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Agrobacterium-Mediated Genetic Transformation: History and Progress

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1. Introduction

Agrobacterium tumefaciens is a Gram-negative soil phytopathogenic bacterium that causes the crown gall disease of dicotyledonous plants, which is characterized by a tumorous phenotype. It induces the tumor by transferring a segment of its Ti plasmid DNA (transferred DNA, or T-DNA) into the host genome and genetically transforming the host. One century has past after *A. tumefaciens* was firstly identified as the causal agent of crown gall disease (Smith & Townsend, 1907). However, *A. tumefaciens* is still central to diverse fields of biological and biotechnological research, ranging from its use in plant genetic engineering to representing a model system for studies of a wide variety of biological processes, including bacterial detection of host signaling chemicals, intercellular transfer of macromolecules, importing of nucleoprotein into plant nuclei, and interbacterial chemical signaling via autoinducer-type quorum sensing (McCullen & Binns, 2006; Newton & Fray, 2004; Pitzschke & Hirt, 2010). Therefore, the molecular mechanism underlying the genetic transformation has been the focus of research for a wide spectrum of biologists, from bacteriologists to molecular biologists to botanists.

1.1 History of *Agrobacterium tumefaciens* research

A. tumefaciens is capable of inducing tumors at wound sites of hundreds of dicotyledonous plants, and some monocots and gymnosperms (De Cleene and De Ley, 1976), which may happen on the stems, crowns and roots of the host. At the beginning of the last century, crown gall disease was considered a major problem in horticultural production. This disease caused significant loss of crop yield in many perennial horticultural crops (Kennedy, 1980), such as cherry (Lopatin, 1939), apple (Ricker et al., 1959), and grape (Schroth et al., 1988). All these horticultural crops are woody species and propagated by grafting scions onto rootstocks. The grafting wounds are usually covered by soil and thus provide an excellent infection point for the soil-borne *A. tumefaciens*. In 1941, it was proved that crown gall tumor tissue could be permanently transformed by only transient exposure to the pathogen of *A. tumefaciens* (White and Braun, 1941). Thereafter, a 'tumor-inducing capacity' was proposed to be transmitted from *A. tumefaciens* to plant tissue (Braun, 1947; Braun and Mandle, 1948). Twenty years late, molecular techniques provided the first evidence that crown gall tumors,

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cultured axenically, contained DNA of *A. tumefaciens* origin, which implied that host cells were genetically transformed by *Agrobacterium* (Schilperoort et al., 1967). In 1974, the tumor-inducing (Ti) plasmid was identified to be essential for the crown gall-inducing ability (Van Larebeke et al., 1974; Zaenen et al., 1974). Southern hybridization turned out to prove that the bacterial DNA transferred to host cells originates from the Ti plasmid and ultimately resulted in the discovery of T-DNA (transferred DNA), specific segments transferred from *A. tumefaciens* to plant cells (Chilton et al., 1977; Chilton et al., 1978; Depicker et al., 1978). The T-DNA is referred to as the T-region when located on the Ti-plasmid. The T-region is delimited by 25-bp directly repeated sequences, which are called T-DNA border sequences. The T-DNAs, when transferred to plant cells, encode enzymes for the synthesis of (1) the plant hormones auxin and cytokinin and (2) strain-specific low molecular weight amino acid and sugar phosphate derivatives called opines. The massive accumulation of auxin and cytokinin in transformed plant cells causes uncontrolled cell proliferation and the synthesis of nutritive opines that can be metabolized specifically by the infecting *A. tumefaciens* strain. Thus, the opine-producing tumor effectively creates an ecological niche specifically suited to the infecting *A. tumefaciens* strain (Escobar & Dandekar, 2003; Gelvin, 2003). Besides the T-DNAs, Ti-plasmid also contains most of the genes that are required for the transfer of the T-DNAs from *A. tumefaciens* to the plant cell.

Initial study of these plant tumors was intended to reveal the molecular mechanism that may be relevant to animal neoplasia. Although no relationship was found between animal and plant tumors, *A. tumefaciens* and plant tumor were proved to be of intrinsic interest because the tumorous growth was shown to result from the transfer of T-DNA from bacterial Ti-plasmid to the plant cell and the stable integration of the T-DNA to plant genome. The demonstration that wild-type T-DNA coding region can be replaced by any DNA sequence without any effect on its transfer from *A. tumefaciens* to the plant inspired the promise that *A. tumefaciens* might be used as gene vector to deliver genetic material into plants. In the early of 1980's, two events about *A. tumefaciens* mediated genetic transformation signaled the beginning of the era of plant genetic engineering. First, *A. tumefaciens* and its Ti-plasmid were used as a gene vector system to produce the first transgenic plant (Zambryski et al., 1983). The healthy transgenic plants had the ability to transmit the disarmed T-DNA, including the foreign genes, to their progeny. Second, non-plant antibiotic-resistance genes, for example, a bacterial kanamycin-resistance gene, could be instructed to function efficiently in plant cells by splicing a plant-active promoter to the coding region of the bacterial genes. This enabled accurate selection of transformed plant cells (Beven, 1984). The eventual success of using *A. tumefaciens* as a gene vector to create transgenic plants was viewed as a prospect and a "wish". The future of *A. tumefaciens* as a gene vector for crop improvement began to look bright. During the 1990's, maize, a monocot plant species that was thought to be outside the *A. tumefaciens* "normal host range", was successfully transformed by *A. tumefaciens* (Chilton, 1993). Today, many agronomically and horticulturally important plant species are routinely transformed by *A. tumefaciens*, and the list of plant species that can be genetically transformed by *A. tumefaciens* seems to grow daily (Gelvin, 2003). At present, many economically important crops, such as corn, soybean, cotton, canola, potatoes, and tomatoes, were improved by *A. tumefaciens*–mediated genetic transformation and these transgenic varieties are growing worldwide (Valentine, 2003). By now, the species that are susceptible to *A. tumefaciens*–mediated transformation were broadened to yeast, fungi, and mammalian cells (Lacroix et al., 2006b).

In the new century, intrests of most *Agrobacterium* community shifted to the transfer channel and host. Most recent important articles on *Agrobacterium*-mediated T-DNA transfer are to explore the molecular mechanism of T-complex targeting to plant nucleus. Recent progresses of these aspects of *Agrobacterium*-mediated genetic transformation will be the emphases of this chapter and be discussed in the following related sections.

