunt for new partners**. *Trends Genet* 2005, **22**:29-37.

This review describes the recent advances in *Agrobacterium*-mediated genetic transformation of non-plant species. It emphasizes the unique features of non-plant species transformation protocols, the advantages of *Agrobacterium* over alternative transformation methods, and the potential biotechnological applications of *Agrobacterium* in the production of genetically engineered non-plant species.

38. Gelvin SB: ***Agrobacterium* and plant genes involved in T-DNA transfer and integration.**. *Annu Rev Plant Physiol Plant Mol Biol* 2000, **51**:223-256.

39. • Zhu Y, Nam J, Humara JM, Mysore K, Lee LY, Cao H, Valentine L, Li J, Kaiser A, Kopecky A *et al.*: **Identification of *Arabidopsis rat* mutants**. *Plant Physiol* 2003, **132**:494-505.
This paper describes the unique screening method developed to identify *Arabidopsis* mutants that are 'resistant to *Agrobacterium* transformation' ('*rat*' mutants). The paper is also an excellent source of data on the *rat* mutants, providing a comprehensive list of the mutants, identifying the mutated genes, and describing the specific step of the transformation process compromised in each *rat* mutant.

40. Zhu Y, Nam J, Carpita NC, Matthysse AG, Gelvin SB: ***Agrobacterium*-mediated root transformation is inhibited by mutation of an *Arabidopsis* cellulose synthase-like gene**. *Plant Physiol* 2003, **133**:1000-1010.

41. Vergunst AC, van Lier MC, den Dulk-Ras A, Grosse Stuve TA, Ouwehand A, Hooykaas PJ: **Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of Agrobacterium**. *Proc Natl Acad Sci USA* 2005, **102**:832-837.

42. Puchta H: **Towards the ideal GMP: homologous recombination and marker gene excision**. *J Plant Physiol* 2003, **160**:743-754.

43. Kempin SA, Liljegren SJ, Block LM, Rounsley SD, Yanofsky MF, Lam E: **Targeted disruption in *Arabidopsis***. *Nature* 1997, **389**:802-803.

44. Kohler F, Cardon G, Pohlman M, Gill R, Schider O: **Enhancement of transformation rates in higher plants by low-dose irradiation: are DNA repair systems involved in incorporation of exogenous DNA into the plant genome?** *Plant Mol Biol* 1989, **12**:189-199.

45. Gorbunova VV, Levy AA: **How plants make ends meet: DNA double-strand break repair**. *Trends Plant Sci* 1999, **4**:263-269.

46. Gherbi H, Gallego ME, Jalut N, Lucht JM, Hohn B, White CI: **Homologous recombination *in planta* is stimulated in the absence of Rad50**. *EMBO Rep* 2001, **2**:287-291.

47. Lyznik LA, Gordon-Kamm WJ, Tao Y: **Site-specific recombination for genetic engineering in plants**. *Plant Cell Rep* 2003, **21**:925-932.

48. Casas-Flores S, Rosales-Saavedra T, Herrera-Estrella A: **Three decades of fungal transformation: novel technologies**. *Methods Mol Biol* 2004, **267**:315-325.

49. Gelvin S: **Improving plant genetic engineering by manipulating the host**. *Trends Biotechnol* 2003, **21**:95-98.

50. •• Broothaerts W, Mitchell HJ, Weir B, Kaines S, Smith LM, Yang W, Mayer JE, Roa-Rodriguez C, Jefferson RA: **Gene transfer to plants by diverse species of bacteria**. *Nature* 2005, **433**:629-633.
This paper shows that the capacity to genetically transform plant species can be expanded from the *Agrobacterium* genus to other bacterial species by acquisition of Ti and binary plasmids. This observation provides a basis for developing an alternative to the over-patented *Agobacterium*-mediated genetic transformation technology.

51. Roa-Rodriguez C, Nottenburg C: **Agrobacterium-mediated transformation of plants**. 2003; URL: CAMBIA technology landscape paper http://www.bios.net/Agrobacterium.

52. • Li J, Vaidya M, White C, Vainstein A, Citovsky V, Tzfira T: **Involvement of KU80 in T-DNA integration in plant cells**. *Proc Natl Acad Sci USA* 2005, **102**:19231-19236.
This report studied the interaction between double-stranded T-DNA molecules and the plant DNA repair machinery. The authors demonstrated that KU80, a key protein in the NHEJ double-stranded DNA break repair, is involved in the T-DNA integration process and suggested that KU80 may bridge between double-stranded T-DNA integration intermediates and DSBs in the host cell genome.

53. • Shaked H, Melamed-Bessudo C, Levy AA: **High frequency gene targeting in *Arabidopsis* plants expressing the yeast RAD54 gene**. *Proc Natl Acad Sci USA* 2005, **102**:12265-12269.
This exciting report demonstrated that genetic manipulation of the host genome can affect the route of T-DNA integration. Using a novel, high-throughput green fluorescence protein-based gene targeting assay, the authors showed that overexpression of the yeast RAD54 gene, a member of a chromatin remodeling gene family, in *Arabidopsis* plants can stimulate the integration of T-DNA by homologous recombination.