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

The process of *A. tumefaciens*–mediated genetic transformation is a long journey. For the sake of description, many authors divided this process into several steps (Guo et al., 2009a; Guo, 2010; McCullen & Binns, 2006; Pitzschke & Hirt, 2010). Here, we arbitrarily and simply split it into five steps: (1) Sensing of plant chemical signals and inducing of virulence (vir) proteins. The chemical signals released by wounded plant are perceived by a VirA/VirG two-component system of *A. tumefaciens*, which leads to the transcription of virulence (vir) gene promoters and thus the expression of vir proteins. (2) T-DNA processing. T-DNA is nicked by VirD2/VirD1 from the T-region of Ti plasmid and forms a single-stranded linear T-strand with one VirD2 molecule covalently attached to the 5′ end of the T-strand. (3) Attaching of *A. tumefaciens* to plant and transferring of T-complex to plant cell. *A. tumefaciens* cell attaches to plant and transfers the T-complex from *A. tumefaciens* to plant cell by a VirD4/B T4SS transport system. (4) Targeting of T-complex to plant cell nucleus and integrating of T-DNA into plant genome. The T-complex is transported into the nucleoplasm under the assistance of some host proteins and then integrated into plant genomic DNA. (5) Expressing of T-DNA in plant cell and inducing of plant tumor. The T-DNA genes encode phytohormone synthases that lead to the uncontrolled proliferation of plant cell and opine synthases that provide nutritive compounds to infecting bacteria.

2. Events happening in *Agrobacteriun*

A. tumefaciens can perceive the signal molecules from plants and recognize the competent hosts. To fulfill the infection, *Agrobacterium* must respond to the signal molecules. The respondence occuring in *Agrobacterium* includes host recognition, virulence gene expression, and T-DNA processing.

2.1 Sensing of plant signal molecules and *vir* gene induction

Many genes are involved in *A. tumefaciens*-mediated T-DNA transfer, but most of the genes required for T-DNA transfer are found on the *vir* region of Ti plasmid. This *vir* region comprises at least six essential operons (*virA*, *virB*, *virC*, *virD*, *virE*, and *virG*,) and two non-essential operons (*virF* and *virH*) encoding approximate 25 proteins (Gelvin, 2000; Zhu et al., 2000; Ziemienowicz, 2001). These proteins are termed virulence (vir) proteins and required for the sensing of plant signal molecules as well as the processing, transfer, and nuclear localization of T-DNA, and the integration of T-DNA into the plant genome. The protein number encoded by each operon differs; *virA*, *virG* and *virF* encode only one protein; *virE*, *virC*, and *virH* encode two proteins; *virD* encodes four proteins and virB encodes eleven proteins. Only *virA* and *virG* are constitutively transcripted. The transcription of all other *vir* operons in *vir* region is coordinately induced during infection by a family of host-released phenolic compounds in combination with some monosaccharides and extracellular acidity in the range of pH 5.0 to 5.8. Virtually all of the genes in the *vir* region are tightly regulated by two proteins VirA and VirG encoded by *virA* operon and *virG* operon (Lin et al., 2008).

The inducible expression of *vir* operons was first found by using the cocultivation of *A. tumerfaciens* with mesophyll protoplasts, isolated plant cells or cultured tissues (Stachel et al., 1986). In vegetatively growing bacteria, only *virA* and *virG* are expressed at significant level. However, when *Agrobacteria* are cocultivated with the susceptible plant cells, the expression of *virB*, *virC*, *virD*, *virE* and *virG* are induced to high levels (Engstrom et al., 1987). The partially purified extracts of conditioned media from root cultures can also induce the expression of *vir* operons, demonstrating that the *vir*-inducing factors are some diffusible plant cell metabolites. By screening 40 plant-derived chemicals, Bolton et al. (1986) identified seven simple plant phenolic compounds that possess the *vir*-inducing activity. Most of these *vir*-inducing phenolic compounds are needed to make lignin, a plant cell wall polymer. The best characterized and most effective *vir* gene inducers are acetosyringone (AS) and hydroxy-acetosyringone from tobacco cells or roots (Stachel et al., 1985). The specific composition of phenolic compounds secreted by wounded plants is thought to underlie the host specificity of some *Agrobacterium* strains. Besides phenolic compounds, other inducing factors include aldose monosaccharides, low pH, and low phosphate (Brencic & Winans, 2005; McCullen & Binns, 2006; Palmer et al., 2004). However, phenols are indispensable for *vir* gene induction, whereas the other inducing factors sensitise *Agrobacteria* to phenols.

2.2 Regulation of *vir* gene induction

The regulatory pathway for *vir* gene induction by phenolic compounds is mediated by the VirA/VirG two-component system, which has structural and functional similarities to other already described for other cellular regulation mechanisms (Bourret et al., 1991; Nixon et al., 1986). Two component regulatory systems comprise two core components, a sensor kinase and an intracellular response regulator. The sensor kinase responds to signal input and mediates the activation of the intracellular response regulator by controlling the latter's phoshporylation status (Brencic & Winans, 2005; McCullen & Binns, 2006). For the *Agrobacterium* VirA/VirG two-component system, VirA is a membrane-bound sensor kinase. The presence of acidic environment and phenolic compounds at a plant wound site may directly or indirectly induce autophosphorylation of VirA. The phosphorylated VirA can transfer its phosphate to the cytoplasmic VirG to activate VirG. The activated VirG binds to the specific 12bp DNA sequences called *vir* box enhancer elements that are found in the promoters of the *virA*, *virB*, *virC*, *virD*, *virE* and *virG* operons, and then upregulates the transcription of these operons (Winans, 1992).

Octopine-type Ti plasmid encoded VirA protein has 829 amino acids. VirA is a member of the histidine protein kinase class and able to autophosphorylate. When VirA autophosphorylates *in vitro*, the phosphate was found to bind to histidine residue 474, a histidine residue that is absolutely conserved among homologous proteins (Jin et al., 1990). VirA protein can be structurally divided into a number of domains. In an order from N-terminus to C-terminus, these domains are defined as transmembrane domain 1 (TM1), periplasmic domain, transmembrane domain 2 (TM2), linker domain, kinase domain and receiver domain (Lee et al., 1996). The periplasmic domain is required for the interaction with ChvE, the sugar-binding protein that responds to the *vir*-inducing sugars. The linker domain is located on the region of amino acid 280~414, which was supposed to interact with the *vir* gene inducing phenolic compounds (Chang & Winans 1992). A highly amphipathic helix sequence of 11 amino acids was identified in the region of amino acid 278-288. This amphipathic sequence is highly

conserved in a large number of chemoreceptor proteins and thus was supposed to be the receptor site for phenolic inducers (Turk et al., 1994). However, it is unclear whether the phenolic inducers interact with VirA directly or indirectly. The kinase domain is a highly conserved domain that presents in the family of the sensor proteins and contains the conserved histidine residue 474 that is the autophosphorylation site. Site-directing mutation of this His 474 results in avirulence and the lost of *vir* gene inducing expression in the presence of plant signal molecules (Jin et al., 1990). The receiver domain shows an unusual feature that is homologous to a region of VirG. Similar receiver domains are present in a small number of homologous histidine protein kinases, but the function of this domain is unclear.

VirG is a transcriptional activator of 241 amino acid residues. It is composed of two main domains, N-terminal domain and C-terminal domain. The aspartic acid 52 in the N-terminal domain of VirG can be phosphorylated by the phosphorylated VirA (Jin et al, 1993). The phosphorylation of N-terminal domain is thought to induce the conformation change of C-terminal domain. The C-terminal domain of VirG possesses the DNA-binding function, resulting in VirG specifically binding to the *vir* box sequence that is found within 80 nucleotides upstream from the transcription initiation sites of *vir* genes. Phosphorylation is required for the transcriptional activation function of VirG, but how phosphorylation modulates the properties of VirG is unknown. Some models suggested that phosphorylation might increase the affinity of VirG to its binding sites or promote the ability of VirG to contact RNA polymerase (Lin et al., 2008; McCullen & Binns, 2006; Wang et al., 2002).

2.3 T-DNA processing

The activation of *vir* genes initiates a cascade of events. Following the expression of *vir* genes, some Vir proteins produce the transfer intermediate, a linear single stranded (ss) DNA called T-DNA or T-strand that is derived from the bottom (coding) strand of the T-region of the Ti plasmid. T-region is flanked by two 25 bp long imperfect direct repeats, termed border sequences. VirD2/VirD1 is able to recognize the border sequences and cleave the bottom strand of T-region at identical positions between bp 3 and 4 from the left end of each border (Sheng & Citovsky, 1996). Upon the cleavage of T-DNA border sequence, VirD2 remains covalently associated with the 5´-end of the ssT-strand *via* tyrosine residue 29 (Vogel & Das, 1992). The excised ssT-strand is removed, and the resulting single-stranded gap in the T-region is repaired, most likely replaced by a newly synthesizing DNA strand. The association of VirD2 with the 5´-end of the ssT-strand is believed to prevent the exonucleolytic attack to the 5´-end of the ssT-strand (Durrenberger et al., 1989) and to distinguish the 5´-end as the leading end of the T-DNA complex during transfer.

One report indicated that VirD1 possesses a topoisomerase-like activity (Ghai and Das, 1989). VirD1 appears to be a type I DNA topoisomerase that do not require ATP for activity. However, a late study (Scheiffele et al., 1995) contradicted this conclusion. The VirD1 protein purified by Scheiffele et al. (1995) never showed any topoisomerase activity. It was speculated that the topoisomerase activity observed by Ghai and Das (1989) might originate from VirD2. Mutational study of VirD1 showed that a region from amino acids 45~60 is important for VirD1 activity. Sequence comparison of this fragment with the functionally analogous proteins of conjugatable bacterial plasmids showed that this region is a potential DNA-binding domain (Vogel & Das, 1994).

The nopaline Ti plasmid encoded VirD2 consists of 447 amino acids with a molecular weight of 49.7 kDa. Deletion analysis of VirD2 demonstrated that the C-terminal 50% of VirD2 could be deleted or replaced without affecting its endonuclease activity. Sequence

comparison of VirD2 from different *Agrobacterium* species shows that the N-terminus is highly conserved with 90% homology, whereas only 26% homology is found in the C-terminus (Wang et al., 1990). A sequence comparison of VirD2 protein with its functionally homologous proteins in bacterial conjugation and in rolling circle replication revealed that a conserved 14-residue motif lies in the residues 126~139 of VirD2. This motif contains the consensus sequence HxDxD(H/N)uHuHuuuN (invariant residues in capital letters; x, any amino acid; u, hydrophobic residue) (Ilyina & Koonin, 1992). Mutational analysis indicated that all the invariant residues except for the last asparagine (N) in this motif are important for the endonuclease activity of VirD2. The second aspartic acid (D) and three nonconserved residues in this motif are also essential for the endonuclease activity of VirD2 (Vogel et al., 1995). This motif is believed to coordinate the essential cofactor Mg^{2+} by the two histidines in the hydrophobic region of the motif (Ilyina & Koonin, 1992). The poorly conserved C-terminal halves of VirD2 from different *Agrobacterium* species displayed a very similar hydropathy profile (Wang et al., 1990). The C-terminal domain of VirD2 is thought to guide the T-complex to the plant nucleus. The sequence characterization and function of this region of VirD2 will be discussed in a late section of this chapter.

3. Contact of *Agrobacterium* with plant and transfer of *Agrobacterial* molecules to plant

3.1 Chemotaxis of *A. tumefaciens*

A. tumefaciens is a motile organism, with peritrichous flagellae, that possesses a highly sensitive chemotaxis system. It could respond to a range of sugars and amino acids and be attracted to these sugars and amino acids (Loake et al., 1988). *A. tumefaciens* mutants deficient in motility and in chemotaxis were fully virulent when inoculated directly. However, when used to inoculate soil, which was air-dried and then used to grow plants, these mutants were completely avirulent. These results indicated that the motility and chemotaxis are critical to *A. tumefaciens* infection under natural conditions (Hawes & Smith, 1989). Wild-type *A. tumefaciens* strains both containing and lacking Ti plasmid exhibited chemotaxis toward excised root tips from all plant species tested and toward root cap cells of pea and maize, suggesting that the majority of chemotactic responses in *A. tumefaciens* appear to be chromosomally encoded (Loake et al., 1988; Parke et al., 1987). However, the chemotactic response to some phenolic compounds, for example acetosyringone, which were identified as strong *vir* gene inducers, is controversial. Some reports showed that chemotaxis toward acetosyringone requires the presence of a Ti plasmid, specifically the regulatory genes *virA* and *virG*, and occurs with a threshold sensitivity of $< 10^{-8}$ M, some 1000-fold below the maximal *vir*-inducing concentration (Ashby et al., 1988; Shaw et al., 1989). Whereas, reports from other groups indicated that acetosyringone did not elicit chemotaxis at any concentration (Hawes & Smith, 1989) and chemotaxis toward related compounds did not require the Ti plasmid (Park et al., 1987). So, it does seem difficult to rationalize a role for acetosyringone and the regulatory genes *virA* and *virG* in chemotaxis.

3.2 Attachment of *A. tumefaciens* to plant

It is reasonable that an intimate association between pathogen and host cells is required for the transfer of T-DNA and virulence proteins from *A. tumefaciens* to plant cells. *A. tumefaciens* can efficiently attach to both plant tissues and abiotic surfaces, and establish

complex biofilms at colonization sites. Microscopic observation of bacteria interacting with the plant cells demonstrates a significant propensity to attach in a polar fashion (Smith & Hindley, 1978; Tomlinson & Fuqua, 2009). All *Agrobacterium* mutants deficient in attachment to plant cells are either avirulent or extremely attenuated in virulence (Cangelosi et al., 1989; Douglas et al., 1982, 1985; Matthysse & McMahan, 2001; O'Connell & Handelsman, 1989). Although obviously critical, the attachment process is one of the least-characterized sets of cellular processes in the entire interaction. Little progress on this area was made in recent years (Tomlinson & Fuqua, 2009).

3.2.1 Bacterial genes involved in the attachment of *A. tumefaciens* to plant

The binding of *A. tumefaciens* to host plant cells seems to require the participation of specific receptors that may exist on the bacterial and plant cell surface because the binding of *A. tumefaciens* to host plant cells is saturable and unrelated bacteria fail to inhibit the binding of *A. tumefaciens* to host plant cells (B.B. Lippincot & J.A. Lippincot, 1969). A number of *A. tumefaciens* mutants reported to affect the attachment of bacteria to plant cells have been isolated. Some related genes are identified and sequenced (Matthysse et al., 2000; Reuhs et al., 1997). However, it is surprising that a large number of genes are involved in the bacterial attachment to host cells and the actual functions of most genes are unclear (Matthysse et al., 2000). All the genes reported to affect the bacterial attachment to host cells are chromosomal genes.The genes involved in the binding of bacteria to host plant cells are identified to mainly locate on two regions of the bacterial chromosome.

The binding of bacteria to host cells is thought to be a two-step process (Matthysse & McMahan, 1998). The binding in the first step is loose and reversible because the bound bacteria are easy to being washed from the binding sites by shear forces, such as water washing or vortexing of tissue culture cells. Genes involved in this step are identified to locate on the *att* gene region (more than 20 kb in size) of the bacterial chromosome. Gene mutations in this region abolish virulence. The mutants in the *att* gene region can be divided into two groups. The first group can be restored to attachment and virulence by the addition of conditioned medium. This group appears to be altered in signal exchange between the bacterium and the host. Mutations in this group of mutants occur in the genes homologous to ABC transporters and transcriptional regulator as well as some closely linked downstream genes (Matthysse et al., 2000; Matthysse & McMahan, 1998; Reuhs et al., 1997). The second group of mutants in the *att* gene region is not affected by the presence of conditioned medium. This mutant group appears to affect the synthesis of surface molecules, which may play a role in the bacterial attachment to the host. This group includes mutants in the genes homologous to transcriptional regulator and ATPase as well as a number of biosynthetic genes, which include the transacetylase required for the formation of an acetylated capsular polysaccharide. The acetylated capsular polysaccharide is required for the bacterial attachment to some plants because the production of the acetylated capsular polysaccharide is correlated to the attachment of wild-type strain C58 to the host cells and the purified acetylated capsular polysaccharide from wild-type strains blocks the binding of the bacteria to some host cells (Matthysse et al., 2000; Matthysse & McMahan, 1998, 2001; Reuhs et al., 1997).

The second step in the bacterial attachment to the host results in tight binding of the bacteria to the plant cell surface because the bound bacteria can no longer be removed

from the plant cell surface by shear forces. This step requires the synthesis of cellulose fibrils by the bacteria, which recruits larger numbers of bacteria to the wound sites. Cellulose-minus bacterial mutants show reduced virulence (Minnemeyer et al., 1991). The genes required for the synthesis of bacterial cellulose fibrils (*cel* genes) are identified to locate on the bacterial chromosome near, but not contiguous with the *att* gene region (Robertson et al., 1988).

Some other chromosomal virulence genes *chvA*, *chvB*, and *pscA* (*exoC*) are believed to be involved indirectly in bacterial attachment to host (Cangelosi et al., 1987; Douglas et al., 1982; O'Connell & Handelsman, 1989). These genes are involved in the synthesis, processing, and export of a cyclic β-1,2-glucan, which has been implicated in the bacterial binding to plant cells. Mutations in *chvA*, *chvB*, and *pscA* (*exoC*) cause a 10-fold decrease in binding of bacteria to zinnea mesophyll cells and strongly attenuate virulence (Douglas et al., 1985; Kamoun et al., 1989; Thomashow et al., 1987). ChvB is believed to be involved in the synthesis of the cyclic β-1,2-glucan (Zorreguieta & Ugalde, 1986). ChvA is homologous to a family of membrane-bound ATPases and appears to be involved in the export of the cyclic β-1,2-glucan from the cytoplasm to the periplasm and extracellular fluid (Cangelosi et al., 1989; De Iannino & Ugalde, 1989). However, the virulence of *chvB* mutants is temperature sensitive (Banta et al., 1998). At lower temperature (16 ^{o}C), *chvB* mutants became virulent and were able to attach to plant roots (Bash & Matthysse, 2002).

3.2.2 Plant factors involved in the attachment of *A. tumefaciens* to plant

In addition to bacterial factors, some plant factors are essential for the attachment of *A. tumefaciens* to plant cells. Two plant cell wall proteins: a vitronectin-like protein (Wagner & Matthysse, 1992) and a rhicadhesin-binding protein (Swart et al., 1994) have been proposed to mediate the bacterial attachment to plant cells. Vitronectin is an animal receptor that is specifically utilized by different pathogenic bacteria (Burridge et al., 1988). A plant vitronectin-like protein is reported to occur in several *A. tumefaciens* host plant (Sanders et al., 1991). Human vitronectin and antivitronectin antibodies were shown to inhibit the binding of *A. tumefaciens* to plant tissues. Nonattaching *A. tumefaciens* mutants, such as *chvB*, *pscA* and *att* mutants, showed a reduction in the ability to bind vitronectin. Therefore, the plant vitronectin-like protein was proposed to play a role in *A. tumefaciens* attachment to its host cells (Wagner & Matthysse, 1992). However, a recent report argues against the role of the vitronectinlike protein in bacterial attachment and *Agrobacterium*-mediated transformation (Clauce-Coupel et al., 2008).

Genetic studies showed that additional plant cell-surface proteins might play a role in *A. tumefaciens* attachment. Two *Arabidopsis* ecotypes, B1-1 and Petergof, which are highly recalcitrant to *Agrobacterium*-mediated transformation, were proposed to be blocked at an early step of the binding (Nam et al., 1997). Two *Arabidopsis* T-DNA insertion mutants of the ecotype Ws, *rat1* and *rat3*, which are resistant to *Agrobacterium* transformation (*rat* mutants), are deficient in *A. tumefaciens* binding to cut root surfaces (Nam et al., 1999). DNA sequence analysis indicated that *rat1* and *rat3* mutations affect an arabinogalactan protein (AGP) and a potential cell-wall protein, respectively. AGPs were confirmed to be involved in *A. tumefaciens* transformation (Nam et al., 1999). Interestingly, AGP17 (*rat1* mutant) appears to be involved in host defense reactions and signaling (Gaspar et al., 2004; Gelvin, 2010a). Other two *rat* mutans, *rat*T8 and *rat*T9, were identified to be mutated in the genes coding for receptor-like protein kinases (Zhu et al., 2003).

3.3 Transfer of *Agrobacterial* molecules to plant

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

3.3.1 Transfer apparatus

A. tumefaciens uses type IV secretion system (T4SS) to transfer T-DNA and effector proteins to its host cells (Cascales & Christie, 2003, 2004). The T4SS was initially defined to be a class of DNA transporters whose components are highly homologous to the conjugal transfer (tra) system of the conjugative IncN plasmid pKM101 and the *A. tumefaciens* T-DNA transfer system (Burns, 2003; Christie & Vogel, 2000). T4SS, also known as the mating pair formation (Mpf) apparatus, is a cell envelope-spanning complex (composed of 11-13 core proteins) that is believed to form a pore or channel through which DNA and/or protein is delivered from the donor cell to the recipient cell. Recently the members of T4SS have steady increased, with the identification of additional systems involved in DNA and protein translocation (Alvarez-Martinez & Christie, 2009; Cascales & Christie, 2003; Christie & Vogel, 2000; Gillespie, 2010). However, the best-studied T4SS member is the VirB/D4 transporter of *A. tumefaciens*. In the past decade, much of the research on *Agrobacterium*-mediated T-DNA transfer focused on the *vir*-specific T4SS, the T-complex transporter. Therefore, the *A. tumefaciens* T-complex transporter has become a paradigm of T4SS (Alvarez-Martinez & Christie, 2009; Cascales & Christie, 2003).

The VirB/D4 T4SS is assembled from 11 proteins (VirB1 to VirB11) encoded by the *virB* operon, and VirD4. At least 10 of the 11 VirB proteins are believed to be the structural subunits of the T-pilin and associated transport apparatus that spans from the cytoplasm of the cell, through the inner membrane, periplasmic space and outer membrane, to the outside of the cell. In the past few years, work in identifying the interactions among the VirB protein subunits and defining the steps in the transporter assembly pathway has extended our knowledge of the structure of the VirB transport apparatus. To demonstrate the architecture of the VirB/D4 transporter, a model that depicts the subcellular locations and interactions of the VirB and VirD4 subunits of the *A. tumefaciens* VirB/D4 T4SS was proposed (Alvarez-Martinez & Christie, 2009; Cascales & Christie, 2004). Recently, VirB7, VirB9, and VirB10 homologs from the pKM101 T4SS were purified and the cryoEM structure of a core complex composed of pKM101 VirB7-like TraN, VirB9-like TraO, and VirB10-like TraF was revealed (Fronzes et al., 2009).

Agrobacterium-mediated T-DNA transfer to plant shows striking similarities to the plasmid interbacterial conjugation (Ream, 1989; Stachel & Zambryski, 1986). Bacterial conjugation can be visualized as the merging of two ancient bacterial systems: the DNA rolling-circle replication system and type IV secretion system (T4SS) (Llosa et al., 2002). The DNA rolling-circle replication system in plasmid conjugation was also known as the DNA transfer and replication (Dtr) system. The Dtr system corresponds to the T-DNA relaxase nucleoprotein complex. The T4SS responding for the plasmid conjugation was initially called mating pair formation (Mpf) system. In order to recognize these two systems and link them, a protein is normally required for many conjugal plasmids to couple the Dtr to the Mpf. This protein was called coupling protein as its function (Gomis-Ruth et al., 2002).

VirD4 is a homologue of coupling protein family and is believed to be the coupling protein that links the transferred molecules and T4SS transporter. VirD4 is an inner membrane

protein with potential DNA binding ability and ATPase activity. Membrane topology analysis of VirD4 revealed that VirD4 contains an N-terminal-proximal region, which includes two transmembrane helices and a small periplasmic domain, and a large C-terminal cytoplasmic domain (Cascales & Christie, 2003; Das & Xie, 1998). VirD4 localizes to the cell pole. The polar location of VirD4 was not dependent on T-DNA processing, the assembly of T4SS transporter and the expression of other Vir proteins. Both the small periplasmic domain and the cytoplasmic nucleotide-binding domain are required for the polar localization of VirD4 and essential for T-DNA transfer. VirD4 forms a large oligomeric complex (Kumar & Das, 2002). VirD4 can recruit VirE2 to the cell poles (Atmakuri et al., 2003) and weakly interact with VirD2-T-strand complex (Cascales & Christie, 2004). Although VirD4 is essential for coordinating the T4SS to drive T-DNA transfer, it has been unclear whether VirD4 physically/directly interacts with the T4SS transporter. However, the interaction between VirD4 homologues and the protein components of Dtr system exhibits specificity. It was supposed that VirD4 protein might recruit T-complex to the T4SS transporter through contacts with the T-complex protein and then through the contacts with VirB10 coordinate the passage of T-complex through the T4SS channel (Cascales & Christie, 2003; Llosa et al., 2003). However, it should be pointed out that the recruitment of T-complex might be much more difficult than the recruitment of single VirE2 molecule due to the difference of molecular size between T-complex and VirE2. Recently, two cytoplasmic proteins, VBP (VirD2-binding protein) (Guo et al., 2007a, 2007b) and VirC1 (Atmakuri et al., 2007) were reported to be involved in the recruitment of the T-complex to T4SS. Genome-wide sequence analysis showed that *A. tumefaciens* contains three *vbp* homologous genes. Reverse genetic study showed that mutatons of three *vbp* genes highly attenuated the bacterial ability to cause tumors on plants (Guo et al., 2007a, 2009b).

3.3.2 *Agrobacterial* molecules transported to plant

Agrobacterial molecules transported into host cells by VirB/D4 T4SS include the VirD2-T-strand complex, VirE2, VirE3, VirF, and VirD5. VirD2 is covalently bound to the 5′ end of the T-strand. The bound VirD2, probably in conjunction with other protein components, such as VBP (Guo et al., 2007a, 2007b) and VirC1 (Atmakuri et al., 2007), confers recognition of the VirD2-T-strand complex by the VirB/D4 T4SS. VirD2 also "pilots" the T-strand through the translocation channel. It was supposed that the VirB/D4 T4SS is actually a protein transporter and the T-strand is the "hitchhiker" (Cascales & Christie, 2004).

VirE2 is a single-stranded DNA-binding protein (Christie et al., 1988; Citovsky et al., 1988) that can bind single-stranded DNA without sequence specificity, and is supposed to protect the T-strand from the nucleolytic degradation because single-stranded T-DNA is believed to be susceptible to nucleases. The binding of VirE2 to single-stranded DNA is strong and co-operative, suggesting that VirE2 coats the T-strand along its length (Citovsky et al., 1989). Another possible function of VirE2 is to guide the nuclear import of T-DNA (Ziemienowicz et al., 1999, 2001). This will be discussed in the following section of this chapter. Induced *Agrobacterium* cell can produce sufficient VirE2 to bind all intracellular single-stranded T-DNA. When bound to single-stranded DNA, VirE2 can alter the ssDNA from a random-coil conformation to a telephone cord-like coiled structure and increases the relative rigidity (Citovsky et al., 1997). Initial hypothesis is that the protective role of VirE2 is required to function in both bacteria and plant cells. So, the prevailing view on the T-DNA transfer is that a packaged nucleoprotein complex, the T-complex, composed of the T-strand DNA

containing the 5´-associated VirD2 and coated with VirE2 along its length, is the transfer intermediate (Howard & Citovsky, 1990; Zupan & Zambryski, 1997). This T-complex structure model implies that both VirD2 and VirE2 together with the T-strand are transported into plant cell in the same time. This idea makes biological sense because it is likely that VirE2 with a high affinity to ssDNA may form a complex with the T-strand already inside *Agrobacterium* cell, especially if both VirE2 and the T-strand are transported through the same channel (Binns et al., 1995). Indeed, the T-complex, which contains T-strand, VirD2 and VirE2, was observed in the crude extracts from *vir*-induced *Agrobacterium* by using anti-VirE2 antibodies to co-immunoprecipitate both T-strand and VirE2 (Christie et al., 1988).

However, two kinds of evidence argued against that the protective role of VirE2 is required to function inside bacterial cells. The first is the observation that a strain expressing *virE2* but lacking T-DNA can complement a *virE2* mutant in a tumor formation assay (Otten et al., 1984) and the T-strand accumulates to wild-type levels in *virE2* mutants (Stachel et al., 1987; Veluthambi et al., 1988). The second kind of evidence is that *virE2* expression in transgenic tobacco plants restores the infectivity of a VirE2-deficient *Agrobacterium* strain (Citovsky et al., 1992). In addition, the observation that *virE2* mutants can transfer T-DNA into plant cells (Yusibov et al., 1994) also proved that VirE2 is not essential for the export of T-DNA. All these data appear to support that T-DNA may not be packaged by VirE2 in the bacterial cells, at least, the packaging of T-DNA inside bacterial cells by VirE2 is not necessary for the tumor formation. VirE2 can be transported independently, but the transportation of VirE2 requires the activities of VirE1. VirE1 is a chaperone and is necessary for VirE2 translation and stability but not essential for the recognition of the translocation signal of ViE2 by the transport machinery and the subsequent translocation of VirE2 into plant cells, indicating that the role of VirE1 playing in the export process of VirE2 seems restricted to the stabilization of VirE2 by preventing VirE2 from the premature interactions in the bacterial cell before translocation into plant cells (Vergunst et al., 2003).

Like VirD2 and VirE2, agrobacterial protein VirF can also be exported to plant cell (Vergunst et al., 2000). *virF* gene is found only in the octopine-specific Ti plasmid. It is not essential for T-DNA transfer. Initially, VirF is thought to be a host-range factor of *Agrobacterium* (Regensburg-Tuink & Hooykaas, 1993). A more recent report showed that VirF interacts with an *Arabidopsis* Skp1 protein (Schrammeijer et al., 2001). Yeast Skp1 protein and its animal and plant homologs are subunits of the complexes involved in targeted proteolysis. This targeted proteolysis can regulate the plant cell cycle. So, it was suggested that VirF may function in setting the plant cell cycle to effect better transformation (Gelvin, 2003; Tzfira & Citovsky, 2002).

Protein truncation and fusion of T4SS substrates demonstrated that certain C-terminal motifs were required for the export of targeted substrates. The C-terminal 37 amino acids of VirF and the C-terminal 50 amino acids of VirE2 and VirE3 are sufficient to mediate transport of these fusion proteins to plants (Vergunst et al., 2000, 2003). The minimal size of VirF required to direct the translocation of VirF-fusion protein to plants is the C-terminal 10 amino acids. Site-directed mutations showed that several arginines within this region are required for transport (Vergunst et al. 2005). These export signals mediate the recognition of substrates by the VirB/D4 T4SS. A possible consensus sequence R-x(7)-R-x-R-x-R (x, any amino acid) was identified in the C termini of substrates secreted by the VirB/D4 T4SS.

4. Events happening in host

Following the entry of agrobacterial molecules in plant cell cytoplasm, the VirD2-T-strand interacts with VirE2 and plant proteins, likely forming "super-T-complex", which is responsible for subcellular travelling of T-strand from cytoplasm through nuclear membrame into nucleus, and to the chromatin, thus facilitating T-DNA integration into host genome. All these biological processes occurring in host cells require the involvement of many host factors.

4.1 Nuclear targeting of T-complex

The dense structure of the cytoplasm, which greatly restricts the free diffusion of macromolecules, and the size of the "super-T-complex", which far exceeds the 60 kDa size-exclusion limit of the nuclear pore (Lacroix et al., 2006a), indicate that active transport processes are required for the nuclear import of T-complex. As a rule, active nuclear import of proteins requires a specific nuclear localization signal (NLS). Typical nuclear localization signals are short regions rich in basic amino acids (Silver, 1991).

4.1.1 Nuclear localization signals in *Agrobacterial* molecules

Because T-strand is presumed to be completely coated with proteins inside plant cells it is impossible for T-strand itself to carry NLSs. Thus, the NLSs that guide T-complex nuclear import most likely reside in its associated proteins, VirD2 and VirE2. Sequence analysis reveals that both VirD2 and VirE2 contain NLSs. Two NLSs are found in VirD2. One is the typical bipartite NLS that resides in residues 396~413. The nuclear localizing function of this bipartite NLS was confirmed by the observation that VirD2-GUS fusion protein, when expressed in tobacco protoplasts, can target to plant cell nuclei (Howard et al., 1992; Tinland et al., 1992). However, mutations that destroy this bipartite NLS attenuate, and do not abolish tumorigenesis, indicating that although this NLS plays a role in T-DNA transfer, it is not essential (Rossi et al., 1993; Shurvinton et al., 1992). Another NLS in VirD2 is found in residues 32~35, adjacent to the active site in the endonuclease domain (Tinland et al., 1992). This NLS is a monopartite NLS. GUS proteins fused with this NLS accumulate in plant nuclei, but this NLS does not play a role in T-DNA nuclear localization (Shurvinton et al., 1992). The sequences of residues 419~423 at the C-terminus of VirD2, known as the ω domain, are important for tumorigenesis, but do not contribute to nuclear localization activity despite its proximity to the bipartite NLS. The ω domain was supposed to be involved in T-DNA integration (Mysore et al., 1998).

VirE2, the most abundant protein component of the T-complex, contains two bipartite NLSs in its central region (residues 205~221, and residues 273~287). When fused to GUS, each VirE2 NLS is capable of directing the fusion protein to the nucleus of a plant cell, but the maximum accumulation in the nucleus requires both VirE2 NLSs (Citovsky et al., 1992). Because these two NLSs overlap with the DNA binding domains, mutations of *virE2* that abolish the activity of one of these NLSs will also eliminate the DNA binding activity. So, no genetic evidence can be provided to verify the function of these two VirE2 NLSs in T-complex nuclear localization. When VirE2 binds to T-strand, the NLSs of VirE2 may be occluded and inactive. It has been observed that for the nuclear import of short ssDNA, VirD2 was sufficient, whereas import of long ssDNA additionally required VirE2 (Ziemienowicz et al., 2001). Although predominantly nuclear localization of VirE2 was

observed in earlier studies, several recent reports demonstrated the cytoplasmic localization of VirE2 (Bhattacharjee et al., 2008; Grange et al., 2008). Indeed, results from different research groups indicate that VirE2 localizes to different subcellular compartments in different tissues (Gelvin, 2010b). All the evidence argued against the function of VirE2 in T-complex nuclear localization. RecA, a NLS-lacking ssDNA binding protein, could substitute for VirE2 in the nuclear import of T-strand, further demonstrating that VirE2 functions not in the nuclear localization, possibly in mediating the passage of T-strand through the nuclear pore (Ziemienowicz et al., 2001). VirE2 was assumed to shape the T-complex such that it is accepted for translocation into the nucleus.

4.1.2 Plant proteins involved in T-complex nuclear targeting

Besides the agrobacterial proteins VirD2 and VirE2, some plant proteins were supposed to be involved in the T-complex nuclear translocation. Early yeast two-hybrid screen identified an *A. thaliana* importin-α (AtKAP, now known as importin-α1) that interacts with VirD2 (Ballas & Citovsky, 1997). Importin-α proteins interact with NLS-containing proteins and guide the nuclear translocation of these proteins. Importin-α proteins constitute a protein family and *Arabidopsis* encodes at least nine of these proteins (Gelvin, 2003). Interaction between VirD2 and importin-α1 was verified to be VirD2 NLS dependent (Ballas & Citovsky, 1997). The importance of importin-α proteins in the T-complex transfer process was confirmed by the genetic evidence that a T-DNA insertion into the importin-α7 gene, or antisense inhibition of expression of the importin-α1 gene, highly reduces the transformation efficiency (Gelvin, 2003). Importin-α1, as well as all other investigated importin-α family members, also interacts with VirE2 (Bhattacharjee et al., 2008) and VirE3 (Garcia-Rodriguez et al., 2006).

Other plant proteins that were identified to interact with VirD2 include several cyclophilins, a kinase CAK2M, and a protein phosphatase 2C (PP2C). Deng et al. (1998) showed that an *Arabdopsis* cyclophilin interacted strongly with VirD2. They further characterized the interaction domain of VirD2 and found that a central domain of VirD2 (residues 274~337) was involved in the interaction with cyclophilin. No previous function of VirD2 had been ascribed to this central region. Cyclosporin A, an inhibitor of VirD2-cyclophilin interaction, inhibits *Agrobacterium*-mediated transformation of *Arabidopsis* and tobacco (Deng et al., 1998). Cyclophilin were presumed to serve as a molecular chaperone to help in T-complex trafficking within the plant cell. Cyclin-dependent kinase-activating kinase CAK2M interacts with VirD2 and catalyzes the phosphorylation of VirD2 *in vivo*. CAK2M may target VirD2 to the C-terminal regulatory domain of RNA polymerase II large subunit (RNApolII CTD) (Pitzschke & Hirt, 2010). A tomato type 2C protein phosphatase (PP2C) that was identified to interact with VirD2 can catalyze the dephosphorylation of VirD2. This phosphatase was assumed to be involved in the phosphorylation and dephosphorylation of a serine residue near the C-terminal NLS in the VirD2. Overexpression of this phosphatase decreased the nuclear targeting of a GUS-VirD2-NLS fusion protein, suggesting that phosphorylation of the C-terminal NLS region may affect the nuclear targeting function of VirD2 (Tao et al, 2004).

Two VirE2-interacting proteins were designated VIP1 and VIP2. VIP1 was showed to facilitate the VirE2 nuclear import in yeast and mammalian cells. Tobacco VIP1 antisense plants were highly resistant to *A. tumefaciens* infection (Tzfira et al., 2001), whereas,

transgenic plants that overexpress VIP1 are hypersusceptible to *A. tumefaciens* transformation (Tzfira et al., 2002). VIP1 is a basic leucine zipper (bZIP) motif protein and shows no significant homology to known animal or yeast proteins (Tzfira et al., 2001). So, how VIP1 facilitates the nuclear import of VirE2 remains unclear. Unlike VIP1, VIP2 was unable to mediate VirE2 into the yeast cell nucleus. However, VIP1 and VIP2 interacted with each other. Thus, VIP1, VIP2 and VirE2 were assumed to function in a multiprotein complex (Tzfira & Citovsky, 2000, 2002). A recent paper showed that VIP1 is phosphorylated by the mitogenactivated protein kinase MPK3 and the VIP1 phosphorylation affects both nuclear localization of VIP1 and *Agrobacterium*-mediated transformation, implying that VIP1 phosphorylation is important for super-T-complex nuclear targeting (Djamei et al., 2007).

4.2 Integration of T-DNA into plant genome

The integration of the incoming ssT-strand of the T-complex into plant genome is the final step of the *Agrobacterium*-mediated genetic transformation. Whether or not the host can be successfully transformed is highly dependent on whether the T-DNA could be integrated into the suitable sites of the host genome.

4.2.1 Integration site

The DNA sequence analysis of several T-DNA host DNA junctions revealed that these junctions, in general, appear more variable than the junctions created by insertions of transposons, retroviruses, or retrotransposons (Gheysen et al., 1987). A statistical analysis of 88,000 T-DNA genome-wild insertions of *Arabidopsis* revealed the existence of a large integration site bias at both the chromosome and gene levels (Alonso et al., 2003). At the chromosomal level, fewer T-DNA insertions were found at the centromeric region. At the gene level, insertions within promotor and coding exons make up nearly 50% of all insertion sites. However, these statistical results may be skewed by the antibiotic resistance selection of transformed plants (T1 plants) because only the T1 plants with transcriptionally active T-DNA insertions can be selected (Alonso et al., 2003; Valentine, 2003). Recently, a genome-wide analysis of T-DNA-integration sites in *Arabidopsis* performed under non-selective conditions showed that T-DNA integration occurs rather randomly (Kim et al., 2007). Another statistical analysis of 9000 flanking sequence tags characterizing transferred DNA (T-DNA) transformants in *Arabidopsis* showed that there are microsimilarities involved in the integration of both the right and left borders of the T-DNA insertions. These microsimilarities occur only in a stretch of 3 to 5 bp and can be between any T-DNA and genomic sequence. This mini-match of 3 to 5 bp basically allows T-DNA to integrate at any locus in the genome. It was also showed that T-DNA integration is favored in plant DNA regions with an A-T-rich content (Brunaud et al., 2002).

4.2.2 Integration mechanism

The observation of the random, as opposed to targeted, nature of T-DNA integration indicated that the integration occurs in illegitimate recombination. To date, it has not been possible to target T-DNA to any particular locus in the genome with any great efficiency. So, the T-DNA integration has been one of the motives of intense investigation of *A. tumefaciens*. But, the molecular mechanism of the T-DNA integration remains largely unknown. Two major models for T-DNA integration have been proposed: single-strand-gap repair model

and double-strand-break repair model (Gelvin, 2010a; Mayerhofer et al., 1991; Tzfira et al., 2004). In the single-strand-gap repair model, VirD2-T-strands invade regions of microhomology between T-DNA and plant DNA sequences and partially anneal to the microhomologous regions. VirD2 on the 5′-end of the T-strand causes a nick in one strand of plant DNA and ligates the T-strand to the nick. Following the ligation of the T-strand to the target DNA, a nick is introduced in the second strand of the target DNA and extended to a gap by exonucleases. During the gap repairing, the complementary strand of T-DNA is synthesized, resulting in incorporation of a double-strand copy of the T-strand into the plant genome. The double-strand-break repair model hypothesizes that single-strand T-strands are replicated in the plant nucleus to a double-strand form and then the double-strand T-DNA is integrated into double-strand breaks in the target DNA. The double-strand-break repair model requires the T-DNA to be converted to a double-strand form before its integration into the double-strand breaks. However, there are more results that strongly support the double-strand-break repair model (Lacroix et al., 2006a). It seems that double-stranded T-DNA integration is the native model of T-DNA integration.

4.2.3 Plant proteins involved in the T-DNA integration

The plant proteins that may be involved in the T-DNA integration process are only now beginning to be defined. As mentioned before, CAK2M phosphorylates VirD2 and targets VirD2 to the C-terminal regulatory domain of RNA polymerase II large subunit (RNApolII CTD), a factor that is responsible for recruiting TATA-box binding proteins (TBP) to actively transcribed regions. CAK2M can also phosphorylate CTD. By associating with VirD2, TBP may guide T-strands to transcriptionally active regions of chromatin for integration. It was supposed that TBP or CAK2M may target VirD2 to the CTD, thereby controlling T-DNA integration (Bako et al., 2003). These nuclear VirD2-binding factors provide a link between T-DNA integration and transcription-coupled repair, suggesting that transcription and transcription-coupled repair may play a role in T-DNA integration (Bako et al., 2003).

To integrate into plant chromosomal DNA, T-DNA must interact with chromatin. More than 109 chromatin genes of 15 gene families were identified to be related to the transformation susceptibility (Crane and Gelvin, 2007). As T-DNA integrates into the plant genome by illegitimate recombination (Mayerhofer et al, 1991), proteins involved in DNA repair and recombination should also be involved in T-DNA integration. Non-homologous end-joining proteins, including Ku70, Ku80, Rad50, Mre11, Xrs2, and Sir4, were identified to be required for T-DNA intigration (Gelvin, 2010a; Tzfira and Citovsky, 2006; Van Attikum et al., 2001).

5. Conclusions

Several decades of intensive studies on *Agrobacterium* make the transformation of many plant and non-plant species by *Agrobacterium*-mediated transformation protocols become routine. The ability of *Agrobacterium* to genetically transform a wide variety of plant and non-plant species has earned it an honour of "nature's genetic engineer" and placed it at the forefront of future biotechnological applications (Rao et al., 2009). However, the *Agrobacterium*-mediated genetic transformation is still an extremely inefficient process, in which only few of the host cells can be infected, and T-DNA integration and stable expression occur in an even smaller fraction of the infected cells. Out of question, a better understanding of the fundamental mechanisms of *Agrobacterium*-mediated genetic

transformation is essential for improving the biotechnological applications of this bacterium as a gene vector for genetic transformation of plant and non-plant species. In addition, *Agrobacterium*-mediated genetic transformation serves as an important model system for studying host-pathogen recognition and delivery of macromolecules into target cells and thus the in-depth study and molecular analysis of *Agrobacterium*-mediated transformation will also add to our understanding of all the biological processes involved in the *Agrobacterium*-mediated genetic transformation.

Agrobacterium-mediated genetic transformation is a complex process that involves *Agrobacterium* reactions to wounded plant, T-DNA transfer in both bacteria and host cells, host reactions to *Agrobacterium* infection, and genetic transformation of host cells. This complex process requires the concerted function of both *Agrobacterium* and host. The golden period of *Agrobacterium* research led us to understand many of the *Agrobacterium*'s biological processes and mechanisms, such as virulence protein inducing, T-DNA processing, and macromolecule exporting by T4SS. However, many key steps of *Agrobacterium*-mediated genetic transformation still remain poorly understood and require further investigation. Particularly the events happening in the host infected by *Agrobacterium* are relatively more poorly understood.

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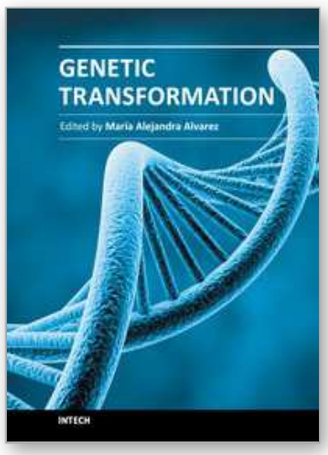

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Genetic transformation of plants has revolutionized both basic and applied plant research. Plant molecular biology and physiology benefit from this power fool, as well as biotechnology. This book is a review of some of the most significant achievements that plant transformation has brought to the fields of Agrobacterium biology, crop improvement and, flower, fruit and tree amelioration. Also, it examines their impact on molecular farming, phytoremediation and RNAi tools.

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